Death Receptors and Colorectal Liver Metastases

CD95/FAS as a pro-tumorigenic receptor

Frederik J.H. Hoogwater

Death Receptors and Colorectal Liver Metastases
CD95/FAS as a pro-tumorigenic receptor

Thesis, Utrecht University, The Netherlands

The work described in this thesis was made possible by a personal grant from the Wijnand M. Pon foundation.

Publication of this thesis was financially supported by: Sanofi-Aventis, Chirurgisch fonds UMC Utrecht, GlaxoSmithKline, ChipSoft, Boston Scientific Nederland B.V., Nycomed, Olympus Nederland B.V., Nederlandse Vereniging voor Gastroenterologie
Dit proefschrift werd mede mogelijk gemaakt met financiële steun van: Sanofi-Aventis, Chirurgisch fonds UMC Utrecht, GlaxoSmithKline, ChipSoft, Boston Scientific Nederland B.V., Nycomed, Olympus Nederland B.V., Nederlandse Vereniging voor Gastroenterologie

Cover: F.J.H. Hoogwater, M.W. Nijkamp, www.**wenz**iD.nl.
TNF-related apoptosis-inducing ligand (TRAIL) treatment stimulates invasion rather than apoptosis in liver metastases of colorectal cancer cells (green) as tumor cells detach from the core of the metastases and invade the surrounding liver parenchyma (red). See page 60.
Lay out: www.**wenz**iD.nl
Printer by: Gildeprint Drukkerijen BV, Enschede

ISBN/EAN 9789461081254

Death Receptors and Colorectal Liver Metastases

CD95/FAS as a pro-tumorigenic receptor

Death Receptoren en Colorectale Levermetastasen

CD95/Fas als een pro-tumorigene receptor
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
vrijdag 21 januari 2011 des middags te 2.30 uur

door

Frederik Jan Hendrik Hoogwater

geboren 2 november 1979 te Dokkum

Promotor: Prof. dr. I.H.M. Borel Rinkes
Co-promotor: dr. O. Kranenburg

voor heit en mem
voor Marjolein

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Apoptosis

Apoptosis is derived from the ancient Greek word "απόπτωσις" which illustrates the process of "leaves falling off from a tree" (apó meaning away or from, ptosis meaning falling down). The phenomenon of apoptosis was first described by Carl Christoph Vogt in 1842, but was introduced in the field of biology by John Foxton Ross Kerr in 1972[1,2]. Apoptosis is the best characterized form of programmed cell death and is an essential cellular homeostasis mechanism that ensures correct development and function of multi-cellular organisms. Probably the most popular example highlighting the importance of cell death in the development of embryonic tissues is the 'sculpting' of our hands and fingers by the elimination of cells located in between digits. The fundamental importance of the correct execution of apoptosis is evident from the many human diseases with aberrancies in apoptosis, including cancer. Apoptosis is essential to maintain appropriate cell numbers and remove damaged cells. If the subtle balance between proliferation and cell death is disturbed then ultimately tumor formation can occur[3]. Furthermore, apoptosis is critical for the shutdown of chronic immune responses and for the prevention of autoimmunity. Inappropriate activation of apoptosis is also associated with neurodegenerative disorders such as Parkinson's and Alzheimer's disease, or myocardial damage seen after reperfusion of cardiac tissue following infarct[4]. The awareness that cell death can be initiated by genetically controlled processes has enabled progress in unravelling the mechanisms of many diseases and this knowledge has led to the development of pharmacologic agents that inhibit or initiate programmed cell death[5-8].

Apoptosis refers to a distinct type of cell death morphologically characterized by cell shrinkage and rounding, enhanced cytoplasm density, condensation of the chromatin and cellular fragmentation into so-called apoptotic bodies. These apoptotic bodies are taken up by neighbouring or specialised cells, thereby avoiding the initiation of an inflammatory response that would occur after spilling of intracellular material. These macroscopic changes are preceded by biochemical events. The central event in apoptotic cell death is a proteolytic system involving the activation of caspases, a family of cysteine proteases with strict cleavage specificity at aspartic acid residues. These enzymes participate in a cascade that is triggered in response to pro-apoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell[9].

There are two distinct, but interconnected, molecular signaling pathways through which apoptosis can be initiated. First, via the intrinsic (or mitochondrial) apoptotic pathway in which pro-apoptotic signaling is triggered in response to a variety of stress conditions such as UV irradiation, growth factor deprivation, abnormal mitosis, hypoxia, chemotherapy or ionizing radiation[10]. These stress conditions cause the disturbance of the mitochondrial membrane and the release of cytochrome-c into the cytoplasm and the activation of caspase-9. The p53

tumor suppressor gene is a key molecule upstream of mitochondrial perturbation in the intrinsic apoptotic cascade and is therefore called the "guardian of the genome"[5]. The intrinsic pathway is largely mediated and controlled by interactions of pro-apoptotic and anti-apoptotic members of the B-cell leukemia/lymphoma 2 (Bcl-2) protein family.

The second mechanism by which apoptosis can be induced is activated by extracellular signals and is therefore referred to as the extrinsic apoptotic pathway. This pathway is initiated by the activation of specialized cell surface receptors, so called "death receptors"[6].

Death receptors

Death receptors are a unique class of cell-surface receptors which are best known for their ability to induce apoptosis upon binding their respective ligands. Death receptors (DRs) are members of the tumor necrosis factor receptor (TNF-R) superfamily. Members of this family are diverse in primary structure, but all of them contain similar cysteine-rich extracellular subdomains. This is a unique structural feature that allows them to recognize their ligands with specificity and in most cases, exclusivity. DRs also contain a homologous cysteine-rich cytoplasmic region known as the "death domain" (DD) which is required for the transmission of the cytotoxic signal[11-15].

Eight members of the DR-family have been characterized so far: CD95, also called FS7-associated surface antigen (FAS) or apoptosis-inducing protein 1 (APO-1/DR2), is the most extensively studied death receptor and was originally discovered in the early 1990s as the target of two monoclonal antibodies that trigger apoptotic cell death in certain human tumor-derived cell lines in culture or as xeno-transplants in immunodeficient mice[16]. Other members of the TNF-R superfamily are TNF-receptor 1 (TNF-R1/p55/p60/CD120a/DR1), DR3 (APO-3/LARD/TRAMP/WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1/APO-2/DR4), TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2/KILLER/TRICK2/DR5), DR6 (TNFRSF21), ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR)[17].
Death receptors are activated by their physiological ligands, a group of complementary cytokines that belong to the TNF cytokine family. They are respectively CD95 ligand (CD95L), TNF-α, APO-3 ligand (APO-3L), TNF-related apoptosis-inducing ligand (TRAIL), EDA ligand (EDAL) and NGF.

Death ligands also interact with decoy receptors (DcRs) that do not posses DDs and so cannot form signaling complexes. Until now four decoy receptors have been characterized: TRAILR3 (DcR1), TRAILR4 (DcR2), DcR3 and osteoprotegrin (OPG).
On ligand-mediated activation of CD95, the conserved intracellular death domain attracts the intracellular adapter molecule, Fas-associated death domain (FADD). The adapter molecule recruits the initiator caspases -8 and -10 to the death receptor, forming the death-inducing

signal complex (DISC), where they proteolytically auto-activate themselves and initiate apoptosis by subsequent cleavage of the downstream effector capspases -3, -6 and -7[18]. Two types of CD95 signaling have been established. Type I cells are characterized by high levels of DISC formation generating high amounts of active caspase-8, which directly activates downstream effector caspases[19]. In type II cells, there are lower levels of CD95 DISC formation and lower levels of active caspase-8. In this case, effector caspase activation requires an additional amplification loop that involves the cleavage of the Bcl-2-family protein Bid by caspase-8 to generate truncated (t) Bid. Subsequently, tBid mediates the release of the apoptosis initiating factors cytochrome-c and second mitochondria-derived activator of caspases (SMAC/DIABLO). Cytochrome-c drives the formation of the caspase-9-activating apoptosome-complex[19]. Active caspase-9 activates the executioner caspase-3, which in turn activates caspase-8 outside the DISC, thereby completing a positive feedback loop (Figure1) [19,20]. Signaling downstream of TRAILR1/R2 receptors is similar to CD95 signaling[21].

Death receptors and cancer

Several characteristics render DR agonists attractive from a therapeutic perspective. First, although DRs are expressed on a wide variety of normal and tumor cell types, there is evidence that DR ligands preferentially induce apoptosis in tumor cells[22]. Second, the capacity of DR agonists to induce apoptosis is independent of the tumor suppressor p53. Because p53 is frequently inactivated in human tumors, DR agonists may have the ability to induce apoptosis in tumors that have acquired either full or partial resistance to chemotherapy[23,24].

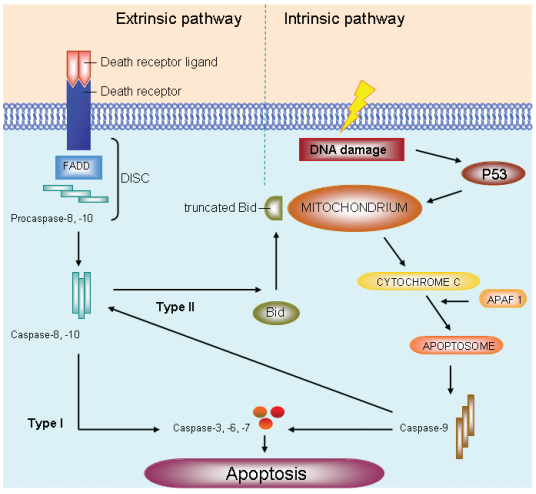

Figure 1. Model of death receptor signaling ipv Model of CD95 signaling

Third, because of the relationship between the extrinsic and intrinsic apoptotic pathways, DR agonists may display enhanced activity when combined with a variety of conventional chemotherapeutics and targeted therapeutics antagonizing cell growth and survival pathways. Currently, clinical trials are performed in which the safety and efficacy of TRAIL and agonistic antibodies to TRAIL-R1 and TRAIL-R2 as novel anti-cancer drugs are being evaluated.[7,25,26]. Unfortunately, CD95L causes severe liver toxicity due to the high presence of CD95 on hepatocytes, which limits its use in anti-tumor therapy[27]. The safety of megaFasL (APO010), a hexameric version of CD95L/FasL with reduced hepatotoxicity is currently being tested in a phase 1 clinical trial (www.clinicaltrials.gov).

Although death receptors are best known for their ability to induce apoptosis and thereby limit tumor formation, accumulating evidence has firmly established that death receptors and their ligands can also act in a pro-tumorigenic fashion by stimulating tumor cell proliferation, motility and invasion[28-34]. For a detailed review about CD95 in colorectal cancer see chapter 2.

Death receptors and metastasis

Death receptors play an important role in the anti-tumor immune surveillance and various studies point to a strong anti-metastatic effect of DR signaling[35-40]. CD95L is expressed in T lymphocytes, such as cytotoxic T cells (CTLs), in tumor-infiltrating T cells (TILs) and in Natural Killer (NK) cells ('pit cells') and macrophages (Kuppfer cells), which are found in the liver[41,42]. These cells of the local immune system encounter and attack tumor cells while sparing normal cells. In order to metastasize to the liver, tumor cells have to escape this first line of defense. During cancer progression CD95 is frequently downregulated, which raises the possibility that loss of CD95 may contribute to tumor immune evasion[31,43]. Moreover, *in vivo* studies have demonstrated that tumor progression and metastasis formation in different types of tumors requires resistance of tumor cells to CD95-induced apoptosis[44-48].

However, complete loss of CD95 is rarely seen in human cancers and many cancer cells still express considerable levels of CD95[31,49]. Furthermore, elevated levels of CD95L are frequently seen in various cancer patients and are associated with metastasis formation, possibly by eliminating tumor-infiltrating immune cells, and/or facilitating tissue destruction during tumor invasion[50,51]. High levels of CD95L in the circulation are related to poor prognosis in a variety of cancers[52,53]. The pro-inflammatory role of CD95L may also contribute to metastasis formation by creating a microenvironment favourable for outgrowth of tumor cells[54,55]. Recently, it has been demonstrated that CD95 acts as a tumor promoter *in vivo* for glioblastoma, lung, ovarian and liver cancer[30,49,56]. Taken together, these studies suggest that, at least under some circumstances, death receptors act in a pro-metastatic fashion.

Metastasis in colorectal cancer: Formation

Colorectal carcinoma (CRC) poses a serious threat to public health as it is one of the most common malignancies in the Western world with over one million new cases each year[57]. Death from this disease is primarily due to the consequences of the formation of liver metastases. They develop in approximately 60% of CRC patients[58,59].

The formation of distant metastases is a complex multistage process[60,61]. First, the interaction between tumor cells within a solid tumor mass needs to be disrupted. Detached cells encounter a tumor-surrounding basement membrane which they have to degrade to disseminate from the primary tumor. Then the tumor cells invade the local stroma to gain access to the blood and/or lymphatic vessels[62]. After migration through the endothelial cell layer, tumor cells enter the circulation (intravasation)[63,64]. Tumor cells disseminated from colorectal cancer travel through the gut-draining mesenteric veins and enter the liver via the portal system[65]. Within the sinusoids of the liver, tumor cells encounter cells of the local immune system (i.e. NK cells). These cytotoxic lymphocytes kill their target cells via two distinct pathways. First, the perforin/granzyme pathway in which perforin promotes 'perforation' of the target cell membrane which enables transfer of apoptosis-inducing granzymes to the target cells. Second, the death receptor pathway in which apoptosis is induced through cytokines like TNFα, TRAIL and CD95L which specifically bind to the death receptors on the target cells[66]. Tumor cells have to evade these defense mechanisms in order to form metastases. Next, tumor cells need to migrate through the sinusoidal endothelial layer and degrade the basement membrane before reaching the liver parenchyma[67]. Further outgrowth of the tumor cells requires the formation of new blood vessels, providing nutrients and oxygen[68].

The current dogma is that colorectal tumors are hierarchically organized tissues in which a tumorigenic 'cancer stem cell' compartment generates differentiated offspring with reduced tumorigenic capacity. Cancer stem cells (CSCs) are defined by their ability to initiate new tumors, their ability to self-renew and their ability to generate differentiated tumor cell types. The cancer stem cell model thus implies that CSCs are the most likely candidates to initiate metastasis and, therefore, could be the most relevant targets for cancer therapy[69]. CSCs are generally believed to be intrinsically resistant to apoptotic stimuli, possibly due to inheritance of the self-maintenance properties of the stem cells from which they originated. Currently, differentiation of cancer stem cells is considered as anti-metastatic therapy[70].

Metastasis in colorectal cancer: Treatment and recurrence

Patients with colorectal liver metastases have a poor prognosis when left untreated, with a median survival between 3 and 12 months[71-73]. Despite different new chemotherapeutic approaches, surgical resection is still the only curative treatment option showing overall

5-year survival rates of 36-58%[74,75]. Unfortunately, not all patients are eligible for partial liver resection. Radiofrequency ablation (RFA) offers an alternative treatment option for patients with colorectal liver metastases which cannot be resected. RFA involves induction of heat coagulation to eradicate tumor tissue. The electrodes placed within the tumor tissue induce alternating electrical currents in the range of radiofrequency waves. These currents result in friction heat around the electrode due to ion movement which destroys the cancer cells. Cell death is eventually the result of coagulation necrosis, the irreversible thermal damage of tissue proteins at temperatures over 55-60°C[76,77].

Surgery can offer cure in patients with colorectal liver metastases. However, during the first two years following surgery, tumor recurrence occurs in approximately 60-70% of cases, both following surgical resection and RFA[78-80]. Recurrence may either develop from circulating tumor cells possibly induced by the surgical procedure[81-83] or from residual tumor deposits remaining after surgery[74,75,84-86]. Furthermore, occult hepatic micrometastases can be found in 26-70% of patients with colorectal liver metastases[87-90]. These micrometastases can exist within the liver tissue in a dormant state for years[91,92]. A balance between apoptosis and proliferation in combination with the suppression of angiogenesis may result in non-growing micrometastases[91,93]. In addition, the adaptive immune system can hold micrometastases in a dormant state[94]. Surgery may be an event which disturbs this balance, resulting in outgrowth of local pre-existing micrometastases.

Vascular clamping of the hepatic inflow during liver surgery is often applied during both surgical interventions, either to control blood loss (partial liver resection) or to increase lesion size (RFA)[95-97]. Vascular clamping can cause ischemia-reperfusion (I/R) injury to the liver. I/R not only causes tissue injury, but also has a growth-stimulatory effect on residual tumor tissue in the liver[98-100]. Moreover, accelerated outgrowth of micrometastases following I/R injury is related to chronic tissue hypoxia[101]. Hypoxia can promote tumor cell survival by apoptosis resistance as a result of adaptive changes in tumor cells[102]. Furthermore, hypoxia can stimulate invasion and migration of tumor cells[103,104]. In this thesis, a possible role for death receptors in liver surgery-stimulated outgrowth of colorectal micrometastases is investigated.

Metastasis control by the KRAS oncogene

The KRAS oncogene is one of the most frequently mutated oncogenes in human cancer with a prevalence of approximately 35-45% in colorectal cancer[105,106]. The Ras family members (K-Ras, N-Ras, H-Ras) are guanine nucleotide (GDP/GTP)-binding proteins that act as self-inactivating signal transducers, located at the inner surface of the plasma membrane. The Ras proto-oncogenes control transduction of extracellular signals to intracellular effector pathways required for differentiation, proliferation and survival. Normal Ras proteins cycle between active GTP-bound state and inactive GDP-bound state. They are activated by guanine

exchange factors (GEF) that promote the exchange of GDP for GTP. GTPase-activating proteins (GAP) that stimulate GTP hydrolysis terminate signaling. Ras oncogenes harbour point mutations that result in amino acid substitutions (commonly in codons 12, 13 or 61) generating proteins with strongly reduced GTPases activity. Mutant Ras proteins are thereby locked in a constitutively activated GTP-bound state, thus favouring the constant transmission of a positive signal for cell growth[107]. Activating mutations in the KRAS oncogene contribute to the formation of colorectal tumors. However, much less is known about the impact of these mutations on metastasis formation and resistance to treatment. It is known that Ras signaling can stimulate cell migration and invasion, reduce sensitivity to anoikis (detachment cell death) and modulates death receptor signaling[61,108-110]. The majority of the reports however, use overexpression models of (K)RAS. To what extent endogenous oncogenic KRAS controls these phenomena in colorectal tumor cells during metastasis formation is studied in this thesis.

OUTLINE OF THE THESIS

Accumulating evidence suggests that death receptors can have pro-tumorigenic activity. In this thesis, the role of death receptors in the development and outgrowth of colorectal liver metastases was investigated.

First, the role of CD95 in colorectal cancer is evaluated in **chapter 2**. We give an overview of the existing literature of both experimental (*in vitro* and *in vivo*) and clinical studies addressing this issue and we focus on both the apoptotic as well as the non-apoptotic activities of CD95.

In **chapter 3**, we set out to determine the role of oncogenic K-Ras in metastasis formation by colorectal tumor cells in the liver, since almost half of the primary colon carcinoma cells harbour mutations in the K-Ras oncogene, but relatively little is known about K-Ras in metastases. In this chapter, we study how K-Ras alters death receptor function on colon carcinoma cells in the hepatic micro-environment.

In the ensuing chapters we focus on how surgery affects residual tumor tissue through death receptor signaling. Hypoxia is known to play an important role in ischemia/reperfusion-induced outgrowth of colorectal liver metastases. Since hypoxia can induce rapid adaptations in tumor cells, we study the early effects of liver surgery on tumor cells and provide mechanistic insight into how surgery-induced hypoxia affects the behaviour of micrometastases in the liver. For this purpose, a murine model of colorectal micrometastases in the liver is used. Two different surgical techniques are used to assess the effects of surgery on micrometastatic

outgrowth: radiofrequency ablation (RFA) in **chapter 4** and ischemia-reperfusion (I/R) injury due to vascular clamping of the hepatic inflow in **chapter 5**.

Only a subgroup of patients with synchronous colorectal liver metastases benefits from liver surgery. Currently, there are no reliable tools to identify such patients. In **chapter 6** we evaluate the potential of circulating CD95L levels to serve as a prognostic factor for disease-free survival following surgery in patients with synchronous liver metastases.

Finally, cancer stem cells are generally believed to be apoptosis resistant to apoptotic stimuli but the relationship between apoptosis resistance and differentiation status has not been addressed. In **chapter 7**, we set out to asses whether differentiation status affects colorectal tumor cell sensitivity to CD95L.

REFERENCES

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-257.
2. Vogt C. Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte (Alytes obstetricians). Jent & Gassmann, Solothurn. 1842.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.
4. Rowinsky EK. Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. J Clin Oncol 2005;23:9394-9407.
5. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N Engl J Med 2009;361:1570-1583.
6. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277-288.
7. Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat Clin Pract Oncol 2006;3:388-398.
8. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456-1462.
9. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312-1316.
10. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-1312.
11. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.
12. French LE, Tschopp J. Protein-based therapeutic approaches targeting death receptors. Cell Death Differ 2003;10:117-123.
13. Nagata S. Apoptosis by death factor. Cell 1997;88:355-365.
14. Smith CA, Farrah T, Goodwin RG. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 1994;76:959-962.
15. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell 1993;74:845-853.
16. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:233-243.
17. Lavrik I, Krueger A, Schmitz I, Baumann S, Weyd H, Krammer PH, Kirchhoff S. The active caspase-8 heterotetramer is formed at the CD95 DISC. Cell Death Differ 2003;10:144-145.
18. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26-35.
19. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17:1675-1687.
20. geciras-Schimnich A, Pietras EM, Barnhart BC, Legembre P, Vijayan S, Holbeck SL, Peter ME. Two CD95 tumor classes with different sensitivities to antitumor drugs. Proc Natl Acad Sci U S A 2003;100:11445-11450.
21. Lavrik I, Golks A, Krammer PH. Death receptor signaling. J Cell Sci 2005;118:265-267.
22. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155-162.
23. Ashkenazi A, Herbst RS. To kill a tumor cell: the potential of proapoptotic receptor agonists. J Clin Invest 2008;118:1979-1990.
24. Ravi R, Jain AJ, Schulick RD, Pham V, Prouser TS, Allen H, Mayer EG, Yu H, Pardoll DM, Ashkenazi A, Bedi A. Elimination of hepatic metastases of colon cancer cells via p53-independent cross-talk between irinotecan and Apo2 ligand/TRAIL. Cancer Res 2004;64:9105-9114.
25. Papenfuss K, Cordier SM, Walczak H. Death receptors as targets for anti-cancer therapy. J Cell Mol Med 2008;12:2566-2585.
26. Duiker EW, Mom CH, de JS, Willemse PH, Gietema JA, van der Zee AG, de Vries EG. The clinical trail of TRAIL. Eur J Cancer 2006;42:2233-2240.
27. Tanaka M, Suda T, Yatomi T, Nakamura N, Nagata S. Lethal effect of recombinant human Fas ligand in mice pretreated with Propionibacterium acnes. J Immunol 1997;158:2303-2309.

28. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. Immunity 2009;30:180-192.
29. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J 2004;23:3175-3185.
30. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, Thiemann M, Mueller W, Sykora J, Kuhn A, Schreglmann N, Letellier E, Zuliani C, Klussmann S, Teodorczyk M, Grone HJ, Ganten TM, Sultmann H, Tuttenberg J, von DA, Regnier-Vigouroux A, Herold-Mende C, Martin-Villalba A. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 2008;13:235-248.
31. Peter ME, Legembre P, Barnhart BC. Does CD95 have tumor promoting activities? Biochim Biophys Acta 2005;1755:25-36.
32. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zornig M, Lynch DH. The CD95 receptor: apoptosis revisited. Cell 2007;129:447-450.
33. Trauzold A, Roder C, Sipos B, Karsten K, Arlt A, Jiang P, Martin-Subero JI, Siegmund D, Muerkoster S, Pagerols-Raluy L, Siebert R, Wajant H, Kalthoff H. CD95 and TRAF2 promote invasiveness of pancreatic cancer cells. FASEB J 2005;19:620-622.
34. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D, Emme D, Roder C, Kalthoff H, Wajant H. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006;25:7434-7439.
35. Van Geelen CM, de Vries EG, de JS. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. Drug Resist Updat 2004;7:345-358.
36. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999;5:157-163.
37. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ, Walczak H. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J Clin Invest 2008;118:100-110.
38. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol 2002;168:1356-1361.
39. Seki N, Hayakawa Y, Brooks AD, Wine J, Wiltrout RH, Yagita H, Tanner JE, Smyth MJ, Sayers TJ. Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. Cancer Res 2003;63:207-213.
40. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, Okumura K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94-100.
41. Kenna T, Golden-Mason L, Norris S, Hegarty JE, O'Farrelly C, Doherty DG. Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver. Clin Immunol 2004;113:56-63.
42. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses. Immunol Rev 2000;174:35-46.
43. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. Oncogene 2004;23:2950-2966.
44. Liu K, Abrams SI. Alterations in Fas expression are characteristic of, but not solely responsible for, enhanced metastatic competence. J Immunol 2003;170:5973-5980.
45. Maecker HL, Yun Z, Maecker HT, Giaccia AJ. Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. Cancer Cell 2002;2:139-148.
46. Medema JP, de JJ, van HT, Melief CJ, Offringa R. Immune escape of tumors *in vivo* by expression of cellular FLICE-inhibitory protein. J Exp Med 1999;190:1033-1038.
47. Owen-Schaub LB, van Golen KL, Hill LL, Price JE. Fas and Fas ligand interactions suppress melanoma lung metastasis. J Exp Med 1998;188:1717-1723.

48. Owen-Schaub LB. Fas function and tumor progression: use it and lose it. Cancer Cell 2002;2:95-96.
49. Chen L, Park SM, Tumanov AV, Hau A, Sawada K, Feig C, Turner JR, Fu YX, Romero IL, Lengyel E, Peter ME. CD95 promotes tumour growth. Nature 2010;465:492-496.
50. Li Q, Peng J, Li XH, Liu T, Liang QC, Zhang GY. Clinical significance of Fas and FasL protein expression in gastric carcinoma and local lymph node tissues. World J Gastroenterol 2010;16:1274-1278.
51. Mullauer L, Mosberger I, Grusch M, Rudas M, Chott A. Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. J Pathol 2000;190:20-30.
52. Mizutani Y, Hongo F, Sato N, Ogawa O, Yoshida O, Miki T. Significance of serum soluble Fas ligand in patients with bladder carcinoma. Cancer 2001;92:287-293.
53. Tsutsumi S, Kuwano H, Shimura T, Morinaga N, Mochiki E, Asao T. Circulating soluble Fas ligand in patients with gastric carcinoma. Cancer 2000;89:2560-2564.
54. Whiteside TL. The role of death receptor ligands in shaping tumor microenvironment. Immunol Invest 2007;36:25-46.
55. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;27:5904-5912.
56. Lee JK, Sayers TJ, Back TC, Wigginton JM, Wiltrout RH. Lack of FasL-mediated killing leads to *in vivo* tumor promotion in mouse Lewis lung cancer. Apoptosis 2003;8:151-160.
57. American Cancer Society. Cancer Facts & Figures 2008. Atlanta: American Cancer Society 2008. 2010.
58. Christoforidis D, Martinet O, Lejeune FJ, Mosimann F. Isolated liver perfusion for non-resectable liver tumours: a review. Eur J Surg Oncol 2002;28:875-890.
59. Steele G, Jr., Ravikumar TS. Resection of hepatic metastases from colorectal cancer. Biologic perspective. Ann Surg 1989;210:127-138.
60. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 2003;3:453-458.
61. Smakman N, Borel R, I, Voest EE, Kranenburg O. Control of colorectal metastasis formation by K-Ras. Biochim Biophys Acta 2005;1756:103-114.
62. Liotta LA. Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. Cancer Res 1986;46:1-7.
63. Fisher B, Fisher ER. The interrelationship of hematogenous and lymphatic tumor cell dissemination. Surg Gynecol Obstet 1966;122:791-798.
64. Fisher ER, Fisher B. Recent observations on concepts of metastasis. Arch Pathol 1967;83:321-324.
65. Welch JP, Donaldson GA. The clinical correlation of an autopsy study of recurrent colorectal cancer. Ann Surg 1979;189:496-502.
66. Janssen EM, Lemmens EE, Gour N, Reboulet RA, Green DR, Schoenberger SP, Pinkoski MJ. Distinct roles of cytolytic effector molecules for antigen-restricted killing by CTL *in vivo*. Immunol Cell Biol 2010.
67. Nicolson GL. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim Biophys Acta 1988;948:175-224.
68. Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. Cancer Res 1986;46:467-473.
69. Todaro M, Perez AM, Scopelliti A, Medema JP, Stassi G. IL-4-mediated drug resistance in colon cancer stem cells. Cell Cycle 2008;7:309-313.
70. Bertrand J, Begaud-Grimaud G, Bessette B, Verdier M, Battu S, Jauberteau MO. Cancer stem cells from human glioma cell line are resistant to Fas-induced apoptosis. Int J Oncol 2009;34:717-727.
71. Bengmark S, Hafstrom L. The natural history of primary and secondary malignant tumors of the liver. I. The prognosis for patients with hepatic metastases from colonic and rectal carcinoma by laparotomy. Cancer 1969;23:198-202.
72. Bengtsson G, Carlsson G, Hafstrom L, Jonsson PE. Natural history of patients with untreated liver metastases from colorectal cancer. Am J Surg 1981;141:586-589.
73. de Brauw LM, van d, V, Bouwhuis-Hoogerwerf ML, Zwaveling A. Diagnostic evaluation and survival analysis of colorectal cancer patients with liver metastases. J Surg Oncol 1987;34:81-86.

74. Choti MA, Sitzmann JV, Tiburi MF, Sumetchotimetha W, Rangsin R, Schulick RD, Lillemoe KD, Yeo CJ, Cameron JL. Trends in long-term survival following liver resection for hepatic colorectal metastases. Ann Surg 2002;235:759-766.
75. Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg 1999;230:309-318.
76. Heisterkamp J, van HR, Ijzermans JN. Interstitial laser coagulation for hepatic tumours. Br J Surg 1999;86:293-304.
77. Nikfarjam M, Muralidharan V, Christophi C. Mechanisms of focal heat destruction of liver tumors. J Surg Res 2005;127:208-223.
78. de Jong MC, Pulitano C, Ribero D, Strub J, Mentha G, Schulick RD, Choti MA, Aldrighetti L, Capussotti L, Pawlik TM. Rates and patterns of recurrence following curative intent surgery for colorectal liver metastasis: an international multi-institutional analysis of 1669 patients. Ann Surg 2009;250:440-448.
79. Simmonds PC, Primrose JN, Colquitt JL, Garden OJ, Poston GJ, Rees M. Surgical resection of hepatic metastases from colorectal cancer: a systematic review of published studies. Br J Cancer 2006;94:982-999.
80. Kosari K, Gomes M, Hunter D, Hess DJ, Greeno E, Sielaff TD. Local, intrahepatic, and systemic recurrence patterns after radiofrequency ablation of hepatic malignancies. J Gastrointest Surg 2002;6:255-263.
81. Fruhauf NR, Kasimir-Bauer S, Gorlinger K, Lang H, Kaudel CP, Kaiser GM, Oldhafer KJ, Broelsch CE. Peri-operative filtration of disseminated cytokeratin positive cells in patients with colorectal liver metastasis. Langenbecks Arch Surg 2005;390:15-20.
82. Koch M, Kienle P, Hinz U, Antolovic D, Schmidt J, Herfarth C, von Knebel DM, Weitz J. Detection of hematogenous tumor cell dissemination predicts tumor relapse in patients undergoing surgical resection of colorectal liver metastases. Ann Surg 2005;241:199-205.
83. Topal B, Aerts JL, Roskams T, Fieuws S, Van PJ, Vandekerckhove P, Penninckx F. Cancer cell dissemination during curative surgery for colorectal liver metastases. Eur J Surg Oncol 2005;31:506-511.
84. Nuzzo G, Giuliante F, Ardito F, Vellone M, Giovannini I, Federico B, Vecchio FM. Influence of surgical margin on type of recurrence after liver resection for colorectal metastases: a single-center experience. Surgery 2008;143:384-393.
85. Rees M, Tekkis PP, Welsh FK, O'Rourke T, John TG. Evaluation of long-term survival after hepatic resection for metastatic colorectal cancer: a multifactorial model of 929 patients. Ann Surg 2008;247:125-135.
86. Wei AC, Greig PD, Grant D, Taylor B, Langer B, Gallinger S. Survival after hepatic resection for colorectal metastases: a 10-year experience. Ann Surg Oncol 2006;13:668-676.
87. Linnemann U, Schimanski CC, Gebhardt C, Berger MR. Prognostic value of disseminated colorectal tumor cells in the liver: results of follow-up examinations. Int J Colorectal Dis 2004;19:380-386.
88. Nanko M, Shimada H, Yamaoka H, Tanaka K, Masui H, Matsuo K, Ike H, Oki S, Hara M. Micrometastatic colorectal cancer lesions in the liver. Surg Today 1998;28:707-713.
89. Wakai T, Shirai Y, Sakata J, Valera VA, Korita PV, Akazawa K, Ajioka Y, Hatakeyama K. Appraisal of 1 cm hepatectomy margins for intrahepatic micrometastases in patients with colorectal carcinoma liver metastasis. Ann Surg Oncol 2008;15:2472-2481.
90. Yokoyama N, Shirai Y, Ajioka Y, Nagakura S, Suda T, Hatakeyama K. Immunohistochemically detected hepatic micrometastases predict a high risk of intrahepatic recurrence after resection of colorectal carcinoma liver metastases. Cancer 2002;94:1642-1647.
91. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1995;1:149-153.
92. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol 1998;153:865-873.
93. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315-328.

94. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903-907.
95. Ritz JP, Lehmann KS, Isbert C, Reissfelder C, Albrecht T, Stein T, Buhr HJ. In-vivo evaluation of a novel bipolar radiofrequency device for interstitial thermotherapy of liver tumors during normal and interrupted hepatic perfusion. J Surg Res 2006;133:176-184.
96. Smyrniotis V, Farantos C, Kostopanagiotou G, Arkadopoulos N. Vascular control during hepatectomy: review of methods and results. World J Surg 2005;29:1384-1396.
97. Wiersinga WJ, Jansen MC, Straatsburg IH, Davids PH, Klaase JM, Gouma DJ, van Gulik TM. Lesion progression with time and the effect of vascular occlusion following radiofrequency ablation of the liver. Br J Surg 2003;90:306-312.
98. Man K, Ng KT, Lo CM, Ho JW, Sun BS, Sun CK, Lee TK, Poon RT, Fan ST. Ischemia-reperfusion of small liver remnant promotes liver tumor growth and metastases--activation of cell invasion and migration pathways. Liver Transpl 2007;13:1669-1677.
99. Nicoud IB, Jones CM, Pierce JM, Earl TM, Matrisian LM, Chari RS, Gorden DL. Warm hepatic ischemia-reperfusion promotes growth of colorectal carcinoma micrometastases in mouse liver via matrix metalloproteinase-9 induction. Cancer Res 2007;67:2720-2728.
100. van der Bilt JD, Kranenburg O, Nijkamp MW, Smakman N, Veenendaal LM, Te Velde EA, Voest EE, van Diest PJ, Borel R, I. Ischemia/reperfusion accelerates the outgrowth of hepatic micrometastases in a highly standardized murine model. Hepatology 2005;42:165-175.
101. van der Bilt JD, Soeters ME, Duyverman AM, Nijkamp MW, Witteveen PO, van Diest PJ, Kranenburg O, Borel R, I. Perinecrotic hypoxia contributes to ischemia/reperfusion-accelerated outgrowth of colorectal micrometastases. Am J Pathol 2007;170:1379-1388.
102. Dong Z, Nishiyama J, Yi X, Venkatachalam MA, Denton M, Gu S, Li S, Qiang M. Gene promoter of apoptosis inhibitory protein IAP2: identification of enhancer elements and activation by severe hypoxia. Biochem J 2002;364:413-421.
103. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38-47.
104. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721-732.
105. Loupakis F, Pollina L, Stasi I, Ruzzo A, Scartozzi M, Santini D, Masi G, Graziano F, Cremolini C, Rulli E, Canestrari E, Funel N, Schiavon G, Petrini I, Magnani M, Tonini G, Campani D, Floriani I, Cascinu S, Falcone A. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. J Clin Oncol 2009;27:2622-2629.
106. Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, Dietrich D, Biesmans B, Bodoky G, Barone C, Aranda E, Nordlinger B, Cisar L, Labianca R, Cunningham D, Van CE, Bosman F. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 2010;28:466-474.
107. Kranenburg O. The KRAS oncogene: past, present, and future. Biochim Biophys Acta 2005;1756:81-82.
108. Drosopoulos KG, Roberts ML, Cermak L, Sasazuki T, Shirasawa S, Andera L, Pintzas A. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. J Biol Chem 2005;280:22856-22867.
109. Kazama H, Yonehara S. Oncogenic K-Ras and basic fibroblast growth factor prevent Fas-mediated apoptosis in fibroblasts through activation of mitogen-activated protein kinase. J Cell Biol 2000;148:557-566.
110. Rosen K, Rak J, Leung T, Dean NM, Kerbel RS, Filmus J. Activated Ras prevents downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. J Cell Biol 2000;149:447-456.

CHAPTER 2

CD95 IN COLORECTAL CANCER: FRIEND OR FOE?

Review article

Frederik J.H. Hoogwater
Ernst J.A. Steller
B. Florien Westendorp
Inne H.M. Borel Rinkes
Onno Kranenburg

Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands

INTRODUCTION

CD95 and TRAIL-receptors belong to the family of death receptors. This receptor system controls development, differentiation, inflammation and immunity in vertebrate species[1]. Death receptors derive their name from a shared ability to induce programmed cell death (apoptosis) upon binding their respective ligands. The signal transduction pathways that cause death receptor-induced apoptosis have largely been elucidated[2]. In short, clusters of ligand-activated death receptors are assembly sites for the death-inducing signaling complex (DISC) which contains adaptor molecules (FADD) and the initiator caspases-8 and/or -10. While caspase-8/10 activation in the DISC is sufficient to stimulate apoptosis in some cell types, others require amplification of the signal via the mitochondrial activation of caspase-9[2]. Activation of the executioner caspase-3 by caspase-8/10 and caspase-9 in most cells represents a point of no return after which cell death rapidly ensues (See also Figure 1, Chapter 1).
The finding that many tumors, including those of colorectal origin, express functional death receptors has raised enormous interest in exploiting them as targets for anti-cancer therapy, aiming to induce tumor cell apoptosis[3]. Although several death receptor agonists are in clinical development, clinical responses have so far been minimal[3,4].
Here we set out to evaluate the role of CD95 in colorectal cancer. We give an overview of existing literature of both experimental and clinical studies on this topic and we focus on apoptotic as well as non-apoptotic functions of CD95.

CD95 AND CD95 LIGAND (CD95L) EXPRESSION PATTERNS

CD95 is ubiquitously expressed in various tissues including the intestines, liver, heart, kidney, pancreas, brain, thymus, lymphoid tissues and activated mature T lymphocytes. It is mainly localized to the plasma membrane, although alternative mRNA-splicing can result in soluble forms of the receptor lacking the transmembrane domain[5]. These soluble forms of the receptor appear to have a negative regulatory effect on membrane-bound CD95 *in vitro*, most likely due to a competitive binding to CD95L[6]. CD95L is mainly expressed in preassociated homotrimeric structures on the plasma membrane of activated T cells. Membrane-bound CD95L (mCD95L) can be cleaved by metalloproteinases in its extracellular domain, generating a soluble form (sCD95L) which can be detected in the circulation[7]. Only mCD95L is able to induce apoptosis[8].

In the normal intestine, during colitis and during colorectal cancer development

CD95 is uniformly expressed at the basolateral cell surface in all epithelial cells of the colon epithelium, but expression of its ligand is confined to the differentiated Paneth cells[9-11]. Intestinal epithelial cells divide at the base of the crypts, migrate up along the crypt-villus

axis and are eliminated by apoptosis at the luminal surface epithelium of the colon[12]. Regardless of their position along the crypt(villus) axis, epithelial cells are equally sensitive to CD95-induced apoptosis *in vitro*[11]. Furthermore, mice lacking either CD95 or functional CD95 ligand display normal intestinal morphology and apoptotic cell count[11]. Together these findings suggest that mechanisms other than the CD95/CD95L system control the physiological turnover of the intestinal epithelium. CD95 is abundantly expressed in intestinal epithelial cells and epithelial apoptosis is increased in inflammatory bowel disease (IBD; i.e. colitis)[13,14]. Therefore, the CD95/CD95L system could play a role in the etiology of IBD. However, mice lacking CD95 activity in the colon were hypersensitive to colitis, rather than protected. This suggests that CD95 does not contribute to intestinal epithelial cell loss during IBD and may even stimulate survival pathways rather than apoptosis[15]. CD95L is implicated in the maintenance of immune privilege, particularly in the testis and the chamber of the eye, by inducing apoptosis of activated infiltrating T lymphocytes[16,17]. By expressing high levels of CD95L tumors might develop as immune privileged sites[17]. However, this theory has been disputed, since animal studies failed to demonstrate that CD95L overexpression in tumors generates immune privilege[18]. During colorectal tumorigenesis the expression of CD95 is diminished or lost in a subpopulation of tumors[19,20], but the expression of CD95L is increased[21-23]. In fact, expression of CD95L is higher in metastases than in matched primary tumors and is related to poor prognosis[22,24]. In addition, our own results show that high levels of circulating CD95L predict early tumor recurrence following surgery (Chapter 6).

CD95 AND CD95L EXPRESSION AND ASSOCIATION WITH PROGNOSIS IN CRC

Several studies in mice indicate that resistance to CD95 induced apoptosis of tumor cells is associated with increased local growth and a more aggressive phenotype[25-29]. However, studies correlating CD95 expression with clinical outcome of patients suffering from large bowel cancer are lacking[30]. Recently, it was shown that impaired CD95 expression predisposes for recurrence in curatively resected colon carcinoma providing clinical evidence for immunoselection and CD95L mediated control of minimal residual disease[30]. This association was maintained in multivariate analysis suggesting that CD95 expression is an independent prognostic factor in colon carcinoma. CD95 expression was previously shown to be associated with carcinoembryonic antigen doubling time (CEA, a widely used tumor marker to identify recurrences after surgical resection) in patients undergoing resection for colorectal metastases[31].There are other mechanisms than reduced CD95 expression that may contribute to apoptosis resistance in colon carcinoma cells. For example, loss of caspase-8 activity by inactivating mutations or by high expression of cFLIP[32,33]. Surprisingly, in a recent paper it was shown that high levels of caspase-8 and caspase-9 were found to be associated with poor prognosis in stage II/III colon carcinoma[34].

Accumulating evidence suggests a tumor-propagating role for (s)CD95L in malignancies including colorectal cancer[8,21,35]. Elevated serum sCD95L concentrations correlate with poor prognosis in large granular lymphocytic leukaemia, NK lymphoma, bladder carcinoma, gastric carcinoma, hepatocellular carcinoma and breast carcinoma[36-42]. In line with these reports we have shown a correlation between elevated serum sCD95L levels and poor DFS in colorectal cancer (Chapter 6). Conversely, in squamous cell carcinoma of the oesophagus, sCD95L levels had no significant prognostic effect on DFS, suggesting that the value of sCD95L measurements may be tumor-specific[43].

CD95 AS A SUPPRESSOR OF COLORECTAL TUMOR GROWTH AND METASTASIS FORMATION

CD95/apoptosis sensitivity in colorectal cancer cell lines

CD95L (and TRAIL) can trigger apoptosis in a many types of different cells, including a variety of colorectal tumor cells[44-49]. However, many colon tumor derived cell lines show loss of sensitivity to CD95L-mediated apoptosis *in vitro*[35,50,51]. A decreased CD95L sensitivity of coloncytes to CD95L-induced apoptosis during malignant transformation may have multiple causes, including downregulation of receptor expression and overexpression of Bcl-2 and/or FLICE inhibitory protein (FLIP)[33,50,51].

Studies in mice have shown that tumor progression and metastatic spread of different types of tumors requires tumor cells to escape from CD95-induced apoptosis[26-29,52]. Taken together, the emerging picture is one in which tumor cells have to escape CD95-mediated apoptosis in order to metastasize and some do so by downregulating CD95.

