Ischemia/reperfusion

and

colorectal liver metastases

Jarmila DW van der Bilt

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Ischemia/reperfusion and colorectal liver metastases

Ischemie/reperfusie en colorectale levermetastasen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht,
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door

Jarmila Dagmara Wendelien van der Bilt
geboren op 20 februari 1976 te 's-Gravenhage

Promotor Prof. dr. I.H.M. Borel Rinkes

Co-promotores Dr. O. Kranenburg
Dr. R. van Hillegersberg

Laugh, and the world laughs with you

Weep, and you weep alone

For the sad old world must borrow its mirth

But has trouble enough of its own

Aan mijn opa

Contents

Chapter 1

General introduction and outline of the thesis

Surgical treatment of colorectal liver metastases

The prevalence of colorectal cancer is among the highest of malignancies in the western world.[1] Hepatic metastases will eventually develop in as many as 50-80% of colorectal cancer patients and the liver is the sole site of dissemination in 30%.[2,3] When untreated, the presence of liver metastases is associated with a very poor prognosis.[4-6] Novel chemotherapy regimes using oxaliplatin or irinotecan have doubled the median overall survival from 10 to 20 months, but still, virtually all patients will eventually succumb to their disease within 5 years.[7-10]
Currently, surgical resection remains the only hope for cure, offering 5 and 10-year survival rates of 30-40% and 20-25% respectively in selected patient groups.[11-14] Recent developments have made an increasing number of patients amenable to this potentially curative treatment.[15-17] For instance, although approximately 10-15% of patients are regarded eligible for an intentionally curative resection upon presentation, oxaliplatin or irinotecan-based neoadjuvant chemotherapy has increased resectability rates with an estimated 10-15%.[18-20] Moreover, multiple metastases or simultaneous resection of limited extrahepatic metastases is now no longer regarded as a contra-indication for hepatic resection, as long as an R0 resection can be achieved.[21-26] Finally, due to technical advances (e.g. portal vein embolization[27,28]) and a more aggressive approach (including two staged resections[29,30] and repeat hepatic resection[31-33]), an increasing number of patients may be offered long-term survival by partial liver resection.
Despite these advances, still many patients with hepatic metastases are not candidates for surgical resection because of extensive disseminated disease, an expected inadequate postoperative hepatic reserve or a poor medical condition. For nonresectable metastases confined to the liver, thermal destruction therapies, such as radiofrequency ablation (RFA) or laser-induced thermotherapy (LITT), have emerged as effective strategies to achieve tumor clearance. With 3-year survival rates of 37-58%, these treatments have generated encouraging results and potentially increase life-expectancy in selected patients.[34-40]

Recurrences and the importance of residual micrometastases

Unfortunately, even after an apparently radical tumor removal or complete tumor destruction, the majority of patients (about two-thirds) develop recurrent disease within the first two years after surgery, predominantly in the liver.[14,41,42] Intrahepatic recurrences may either develop from circulating tumor cells that are released into the circulation through surgical manipulation or from pre-existent microscopic tumor residues in the liver. In hepatic surgery, circulating tumor cells are detected peri-operatively in 15-44% of patients.[43-45] However, the majority of circulating tumor cells have limited life-span, and thus, due to this metastatic inefficiency their detection is of limited prognostic value.[46-50]
In contrast, residual microscopic tumor cell deposits that are undetected at the time of surgery are a more likely source for tumor recurrence, as they have already passed the first steps of the metastatic process. Intrahepatic micrometastatic colorectal cancer lesions are detected in 26-70% of randomly selected biopsies and are strongly associated with a poor outcome.[51-54] Moreover, two other obvious sources for residual metastatic disease are micro-satellite lesions and a positive resection margin, which are both highly indicative for early tumor recurrence.

In thermal destruction therapies, residual tumor tissue may be caused by incomplete heat-destruction, resulting in the development of local recurrences around the thermally induced lesion in up to 60% of treated tumors.[42,55,56] Insufficient heat-diffusion at the tumor periphery may cause unsuccessful treatment, especially in tumors greater than 4 centimeter or during the percutaneous approach.[35,57-59] Local recurrence may also develop from viable tumor cells that survive around blood vessels due to the cooling effect of the blood stream, i.e. the 'heat sink' effect.[60-63]
Thus, the development of recurrent disease is strongly associated with the presence of intrahepatic residual micrometastatic disease. The biological behavior of these micrometastases largely determines the time to develop recurrence, which is crucial for the further course of the disease and ultimately has great impact on patient survival.