Recruitment of tumoricidal neutrophils by CD95L

It is well recognized that CD95L is a potent chemoattractant for neutrophils *in vitro* and *in vivo* and that ligation of CD95 on neutrophils results in their attraction and activation[53-56]. When pancreatic islets or islet cells engineered to express CD95L on their surface were transplanted into mice, islet cells were rejected even more rapidly than untransfected controls due to an invasion of granulocytes[57,58]. Moreover, CD95L overexpression by tumors is associated with their *in vivo* eradication involving neutrophils[59-61]. In addition, CD95-resistant colon tumor cells C26 (CT26), which were subcutaneously injected in mice, rapidly regressed when infected with an adenoviral vector encoding CD95L due to infiltration of the tumor by tumoricidal neutrophils[62].

Stimulation of anti-tumor immunity by CD95L

The CD95/CD95L system has been implicated in the suppression and stimulation of immune responses and anti-tumorigenic effects by inflammatory cells have been reported[63].

Neutrophils attracted by CD95L may impair tumor cells not only through direct cytotoxic effects, but also by promoting the development of $CD8^+$ T cell-dependent antitumor immunity, leading to eradication of tumor cells[59-61,64]. Activation of CD95 on dendritic cells induces their maturation and promotes the secretion of chemokines and cytokines that attract neutrophils and T cells[65]. In addition, CD95 expressed on helper T cells is critical for generating a cytotoxic T lymphocyte (CTL) response[66]. These phenomena could help boost anti-tumor T cell responses, but their contribution to the generation of anti-tumor immunity in CD95L-expressing tumors is currently unknown.

Mouse models: the effect of CD95 or CD95L deficiency on intestinal lesion formation

A widely used system to study the involvement of CD95 signaling in disease states are mice lacking either functional CD95L (*gld/gld; generalized lymphoproliferative disease*), or lacking CD95 (*lpr/lpr; lymphoproliferation*)[67,68]. Both mouse strains are characterized by progressive lymphadenopathy, splenomegaly and autoimmune disease, at least in part due to defective elimination of activated T cells and autoreactive B cells[69]. Young *lpr* and *gld* mice (6-12 weeks) have been frequently used for studying the contribution of CD95 signaling to inflammatory disorders[70-73]. Such studies have demonstrated the pivotal role of CD95 signaling in establishing the inflammatory response following hepatocyte injury, either caused by acetominophen (APAP; a commonly used analgesic drug) or by concanavalin A (conA; glycoprotein stimulating T cell production)[71,74]. In addition, *gld* mice are also protected from dextran sodium sulfate (DSS)-induced inflammation of the colon and from silica-induced inflammation of the lungs[70,75]. By contrast, lpr^{cg} mice with defective CD95 in intestinal epithelial cells displayed hypersensitivity to DSS-induced colitis[15]. lpr^{cg} mice differ from the originally described *lpr* strain in that CD95 is normally expressed but signaling-deficient. Furthermore, CD95 signaling-deficient mice with a wild-type hematopoietic system were prone to hepatic neoplasia[76]. In addition, loss of functional CD95L enhanced intestinal tumorigenesis in *Min* mice, which have a mutation in the Apc tumor suppressor gene[75,77]. Membrane-bound CD95L is essential for cytotoxic activity and protects against lymphadenopathy, autoimmunity and cancer (via apoptosis), whereas soluble CD95L promotes autoimmunity and tumorigenesis of hystiocytic sarcoma in the liver with deposits in the spleen and lung through non-apoptotic activities[8]. Whether there is an effect on the intestines in mice lacking either sCD95L or mCD95L was not described.

CD95 AS A STIMULATOR OF COLORECTAL TUMOR GROWTH AND METASTASIS FORMATION

Several observations suggest that CD95 could play a pro-tumorigenic role during the metastatic progression of colorectal cancer (CRC). First, CD95L expression increases early during tumor development and CRC metastases often co-express CD95 and CD95L without undergoing apoptosis[17,22]. Second, CD95L is a strong pro-inflammatory cytokine, which attracts pro-tumorigenic inflammatory cells[78]. Third, work from our group has shown that CD95, under the influence of the common *KRAS* oncogene, stimulates tumor cell invasion rather than apoptosis[35] (Chapter 3). Fourth, following liver surgery, micrometastases in the remnant liver rapidly adopt a highly invasive phenotype and this depends on autocrine activation of CD95 on tumor cells[79] (Chapter 4). Fifth, continuous CD95 stimulation on colon carcinoma cells selects highly metastatic variants, which could not be explained solely by resistance to apoptosis[80]. Sixth, overexpression of CD95L in human and mouse colon cancer cells showed an increase in local tumor growth and enhancement of liver metastasis formation[81].

Apoptosis in tumor infiltrating lymphocytes (TIL) – "counterattack"

Activated T cells are highly sensitive to killing by CD95L which is important for T cell contraction during a normal T cell response. It has been proposed that CD95L-expressing tumors can kill tumor-infiltrating activated T cells in a CD95-dependent manner[23,82-84]. This has been referred to as the tumor "counterattack". However, the relevance of the CD95L-mediated counterattack *in vivo* is debated[18,85]. This is primarily based on results showing that over-expressed CD95L in tumor cells caused tumor rejection and development of anti-tumor immunity, rather than tolerance through induction of T cell apoptosis[53,61,85]. Recent data now suggest that tumor cell-produced CD95L acts as an autocrine and paracrine growth factor, rather than a death signal[13,86].

Apoptosis in hepatocytes

Injection of the CD95-activating antibody in mice causes severe hepatitis[87]. This is due to the exquisite sensitivity of hepatocytes to CD95-induced apoptosis[88]. CD95 also mediates hepatocyte apoptosis induced by concanavalinA and APAP, but not by ischemia/reperfusion[71,73,89]. Colorectal liver metastases express high levels of CD95L and may therefore induce apoptosis in nearby hepatocytes[22,90]. Indeed, apoptotic hepatocytes are frequently detected at the tumor margin of colorectal liver metastases. The inflammatory response at the margin of colorectal liver metastases may induce CD95 expression in surrounding hepatocytes, allowing them to be killed by CD95-L-bearing tumor infiltrating lymphocytes and/or tumor cells. A direct consequence of hepatocyte killing by invasive tumor cells could be that tumor invasion into the surrounding liver tissue is facilitated[90].

Attraction of pro-tumorigenic inflammatory cells

CD95 ligand is a potent inducer of inflammation by stimulating the expression and release of inflammatory factors such as interleukin 1β, interleukin 8, MCP1 (monocyte chemotactic protein-1), IP10 (interferon-gamma-induced protein 10), MIG (monokine induced interferon-gamma), keratinocyte-derived chemokine, MIP1α and 1β and MIP2 (macrophage inflammatory protein 2) from hepatocytes, monocytes and monocyte-derived macrophages[91-95]. These inflammatory cytokines and chemokines in turn promote influx and activation of neutrophils, which can subsequently promote tumor rejection[53,61,85,92-94]. However, influx of inflammatory cells into tumors can also generate a pro-tumorigenic microenvironment characterized by high levels of growth factors, pro-angiogenic factors and metalloproteinases[78]. Indeed, chronically inflamed tissue is a pre-condition for tumor development in several organs, including the liver and the intestines[96,97]. Interestingly, anti-CD95L antibodies prevented inflammation-associated hepatocarcinogenesis induced by the hepatitis B surface antigen[98]. In this model, anti-CD95L prevented both hepatocyte apoptosis and inflammation[98]. Thus, CD95L is a critical regulator of inflammation-associated tumor formation in the liver. Conversely, the related cytokine TRAIL has not been so clearly indicated as an inducer of pro-inflammatory cytokines. Moreover, signaling by TRAIL suppresses inflammation-associated tumor formation in the liver[99].

Non-apoptotic signaling modes in colorectal tumor cells and lessons from other cell types

Many tumor cells display intrinsic resistance to apoptosis induced by CD95 and/or TRAIL-receptors[45,100]. Tumor cell sensitivity (or resistance) to death receptor-induced apoptosis depends on multiple factors, including the levels of expression of pro-apoptotic proteins (death receptors, caspase-8, Bax) and anti-apoptotic proteins (decoy receptors, cFLIP, XIAP, Bcl-2) as well as the localization and post-translational modification of death receptors[45,100].

Apoptosis is only one of a variety of potential outcomes of death receptor activation on tumor cells. Alternative outcomes include proliferation, invasion, differentiation and cell survival [1,101,102]. In a recent landmark study it was shown that CD95 signaling is required for the maintenance of cell proliferation in tumor cell lines of diverse origin and for the initiation of liver tumors and endometroid tumors driven by oncogenic KRAS[86]. A proliferation-stimulating function of CD95 was first demonstrated in a study showing that CD95 engagement accelerated liver regeneration after partial hepatectomy[103]. A recent report showed that cancer cells and the regenerating liver require CD95 to activate Jun kinase (JNK) to drive cell proliferation[86]. In addition, CD95 ligand can be a proliferative and anti-apoptotic signal in quiescent hepatic stellate cells[104].

The capacity of tumor cells to invade the surrounding tissue and basement membrane is essential for metastasis formation. CD95 activation can stimulate tumor cell migration and invasion (reviewed in[102]). Interestingly, during embryonic neural development and in adult

neural progenitor cells, CD95 is constitutively expressed and promotes the branching and outgrowth of neurites rather than apoptosis[105-107]. The molecular mechanisms driving neurite outgrowth share many features with the mechanisms driving (tumor) cell migration. Both processes are orchestrated by the coordinated activation and inactivation of the Rho family GTPases RhoA, Rac and CDC42[108]. Both Rac and CDC42 can be activated by CD95[105,109]. Moreover, Rac activation plays an essential role during CD95-dependent stimulation of neurite outgrowth[105]. In glioma cells, CD95 stimulates invasion by activating a tyrosine kinase pathway leading to activation of PI(3) kinase and, ultimately, expression of the matrix-degrading metalloproteinases MMP2 and MMP9[110]. Furthermore, in a panel of apoptosis-resistant tumor cells CD95 signals migration via caspase-8 and ERK activation[111]. Our own unpublished data show that CD95 activates the cofilin pathway to stimulate actin-driven formation of cell protrusions and tumor cell invasion. Taken together, it appears that CD95 controls the invasive phenotype of tumor cells at multiple levels. Which of these signals is/are the predominant force in CD95-stimulated invasion is likely to be cell type-specific.
Activation of NFκB is one of the non-apoptotic pathways emanating from CD95[112-114]. NFκB activation in tumor cells is generally considered a survival signal and this transcription factor has been implicated in colorectal tumorigenesis and tumor maintenance[97,115-118]. Hence, CD95 may primarily promote colorectal tumor cell survival by activating the NFκB pathway.

CD95 AND HYPOXIA

Inflammation and hypoxia are intricately related phenomena. Hypoxia induces expression of several pro-inflammatory cytokines and chemokines and hypoxic tissue is rapidly infiltrated by inflammatory cells. CD95 expression in colon cancer cells is strongly increased under conditions of hypoxia, while CD95L appears to be mainly induced after reoxygenation and radical oxygen formation[119,120]. Furthermore, metalloproteases, which are highly produced by inflammatory cells, can mediate the shedding of membrane-bound CD95L to yield sCD95L[96]. This may either positively or negatively influence its ability to induce apoptosis[121-125]. Recently it was shown that sCD95L promotes tumor progression and that membrane-bound CD95L only is essential for CD95-induced apoptosis[8].
We have shown that hypoxia induces autocrine CD95 stimulation on colorectal tumor cells[79]. This was associated with increased tumor cell invasion and outgrowth of liver metastases, but it did not result in apoptosis.

DETERMINANTS OF CD95 SIGNALING OUTPUT

Cellular FLICE-inhibitory Protein (cFLIP)

Activation of caspase-8 and 10 at the DISC can be influenced by cFLIP[126]. Alternative mRNA splicing may result in three cFLIP variants: cFLIP long ($cFLIP_L$), cFLIPshort ($cFLIP_s$), and a short variant recently cloned from the Raji B-cell line ($cFLIP_R$)[127,128]. The role of $cFLIP_S$ in inhibiting death receptor-mediated apoptosis is well established[128,129]. It blocks caspase-8 activation at the DISC, most probably by competing for binding and recruitment to FADD. The structure of $cFLIP_R$ resembles the structure of $cFLIP_S$ and is likely to inhibit apoptosis through similar mechanisms. However, the role of $cFLIP_L$ at the DISC is controversial[128,130]. Initially, $cFLIP_L$ was described as an anti-apoptotic molecule inhibiting death receptor-induced apoptosis by interfering with the activation of caspase-8 at the DISC[131,132]. Experiments with overexpressed $cFLIP_L$ resulted in recruitment of both $cFLIP_L$ and caspase-8 to the DISC but was followed by defective processing of caspase-8[129]. Furthermore, several reports have implicated $cFLIP_L$ in the activation of survival signaling pathways, such as NFκB and ERK, upon death receptor stimulation[133,134]. Recently, RNA interference (RNAi) identified a critical role for cFLIPL in CD95-mediated apoptosis resistance in human colon cancer cells[135].

On the contrary, $cFLIP_L$ has also been reported to have pro-apoptotic functions by forming caspase-8:$cFLIP_L$ heterodimers, which actually promote activation of caspase-8 at the DISC[136,137]. Furthermore, the pro-apoptotic role of $cFLIP_L$ is supported by the fact that mice lacking $cFLIP_L$ show the same phenotype as mice lacking caspase-8 or FADD. In addition, all three proteins are required for embryonic development[132,138-140]. Together, these results implicate that the function $cFLIP_L$ is twofold, either as inhibitor or promoter of caspase activation, and that its role is being determined by a variety of factors, including its expression levels relative to caspase-8[128,130].

Modification and Localization

Palmitoylation (the covalent binding of fatty acids to cysteine residues of membrane proteins) of CD95 on the membrane-proximal residue Cys 199 directs it into membrane subdomains that are characterized by high glycolipid content[141,142]. Such subdomains are frequently referred to as 'rafts'. Interestingly, CD95 localization to lipid rafts is not only determined by its palmitoylation, but also by an extracellular domain that directly interacts with the glycosphingolipids that are enriched in the rafts[143]. Localization of CD95 to rafts is essential for efficient stimulation of apoptosis[144,145]. Palmitoylation of CD95 also promotes clustering of CD95 into high molecular weight complexes that recruit and activate caspase-8[142]. Indeed, caspase-8 activation occurs primarily in raft-like membrane subdomains[146]. In contrast, CD95 clustering and raft localization do not appear to be required for the activation of, at least some, non-apoptotic signaling pathways[111,143,147]. Internalization of activated (raft-localized) CD95 is required for efficient DISC formation and apoptosis, but not for activation of ERK and

NFκB[141,147]. Furthermore, mutation of the glycosphingolipid interaction motif disables apoptosis signaling but promotes non-apoptotic signaling and this is associated with altered trafficking/internalization of activated CD95[143]. In addition, phosphatidylinositol 3'-kinase blocks CD95 aggregation and caspase-8 cleavage at the death inducing signaling complex by modulating lateral diffusion of the receptor[148]. Together these studies suggest that the localization of CD95 to specific membrane subdomains may specify both its trafficking and signaling output upon receptor stimulation.

KRAS

In the presence of mutant K-Ras, CD95 and TRAIL-receptors preferentially stimulate migration and invasion of colorectal tumor cells, both *in vitro* and in liver metastases *in vivo*. However, deletion (or suppression) of mutant K-Ras is sufficient to switch these receptors back into apoptosis mode[35]. The K-Ras deleted/suppressed cells that were re-sensitized to CD95L and TRAIL had also lost their ability to form liver metastases and this was accompanied by tumor cell apoptosis in the liver[35].

Expression of dominant negative FADD, which prevents assembly of the classical death-inducing signalling complex (DISC) at the death domain, did not interfere with CD95-stimulated invasion, suggesting that other domains must be involved[35].

Sequence alignment of mouse and human CD95 and TRAIL-receptors shows that the intracellular portion of these receptors contains 6 conserved domains outside the death domain (domains I-VI). Interestingly, domain I is exactly the same region as the 'membrane proximal domain (MPD)', which was recently identified as an essential domain in CD95-mediated activation of the small GTPase Rac in neuronal cells[105].

The function of domains II-VI is unknown. Our preliminary results have shown that CD95-stimulated invasion is not affected by blocking the death domain. Therefore, we propose that signals generated by one or more of the non-DD conserved domains (e.g. activation of Rac1 via domain I), mediate tumor cell invasion in response to CD95L.

Raf1 suppression of ROK and LIMK1

Raf1 is perhaps best known as the Ras effector that mediates activation of the classical ERK pathway. However, the physiological function of Raf1 is to suppress apoptosis[149,150]. Strikingly, this does not require Raf1 kinase activity and is independent of its ability to activate the ERK pathway[149,150]. We identified Raf1 as the critical K-Ras effector in switching CD95 signalling output, in preserving tumor cell viability in the liver, and for metastatic potential[35]. This suggests that the apoptosis-suppressing function of Raf1 during embryonic development is preserved during the pathophysiology of metastasis formation.

Raf1 binds and inactivates the pro-apoptotic kinases ASK1 and MST2 in a kinase-independent manner[151,152]. Activation of CD95 causes disruption of the RAF1-MST2 complex, which leads to activation of MST2. Interestingly, this was required for efficient apoptosis induction by

CD95L[152]. Raf1 also binds to and suppresses the activity of ROK in a kinase-independent manner[153,154]. Our own data have shown that oncogenic KRAS and Raf1 are very potent suppressors of ROK in colorectal cancer cells[35]. One of the prime substrates of ROK is the membrane-actin linker protein Ezrin. CD95 binds to ROK-phosphorylated Ezrin and is thereby targeted to the cortical actin cytoskeleton[155]. Cortical actin filaments provide membrane rigidity, facilitate the formation of higher-order CD95 complexes, and promote their internalization via clathrin-coated pits[147,156]. Actin-dependent internalization of activated CD95 facilitates DISC formation and apoptosis[147]. Furthermore, disruption of the actin cytoskeleton in T lymphocytes interferes with apoptosis induction by CD95[141,147,156-158]. By inhibiting ROK and ROK-dependent Ezrin phosphorylation Raf1 reduces CD95 targeting to cortical actin and suppresses CD95-mediated apoptosis[154].

An important consideration is that the dependency of apoptosis signaling on intact actin cytoskeleton appears not to hold for epithelial (tumor) cells[159-162]. Likewise, it is unknown whether the ezrin-CD95 interaction controls CD95 function in cell types that do not require an intact actin cytoskeleton for apoptosis.

Apart from Ezrin, ROK phosphorylates a number of other substrates with distinct functional consequences[163]. In colorectal cancer cells we have found that one of the ROK-substrates, LIM-kinase 1 (LIMK1), is a critical determinant of CD95-induced apoptosis[35]. Raf1-mediated suppression of ROK and LIMK1 not only blocked apoptosis signaling by CD95 but also allowed CD95 to stimulate tumor cell invasion and increase metastatic potential[35]. Surprisingly little is known about the LIMK1-cofilin pathway in colorectal cancer. Immunohistochemistry studies have indicated that the Arp2/3 complex is increasingly expressed during colorectal carcinogenesis[164].

However, functional studies implicating LIMK1 or other components of the cofilin pathway in colorectal carcinogenesis or metastasis formation are currently lacking. Nothing is known about a potential role for cortical actin filaments, or the dynamics of their turnover, in controlling CD95-stimulated non-apoptotic signalling pathways.

CD95 IN COLORECTAL CANCER TREATMENT

Activation of CD95 and TRAIL-receptors in cancer therapy

Pre-clinical studies showed that systemic activation of CD95 in mice caused massive hepatocyte apoptosis resulting in fatal hepatitis[87,165]. However, a phase I study is being conducted in patients with solid tumors determining the safety and tolerability of APO010, which is a humanized, recombinant mega-CD95L (www.clinicaltrials.gov). It shows anticancer activity *in vitro* and in animal models carrying a human xenograft of a variety of cancers, including multiple myeloma, non-small cell lung cancer (NSCLC) and ovarian cancer and appears to be synergistic with a variety of commonly used anticancer drugs[166]. Pre-clinical studies show

that APO010 may cause moderate and reversible liver toxicity and a drop in platelets that recover within 5 days[166,167].
A number of pre-clinical studies have shown the efficacy of using TRAIL-receptors as targets for agonistic therapy, aiming to induce apoptosis in tumor cells expressing these receptors[3,4,168]. From at least 15 completed phase I and phase II clinical trials it has become clear that agonistic TRAIL-receptor therapy appears to be generally safe and well-tolerated in most patients, but tumor responses were generally minimal. Single-agent phase 2 trials of the human DR4 agonist antibody mapatumumab have been completed in NSCLC, colorectal cancer (CRC) and non-Hodgkin lymphoma (NHL)[169]. Two partial responses and one complete response among 40 patients with pretreated follicular NHL were observed, however there were no objective responses in treatment-refractory NSCLC and CRC patients[169].

Neutralization of CD95L in preclinical studies
Suppression of CD95 signaling offers the possibility of limiting inflammation and, possibly, inflammation-associated tumor growth. This has proven to be an effective strategy in the prevention of inflammation-associated hepatocarcinogenesis in mice[98]. In addition, suppression of CD95 signaling *in vivo* has also shown great therapeutic potential in limiting spinal cord injury, inflammatory lung disease, hepatitis, graft-versus-host disease and amyloid-β-associated neurodegeneration[70,122,170-172]. In these pre-clinical studies CD95L-neutralizing agents have proven to be non-toxic and well-tolerated. Currently, a phase 1 clinical trial is ongoing to test the safety and tolerability of CD95-Fc in healthy volunteers and patients with spinal cord injury[173]. To our knowledge there are no ongoing clinical trials in which such compounds are being tested for their efficacy as anti-cancer agents.

CONCLUSIONS AND FUTURE PERSPECTIVES

Activation of death receptors is a potentially effective therapeutic opportunity in apoptosis-sensitive tumor types. However, most colorectal tumors are apoptosis resistant and severe systemic and liver toxicity are major concerns when considering CD95-activating therapeutics. In colorectal cancer it is therefore perhaps more appealing to assess whether CD95 inhibition could be effective. To address this question we need to use translational models (for instance those that make use of tumor-derived colonosphere cultures) to assess the effects of CD95 neutralization on tumor behavior, either alone or in combination with chemotherapy.
CD95 signaling output may change during the progression of colorectal cancer development, for instance before and after the acquisition of activating mutations in KRAS. Any therapeutic approach targeting CD95 and other death receptors should take this into account. Whether this holds for other cancer types with KRAS mutations needs further investigation.
Still, very little is known about the signals that determine the cellular response to CD95

activation. To what extent the non-apoptotic modes of CD95 signaling govern (colorectal) tumorigenesis, metastasis formation and response to therapy needs to be elucidated. We suggest that identifying compounds that switch death receptor function would be of greater interest than those that inhibit or stimulate them. The determination of such factor(s) could be the ultimate antidote that prevents “Dr. Jekyll” from turning back into “Mr. Hyde”.

REFERENCES

1. Bridgham,J.T., Wilder,J.A., Hollocher,H., & Johnson,A.L. All in the family: evolutionary and functional relationships among death receptors. Cell Death. Differ. 10, 19-25 (2003).
2. Strasser,A., Jost,P.J., & Nagata,S. The many roles of FAS receptor signaling in the immune system. Immunity. 30, 180-192 (2009).
3. Reed,J.C. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat. Clin. Pract. Oncol. 3, 388-398 (2006).
4. Ashkenazi,A., Holland,P., & Eckhardt,S.G. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J. Clin. Oncol. 26, 3621-3630 (2008).
5. Cascino,I., Fiucci,G., Papoff,G., & Ruberti,G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J. Immunol. 154, 2706-2713 (1995).
6. Cheng,J. et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 263, 1759-1762 (1994).
7. Tanaka,M. et al. Fas ligand in human serum. Nat. Med. 2, 317-322 (1996).
8. O' Reilly,L.A. et al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature 461, 659-663 (2009).
9. Moller,P., Walczak,H., Reidl,S., Strater,J., & Krammer,P.H. Paneth cells express high levels of CD95 ligand transcripts: a unique property among gastrointestinal epithelia. Am. J. Pathol. 149, 9-13 (1996).
10. Strater,J. & Moller,P. Expression and function of death receptors and their natural ligands in the intestine. Ann. N. Y. Acad. Sci. 915, 162-170 (2000).
11. Strater,J. & Moller,P. CD95 (Fas/APO-1)/CD95L in the gastrointestinal tract: fictions and facts. Virchows Arch. 442, 218-225 (2003).
12. Eastwood,G.L. Gastrointestinal epithelial renewal. Gastroenterology 72, 962-975 (1977).
13. Chen,L., Park,S.M., Turner,J.R., & Peter,M.E. Cell death in the colonic epithelium during inflammatory bowel diseases: CD95/Fas and beyond. Inflamm. Bowel. Dis.(2010).
14. Strater,J. et al. CD95 (APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis. Gastroenterology 113, 160-167 (1997).
15. Park,S.M. et al. CD95 is cytoprotective for intestinal epithelial cells in colitis. Inflamm. Bowel. Dis.(2010).
16. Li,J.H., Rosen,D., Sondel,P., & Berke,G. Immune privilege and FasL: two ways to inactivate effector cytotoxic T lymphocytes by FasL-expressing cells. Immunology 105, 267-277 (2002).
17. Houston,A. et al. Fas ligand expressed in colon cancer is not associated with increased apoptosis of tumor cells in vivo. Int. J. Cancer 107, 209-214 (2003).
18. Restifo,N.P. Countering the 'counterattack' hypothesis. Nat. Med. 7, 259 (2001).
19. Moller,P. et al. Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int. J. Cancer 57, 371-377 (1994).
20. Peduto,E.L. et al. Fas and Fas ligand expression in tumor cells and in vascular smooth-muscle cells of colonic and renal carcinomas. Int. J. Cancer 81, 772-778 (1999).
21. Bennett,M.W. et al. Fas ligand upregulation is an early event in colonic carcinogenesis. J. Clin. Pathol. 54, 598-604 (2001).
22. Mann,B. et al. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. Br. J. Cancer 79, 1262-1269 (1999).
23. O'Connell,J. et al. Fas ligand expression in primary colon adenocarcinomas: evidence that the Fas counterattack is a prevalent mechanism of immune evasion in human colon cancer. J. Pathol. 186, 240-246 (1998).
24. Nozoe,T., Yasuda,M., Honda,M., Inutsuka,S., & Korenaga,D. Fas ligand expression is correlated with metastasis in colorectal carcinoma. Oncology 65, 83-88 (2003).
25. Djerbi,M. et al. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J. Exp. Med. 190, 1025-1032 (1999).

26. Liu,K. & Abrams,S.I. Alterations in Fas expression are characteristic of, but not solely responsible for, enhanced metastatic competence. J. Immunol. 170, 5973-5980 (2003).
27. Maecker,H.L., Yun,Z., Maecker,H.T., & Giaccia,A.J. Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. Cancer Cell 2, 139-148 (2002).
28. Medema,J.P., de,J.J., van,H.T., Melief,C.J., & Offringa,R. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. J. Exp. Med. 190, 1033-1038 (1999).
29. Owen-Schaub,L.B., van Golen,K.L., Hill,L.L., & Price,J.E. Fas and Fas ligand interactions suppress melanoma lung metastasis. J. Exp. Med. 188, 1717-1723 (1998).
30. Strater,J. et al. Impaired CD95 expression predisposes for recurrence in curatively resected colon carcinoma: clinical evidence for immunoselection and CD95L mediated control of minimal residual disease. Gut 54, 661-665 (2005).
31. Onodera,H. et al. Fas/CD95 signaling rather than angiogenesis or proliferative activity is a useful prognostic factor in patients with resected liver metastases from colorectal cancer. Int. J. Colorectal Dis. 20, 477-484 (2005).
32. Kim,H.S. et al. Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology 125, 708-715 (2003).
33. Ryu,B.K., Lee,M.G., Chi,S.G., Kim,Y.W., & Park,J.H. Increased expression of cFLIP(L) in colonic adenocarcinoma. J. Pathol. 194, 15-19 (2001).
34. Strater,J. et al. Expression and prognostic significance of APAF-1, caspase-8 and caspase-9 in stage II/III colon carcinoma: caspase-8 and caspase-9 is associated with poor prognosis. Int. J. Cancer 127, 873-880 (2010).
35. Hoogwater,F.J. et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 138, 2357-2367 (2010).
36. Hasegawa,D. et al. Elevation of the serum Fas ligand in patients with hemophagocytic syndrome and Diamond-Blackfan anemia. Blood 91, 2793-2799 (1998).
37. Kato,K. et al. Elevated serum soluble Fas ligand in natural killer cell proliferative disorders. Br. J. Haematol. 103, 1164-1166 (1998).
38. Lim,S.C. Expression of Fas ligand and sFas ligand in human gastric adenocarcinomas. Oncol. Rep. 9, 103-107 (2002).
39. Mizutani,Y. et al. Significance of serum soluble Fas ligand in patients with bladder carcinoma. Cancer 92, 287-293 (2001).
40. Nagao,M. et al. The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo? Hepatology 30, 413-421 (1999).
41. Reimer,T. et al. FasL:Fas ratio--a prognostic factor in breast carcinomas. Cancer Res. 60, 822-828 (2000).
42. Tsutsumi,S. et al. Circulating soluble Fas ligand in patients with gastric carcinoma. Cancer 89, 2560-2564 (2000).
43. Kozlowski,M. et al. Serum soluble Fas ligand (sFasL) in patients with primary squamous cell carcinoma of the esophagus. Folia Histochem. Cytobiol. 45, 199-204 (2007).
44. Pitti,R.M. et al. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 271, 12687-12690 (1996).
45. Van Geelen,C.M., de Vries,E.G., & de,J.S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. Drug Resist. Updat. 7, 345-358 (2004).
46. Wiley,S.R. et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 3, 673-682 (1995).
47. Finnberg,N. et al. Non-invasive fluorescence imaging of cell death in fresh human colon epithelia treated with 5-Fluorouracil, CPT-11 and/or TRAIL. Cancer Biol. Ther. 4, 937-942 (2005).
48. Yao,H., Song,E., Chen,J., & Hamar,P. Expression of FAP-1 by human colon adenocarcinoma: implication for resistance against Fas-mediated apoptosis in cancer. Br. J. Cancer 91, 1718-1725 (2004).
49. Oikonomou,E. et al. Newly established tumourigenic primary human colon cancer cell lines are sensitive to TRAIL-induced apoptosis in vitro and in vivo. Br. J. Cancer 97, 73-84 (2007).
50. O'Connell,J. et al. Interferon-gamma sensitizes colonic epithelial cell lines to physiological and therapeutic inducers of colonocyte apoptosis. J. Cell Physiol 185, 331-338 (2000).

51. von,R.U. et al. Colon carcinoma cells use different mechanisms to escape CD95-mediated apoptosis. Cancer Res. 58, 526-534 (1998).
52. Owen-Schaub,L.B. Fas function and tumor progression: use it and lose it. Cancer Cell 2, 95-96 (2002).
53. Chen,J.J., Sun,Y., & Nabel,G.J. Regulation of the proinflammatory effects of Fas ligand (CD95L). Science 282, 1714-1717 (1998).
54. Ottonello,L., Tortolina,G., Amelotti,M., & Dallegri,F. Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. J. Immunol. 162, 3601-3606 (1999).
55. Buonocore,S. et al. Neutrophil-dependent tumor rejection and priming of tumoricidal CD8+ T cell response induced by dendritic cells overexpressing CD95L. J. Leukoc. Biol. 84, 713-720 (2008).
56. Buonocore,S. et al. Dendritic cells overexpressing CD95 (Fas) ligand elicit vigorous allospecific T-cell responses in vivo. Blood 101, 1469-1476 (2003).
57. Allison,J., Georgiou,H.M., Strasser,A., & Vaux,D.L. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. Proc. Natl. Acad. Sci. U. S. A 94, 3943-3947 (1997).
58. Kang,S.M. et al. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. Nat. Med. 3, 738-743 (1997).
59. Cairns,C.M. et al. Lymphotactin expression by engineered myeloma cells drives tumor regression: mediation by CD4+ and CD8+ T cells and neutrophils expressing XCR1 receptor. J. Immunol. 167, 57-65 (2001).
60. Graf,M.R., Prins,R.M., & Merchant,R.E. IL-6 secretion by a rat T9 glioma clone induces a neutrophil-dependent antitumor response with resultant cellular, antiglioma immunity. J. Immunol. 166, 121-129 (2001).
61. Seino,K., Kayagaki,N., Okumura,K., & Yagita,H. Antitumor effect of locally produced CD95 ligand. Nat. Med. 3, 165-170 (1997).
62. Arai,H., Gordon,D., Nabel,E.G., & Nabel,G.J. Gene transfer of Fas ligand induces tumor regression in vivo. Proc. Natl. Acad. Sci. U. S. A 94, 13862-13867 (1997).
63. Zitvogel,L., Tesniere,A., & Kroemer,G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat. Rev. Immunol. 6, 715-727 (2006).
64. Shimizu,M. et al. Induction of antitumor immunity with Fas/APO-1 ligand (CD95L)-transfected neuroblastoma neuro-2a cells. J. Immunol. 162, 7350-7357 (1999).
65. Ullrich,E. et al. Dendritic cells and innate defense against tumor cells. Cytokine Growth Factor Rev. 19, 79-92 (2008).
66. Puliaeva,I., Puliaev,R., Shustov,A., Haas,M., & Via,C.S. Fas expression on antigen-specific T cells has costimulatory, helper, and down-regulatory functions in vivo for cytotoxic T cell responses but not for T cell-dependent B cell responses. J. Immunol. 181, 5912-5929 (2008).
67. Nagata,S. & Suda,T. Fas and Fas ligand: lpr and gld mutations. Immunol. Today 16, 39-43 (1995).
68. Cohen,P.L. & Eisenberg,R.A. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu. Rev. Immunol. 9, 243-269 (1991).
69. Rathmell,J.C. & Goodnow,C.C. Autoimmunity. The Fas track. Curr. Biol. 5, 1218-1221 (1995).
70. Borges,V.M. et al. Fas ligand triggers pulmonary silicosis. J. Exp. Med. 194, 155-164 (2001).
71. Liu,Z.X., Govindarajan,S., & Kaplowitz,N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. Gastroenterology 127, 1760-1774 (2004).
72. Nakashima,H. et al. Activation of mouse natural killer T cells accelerates liver regeneration after partial hepatectomy. Gastroenterology 131, 1573-1583 (2006).
73. Tagawa,Y., Kakuta,S., & Iwakura,Y. Involvement of Fas/Fas ligand system-mediated apoptosis in the development of concanavalin A-induced hepatitis. Eur. J. Immunol. 28, 4105-4113 (1998).
74. Takeda,K. et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med. 7, 94-100 (2001).
75. Fingleton,B., Carter,K.J., & Matrisian,L.M. Loss of functional Fas ligand enhances intestinal tumorigenesis in the Min mouse model. Cancer Res. 67, 4800-4806 (2007).
76. Park,S.M. et al. CD95 signaling deficient mice with a wild-type hematopoietic system are prone to hepatic neoplasia. Apoptosis. 13, 41-51 (2008).

77. Guillen-Ahlers,H., Suckow,M.A., Castellino,F.J., & Ploplis,V.A. Fas/CD95 deficiency in ApcMin/+ mice increases intestinal tumor burden. PLoS. One. 5, e9070 (2010).
78. Whiteside,T.L. The role of death receptor ligands in shaping tumor microenvironment. Immunol. Invest 36, 25-46 (2007).
79. Nijkamp,M.W. et al. CD95 is a key mediator of invasion and accelerated outgrowth of mouse colorectal liver metastases following radiofrequency ablation. J. Hepatol.(2010).
80. Liu,K., McDuffie,E., & Abrams,S.I. Exposure of human primary colon carcinoma cells to anti-Fas interactions influences the emergence of pre-existing Fas-resistant metastatic subpopulations. J. Immunol. 171, 4164-4174 (2003).
81. Li,H. et al. Human and mouse colon cancer utilizes CD95 signaling for local growth and metastatic spread to liver. Gastroenterology 137, 934-44, 944 (2009).
82. O'Connell,J., O'Sullivan,G.C., Collins,J.K., & Shanahan,F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J. Exp. Med. 184, 1075-1082 (1996).
83. Houston,A., Bennett,M.W., O'Sullivan,G.C., Shanahan,F., & O'Connell,J. Fas ligand mediates immune privilege and not inflammation in human colon cancer, irrespective of TGF-beta expression. Br. J. Cancer 89, 1345-1351 (2003).
84. Ryan,A.E., Shanahan,F., O'Connell,J., & Houston,A.M. Fas ligand promotes tumor immune evasion of colon cancer in vivo. Cell Cycle 5, 246-249 (2006).
85. Igney,F.H. & Krammer,P.H. Tumor counterattack: fact or fiction? Cancer Immunol. Immunother. 54, 1127-1136 (2005).
86. Chen,L. et al. CD95 promotes tumour growth. Nature 465, 492-496 (2010).
87. Ogasawara,J. et al. Lethal effect of the anti-Fas antibody in mice. Nature 364, 806-809 (1993).
88. Galle,P.R. et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J. Exp. Med. 182, 1223-1230 (1995).
89. Rudiger,H.A. & Clavien,P.A. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. Gastroenterology 122, 202-210 (2002).
90. Yoong,K.F., Afford,S.C., Randhawa,S., Hubscher,S.G., & Adams,D.H. Fas/Fas ligand interaction in human colorectal hepatic metastases: A mechanism of hepatocyte destruction to facilitate local tumor invasion. Am. J. Pathol. 154, 693-703 (1999).
91. Arai,K., Liu,Z.X., Lane,T., & Dennert,G. IP-10 and Mig facilitate accumulation of T cells in the virus-infected liver. Cell Immunol. 219, 48-56 (2002).
92. Faouzi,S. et al. Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-kappa B-independent, caspase-3-dependent pathway. J. Biol. Chem. 276, 49077-49082 (2001).
93. Hohlbaum,A.M., Gregory,M.S., Ju,S.T., & Marshak-Rothstein,A. Fas ligand engagement of resident peritoneal macrophages in vivo induces apoptosis and the production of neutrophil chemotactic factors. J. Immunol. 167, 6217-6224 (2001).
94. Miwa,K. et al. Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. Nat. Med. 4, 1287-1292 (1998).
95. Park,D.R. et al. Fas (CD95) induces proinflammatory cytokine responses by human monocytes and monocyte-derived macrophages. J. Immunol. 170, 6209-6216 (2003).
96. Coussens,L.M. & Werb,Z. Inflammation and cancer. Nature 420, 860-867 (2002).
97. Greten,F.R. et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118, 285-296 (2004).
98. Nakamoto,Y. et al. Prevention of hepatocellular carcinoma development associated with chronic hepatitis by anti-fas ligand antibody therapy. J. Exp. Med. 196, 1105-1111 (2002).
99. Finnberg,N., Klein-Szanto,A.J., & El-Deiry,W.S. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J. Clin. Invest 118, 111-123 (2008).
100. O'Connell,J. et al. Altered mechanisms of apoptosis in colon cancer: Fas resistance and counterattack in the tumor-immune conflict. Ann. N. Y. Acad. Sci. 910, 178-192 (2000).

101. Peter,M.E., Legembre,P., & Barnhart,B.C. Does CD95 have tumor promoting activities? Biochim. Biophys. Acta 1755, 25-36 (2005).
102. Peter,M.E. et al. The CD95 receptor: apoptosis revisited. Cell 129, 447-450 (2007).
103. Desbarats,J. & Newell,M.K. Fas engagement accelerates liver regeneration after partial hepatectomy. Nat. Med. 6, 920-923 (2000).
104. Reinehr,R., Sommerfeld,A., & Haussinger,D. CD95 ligand is a proliferative and antiapoptotic signal in quiescent hepatic stellate cells. Gastroenterology 134, 1494-1506 (2008).
105. Ruan,W., Lee,C.T., & Desbarats,J. A novel juxtamembrane domain in tumor necrosis factor receptor superfamily molecules activates Rac1 and controls neurite growth. Mol. Biol. Cell 19, 3192-3202 (2008).
106. Zuliani,C. et al. Control of neuronal branching by the death receptor CD95 (Fas/Apo-1). Cell Death. Differ. 13, 31-40 (2006).
107. Desbarats,J. et al. Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat. Cell Biol. 5, 118-125 (2003).
108. von,P.A. & Bastmeyer,M. Mechanisms of gradient detection: a comparison of axon pathfinding with eukaryotic cell migration. Int. Rev. Cytol. 263, 1-62 (2007).
109. Subauste,M.C. et al. Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. J. Biol. Chem. 275, 9725-9733 (2000).
110. Kleber,S. et al. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 13, 235-248 (2008).
111. Barnhart,B.C. et al. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J. 23, 3175-3185 (2004).
112. Kreuz,S. et al. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J. Cell Biol. 166, 369-380 (2004).
113. Ponton,A., Clement,M.V., & Stamenkovic,I. The CD95 (APO-1/Fas) receptor activates NF-kappaB independently of its cytotoxic function. J. Biol. Chem. 271, 8991-8995 (1996).
114. Legembre,P., Barnhart,B.C., & Peter,M.E. The relevance of NF-kappaB for CD95 signaling in tumor cells. Cell Cycle 3, 1235-1239 (2004).
115. Lin,A. & Karin,M. NF-kappaB in cancer: a marked target. Semin. Cancer Biol. 13, 107-114 (2003).
116. Karin,M. & Lin,A. NF-kappaB at the crossroads of life and death. Nat. Immunol. 3, 221-227 (2002).
117. Karin,M. Nuclear factor-kappaB in cancer development and progression. Nature 441, 431-436 (2006).
118. Pikarsky,E. et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 431, 461-466 (2004).
119. Liu,T. et al. Hypoxia induces p53-dependent transactivation and Fas/CD95-dependent apoptosis. Cell Death. Differ. 14, 411-421 (2007).
120. Vogt,M., Bauer,M.K., Ferrari,D., & Schulze-Osthoff,K. Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells. FEBS Lett. 429, 67-72 (1998).
121. Ahn,J.H. et al. Non-apoptotic signaling pathways activated by soluble Fas ligand in serum-starved human fibroblasts. Mitogen-activated protein kinases and NF-kappaB-dependent gene expression. J. Biol. Chem. 276, 47100-47106 (2001).
122. Ethell,D.W., Kinloch,R., & Green,D.R. Metalloproteinase shedding of Fas ligand regulates beta-amyloid neurotoxicity. Curr. Biol. 12, 1595-1600 (2002).
123. Powell,W.C., Fingleton,B., Wilson,C.L., Boothby,M., & Matrisian,L.M. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr. Biol. 9, 1441-1447 (1999).
124. Tanaka,M., Itai,T., Adachi,M., & Nagata,S. Downregulation of Fas ligand by shedding. Nat. Med. 4, 31-36 (1998).
125. Vargo-Gogola,T., Crawford,H.C., Fingleton,B., & Matrisian,L.M. Identification of novel matrix metalloproteinase-7 (matrilysin) cleavage sites in murine and human Fas ligand. Arch. Biochem. Biophys. 408, 155-161 (2002).
126. Tschopp,J., Irmler,M., & Thome,M. Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. 10, 552-558 (1998).

127. Golks,A., Brenner,D., Fritsch,C., Krammer,P.H., & Lavrik,I.N. c-FLIPR, a new regulator of death receptor-induced apoptosis. J. Biol. Chem. 280, 14507-14513 (2005).
128. Guicciardi,M.E. & Gores,G.J. Life and death by death receptors. FASEB J. 23, 1625-1637 (2009).
129. Krueger,A., Schmitz,I., Baumann,S., Krammer,P.H., & Kirchhoff,S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem. 276, 20633-20640 (2001).
130. Chang,D.W. et al. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 21, 3704-3714 (2002).
131. Irmler,M. et al. Inhibition of death receptor signals by cellular FLIP. Nature 388, 190-195 (1997).
132. Rasper,D.M. et al. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. Cell Death. Differ. 5, 271-288 (1998).
133. Hu,W.H., Johnson,H., & Shu,H.B. Activation of NF-kappaB by FADD, Casper, and caspase-8. J. Biol. Chem. 275, 10838-10844 (2000).
134. Kataoka,T. et al. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Curr. Biol. 10, 640-648 (2000).
135. Zang,F. et al. Critical role for c-FLIP(L) on Fas resistance in colon carcinoma cell line HT-29. Cell Biol. Int. 32, 329-336 (2008).
136. Dohrman,A. et al. Cellular FLIP long form augments caspase activity and death of T cells through heterodimerization with and activation of caspase-8. J. Immunol. 175, 311-318 (2005).
137. Micheau,O. et al. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J. Biol. Chem. 277, 45162-45171 (2002).
138. Peter,M.E. & Krammer,P.H. The CD95(APO-1/Fas) DISC and beyond. Cell Death. Differ. 10, 26-35 (2003).
139. Yeh,W.C. et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279, 1954-1958 (1998).
140. Yeh,W.C. et al. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity. 12, 633-642 (2000).
141. Chakrabandhu,K. et al. Palmitoylation is required for efficient Fas cell death signaling. EMBO J. 26, 209-220 (2007).
142. Feig,C., Tchikov,V., Schutze,S., & Peter,M.E. Palmitoylation of CD95 facilitates formation of SDS-stable receptor aggregates that initiate apoptosis signaling. EMBO J. 26, 221-231 (2007).
143. Chakrabandhu,K. et al. The extracellular glycosphingolipid-binding motif of Fas defines its internalization route, mode and outcome of signals upon activation by ligand. Cell Death. Differ. 15, 1824-1837 (2008).
144. Gajate,C. & Mollinedo,F. Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy. J. Biol. Chem. 280, 11641-11647 (2005).
145. Hueber,A.O., Bernard,A.M., Herincs,Z., Couzinet,A., & He,H.T. An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. EMBO Rep. 3, 190-196 (2002).
146. Eramo,A. et al. CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells. Eur. J. Immunol. 34, 1930-1940 (2004).
147. Lee,K.H. et al. The role of receptor internalization in CD95 signaling. EMBO J. 25, 1009-1023 (2006).
148. Varadhachary,A.S. et al. Phosphatidylinositol 3'-kinase blocks CD95 aggregation and caspase-8 cleavage at the death-inducing signaling complex by modulating lateral diffusion of CD95. J. Immunol. 166, 6564-6569 (2001).
149. Huser,M. et al. MEK kinase activity is not necessary for Raf-1 function. EMBO J. 20, 1940-1951 (2001).
150. Mikula,M. et al. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J. 20, 1952-1962 (2001).
151. Chen,J., Fujii,K., Zhang,L., Roberts,T., & Fu,H. Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc. Natl. Acad. Sci. U. S. A 98, 7783-7788 (2001).

152. O'Neill,E., Rushworth,L., Baccarini,M., & Kolch,W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science 306, 2267-2270 (2004).
153. Ehrenreiter,K. et al. Raf-1 regulates Rho signaling and cell migration. J. Cell Biol. 168, 955-964 (2005).
154. Piazzolla,D., Meissl,K., Kucerova,L., Rubiolo,C., & Baccarini,M. Raf-1 sets the threshold of Fas sensitivity by modulating Rok-alpha signaling. J. Cell Biol. 171, 1013-1022 (2005).
155. Fais,S., De,M.A., & Lozupone,F. The role of FAS to ezrin association in FAS-mediated apoptosis. Apoptosis. 10, 941-947 (2005).
156. geciras-Schimnich,A. et al. Molecular ordering of the initial signaling events of CD95. Mol. Cell Biol. 22, 207-220 (2002).
157. Gajate,C. et al. Intracellular triggering of Fas aggregation and recruitment of apoptotic molecules into Fas-enriched rafts in selective tumor cell apoptosis. J. Exp. Med. 200, 353-365 (2004).
158. Parlato,S. et al. CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway. EMBO J. 19, 5123-5134 (2000).
159. Celeste,M.S., Sun,G.P., & Bierer,B.E. Inhibition of actin polymerization enhances commitment to and execution of apoptosis induced by withdrawal of trophic support. J. Cell Biochem. 88, 1066-1076 (2003).
160. geciras-Schimnich,A. & Peter,M.E. Actin dependent CD95 internalization is specific for Type I cells. FEBS Lett. 546, 185-188 (2003).
161. Kulms,D. et al. Apoptosis induced by disruption of the actin cytoskeleton is mediated via activation of CD95 (Fas/APO-1). Cell Death. Differ. 9, 598-608 (2002).
162. Meng,Y., Kang,S., & Fishman,D.A. Lysophosphatidic acid inhibits anti-Fas-mediated apoptosis enhanced by actin depolymerization in epithelial ovarian cancer. FEBS Lett. 579, 1311-1319 (2005).
163. Amano,M., Fukata,Y., & Kaibuchi,K. Regulation and functions of Rho-associated kinase. Exp. Cell Res. 261, 44-51 (2000).
164. Otsubo,T. et al. Involvement of Arp2/3 complex in the process of colorectal carcinogenesis. Mod. Pathol. 17, 461-467 (2004).
165. Papenfuss,K., Cordier,S.M., & Walczak,H. Death receptors as targets for anti-cancer therapy. J. Cell Mol. Med. 12, 2566-2585 (2008).
166. Russo,M., Mupo,A., Spagnuolo,C., & Russo,G.L. Exploring Death Receptor Pathways as Selective Targets in Cancer Therapy. Biochem. Pharmacol.(2010).
167. Verbrugge,I. et al. Combining radiotherapy with APO010 in cancer treatment. Clin. Cancer Res. 15, 2031-2038 (2009).
168. Koschny,R., Walczak,H., & Ganten,T.M. The promise of TRAIL--potential and risks of a novel anticancer therapy. J. Mol. Med. 85, 923-935 (2007).
169. Wiezorek,J., Holland,P., & Graves,J. Death receptor agonists as a targeted therapy for cancer. Clin. Cancer Res. 16, 1701-1708 (2010).
170. Demjen,D. et al. Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. Nat. Med. 10, 389-395 (2004).
171. Kondo,T., Suda,T., Fukuyama,H., Adachi,M., & Nagata,S. Essential roles of the Fas ligand in the development of hepatitis. Nat. Med. 3, 409-413 (1997).
172. Miwa,K. et al. Therapeutic effect of an anti-Fas ligand mAb on lethal graft-versus-host disease. Int. Immunol. 11, 925-931 (1999).
173. Fischer,U. & Schulze-Osthoff,K. Apoptosis-based therapies and drug targets. Cell Death. Differ. 12 Suppl 1, 942-961 (2005).

CHAPTER 3

ONCOGENIC K-RAS TURNS DEATH RECEPTORS INTO METASTASIS-PROMOTING RECEPTORS IN HUMAN AND MOUSE COLORECTAL CANCER CELLS

Gastroenterology. 2010 Jun; 138(7):2357-67

Frederik J.H. Hoogwater[1]
Maarten W. Nijkamp[1*]
Niels Smakman[1*]
Ernst J.A. Steller[1]
Benjamin L. Emmink[1]
B. Florien Westendorp[1]
Danielle A.E. Raats[1]
Martin R. Sprick[2]
Uta Schaefer[3]
Winan J. van Houdt[1]
Menno T. de Bruijn[1]
Ron C.J. Schackmann[1]
Patrick W.B. Derksen[1]
Jan-Paul Medema[3]
Henning Walczak[4]
Inne H.M. Borel Rinkes[1]
Onno Kranenburg[1]

[*] *these authors contributed equally*

Departments of [1]Surgery and [2]Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[3]Laboratory of Experimental Oncology and Radiobiology, Academic Medical Center, Amsterdam, The Netherlands
[4]Department of Immunology, Division of Medicine, Imperial College London, London, UK

ABSTRACT

Background

Death receptors expressed on tumor cells can prevent metastasis formation by inducing apoptosis, but they can also promote migration and invasion. The determinants of death receptor signaling output are poorly defined. Here we investigated the role of oncogenic K-Ras in determining death receptor function and metastatic potential.

Methods

Isogenic human and mouse colorectal cancer cell lines differing only in the presence or absence of the K-Ras oncogene were tested in apoptosis and invasion assays using CD95 ligand and TRAIL as stimuli. Metastatic potential was assessed by intrasplenic injections of GFP- or luciferase-expressing tumor cells, followed by intravital fluorescence microscopy or bioluminescence imaging, and confocal microscopy and immunohistochemistry. Ras-effector pathway control of CD95 output was assessed by an RNA-interference and inhibitor-based approach.

Results

CD95 ligand and TRAIL stimulated invasion of colorectal tumor cells and liver metastases in a K-Ras-dependent fashion. Loss of mutant K-Ras switched CD95 and TRAIL receptors back into apoptosis mode and abrogated metastatic potential. Raf1 was essential for the switch in CD95 function, for tumor cell survival in the liver and for K-Ras-driven formation of liver metastases. K-Ras and Raf1 suppressed ROCK/LIM kinase-mediated phosphorylation of the actin-severing protein cofilin. Overexpression of ROCK or LIM kinase allowed CD95L to induce apoptosis in K-Ras-proficient cells and prevented metastasis formation, while their suppression protected K-Ras-deficient cells against apoptosis.

Conclusions

Oncogenic K-Ras and its effector Raf1 convert death receptors into invasion-inducing receptors by suppressing the ROCK/LIM kinase pathway and this is essential for K-Ras/Raf1-driven metastasis formation.

INTRODUCTION

Metastasis formation by colorectal tumors occurs primarily in the liver and is the major determinant of patient survival. The mutations that drive initiation and progression of primary colorectal carcinomas have been well documented[1]. Mutational inactivation of the *APC* and *TP53* tumor suppressor genes and activating mutations in the *K-Ras* proto-oncogene are by far the most frequently occurring mutations driving human colorectal tumorigenesis[1,2]. Large-scale sequence analysis of paired primary and metastatic tumors has shown that invasive carcinomas and metastases share the vast majority of their mutations, and that once an invasive carcinoma has formed, metastasis formation ensues relatively rapidly, possibly without the requirement for additional mutations[3]. This implies that (part of) the genetic changes that cause the formation of invasive primary carcinomas may also drive metastasis formation, at least in colorectal cancer[3].

One of the first genes to be mutated during the course of colorectal cancer development in humans is the proto-oncogene *K-Ras*. Activated Ras genes promote tumor cell motility, invasion and survival in a number of distinct cell types[4]. However, it remains unclear how endogenous oncogenic *K-Ras* alleles, in the presence of a multitude of other genetic alterations, contribute to the metastatic potential of colorectal tumor cells. Metastasis formation can be suppressed by the activation of death receptors on tumor cells[5-8]. Among the best studied death receptors are CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptors[9]. Apoptosis has long been thought to be the primary outcome of death receptor activation, and this has lead to the development of death receptor-stimulating agents as anti-tumor therapeutics[9]. However, more recent data suggest that CD95 and TRAIL receptors can also act in a pro-tumorigenic fashion by stimulating tumor cell proliferation, survival and invasion[10-15]. The determinants of death receptor signalling output are poorly defined. Here we provide evidence that endogenous oncogenic K-Ras transforms anti-metastatic death receptors into invasion-stimulating pro-metastatic ones. We further show that Raf1-mediated suppression of the Rho kinase (ROCK)/LIM kinase pathway is responsible for death receptor transformation downstream of K-Ras and for K-Ras-driven metastasis formation.

MATERIALS AND METHODS

Cell lines and cell cultures

C26 cells were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). C26 cell lines in which the endogenous K-Ras^{D12} allele is stably suppressed by RNA interference (C26-KrasKD) and control cells (C26-pLL) were described before[16]. HCT116 and DLD1 cells and their isogenic derivatives lacking K-Ras^{D13} (Hkh2 and DKO4 respectively) were kindly provided by Dr Shirasawa and were described before[17]. HCT116 cells lacking β-catenin$^{\Delta S45}$

and p53 were kindly provided by Dr B Vogelstein. C26 cells expressing the firefly luciferase gene (C26-luc) were described previously[18]. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. A manuscript describing the isolation and characterization of the patient-derived colorectal spheroid cultures is currently in preparation.

Lentiviral transduction

Lentiviral transductions were performed as described[18]. All lentiviral constructs are specified below.

Animals and surgery

Colorectal liver metastases were induced as described, using male BALB/c or BALB/c-AnNCrl-*Nu*BR mice aged 8-10 weeks (Charles River Laboratories, Maastricht, The Netherlands)[18]. In separate experiments C26-luc, C26-KrasKD-luc, DLD1-luc, or DKO4-luc cells, suspended in 50 µl PBS, were injected just under the capsule of the left liver lobe after a midline abdominal incision.

Intravital microscopy

Liver metastases were induced by intrasplenic injection as described above. On post-operative days (POD) 0, 3, and 7 (n=3/day/group) the liver was exposed through a midline abdominal incision. Intravital fluorescence microscopy was performed using a Nikon TE-300 inverted microscope (Uvikon, The Netherlands). 10 random fields (magnification 100X) per animal were recorded digitally with a charge coupled device camera (Exwave HAD, Sony, The Netherlands) and stored for off-line data analysis. Data are average counts performed by two independent observers blinded to treatment.

Confocal microscopy

Cells were fixed in 3.7% formaldehyde for 10 minutes and were subsequently permeabilized (PBS 0.05% TritonX100; 1% BSA; 2 min) and blocked (PBS 3% BSA; 1h). Anti-CD95 primary antibody and FITC-coupled secondary antibody with Alexa-568- conjugated phalloidin were incubated for 1hour. Coverslips were mounted in Vectashield with DAPI (H-1200, Vectorlabs). Image acquisition and analysis was performed using a Zeiss Axiovert 200M and Zeiss LSM 510 Software.

Viability assays

Cells (5000 cells/96-well) were stimulated with CD95L (2 ng/ml to 10 ng/ml) or TRAIL (25 ng/ml to 100 ng/ml) and viability was analyzed 24h later by 3-(4,5dimethylthiazolyl-2)-2,5-

diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) according to the manufacturer's instructions. For anoikis assays the cells were cultured under non-adherent conditions at 37°C in a humidified atmosphere under constant low speed rotation. Cells were collected by centrifugation (1500 rpm, 3 min) at the indicated time points and cell viability was subsequently assessed by MTT assays.

CD95 ligand, TRAIL and anti-CD95

The following reagents were used in this study: CD95 Ligand (FasL), membrane bound (#01-210) from Upstate Cell Signaling Solutions, Lake Placid, NY. HisTRAIL: The bacterial expression plasmid pETdwHisTRAIL(114-281) for human TRAIL was kindly provided by Dr. D.W. Seol. His-TRAIL was purified by using the His-bind Resins beads and buffer systems from Novagen. Recombinant isoleucine zipper-tagged murine TRAIL (izmuTRAIL) was generated and purified as described[19]. To remove endotoxins, an additional washing step with 0.1% Triton-X-114 was used during the purification procedure. The protocol for endotoxin removal was adapted from[20]. Anti-CD95 CH11 was used at a concentration of 10 ng/ml.

TRAIL treatment *in vivo*

Mice carrying 2-day micrometastases received isoleucine zipper-tagged murine TRAIL (iz-muTRAIL; 6 mg/kg bodyweight/day) (n=4) or vehicle (n=4) for 2 days via tail vein injections. Livers were then harvested and analyzed by confocal microscopy. The length of the longest distance between tumor cells in each metastasis was measured in 10 randomly chosen metastases per mouse using Zeiss LSM image examiner software. These values were used to calculate the average longest trail-length within metastases in control- and TRAIL-treated animals.

Live cell imaging and motility assays

Cells were seeded in a Lab-Tek® Chambered #1.0 Borosilicate Coverglass System (Nalgene Nunc International, Rochester, NY14625, USA) and were mounted on a Zeiss Axiovert 200M microscope for live cell imaging (5% CO2; 37°C) overnight. Phase contrast images were captured every 5 min using a Photometrics Coolsnap CCD camera (Scientific, Tucson, AZ). Images were processed using Metamorph software (Universal Imaging, Downington, PA). The number of cells displaying membrane ruffling was scored by off-line analysis of the generated videos and plotted as means ± SEM from all cells in 20 videos from 3 independent dishes. For motility assays cells were filmed for 48h (20 videos; 3 dishes). The average speed of tumor cells in each dish was then quantified in pixels per hour. For studying directed cell migration either CD95L or isoleucine-zipper-tagged murine TRAIL (iz-muTRAIL) were mixed with soluble Matrigel which was then allowed to solidify in a small droplet in the middle of a culture dish in 24 well format. Cells were seeded into the dish and allowed to migrate towards, or away from, the Matrigel during 24h incubation. Cells were then fixed and stained with crystal violet.

Invasion assay

Invasion was measured using 24-well BioCoat Matrigel invasion chambers (BD Biosciences, Alphen aan den Rijn, The Netherlands) according to the manufacturer's instructions using CD95L (20 ng/ml) or iz-muTRAIL (200 ng/ml) as stimuli.

Immunohistochemistry

Mouse livers were harvested and fixed in 4% buffered formaldehyde and embedded in paraffin for further immunohistochemical processing. Anti-active caspase 3 was used for assessment of apoptotic tumor cells. As secondary antibody PowerVision+ (Immunologic, Duiven, The Netherlands) with 2% mouse serum was used. Reactions were developed using diaminobenzidine/ H_2O_2 as a chromogen substrate. Primary antibody-omitted negative controls were treated with the antibody diluent alone and were all free of non-specific background staining. Quantification was performed by counting the number of active capase-3-positive cells in 20 randomly chosen high-power fields in 3 non-consecutive tissue sections in 4 mice. The bar diagrams represent the average numbers of caspase-3-positive cells per field in 20x3x4 fields.

FACS analysis

For cell surface CD95 expression, the cells were plated onto six-well plates at a density of $4x10^5$ cells/well. 24 Hours after plating the cells were trypsinized, washed in PBS/BSA 1% and incubated for 1 hour on ice with anti-CD95 (CH11). Secondary antibodies were fluorescein isothiocyanate-conjugated anti-mouse IgM. For sub-G1 DNA content analysis, cells were stimulated with either CD95L or TRAIL and were harvested by centrifugation, washed with PBS and then fixed in chilled 80% ethanol on ice. The cells were washed and suspended in PBS and treated with RNase A (200 µg/ml) at room temperature for 30 min. The cells were then stained with Propidium Iodide (50 µg/ml) for 30 min. All samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

C26-KrasKD cells were transfected with the dominant-negative GFP-LIMK-kinase dead mutant and cells were either left untreated or were stimulated with CD95L (10 ng/ml) overnight. Cells were trypsinized and stained with propidium iodide to determine the amount of apoptotic (PI-positive) cells by FACS.

Cell fractionation

Cells were lysed in 0.1% Triton buffer (20 mM Hepes pH7.4, 0.1% TrirtonX100, 150 mM NaCl, 5 mM MgCl2, 10% glycerol). Lysates were then cleared by sequential low speed (13.000 rpm Eppendorff tablecentrifuge) and high speed (Beckmann airfuge, 100.000g) centrifugation at 4°C.

Antibodies and reagents

The following antibodies were obtained from Cell Signaling Technology Inc., Danvers, MA: mouse anti-caspase-8 (1C12, #9746), rabbit anti-FADD (human specific, #2782), anti-Raf1

(#9422), anti-A-Raf (#4432) anti-B-Raf (L12G7, #9434), phospho-ERK1/2 T202/T204 (#9101), phospho-Akt Ser473 (193H12, #4058), anti-Akt (#9272), phospho-cofilin S3 (#3311), cofilin (#3312), phospho-ezrin Thr567 (#3141), ezrin (#3145), and anti-RalB (#35235). Mouse anti-Fas (human, activating), clone CH11 (#05-201) was obtained from Upstate Cell Signaling Solutions, Lake Placid, NY. Anti-human TRAILR1/DR4 was obtained from R&D Systems Europe Ltd., Abingdon, UK (#AF347). Anti-DR5 (# 160770) was obtained from Cayman Chemical, Ann Arbor, MI. Anti-FLIP (F9800) was obtained from Sigma-Aldrich, Saint Louis, Missouri. Mouse anti-Bcl2 (#M0887, clone 124) was obtained from DAKO, DK-2600 Glostrup, Denmark. Anti-active caspase 3 for immunohistochemistry (BD559565), anti-RalA (BD610221) and anti-ROCK (BD611136) were obtained from BD Biosciences, San Jose, USA. Mouse anti-β-actin was purchased from Novus Biological, Littleton, CO (NB 600-501).

Expression vectors

The cDNA encoding dominant-negative FADD (DN-FADD) was cut from pRVQ7- FADD-DN (kindly provided by Dr. J. Borst) by using BamH1/Xho1, and ligated into BamH1/Sal1-cut pWPT-GFP to generate pWPT-dnFADD. pWPT-GFP and the lentiviral packaging system were kindly provided by Prof D. Trono. All lentiviral knockdown vectors were in the pLKO1 background and were purchased from Open Biosystems (Huntsville Al, USA). A panel of 5 constructs was tested for each set. The following vectors were used for stable knockdown of Raf1 (TRCN0000055140 (#1), TRCN0000055138 (#2),TRCN0000055142 (#3)), B-Raf (TRCN0000054345), RalA (TRCN0000004867, TRCN0000004868), RalB (TRCN0000072954, TRCN0000072955). The ROCK targeting sequences were: TGTCGAAGATGCCATGTTA (#1) and GACCTTCAAGCACGAATTA (#2) were cloned into the lentiviral shRNA vector pLV-THM (AddGene). The lentiviral constructs targeting Erk1 and Erk2 were kindly provided by Dr Riccardo Brambilla and were described before[21]. pREP4-Bcl2 was kindly provided by Dr. A Shvarts. The caspase-8a and –b open reading frames were excised from pIRES2-GFP-caspase-8 together with parts of the GFP and the CMV-promoter sequence. Subsequently, they were ligated into a modified pWPTS (Addgene). The final constructs were verified by sequencing. pRRL-CMV-luciferase was kindly provided by Dr. RC Hoeben. Expression constructs for GFP-LIMK and GFP-LIMKkinase-dead[22] were kindly provided by Prof John Condeelis. The expression construct for constitutively active ROCK (ROCKΔ3)[23] was kindly provided by Prof Christopher Chen.

Statistical Analysis

Differences between groups were evaluated using the Mann-Whitney test. Results are presented as means ± standard error of the mean. All P values were two-tailed. $P < 0.05$ was considered statistically significant.

RESULTS

An essential role for oncogenic K-Ras in liver metastasis formation

To assess the contribution of endogenous oncogenic K-Ras to liver metastasis formation we analyzed how deletion or suppression of endogenous oncogenic *K-Ras* alleles from colorectal tumor cells affected tumor growth in the liver when compared to subcutaneous tumor growth.

DLD1 cells and isogenic cells lacking *K-Ras*^{D13} (DKO4) were injected into the liver parenchyma. DLD1 cells formed liver tumors in all injected mice, but DKO4 cells were unable to form liver tumors (Figure 1A, Table 1). However, the *in vitro* proliferation rate for this pair of isogenic cells was very similar and both cell lines formed subcutaneous tumors with an incidence of 100% (Table 1). Subcutaneous DKO4 tumors did not acquire de novo mutations in the remaining wild type allele during tumor formation (data not shown).

Next, we tested whether suppression of K-Ras^{D12} in C26 cells (C26-KrasKD) had a similar selective effect on liver tumor formation. C26 control cells formed tumors both in the liver and under the skin with 100% incidence (Figure 1B, Table 1). C26-KrasKD cells failed to form liver tumors, either when cells were injected via the spleen or directly into the liver parenchyma (Figure 1B, Table 1), but were able to form benign, non-invasive subcutaneous tumors in 30-40% of the mice (Table 1, Smakman et al[16]).

Table 1. Deletion of oncogenic K-Ras abrogates liver tumor formation but not subcutaneous tumor growth

Cell line	KRAS/Kras status	Injection site	Incidence (%)	Invasion	Morbidity
DLD1	D13/wt	subcutaneous	100 (7/7)	yes	yes
DKO4	Δ/wt		100 (7/7)	no	no
C26-pLL	D12/-		100 (7/7)	yes	yes
C26-KrasKD	D12-KD/-		30 (3/10)	no	no
DLD1	D13/wt	spleen	0 (0/7)	-	-
DKO4	Δ/wt		0 (0/7)	-	-
C26-pLL	D12/-		100 (7/7)	yes	yes
C26-KrasKD	D12-KD/-		0 (0/6)	-	-
DLD1	D13/wt	liver	100 (6/6)	yes	yes
DKO4	Δ/wt		42 (3/7)*	NA	no
C26-pLL	D12/-		100 (6/6)	yes	yes
C26-KrasKD	D12-KD/-			-	-

DLD1, DKO4, C26-pLL and C26-KrasKD cells were injected either subcutaneously, into the spleen, or under the liver capsule. The effect of tumor growth on health score was monitored as in[3], and *post mortem* analysis of skin invasion was performed by Haematoxilin and Eosin histochemistry. Subcutaneous tumor growth was measured by standard caliper measurements; liver tumor growth was measured by bioluminescence. D12-KD: Stable knockdown of the Kras^{D12} mRNA[3]. C26 cells have lost the wt Kras allele. *Although very low bioluminescence signals were detected in 3/7 livers injected with DKO4 cells, the tumors remained extremely small over time and had no effect on the health status of the mice (See Figure 1A). NA: not analyzed.

Intravital microscopy showed that the inability of C26-KrasKD cells to form liver tumors was caused by a rapid clearance of tumor cells from the liver (Figure 1C). Immunohistochemistry on liver tissue sections revealed the presence of high numbers of apoptotic (active caspase-3-positive) tumor cells in mice injected with C26-KrasKD cells but not in mice injected with control C26 cells (Figure 1D). These results suggest that endogenous oncogenic K-Ras is required for tumor cell resistance to apoptotic stimuli in the liver.

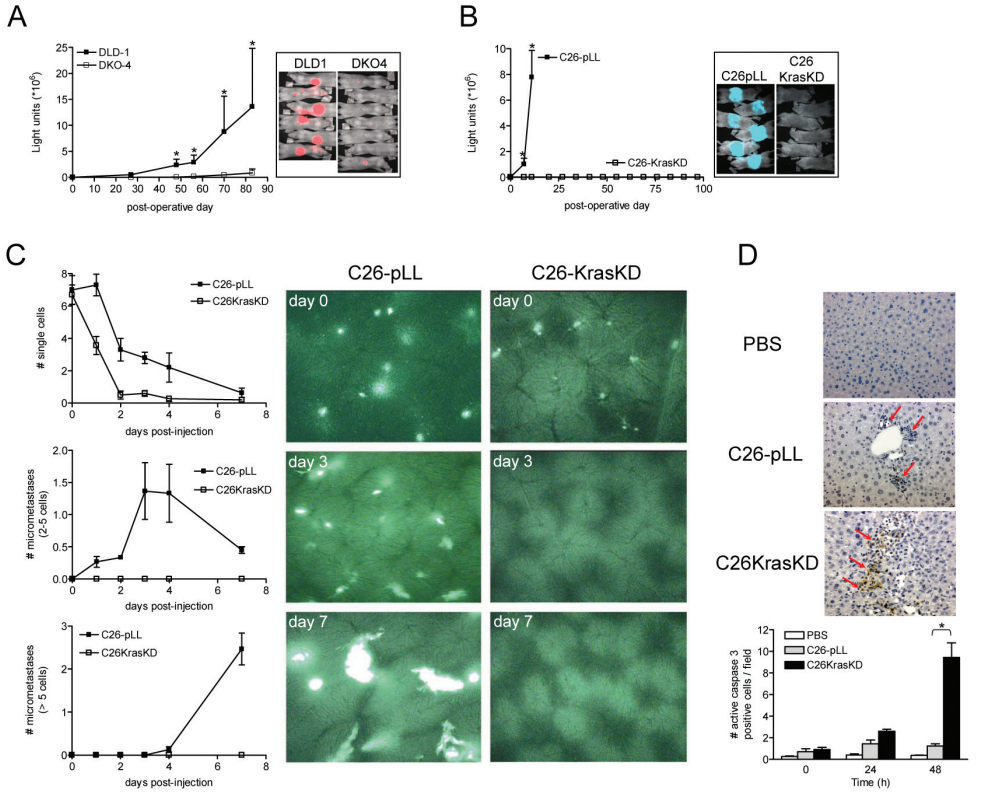

Figure 1. Oncogenic Kras is essential for liver metastasis formation and for tumor cell survival in the sinusoids. (**A**) Luciferase-expressing DLD1 and DKO4 cells were injected directly into the liver parenchyma of immune deficient BALB/c$^{nu/nu}$ mice (n=7) and bioluminescence imaging was used to detect tumor growth over time. (**B**) Luciferase-expressing C26-pLL and C26-KrasKD cells were injected into the spleen of syngenic BALB/c mice (n=6) and liver metastasis formation was assessed over time by bioluminescence imaging. (**C**) EGFP-expressing C26-pLL and C26-KrasKD cells were injected into the spleens of BALB/c mice (n=3) and tumor cell fate in the liver sinusoids was followed by intravital microscopy. The number of single cells in the liver sinusoids and the development of micrometastases (2-5 cells and >5 cells) was then scored by off-line analysis of the generated videos. Quantification of these data is shown in the left panel. Stills from representative videos are shown in the right panel. (**D**) After intrasplenic injection of C26-pLL and C26-KrasKD cells, the livers were harvested at the indicated time points. The number of apoptotic cells was then assessed by immunohistochemistry using an antibody recognizing activated (cleaved) caspase 3, and the number of active caspase-3 positive cells was quantified as detailed in the M&M section. * $p<0.05$. All data are presented as means ± SEM.

Oncogenic K-Ras provides resistance to apoptosis induced by death receptor ligands

Metastatic tumor cells have to survive the apoptotic stimulus that results from loss of adhesion (anoikis). In addition, immune cells in the liver attack tumor cells, in part by producing death receptor ligands like CD95L and TRAIL.

The K-Ras status had no discernable effect on anoikis in DLD1 and C26 cells (Figure 2A). However, tumor cells lacking oncogenic K-Ras (DKO4, Hkh2 and C26-KrasKD) were highly sensitive to apoptosis induction by CD95L and TRAIL and by the CD95 agonistic antibody CH11 (Figures 2B, 3A and 3B). Re-expression of oncogenic K-Ras in Hkh2 cells provided renewed resistance to CD95L-induced apoptosis (Figure 2C).

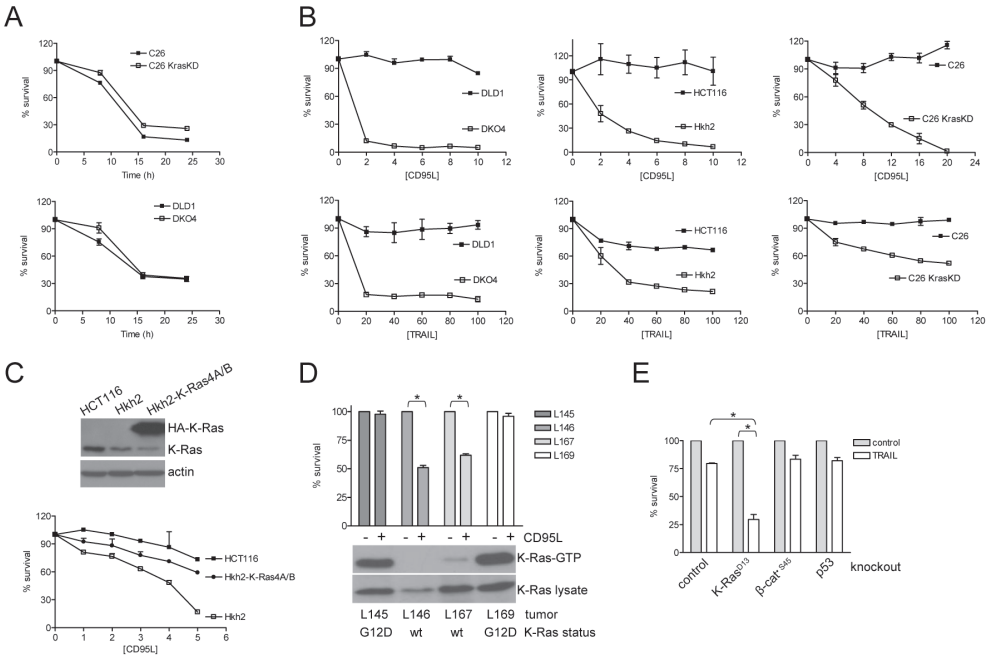

Figure 2. Oncogenic K-Ras provides resistance to apoptosis induced by death receptor ligands, but not by anoikis. (**A**) C26, C26-KrasKD, DLD1 and DKO4 cells were cultured under non-adherent conditions for the indicated periods of time and cell viability was then measured by MTT assays. The data shown represent means of triplicates. (**B**) DLD1 and DKO4, HCT116 and Hkh2, and C26 and C26-KrasKD cells were treated with CD95L (2-10 and 4-20 ng/ml), hisTRAIL (20-100 ng/ml) or iz-muTRAIL (20-100 ng/ml) for 24 hours. Cell viability was then assessed by MTT assays. (**C**) Hkh2 cells were transduced with lentiviral expression vectors for HA-tagged K-Ras4A and 4B. These cells were exposed to CD95L and cell viability was measured as in B. (**D**) Spheroid cultures were established from biopsies of liver metastases of 4 different colon cancer patients. Single cell suspensions were prepared and these were stimulated overnight with CD95L (6 ng/ml). Cell viability was assessed as above. The presence of active (GTP-bound) K-Ras was analyzed in parallel using lysates of the same cell lines (lower panel). K-Ras mutation status was analyzed by sequencing and is indicated at the bottom. (**E**) HCT116, Hkh2, HCT116$^{\Delta-\beta CAT\Delta S45}$ and HCT116$^{p53-/-}$cells were treated with hisTRAIL (100 ng/ml) for 24h and cell viability was assessed as above. Data are presented as means ± SEM. * p<0.05.

We next used four low-passage spheroid cultures that were established from freshly resected liver metastases of colorectal cancer patients. Two of these cultures expressed wild type K-Ras, and two expressed constitutively active oncogenic K-Ras (G12D) (Figure 2D). Spheroid cultures expressing wildtype K-Ras rapidly lost viability (~50%) following overnight exposure to CD95L (Figure 2D), whereas those expressing oncogenic K-Ras were resistant.
Deletion of oncogenic K-Ras, but not β-catenin$^{\Delta S45}$ or p53, sensitized HCT116 cells to TRAIL-induced apoptosis (Figure 2E). Thus, of the three most common genetic alterations in colorectal tumors, only the *K-Ras* oncogene provides resistance to apoptosis induction by death receptor activation.

Deletion of oncogenic K-Ras allows caspase-8 processing by death receptor ligands

Activation of caspase-8 in the death-inducing signaling complex (DISC) is essential for apoptosis induction by CD95L[24] and TRAIL[25]. DLD1 cells express low levels of caspase-8 but re-expression of caspase-8 did not sensitize these cells to CD95L or TRAIL (Figures 4A, 4B and not shown). Resistance was accompanied by a failure to process caspase-8 in the DISC, despite expression of CD95, TRAIL-R1, TRAIL-R2, cFLIP and FADD. (Figures 3C, 3D and 4A). Deletion of oncogenic K-Ras allowed CD95L-stimulated caspase-8 processing (Figures 4A and 4B) but had no effect on caspase-8 processing during anoikis (Figure 4C).

CD95L is an attractant for tumor cells expressing oncogenic K-Ras

The apoptosis-resistant cell lines that were exposed to CD95L displayed extensive membrane ruffling but their derivatives expressing only wildtype K-Ras did not (Figure 5A and not shown). This suggests that CD95 signaling is not simply reduced in cells expressing oncogenic K-Ras, but

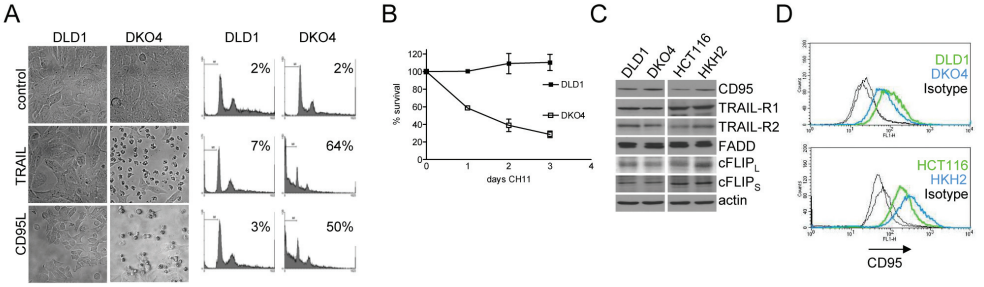

Figure 3. Oncogenic K-Ras protects colorectal cancer cells from CD95-induced apoptosis without affecting expression of DISC components. (**A**) DLD1 and DKO4 cells were treated with CD95L (6 ng/ml) or TRAIL (20 ng/ml) overnight. Cells were then photographed (left panels), stained with propidium iodide and analyzed by FACS (right panels). The percentage of apoptotic cells (with sub-G1 DNA content) is indicated in the graphs. (**B**) DLD1 and DKO4 cells were treated with the agonistic anti-CD95 CH11 antibody (10 ng/ml) for 3 consecutive days and cell viability was then assessed by standard MTT assays. (**C**) DLD1, DKO4, HCT116 and HKH2 cells were lysed and the steady state levels of FADD, cFLIP$_L$, and cFLIP$_S$ were analyzed by Western blotting. (**D**) Cell surface levels of CD95 on HCT116, Hkh2, DLD1 and DKO4 cells were tested by FACS analysis.

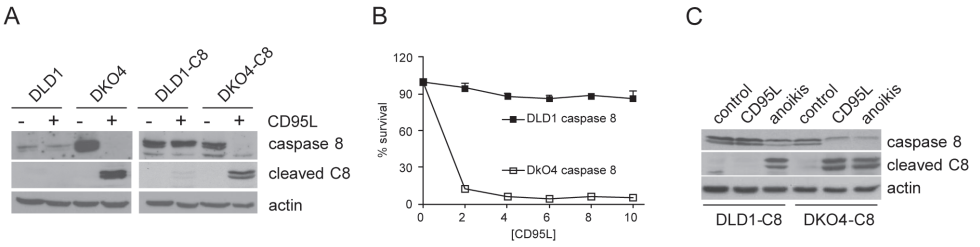

Figure 4. Oncogenic K-Ras prevents caspase-8 cleavage in response to CD95L, but not during anoikis. (**A**) DLD1 and DKO4 cells and DLD1 and DKO4 cells ectopically expressing caspase-8a and 8b (DLD1-C8, DKO4-C8) were either left untreated or were treated with CD95L (2 ng/ml) for 24 hours. Caspase-8 cleavage was then assessed by Western blot analysis. (**B**) DLD1-C8 and DKO4-C8 cells were exposed to CD95L (2 -10 ng/ml) for 24h. Cell viability was assessed by MTT assays and caspase 8 processing by Western blot analysis. Data are presented as means ± SEM. (**C**) DLD1-C8 and DKO4-C8 cells were either stimulated with CD95L (2 ng/ml) or were grown under non-adherent conditions to promote anoikis. Caspase-8 processing was then assessed by Western blot analysis.

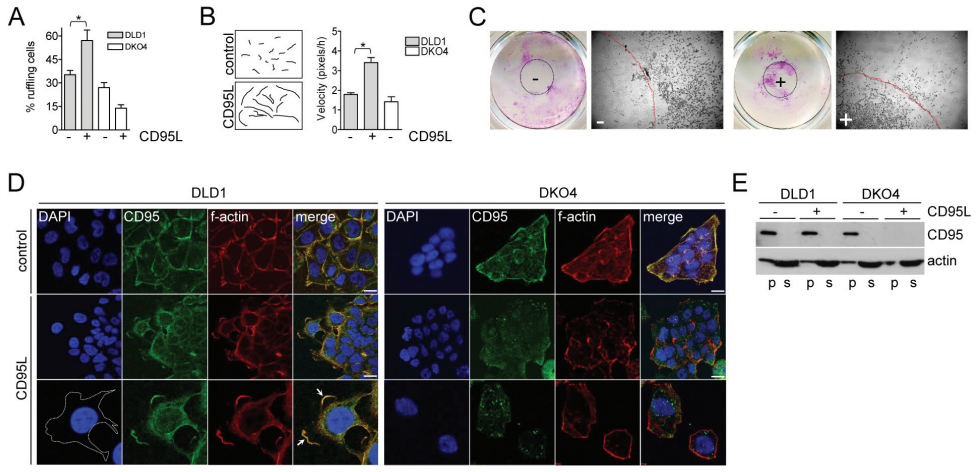

Figure 5. CD95 ligand is an attractant for tumor cells expressing oncogenic K-Ras. (**A**) DLD1 and DKO4 cells were stimulated with CD95L (2 ng/ml) and the number of cells with extensive ruffling was analyzed by live cell imaging. (**B**) DLD1 and DKO4 cells were treated with CD95L (2 ng/ml) for 48 hours and were analyzed by live cell imaging. The migration trails of single tumor cells are depicted in the left panel. The right panel shows a quantification of average tumor cell speed. CD95L-stimulated DKO4 cells failed to migrate but underwent apoptosis. (**C**) A droplet of Matrigel containing no (-) or 20 ng/ml CD95L (+) was allowed to set in the middle of a tissue culture dish. DLD1 cells were plated and after 24h cells were stained with crystal violet and fixed. Representative images are shown. The borders of the Matrigel drops are indicated by red dotted lines. (**D**) Immunofluorescence analysis of CD95 (green) and f-actin (red) in DLD1 cells (left panel) and DKO4 cells (right panel). Cells were left untreated or were stimulated with CD95L (2 ng/ml) for 2 hours. Arrows indicate CD95 at the leading edge in CD95L-stimulated DLD1 cells. (**E**) DLD1 and DKO4 cells were treated overnight with CD95L (6 ng/ml). Detergent lysates were prepared and cleared by high speed centrifugation. CD95 levels in the high-speed pellet (p) and sup (s) fractions were subsequently analyzed by Western blotting. * $p<0.05$.

that alternative pathways may be activated. Live cell imaging showed that exposure of DLD1 cells to CD95L increased motility by approximately 2-fold (Figure 5B). In addition, CD95L stimulated the closure of monolayer wounds in scratch assays (data not shown). Directed migration assays showed that CD95L acts as an attractant for apoptosis-resistant tumor cells (Figure 5C).
Apoptosis signaling by CD95 is associated with its internalization[26]. Immuno-fluorescence analysis showed that CD95 is localized at the cell periphery both in DLD1 and DKO4 cells (Figure 5D). Furthermore, FACS analysis showed that cell surface expression of CD95 was unaffected by K-Ras status (Figure 3D). CD95L stimulation induced internalization of CD95 in DKO4 cells. In DLD1 cells however, activated CD95 localized to cortical f-actin-rich plasma membrane areas, including those at the leading edge of migrating cells (Figure 5D). In DKO4 cells, internalized CD95 was completely degraded after overnight stimulation, but CD95 in DLD1 cells was resistant to degradation (Figure 5E). Similar results were obtained in the HCT116/Hkh2 cell system (not shown). Thus, oncogenic K-Ras prevents CD95 from joining the classical internalization/degradation pathway.

Systemic administration of TRAIL stimulates invasion of K-RasD12-expressing micrometastases in the liver

The safety and efficacy of recombinant TRAIL or agonistic TRAIL-R2 antibodies is currently being tested in different patient groups, including those with metastatic colorectal cancer. Both CD95L and TRAIL induced invasion of C26 cells in Matrigel *in vitro* (Figure 6A). Therefore, we assessed whether systemic administration of TRAIL affected the invasive behavior of micrometastases in the liver. Micrometastases in control mice displayed a compact morphology with sharply demarcated edges (Figure 6B). In contrast, micrometastases in TRAIL-treated mice displayed a highly invasive phenotype with cells detaching from the core of the metastasis and invading the surrounding parenchyma (Figure 4B). TRAIL treatment increased the average distance between tumor cells at the extreme edges of individual metastases by approximately 3-fold (Figure 6B). TRAIL treatment had no apparent effect on the number of liver metastases, nor did we observe apoptotic GFP-positive tumor cells. Thus, rather than promoting their clearance, TRAIL induced dissemination of micrometastatic tumor cells through the liver parenchyma.

Invasion of apoptosis-resistant tumor cells requires oncogenic K-Ras but not capsase-8 recruitment

We next tested whether caspase-8 recruitment was required for CD95-mediated invasion. To this end, dnFADD was expressed in DLD1 and DKO4 cells. dnFADD abrogated CD95L-stimulated caspase-8 processing and apoptosis in DKO4 cells (Figures 7A and 7B). In contrast, neither dnFADD nor overexpression of capsase-8 affected CD95L-stimulated invasion of DLD1 cells (Figure 6C). Thus, caspase-8 recruitment is not required for CD95L-stimulated invasion of DLD1 cells.