Surgery-induced tumor growth

Despite the curative intent of surgery, it has been long suggested that surgical trauma may enhance the outgrowth of cancer cells.[64] Pre-existent micrometastases may stay dormant for years due to balanced apoptosis and proliferation and angiogenesis suppression,[65] until surgically induced micro-environmental stimuli may evoke their uncontrolled growth early in the postoperative period. The first experimental evidence of surgery-induced tumor growth originates from 1959 by Fisher and Fisher, showing enhanced micrometastasis outgrowth after subsequent relaparotomies and organ manipulation.[66] Many experimental studies on accelerated tumor growth and clinical reports on early recurrence after surgery have been reported since.[67-71] As neovascularization (or angiogenesis) is an integral part of physiological tissue repair, it has been implicated in the generation of the unfavorable side-effects of oncological surgery.[72]
Other than surgical injury and wound healing in general, some procedures related to hepatic surgery have been specifically associated with enhanced tumor growth and early tumor recurrence, such as blood transfusion and hepatectomy.[73-78] This thesis focuses on two particular surgical interventions that may adversely affect outcome by stimulating tumor growth following hepatic surgery: vascular clamping and thermal destruction therapy.

Vascular clamping and ischemia/reperfusion during hepatic surgery

The prime concern of hepatic surgeons is to safely perform a curative resection, without excessive blood loss. Intra-operative hemorrhage during hepatectomy is common, often necessitating blood transfusion, which is associated with unfavorable short and long-term outcome.[73-76] Therefore, approaches to reduce intra-operative blood loss are applied worldwide and include vascular clamping methods.[79-82]
During thermal destruction therapies, hepatic blood flow transports heat away from the probe resulting in a smaller lesions, known as the 'heat sink' effect. For this reason, vascular clamping is advised by many authors, as it reduces dissipation of the generated heat, providing increased destruction volumes and greater tumor free margins.[56,83-85]
A major disadvantage of temporary vascular clamping is ischemia and subsequent reperfusion injury to the remaining liver parenchyma.[86] The local events after ischemia/reperfusion (I/R) that induce liver tissue damage are complex, but can grossly be divided in two distinct phases. In the acute phase, oxygen radicals, proteases and inflammatory

cytokines are generated shortly after reperfusion and contribute to early hepatocellular damage.[87] The late phase is characterized by an imbalance of vasoconstrictors (e.g. endothelin-1) and vasodilators (e.g. nitric oxide), causing microcirculatory disturbances and prolonged tissue hypoxia.[88-91] Moreover, the accumulation of neutrophils induces delayed perfusion failure by plugging of the hepatic sinusoids, which further aggravates the ischemic damage and finally results in microscopic tissue necrosis.[92] Consequently, I/R may contribute to postoperative liver dysfunction and morbidity. Several therapeutic strategies have been successfully developed to prevent liver tissue damage following I/R.[93,94]

The degree of ischemic injury largely depends on the type and duration of vascular occlusion and is influenced by several (patho)physiological parameters, including hemodynamic stability, body temperature, age, gender and the presence of underlying liver disease.[95-100]

Several different clamping techniques have been described, each with its advantages and disadvantages with respect to hemodynamic stability, the duration of the procedure, the amount of blood loss and the magnitude of I/R damage.[79-82] Although the vascular clamping technique used during hepatic surgery depends on the individual surgeon's judgment and preference, good knowledge of all the benefits and drawbacks of the different techniques available is a prerequisite for appropriate individualized application of vascular clamping during hepatic resection and thermal ablation.

Vascular clamping techniques

Portal triad clamping, i.e. the Pringle Maneuver, is the oldest and the simplest technique first described in 1909 by James Hogarth Pringle.[101] The Pringle Maneuver results in complete arterial and portal inflow occlusion, leaving back flow from the hepatic veins intact. For more complex major liver resections, inflow occlusion may be combined with occlusion of the supra and infrahepatic inferior caval vein, resulting in total vascular occlusion.[102,103] When caval flow is preserved, inflow occlusion combined with selective control of major hepatic veins results in selective vascular exclusion. In addition, selective hemihepatic or segmental vascular occlusion techniques have been successfully developed to minimize ischemic damage to the contralateral lobe. Selective clamping of the portal, arterial or venous flow has also been described. Portal clamping may be advantageous in thermal destruction techniques, as it provides an increase in lesion size, but minimizes ischemic injury.[83,85,104,105] Finally, as a result of several advances in parenchymal transection devices, improved visualization of hepatic vascularization by intra-operative ultra-sonography, and the maintenance of low central venous pressure, major liver resection may even be performed without vascular clamping.