The finding that CD95L causes invasion of apoptosis-resistant tumor cells raises the question whether in apoptosis-prone cells the induction of apoptosis overrides the invasion signal, or whether these are two independent modes of receptor signaling. We tested this by expressing Bcl-2 in apoptosis-prone DKO4 cells. DKO4-Bcl-2 cells were protected from CD95L-induced apoptosis (Figure 7C), but they failed to undergo invasion in response to CD95L (Figure 6C). Moreover, DKO4 cells that undergo apoptosis do not show CD95L-induced ruffling in the period preceding cell death (data not shown).

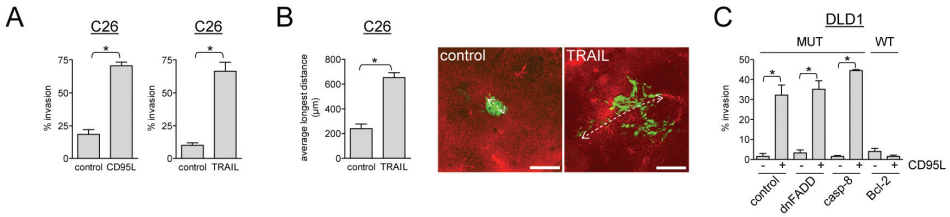

Figure 6. Oncogenic K-Ras allows death receptors to signal invasion. (**A**) Transwell invasion assay using C26 cells stimulated with either CD95L or TRAIL. Graphs represent means of triplicates. (**B**) C26-GFP cells were injected into the spleen and micrometastases were allowed to develop for 2 days, prior to treatment with TRAIL (6 mg/kg body weight/day), or vehicle, for 2 days. All mice were injected with Rhodamine-conjugated dextran to visualize the microvasculature. The livers were harvested and analyzed by confocal fluorescence microscopy. Representative examples of micrometastases in control-treated (left panel) and TRAIL-treated (right panel) mice are shown. (Scale bars: 150 μm). The lengths of the longest tumor cell trails in each of the metastases were measured (10 metastases per liver) and plotted. Data are presented as means ± SEM. *denotes statistical significance ($p<0.05$). (**C**) Transwell invasion assay using DLD1 control cells, DLD1 cells expressing dnFADD or caspase-8, and DKO4 cells expressing Bcl-2. * $p<0.05$.

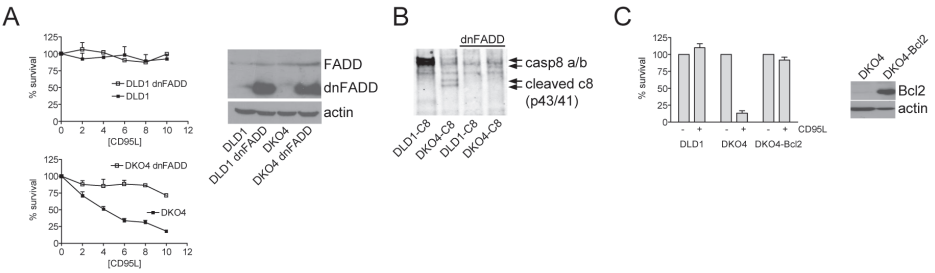

Figure 7. Protection of DKO4 cells from CD95-induced apotposis by dnFADD and Bcl2. (**A**) DLD1-caspase-8 and DKO4-caspase-8 cells were transduced with a lentiviral vector driving expression of dnFADD. Cells were stimulated with CD95L overnight (2 ng/ml) and cell viability was measured using MTT assays. dnFADD protected DKO4 cells from CD95L-induced apoptosis. (**B**) DLD1, DLD1-dnFADD, DKO4 and DKO4-dnFADD cells were stimulated with FLAG-TRAIL (5 μg/ml) for 15 minutes. Cell extracts were prepared and anti-Flag immunoprecipitation was performed overnight. The presence of caspase-8 in the immunocomplexes was then analyzed by Western blotting. (**C**) DKO4 cells were transfected with pREP4-Bcl2 or empty pREP4, and stable polyclonal cell lines were generated by selection in Hygromycin B medium. DLD1, DKO4-pREP4, and DKO4-pREP4-Bcl2 cells were then stimulated with CD95L overnight (2 ng/ml) and cell survival was measured by MTT assays. Bcl2 expression protected DKO4 cells from CD95L-induced apoptosis.

Oncogenic K-Ras requires Raf1 to prevent apoptosis induction by CD95

K-Ras signals through several distinct effector pathways. We took an RNAi- and inhibitor-based approach to assess which of these pathways mediates the switch in CD95 signaling output. Lentivirus-mediated RNAi-suppression of A-Raf, B-Raf, ERK1+ERK2, RalA, or RalB, or inhibition of MEK (with U0126) or PI(3)-kinase (with LY294002) failed to sensitize tumor cells to CD95L-stimulated apoptosis in the presence of oncogenic K-Ras (Figure 8A). However, suppression of Raf1 using 2 independent targeting sequences sensitized C26 cells to apoptosis induction by CD95L (Figures 8A and 8B). This was accompanied by activation (cleavage) of caspase-3, but not by reduced phosphorylation of ERK1 or ERK2 (Figure 8C). Raf1 suppression did not further sensitize C26-KrasKD cells to CD95L (Figure 8D). Thus, Raf1 suppression primarily affects tumor cell sensitivity to CD95L in the presence of oncogenic K-Ras.

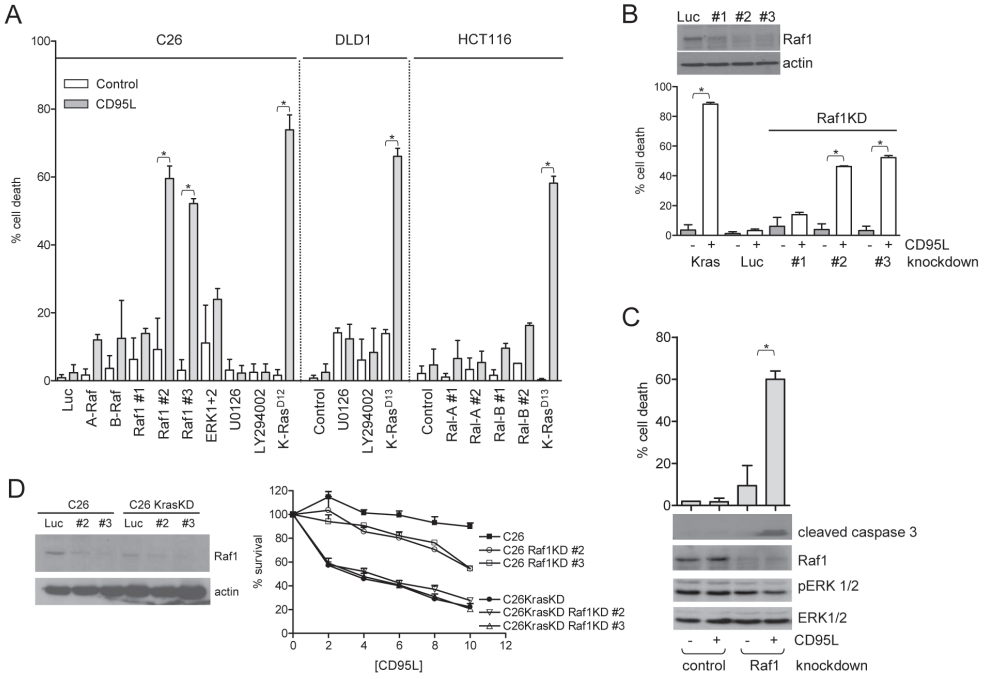

Figure 8. Raf-1 mediates K-Ras-dependent apoptosis resistance. (**A**) C26, DLD1 and HCT116 cells were infected with lentiviral RNA-interference constructs targeting the indicated genes. In addition, cells were treated with the MEK inhibitor U0126 or with the PI(3) kinase inhibitor LY294002 as indicated. Cells were then stimulated with CD95L and cell viability was measured as in Figure 2. (**B**) C26 cells were infected with three different Raf1-targeting lentiviral RNAi constructs. Cells were stimulated with CD95L and cell viability was measured as above. Knockdown efficiency was analyzed by Western blotting. (**C**) Control and Raf1-knockdown cells were stimulated with CD95L overnight. Cells were then analyzed for viability as above. In addition, the effect of Raf1 knockdown on caspase-3 cleavage (activation) and ERK phosphorylation was assessed by Western blotting. (**D**) C26 control and C26-KrasKD cells were transduced with control (Luc) or Raf1 knockdown constructs (#2, #3) as indicated and Raf1 expression was assessed by Western blotting. Cells were stimulated with the indicated concentrations of CD95L overnight and cell viability was assessed as above. * p<0.05.

Raf1 is critical for K-Ras-driven invasion and liver metastasis formation

To test whether the restoration of apoptosis signaling by CD95 in Raf1-suppressed cells would be accompanied by loss of its ability to stimulate invasion, Raf1 knockdown cells were treated with the caspase inhibitor z-VAD. Raf1 knockdown cells, protected by z-VAD, displayed a greatly reduced potential to invade Matrigel in response to CD95L (Figure 9A). z-VAD had no effect on C26 control cells (Figure 9A). Thus, Raf1 knockdown switched the signaling output of activated CD95 from invasion mode back to apoptosis mode, in the presence of K-Ras^{D12}. Importantly, knockdown of Raf1 also strongly reduced the metastatic potential of C26 tumor cells, despite the continued presence of oncogenic K-Ras^{D12} (Figure 9B). This was due to massive apoptosis of tumor cells in the liver (Figure 9C), similar to K-Ras-suppressed cells (Figure 1D). These results identify Raf1 as a critical mediator of the survival of metastatic tumor cells in the liver and as a critical K-Ras effector during liver metastasis formation.

KRAS and Raf1 subvert CD95 signaling by suppressing the Rho/ROCK/LIM kinase pathway

We noted that knockdown of K-Ras and Raf1, but not B-Raf, caused a massive increase in the number of actin stress fibers, indicating activation of the Rho/ROCK pathway (Figure 10). In fibroblasts, ROCK facilitates apoptosis induction by CD95, possibly by phosphorylation of ezrin on T567[27]. ROCK also activates LIM kinase to phosphorylate the actin-severing protein cofilin on S3. Raf1 or K-Ras knockdown in C26 cells dramatically increased phosphorylation of cofilin S3, but did not affect ezrin T567 phosphorylation (Figure 11A). Overexpression of ROCK restored cofilin phosphorylation (data not shown) and sensitized C26 cells to CD95L-induced apoptosis (Figure 11B). Likewise, overexpression of LIM kinase sensitized both C26 and DLD1 cells to CD95L-induced apoptosis (Figure 11C). Restoration of apoptosis signaling was accompanied by stimulus-dependent degradation of CD95 (Figure 11D), similar to what was observed in K-Ras knockout cells (Figure 5E). Importantly, the metastasis-forming potential of LIM kinase-expressing C26 cells was strongly reduced (Figure 11E). Furthermore, RNAi-mediated suppression of ROCK or expression of a kinase-dead LIM kinase mutant in K-Ras knockdown cells protected these cells from CD95L-stimulated apoptosis (Figure 11F). Taken together, the results suggest that suppression of ROCK/LIM kinase by K-Ras/Raf1 is essential for altering CD95 functionality and for metastasis formation.

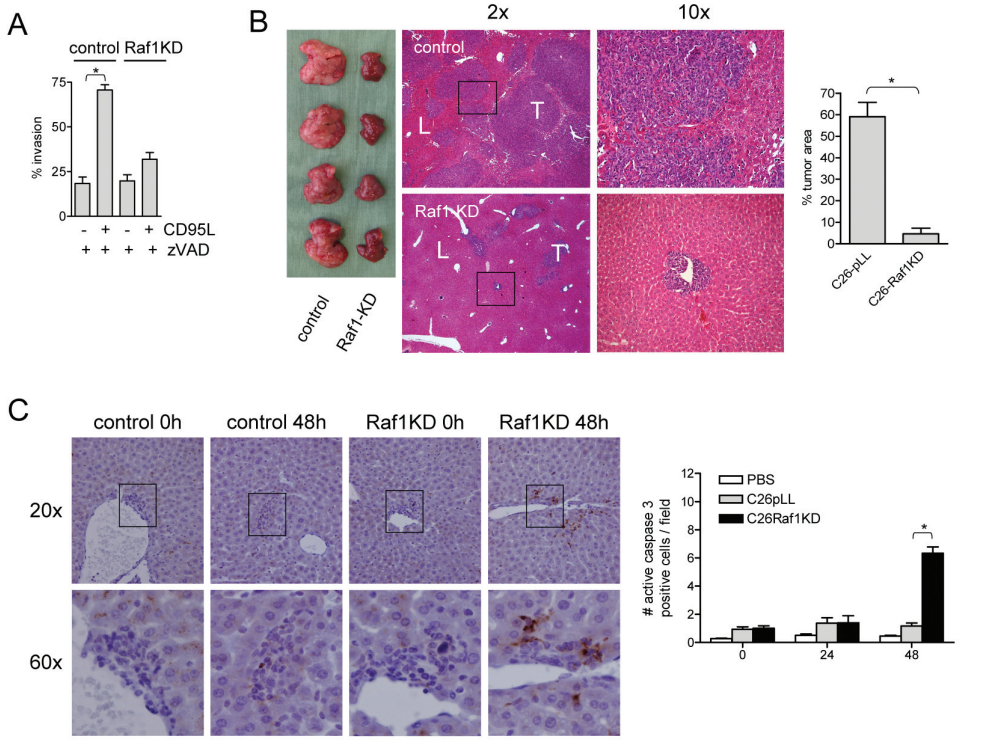

Figure 9. Raf1 is required for CD95L-stimulated invasion and K-Ras-driven metastasis formation. (**A**) Transwell invasion assay using C26 cells expressing shRNA's targeting luciferase or Raf1 in the presence of the apoptosis-inhibiting peptide z-VAD (20 µM). (**B**) C26 control and Raf1 knockdown cells were injected into the spleens of BALB/c mice (n=4) and liver metastasis formation was then assessed by morphometric assessment of the tumor-containing areas on liver tissue sections. Quantification of the % tumor areas are shown in the graph. (**C**) Micrometastases were established using either control (Luc knockdown) or Raf1 knockdown C26 cells. Apoptotic tumor cells were then quantified by anti-active caspase-3 immunohistochemistry as in Figure 1D. * p<0.05.

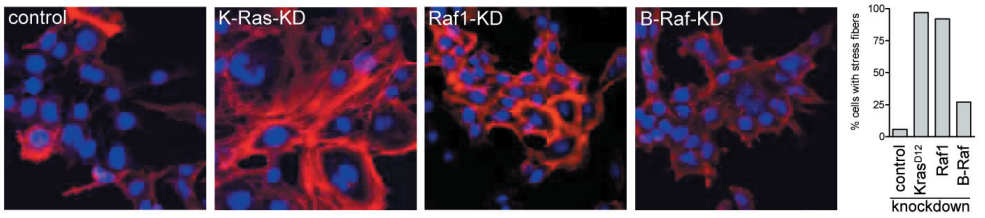

Figure 10. Stress fiber formation following Kras or Raf1 knockdown. C26 cells transduced with a lentiviral control shRNA (Luc) or with Kras-, Raf1-, or BRAF-targeting shRNA's were grown on glass coverslips. After fixation, the actin cytoskeleton was stained with phalloidin texas-red. Nuclei were counterstained with DAPI. The % of cells with stress fibers was then quantified without knowledge of the slide's identities.

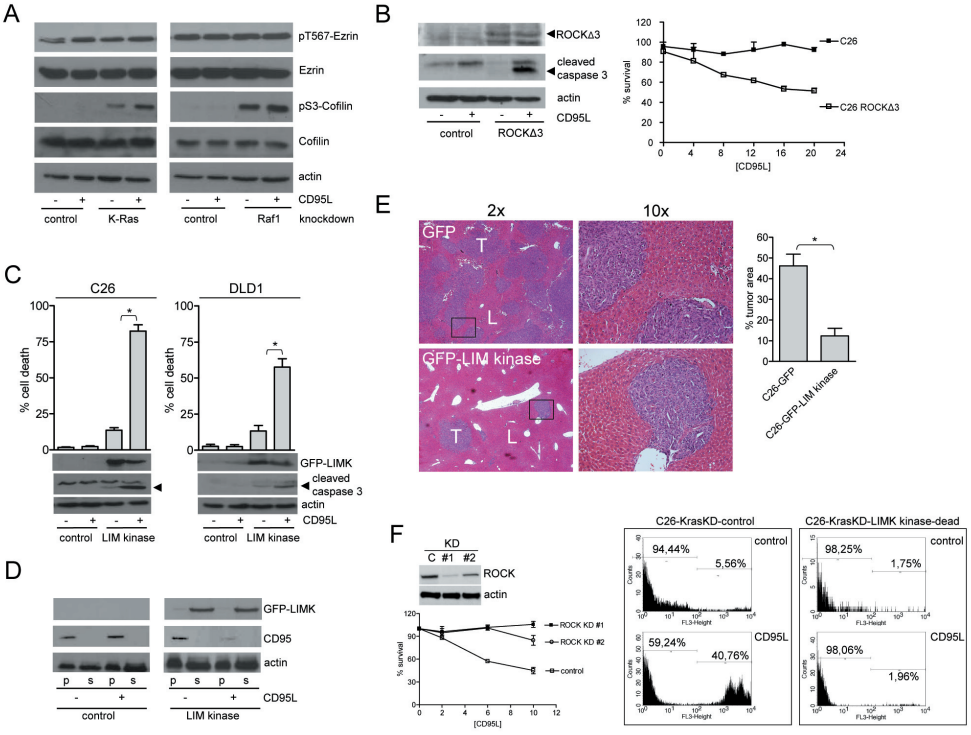

Figure 11. K-Ras-induced subversion of CD95 signaling and metastasis formation requires suppression of the Rho kinase/LIM kinase pathway. (**A**) Control, K-Ras knockdown and Raf1 knockdown C26 cells were either left unstimulated or were stimulated with CD95L 24h (10 ng/ml). Cell lysates were then analyzed for the presence of pT567-ezrin, ezrin, pS3-cofilin and cofilin. (**B**) C26 cells overexpressing ROCKΔ3 or a control vector were exposed to CD95L (20 ng/ml) for 24h. Caspase-3 processing and ROCKΔ3 expression was assessed by Western blot analysis. Cell viability was assessed by MTT assays. Data are presented as means ± SEM. (**C**) C26 and DLD1 cells were grown on glass coverslips (n=3), were transfected with an expression construct for GFP (control) or GFP-tagged LIMK, and were stimulated 24h with CD95L (10 ng/ml). Immunofluorescence analysis for active casase-3 was then performed to assess the number of apoptotic (a-casp3-positive) green cells under all conditions. Detergent lysates were prepared in parallel and were blotted for LIMK expression and active caspase-3. (**D**) DLD1 cells were transfected with an expression construct for GFP-tagged LIMK. Cells were stimulated 24h with CD95L (6 ng/ml). Detergent lysates were prepared and cleared by high speed centrifugation. CD95 levels in the high-speed pellet (p) and sup (s) fractions were subsequently analyzed by Western blotting. (**E**) FACS-sorted C26-GFP and C26-LIMK-GFP cells were injected into the spleens of BALB/c mice (n=4) and liver metastasis formation was then assessed as in Figure 7. (**F**) C26-KrasKD cells were transduced with lentiviral vectors targeting luciferase (control) or ROCK (#1 and #2). Cells were stimulated with CD95L (2-10 ng/ml) for 24 hours and cell viability was assessed by MTT assays as above. In addition, C26-KrasKD cells were transfected with an expression construct for GFP-tagged dominant negative LIMK. Cells were stimulated with CD95L for 24 hours (10 ng/ml). Cell viability of GFP-LIMK-positive and negative populations was then assessed by propidium iodide exclusion by FACS. * p<0.05.

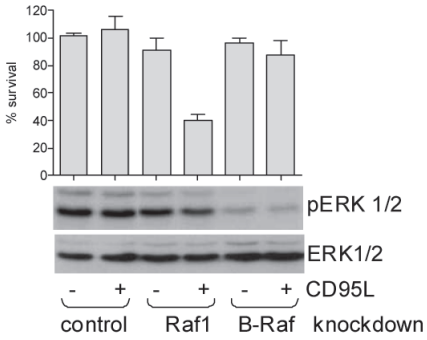

Figure 12. BRAF, but not Raf1, is required for ERK phosphorylation. C26 cells were transduced with lentiviral RNAi vectors targeting either Raf1 or B-Raf. Cells were then stimulated with CD95L (10 ng/ml) overnight and cell viability was determined by MTT assays. Parallel samples were analyzed for the effect of Raf1 and B-Raf knockdown on the levels of phosphorylated and total ERK1 and ERK2.

DISCUSSION

The factors that determine cell fate following death receptor stimulation (i.e. apoptosis, proliferation, or invasion) are poorly defined. In this manuscript we identify oncogenic K-Ras and its effector Raf1 as critical determinants of death receptor function in metastatic colorectal cancer cells. K-Ras and Raf1 do not simply suppress CD95-mediated apoptosis, but alter its signaling output to generate an invasion signal. Invasion signaling by CD95 required the continued presence of oncogenic K-Ras and Raf1 and was not secondary to apoptosis resistance. Thus, CD95 is primed to signal either apoptosis or invasion. Previous reports have uncovered death domain-dependent pathways of CD95-stimulated tumor cell invasion[10,11]. Our results show that in metastatic colorectal cancer cells CD95 retains the capacity to stimulate invasion even when its death domain is blocked. CD95 may therefore stimulate invasion via distinct pathways in distinct cell types.

The K-Ras-controlled switch in death receptor function turns one of the most potent tumor suppressor mechanisms into one stimulating tumor cell invasion. To our knowledge this is the first demonstration of such a dramatic switch in death receptor function induced by a single common oncogene. When instructed to signal invasion, CD95 and TRAIL receptors may contribute to tumor progression and metastasis formation. Indeed, components of these signaling systems are not simply lost or inactivated during tumor progression[28-35]. In fact, expression of CD95L is increased during colorectal tumorigenesis[28,31,32], is higher in metastases than in matched primary tumors[31] and is related to poor prognosis[36]. Furthermore, CD95 and CD95L are frequently co-expressed in primary colorectal tumors and liver metastases[31,37].

CD95L could contribute to tumor progression, either by promoting the expression of pro-inflammatory cytokines from neighbouring cells, by stimulating apoptosis in hepatocytes[35,37] and/or by activating non-apoptotic CD95 signalling pathways in tumor cells that retain CD95[12,13]. The latter possibility is supported by the finding that continuous CD95 stimulation on colon carcinoma cells selects highly metastatic variants[38] and overexpression of CD95 ligand in apoptosis-resistant colorectal cancer cells promotes liver metastasis formation[37].
An immediate implication of our finding that oncogenic K-Ras alters CD95 signaling output is that therapeutic targeting of death receptors by TRAIL or by agonistic TRAIL receptor antibodies could have adverse effects on disease progression by promoting invasion and dissemination of micrometastases instead of clearing them. This is a major concern, at least when considering such compounds in the treatment of tumors harboring activating mutations in the *K-Ras* gene. Currently, the safety and efficacy of recombinant TRAIL and TRAIL-receptor-activating antibodies are being tested in phase I and phase II clinical trials. These include trials in patients with metastatic colorectal, pancreatic, and non-small cell lung cancer, tumor types in which activating mutations in K-Ras are frequently observed[39]. Our results provide a rationale for testing the tumors of the patients in these trials for activating mutations in K-Ras.

The physiological function of Raf1 is to suppress apoptosis[40,41]. Strikingly, this does not require Raf1 kinase activity and is independent of its ability to activate the ERK pathway[40,41]. Our results show that this function extends to the pathophysiology of metastasis formation, as Raf1 is required for the survival of metastatic colorectal cancer cells in the liver without modulating ERK phosphorylation. Rather, B-Raf is the most effective activator of the MEK/ERK pathway (reviewed Niault and Baccarini[42]). Likewise, in the colorectal cancer cells studied here, only BRAF was essential for maintaining ERK phosphorylation (Figure 12). A-Raf is required for normal neural and intestinal development in mice[43], but it had no significant impact on the survival of colorectal cancer cells. The role of A-Raf in colorectal cancer cells is presently unknown. Taken together, the Raf kinases appear to fulfil distinct functions in colorectal cancer cells.

Raf1 suppresses apoptosis by binding and inactivating the pro-apoptotic kinases ASK1 and MST2[44,45]. CD95 activation causes disruption of the RAF1-MST2 complex, which leads to activation of MST2, and this is required for efficient apoptosis induction by CD95L[45]. A role for (disruption of) the Raf1-Ask1 complex in CD95-induced apoptosis is less clear. Raf1 also binds to and suppresses the activity of ROCK, which impairs CD95-induced apoptosis[27]. All of these studies firmly implicate Raf1 in the control of (CD95-induced) apoptosis. Our results suggest that suppression of the ROCK/LIM kinase/cofilin pathway by K-Ras/Raf1 is essential for specifying the signaling output of CD95. The cofilin pathway is a critical regulator of cortical actin dynamics, tumor cell migration, invasion and metastasis formation[46,47]. Therefore, the

control of cortical actin dynamics by K-Ras/Raf1 may underlie subversion of CD95 trafficking and signaling output. Our results add a novel dimension to the control of metastatic capacity by the cofilin pathway. By keeping ROCK and LIM kinase activities suppressed, K-Ras and Raf1 turn death receptors into pro-metastatic receptors.

REFERENCES

1. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. Science 2006; 314: 268-274.
2. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B. The genomic landscapes of human breast and colorectal cancers. Science 2007; 318: 1108-1113.
3. Jones S, Chen WD, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, Traulsen A, Nowak MA, Siegel C, Velculescu VE, Kinzler KW, Vogelstein B, Willis J, Markowitz SD. Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A 2008; 105: 4283-4288.
4. Smakman N, Borel Rinkes IH, Voest EE, Kranenburg O. Control of colorectal metastasis formation by K-Ras. Biochim Biophys Acta 2005; 1756: 103-114.
5. Koshkina NV, Khanna C, Mendoza A, Guan H, DeLauter L, Kleinerman ES. Fas-negative osteosarcoma tumor cells are selected during metastasis to the lungs: the role of the Fas pathway in the metastatic process of osteosarcoma. Mol Cancer Res 2007; 5: 991-999.
6. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, Yagita H, Okumura K. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J Exp Med 2001; 193: 661-670.
7. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, Okumura K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001; 7: 94-100.
8. Yang D, Stewart TJ, Smith KK, Georgi D, Abrams SI, Liu K. Downregulation of IFN-gammaR in association with loss of Fas function is linked to tumor progression. Int J Cancer 2008; 122: 350-362.
9. Papenfuss K, Cordier SM, Walczak H. Death receptors as targets for anti-cancer therapy. J Cell Mol Med 2008; 12: 2566-2585.
10. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J 2004; 23: 3175-3185.
11. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, Thiemann M, Mueller W, Sykora J, Kuhn A, Schreglmann N, Letellier E, Zuliani C, Klussmann S, Teodorczyk M, Grone HJ, Ganten TM, Sultmann H, Tuttenberg J, von DA, Regnier-Vigouroux A, Herold-Mende C, Martin-Villalba A. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 2008; 13: 235-248.
12. Peter ME, Legembre P, Barnhart BC. Does CD95 have tumor promoting activities? Biochim Biophys Acta 2005; 1755: 25-36.
13. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zornig M, Lynch DH. The CD95 receptor: apoptosis revisited. Cell 2007; 129: 447-450.
14. Trauzold A, Roder C, Sipos B, Karsten K, Arlt A, Jiang P, Martin-Subero JI, Siegmund D, Muerkoster S, Pagerols-Raluy L, Siebert R, Wajant H, Kalthoff H. CD95 and TRAF2 promote invasiveness of pancreatic cancer cells. FASEB J 2005; 19: 620-622.
15. Trauzold A, Siegmund D, Schniewind B, Sipos B, Egberts J, Zorenkov D, Emme D, Roder C, Kalthoff H, Wajant H. TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. Oncogene 2006; 25: 7434-7439.
16. Smakman N, Veenendaal LM, van DP, Bos R, Offringa R, Borel R, I, Kranenburg O. Dual effect of Kras(D12) knockdown on tumorigenesis: increased immune-mediated tumor clearance and abrogation of tumor malignancy. Oncogene 2005; 24: 8338-8342.

17. Shirasawa S, Furuse M, Yokoyama N, Sasazuki T. Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science 1993; 260: 85-88.
18. Smakman N, Martens A, Kranenburg O, Borel R, I. Validation of bioluminescence imaging of colorectal liver metastases in the mouse. J Surg Res 2004; 122: 225-230.
19. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ, Walczak H. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J Clin Invest 2008; 118: 100-110.
20. Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. Protein Expr Purif 2006; 46: 483-488.
21. Vantaggiato C, Formentini I, Bondanza A, Bonini C, Naldini L, Brambilla R. ERK1 and ERK2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J Biol 2006; 5: 14.
22. Wang W, Mouneimne G, Sidani M, Wyckoff J, Chen X, Makris A, Goswami S, Bresnick AR, Condeelis JS. The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. J Cell Biol 2006; 173: 395-404.
23. Pirone DM, Liu WF, Ruiz SA, Gao L, Raghavan S, Lemmon CA, Romer LH, Chen CS. An inhibitory role for FAK in regulating proliferation: a link between limited adhesion and RhoA-ROCK signaling. J Cell Biol 2006; 174: 277-288.
24. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 1997; 16: 2794-2804.
25. Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 2000; 12: 599-609.
26. Lee KH, Feig C, Tchikov V, Schickel R, Hallas C, Schutze S, Peter ME, Chan AC. The role of receptor internalization in CD95 signaling. EMBO J 2006; 25: 1009-1023.
27. Piazzolla D, Meissl K, Kucerova L, Rubiolo C, Baccarini M. Raf-1 sets the threshold of Fas sensitivity by modulating Rok-alpha signaling. J Cell Biol 2005; 171: 1013-1022.
28. Bennett MW, O'Connell J, Houston A, Kelly J, O'Sullivan GC, Collins JK, Shanahan F. Fas ligand upregulation is an early event in colonic carcinogenesis. J Clin Pathol 2001; 54: 598-604.
29. Houston A, Waldron-Lynch FD, Bennett MW, Roche D, O'Sullivan GC, Shanahan F, O'Connell J. Fas ligand expressed in colon cancer is not associated with increased apoptosis of tumor cells in vivo. Int J Cancer 2003; 107: 209-214.
30. Koornstra JJ, Kleibeuker JH, van Geelen CM, Rijcken FE, Hollema H, de Vries EG, de JS. Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. J Pathol 2003; 200: 327-335.
31. Mann B, Gratchev A, Bohm C, Hanski ML, Foss HD, Demel G, Trojanek B, Schmidt-Wolf I, Stein H, Riecken EO, Buhr HJ, Hanski C. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. Br J Cancer 1999; 79: 1262-1269.
32. O'Connell J, Bennett MW, O'Sullivan GC, Roche D, Kelly J, Collins JK, Shanahan F. Fas ligand expression in primary colon adenocarcinomas: evidence that the Fas counterattack is a prevalent mechanism of immune evasion in human colon cancer. J Pathol 1998; 186: 240-246.
33. Strater J, Hinz U, Walczak H, Mechtersheimer G, Koretz K, Herfarth C, Moller P, Lehnert T. Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter. Clin Cancer Res 2002; 8: 3734-3740.
34. van Geelen CM, Westra JL, de Vries EG, Boersma-van EW, Zwart N, Hollema H, Boezen HM, Mulder NH, Plukker JT, de JS, Kleibeuker JH, Koornstra JJ. Prognostic significance of tumor necrosis factor-related apoptosis-inducing ligand and its receptors in adjuvantly treated stage III colon cancer patients. J Clin Oncol 2006; 24: 4998-5004.
35. Yoong KF, Afford SC, Randhawa S, Hubscher SG, Adams DH. Fas/Fas ligand interaction in human colorectal hepatic metastases: A mechanism of hepatocyte destruction to facilitate local tumor invasion. Am J Pathol 1999; 154: 693-703.

36. Nozoe T, Yasuda M, Honda M, Inutsuka S, Korenaga D. Fas ligand expression is correlated with metastasis in colorectal carcinoma. Oncology 2003; 65: 83-88.
37. Li H, Fan X, Stoicov C, Liu JH, Zubair S, Tsai E, Ste MR, Wang TC, Lyle S, Kurt-Jones E, Houghton J. Human and mouse colon cancer utilizes CD95 signaling for local growth and metastatic spread to liver. Gastroenterology 2009; 137: 934-944.
38. Liu K, McDuffie E, Abrams SI. Exposure of human primary colon carcinoma cells to anti-Fas interactions influences the emergence of pre-existing Fas-resistant metastatic subpopulations. J Immunol 2003; 171: 4164-4174.
39. Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989; 49: 4682-4689.
40. Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S, Sun XM, Brown J, Marais R, Pritchard C. MEK kinase activity is not necessary for Raf-1 function. EMBO J 2001; 20: 1940-1951.
41. Mikula M, Schreiber M, Husak Z, Kucerova L, Ruth J, Wieser R, Zatloukal K, Beug H, Wagner EF, Baccarini M. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J 2001; 20: 1952-1962.
42. Niault T and Baccarini M. Targets of Raf in tumorigenesis. Carcinogenesis 2010; [Epub ahead of print].
43. Pritchard CA, Bolin L, Slattery R, Murray R, McMahon M. Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. Curr Biol 1996; 6: 614-617.
44. Chen J, Fujii K, Zhang L, Roberts T, Fu H. Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci U S A 2001; 98: 7783-7788.
45. O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science 2004; 306: 2267-2270.
46. Scott RW and Olson MF. LIM kinases: function, regulation and association with human disease. J Mol Med 2007; 85: 555-568.
47. Wang W, Eddy R, Condeelis J. The cofilin pathway in breast cancer invasion and metastasis. Nat Rev Cancer 2007; 7: 429-440.

CHAPTER 4

CD95 IS A KEY MEDIATOR OF INVASION AND ACCELERATED OUTGROWTH OF COLORECTAL LIVER METASTASES FOLLOWING RADIOFREQUENCY ABLATION

Journal of Hepatology. 2010 Dec; 53(6):1069-77

Frederik J.H. Hoogwater*
Maarten W. Nijkamp*
Ernst J.A. Steller
B. Florien Westendorp
Taco A. van der Meulen
Martijn W.H. Leenders
Inne H.M. Borel Rinkes
Onno Kranenburg

* *these authors contributed equally*

Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands

ABSTRACT

Background

Recently, we have shown that micrometastases in the hypoxic transition zone surrounding lesions generated by radiofrequency ablation (RFA) display strongly accelerated outgrowth. CD95 is best known for its ability to induce apoptosis but can also promote tumorigenesis in apoptosis-resistant tumor cells. Therefore, we tested whether CD95 signaling plays a role in accelerated outgrowth of colorectal liver metastases following RFA.

Methods

Hypoxia-induced invasion was assessed in three-dimensional EGFP-expressing C26 tumor cell cultures by confocal microscopy. CD95 localization was tested by immunofluorescence. Invasion and outgrowth of liver metastases following RFA was analyzed by post-mortem confocal microscopy and by morphometric assessment of tumor load. Neutralization of CD95L was performed by using antibody MFL4. CD95 was suppressed by lentiviral RNA interference. The role of host CD95L was assessed using gld mice.

Results

Micrometastases in the hypoxic transition zone following RFA displayed a highly invasive phenotype and increased expression of CD95 and CD95L. Hypoxia induced tumor cell invasion *in vitro*, increased expression of CD95 and CD95L and induced translocation of CD95 to the invasive front. *In vitro* invasion, metastasis invasion and accelerated tumor growth in the transition zone were strongly suppressed by neutralizing CD95L or by suppressing tumor cell CD95. In contrast, metastasis invasion and outgrowth were unaffected in *gld* mice.

Conclusions

Hypoxia causes autocrine activation of CD95 on colorectal tumor cells, thereby promoting local invasion and accelerated metastasis outgrowth in the hypoxic transition zone following RFA. Further pre-clinical work is needed to assess the role of CD95L neutralization, either alone or in combination with chemotherapy, in limiting aggressive recurrence of liver metastases following RFA.

INTRODUCTION

Radiofrequency ablation (RFA) is a local thermal destruction therapy that is used as an alternative treatment option for patients with non-resectable colorectal liver metastases. Local tumor recurrence following RFA treatment occurs in approximately 15% of all cases. However, with increased tumor size, or when a percutaneous approach is used, local recurrence rates of up to 60% are reported[1-4].

Recently, we have shown in two murine models that RFA can stimulate the outgrowth of colorectal liver metastases. The RFA-generated necrotic lesion is surrounded by a rim of chronically hypoxic liver tissue which is usually referred to as the 'transition zone' (TZ). Micrometastases that happen to be present in the transition zone grow out approximately three to four times faster than those in the normoxic reference zone (RZ) further away from the lesion[5]. Interestingly, up to 70% of patients with colorectal liver metastases contain micrometastases that may result in (local) tumor recurrence[6].

Hypoxia can contribute to tumor cell invasion and metastatic progression and is generally associated with a more aggressive tumor phenotype[7,8]. Several genes and receptor systems have been implicated in hypoxia-stimulated invasion and metastasis formation, including hepatocyte growth factor receptor (c-Met), vascular endothelial growth factor receptors (VEGFR) and increased expression of proteolytic enzymes[8-10]. Additionally, hypoxia can cause activation of the CD95 death receptor system, leading to CD95-induced apoptosis[11-13]. However, in apoptosis-resistant cells, CD95 can activate alternative signaling pathways which may cause invasion, differentiation, survival or proliferation[14-18].

In colorectal cancer, the expression of CD95 ligand (CD95L) is correlated with metastasis formation[19] Recent evidence shows that enforced expression of CD95L promotes the metastatic capacity of apoptosis-resistant colorectal cancer cells[20] In addition, we have recently shown that stimulation of CD95 on such cells predominantly leads to tumor cell invasion and that signaling by the KRAS oncogene is a critical determinant of CD95 signaling output[15].

The combined observations that i) CD95 signaling is regulated by hypoxia and that ii) CD95L can promote invasion and metastatic capacity, prompted us to assess whether activation of the CD95 system contributes to local outgrowth of liver metastases following RFA. We found that CD95 and CD95L were selectively and strongly upregulated in the hypoxic transition zone following RFA and that this was correlated with a dramatic invasive tumor phenotype. *In vitro* hypoxia was sufficient to cause autocrine CD95 activation and CD95-dependent tumor cell invasion. Autocrine CD95 activation also mediated invasion and outgrowth of micrometastases following RFA. Our results identify CD95 as a major participant in local tumor cell invasion and accelerated outgrowth of micrometastases following RFA.

MATERIALS AND METHODS

Cell lines and cell culture

C26 mouse colon carcinoma cells were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). C26-EGFP cells were generated by lentiviral transduction using pWPT-GFP (kindly provided by Professor Didier Trono). A panel of five lentiviral pLKO1 constructs targeting CD95 (Open Biosystems; Huntsville, AL, USA) was tested. Of these five, two gave the best knockdown and were used for generating stable C26-CD95-knockdown cell lines (TCRN0000012328 (#1) and (TCRN0000012331 (#2)). As a control, C26 cells were transduced with luciferase-targeting lentiviral shRNA vectors (C26-Luc-kd). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg/ml steptomycin and 100 U/ml penicillin. Cells were kept at 37°C in a humidified atmosphere containing 5% CO2.

Antibodies and reagents

The following primary antibodies were used: anti-mouse CD95 (clone M-20, #sc-716) Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Anti-CD95 Ligand (ab15285), Abcam, Cambridge, MA, USA. Anti-HIF-1 alpha (NB100-449) and anti-Beta-Actin (AC-15, NB600-501), Novus Biologicals, LLC, Littleton, CO, USA. Secondary antibodies were anti-rabbit IgG, HRP-linked (#7074), Cell Signaling, Danvers, MA, USA. Polyclonal rabbit anti-mouse immunoglobulins, HRP (p0260), DAKO, Glostrup, Denmark. Goat anti-hamster IgG-HRP (sc2493), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The following reagents were used in this study: CD95 Ligand (FasL), membrane bound (#01-210) from Upstate Cell Signaling Solutions (now Millipore), Lake Placid, NY, USA. Functional Grade purified anti-mouse CD95 Ligand (Fas Ligand, CD178), clone MFL4 (#16-5912), from eBioscience, Inc., San Diego, CA, USA.

MTT assay

To test the neutralizing effect of MFL4 on CD95L, C26 cells and apoptosis-prone C26KrasKD cells[15] were plated at a density of 5000 cells/well in 96-well plates. Cells were either pre-treated with MFL4 (6 ng/ml) for 2h or directly stimulated with increasing concentrations of CD95L (2 ng/ml to 10 ng/ml). Mitochondrial activity in each well was analyzed after 24h stimulation by standard 3- (4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) according to the manufacturer's instructions.

Invasion (colony scatter) assay under hypoxia

To investigate the phenotype of cells under normoxic and hypoxic conditions $3.5x10^4$ C26-EGFP cells were plated on coverslips with matrigel. Micro-colonies were allowed to form for 72 hours under normoxic (21% O_2) or hypoxic (1% O_2 Invivo2 Hypoxia Workstation 1000 (Biotrace

International, UK)) conditions in a humidified atmosphere at 37°C. Coverslips were mounted using Vectashield mounting fluid (H-1200, Vectorlabs). Confocal images were then acquired on a Zeiss Axiovert 200M. Image acquisition and analysis were performed using Zeiss LSM 510 Software. The length of the longest diameter of each colony was measured in 10 randomly chosen fields per coverslip. All assays were performed in duplicate and repeated twice.

Confocal microscopy

Control cells and cells treated with CD95L and MFL4 (isotype control with IgG; 6 ng/ml) were fixed in 3.7% formaldehyde for 10 minutes and were subsequently permeabilized with 0.05% Triton X-100 in 1% PBS/BSA. Coverslips were then blocked in PBS with 3% BSA for 1h. Anti-CD95 primary antibody (isotype control with IgG, 10 ng/ml) was incubated at room temperature for 1 hour and secondary antibodies (FITC and Alexa-568-conjugated phalloidin) were incubated for 30 minutes at room temperature. Coverslips were mounted using Vectashield mounting fluid (H-1200, Vectorlabs) with DAPI. Confocal images were acquired on a Zeiss Axiovert 200M microscope. Image acquisition and analysis were performed using Zeiss LSM-510 software.

Animals and surgery

Male BALB/c mice (10-12 weeks) were purchased from Charles River (Sulzfeld, Germany). BALB/c-*gld/gld* (CPt.C3-Faslgld/J) mice carrying homozygous loss-of-function mutation in CD95L (hereafter referred to as *gld*-mice) were purchased from The Jackson Laboratory (Bar Harbor, USA). In experiments using *gld*-mice, control BALB/c mice were also from The Jackson Laboratory. Mice were housed under standard laboratory conditions and received food and water ad libitum. All surgical procedures were performed under isoflurane inhalation anesthesia. Prior to surgery, buprenorfine was administered intramuscularly to provide sufficient peri-operative analgesia. All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands.

Induction of micrometastases and radiofrequency ablation

Induction of colorectal micrometastases and subsequent radiofrequency ablation were performed as previously described[5]. In brief, through a left lateral flank incision, $5x10^4$ C26 murine colon carcinoma cells were injected into the splenic parenchyma, followed by removal of the spleen after ten minutes to prevent intrasplenic tumor growth. For tumor cell invasion experiments, EGFP-expressing C26 cells were used. This resulted in a homogeneous spread of diffuse intrahepatic micrometastases, which were allowed to grow out for three days. Three days after the intrasplenic injection, RFA was performed using the CELON Power System (Celon AG, Teltow, Germany). A single non-cooled bipolar electrode (outer diameter 1.0 mm, active length 10 mm, Celon AG, Teltow, Germany) was used for RFA of the left liver lobe at 2

Watts for 50 seconds (100 Joules), resulting in a lesion of approximately 8 by 15 mm. Since RFA was performed in a liver containing homogeneous spread of numerous diffuse micrometastases, the effect of RFA on residual tumor cells could be investigated.