Vascular clamping may be applied either continuously or intermittently. Intermittent clamping allows the liver parenchyma to be reperfused shortly in-between clamping periods, which protects against ischemic damage.[106-108] Moreover, the application of a short occlusion period before prolonged vascular clamping, called ischemic preconditioning, can render liver tissue less vulnerable to a sustained ischemic insult by triggering hepatocellular defense mechanisms.[86,109,110]

Peri-operative tolerance to hepatic ischemia also correlates with the duration of vascular occlusion. In general, occlusion periods of up to 60 minutes for continuous clamping, and 120 minutes for intermittent clamping can be safely performed in normal livers, i.e. without major postoperative morbidity or mortality.[111-113]

In recent years, the adverse effects of I/R resulting from vascular clamping on hepatocellular function have been well documented. Strikingly, the influence of I/R on the outgrowth of residual colorectal micrometastases has been underexposed. We performed an extensive systematic review among more than 1500 papers, searching for all studies comparing different clamping techniques and all papers describing prognostic factors of recurrence and survival after partial liver resection for colorectal liver metastases, and found only four studies evaluating long-term outcome after vascular clamping.[114-117] The patient groups included and the clamping techniques studied were highly heterogeneous and the studies lacked a sufficiently detailed evaluation to draw any firm conclusion. Moreover, the only preclinical studies available show that I/R, when applied prior to a challenge with tumor cells, stimulates tumor cell adhesion and promotes the incidence of metastases formation.[118,119] However, these studies may only be relevant for the implantation of tumor cells that are shed into the blood circulation during surgical manipulation. It is currently unknown how I/R, such as frequently encountered during liver surgery, affects the outgrowth of pre-existent hepatic micrometastases and how this influences the time to develop (liver) recurrence and survival.

Outline and central questions of this thesis

The central theme of this thesis is surgery-induced tumor growth. In **chapter 2**, the role of angiogenesis (in response to tissue injury and hypoxia) in surgery-induced tumor growth is reviewed and its mechanistic overlap with tumor associated neovascularization is discussed. In this review, we also address the influence of antiangiogenic therapy on angiogenesis-dependent phenomena such as wound repair, healing of intestinal anastomoses and liver regeneration.
The impact of two key procedures in hepatic surgery on the outgrowth of micrometastases have been examined in detail in this work. We mainly focused on the adverse effects of I/R resulting from vascular clamping on the outgrowth of existent hepatic colorectal micrometastases (**chapters 3-8**). In addition, the effects of thermal destruction therapy on the outgrowth of perilesional micrometastastic tumor cell deposits was studied (c**hapter 9**).

The studies presented in this thesis were guided by the following research questions:

- **I** How often and to what extent are vascular clamping methods currently used by hepatic surgeons in and around Europe? (**chapter 3**)
- **II** Does I/R resulting from vascular inflow occlusion promote the outgrowth of residual micrometastases in the liver and how does this affect prognosis? (**chapters 4 and 5**)
- **III** How does ischemia time affect the outgrowth of micrometastases after I/R? (**chapter 6**)
- **IV** Are the adverse effects of vascular clamping on tumor growth influenced by age, gender and hepatic steatosis? (**chapters 5 and 6**)
- **V** What alternative clamping methods can be used to circumvent the putative adverse effects of vascular clamping on tumor growth? (**chapters 4 and 7**)
- **VI** What mechanisms contribute to the stimulated micrometastasis outgrowth after I/R and how can these be mediated by pharmacological interventions? (**chapters 4 and 8**)
- **VII** Does thermal ablation, which is associated with pathophysiological events similar to I/R, also stimulate the outgrowth of residual tumor cell deposits and how can this be inhibited? (**chapter 9**)

To study the effects of I/R on metastasis outgrowth a standardized murine model of partial hepatic I/R with pre-established colorectal micrometastases was developed (**chapters 4,6-8**). As hypotension, systemic anoxia and hypothermia all may affect I/R damage, special attention was paid to anesthetic management, hemodynamic stability and body temperature in this model. Similarly, two animal models with established colorectal micrometastases were used to study the effect of radiofrequency ablation and laser-induced thermotherapy on the outgrowth of tumor cell clusters at the lesion periphery. Finally, the preclinical studies were strengthened by a descriptive survey (**chapter 3**) and a retrospective patient analysis (**chapter 5**).

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Chapter 2

Surgery and angiogenesis

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Abstract

Surgery may be regarded as an angiogenesis-inducing condition since it evokes the release of many angiogenic factors. Regarding the mechanistic overlap between tumor-associated neovascularization and (physiological) angiogenesis in response to injury and hypoxia, surgery may promote the uncontrolled growth of residual dormant tumor cells. With the advent of anti-angiogenic agents, surgeons will be faced with more patients undergoing surgery for primary and secondary tumors under anti-angiogenic treatment. This could present problems with regard to angiogenesis-dependent phenomena such as wound repair, healing of intestinal anastomoses and liver regeneration. In this review we will discuss these matters from a biomedical and clinical point of view.

Introduction

Although research on angiogenesis has particularly evolved around its pivotal role in tumor propagation, it should presently be considered equally important in physiological processes involving tissue repair, such as wound healing, liver regeneration, and the female reproductive cycle.[1,2] Angiogenesis is a complex, multi-step process involving a multitude of humoral and cellular regulatory components. As a consequence, a clear mechanistic distinction between tumor-induced and physiological angiogenesis cannot be made. Moreover, the majority of the rapidly increasing number of pro- (and anti-) angiogenic factors that are being discovered appear to be up-regulated regardless of the condition that stimulates their release.[3] Nonetheless, tumor-induced angiogenesis is characterized by the development of a highly tortuous, dilated and disorganized microvascular network,[3] which is associated with increased vascular leakiness,[4] influx of inflammatory cells and fibrin-deposition.[5] Furthermore, tumor-induced angiogenesis is an ongoing, uncontrolled phenomenon, as opposed to the self-limiting nature of undisturbed wound healing and regeneration. In this respect, cancer has been referred to as a never healing wound.[6] Over the last decades, questions have been raised regarding possible adverse effects of surgery on the outcome in cancer patients. Rapid acceleration of tumor growth following surgery for primary or secondary malignancy is a concern expressed by both surgeons and patients. Although speculative, some circumstantial evidence exists on this matter. As surgery inflicts wounding, tissue repair is an integral part of any surgical treatment, including that of solid tumors. For many years, a role for wound healing in favoring local tumor recurrence has been implied, based on observations in clinical and experimental studies.[7] Moreover, surgery may accelerate the growth of residual disease, not only by removal of anti-angiogenic factors produced by the primary tumor, but also by the production of pro-angiogenic factors.[8,9] These issues become all the more prominent because, in addition to surgical removal of solid primary tumors with curative intent, surgery is becoming more important in patients with disseminated disease. Despite recent advances in systemic therapies,[10,11] partial liver resection for secondary colorectal malignancies still offers the only hope for cure.[12] In addition, local tumor destruction methods, in which the tumor is heat-destructed by laser-light or radiofrequency waves, are increasingly performed in patients with irresectable colorectal liver metastases.[13,14] Similarly, metastases confined to the lung are also treated more aggressively by surgical removal.[15-17] Still, the outgrowth of occult micrometastases often present in the remnant liver or lung will eventually lead to recurrence and death in the vast majority of patients. In addition, during oncologic surgical procedures (temporary), vascular clamping is routinely employed in order to safely and effectively resect malignant tumors. Such vascular inflow occlusion is accompanied by tissue hypoxia, which may, in turn, contribute to the induction of angiogenesis. Such potentially adverse aspects of oncologic surgery through enhancement of the many factors involved in angiogenesis are addressed here. Clearly, the discovery of the dependency of tumor outgrowth on angiogenesis has created an avalanche of novel antitumor agents, whose common denominator is their anti-angiogenic mode of action. It is well established that, under normal circumstances, less than 0.01% of endothelial cells are in S-phase at any given time, while all others are at rest.[18] Based on the assumption that suppression of tumor-induced endothelial cell proliferation does not affect resting endothelium, serious adverse effects of anti-angiogenic treatment were not anticipated.[19] Nevertheless, it seems likely that repair processes following surgery, involving "physiological" angiogenesis, may be hampered at least in part by such treatment. As so many new anti-angiogenic compounds

emerge, the relevance of this issue becomes more and more obvious. However, experimental studies on the subject have thus far produced contradictory results, which has led us to investigate these phenomena in somewhat greater detail. This work is discussed in the latter part of this review.