Assessment of the tumor cell phenotype in the transition zone following RFA

Mice bearing EGFP-expressing tumor cells were sacrificed at t=0, t=6 hours, t=12 hours, t=24 hours and t=7 days following RFA of the left liver lobe (n=4 mice each group). The liver was excised and placed on a coverslip using immersion oil to improve visualization. The livers were visualized using combined bright field/fluorescence settings on the confocal microscope to localize the transition zone, using a 10x magnification[5]. To visualize the tumor cells, EGFP was excited at 488nm. Tumor cell invasiveness was assessed by creating confocal image stacks in axial dimension. At least 15 randomly chosen tumor cell clusters in the transition zone and the reference zone were visualized and relayed to a personal computer for off line analysis using Zeiss LSM 510 Software. Tumor cell invasion was assessed by creating a two-dimensional stack projection and was defined as the average longest distance between cells making up a single cluster/metastasis. Analysis of tumor cell invasion was performed by two independent observers on liver tissue 24 hours following RFA.

Immunohistochemistry

Tumor-bearing and non-tumor-bearing mice were sacrificed 24 hours and 7 days after RFA (n=4 each group). To visualize hypoxia, these mice were injected intravenously with 60mg/kg pimonidazole hydrochloride (Hypoxyprobe-1,90201; Chemicon International, Temecula, CA) prior to their sacrifice. After harvesting, the livers were fixed in formaldehyde and embedded in paraffin. Tissue sections (4µm) were used for the immunohistochemical staining of CD95L, using a rabbit polyclonal antibody to CD95L (Abcam, Cambridge, USA). As secondary antibody PowerVision+ (Immunologic, Duiven, The Netherlands) with 2% mouse serum was used. Hypoxia was visualized using anti-pimonidazole, according to the manufacturer's instructions. Reactions were developed using diaminobenzidine/H_2O_2 as a chromogen substrate. Negative controls were treated with isotype control antibody and were all free of nonspecific background staining.

Analysis of tumor load

Tumor load in the liver was scored as hepatic replacement area (HRA), i.e. the percentage of liver tissue that had been replaced by tumor tissue. HRA was measured in the reference zone and the transition zone, defined as the area stretching 2 mm outside the necrotic central area, as previously described[5]. In brief, on hematoxylin and eosin (H&E) stained sections, at least 100 fields were selected using an interactive video overlay system, including an automated microscope (Q-Prodit; Leica Microsystems, Rijswijk, The Netherlands) at a 40x magnification. Using a four-points grid overlay, the ratio of tumor cells versus normal

hepatocytes was determined for each field. Tumor load (HRA) was expressed as the average area ratio of all fields.

Experimental design of *in vivo* studies

First, the effects of RFA on the tumor cell phenotype in the transition zone were assessed. Mice (n=4 each group) were euthanized at five different time points following RFA (t=0, 6 hours, 12 hours, 24 hours and 7 days) and analyzed for tumor cell phenotype as mentioned. Next, the role of CD95/CD95L system in RFA-induced tumor cell invasion was assessed 24 hours post-RFA by using MFL4 as CD95L neutralizing antibody or IgG isotype control (2 intraperitoneal doses of 20mg/kg, 12 hours before and 12 hours after RFA), CD95 knockdown (KD) cells using shRNA-interference or *gld*-mice that lack functional CD95L. Finally, the influence of CD95 interference on accelerated outgrowth of micrometastases following RFA was investigated using CD95KD cells or *gld*-mice. For mice injected with C26-CD95kd cells, mice injected with C26 shRNA luc served as controls.

Statistical analysis

Statistical differences between the transition zone and the reference zone were analyzed by a paired t-test or Wilcoxon signed rank test when appropriate. Differences between groups were analyzed by ANOVA or Kruskal-Wallis test when appropriate. Data are expressed as mean +/- SEM. A p-value < 0.05 was considered statistically significant.

RESULTS

Micrometastases in the hypoxic transition zone following RFA rapidly acquire an invasive phenotype

Metastases in the reference zone grew as compact spherical tumor cell clusters. However, micrometastases in the transition zone displayed a characteristic invasive growth pattern which was first observed 6 hours post-RFA, and which further increased over time (Figure 1A). At 24 hours after RFA, the average longest distance of tumor cells making up a single scattered micrometastasis in the transition zone (569 ± 62 µm) was significantly longer when compared to micrometastases in the reference zone (166 ± 13 µm; p=0.0007) (Figure 1B). During the ensuing 7 days, micrometastases in the reference zone grew but retained their compact phenotype with sharply demarcated edges. In contrast, micrometastases in the transition zone eventually formed a confluent rim of invasive tumor tissue (Figure 2A). To asses the relationship between the invasive phenotype of the micrometastases and tissue hypoxia, we made use of pimonidazole staining. Interestingly, tumor cell invasion was primarily observed at the hypoxic side of the tumor rim facing the necrosis, while tumor cells facing the normoxic reference zone were relatively non-invasive (Figures 2B and 2C).

Hypoxia promotes matrix invasion of C26 cells *in vitro*

In vitro hypoxia for 24 hours was sufficient to induce a drastic change in the growth pattern of tumor cell clusters. Normoxic clusters remained spherical and compact, but hypoxic clusters grew in a scattered fashion with tumor cells detaching from the center of the cluster and invading the surrounding matrix, resulting in a 3.8 fold increase of longest tumor diameter (p=0.0001, figure 3A).

Hypoxia stimulates expression of CD95 and CD95L and causes translocation of CD95 to cell protrusions

Hypoxia induced the expression of HIF1α, CD95 and CD95L *in vitro* (Figure 3B). Immunofluorescence analysis showed that upon CD95L stimulation a subpopulation of CD95 was associated with the leading edges of migrating C26 cells, similar to what was shown for CD95L-stimulated DLD1 cells[15] (Figure 3C). Interestingly, hypoxia-induced tumor cell invasion was also associated with translocation of CD95 to the leading edges of migrating cells (Figure 3C). Neutralization of CD95L with MFL4 completely blocked CD95 translocation. The ability of MFL4 to neutralize CD95L was validated in a control experiment using apoptosis-prone cells stimulated with CD95L (data not shown).

Neutralization of CD95L or suppression of CD95 reduces hypoxia-induced invasion

Using lentiviral shRNA constructs we generated two stable CD95 knockdown cell lines and a control cell line expressing luciferase-targeting shRNAs (shLuc) (Figure 4A).

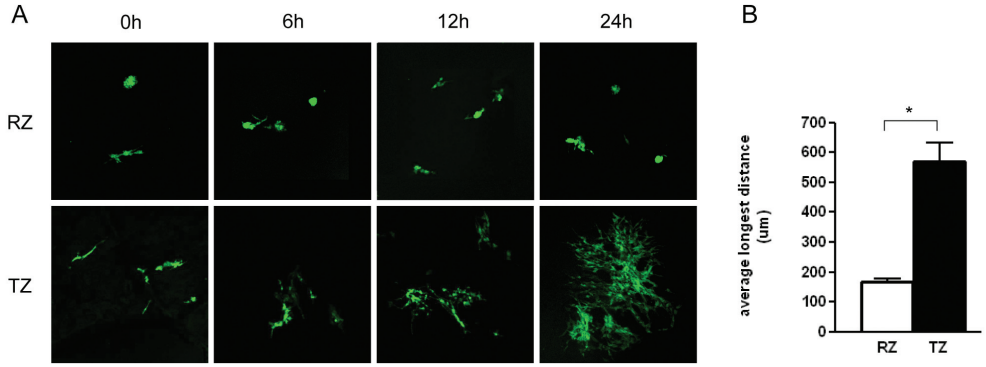

Figure 1. Tumor cell invasion in the hypoxic transition zone following RFA. (**A**) Mice carrying EGFP-expressing micrometastases were subjected to RFA and were analyzed by confocal microscopy at the indicated time points following RFA. Image z-stacks were created in axial dimension to visualize tumor cell invasion, defined as the average longest distance between tumor cells making up a single cluster/metastasis. All metastasis diameters in the transition zone were compared to those of metastases located in the reference zone of the same liver lobe. (**B**) Quantification of metastasis diameters using projections of z-stacks generated 24 hours following RFA (≥15/mice; n=4 mice). TZ transition zone; RZ reference zone; * p<0.05.

When exposed to hypoxia, control (shLuc) cells displayed the scatter-like growth pattern as expected (Figures 4B and 4C). However, neutralization of CD95L using MFL4 or knockdown of tumor cell CD95 was sufficient to completely prevent the scatter response (Figures 4B and 4C).

Increased expression of CD95 and CD95L in the transition zone following RFA

In tumor-bearing mice, western blotting showed a clear increase in the levels of CD95L and CD95 in the transition zone of all mice when compared to the reference zone (Figure 5A). However, this difference could not be observed in non-tumor-bearing mice (Figure 5B).

Next, to analyze the localization of CD95L, immunohistochemistry was performed on liver tissue sections of tumor-bearing mice 24 hours and 7 days following RFA. At 24 hours after RFA, CD95L levels were below detection level for immunohistochemistry (data not shown).

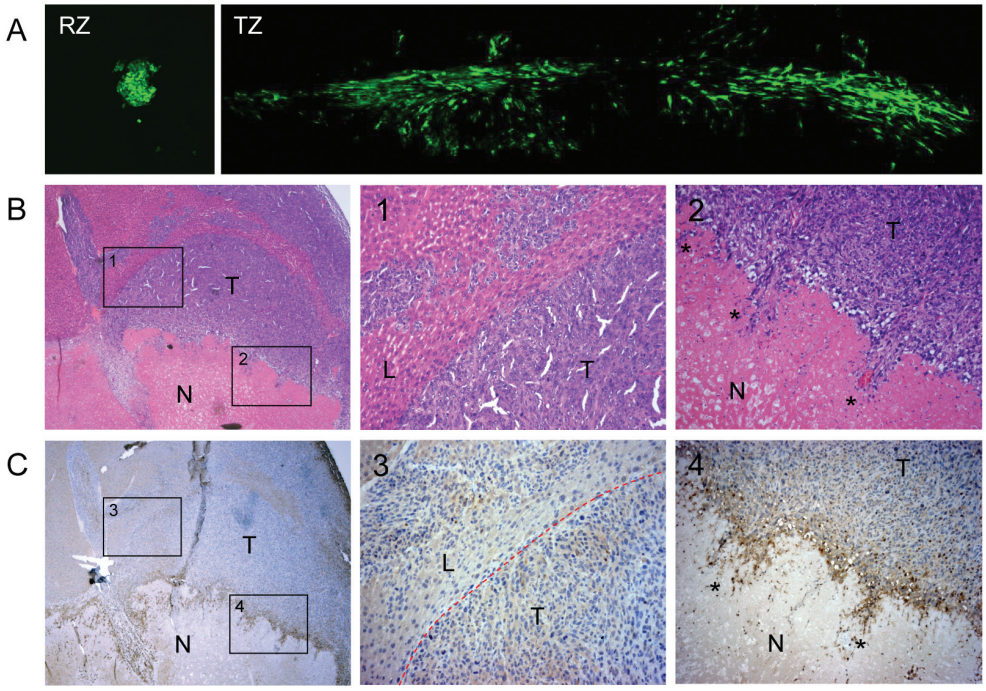

Figure 2. Hypoxia in the transition zone is associated with tumor cell invasion. Mice carrying EGFP-expressing liver metastases were subjected to RFA and the livers were harvested seven days later. To visualize hypoxia, all mice received pimonidazole 60 minutes prior to sacrifice. The growth pattern of metastases in the reference zone and the transition zone was analyzed (**A**) by confocal microscopy on non-fixed livers, and (**B**) by haematoxylin and eosin staining and (**C**) anti-pimonidazole immunohistochemistry on formalin-fixed paraffin-embedded tissue. The right panels show higher magnifications of the normoxic tumor-liver border (1 and 3) and the hypoxic tumor-necrosis border (2 and 4). Invasive tumor cell clusters at the hypoxic tumor-necrosis border are indicated by asterisks. TZ transition zone; RZ reference zone; T tumor; L liver; N necrosis;

Interestingly, 7 days after RFA CD95L was strongly expressed in the transition zone in tumor-bearing mice, but was hardly detectable in non tumor-bearing mice (Figure 5C). These results suggest that tumor cells could be the source of CD95L. CD95L was selectively localized at the invasive hypoxic side of the tumor rim facing the generated lesion, but not in the non-invasive normoxic side facing the liver parenchyma. This is in line with a potential role for this cytokine in local, hypoxia-associated, tumor cell invasion (Figure 5C central panel, see also pimonidazole staining for hypoxia at the tumor-necrosis interface in figure 2). No immunohistochemical differences could be observed for CD95 at the mentioned time points (data not shown).

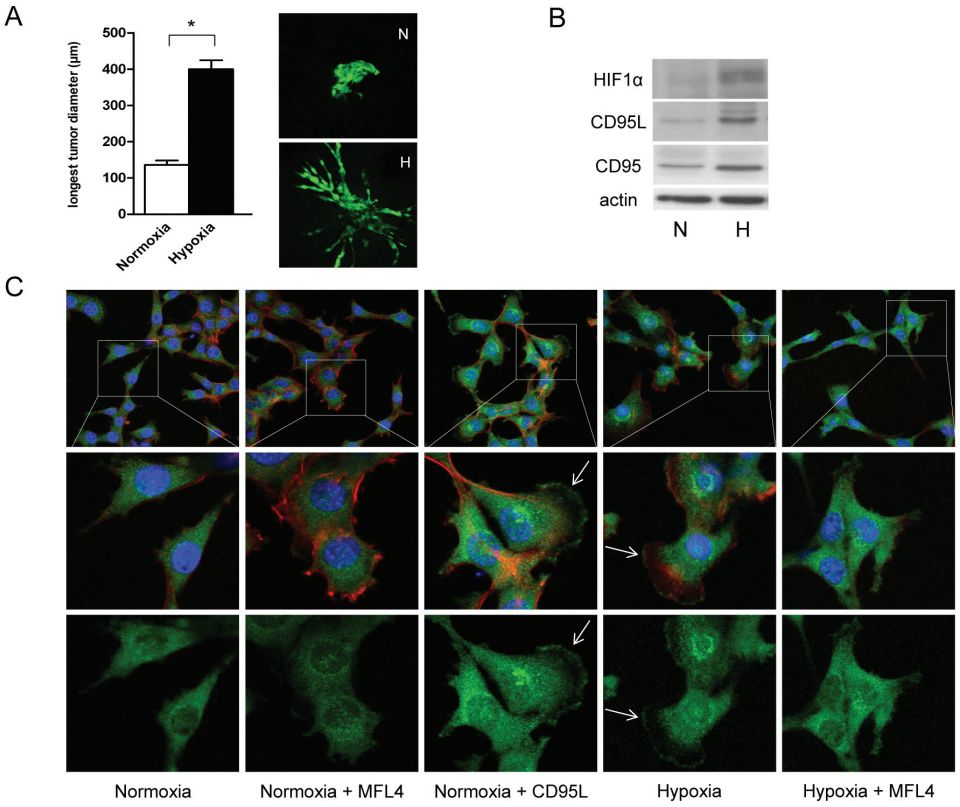

Figure 3. Hypoxia stimulates expression of CD95 and CD95L and causes activation of CD95. (**A**) EGFP-expressing tumor cells were cultured as three-dimensional clusters in Matrigel and were exposed to normoxia or hypoxia for 24 hours. Confocal microscopy was used to analyze the longest diameter of all tumor cell clusters. The bar graph shows a quantification of the results. (**B**) C26 cells were incubated under normoxia or hypoxia for 24 hours and cell lysates were analyzed by Western blotting for HIF1a, CD95 and CD95L. (**C**) C26 cells were grown on glass coverslips under normoxia or hypoxia for 24 hours either in the presence or absence of MFL4 (6 ng/ml). Stimulation under normoxia with CD95L (10ng/ml for 1h) was used as a positive control. Cells were then fixed and processed for immunofluorescence using anti-CD95 (green) and Alexa568-phalloidin (filamentous actin). Stained coverslips were then analyzed by confocal microscopy. N normoxia; H hypoxia; * $p<0.05$. Arrows indicate the leading edges of migrating cells.

Autocrine CD95 signaling is required for tumor cell invasion in the transition zone following RFA

Neutralizing CD95L using MFL4 caused an impressive (~60%) reduction in tumor cell invasion in the transition zone when compared to mice treated with isotype IgG (230 ± 35 µm versus 570 ± 55 µm; p=0.007, figure 6). Additionally, CD95 knockdown (C26-CD95kd, figure 4A) had a strong suppressive effect on metastasis invasion in the transition zone, when compared to control metastases (C26-Luc-kd, 232 ± 34 µm versus 586 ± 64 µm, respectively; p=0.0012, figure 6). Thus, both CD95L neutralization and knockdown of CD95 in tumor cells prevented

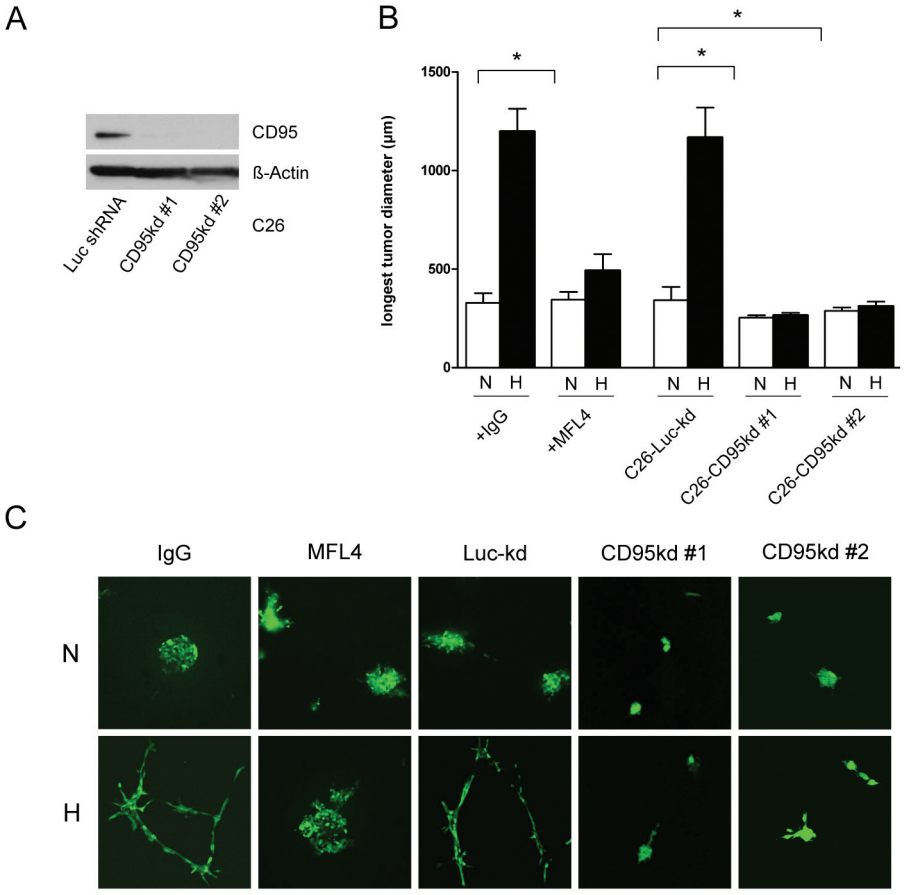

Figure 4. CD95 is required for hypoxia-induced tumor cell invasion. (**A**) Western blot showing efficient CD95 knockdown by two independent constructs. (**B**) EGFP-expressing tumor cells were cultured as three-dimensional clusters in Matrigel and were exposed to normoxia or hypoxia for 24 hours. Cultures were incubated either with IgG (6ng/ml), or with MFL4 (6ng/ml) for 24 hours. In addition, C26 cells stably expressing shRNA's targeting either Luciferase (Luc) or CD95 were used. Confocal microscopy was used to analyze the longest diameter of all tumor cell clusters. The bar graph shows means and SEM of 10 tumor cell clusters in three independent cultures. (**C**) Representative confocal microscopic images of tumor cell clusters. N normoxia; H hypoxia; * p<0.05

RFA-induced invasion of micrometastases in the transition zone. This indicates that the tumor cell invasion is based on a direct effect of CD95L on tumor cells rather than an indirect effect by, for instance, suppressing hepatocyte apoptosis or local inflammation.

To analyze the contribution of host CD95L, we made use of mice lacking functional CD95L (gld). Invasion of micrometastases in the transition zone following RFA was not significantly different between gld-mice and control mice (491 ± 58 µm versus 554 ± 56 µm; p=0.53, figure 6). This suggests that tumor cell invasion following RFA is most likely due to autocrine activation of CD95 on tumor cells.

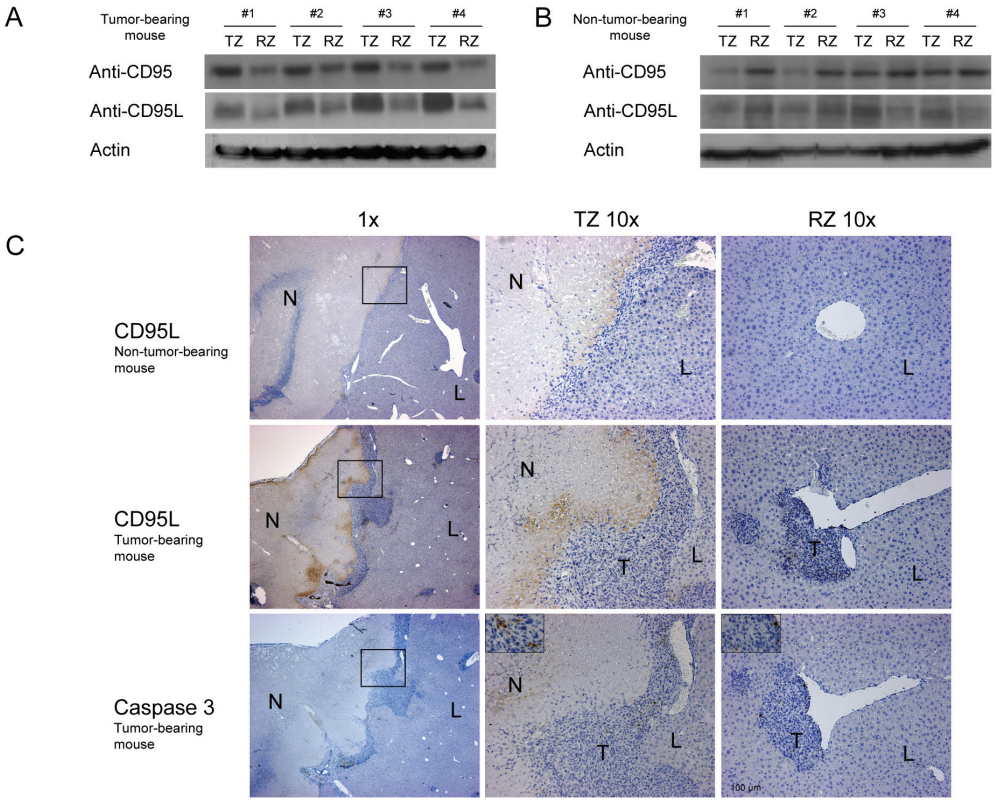

Figure 5. Upregulation of CD95 and CD95L in the transition zone following RFA. TZ-tissue and RZ-tissue were separated from frozen (**A**) tumor-bearing and (**B**) non-tumor-bearing livers 24 hours after RFA using a dissection microscope. Tissue (TZ and RZ) lysates were prepared and were analyzed by Western blotting using specific antibodies directed against CD95, CD95L and actin as a loading control. (**C**) Immunohistochemistry of tumor bearing livers (T+) and non-tumor bearing livers (T-) 7 days following RFA, using anti-CD95L and anti-active caspase-3. TZ transition zone; RZ reference zone; T+ tumor bearing; T- non-tumor bearing; T tumor; L liver; N necrosis.

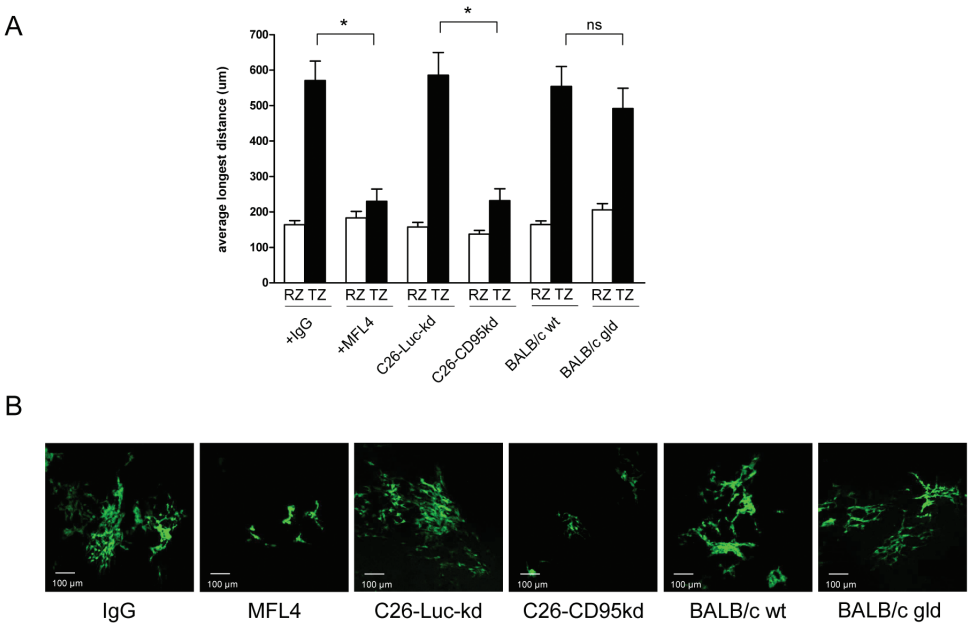

Figure 6. Metastasis invasion in the transition zone requires autocrine CD95 signaling. Mice carrying EGFP-expressing C26 micrometastases were treated either with the CD95L-neutralizing antibody MFL4 or with control IgG prior to the RFA procedure. In addition, RFA was performed on mice carrying C26-Luc-kd or C26-CD95kd metastases. Finally, EGFP-expressing C26 cells were allowed to form metastases in control mice and in mice lacking functional CD95L (*gld*). (**A**) Metastasis diameters in the RZ (white bars) and the TZ (black bars) were then determined 24 hours following RFA, as in Figure 1 (n≥15 metastases). (**B**) Representative confocal microscopic images of transition zone metastases. TZ transition zone; RZ reference zone; kd knock down; * p<0.05; ns not significant

Suppression of CD95 signaling reduces accelerated tumor outgrowth in the transition zone following RFA

In mice carrying C26-Luc control metastases, tumor growth in the transition zone was stimulated 3.1-fold when compared to tumor growth in the reference zone (18± 3% versus 47± 5%; p<0.0001, figures 7A-C). However, in mice carrying C26-CD95kd metastases, tumor load in the transition zone was strongly and significantly reduced when compared to C26-Luc metastases (16 ± 3% versus 47± 5%, p<0.0001 respectively). The acceleration of tumor growth, as indicated by the ratio between HRA values of transition zone and reference zone, was significantly reduced by ~60% from 3.1 for C26-Luc cells to 1.7 for C26-CD95kd cells (p=0.0048, figure 7B).

Finally, we tested whether host CD95L was instrumental in accelerating transition zone tumor growth following RFA. Tumor load in the transition zone was not significantly different in gld-mice lacking functional CD95L when compared to control mice (44 ± 6% versus 43± 5%, p=0.91, respectively). Consequently, tumor growth acceleration, as measured by HRA ratios, was similar in both genetic backgrounds (2.8 ± 0.3 versus 3.0 ± 0.35; p=0.75, figures 7A-C).

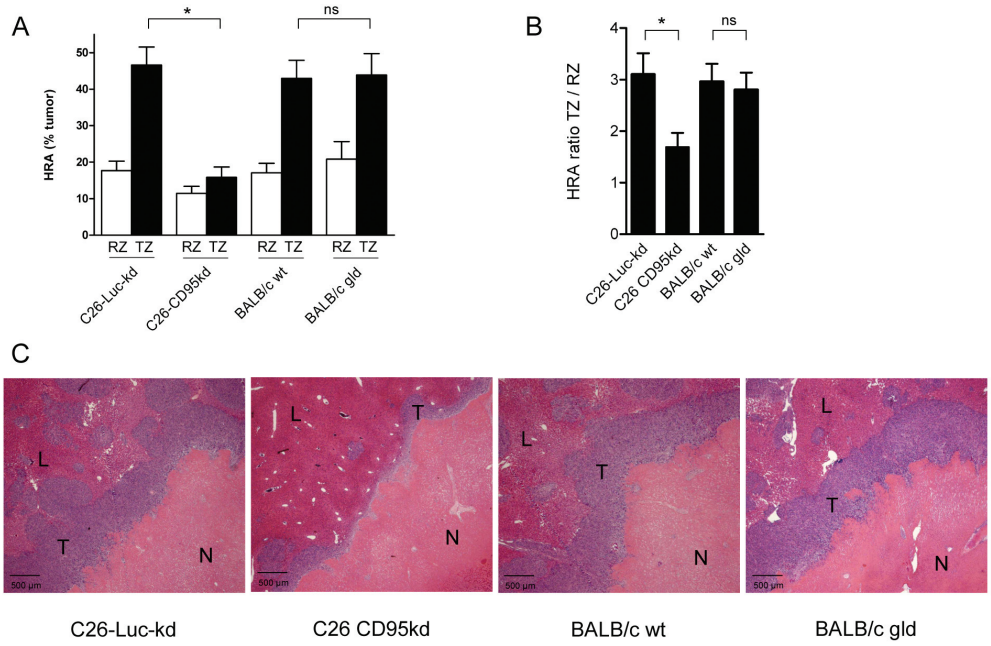

Figure 7. Tumor-cell CD95 mediates accelerated outgrowth of liver metastases following RFA. RFA was performed on mice carrying C26-Luc-kd or C26-CD95kd metastases. In addition, RFA was performed on tumor-bearing control mice and on mice lacking functional CD95L (*gld*). All mice were sacrificed 7 days after the RFA procedure. (**A**) The livers were harvested and tumor load in the reference zone (RZ) (white bars) and in the transition zone (TZ) (black bars) was analyzed by morphometric measurement of the hepatic replacement areas (HRA). (**B**) HRA ratio values were determined and plotted. These values indicate the acceleration of tumor growth in the transition zone relative to the reference zone. CD95kd reduced growth acceleration by ~ 60%. (**C**) Representative microscopic images of tumor growth (T) in the transition zone by H&E histochemistry. TZ transition zone; RZ reference zone; T tumor; L liver; N necrosis; * $p<0.05$; ns not significant

DISCUSSION

In the present study, we show that RFA results in rapid CD95-dependent invasion and accelerated outgrowth of micrometastases in the hypoxic transition zone. These results couple hypoxia-activated CD95 signaling to invasive tumor recurrence in the liver following RFA.

Expression of both CD95 and CD95L is increased in the brain and in the heart following ischemia. In these post-ischemic tissues CD95 is instrumental in inducing apoptosis[11,13,21]. Similarly, hypoxia induces CD95 expression in colorectal cancer cells, which can result in CD95-dependent apoptosis[12]. However, many colorectal tumor cells display inherent resistance to apoptosis induction by CD95. In recent years it has become clear that CD95 can activate a number of non-apoptotic pathways that stimulate proliferation and invasion[14-18].

Although CD95 is expressed on the majority of colonic epithelial cells, its primary function in these cells does not appear to be apoptosis induction[22,23]. Remarkably, it was shown in a recent study that CD95 on intestinal epithelial cells is cytoprotective rather than cytotoxic[23]. The non-apoptotic function of CD95 may therefore be conserved in colon tumors. Indeed, chronic CD95 stimulation of colorectal tumor cells selects for highly metastatic variants[24]. Moreover, forced expression of CD95L in apoptosis-resistant tumor cells can promote liver metastasis formation[20]. During colorectal cancer progression, expression of CD95L increases and correlates with metastasis formation[19,25]. In addition, we have shown recently that the KRAS oncogene, which is present in approximately 40% of all colorectal tumors, is a major determinant of CD95 signaling output and forces it to signal invasion, rather than apoptosis[15]. In the present study, we demonstrate that hypoxia, generated following the RFA procedure, stimulates CD95-dependent outgrowth of colorectal micrometastases. Taken together, it appears that CD95 activation on colorectal tumor cells promotes tumor progression rather than tumor clearance. Our data suggest that this is especially relevant during conditions of hypoxia, which causes autocrine activation of the CD95 system and CD95-dependent invasion.

High levels of tumor-produced CD95L in the hypoxic transition zone could affect tumor progression in different ways. First, it can cause apoptosis of infiltrating lymphocytes (a phenomenon known as the 'tumor counterattack')[26]. Second, it can induce apoptosis in surrounding hepatocytes, which could lead to facilitated tumor cell invasion[27]. Third, our results (based on the use of CD95 knockdown tumor cells and CD95L-deficient mice) strongly suggest that autocrine activation of the CD95 system on tumor cells mediates aggressive outgrowth of liver metastases following RFA.

It has recently been demonstrated that hypoxia leads to reduced cell proliferation and increased survival of colorectal cancer cells. This was due to HIF-1α stabilization followed by the formation of a HIF1-ß-catenin complex at the expense of the TCF-ß-catenin complex[28]. However, hypoxia is generally associated with metastatic spread and poor prognosis[7,29]. Also in colorectal tumors, expression of different HIF targets is associated with disease progression and poor patient prognosis[30,31]. It therefore seems unlikely that hypoxia-induced cell cycle arrest plays an important role during aggressive tumor recurrence in the transition zone following RFA. In accordance with our findings, pre-clinical studies have shown that tumor cell proliferation is increased, not decreased, following local ablative therapies like RFA[32,33].

Many surgical procedures generate tissue hypoxia. We have recently shown that vascular clamping, which is frequently applied to prevent excessive blood loss during partial liver resection, generates chronically hypoxic liver tissue that is associated with aggressive tumor recurrence in pre-clinical mouse models[34,35]. In colorectal cancer patients, prolonged clamping times during liver surgery were significantly associated with decreased time to tumor progression in the liver[36]. In addition, we have recently found that the levels of circulating CD95L were significantly increased following RFA treatment of liver metastases

in CRC patients (FJHH, ms in preparation). Taken together, we propose that CD95 signaling may contribute to tumor recurrence in livers treated with hypoxia-generating (surgical) procedures.

Patient selection for RFA of colorectal liver metastases is essential for optimal response to the treatment, making it a potentially curative treatment option. The mechanistic understanding of local recurrences might be important for making RFA applicable to more patients with colorectal liver metastases. Currently, clinical observations show highly aggressive local tumor recurrence in patients treated with local ablative therapies[37,38]. In the present study we have demonstrated that the hypoxic rim of RFA-generated lesions is characterized by aggressive CD95-dependent outgrowth of colorectal micrometastases. Although neutralization of CD95L would not kill remaining tumor cells, it could be of therapeutic benefit in patients with colorectal liver metastases treated with RFA by reducing tumor cell invasion and outgrowth. Prolonged treatment with CD95L-neutralizing agents holds the potential danger of inducing lymphoproliferative disorders and autoimmunity[39,40], short-term peri-operative treatment however may prove to be feasible. Whether CD95L-neutralizing agents, either alone or in combination with chemotherapy, can be of added value in preventing aggressive tumor recurrence following RFA requires further pre-clinical work. This work should initially focus on tumors with mutant KRAS as such tumors are prone to respond aggressively to CD95L[15].

REFERENCES

1. Abdalla EK, Vauthey JN, Ellis LM, Ellis V, Pollock R, Broglio KR, Hess K, Curley SA. Recurrence and outcomes following hepatic resection, radiofrequency ablation, and combined resection/ablation for colorectal liver metastases. Ann Surg 2004; 239: 818-825.
2. Mulier S, Ni Y, Jamart J, Ruers T, Marchal G, Michel L. Local recurrence after hepatic radiofrequency coagulation: multivariate meta-analysis and review of contributing factors. Ann Surg 2005; 242: 158-171.
3. Siperstein AE, Berber E, Ballem N, Parikh RT. Survival after radiofrequency ablation of colorectal liver metastases: 10-year experience. Ann Surg 2007; 246: 559-567.
4. Solbiati L, Livraghi T, Goldberg SN, Ierace T, Meloni F, Dellanoce M, Cova L, Halpern EF, Gazelle GS. Percutaneous radio-frequency ablation of hepatic metastases from colorectal cancer: long-term results in 117 patients. Radiology 2001; 221: 159-166.
5. Nijkamp MW, van der Bilt JD, de Bruijn MT, Molenaar IQ, Voest EE, van Diest PJ, Kranenburg O, Borel Rinkes IH. Accelerated perinecrotic outgrowth of colorectal liver metastases following radiofrequency ablation is a hypoxia-driven phenomenon. Ann Surg 2009; 249: 814-823.
6. Yokoyama N, Shirai Y, Ajioka Y, Nagakura S, Suda T, Hatakeyama K. Immunohistochemically detected hepatic micrometastases predict a high risk of intrahepatic recurrence after resection of colorectal carcinoma liver metastases. Cancer 2002; 94: 1642-1647.
7. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2002; 2: 38-47.
8. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3: 721-732.
9. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006; 441: 437-443.
10. Wey JS, Fan F, Gray MJ, Bauer TW, McCarty MF, Somcio R, Liu W, Evans DB, Wu Y, Hicklin DJ, Ellis LM. Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. Cancer 2005; 104: 427-438.
11. Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, Debatin KM. Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. Circulation 2000; 102: 915-920.
12. Liu T, Laurell C, Selivanova G, Lundeberg J, Nilsson P, Wiman KG. Hypoxia induces p53-dependent transactivation and Fas/CD95-dependent apoptosis. Cell Death Differ 2007; 14: 411-421.
13. Martin-Villalba A, Herr I, Jeremias I, Hahne M, Brandt R, Vogel J, Schenkel J, Herdegen T, Debatin KM. CD95 ligand (Fas-L/APO-1L) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. J Neurosci 1999; 19: 3809-3817.
14. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J 2004; 23: 3175-3185.
15. Hoogwater FJ, Nijkamp MW, Smakman N, Steller E.J., Emmink B.L., B.F.Westendorp, Raats D.A., Sprick M.R., Schaeffer U., van Houdt WJ, de Bruijn MT, Schackmann R.C., Derksen P.W., Medema J.P., Walczak H, Borel Rinkes I.H., Kranenburg O. Oncogenic K-Ras Turns Death Receptors Into Metastasis-Promoting Receptors in Human and Mouse Colorectal Cancer Cells. Gastroenterology 2010; 137: 2357-2367.
16. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, Thiemann M, Mueller W, Sykora J, Kuhn A, Schreglmann N, Letellier E, Zuliani C, Klussmann S, Teodorczyk M, Grone HJ, Ganten TM, Sultmann H, Tuttenberg J, von DA, Regnier-Vigouroux A, Herold-Mende C, Martin-Villalba A. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 2008; 13: 235-248.
17. Trauzold A, Roder C, Sipos B, Karsten K, Arlt A, Jiang P, Martin-Subero JI, Siegmund D, Muerkoster S, Pagerols-Raluy L, Siebert R, Wajant H, Kalthoff H. CD95 and TRAF2 promote invasiveness of pancreatic cancer cells. FASEB J 2005; 19: 620-622.
18. Wisniewski P, Ellert-Miklaszewska A, Kwiatkowska A, Kaminska B. Non-apoptotic Fas signaling regulates invasiveness of glioma cells and modulates MMP-2 activity via NFkappaB-TIMP-2 pathway. Cell Signal 2010; 22: 212-220.19. Nozoe T, Yasuda M, Honda M, Inutsuka S, Korenaga D. Fas ligand expression is correlated with metastasis in colorectal carcinoma. Oncology 2003; 65: 83-88.

20. Li H, Fan X, Stoicov C, Liu JH, Zubair S, Tsai E, Ste MR, Wang TC, Lyle S, Kurt-Jones E, Houghton J. Human and mouse colon cancer utilizes CD95 signaling for local growth and metastatic spread to liver. Gastroenterology 2009; 137: 934-44, 944.
21. Sairanen T, Karjalainen-Lindsberg ML, Paetau A, Ijas P, Lindsberg PJ. Apoptosis dominant in the periinfarct area of human ischaemic stroke--a possible target of antiapoptotic treatments. Brain 2006; 129: 189-199.
22. Abreu-Martin MT, Palladino AA, Faris M, Carramanzana NM, Nel AE, Targan SR. Fas activates the JNK pathway in human colonic epithelial cells: lack of a direct role in apoptosis. Am J Physiol 1999; 276: G599-G605.
23. Park SM, Chen L, Zhang M, Ashton-Rickardt P, Turner JR, Peter ME. CD95 is cytoprotective for intestinal epithelial cells in colitis. Inflamm Bowel Dis 2010; 16: 1063-1070.
24. Liu K, McDuffie E, Abrams SI. Exposure of human primary colon carcinoma cells to anti-Fas interactions influences the emergence of pre-existing Fas-resistant metastatic subpopulations. J Immunol 2003; 171: 4164-4174.
25. Mann B, Gratchev A, Bohm C, Hanski ML, Foss HD, Demel G, Trojanek B, Schmidt-Wolf I, Stein H, Riecken EO, Buhr HJ, Hanski C. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. Br J Cancer 1999; 79: 1262-1269.
26. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996; 184: 1075-1082.
27. Yoong KF, Afford SC, Randhawa S, Hubscher SG, Adams DH. Fas/Fas ligand interaction in human colorectal hepatic metastases: A mechanism of hepatocyte destruction to facilitate local tumor invasion. Am J Pathol 1999; 154: 693-703.
28. Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. Nat Cell Biol 2007; 9: 210-217.
29. Gort EH, Groot AJ, van der Wall E., van Diest PJ, Vooijs MA. Hypoxic Regulation of Metastasis via Hypoxia-Inducible Factors. Curr Mol Med 2008; 8: 60-67.
30. Chung FY, Huang MY, Yeh CS, Chang HJ, Cheng TL, Yen LC, Wang JY, Lin SR. GLUT1 gene is a potential hypoxic marker in colorectal cancer patients. BMC Cancer 2009; 9: 241.
31. Koukourakis MI, Giatromanolaki A, Sivridis E, Gatter KC, Harris AL. Lactate dehydrogenase 5 expression in operable colorectal cancer: strong association with survival and activated vascular endothelial growth factor pathway--a report of the Tumour Angiogenesis Research Group. J Clin Oncol 2006; 24: 4301-4308.
32. Ohno T, Kawano K, Yokoyama H, Tahara K, Sasaki A, Aramaki M, Kitano S. Microwave coagulation therapy accelerates growth of cancer in rat liver. J Hepatol 2002; 36: 774-779.
33. von Breitenbuch P, Kohl G, Guba M, Geissler E, Jauch KW, Steinbauer M. Thermoablation of colorectal liver metastases promotes proliferation of residual intrahepatic neoplastic cells. Surgery 2005; 138: 882-887.
34. van der Bilt JD, Kranenburg O, Nijkamp MW, Smakman N, Veenendaal LM, Te Velde EA, Voest EE, van Diest PJ, Borel Rinkes IH. Ischemia/reperfusion accelerates the outgrowth of hepatic micrometastases in a highly standardized murine model. Hepatology 2005; 42: 165-175.
35. van der Bilt JD, Soeters ME, Duyverman AM, Nijkamp MW, Witteveen PO, van Diest PJ, Kranenburg O, Borel Rinkes IH. Perinecrotic hypoxia contributes to ischemia/reperfusion-accelerated outgrowth of colorectal micrometastases. Am J Pathol 2007; 170: 1379-1388.
36. Nijkamp MW, van der Bilt JD, Snoeren N, Hoogwater FJ, van Houdt WJ, Molenaar IQ, Kranenburg O, van Hillegersberg R, Borel Rinkes IH. Prolonged portal triad clamping during liver surgery for colorectal liver metastases is associated with decreased time to hepatic tumour recurrence. Eur J Surg Oncol 2009; 36: 182-188.
37. Portolani N, Tiberio GA, Ronconi M, Coniglio A, Ghidoni S, Gaverini G, Giulini SM. Aggressive recurrence after radiofrequency ablation of liver neoplasms. Hepatogastroenterology 2003; 50: 2179-2184.
38. Ruzzenente A, Manzoni GD, Molfetta M, Pachera S, Genco B, Donataccio M, Guglielmi A. Rapid progression of hepatocellular carcinoma after Radiofrequency Ablation. World J Gastroenterol 2004; 10: 1137-1140.
39. Cohen PL and Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu Rev Immunol 1991; 9:243-69.: 243-269.
40. Jackson CE and Puck JM. Autoimmune lymphoproliferative syndrome, a disorder of apoptosis. Curr Opin Pediatr 1999; 11: 521-527.

CHAPTER 5

A ROLE FOR CD95 SIGNALING IN ISCHEMIA/ REPERFUSION-INDUCED INVASION AND OUT-GROWTH OF COLORECTAL MICROMETASTASES IN MOUSE LIVER

Submitted

Maarten W. Nijkamp
Frederik J.H. Hoogwater
Klaas M. Govaert
Ernst J.A. Steller
Andre Verheem
Onno Kranenburg
Inne H.M. Borel Rinkes

Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands

ABSTRACT

Background

Ischemia/reperfusion (I/R) injury is frequently caused by hepatic surgery due to clamping of the vascular inflow of the liver. I/R injury generates hepatocellular damage but is also associated with accelerated outgrowth of micrometastases. Recently, we demonstrated that CD95 is a key mediator of tumor cell invasion and outgrowth following radiofrequency ablation. Here, we tested whether CD95 signaling plays a role in accelerated outgrowth of colorectal liver metastases following I/R.

Methods

Mice underwent vascular clamping five days after induction of colorectal liver metastases. Invasion and outgrowth of micrometastases following I/R were analyzed by post-mortem confocal microscopy (36 hours post-I/R) and by morphometric assessment of tumor load (5 days post-I/R), respectively. Tumor cell CD95 was suppressed by lentiviral RNA interference. The contribution of host CD95L was assessed by using gld-mice lacking functional CD95L.

Results

I/R induced invasion of micrometastases selectively in the perinecrotic regions. CD95 knockdown in tumor cells strongly reduced invasion and largely prevented accelerated outgrowth of perinecrotic liver metastases following I/R. I/R-induced liver necrosis and necrosis-associated accelerated tumor growth were reduced in *gld*-mice. However, the remaining perinecrotic tumor cell clusters in *gld*-mice still displayed an invasive phenotype.

Conclusions

I/R induces invasion and accelerated outgrowth of pre-established metastases in a CD95-dependent manner. Activation of the CD95 system following I/R not only contributes to liver injury, but may also promote aggressive tumor recurrence.

INTRODUCTION

Approximately 50% of colorectal cancer patients develop liver metastases. For these patients, partial liver resection is the only curative treatment option, leading to a 5 years survival of approximately 40-60%[1,2]. Nonetheless, tumor recurrence following partial liver resection is observed in ~60% of cases[2,3]. Vascular clamping is frequently applied to limit excessive blood loss during hepatic resection and can cause ischemia/reperfusion (I/R) injury to the liver. I/R not only causes tissue injury, but also has a growth-stimulatory effect on residual tumor tissue in the liver[4-6]. Indeed, in colorectal cancer patients receiving liver surgery for colorectal liver metastases, prolonged vascular clamping is associated with a reduced time to tumor recurrence in the liver[7].

I/R in the liver generates long-term microcirculatory disturbances and chronic hypoxia, which play an important role in accelerated tumor outgrowth following hepatic I/R[8]. Hypoxia can stimulate motility and invasion of tumor cells through induction of matrix-metalloproteinases (MMPs), urokinase plasminogen activator receptor (uPAR) and/or c-Met[9-11]. Recently, we demonstrated that hypoxia also activates the CD95/CD95L system in apoptosis-resistant colorectal tumor cells and that this mediates tumor cell invasion[12]. Moreover, radiofrequency ablation (RFA) resulted in invasion and accelerated outgrowth of tumor cells in the hypoxic tissue areas surrounding RFA lesions and this could be abrogated by suppressing the CD95/CD95L system[12]. CD95 is also activated during liver I/R and mediates tissue injury[13,14]. This is most likely due to the fact that hepatocytes express CD95 and are highly sensitive to CD95-mediated apoptosis[15]. However, CD95 was not a critical mediator of liver injury following I/R in another study[16].

Based on these observations, we hypothesized that CD95 activation during I/R may contribute to invasion and outgrowth of hepatic micrometastases. In the present study we demonstrate that CD95 and CD95L are upregulated following hepatic I/R and that this contributes to invasion and outgrowth of micrometastases. Our results identify CD95 as an important participant in local tumor cell invasion and accelerated outgrowth of micrometastases following liver I/R.

MATERIALS AND METHODS

Animals and surgery

All experiments were performed in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Male BALB/c mice (10-12 weeks) were purchased from Charles River (Sulzfeld, Germany). BALB/c-gld/gld (CPt. C3-Fasl^{gld}/J) mice carrying a homozygous loss-of-function mutation in CD95L (hereafter referred to as *gld*-mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

When *gld*-mice were used, control BALB/c mice were purchased from The Jackson Laboratory as well. Mice were housed under standard laboratory conditions and received food and water *ad libitum*. All surgical procedures were performed under isoflurane inhalation anesthesia. Prior to surgery, buprenorfine was administered intramuscularly to provide sufficient peri-operative analgesia. During surgery, body temperature was maintained at 36,5˚C to 37,5˚C by placing the mice on a heated table and covering them with aluminium foil.

Induction of micrometastases and ischemia/reperfusion injury

C26 mouse colon carcinoma cells and its derivatives, including Green Fluorescent Protein (GFP)-expressing C26 cells, C26-CD95 knockdown cells and C26-Luciferase-knockdown control cells were cultured exactly as previously described[12]. For tumor cell invasion experiments, GFP-expressing C26 cells were used. When C26-CD95 knockdown (kd) cells were used, cells expressing shRNA's directed at firefly luciferase (sh-Luc) were used as control cells. For induction of colorectal micrometastases we used our previously described mouse model[5,8.] In brief, through a left lateral flank incision, $5x10^4$ routinely cultured C26 colon carcinoma cells were injected into the splenic parenchyma, followed by removal of the spleen after ten minutes to prevent intrasplenic tumor growth.

Diffuse intrahepatic micrometastases were allowed to grow out for five days, followed by partial hepatic ischemia. After laparotomy, the liver hilus was exposed and the vascular inflow to the left lateral lobe was clamped for 45 minutes. Tumor cell invasion was assessed 36 hours later in both the clamped and non-clamped lobes. Tumor outgrowth was assessed 5 days following I/R. Sham-operated animals underwent laparotomy with subsequent exposure of the liver hilus, but without clamping the hepatic blood inflow.

Liver enzymes

Early liver damage was assessed by plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Blood was drawn from mice 6 hours following surgery and centrifuged at 14000 rpm for 10 minutes (n=8 I/R groups, n=4 sham groups). Plasma levels of ALT and AST were automatically analyzed (Beckman Coulter UniCel® DxC 600, Beckman Coulter B.V. Woerden, The Netherlands) and were expressed as units per liter (U/L).

Assessment of the perinecrotic tumor cell phenotype following I/R

Mice bearing GFP-expressing tumor cells were sacrificed at 36 hours following I/R of the left liver lobe. Prior to sacrifice, mice were injected intravenously with rhodamine(RITC)-labeled dextran (MW 2.000.000; Invitrogen, Carlsbad, CA) to identify the perinecrotic region. The non-clamped and clamped liver lobes were harvested and placed on a coverslip using immersion oil to improve visualization. The livers were imaged with a Zeiss LSM510 Meta confocal microscope using 10x magnification (n=4 mice each group). As previously described, the perinecrotic region is characterized by microcirculatory disturbances[8,17], which could be

visualized by exciting RITC at 561 nm. GFP was excited at 488nm to visualize the tumor cells. Tumor cell invasion was assessed using confocal image stacks in axial dimension and was defined as the average longest distance between cells making up a single cluster metastasis, exactly as previously described[12]. At least 15 randomly chosen tumor cell clusters in clamped and non-clamped liver lobes were visualized and relayed to a personal computer for off line analysis. Analysis of tumor cell invasion was performed by two independent observers.

Western blot analysis of tissue extracts

Tumor-bearing mice (n=4) were sacrificed 36 hours following surgery. After harvesting the livers, the clamped and non-clamped liver lobes were divided. Next, the liver sections were minced and homogenized. Homogenized tissue samples were then lysed in a buffer containing 20 mM HEPES pH7.4, 1% NP40, 150 mM NaCl, 5 mM MgCl2 and 10% glycerol. Lysates were cleared by centrifugation (Eppendorf, 13.000 rpm) and analyzed by Western blotting using anti-CD95 (clone M-20, #sc-716, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-CD95L (ab15285, Abcam, Cambridge, MA, USA) and anti-ß-actin (AC-15, NB600-501, Novus Biologicals, Littleton, CO, USA).

Immunohistochemistry

Tumor-bearing mice (n=4 each time point) were sacrificed 36 hours and 5 days after I/R. After harvesting, livers were fixed in formaldehyde and embedded in paraffin. Tissue sections (4µm) were used for the immunohistochemical staining of CD95L (ab15285, Abcam, Cambridge, MA, USA). As secondary antibody PowerVision+ (Immunologic, Duiven, The Netherlands) with 2% mouse serum was used. Reactions were developed using diaminobenzidine/H_2O_2 as a chromogen substrate. Negative controls were stained with isotype control antibody and were all free of nonspecific background staining.

Analysis of tumor load and hepatocellular necrosis

Tumor load in the liver was assessed in both the clamped and the non-clamped liver lobes. Tumor load was scored as hepatic replacement area (HRA), i.e. the percentage of liver tissue that had been replaced by tumor tissue[5,8]. In brief, on hematoxylin and eosin (H&E) stained sections, at least 100 fields were selected using an interactive video overlay system, including an automated microscope (Q-Prodit; Leica Microsystems, Rijswijk, The Netherlands) at a 40x magnification. Using a four-points grid overlay, the ratio of tumor cells versus normal hepatocytes plus necrotic cells was determined for each field. Tumor load (HRA) was expressed as the average area ratio of all fields.

The percentage of hepatocellular necrosis was scored simultaneously with tumor HRA analysis. The ratio of necrotic cells versus normal hepatocytes plus tumor cells was determined for each field. The percentage of hepatocellular necrosis was expressed as the average area ratio of all fields.

Statistical analysis

Statistical differences between the clamped and the unclamped lobes were analyzed by a paired t-test or Wilcoxon signed rank test when appropriate. Differences between groups were analyzed by ANOVA or Kruskal-Wallis test when appropriate. Data are expressed as mean +/- SEM. A p-value < 0.05 was considered statistically significant.

RESULTS

Hepatic I/R induces scattering of micrometastases in perinecrotic liver tissue

Micrometastases in the livers from sham-operated mice grew non-invasively, both in the median and left liver lobe (Figure 1). Necrotic tissue areas had formed in the clamped but not in the non-clamped liver lobes 36 hours following I/R. Micrometastases surrounding these

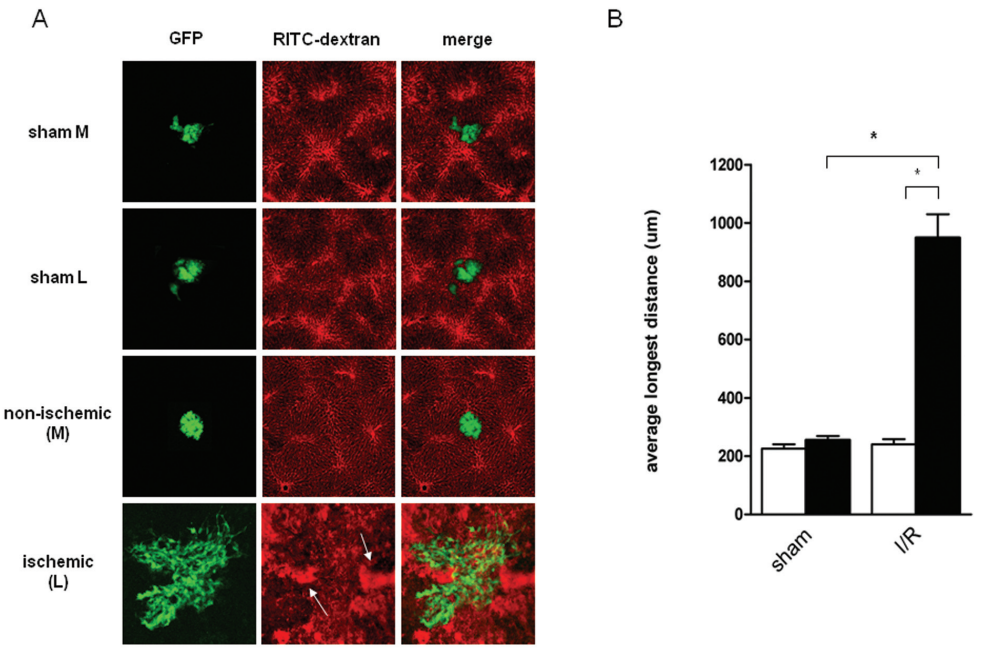

Figure 1. Perinecrotic tumor cell invasion in the clamped liver lobes following I/R. (**A**) Mice carrying EGFP-expressing micrometastases were subjected to I/R or sham operation and were analyzed by confocal microscopy 36 hours later. The perinecrotic area was identified by microcirculatory disturbances (arrow). Image z-stacks were created in axial dimension to visualize tumor cell invasion, defined as the average longest distance between tumor cells making up a single cluster/metastasis. (**B**) Quantification of metastasis diameters in mice that underwent sham operation or I/R (≥15/mice; n=4 mice each group). In I/R mice, black bars represent clamped (ischemic) liver lobes, white bars represent unclamped (non-ischemic) liver lobes. In sham-operated mice, black bars represent left liver lobes, white bars represent median liver lober. M median liver lobe; L left liver lobe; * p<0.05.

necrotic tissue areas displayed a characteristic scatter response, which is indicative of tumor cell invasion. Quantification of the average longest distance between tumor cells making up a single metastasis revealed that peri-necrotic 'scattered' metastases in the clamped (left) liver lobes had an average diameter of 949 ± 81 µm. This was significantly longer than the diameter of metastases in the unclamped median lobes (241 ± 18 µm; p=0.0008, figure 1) and in the left lobe of sham-operated mice (256 ± 15 µm; p=0.0001, figure 1).

Induction of CD95 and CD95L following hepatic I/R

At 36 hours following I/R, the liver lobes were harvested, lysed and analyzed for expression of CD95 and CD95L by Western blotting. CD95 was induced in the clamped liver lobes compared to the unclamped liver lobes in 3 out of 4 mice. Moreover, CD95L was induced in the clamped liver lobes of all 4 mice (Figure 2A). Next, immunohistochemistry was performed on liver tissue sections of tumor-bearing mice 36 hours and 5 days following I/R. The induction of CD95L in the clamped liver lobes 36 hours following I/R, as it was observed on the Western blots, could not be detected by immunohistochemistry (data not shown). However, 5 days following I/R, CD95L was strongly expressed in the rim of invasive tumor tissue facing the areas of I/R-induced necrosis (Figure 2B).

I/R-injury is reduced in gld-mice

In wildtype mice, I/R induced early hepatocelular damage as evidenced by elevated plasma ALT and AST levels (Figures 3A and 3B). *Gld*-mice, however, were partly protected from I/R-induced

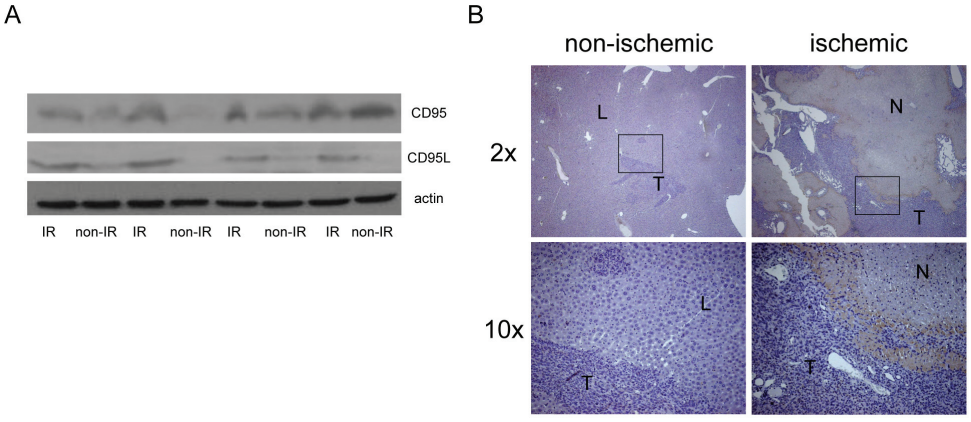

Figure 2. Upregulation of CD95 and CD95L in the clamped liver lobes following I/R. (**A**) Tissue lysates were prepared from clamped (ischemic)- and unclamped (non-ischemic) liver lobes 36 hours after I/R and were analyzed by Western blotting using specific antibodies directed against CD95 and CD95L. Actin was used as a loading control. (**B**) Immunohistochemichal staining of clamped (ischemic)- and unclamped (non-ischemic) tumor-bearing liver lobes 5 days following I/R, using anti-CD95L antibody. IR ischemic liver lobe; non-IR non-ischemic liver lobe; T tumor; L liver; N necrosis.

liver damage as plasma ALT levels were reduced by ~55% (3145 ± 864 U/L versus 6891 ± 1310 U/L in wildtype mice, p=0.036, figure 3A) and plasma AST levels were reduced by ~35% (2124 ± 412 U/L versus 3241 ± 446 U/L in wildtype mice, p=0.083, figure 3B). In addition, the formation of necrotic tissue areas 5 days post-I/R was also significantly reduced in gld-mice when compared to wildtype mice (9.1 ± 4.3% vs 22.8 ± 3.8%, p=0.035, figure 3C).

CD95 knockdown prevents invasion of perinecrotic metastases following I/R

Since I/R-injury was reduced in *gld*-mice, we investigated whether tumor cell invasion following I/R would also be reduced in these mice. However, micrometastases surrounding the necrotic regions in *gld*-mice still displayed the invasive phenotype that was also observed in wildtype mice (1043 ± 124 µm versus 867 ± 92 µm p=0.29, respectively; figure 4).

Next, we tested whether tumor cell CD95 was essential for the invasive phenotype of perinecrotic metastases. The diameter of perinecrotic metastases in clamped liver lobes was significantly reduced from 824 ± 31 µm in mice bearing C26 shRNA-luc control cells to 481 ± 31 µm in mice bearing C26-CD95kd cells (p=0.0003, figure 4).

CD95 signaling is required for accelerated tumor outgrowth following I/R

Following I/R in control mice, increased tumor load (expressed as the hepatic replacement area (HRA)) was observed in the clamped liver lobes compared to unclamped lobes, as expected (Figures 5A and 5C)[5,8]. In *gld*-mice, the increased tumor load in the clamped lobes was reduced when compared to that in wildtype mice, although this did not reach statistical significance (26.8 ± 4.9% vs 38.4 ± 6.0% p=0.14, respectively; figures 5A and 5C). However, the acceleration of tumor growth, as expressed by the ratio of HRA values in the clamped versus the non-clamped lobes, was significantly reduced in *gld*-mice (HRA ratio 4.9 ± 1.9 for gld-mice versus 8.3 ± 1.4 for control mice, p=0.035; figure 5B).

Next, we tested whether the accelerated tumor growth in clamped liver lobes 5 days following I/R was reduced when interfering with CD95 signaling in tumor cells (CD95kd cells). The increase in tumor load in clamped liver lobes of mice bearing C26-CD95kd cells was significantly lower when compared to that of mice bearing C26 shRNA-luc control cells (21.1 ± 6.2% versus 34.5 ± 3.2% p=0.049, respectively; figure 5A and 5C). In addition, tumor growth acceleration (HRA ratio) was significantly reduced using CD95kd cells, from 7.8 ± 1.1 for C26 shRNA-luc control cells to 4.7 ± 0.6 for C26-CD95kd cells (p=0.0081, figure 5B).

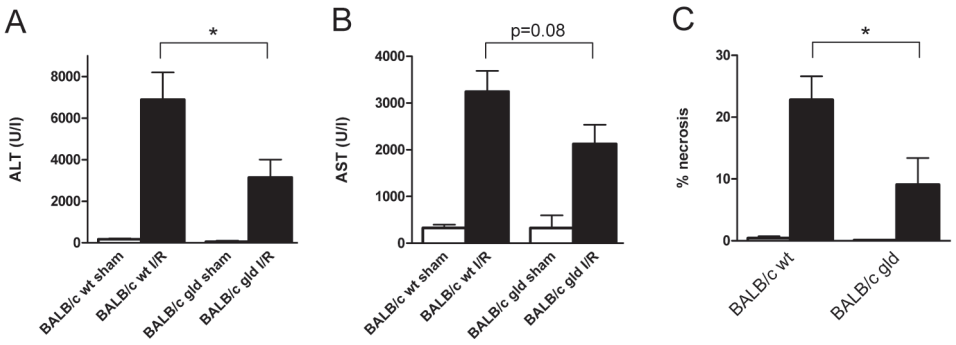

Figure 3. I/R-injury is reduced in *gld*-mice. Plasma levels of (**A**) alanine aminotransferase (ALT) and (**B**) aspartate aminotransferase (AST) from wildtype (wt) mice and *gld*-mice 6 hours after I/R or sham operation. (**C**) Percentage of liver tissue necrosis in wildtype mice and *gld*-mice 5 days after I/R. White bars indicate unclamped liver lobe, black bars indicate clamped liver lobe. * p<0.05.

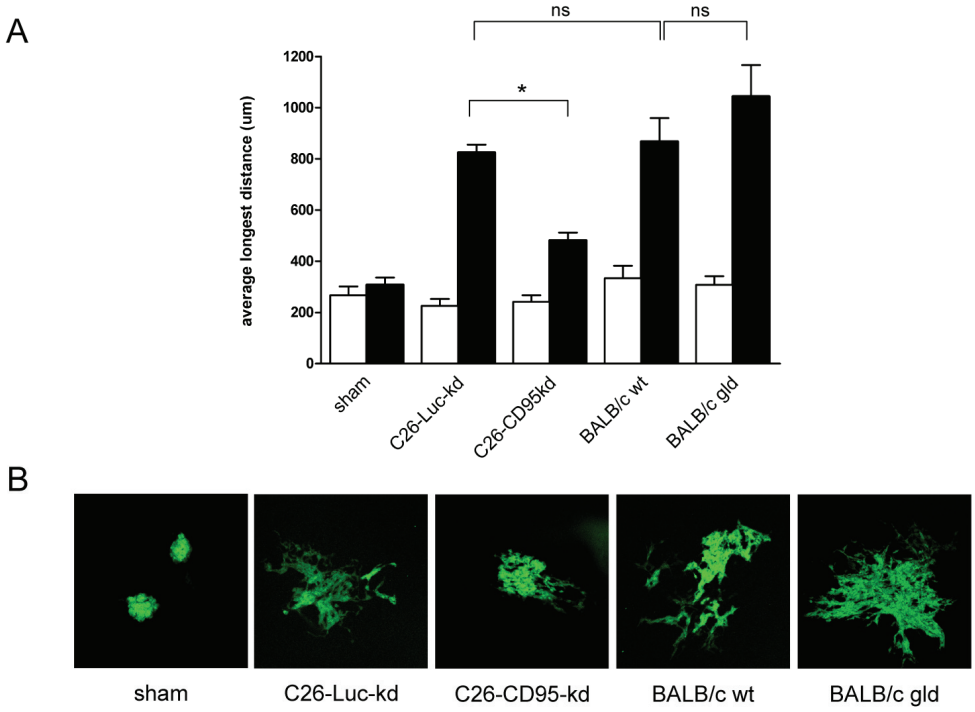

Figure 4. Metastasis invasion following I/R requires autocrine CD95 signaling. I/R was performed in mice carrying C26-Luc-kd or C26-CD95kd metastases. Additionally, EGFP-expressing C26 cells were allowed to form metastases in control mice and in gld-mice lacking functional CD95L. (**A**) Metastasis diameters in the unclamped non-ischemic (white bars) and the clamped ischemic liver lobes (black bars) determined 36 hours following I/R, as in Figure 1 (n≥15 metastases). (**B**) Representative confocal microscopic images of metastases located in the clamped liver lobes. kd knock down; * p<0.05; ns not significant

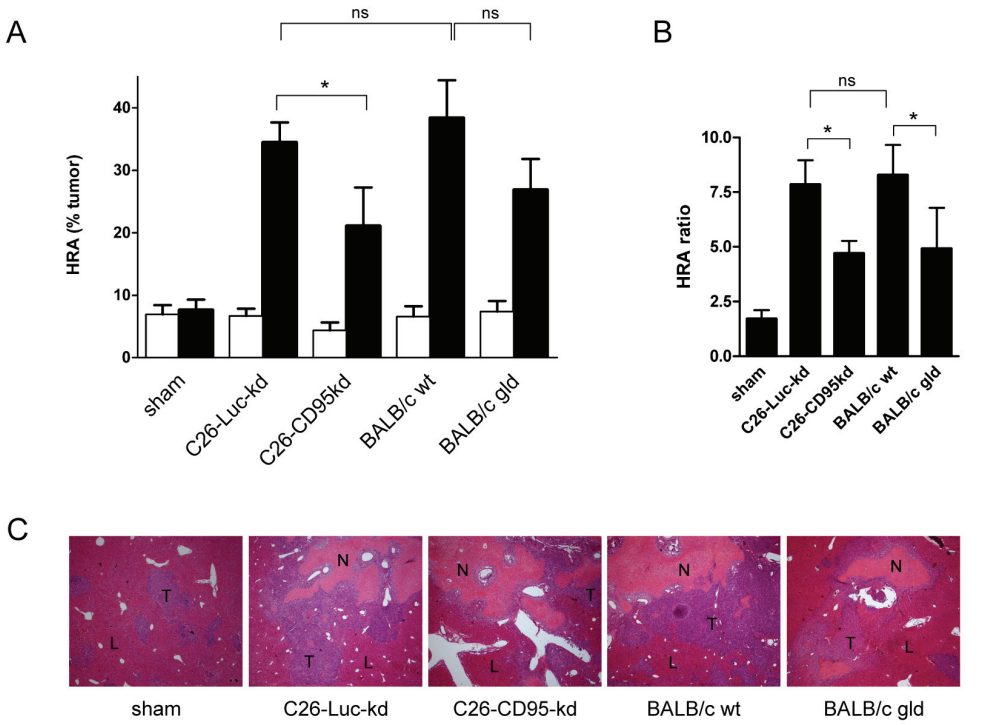

Figure 5. Accelerated outgrowth of liver metastases following I/R is reduced by inhibiting CD95 signaling in tumor cells. I/R was performed on mice carrying C26-Luc-kd or C26-CD95kd metastases. Additionally, I/R was performed on tumor-bearing control mice and on *gld*-mice. All mice were sacrificed 5 days following I/R. (**A**) The livers were harvested and tumor load in unclamped non-ischemic (white bars) and in the clamped ischemic liver lobes (black bars) was analyzed by morphometric measurement of the hepatic replacement areas (HRA). (**B**) HRA ratio values were determined and plotted. These values indicate the acceleration of tumor growth in the clamped ischemic liver lobe relative to the unclamped non-ischemic liver lobe. (**C**) Representative microscopic images of tumor growth in the clamped ischemic liver lobe by H&E histochemistry. wt wildtype; kd knockdown; T tumor; L liver; N necrosis; * $p<0.05$; ns not significant

DISCUSSION

In the present study we have demonstrated that CD95 signaling in tumor cells contributes to I/R-induced invasion and outgrowth of micrometastases. These results are in accordance with our previous results demonstrating that tumor cell CD95 mediates accelerated invasive outgrowth of liver metastases in the hypoxic transition zone surrounding radiofrequency ablation (RFA)-generated lesions[12]. Likewise, accelerated tumor outgrowth following I/R is exclusively observed in areas of prolonged microcirculatory disturbances and chronic hypoxia[8].

Tumor growth may be stimulated by CD95/CD95L signaling in several ways. First, CD95 may induce apoptosis in hepatocytes, thereby facilitating tumor cell invasion and outgrowth[18].

Second, CD95 may cause apoptosis of infiltrating cytotoxic lymphocytes, resulting in a reduction of the anti-tumor immune response. This phenomenon is known as the 'tumor counterattack'[19]. Third, CD95 may activate non-apoptotic pro-tumorigenic signaling pathways in tumor cells. Evidence is accumulating that CD95 signaling can promote tumor cell invasion[20-22]. We have recently demonstrated that the KRAS oncogene switches CD95 from a death receptor into an invasion-inducing receptor[22]. Interestingly, *in vitro* hypoxia alone was sufficient to induce CD95-dependent invasion[12].

In the present study, mice lacking functional CD95L (*gld*-mice) showed reduced I/R injury as demonstrated by reduced post-operative ALT and AST plasma levels and reduced tissue necrosis following I/R. These results are in accordance with those of other investigators[13,14] and suggest that hepatocyte cell death following I/R is partly mediated by CD95. Whether post-I/R hepatocytes die by apoptosis, by necrosis or by an intermediate form of cell death is still a matter of debate[15,23]. In the present study we have referred to the post-IR lesions of cell debris that were identified by histology as 'necrotic'. Nevertheless, the initial type of hepatocyte cell death that caused the formation of these lesions is at least in part apoptotic since it is characterized by the presence of active caspase 3-positive cells[5] and it is partly dependent on CD95L (present study). In contrast, another study showed that I/R-induced liver injury was CD95/CD95L-independent[16]. Possibly, differences in the genetic backgrounds of the mice (C57Bl6 *versus* BALB/c) may underlie these different dependencies on CD95.

Interestingly, accelerated outgrowth, but not perinecrotic invasion of micrometastases was reduced in *gld*-mice following I/R. Accelerated tumor growth following I/R is exclusively observed in perinecrotic hypoxic tissue areas[5,8]. Since CD95L contributes to I/R injury and necrosis formation, the reduced tumor outgrowth following I/R in *gld*-mice is most likely the result of reduced formation of necrotic tissue, although a direct stimulatory effect of host CD95L on tumor cells cannot be excluded. Nonetheless, the observation that tumor cell invasion in perinecrotic tissue following I/R is similar in *gld*-mice and wildtype mice, but is strongly reduced when using CD95 knockdown tumor cells, strongly suggests that autocrine CD95 signaling in tumor cells mediates this response. This is in line with our previous study showing that autocrine CD95 signaling mediates tumor cell invasion and accelerated outgrowth in hypoxic tissue surrounding RFA-generated lesions[12]. In this study, tumor outgrowth was not affected in the *gld*-mice, most likely because RFA-generated tissue necrosis, unlike that generated by I/R, does not depend on CD95L. Together, these results are in line with a model in which host CD95L contributes to I/R-induced (but not RFA-induced) tissue necrosis and thereby indirectly affects tumor outgrowth. Secondly, autocrine CD95 signaling on tumor cells in hypoxic perinecrotic tissue contributes to the aggressive invasive behaviour of micrometastases in these areas following both surgical procedures (Figure 6).

The mechanisms by which I/R may accelerate the outgrowth of tumour cell deposits are incompletely understood. Nicoud *et al*. showed that vascular clamping was associated with upregulation of matrix-metalloprotease (MMP)-9 and MMP-9 inhibition resulted in reduced

tumor outgrowth[4]. MMP-9 is frequently upregulated in colorectal tumor cells and induces invasion of tumor cells by degrading the extracellular matrix[24,25]. Interestingly, CD95 induces MMP-9 expression to mediate invasion of gliomas[20]. Further research should elucidate whether MMP-9 expression following I/R depends on CD95 activation.

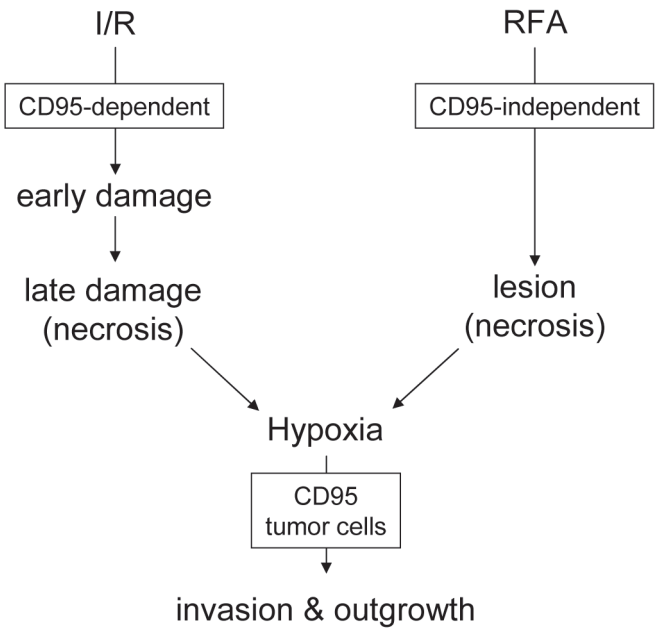

Figure 6. Schematic overview of surgery-induced, CD95-dependent tumor invasion and outgrowth in the liver. CD95 signaling in tumor cells plays a key role in I/R- and RFA-stimulated invasion and outgrowth of micrometastases in the liver. In addition, host CD95L has an indirect effect on outgrowth of micrometastases due to its role in necrosis formation following I/R.$p<0.05$; ns not significant

Hypoxia is an unavoidable consequence of wound healing and surgery. Here, we demonstrate that hepatic I/R injury due to vascular clamping induces aggressive tumor outgrowth which depends on CD95 signaling in tumor cells. Interestingly, prolonged vascular clamping during liver surgery for colorectal liver metastases was significantly associated with decreased time to hepatic tumor recurrence in patients[7]. In addition, high preoperative levels of circulating CD95L were significantly associated with a reduced period of disease free survival following liver surgery in colorectal cancer patients (FJHH, submitted). Taken together, we propose that CD95 signaling may contribute to hepatic tumor recurrence following surgery for colorectal liver metastases. As such, it may form a potential therapeutic target in these patients.

REFERENCES

1. Choti MA, Sitzmann JV, Tiburi MF, Sumetchotimetha W, Rangsin R, Schulick RD, Lillemoe KD, Yeo CJ, Cameron JL. Trends in long-term survival following liver resection for hepatic colorectal metastases. Ann Surg 2002; 235: 759-766.
2. de Jong MC, Pulitano C, Ribero D, Strub J, Mentha G, Schulick RD, Choti MA, Aldrighetti L, Capussotti L, Pawlik TM. Rates and patterns of recurrence following curative intent surgery for colorectal liver metastasis: an international multi-institutional analysis of 1669 patients. Ann Surg 2009; 250: 440-448.
3. Simmonds PC, Primrose JN, Colquitt JL, Garden OJ, Poston GJ, Rees M. Surgical resection of hepatic metastases from colorectal cancer: a systematic review of published studies. Br J Cancer 2006; 94: 982-999.
4. Nicoud IB, Jones CM, Pierce JM, Earl TM, Matrisian LM, Chari RS, Gorden DL. Warm Hepatic Ischemia-Reperfusion Promotes Growth of Colorectal Carcinoma Micrometastases in Mouse Liver via Matrix Metalloproteinase-9 Induction. Cancer Res 2007; 67: 2720-2728.
5. van der Bilt JD, Kranenburg O, Nijkamp MW, Smakman N, Veenendaal LM, Te Velde EA, Voest EE, van Diest PJ, Borel Rinkes IH. Ischemia/reperfusion accelerates the outgrowth of hepatic micrometastases in a highly standardized murine model. Hepatology 2005; 42: 165-175.
6. Man K, Ng KT, Lo CM, Ho JW, Sun BS, Sun CK, Lee TK, Poon RT, Fan ST. Ischemia-reperfusion of small liver remnant promotes liver tumor growth and metastases--activation of cell invasion and migration pathways. Liver Transpl 2007; 13: 1669-1677.
7. Nijkamp MW, van der Bilt JD, Snoeren N, Hoogwater FJ, van Houdt WJ, Molenaar IQ, Kranenburg O, van Hillegersberg R., Borel Rinkes IH. Prolonged portal triad clamping during liver surgery for colorectal liver metastases is associated with decreased time to hepatic tumour recurrence. Eur J Surg Oncol 2009; 36: 182-188.
8. van der Bilt JD, Soeters ME, Duyverman AM, Nijkamp MW, Witteveen PO, van Diest PJ, Kranenburg O, Borel Rinkes IH. Perinecrotic hypoxia contributes to ischemia/reperfusion-accelerated outgrowth of colorectal micrometastases. Am J Pathol 2007; 170: 1379-1388.
9. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2002; 2: 38-47.
10. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3: 721-732.
11. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006; 441: 437-443.
12. Nijkamp MW, Hoogwater FJ, Steller EJ, Westendorp BF, van der Meulen TA, Leenders MW, Borel Rinkes IH, Kranenburg O. CD95 is a key mediator of invasion and accelerated outgrowth of mouse colorectal liver metastases following radiofrequency ablation. J Hepatol 2010; in press.
13. Cursio R, Filippa N, Miele C, Colosetti P, Auberger P, Van OE, Gugenheim J. Fas ligand expression following normothermic liver ischemia-reperfusion. J Surg Res 2005; 125: 30-36.
14. Nakajima H, Mizuta N, Fujiwara I, Sakaguchi K, Ogata H, Magae J, Yagita H, Koji T. Blockade of the Fas/Fas ligand interaction suppresses hepatocyte apoptosis in ischemia-reperfusion rat liver. Apoptosis 2008; 13: 1013-1021.
15. Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 2006; 43: S31-S44.
16. Rudiger HA and Clavien PA. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. Gastroenterology 2002; 122: 202-210.
17. Nijkamp MW, van der Bilt JD, de Bruijn MT, Molenaar IQ, Voest EE, van Diest PJ, Kranenburg O, Borel Rinkes IH. Accelerated perinecrotic outgrowth of colorectal liver metastases following radiofrequency ablation is a hypoxia-driven phenomenon. Ann Surg 2009; 249: 814-823.
18. Yoong KF, Afford SC, Randhawa S, Hubscher SG, Adams DH. Fas/Fas ligand interaction in human colorectal hepatic metastases: A mechanism of hepatocyte destruction to facilitate local tumor invasion. Am J Pathol 1999; 154: 693-703.19. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996; 184: 1075-1082.

19. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996; 184: 1075-1082
20. Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, Thiemann M, Mueller W, Sykora J, Kuhn A, Schreglmann N, Letellier E, Zuliani C, Klussmann S, Teodorczyk M, Grone HJ, Ganten TM, Sultmann H, Tuttenberg J, von DA, Regnier-Vigouroux A, Herold-Mende C, Martin-Villalba A. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 2008; 13: 235-248.
21. Barnhart BC, Legembre P, Pietras E, Bubici C, Franzoso G, Peter ME. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J 2004; 23: 3175-3185.
22. Hoogwater FJ, Nijkamp MW, Smakman N, Steller EJ, Emmink BL, BF Westendorp, Raats DA, Sprick MR, Schaeffer U, van Houdt WJ, de Bruijn MT, Schackmann RC, Derksen PW, Medema JP, Walczak H, Borel Rinkes IH, Kranenburg O. Oncogenic K-Ras Turns Death Receptors Into Metastasis-Promoting Receptors in Human and Mouse Colorectal Cancer Cells. Gastroenterology 2010; 137: 2357-2367.
23. Massip-Salcedo M, Rosello-Catafau J, Prieto J, Avila MA, Peralta C. The response of the hepatocyte to ischemia. Liver Int 2007; 27: 6-16.
24. Waas ET, Wobbes T, Lomme RM, DeGroot J, Ruers T, Hendriks T. Matrix metalloproteinase 2 and 9 activity in patients with colorectal cancer liver metastasis. Br J Surg 2003; 90: 1556-1564.
25. Zucker S and Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev 2004; 23: 101-117.

CHAPTER 6

CIRCULATING CD95 LIGAND AS A POTENTIAL PROGNOSTIC FACTOR FOR DISEASE-FREE SURVIVAL FOLLOWING SURGERY IN PATIENTS WITH SYNCHRONOUS COLORECTAL LIVER METASTASES

Submitted

Frederik J.H. Hoogwater[1*]
Nikol Snoeren[1*]
Maarten W. Nijkamp[1]
Amy C. Gunning[1]
Winan J. van Houdt[1]
Menno T. de Bruijn[1]
Emile E. Voest[2]
Richard van Hillegersberg[1]
Onno Kranenburg[1]
Inne H.M. Borel Rinkes[1]

[*] *these authors contributed equally*

Department of [1]Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
Department of [2]Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands

ABSTRACT

Background

Only a subgroup of patients with synchronous colorectal liver metastases benefits from liver surgery. However, there are currently no reliable tools to identify such patients. Expression of CD95 ligand in colorectal tumor tissue has been associated with a more aggressive tumor phenotype and tumor progression is selectively promoted by the soluble form of CD95 ligand (sCD95L). The aim of this study was to assess whether circulating sCD95L levels in serum are associated with disease-free survival (DFS) in colorectal cancer patients with synchronous liver metastases.

Methods

Blood samples were obtained from 31 patients with synchronous colorectal liver metastases before and after liver surgery. Serum sCD95L levels were determined using Enzyme-Linked Immunosorbent Assays (ELISA's). Cox regression analysis was performed to determine the correlation between sCD95L levels and DFS.

Results

Mean follow-up was 38 months. High pre-operative sCD95L levels were associated with poor DFS in univariable (*p=0.004, HR=5.411, 95%CI: 1.703-17.198*) and multivariable analysis (*p=0.001, HR=8.86, 95%CI: 2.351-33.384*).

Conclusions

Pre-operative sCD95L is a potential prognostic factor for DFS of patients undergoing surgery for synchronous colorectal liver metastases. Low pre-operative sCD95L levels may help identify a subgroup of patients with synchronous liver metastases that are likely to benefit from liver surgery.

INTRODUCTION

The presence of liver metastases is the major determinant of survival in patients with colorectal cancer. Approximately 25% of the patients with colorectal cancer already have liver metastases at diagnosis[1]. Synchronous liver metastases may indicate a more aggressive and unpredictable disease course when compared to metachronous metastases[2]. The optimal strategy for treatment of patients with synchronous liver metastases is currently debated[3]. Three approaches have been advocated. First, after removal of the primary colon tumor, hepatic resection is delayed to identify patients with aggressive early dissemination who do not benefit from liver surgery. Second, the primary colon tumor and the liver metastasis/metastases may be resected simultaneously. Third, in the 'liver first' approach the traditional therapeutic order is reversed and patients first receive liver surgery. The primary colon tumor is removed last to prevent surgery-associated stimulation of metastatic spread. Reliable predictive tools to select patients for any of these three strategies are needed.

CD95 ligand (CD95L/FASL) and its receptor CD95 (APO1/FAS) are transmembrane proteins that play an essential role in lymphocyte cytotoxicity and the maintenance of immunological homeostasis[4]. CD95L can induce tumor cell apoptosis but can also act in a pro-tumorigenic fashion by stimulating tumor cell proliferation, survival and invasion[5, 6]. In colorectal cancer, the expression of CD95L is higher in liver metastases than in matched primary tumors and high expression is related to poor prognosis[7, 8]. Furthermore, we have recently shown that CD95L stimulates migration and invasion of colorectal cancer cells, rather than apoptosis[5]. Membrane-bound CD95L can be cleaved by metalloproteases, which results in a soluble form of CD95L (sCD95L) that can be detected in the circulation[9]. Preclinical studies showed that tumor progression is selectively promoted by this sCD95L[10].

We hypothesized that high levels of sCD95L in patients undergoing surgery for synchronous liver metastases may be associated with an aggressive tumor phenotype and may be used to predict disease-free survival. To test this, we determined the levels of sCD95L in the serum of patients before and after surgery for synchronous colorectal liver metastases and related these values to clinical outcome.

METHODS

Blood samples were obtained from all consecutive patients with colorectal liver metastases before and 24 hours after liver surgery between March 2004 and August 2008 at the University Medical Center Utrecht in The Netherlands. The study protocol was approved by the ethical committee on human research. Written informed consent was obtained from all patients. Patients 18 years or older who underwent resection with curative intent for synchronous colorectal metastases confined to the liver were included in the study. Our surgical routine

has been the classic approach: first resection of the primary tumor followed by delayed liver resection. Patients were excluded in case of extrahepatic disease, treatment with local ablative therapies, or macroscopic residual disease (R2) after surgery. Patient and tumor characteristics as well as surgical characteristics were retrospectively drafted from our prospectively collected liver database.

sCD95L

Venous blood samples were drawn into sterile vacuum tubes before surgery and 24 hours after surgery. Blood samples were centrifuged at 1450g for 15 minutes and immediately frozen at -80°C until assayed. The levels of sCD95L in the sera were determined using a commercially available solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit for the quantitative detection of human sCD95L, recognizing both natural and recombinant human CD95L (ab45907, Abcam, Cambridge, UK). ELISA's were performed according to the manufacturers' protocol.

Follow-up

All patients were subjected to routine follow-up. CT-scans were acquired every 3 months to monitor recurrences. The follow-up data were updated by letters and telephone calls to referring physicians and general practitioners. The duration of the follow-up and the time between surgery and the detection of recurrence were obtained, as well as overall survival data.

Statistical Analyses

Disease-free survival and overall survival were calculated from the day of surgery to the day of the first recurrence, or the day of death, respectively. Mean disease-free survival and overall survival were estimated by the Kaplan-Meier's method. To determine the influence of possible risk factors on disease-free survival a univariable cox regression analysis was performed. A multivariable cox proportional hazards model was used to determine the independent prognostic impact of all variables on disease-free survival. Risk factors were included as continuous variables where possible. Statistical significance was assumed for p-values less than 0.05. Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Thirty-one patients undergoing partial hepatectomy for synchronous colorectal liver metastases with curative intent fulfilled the inclusion criteria and were enrolled in this study (22 male and 9 female patients, with a mean age of 62 years, ranging from 34 to 81 years). Baseline characteristics are shown in Table 1.

Among 31 patients, 14 died during follow up. The remaining 17 patients had a mean follow up time of 38 months. None of the patients were lost to follow-up. Mean DFS, as calculated by the Kaplan-Meier's method, was 18 months and mean overall survival was 42 months. Median pre-operative sCD95L levels were 0.1725 +/- 0.35 ng/ml. Median postoperative levels were 0.137 +/- 0.37 ng/ml.

Clinical factors that were significantly associated with poor DFS in univariable cox regression analysis included the Memorial Sloan-Kettering Cancer Center Clinical Risk Score (MSKCC-CRS) as defined by Fong et al.[11] (*p=0.017, HR=1,776, 95%CI: 1.108-2.847*) and high pre-operative sCD95L levels (*p=0.004, HR=5.411, 95%CI: 1.703-17.198*) (Table 2). For Kaplan-Meier survival curves, patients were divided in high (above median pre-operative levels) and low (below median pre-operative levels) sCD95L groups. Patients in the high pre-operative sCD95L group (n=15) had a mean DFS of 10.1 months (*95% CI: 6.1-14.1*) whereas patients in the low pre-operative sCD95L group (n=16) had a mean DFS of 22.7 months (*95%CI: 15.2-30.2*) (Figure 1). Postoperative levels of sCD95L were not significantly associated with DFS (Table 2).

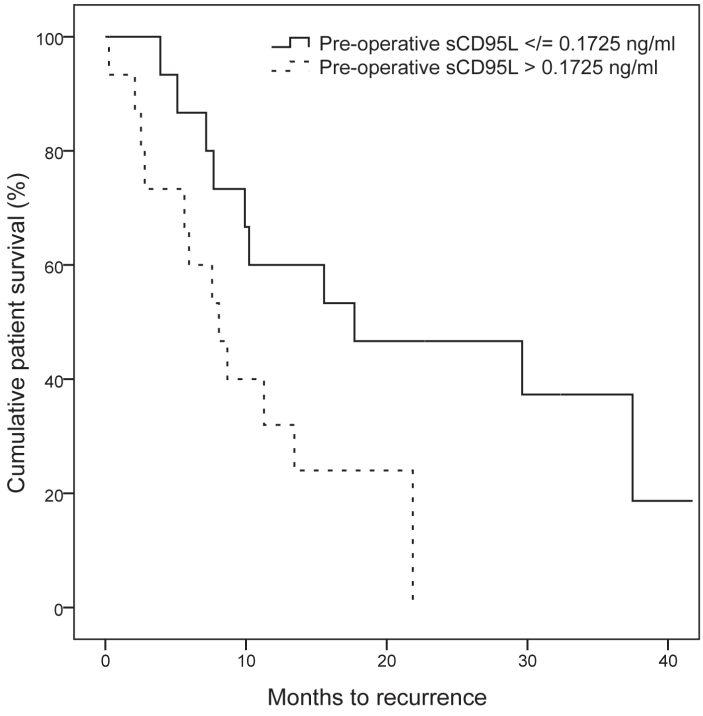

Figure 1. Kaplan-Meier curves illustrating the effects of high pre-operative sCD95L (above median pre-operative value) on disease-free survival (p=0.03, log-rank test)

Table 1. Patient and tumor characteristics

Total number of patients	**31**
Male (n)	22 (71%)
Female (n)	9 (29%)
Age (Mean; SEM; IQR; range; 95%CI)	62; 1.9; 13; 47; (58.14-65.73)
Location primary tumor	
Rectum	11 (35.5%)
Colon	20 (64.5%)
Differentiation primary tumor	
Good	1 (3.2%)
Moderate	27 (87.1%)
Poor	2 (6.5%)
Missing	1 (3.2%)
Nodal status	
N+	16 (51.6%)
N-	15 (48.4%)
Neoadjuvant chemotherapy	
Yes	2 (6.5%)
No	29 (93.5%)
Type of resection	
Minor	18 (58.1%)
Major (3 segments resected or more)	13 (41.9%)
R0/R1 Resection	
R0	30 (96.8%)
R1	1 (3.2%)
Bloodtransfusion	
No	28 (90.3%)
Yes	3 (9.7%)
Mean number of LM/patient	2
Vascular clamping	
No	15 (48.4%)
≤ 20 min	10 (32.3%)
>20 min	6 (19.4%)
Pre-operative CEA (Median; 95%CI)	51.27ng/ml; (38.45-80.11)
Pre-operative sCD95L levels (Median; 95%CI)	0.1725ng/ml; (0.15-0.41)
Postoperative sCD95L levels (Median; 95%CI)	0.137ng/ml; (0.14-0.42)

Next we employed a multivariable cox regression model containing the factors that displayed p-values below 0.1 in univariable analysis. Male sex (*p=0.013, HR =0.202, 95%CI: 0.57-0.719*), location of the primary tumor in the rectum (*p=0.003, HR= 0.139, 95%CI: 0.038-0.512*) and pre-operative sCD95L levels (*p=0.001, HR 8.860, 95%CI: 2.351-33.384*) were found to be independent risk factors for poor DFS. Factors significantly correlating with overall survival (OS) in univariable cox regression analysis were extent of resection and amount of ischemia induced during surgery[12]. Multivariable analysis did not show a significant correlation with any of the factors shown in Table 2. In multivariable analysis high sCD95L levels showed a non-significant trend towards a poor OS (data not shown). To assess whether sCD95L was not merely a reflection of the overall tumor load or influenced by cell death induced by chemotherapy, we performed an additional binary logistic regression analysis comparing sCD95L expression levels with clinicopathological features (Table 3). None of these variables were significantly associated with sCD95L.

Table 2. Risk factors for disease-free survival and overall survival identified by univariable cox regression analysis

	Disease Free Survivall			**Overall Survival**		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
Age	0.957	0.909-1.008	0.098	0.980	0.35-1.027	0.405
Sex	0.386	0.130-1.151	0.088	0.372	0.083-1.670	0.197
Location primary (rectum/colon)	0.724	0.297-1.762	0.476	0.733	0.234-2.301	0.595
Differentiation (good/moderate/poor)	1.321	0.505-3.458	0.570	0.988	0.359-2.718	0.981
Nodal status (N+/N-)	1.391	0.596-3.245	0.445	0.922	0.313-2.716	0.884
Neoadjuvant chemotherapy	0.620	0.083-4.65	0.642	2.195	0.273-17.66	0.460
Blood transfusion	1.130	0.260-4.908	0.871	2.257	0.607-8.399	0.225
Major/Minor resection	2.987	0.972-9.174	0.056	3.148	1.032-9.606	**0.044**
R1/R0	0.765	0.097-5.576	0.765	2.514	0.317-19.90	0.383
Nr of liver metastases	1.206	0965-1.507	0.100	0.945	0.699-1.278	0.713
Ischemia (none/minor/severe)	0.733	0.428-1.255	0.257	0.485	0.239-0.982	**0.044**
Size biggest tumor	1.055	0.891-1.248	0.536	1.145	0.923-1.420	0.217
CEA pre-operative	1.007	0.998-1.016	0.104	0.999	0.988-1.010	0.833
Bilobar distribution	2.503	0.817-7.671	0.108	1.162	0.252-5.359	0.847
MSKCC-CRS	1.776	1.108-2.847	**0.017**	1.141	0.676-1.926	0.621
Iwatsuki score	0.216	0.816-2.453	0.216	1.015	0.539-1.911	0.964
sCD95L pre-operative	5.411	1.703-17.198	**0.004**	0.409	0.067-2.508	0.134
sCD95L postoperative	2.011	0.371-10.894	0.418	4.332	0.954-19.66	0.079

Table 3. Factors associated with levels of pre-operative sCD95L identified by univariable logistic regression analysis.

	Low sCD95L (n=16)	High sCD95L (n=15)	p-value	HR	95%CI
Age	65.4 (SD 7.12)	58.27 (SD12.13)	0.072	0.923	0.846-1.007
Male	11 (68,2%)	11 (73.3%)	0.779	0.800	0.168-3.739
Female	5 (31,8%)	4 (26.7%)			
Location primary					
Rectum	5 (31.3%)	6 (40%)	0.612	0.682	0.156-2.989
Colon	11 (68.8%)	9 (60%)			
Differentiation					
Good	1 (6.3%)	1 (6.7%)	0.931	0.945	0.264-3.383
Moderate	14 (87.9%)	13 (86.7%)			
Poor	1 (6.3%)	1 (6.7%)			
Nodal status					
N+	7 (43.8%)	9 (60%)	0.368	1.929	0.462-8.053
N-	9 (56.3%)	6 (40%)			
Neoadjuvant chemotherapy					
Yes	0 (0%)	2 (13.3%)	0.999	1.988	0.000-0.000
No	16 (100%)	13 (86.7%)			
Blood transfusion					
Yes	2 (12.5%)	1 (6.7%)	0.589	0.500	0.041-6.166
No	14 (87.5%)	14 (93.3%)			
Type of resection					
Minor	13 (81.3%)	13 (86.7%)	0.765	1.250	0.289-5.407
Major	3 (18.8%)	2 (33.3%)			
Bilobar distribution	2 (12.5%)	3 (20%)	0.573	1.740	0.249-12.28
Nr of liver metastases (mean, SD)	1.75 (1.73)	1.87 (1.81)	0.850	1.041	0.689-1.572
Size biggest tumor (mean, SD)	4 cm (1.97)	4.5 cm (2.5)	0.497	1.121	0.807-1.558
Ischemia					
none	8 (50%)	5 (33.3%)	0.809	0.833	0.191-3.644
minor	4 (25%)	4 (26.7%)			
severe	4 (25%)	6 (40%)			
CEA pre-operative (continuous)	57.17 (SD 46.5)	61.5 (67.65)	0.828	1.001	0.989-1.101
Iwatsu score					
Grade 1	4 (25%)	4 (26.7%)	0.371	1.367	0.689-2.711
Grade 2	9 (56.3%)	8 (53.3%)			
Grade 3	2 (12.5%)	2 (13.3%)			
Grade 4	1 (6.3%)	1 (6.7%)			
Fong score					
1	5 (31.3%)	5 (33.3%)	1.000	1.000	0.416-2.404
2	6 (37.5%)	3 (20.0%)			
3	4 (25.0%)	6 (40.0%)			
4	1 (6.25%)	1 (6.67%)			

DISCUSSION

The findings presented point towards a potentially prognostic role for sCD95L regarding the biological behaviour of colorectal cancer presenting with synchronous liver metastases. Our results show that high pre-operative levels of sCD95L upon presentation are associated with an unfavourable outcome, reflected by a DFS of less than 11 months. sCD95L levels were not simply a reflection of overall tumor burden. sCD95L may therefore identify a subgroup of patients that is unlikely to benefit from liver surgery and that should be referred for upfront chemotherapy and/or biological therapies[13]. In contrast, patients presenting with low sCD95L levels might be considered for surgery, including a 'liver first' or 'simultaneous' approach. Poor OS was seen in the high sCD95L group. However, this did not reach statistical significance, presumably due to the small number of patients available for survival analysis. Furthermore, OS might be influenced by subsequent therapies after recurrence, such as repeated chemotherapy.

Although synchronous liver metastasis is seen as a poor prognostic factor in itself, it does not preclude the possibility of long-term survival and 5-year survival rates of up to 40% can still be achieved[11, 14]. Various studies have defined factors predicting DFS in colorectal cancer patients with synchronous liver metastases such as sex, tumor differentiation, postoperative CEA levels, infiltration in other organs, number of metastases and metastatic lymph nodes[15, 16]. Different combinations of these factors have been proposed as clinical prediction models for selecting patients who could benefit from surgery[11, 17-19]. Nonetheless, these models are still far from optimal, as they predict outcome with a considerable degree of variation[20, 21]. This makes it essential to search for new molecular prognostic factors that include actual tumor status and accurately reflect biological behaviour of the disease[15, 22].

Accumulating evidence suggests a tumor-propagating role for (s)CD95L in malignancies including colorectal cancer[5, 7, 10]. Our results are in line with several other studies in which elevated serum sCD95L concentrations are correlated with poor prognosis in large granular lymphocytic leukaemia, NK lymphoma, bladder carcinoma, gastric carcinoma, hepatocellular carcinoma and breast carcinoma[23, 24]. To our knowledge, our results are the first to show a correlation between elevated serum sCD95L and poor DFS in colorectal cancer. Conversely, in squamous cell carcinoma of the oesophagus sCD95L levels had no significant prognostic effect on DFS, suggesting that the merit of sCD95L measurements may be tumor-type-specific [25]. Whether sCD95L is merely associated with decreased DFS or whether it is causally involved in accelerating tumor progression will be the subject of further studies.

Although our study is retrospective in nature and based on a relatively small number of patients, the differences in outcome that are associated with varying sCD95L levels are such that further validation studies are justified. Moreover, analysis of sCD95L levels in clinical practice is appealing as it can simply be measured in patient blood samples pre-operatively by using thoroughly validated ELISA's[9, 26].

In conclusion, our data suggest that high pre-operative levels of sCD95L are associated with poor DFS in patients scheduled for surgery for synchronous colorectal liver metastases. Obviously, these findings should be regarded as hypothesis-generating and require substantiation in larger patient cohorts. Low pre-operative sCD95L levels may help identify a subgroup of patients with synchronous liver metastases that are likely to benefit from liver surgery.

REFERENCES

1. Khatri VP, Petrelli NJ, Belghiti J. Extending the frontiers of surgical therapy for hepatic colorectal metastases: is there a limit? J Clin Oncol 2005;23:8490-8499.
2. Tsai MS, Su YH, Ho MC, Liang JT, Chen TP, Lai HS, Lee PH. Clinicopathological features and prognosis in resectable synchronous and metachronous colorectal liver metastasis. Ann Surg Oncol 2007;14:786-794.
3. Martin RC, Augenstein V, Reuter NP, Scoggins CR, McMasters KM. Simultaneous versus staged resection for synchronous colorectal cancer liver metastases. J Am Coll Surg 2009;208:842-850.
4. Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature 1995;373:438-441.
5. Hoogwater FJ, Nijkamp MW, Smakman N, et. al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 2010;138:2357-2367.
6. Peter ME, Budd RC, Desbarats J, et. al. The CD95 receptor: apoptosis revisited. Cell 2007;129:447-450.
7. Bennett MW, O'Connell J, Houston A, Kelly J, O'Sullivan GC, Collins JK, Shanahan F. Fas ligand upregulation is an early event in colonic carcinogenesis. J Clin Pathol 2001;54:598-604.
8. Mann B, Gratchev A, Bohm C, et.al. FasL is more frequently expressed in liver metastases of colorectal cancer than in matched primary carcinomas. Br J Cancer 1999;79:1262-1269.
9. Tanaka M, Suda T, Haze K, et. al. Fas ligand in human serum. Nat Med 1996;2:317-322.
10. O' Reilly LA, Tai L, Lee L, et. al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature 2009;461:659-663.
11. Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg 1999;230:309-318.
12. Nijkamp MW, van der Bilt JD, Snoeren N, et. al. Prolonged portal triad clamping during liver surgery for colorectal liver metastases is associated with decreased time to hepatic tumour recurrence. Eur J Surg Oncol 2010;36:182-188.
13. Adam R, Haller DG, Poston G, Raoul JL, Spano JP, Tabernero J, Van CE. Toward optimized front-line therapeutic strategies in patients with metastatic colorectal cancer--an expert review from the International Congress on Anti-Cancer Treatment (ICACT) 2009. Ann Oncol 2010.
14. van der Pool AE, de Wilt JH, Lalmahomed ZS, Eggermont AM, Ijzermans JN, Verhoef C. Optimizing the outcome of surgery in patients with rectal cancer and synchronous liver metastases. Br J Surg 2010;97:383-390.
15. Capussotti L, Vigano' L, Ferrero A, Lo TR, Ribero D, Polastri R. Timing of resection of liver metastases synchronous to colorectal tumor: proposal of prognosis-based decisional model. Ann Surg Oncol 2007;14:1143-1150.
16. Konopke R, Kersting S, Distler M, et. al. Prognostic factors and evaluation of a clinical score for predicting survival after resection of colorectal liver metastases. Liver Int 2009;29:89-102.
17. Iwatsuki S, Dvorchik I, Madariaga JR, et. al. Hepatic resection for metastatic colorectal adenocarcinoma: a proposal of a prognostic scoring system. J Am Coll Surg 1999;189:291-299.
18. Mann CD, Metcalfe MS, Leopardi LN, Maddern GJ. The clinical risk score: emerging as a reliable preoperative prognostic index in hepatectomy for colorectal metastases. Arch Surg 2004;139:1168-1172.
19. Nordlinger B, Guiguet M, Vaillant JC, Balladur P, Boudjema K, Bachellier P, Jaeck D. Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association Francaise de Chirurgie. Cancer 1996;77:1254-1262.
20. Chen J, Li Q, Wang C, Wu J, Zhao G. Prognostic Significance of c-erbB-2 and Vascular Endothelial Growth Factor in Colorectal Liver Metastases. Ann Surg Oncol 2010.
21. Eschrich S, Yang I, Bloom G, et. al. Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 2005;23:3526-3535.
22. Poston GJ, Adam R, Alberts S, et. al. OncoSurge: a strategy for improving resectability with curative intent in metastatic colorectal cancer. J Clin Oncol 2005;23:7125-7134.
23. Mizutani Y, Hongo F, Sato N, Ogawa O, Yoshida O, Miki T. Significance of serum soluble Fas ligand in patients with bladder carcinoma. Cancer 2001;92:287-293.

24. Tsutsumi S, Kuwano H, Shimura T, Morinaga N, Mochiki E, Asao T. Circulating soluble Fas ligand in patients with gastric carcinoma. Cancer 2000;89:2560-2564.
25. Kozlowski M, Kowalczuk O, Sulewska A, et. al. Serum soluble Fas ligand (sFasL) in patients with primary squamous cell carcinoma of the esophagus. Folia Histochem Cytobiol 2007;45:199-204.
26. Kayagaki N, Kawasaki A, Ebata T, et. al. Metalloproteinase-mediated release of human Fas ligand. J Exp Med 1995;182:1777-1783.

CHAPTER 7

DIFFERENTIATION OF COLORECTAL TUMOR-INITIATING CELL CULTURES DOES NOT AFFECT THEIR RESISTANCE TO CD95 LIGAND-INDUCED APOPTOSIS

In progress

Frederik J.H. Hoogwater
Benjamin L. Emmink
Winan J. van Houdt
Inne H.M. Borel Rinkes
Onno Kranenburg

Department of Surgery, University Medical Center Utrecht, Utrecht, The Netherlands

ABSTRACT

Background

Colorectal tumors are hierarchically organized tissues in which an undifferentiated tumorigenic 'cancer stem cell' compartment generates differentiated offspring with reduced tumorigenic capacity. Eradication of the cancer stem cell compartment may be essential for effective and lasting anti-tumor therapy. Although cancer stem cells are generally believed to be apoptosis-resistant, the relationship between apoptosis resistance and differentiation status has not been formally addressed. We established low-passage colonosphere cultures, enriched in undifferentiated tumorigenic cancer stem cells, and generated stably differentiated non-tumorigenic cultures from these. CD95 ligand (CD95L) was used as a prototypic apoptosis inducer.

Aim

To assess whether differentiation status affects colorectal tumor cell sensitivity to CD95L.

Materials and methods

We used three pairs of metastasis-derived colonosphere cultures and their stably differentiated progeny. Expression levels of CD95 were determined by Western blot analysis. Tumor cell sensitivity to CD95L was tested using mitochondrial-activity (MTS)-assays and Western blot analysis of caspase-3 cleavage.

Results

CD95 was expressed in all colonospheres and expression was increased upon differentiation in two out of three cultures. Both undifferentiated (tumorigenic) and differentiated colorectal cancer cell cultures were resistant to CD95L-induced apoptosis, using concentrations of up to 20 ng/ml.

Conclusion

The resistance of colorectal tumor cells to CD95L is unaffected by their differentiation status and tumor-initiating capacity.

INTRODUCTION

The concept that cancer arises from a rare population of cells with stem-cell properties was proposed already 150 years ago, but recent advances in stem cell biology have given a new impulse to this research field[1-3]. Cancer stem cells (CSCs) are defined as a small population of tumorigenic cells within a tumor that are able to self-renew and differentiate into one or more cell types[4]. CSCs have been identified and characterized in a large variety of haematological malignancies and solid tumors[5-11]. In colorectal tumors, the tumor-initiating colorectal cancer (CRC) stem cells were identified on the basis of specific cell-surface markers[12-14]. These reports suggest that colorectal tumors are organized in a hierarchical fashion in which only a small subset of tumorigenic tumor cells can give rise to differentiated tumor cells with reduced tumorigenic potential. Although chemotherapy is supposed to kill most cells in a tumor, it is believed that CSCs are relatively resistant to cytotoxic agents and are therefore enriched following chemotherapy. This may be due to high expression levels of ATP-binding cassette (ABC) transporters, active DNA-repair capacity and a general resistance to apoptosis-inducing stimuli[15]. The resistance of colon CSCs to therapy-induced cell death may be due to elevated expression of anti-apoptotic proteins and/or autocrine production of the survival factor IL-4[16,17]. However, IL-4 production and IL-4 receptor expression are not restricted to CSC and the relationship between apoptosis resistance and differentiation status is poorly understood. Recently we have generated low-passage colonosphere cultures from freshly resected liver metastases of colorectal cancer patients. These cultures are highly enriched for CSCs and have high clone- and tumor-initiating capacity. In addition, we have generated stably differentiated tumor cell cultures derived from these colonospheres that have lost tumor-initiating potential (*Emmink et al., submitted*). These culture pairs provide an excellent tool for evaluating the influence of differentiation status on apoptosis sensitivity. CD95 is the prototypic death receptor. Upon binding CD95 ligand (CD95L) this receptor induces apoptosis in a wide variety of cell types, including tumor cells[18]. Here we set out to determine whether differentiation status affects the sensitivity of colorectal tumor cell cultures to CD95L.

MATERIALS AND METHODS

Colonosphere cultures

Colonospheres (*Emmink et al.*) were cultured in advanced DMEM/F12 (Gibco) supplemented with 0,6% glucose (BDH Lab. Supplies), 2 mM L-glutamine (Biowhittaker), 9.6 µg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 µg/ml insulin (Sigma), 100 µg/ml apotransferrin (Sigma), 5 mM hepes (Gibco), 0,005 µg/ml trace element A (Cellgro), 0,01 µg/ml trace element B (Cellgro), 0,01 µg/ml trace element C (Cellgro), 100

µM β-mercaptoethanol (Merck), 10 ml antibiotic-antimycotic (Gibco), 4 µg/ml gentamicine (Invitrogen), 0.002% lipid mixture (Sigma), 5 µg/ml glutathione (Roche) and 4 µg/ml Heparin (Sigma). Growth factors (20 ng/ml EGF (Invitrogen) and 10 ng/ml b-FGF (Abcam)) were added to the cell culture medium freshly each week. All cell culture was carried out in non-tissue culture treated flasks (BD Falcon) at 37° C in a 5% CO_2 humidified incubator.

Generation of differentiated tumor cell cultures

In vitro differentiation was induced by culturing colonospheres for 3 weeks on collagen-coated dishes in DMEM-F12 (GIBCO) supplemented with 20% fetal bovine serum.

Colonosphere-Forming Efficiency (CFE)

Single tumor cell suspensions derived from colonospheres and differentiatied tumor cell cultures were counted and resuspended in Matrigel at 1000 cells/ml. Equal numbers of Matrigel-embedded cells (100 cells in 100 µl) were allowed to set in 48-well plates. After the cell suspension in Matrigel set, stem cell medium with fresh growth factors was added. Clone formation was analyzed after three weeks of culture. Colonies were counted using a Leica DM IRBE microscope and the colonosphere-forming efficiency (CFE) was calculated as the percentage of seeded cells that formed colonospheres.

Tumor Formation

Single cell populations were diluted to 200, 1000 and 10.000 live cells, mixed with BD Matrigel (BD Biosciences) at a 1:1 ratio (total volume 100 µl) and injected subcutaneous into the flanks of 6 weeks old BALB/cnu/nu mice. Injected mice were followed for up to 5 months or when tumors reached a maximum of 1 cm^3. Mice were sacrificed by cervical dislocation. All experiments involving the use of animals were performed in accordance with University of Utrecht institutional animal welfare guidelines.

Antibodies

The anti-β-actinused in this study were: anti-cleaved-caspase-3 (Cell Signaling), anti-ALDH1 (#44; BD Biosciences), anti-cytokeratin 20 (Ks20.8; Dako), anti-β-actin (AC-15; Novis Biologicals), anti-CD95 clone CH11 (Cell Signaling) and anti-CD95L (Santa Cruz).

MTS-Assays

All analyses and cell isolations were performed using freshly dispersed cell suspensions. To obtain single cell suspensions, colonospheres were dissociated in Accumax (Innovative Cell Technologies) plus 2 U/ml DNAse1 (Sigma) for 10 minutes in a rotary incubator at 37°C. The suspension was then filtered though a 40-µm-pore size nylon cell strainer (BD Falcon) to obtain single cells. Cells (10.000 cells/48-well) were stimulated with CD95L (membrane bound (#01-210) from Upstate Cell Signaling Solutions, Lake Placid, NY; 4 ng/ml to 20 ng/ml) and

viability was analyzed 48h later by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assays (Roche Diagnostics) according to the manufacturer's instructions.

Western Blotting

Samples were washed with ice-cold PBS and lysed in lysis buffer (20mM HEPES pH7.4, 1% NP40, 150mM NaCl, 5mM $MgCl^2$, 10% glycerol). Equal amounts of protein (80µg) were analysed on 10% SDS-PAGE gels following standard Western Blot procedures.

RESULTS

Isogenic pairs of undifferentiated and differentiated colorectal tumor cell cultures

Three previously established colonosphere cultures (L145, L167 and L169) were exposed to serum-containing medium. The colonospheres attached to the culture dish and grew out to form adherent cultures with heterogeneous morphology (Figure 1A). Western blot analysis shows that ALDH1, a marker for colorectal cancer stem cells[19, 20], was expressed in all three colonosphere cultures, but expression was strongly reduced in the corresponding adherent cultures. Reduced ALDH1 expression was accompanied by increased expression of the differentiation marker cytokeratin 20 (CK20) (Figure 1B). In addition, all adherent cultures showed a drastically reduced clone-forming capacity and tumor-forming capacity when injected into mice (Table 1, Figure 1C and D). These results are in line with recent reports identifying ALDH1 as a marker for colorectal tumor-initiating cells[19, 20]. Taken together, we have established three isogenic cell pairs of ALDH1-positive, CK20 negative colonospheres (enriched in cancer stem cells), and their stably differentiated ALDH1-negative, CK20 positive progeny.

Differentiation status does not affect resistance to CD95L-induced apoptosis

CD95 was expressed in all colonospheres and expression was increased upon differentiation in 2/3 cultures (L145; L167) (Figure 2A). Single cell cultures derived from colonospheres and differentiated cell cultures were exposed to CD95L (0-20 ng/ml). As a positive control we used DKO4 cells, a CD95L-sensitive human colorectal cancer cell line[21, 22]. MTS assays showed that none of the colonosphere or differentiated tumor cell cultures tested displayed a significant loss of viability following exposure to CD95L, while DKO4 control cells readily died (Figure 2B). L167 spheroid-derived cells did show a minor response to increasing levels of CD95L. However caspase-3 was not processed in these cells, suggesting that the reduction in MTS signal was not due to apoptosis. The Western blot analysis showed that only apoptosis-sensitive DKO4 cells displayed activation of capsase-3 in response to CD95L. Caspase-3 processing was undetectable in all other cultures, irrespective of differentiation status. (Figure 2C).

Table 1. Clone- and tumor-forming potential of human colorectal colonosphere cultures.

	Clone formation		Tumor formation			
	colonosphere cells	differentiated cells	colonosphere cells			differentiated cells
Tumor	*%*		*200*	*1.000*	*10.000*	*10.000*
L145	46	3	3/3	3/3	3/3	0/3
L167	20	1	0/3	1/3	2/3	0/3
L169	62	6	1/3	2/3	3/3	ND

ND = not determined

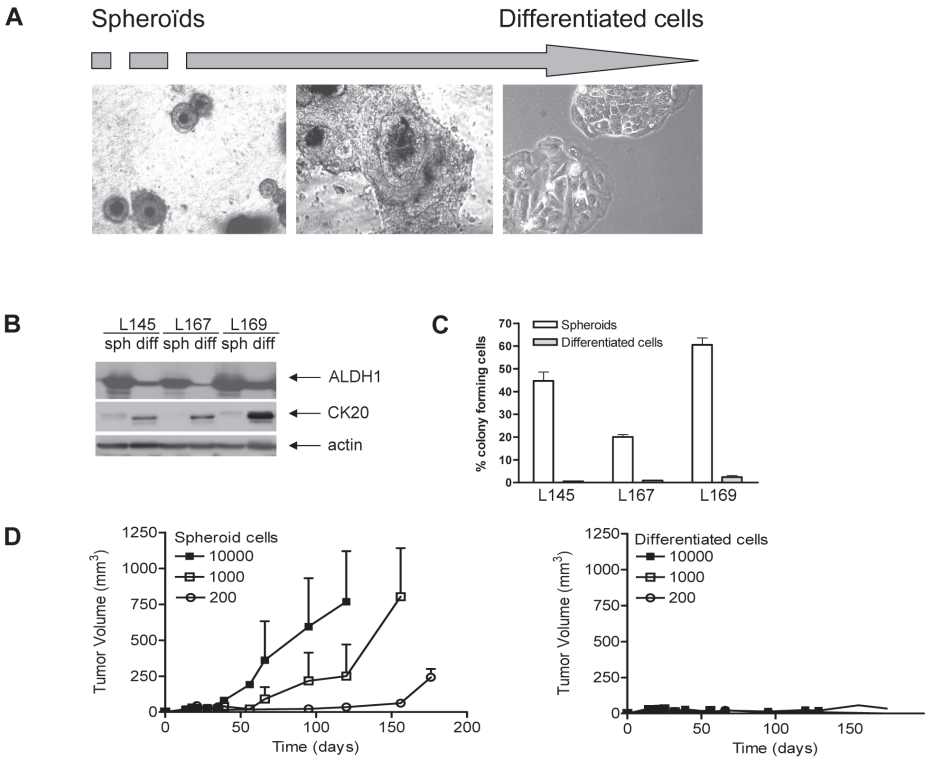

Figure 1. Differentiation of colonosphere-forming cells from human colorectal tumors. (**A**) Light microscopic images of a representative colonosphere and a population of colonosphere-derived adherent tumor cells. Bar 20 µm. (**B**) Western blot analysis of the expression of ALDH1, cytokeratin (CK20) and actin in isogenic colonosphere and adherent tumor cell cultures. (**C**) Single cell populations were generated from the indicated colonosphere lines and from adherent tumor cell cultures and these were suspended in Matrigel at 1000 cells/ml. Equal numbers of Matrigel-embedded cells (n=100 in 100 µl) were allowed to set in 48-well plates and clone formation was analyzed after three weeks of culture. (**D**) Single cell populations were generated from L145 colonospheres and from adherent tumor cell cultures and these were suspended in Matrigel. Mice were injected with 10.000 (n=3), 1000 (n=3), or 200 (n=3) tumor cells as indicated and tumor growth was followed over time by caliper measurements.

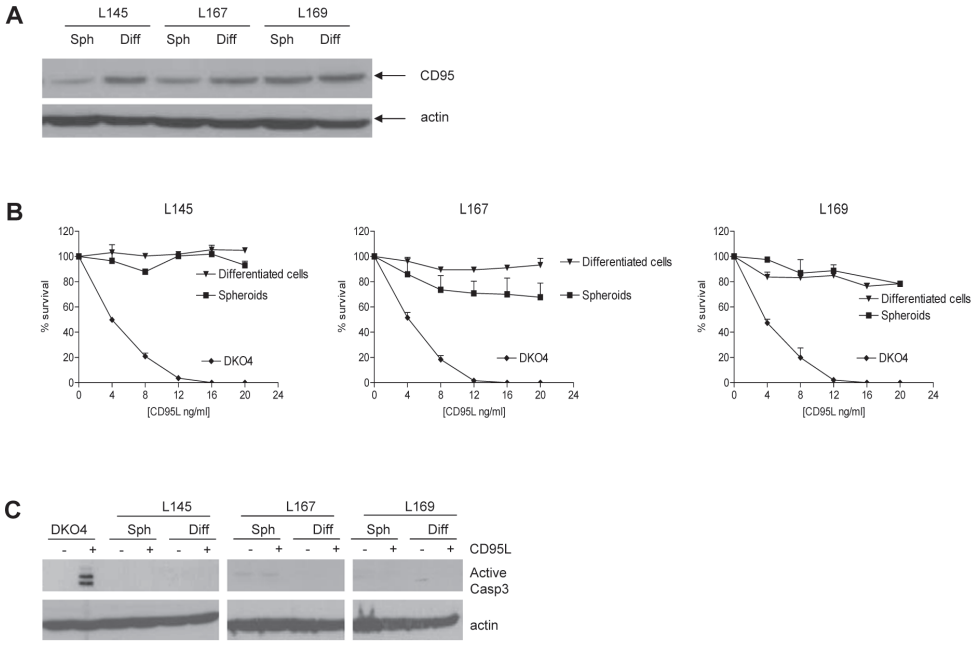

Figure 2. Expression levels of CD95 and assesment of sensitivity for CD95L in colonsphere cultures and their differentiated derivative cells. (**A**) Lysates of paired colonosphere and differentiated cultures derived from liver metastases were analyzed for the expression of CD95 and actin by Western blotting. (**B**) Spheroid cultures and isogenic differentiated tumor cells were seeded in 48-well plates in triplicate and were exposed to the indicated concentrations of CD95L (4-20ng/ml) for 48h. Viability was then tested by standard MTS assays. Absorbance values are expressed as percentages of vehicle-treated control wells. DKO4 cells were used as a positive control. (**C**) Cells were exposed to 20ng/ml CD95L for 48h and then evaluated for Caspase-3-activity by Western blotting

DISCUSSION

The cancer stem cell hypothesis has important implications for understanding key biological processes of carcinogenesis. It also suggests that lasting therapeutic responses presumable require novel therapeutics that selectively target the cancer stem cell population. The current dogma is that cancer stem cells (CSCs) are intrinsically resistant to apoptosis stimuli when compared with proliferating, differentiated cells[15]. This does not appear to hold for the three CRC cultures tested here, since differentiation status had no effect on resistance to CD95L-induced apoptosis. Our results differ from other recent reports in which is described that $CD133^{+}$ colon CSCs are resistant to conventional and to innovative therapies such as TNF-related apoptosis inducing ligand (TRAIL), compared with mature (differentiated) cells[16]. Furthermore, in glioblastoma cells it was shown that differentiation status does affect apoptosis sensitivity to CD95L and that differentiation of stem cells needs to be considered to enhance therapeutic efficiency[23]. Colorectal cancer stem cells may be less sensitive to

apoptosis-inducing agents due to higher levels of anti-apoptotic proteins, including Fas-linked ICE-like protease inhibitor protein (FLIP), Bcl-2 , B cell lymphoma like X (Bcl-XL), PED/PEA-15 and IAP family members[15, 24, 25]. Apoptosis resistance may be overcome by using a combination of cytotoxic drugs and inhibitors of survival pathways. Although our experiments were performed in a limited number of paired colonosphere/differentiated cell cultures using CD95L as a single prototypic apoptosis inducer, the results suggest that cancer stem cells do not need to be universally more resistant to apoptotic stimuli. Additional work using a large panel of paired CSC/differentiated cell cultures and additional death receptor ligands such as TRAIL or TNFα is required to assess whether resistance to death receptor-induced apoptosis is related to differentiation status.

REFERENCES

1. Cohnheim,J. Ueber entzundung und eiterung. Path Anat Physiol Klin Med 40, 1-79 (1867).
2. Durante,F. Nesso fisio-pathologico tra la struttura die nei materni e la genesi di alcuni tumori maligni. 11, 217-226. 1874.
3. Wicha,M.S., Liu,S., & Dontu,G. Cancer stem cells: an old idea--a paradigm shift. Cancer Res. 66, 1883-1890 (2006).
4. Reya,T., Morrison,S.J., Clarke,M.F., & Weissman,I.L. Stem cells, cancer, and cancer stem cells. Nature 414, 105-111 (2001).
5. Todaro,M., Perez,A.M., Scopelliti,A., Medema,J.P., & Stassi,G. IL-4-mediated drug resistance in colon cancer stem cells. Cell Cycle 7, 309-313 (2008).
6. Bonnet,D. & Dick,J.E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med 3, 730-737 (1997).
7. Dontu,G., El-Ashry,D., & Wicha,M.S. Breast cancer, stem/progenitor cells and the estrogen receptor. Trends Endocrinol. Metab 15, 193-197 (2004).
8. Singh,S.K. et al. Identification of human brain tumour initiating cells. Nature 432, 396-401 (2004).
9. Fang,D. et al. A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res. 65, 9328-9337 (2005).
10. Collins,A.T., Berry,P.A., Hyde,C., Stower,M.J., & Maitland,N.J. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 65, 10946-10951 (2005).
11. Bapat,S.A., Mali,A.M., Koppikar,C.B., & Kurrey,N.K. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res. 65, 3025-3029 (2005).
12. Ricci-Vitiani,L. et al. Identification and expansion of human colon-cancer-initiating cells. Nature 445, 111-115 (2007).
13. O'Brien,C.A., Pollett,A., Gallinger,S., & Dick,J.E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445, 106-110 (2007).
14. Dalerba,P. et al. Phenotypic characterization of human colorectal cancer stem cells. Proc. Natl. Acad. Sci. U. S. A 104, 10158-10163 (2007).
15. Dean,M., Fojo,T., & Bates,S. Tumour stem cells and drug resistance. Nat. Rev. Cancer 5, 275-284 (2005).
16. Todaro,M. et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 1, 389-402 (2007).
17. Stassi,G. et al. Thyroid cancer resistance to chemotherapeutic drugs via autocrine production of interleukin-4 and interleukin-10. Cancer Res. 63, 6784-6790 (2003).
18. Barnhart,B.C. et al. CD95 apoptosis resistance in certain cells can be overcome by noncanonical activation of caspase-8. Cell Death. Differ. 12, 25-37 (2005).
19. Dylla,S.J. et al. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. PLoS. One. 3, e2428 (2008).
20. Huang,E.H. et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res. 69, 3382-3389 (2009).
21. Hoogwater,F.J. et al. Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells. Gastroenterology 138, 2357-2367 (2010).
22. Smakman,N. et al. Dual effect of Kras(D12) knockdown on tumorigenesis: increased immune-mediated tumor clearance and abrogation of tumor malignancy. Oncogene 24, 8338-8342 (2005).
23. Bertrand,J. et al. Cancer stem cells from human glioma cell line are resistant to Fas-induced apoptosis. Int. J. Oncol. 34, 717-727 (2009).
24. Liu,G. et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol. Cancer 5, 67 (2006).
25. Ricci-Vitiani,L. et al. Absence of caspase 8 and high expression of PED protect primitive neural cells from cell death. J. Exp. Med. 200, 1257-1266 (2004).

CHAPTER 8

GENERAL DISCUSSION

After triggering death receptors with their ligands they can activate the classical extrinsic apoptotic signaling pathway, but they can also stimulate alternative pathways leading to cell proliferation, invasion and even tumorigenesis (**chapter 2**). Identification of the factors and molecular mechanisms that determine these various outcomes in death receptor signaling may lead to new therapeutic strategies targeting death receptors in (surgical) cancer therapy.

This thesis describes the role of death receptors in the development and outgrowth of colorectal liver metastases. The key findings in this thesis are that:

- Death receptors can be switched into metastasis-promoting receptors by the single common oncogene K-Ras and this is important for survival of metastatic tumor cells and their outgrowth in the liver (**chapter 3**);
- CD95 plays an important role in surgery-stimulated outgrowth of colorectal micrometastases in the liver. Therapy antagonizing death receptor signaling could therefore be of interest in the reduction of accelerated outgrowth (**chapter 4 and 5**);
- Preliminary results indicate that circulating CD95L might be usefull as a prognostic factor contributing to the selection of patients for liver surgery (**chapter 6**);
- Differentiation of colorectal tumor-initiating cell cultures does not affect their resistance to CD95L-induced apoptosis (**chapter 7**).

CD95 as "Dr. Jekyll" or "Mr. Hyde"? Mutant KRAS decides

Although death receptors are best known for their role in apoptosis, the non-apoptotic functions have been relatively underexposed in relation to tumor formation.

In **chapter 3** we show that alternative non-apoptotic signaling of CD95 and TRAILR is important for the formation and survival of colorectal liver metastases. We demonstrate that K-Ras alters the signaling output of CD95 and TRAIL receptors, from apoptosis to invasion, and that this phenomenon is important for metastatic potential. This study has potential clinical relevance. Clinical trials are ongoing that evaluate the safety and efficacy of TRAIL and agonistic death receptor-targeting antibodies. The rationale behind these trials is based on the simplistic idea that TRAIL should induce (only) apoptosis in tumor cells. Although the agents have been well-tolerated at the levels tested, formal evaluation of the safety and clinical efficacy of the DR agonists in a variety of combinations and tumor types is to be anticipated over the next coming years. Noteworthy is that the trials contain patients with metastatic colorectal, pancreatic, and non-small cell lung cancer. These tumor types frequently harbour activating K-Ras mutations in 40%, 95% and 35% respectively[1]. Therefore, testing the K-Ras mutation status in the tumors of the patients in these trials may predict tumor resistance to death receptor-activating therapeutics.

By virtue of the fact that they have tumor-suppressing as well as tumor-promoting capacities, death receptors resemble the “Dr. Jekyll and Mr. Hyde” character in the famous novel by Robert Louis Stevenson (Barnemouth 1885). In this story, the good Dr. Jekyll turns into the murderer Mr. Hyde under the influence of a self-made concoction (i.e. the KRAS oncogene). An increasingly less potent antidote turns Mr. Hyde back into Dr. Jekyll. By analogy, this antidote would represent a potent anti-cancer drug and its diminishing potency would relate to developing drug resistance.

Alternative CD95 signaling resulting in proliferation and invasion has been associated with increased expression of matrix metalloproteinases (MMPs) and/or urokinase plasminogen activator (uPA)[2, 3]. We identified Raf1 as the critical K-Ras effector in switching CD95 signaling output by suppression of Rock and Lim kinase, leading to increased cofilin pathway output. The cofilin pathway is a critical regulator of cortical actin dynamics, tumor cell migration, invasion and metastasis formation[4, 5]. The control of cortical actin dynamics by K-Ras/Raf1 via stimulation of cofilin activity may be the cause of altered CD95 trafficking and signaling output. CD95 undergoes several biophysical alterations upon binding with its ligand. Receptor ligation results in the rapid formation of microaggregates at the cell surface, which precedes DISC formation. Subsequently, the receptor is redistributed over the cell membrane into specific subdomains, called lipid rafts. Receptor compartmentalization is followed by its internalization, which is essential for effective apoptosis signaling[6, 7]. The actin filament system is involved in the ligand-induced internalization of death receptors[6, 8]. (Over)active actin remodeling by oncogenic K-Ras may impair efficient redistribution of the receptor in the lipid rafts, thereby obstructing internalization. Indeed, our results showed reduced internalization in the K-Ras mutated cells after stimulation with CD95L. When internalization of the receptor is abated and DISC formation is reduced (i.e. in caspase 8-defective tumor cells), receptor aggregates activate pro-survival proteins such as NFκB, JNK, PI3K and ERK[3, 7, 9]. The mechanisms by which death receptors activate these pro-survival pathways have not been fully elucidated.

The impact of K-Ras on cortical actin remodeling is likely to also influence other cell surface receptors like epidermal growth factor receptor (EGFR). The exact role of oncogenic K-Ras in modulating the dynamics of the cortical actin cytoskeleton is subject of further investigation.

Based on our results, it seems reasonable that targeting the cofilin pathway may be an effective strategy to diminish metastatic potential. Recent reports on the cofilin pathway implicate that looking at the expression status of a single gene in the pathway can be misleading when interpreting phenotype, since it is the collective activity of multiple genes of the pathway that defines the integrated output of the pathway and therefore phenotype[10].

Combinations of genes must be altered, as has been done for cofilin and LIMK1 in mammary tumors, concerning expression or activity status in order to suppress or activate the output of the cofilin pathway and modulate its initiation of tumor cell migration[5]. Based on these results, inhibitors or activators of this pathway could eventually be developed for clinical practice.

While actin dynamics can alter receptor localization and/or trafficking, recent studies have shown that a conserved extracellular glycosphingolipid-binding motif (GBM) on CD95 also plays an important part in selection of the receptor internalization route[11]. Whether mutant K-Ras is involved in altering glycosphingolipid modification on CD95 remains to be determined.

An outstanding question is the role of the transcription factor nuclear factor-κB (NFκB) in survival and invasiveness of K-Ras mutated tumor cells in relation to death receptor signaling. Several recently published papers have identified genes that are required for the survival of tumor cells with mutant K-Ras, including the non-canonical IκB kinase TBK1[12-14]. In mutant K-Ras cells, TBK1 activated NFκB anti-apoptotic signals (involving Bcl-2) that were essential for survival. Knockdown or inhibition of such genes leads to spontaneous apoptosis in mutant K-Ras cells[12-14]. However, none of these papers have addressed the potential impact of (inhibition) of these genes on specifying the output of CD95 signaling. Studies addressing this interesting topic could give substantial advances in our knowledge of the molecular mechanisms that determine death receptor signaling.

Death receptors in surgery-stimulated outgrowth of micrometastases in the liver

Hypoxia can contribute to tumor cell invasion and metastatic progression and is associated with a more aggressive tumor phenotype[15, 16]. Hypoxia can activate the CD95 death receptor system which generally leads to apoptosis[17, 18]. In **chapter 4** we demonstrate that hypoxia-induced tumor cell invasion *in vitro* is abrogated by inhibiting CD95 signaling. Furthermore, we show in **chapter 4** (RFA) and in **chapter 5** (I/R injury) that tumor cells located in hypoxic liver tissues areas following liver surgery acquired a highly invasive phenotype when compared to tumor cells in normoxic tissue areas. Interfering with CD95 signaling reduced invasion and outgrowth of tumor cells in hypoxic tissue areas. These results suggest that neutralizing CD95/CD95L signaling could be of therapeutic benefit following surgical treatment of colorectal liver metastases.

Although our results are promising, we have to take into account that they are based on one cell line (C26) and on one mouse model (C26-BALB/c). Further pre-clinical work is needed if CD95L-neutralizing agents, either alone or in combination with chemotherapy, can be of added value in preventing aggressive tumor recurrence following RFA. Of note, chemotherapy

can sensitize tumor cells to CD95L-induced apoptosis and combination therapy resulted in a synergistic inhibitory effect on tumor growth[19]. Therefore, blocking CD95 signaling may reduce the efficacy of chemotherapy. Interestingly, the first mouse model for colorectal cancer with spontaneous liver metastasis formation was recently published. In this model oncogenic KRAS and loss of the APC tumor suppressor drive the formation of colon carcinomas that spontaneously metastasize to the liver. This model is ideally suited to study the benefits of CD95L-neutralizing agents in limiting liver metastasis formation and in preventing surgery-stimulated outgrowth of metastases[20].

In addition, it has recently become possible to efficiently establish human colorectal tumors as 3-dimensional colonospheres. The colonosphere cultures are currently believed to be the most relevant cell culture system for human colorectal cancer available and it provides an excellent tool for translational research. Therefore, prior to starting a clinical trial, it would be worthwile to investigate the effects of CD95 suppression and/or CD95L-neutralizing antibodies in a large panel of human tumor-derived colonospheres, similar to the ones used in **chapter 7**. In addition, the effects of CD95 neutralization on chemotherapy efficacy deserves further attention.

Preliminary experimental data from our group reveal that differentiation status did not affect resistance to CD95L-induced apoptosis in colonospheres and their differentiated counterparts. These data need to be confirmed in larger numbers of colorectal tumors and with various apoptotic stimuli. Nevertheless, the data so far indicate that CSCs are not universally more resistant against apoptotic stimuli than their differentiated offspring[21].

Circulating CD95L as a potential prognostic factor in colorectal liver metastases

In **chapter 6** we describe the potential utility of CD95L as a prognostic factor in patients with synchronous colorectal liver metastases. High levels of circulating CD95L were associated with a strongly reduced period of disease-free survival (DFS) after surgery and were not simply a reflection of overall tumor burden. Low levels of circulating CD95L could identify the subgroup of patients with synchronous colorectal liver metastases that benefits from liver surgery. Since there was no significant drop or raise in the levels of sCD95L after surgery, which could be correlated with disease progression or reduction, it does not meet the criteria for a biomarker. Several reports have defined factors predicting DFS in colorectal cancer patients with synchronous liver metastases and different combinations of these factors have been proposed as clinical prediction models for selecting patients who could benefit from surgery[22-24]. In our preliminary results pre-operative levels of carcinoembryonic antigen (CEA), the widely used serum marker for colon cancer, was not related with DFS. Although the sCD95L levels were measured in a relatively small group of patients and patients will never undergo surgery based on one simple feature alone, its role as a

prognostic factor in DFS could be of additional value in the decision tree for selecting patients for surgery and/or chemotherapy.

Reliable tools for determining prognosis and guidance for therapy, including the identification of patient groups for surgery, are still needed. Innovative methods including gene expression profiling and tumor genome sequencing hold great promise in this respect [25, 26]. However, the cost-effectiveness of such methods is still a matter of concern. In the meantime, it might be worthwhile to genotype for mutations in the KRAS gene, since recent data have demonstrated the predictive power of such mutations for resistance against anti-EGFR agents[27, 28]. A potential place for measuring circulating CD95L levels in patients in relation to existing and novel diagnostic tools should be further evaluated in prospective clinical trials.

In summary, this thesis gives us more insight into the role of death receptors in the formation and outgrowth of colorectal liver metastases. The K-Ras oncogene switches death receptor signaling output, which is important for the formation and survival of metastases in the hepatic microenvironment. Furthermore, CD95 turns out to be a key mediator of surgery-stimulated aggressive outgrowth of micrometastases in the liver. Our knowledge about death receptors has increased dramatically in the past decades. However, defining the factor(s) that determine signaling output remains a challenge for future research in the field of death receptors.

REFERENCES

1. Kranenburg,O. The KRAS oncogene: past, present, and future. Biochim. Biophys. Acta 1756, 81-82 (2005).
2. Barnhart,B.C. et al. CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J. 23, 3175-3185 (2004).
3. Kleber,S. et al. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 13, 235-248 (2008).
4. Scott,R.W. & Olson,M.F. LIM kinases: function, regulation and association with human disease. J. Mol. Med. 85, 555-568 (2007).
5. Wang,W., Eddy,R., & Condeelis,J. The cofilin pathway in breast cancer invasion and metastasis. Nat. Rev. Cancer 7, 429-440 (2007).
6. geciras-Schimnich,A. et al. Molecular ordering of the initial signaling events of CD95. Mol. Cell Biol. 22, 207-220 (2002).
7. Lee,K.H. et al. The role of receptor internalization in CD95 signaling. EMBO J. 25, 1009-1023 (2006).
8. Qualmann,B., Kessels,M.M., & Kelly,R.B. Molecular links between endocytosis and the actin cytoskeleton. J. Cell Biol. 150, F111-F116 (2000).
9. Russo,M., Mupo,A., Spagnuolo,C., & Russo,G.L. Exploring Death Receptor Pathways as Selective Targets in Cancer Therapy. Biochem. Pharmacol.(2010).
10. Wang,W. et al. The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. J. Cell Biol. 173, 395-404 (2006).
11. Chakrabandhu,K. et al. The extracellular glycosphingolipid-binding motif of Fas defines its internalization route, mode and outcome of signals upon activation by ligand. Cell Death. Differ. 15, 1824-1837 (2008).
12. Barbie,D.A. et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature 462, 108-112 (2009).
13. Luo,J. et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell 137, 835-848 (2009).
14. Scholl,C. et al. Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. Cell 137, 821-834 (2009).
15. Harris,A.L. Hypoxia--a key regulatory factor in tumour growth. Nat. Rev. Cancer 2, 38-47 (2002).
16. Semenza,G.L. Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer 3, 721-732 (2003).
17. Jeremias,I. et al. Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. Circulation 102, 915-920 (2000).
18. Liu,K. & Abrams,S.I. Alterations in Fas expression are characteristic of, but not solely responsible for, enhanced metastatic competence. J. Immunol. 170, 5973-5980 (2003).
19. Liu,Z. et al. Combination of human Fas (CD95/Apo-1) ligand with adriamycin significantly enhances the efficacy of antitumor response. Cell Mol. Immunol. 6, 167-174 (2009).
20. Hung,K.E. et al. Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. Proc. Natl. Acad. Sci. U. S. A 107, 1565-1570 (2010).
21. Dean,M., Fojo,T., & Bates,S. Tumour stem cells and drug resistance. Nat. Rev. Cancer 5, 275-284 (2005).
22. Iwatsuki,S. et al. Hepatic resection for metastatic colorectal adenocarcinoma: a proposal of a prognostic scoring system. J. Am. Coll. Surg. 189, 291-299 (1999).
23. Mann,C.D., Metcalfe,M.S., Leopardi,L.N., & Maddern,G.J. The clinical risk score: emerging as a reliable preoperative prognostic index in hepatectomy for colorectal metastases. Arch. Surg. 139, 1168-1172 (2004).
24. Nordlinger,B. et al. Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1568 patients. Association Francaise de Chirurgie. Cancer 77, 1254-1262 (1996).
25. Frederiksen,C.M., Knudsen,S., Laurberg,S., & Orntoft,T.F. Classification of Dukes' B and C colorectal cancers using expression arrays. J. Cancer Res. Clin. Oncol. 129, 263-271 (2003).
26. Jones,S. et al. Comparative lesion sequencing provides insights into tumor evolution. Proc. Natl. Acad. Sci. U. S. A 105, 4283-4288 (2008).

27. Amado,R.G. et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J. Clin. Oncol. 26, 1626-1634 (2008).
28. Bokemeyer,C. et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J. Clin. Oncol. 27, 663-671 (2009).

CHAPTER 9

SUMMARY IN DUTCH
NEDERLANDSE SAMENVATTING

K-Ras, dikkedarmkanker en uitzaaiingen naar de lever

Het menselijk lichaam bestaat uit allerlei organen en weefsels die zijn opgebouwd uit miljarden cellen. In de kern van deze cellen ligt het erfelijk materiaal met de erfelijke eigenschappen opgeslagen, het DNA. Elk stukje DNA dat de code bevat voor één van de vele eiwitten waaruit het lichaam is opgebouwd wordt een gen genoemd. In iedere cel treden er continu beschadigingen op van het DNA en komen er veranderingen (mutaties) van het DNA voor. De cel heeft bepaalde verdedigings- en reparatiemechanismen om deze beschadigingen teniet te doen. Wanneer het herstelmechanisme faalt en een aaneenschakeling van dergelijke mutaties zich voordoet op belangrijke plekken in het DNA (genen), dan kan kanker ontstaan. In het geval van dikkedarmkanker (colorectaal carcinoom) is het K-Ras gen één van de genen die vaak gemuteerd is. Een K-Ras mutatie komt in ongeveer 40% van de gevallen van dikkedarmkanker voor. Het K-Ras gen bevat de erfelijke code voor een eiwit dat fungeert als signaaltransductie eiwit van groeistimulerende factoren, dat wil zeggen het eiwit geeft signalen van buiten de cel door naar binnen in de cel. Dit zijn signalen die belangrijk zijn voor de groei (celdeling), verdere ontwikkeling (differentiatie) en overleving van de cel. Normaal kan dit eiwit aan of uit worden gezet, maar indien er een mutatie (K-Ras mutatie) is opgetreden staat het eiwit altijd 'aan'. Hierdoor worden er zelfs in de afwezigheid van groeistimulerende factoren signalen aan de cel doorgegeven hetgeen leidt tot ongeremde celdeling, een belangrijk kenmerk van kanker. Dikkedarmkanker is één van de meest voorkomende vormen van kanker in de westerse wereld. Jaarlijks wordt er bij ongeveer 12.000 nieuwe patiënten in Nederland de diagnose gesteld en overlijden er ongeveer 5.000 mensen aan. In de meeste gevallen overlijden patiënten aan dikkedarmkanker omdat de ziekte is uitgezaaid, meestal gebeurt dit naar de lever (levermetastasen). Het is uitgebreid beschreven dat K-Ras betrokken is bij de ontwikkeling van dikkedarmkanker. Echter, de rol die K-Ras speelt bij de vorming van uitzaaiingen is onduidelijk. Voordat een tumorcel vanuit de primaire tumor (lees dikkedarmkanker) een levermetastase kan vormen, moet de tumorcel een reeks van gebeurtenissen doorstaan. Al deze opeenvolgende gebeurtenissen moeten met succes worden doorlopen voordat een uitzaaiing kan ontstaan. In de eerste plaats moeten de kankercellen zich van de dikkedarmkanker los maken. De kankercellen moeten daarna het basale membraan afbreken dat de kankercellen omringt waarna ze zich naar een bloedvat kunnen begeven om de bloedbaan binnen te dringen (intravasatie). In de bloedbaan moeten kankercellen zonder de steun van andere cellen zien te overleven. De kankercellen bereiken via de darmvenen en de poortader de lever, waar ze vastlopen in kleine leverbloedvaatjes. Door uit deze bloedvaatjes te treden (extravasatie) bereiken ze het leverweefsel. Hier kunnen ze alleen overleven door het immuunsysteem van de lever te overwinnen. Voor de verdere uitgroei tot een grote uitzaaiing moeten de kankercellen nieuwe bloedvaten vormen om de benodigde zuurstof en voedingsstoffen te krijgen. Eén van de eerste verdedigingsmechanismen van het immuunsysteem in de lever om een (uitzaaiende) tumorcel aan te pakken is door het activeren van het zogenaamde 'death receptor' systeem. In **hoofdstuk 3** van dit proefschrift

hebben we gekeken naar de rol van het K-Ras oncogen in de vorming van uitzaaiingen van dikkedarmkanker naar de lever. Hierbij hebben we specifiek gekeken naar de rol van K-Ras bij de overleving van de tumorcellen in de lever.

Apoptose en death receptoren

Apoptose is het proces van geprogrammeerde celdood en speelt een essentiele rol bij de regulatie van celpopulaties in weefsels. Apoptose zorgt ervoor dat ongewenste of beschadigde cellen worden opgeruimd. In de embryonale ontwikkeling zorgt apoptose er bijvoorbeeld voor dat niet noodzakelijke weefselstructuren verdwijnen (denk aan de 'zwemvliezen' tussen de vingers die bij mensen in aanleg aanwezig zijn). In het immuunsysteem worden cellen die het eigen afweersysteem willen aanvallen en dus ongewenst zijn, opgeruimd door apoptose. Als een cel signalen van zijn omgeving ontvangt die de cel tot apoptose dwingen, of als de cel zoveel schade heeft opgelopen dat verder bestaan niet mogelijk is, wordt binnen in de cel een mechanisme geactiveerd. Dit mechanisme leidt uiteindelijk tot de activatie van enzymen, de zogenaamde caspases (cysteïne-aspartylproteases). Deze enzymen zetten de cel aan tot de afbraak van eiwitten en DNA. Apoptose zorgt voor een balans tussen de juiste cel-aantallen en verwijdering van beschadigde cellen. Wanneer er verstoring optreedt in de uitvoering van apoptose kan dit leiden tot ziekte, waaronder kanker.

Eén van de manieren waarop apoptose geïnduceerd kan worden is via gespecialiseerde receptoren aan de buitenkant van de (tumor)cel, zogenaamde 'death receptoren'. Deze death receptoren worden geactiveerd door een eiwit van het immuunsysteem dat een complex aangaat met de receptor, een zogenaamd ligand. CD95 is de bekendste death receptor en CD95-ligand is zijn activator. TNF-related apoptosis-inducing ligand receptor 1 en 2 (TRAIL-R1/2), zijn ook death receptoren en worden geactiveerd door TRAIL. Gezien het feit dat death receptoren heel specifiek tumorcellen kunnen aanvallen, wordt er op dit moment gekeken of death receptoren een goed aanknopingspunt kunnen zijn voor nieuwe therapieën. Op dit moment worden er klinische trials opgezet waarin death receptor-liganden en death receptor-agonisten (stimulerende anti-lichamen) worden getest op veiligheid en effectiviteit in patiënten als anti-kanker middel.

Sinds kort zijn er enkele onderzoeken die laten zien dat death receptoren ook op een heel andere manier kunnen signaleren en in plaats van apoptose juist groei (proliferatie) of invasie kunnen stimuleren. **Hoofdstuk 2** geeft een overzicht van bekende literatuur over de death receptor CD95 in dikkedarmkanker. Op dit moment is het onduidelijk wat de moleculaire mechanismen zijn die bepalen of een death receptor celdood of juist groei of invasiviteit signaleert. In **hoofdstuk 3** van dit proefschrift beschrijft hoe een K-Ras mutatie zorgt voor een switch in de signalering van death receptoren, van apoptose (geprogrammeerde celdood) naar een "agressief/invasief worden"-signaal en dat dit belangrijk is voor de vorming van levermetastasen. Door het uitvoeren van een brede screening onder de eiwitten die K-Ras

kan aansturen, blijkt dat het Raf1 eiwit met deze switch te maken heeft doordat het de cascade Rho-ROCK-Lim kinase-cofilin blokkeert. Hierdoor wordt onder andere de beweeglijkheid van de tumorcellen beïnvloed. CD95 ontpopt zich als een soort "Dr. Jekyll and Mr. Hyde" en laat zich hier als een pro-tumorigene receptor zien. Daarnaast zou de keus van death receptor ligands als potentieel anti-kanker middel in patiënten met een K-Ras mutatie op basis van deze switch wel eens zeer averechts kunnen werken.

Chirurgische behandeling van colorectale levermetastasen

Ongeveer de helft van alle patiënten met dikkedarmkanker krijgt levermetastasen. Met alleen chemotherapie kan het leven van deze patiënten wel verlengd worden van ongeveer 10 naar 20 maanden, echter patiënten kunnen nog niet genezen worden. Het wegsnijden van de uitzaaiing (leverresectie) biedt tot op heden de enige kans op genezing. De 5-jaars overleving van geopereerde patiënten is ongeveer 35-60%, de 10-jaars overleving is ongeveer 25%. Slechts een kwart van de patiënten met levermetastasen komt in aanmerking voor leverresectie. Voor patiënten bij wie de leveruitzaaiing niet weggesneden kan worden, is het soms mogelijk deze te vernietigen met behulp van hitte door middel van zogenaamde radiofrequente ablatie (RFA). Met behulp van een dikke naald die in de metastase wordt gestoken, wordt een wisselstroom teweeg gebracht. De ionen in de uitzaaiing worden door deze wisselstroom dusdanig snel heen en weer bewogen dat frictiehitte ontwikkeld wordt. De temperatuur loopt hierbij op tot boven de 60ºC waardoor de uitgezaaide kankercellen sterven.

Chirurgie-gestimuleerde tumor uitgroei en de rol van CD95

Zowel bij de leverresecties als bij de RFA-behandeling wordt vaak de bloedtoevoer naar de lever afgeklemd. Bij de leverresecties is dat om het bloedverlies te verminderen en bij de RFA-behandeling om te voorkomen dat de hitte die nodig is om al het kankerweefsel te vernietigen, wordt weggevoerd door de bloedstroom. Het tijdelijk afklemmen van de bloedtoevoer naar de lever heeft ook een nadelig effect. Door het afklemmen ontstaat er een zuurstoftekort in de levercellen (ischemie). Wanneer de klem verwijderd wordt en de zuurstoftoevoer naar de lever is hersteld, spreekt men van reperfusie. Het is bekend dat dit mechanisme van ischemie-reperfusie schade kan veroorzaken aan de levercellen. Bovendien is aangetoond dat dit kan leiden tot versnelde tumoruitgroei van achtergebleven micrometastasen. Het werkingsmechanisme hierachter is echter onduidelijk. Deze versnelde tumorgroei is specifiek gelegen in de rand van het dode leverweefsel, na zowel RFA als na het afklemmen van de bloedtoevoer naar de lever. Deze rand van het gedode leverweefsel kenmerkt zich door een verlaagd zuurstofgehalte (hypoxie).

We hebben onderzocht of CD95 een rol speelt in deze versnelde tumor uitgroei van micrometastasen na chirurgie. In **hoofdstuk 4** en **5** laten we zien dat door middel van

respectievelijk RFA (hoofdstuk 4) en het afklemmen van de bloedtoevoer naar de lever (hoofdstuk 5) overgebleven micrometastasen een invasief karakter aannemen. In **hoofdstuk 4** laten we zien dat hypoxie CD95 in de tumorcellen activeert. Het blijkt ook dat CD95 en CD95-ligand meer aanwezig zijn in de rand van het gedode leverweefsel na de RFA behandeling. Vervolgens is op drie verschillende manieren het CD95/CD95-ligand systeem geremd en is gekeken welk effect dit had op het invasieve karakter en de uitgroei van de tumorcellen na RFA. Allereerst werden muizen behandeld met een geneesmiddel dat het CD95-ligand neutraliseert. Het gevolg was dat het invasieve karakter spectaculair werd gereduceerd. Vervolgens hebben we kankercellen gebruikt waarin CD95 genetisch geremd was. Ook hierbij verminderde de invasiviteit en de uitgroei van de tumorcellen, waardoor een direct effect van het CD95/CD95-ligand systeem aangetoond werd. Om tenslotte te onderzoeken wat de bijdrage van het CD95-ligand van de muis zelf was met betrekking tot de invasiviteit en uitgroei van de tumorcellen na RFA, hebben we gekeken naar het effect in muizen die een genetische mutatie hadden ondergaan waardoor zij geen CD95-ligand hadden. In deze muizen bleek de invasiviteit en uitgroei van de tumorcellen na RFA niet verminderd te zijn. Dit suggereert dat de tumorcel in deze hoedanigheid zelf verantwoordelijk is voor CD95-ligand productie en CD95 activatie. In **hoofdstuk 5** onderzoeken we de rol van CD95 in invasiviteit en versnelde tumor uitgroei na afklemmen (ischemie-reperfusie schade). Hier worden vergelijkbare resultaten gevonden als in hoofdstuk 4. De invasiviteit en uitgroei van tumorcellen na het afklemmen van de bloedtoevoer bleek ook verminderd te zijn als we de tumorcellen gebruikten waarin CD95 genetisch geremd was. Als wij vervolgens de muizen gebruikten die geen CD95-ligand hadden dan bleek de invasiviteit van de tumorcellen niet verminderd, wat opnieuw duidt op autoregulatie. Echter, de uitgroei van de tumorcellen was wel verminderd. Gezien het feit dat deze muizen ook een verminderde schade (minder dood leverweefsel) lieten zien na het afklemmen vergeleken met normale muizen, denken wij dat dit de oorzaak is van de verminderde tumor uitgroei.

Al met al zou het dus zo kunnen zijn dat er in de toekomst eerst behandeld moet worden met CD95 blokkerende of CD95-ligand remmende middelen alvorens een patiënt een RFA-behandeling of leverresectie ondergaat. Voordat het zover is, moet er eerst veel meer onderzoek gedaan worden en gekeken worden of de gevonden bevindingen in de humane setting ook standhouden. Een mogelijk model dat hiervoor gebruikt zou kunnen worden, is het model zoals beschreven in **hoofdstuk 7**. Hierbij worden uit humane tumorbiopten (stukjes tumorweefsel uit de mens) zogenaamde kankerstamcellen geïsoleerd. Kankerstamcellen zijn cellen waarvan wij nu denken dat zij de verantwoordelijke cellen zijn voor de opbouw/vorming van een tumor en mogelijk ook de aanjagers zijn voor de vorming van metastasen. Kankerstamcellen vormen zogenaamde spheroiden, een soort 3D-structuur van kankerstamcellen. Een eerste stap zou dan zijn om te zien hoe deze humane spheroiden reageren op CD95 stimulatie danwel blokkades.

CD95-ligand in het bloed als een potentieel prognostische factor

In ongeveer 25% van de patiënten met colorectaal carcinoom worden er ook gelijk al levermetastasen gediagnosticeerd. Slechts een deel van deze patiënten met zogenaamde synchrone levermetastasen heeft (dan nog) profijt van leverchirurgie. Echter, er zijn tot op heden geen betrouwbare voorspellende factoren die deze patiëntengroep kan aanwijzen. In **hoofdstuk 6** laten we zien dat circulerend CD95-ligand (CD95-ligand in het bloed) mogelijk een dergelijke voorspellende factor zou kunnen zijn. De studie die wij gedaan hebben laat zien, dat hoge waardes van circulerend CD95-ligand bij patiënten met synchrone levermetastasen geassocieerd zijn met een lage ziekte-vrije overleving (na de operatie) en dus weinig profijt hebben van een dergelijk zware ingreep. Circulerend CD95-ligand zou dus kunnen helpen bij het selecteren van patiënten die baat hebben bij leverchirurgie. Dergelijke bevindingen zullen moeten worden gestaafd in grotere patiëntengroepen.

Conclusies

Dit proefschrift geeft ons meer inzicht in de rol van death receptoren in de vorming en uitgroei van colorectale levermetastasen. Het K-Ras oncogen zorgt voor een switch in de signalering van death receptors. Dit is belangrijk is voor de formatie en overleving van uitzaaiingen in de lever. Verder blijkt dat CD95 een belangrijke rol speelt in de chirurgie-gestimuleerde tumor uitgroei. Mogelijk kan circulerend CD95-ligand als een prognostische factor dienen voor ziekte-vrije overleving bij patiënten met synchrone levermetastasen. In de toekomst is het belangrijk dat er verder gezocht wordt naar wat de exacte moleculaire mechanismen zijn die de uiteindelijke signalering van de death receptors bepalen. Identificatie hiervan zou kunnen leiden tot nieuwe aanknopingspunten voor therapieën tegen kanker.

CHAPTER 10

ACKNOWLEDGEMENTS - DANKWOORD

LIST OF PUBLICATIONS

CURRICULUM VITAE AUCTORIS

I would like to thank a number of people who contributed, professionally and personally, to the realization of this thesis.
Dit proefschrift is tot stand gekomen met de inzet, hulp en steun van veel mensen. Een aantal personen wil ik in het bijzonder bedanken.

Prof. dr. I.H.M. Borel Rinkes, beste Inne,
Veel dank voor je vertrouwen, je oprechte steun en de kans die je me gegeven hebt om de uitdaging aan te gaan van het schrijven van dit proefschrift. Ik beschouw het als een voorrecht in jouw onderzoeksgroep terecht te zijn gekomen. Je scherpzinnigheid en je enorm positieve instelling werken stimulerend en halen het beste bij mensen naar boven. Het maakt je een zeer prettige begeleider. Onze trips naar Denver en Chamonix zijn memorabel en ik kijk nu al uit naar een volgende. Ik hoop in de toekomst nog veel van je te mogen leren.

Dr. O. Kranenburg, beste Onno,
Jouw inbreng in de totstandkoming van dit proefschrift is voor mij onmisbaar geweest. Dankzij jou heb ik de beginselen van het doen van basaal wetenschappelijk onderzoek onder de knie gekregen. Je enorme betrokkenheid en enthousiasme hebben altijd diepe indruk gemaakt. Met je continue kritische blik heb je ervoor gezorgd dat het onderzoek naar een hoger plan is getild. Ook al heb je daarmee mijn doorzettingsvermogen af en toe als geen ander op de proef gesteld, ik ben je er zeer dankbaar voor ("blijf op de juiste plek tegen die deur aan schoppen, dan gaat ie een keer open…").

Prof. dr. R.H. Medema, beste Rene,
Met veel bewondering kijk ik naar hoe jij je onderzoeksgroep begeleidt en op hoog niveau invulling geeft aan de wetenschap. Dank voor je interesse tijdens de werkbesprekingen en daarbuiten en de beregezellige retraite. Mooi hoe je op precies het goede moment het filmpje over 'reviewer 3' wist te sturen en de dingen weer in perspectief wist te zetten. Als je nog weer een keer een splinter in je hand hebt, hoor ik 't wel. Dank voor je tijd en het kritisch beoordelen van mijn manuscript als voorzitter van de beoordelingscommissie.

Prof. dr. H. Walczak, dear Henning,
I am very grateful for all the help and scientific input you have given to my thesis. It has been a privilege working with you. "Our man in London!" I hope that the collaboration between our groups will continue in the future. Thank you very much for taking place in my thesis committee. It is highly appreciated.

Overige leden van de beoordelingscommissie:
Prof. dr. J.P. Medema, dank voor de belangrijke wetenschappelijke inbreng en de actieve betrokkenheid bij mijn proefschrift. Ook u wil ik hartelijk danken voor uw tijd en het kritisch beoordelen van het manuscript.
Prof. dr. F.L. Moll, dank voor uw tijd en interesse in mijn proefschrift. Ik waardeer het zeer dat u op de dag zelf vanuit het buitenland wilt afreizen om in de oppositie plaats te nemen.

Prof. dr. R. van Hillegersberg, beste Richard,
Binnen de levergroep ben jij het meest klinisch georiënteerd. Je bevlogenheid als chirurg en je enthousiasme werken aanstekelijk en ik hoop nog veel van je te leren in de kliniek. Dank ook voor alle gastvrijheid voor de leuke borrels bij jullie thuis. Ik waardeer het zeer dat je in de oppositie plaats wilt nemen.

Overige leden van de leescommissie:
Prof. dr. Th.J.M.V. van Vroonhoven, Prof. dr. J. Kirpensteijn, hartelijk dank voor het kritisch lezen van mijn proefschrift en dat u in de oppositie plaats wilt nemen.
Dr. P.J. Breslau, met veel plezier denk ik terug aan de tijd in het Rode Kruis Ziekenhuis in Den Haag. U bent één van de aanjagers geweest dat ik graag chirurg wil worden. Dank voor uw interesse in mijn proefschrift en dat u in de oppositie plaats wilt nemen.

Dr. P.W.B. Derksen, beste Patrick,
Dank dat je op het lab en daarbuiten als 'sparring-partner' wilde fungeren. Je commentaren waren altijd opbouwend en realistisch en dat heeft me geholpen mijn prioriteiten in het onderzoek verder te kunnen stroomlijnen. Onze trip naar Cold Spring Harbor was top en ik kijk weer uit naar de etentjes met jou en Winan. Die houden we erin.

Prof. dr. E.E. Voest, beste Emile,
Je bent een meester in het slaan van bruggen tussen onderzoek in het laboratorium en de kliniek. Dank voor je welgemeende interesse en alle adviezen tijdens de werkbesprekingen en de retraite.

Prof. dr. P.J. van Diest en alle medewerkers van de afdeling pathologie, beste Paul,
In het begin van mijn promotietijd heb ik een paar keer met jou achter de microscoop mogen zitten en wist je al snel je enthousiasme voor de pathologie op mij over te brengen. Ik ben zeer dankbaar dat er zo'n geweldige samenwerking bestaat tussen de pathologie en de chirurgie.

Dr. M.R. Vriens, beste Menno,
Heel hartelijk bedankt voor alle coaching, zowel op professioneel als op persoonlijk vlak. Ik heb dit altijd zeer gewaardeerd en kijk er naar uit in de toekomst verder met je samen te werken.

Marielle Hoefakker en Romy Liesdek,
Dank voor alle hulp en ondersteuning, jullie zijn super.

Alle stafleden en arts-assistenten van de afdeling Heelkunde van het UMC Utrecht, dank voor de interesse in mijn onderzoek en de gezelligheid tijdens de extra-curriculaire evenementen.

Wijnand M. Pon stichting, geachte heer en mevrouw Schipper,
Met veel plezier denk ik terug aan onze ontmoeting. Dank dat u het financieel mogelijk heeft gemaakt om het onderzoek, beschreven in dit proefschrift, uit te voeren. Ik beschouw het als een groot voorrecht dat ik op deze manier onderzoek heb mogen doen. Ik ben u dan ook zeer erkentelijk.

Alle collega's van de Medische Oncologie (Annelieke Jaspers, Dorus Mans, Eva Vlug, Jeanine Roodhart, Joost Vermaat, Laura Daenen, Marlies Langenberg, Martijn Lolkema, Miranda van Amersfoort, Rachel Giles, Rhandy Eman, Ron Schackmann, Sander Basten) en van de Urologie (Judith Jans en Stephanie Kroeze)

All colleagues of the Experimental Oncology Department, thanks for the good collaboration, your interest and input during the work discussions, but also for the good times during the 'labborrels' and the retraite. Rob Klompmaker en Livio Kleij: dank voor al jullie hulp en technische ondersteuning.

Dr. Brian Snyder, Ara Nazarian and Robert Fajardo and all other people from the Orthopedic Biomechanics Laboratory, Boston MA, USA. Thank you for teaching me the first steps in science, I really enjoyed it.

Assistenten en stafleden chirurgie van het Diakonessenhuis, alsmede alle SEH-verpleegkundigen, dank voor jullie interesse in mijn onderzoek en dank dat ik mijn eerste stappen in de kliniek bij jullie heb mogen ervaren.
Niels Smakman, Jarmila van der Bilt en Liesbeth Veenendaal, jullie gingen mij voor in het lab en hebben mij de eerste 'tips and tricks' geleerd, dank daarvoor.

Mijn student-onderzoekers: Martijn Leenders en Taco van der Meulen, dank voor al jullie hulp en inzet. Heel erg fijn. Taco, erg mooi om nu in de kliniek ook weer samen te werken. En last but not least Florien Westendorp, “Flo” je bent een topper, dank voor al je hulp binnen en buiten het ziekenhuis. Ik hoop dat ik ook in de toekomst van je enthousiasme mag blijven genieten.

De vrienden van Isengard: Bob Bloemendaal, Eline van Hattum, Falco Hietbrink, Judith Boone, Stijn van Esser, Tjaakje Visser, Anne den Hartog, Charlotte van Kessel, Emily Postma, Jasper van Keulen, Klaas Govaert en Roy Verhage. Het was altijd een feestje om even bij jullie langs te gaan. Dank voor de vele gezellige momenten en dat er nog maar vele mogen volgen!

Andere (oud) mede-onderzoekers van de Heelkunde: Daphne de Groot, Erik Tournoy, Femke Lutgendorff, Janesh Pillay, Joffrey van Prehn, Joris Broeders, Kathelijne Groeneveld, Ralf Sprengers, Rian Nijmeijer, Rob Hurks, Siegrid de Meer, Usama Ahmed Ali, Willem Hellings, Wouter Derksen en Wouter Peeters.

Het Kranenburg-Borel Rinkes lab, ons eigen groeppie:
Benjamin Emmink en Ernst Steller, stelletje koningen. Dank voor de gezelligheid binnen en buiten het lab, de hulp en de vriendschap. Ik heb met heel veel plezier met jullie samengewerkt. Danielle Raats, Jamila Laoukili en Andre Verheem, dank voor al jullie hulp, ondersteuning en gezelligheid.

JC Corleone, “Mannen, jullie worden bedankt”. Dank voor jullie onophoudelijke interesse en broodnodige relativering. Het is beregezellig met jullie samen te zijn en ik hoop op nog vele mooie momenten. Iets zegt me dat dat wel gaat lukken….

Mijn (zeil)vrienden: Peter-Paul Willemse, Pieter van der Woude, Peer van der Zwaal, Mathieu Wijffels, Auke Appelman, Roelof Risselada, Edgar Bothe, Rutger Bakker, het is weer tijd voor ‘skippersborrel’!

Tzigane sinds 1910

Onno ten Berge, vriend voor het leven, my best man! Als geen ander weet jij hoe het is een promotietraject te doorlopen en als geen ander weet jij me af en toe weer even met beide benen op de grond te zetten. Je vriendschap is me ontzettend dierbaar. Dank je wel.

Menno de Bruijn en Winan van Houdt, buddies, samen op het lab begonnen en wat een toptijd hebben we gehad. Dank voor jullie prachtige vriendschap die hier op het lab ontstaan is en ik hoop dat we nog maar vele mooie dingen samen mogen beleven. Menno, je staat

altijd voor iedereen klaar en dat is iets wat ik zeer in jou waardeer. Ik kijk weer uit naar je kookkunsten, heerlijk. Winan, onze trip naar Cold Spring Harbor was briljant en ik denk met veel plezier terug aan alle 'besprekingen' die we hebben gehad. Je bent een topkerel, als jij er bent is het altijd goed.

Mijn paranimfen: Maarten Nijkamp en Nikol Snoeren.
Maarten, mijn grote vriend op het lab en daarbuiten. Vanaf dag één was er de klik en zijn we als broers op het lab aan de slag gegaan ("Fokke en Sukke doen onderzoek…" en dat heeft men geweten!). Samen keihard werken en dan 'heel rustig' een biertje doen... of een long island iced tea… Dank voor de briljante tijd en ik kijk ernaar uit samen te opereren. Je bent gewoon een gouden vent.
Lieve Nikol, met jouw humor en enthousiasme is het altijd een feestje om bij je te zijn. Met veel plezier denk ik terug aan onze experimenten op het lab. Ik heb veel respect voor de onophoudelijke moed en energie die jij in (nieuwe) projecten weet te leggen zowel binnen als buiten het ziekenhuis. Ik ben er trots op dat zo'n lieve vriendin naast me staat op de 21e.

Dhr en mevr. Rauws, Lieve Ed en Dorothy, dank voor al jullie steun en interesse. Ik voel me altijd zeer welkom bij jullie en dat is een ontzettend fijn gevoel. Ik vind het heel bijzonder om zo'n tweede thuis te hebben. Dank jullie wel.

Lieve Marrit en Jan (Dirk), mijn grote kleine zus! Heerlijk om je zo te zien stralen als jonge moeder. Wat ben ik blij voor je dat één en ander weer in rustiger vaarwater is gekomen. Dank voor al je gevraagde en ongevraagde adviezen, die waardeer ik altijd zeer.

Lieve Wim en Leonie (Sep, Julie, Emma), mijn grote broer! Beremooi dat je weer iets dichter in de buurt woont, maar of je nou in Amerika bent of hier, het is een heel fijn gevoel om te weten dat ik altijd op je kan rekenen. Dank je wel.

Lieve heit en mem, zonder jullie was ik nooit zover gekomen. Dankzij jullie onvoorwaardelijke steun en grenzeloze vertrouwen heb ik me zo kunnen ontwikkelen. Ik ben jullie daar ontzettend dankbaar voor! Het is altijd heerlijk om bij jullie in Friesland thuis te komen. Jullie zijn echt geweldige ouders. Dank jullie wel.

Lieve, lieve MARJOLEIN,
Met hoofdletters in mijn proefschrift. En terecht! Je bent ongelooflijk belangrijk voor me en je bent mijn allerliefste schat. Wat ontzettend fijn dat jij er bent. Dank je wel voor alles.

Hoogwater FJH*, Snoeren N*, Nijkamp MW, Gunning AC, van Houdt WJ, de Bruijn MT, Voest EE, van Hillegersberg R, Kranenburg O, Borel Rinkes IHM.
Circulating CD95 ligand as a potential prognostic factor for recurrence-free survival following surgery in patients with synchronous colorectal liver metastases.
(submitted)

Nijkamp MW, Hoogwater FJH, Govaert KM, Steller EJA, Verheem A, Kranenburg O, Borel Rinkes IHM.
A role for CD95 signaling in ischemia/reperfusion-induced invasion and outgrowth of colorectal micrometastases in mouse liver.
(submitted)

Hoogwater FJH*, Nijkamp MW*, Steller EJA, Westendorp BF, van der Meulen TA, Leenders MW, Borel Rinkes IHM, Kranenburg O.
CD95 is a key mediator of invasion and accelerated outgrowth of mouse colorectal liver metastases following radiofrequency ablation.
Journal of Hepatology. 2010 Dec;53(6):1069-77.

Hoogwater FJH, Nijkamp MW*, Smakman N*, Steller EJA, Emmink BL, Westendorp BF, Raats DAE, Sprick MR, Schaefer U, Van Houdt WJ, De Bruijn MT, Schackmann RCJ, Derksen PWB, Medema JP, Walczak H, Borel Rinkes IHM, Kranenburg O.
Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells.
Gastroenterology. 2010 Jun;138(7):2357-67.

van Houdt WJ, Hoogwater FJH, de Bruijn MT, Emmink BL, Nijkamp MW, Raats DAE, van der Groep P, van Diest PJ, Borel Rinkes IHM, Kranenburg O.
Oncogenic KRAS desensitizes colorectal tumor cells to epidermal growth factor receptor inhibition and activation.
Neoplasia. 2010 Jun;12(6):443-52.

Nijkamp MW, Borren A, Govaert KM, Hoogwater FJH, Molenaar IQ, van Diest PJ, Kranenburg O, Borel Rinkes IHM.
Radiofrequency ablation of colorectal liver metastases induces an inflammatory response in distant hepatic metastases but not in local accelerated outgrowth.
Journal of Surgical Oncology. 2010 Jun 1;101(7):551-6.

de Bruijn MT, Raats DAE, Hoogwater FJH, van Houdt WJ, Cameron K, Medema JP, Borel Rinkes IHM, Kranenburg O.
Oncogenic KRAS sensitises colorectal tumour cells to chemotherapy by p53-dependent induction of Noxa.
Br J Cancer. 2010 Apr 13;102(8):1254-64.

Nijkamp MW, van der Bilt JD, Snoeren N, Hoogwater FJH, van Houdt WJ, Molenaar IQ, Kranenburg O, van Hillegersberg R, Borel Rinkes IHM.
Prolonged portal triad clamping during liver surgery for colorectal liver metastases is associated with decreased time to hepatic tumour recurrence.
Eur J Surg Oncol. 2010 Feb;36(2):182-8.

van Houdt WJ, de Bruijn MT, Emmink BL, Raats DAE, Hoogwater FJH, Borel Rinkes IHM, Kranenburg O.
Oncogenic K-ras activates p38 to maintain colorectal cancer cell proliferation during MEK inhibition.
Cellular Oncology. 2010 Jan 1;32(4):245-57.

*These authors contributed equally

CURRICULUM VITAE AUCTORIS

Frederik Hoogwater was born on November 2nd, 1979, in Dokkum, The Netherlands. He graduated from the Stedelijk Gymnasium in Leeuwarden in 1997 and studied Law at Utrecht University from 1997-1999, during which he finished the first year of the program. In 1999 he started medical school at Leiden University. In 2003, before his clinical rotations, he performed his research elective at the Orthopedic Biomechanics Laboratory (director: Dr. B.D. Snyder), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston MA, USA (supervisors: Prof. dr. S.E. Papapoulos , Dr. B.D. Snyder and Dr. A. Nazarian). At the end of 2003 he started his clinical rotations, during which he stayed involved in the research projects of the Orthopedic Biomechanics Laboratory. He obtained his medical degree in November 2005. In January 2006 he joined the research group of Prof. dr. I.H.M. Borel Rinkes at the Department of Surgical Oncology, Utrecht University, and was able to start the research described in this thesis thanks to the financial support of the Wijnand M. Pon foundation. In 2008 he was chairman of the Symposium on Experimental Research for Surgical Specialties (SEOHS) in Utrecht. He and the work described in this thesis have been awarded with the Scholar-in-training Award at the 100th Annual Meeting of the American Association for Cancer Research in Denver CO, USA (2009). After completing his research projects in June 2010, he started working in the Department of Surgery of the Diakonessenhuis, Utrecht (supervisor: Dr. G.J. Clevers). In January 2011 he will start the residency program in general surgery in the St. Antonius Hospital in Nieuwegein (supervisor: Dr. P.M.N.Y.H. Go). The last two years are scheduled in the University Medical Center Utrecht under supervision of Prof. dr. I.H.M. Borel Rinkes.