Surgery as an angiogenic factor

The presumed deleterious effects of surgery may adversely affect the prognosis of cancer patients either by favoring the implantation, survival and outgrowth of tumor cells that are released through surgical manipulation, or by promoting the growth of preexistent micrometastases. Surgical wounds and gastrointestinal anastomoses have been reported to be privileged sites for metastases.[20-23] Reports about the development of port-site metastases have raised questions of general importance about the application and safety of laparoscopy in oncological surgery.[24-27] Many animal studies are available showing increased implantation and growth at the site of injury following injection of tumor cells immediately after surgery.[28-33] As angiogenesis is an integral part of tissue repair, it has been implied to play a role in such unfavorable side effects of oncological surgery.[7] Concomitant with the inflammatory response provoked by wound healing, increased angiogenic stimuli in the postoperative period provide the formation of a perfect microenvironment, allowing newly seeded microscopic tumor deposits to develop.

In addition, patients undergoing surgery for primary or secondary tumors are likely to harbor residual disease.[34] Surgery is believed to enhance the outgrowth of these micrometastases. Interestingly, one of the strongest indications that surgery induces distant tumor growth is offered by the discovery of angiostatin and endostatin, both endogenous anti-angiogenic factors produced by the primary tumor.[35,36] These factors inhibit remote micrometastases from growing by suppressing the formation of their vascular supply. Their discovery was based on the observation that, following the removal of a primary tumor, metastases appeared to develop new vessels and displayed an increased growth rate. These communications were, in fact, among the first to provide evidence that tumors are angiogenesis-dependent. By treating tumor-bearing mice with angiostatin or endostatin, a state of dormancy is attained whereby tumor cell deposits remain viable, but do not progress.[37,38] Accelerated metastatic growth following the removal of the primary tumor has been observed in patients after nephrectomy for renal cell carcinoma and after excision of primary melanomas and breast cancer.[39-41]

Not only the removal of the source of anti-angiogenic factors may lead to subsequent growth of previously dormant micrometastases; the enhancement of angiogenesis by surgery is at least equally important. The angiogenic switch of microscopic tumor nodules to change from a non-vascularized to a vascularized phenotype occurs when a local imbalance arises between angiogenic and anti-angiogenic growth factors, in favor of the pro-angiogenic factors.[9] Considering the fact that many of the locally produced factors are released into the systemic circulation, surgery may modulate the transition of quiescent tumor cells into rapidly growing ones.[8] Clinical evidence to substantiate these phenomena is no more than correlative, because human studies usually lack a non-operated control group. A study comparing different surgical treatments for colon tumors revealed increased recurrence and decreased survival rates in patients undergoing staged resection as compared to one single operation[42]. Already in 1959, Fisher and Fisher provided experimental evidence of increased growth of dormant micrometastases.[43] When rats were injected with as few as 50 tumor cells and then examined five months later for hepatic

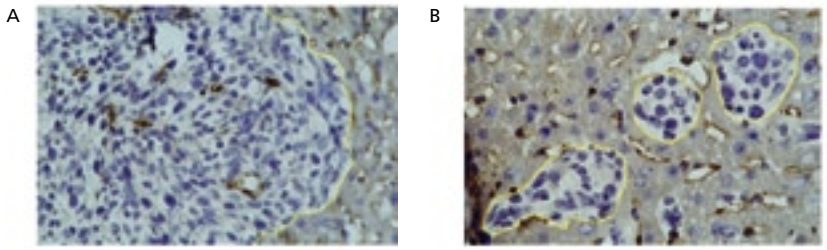

Figure 1. Accelerated metastatic outgrowth in regenerating liver. Histological sections of murine livers, showing microscopic appearance of hepatic metastases 4 days after intrasplenic injection of colorectal tumor cells. In regenerating liver following 70% partial hepatectomy (**A**), tumor area (circumscribed area) is significantly larger in comparison with control nonregenerating liver (**B**).[50]

tumor growth, none was evident. However, when rats were subjected to sequential laparotomies with simple liver manipulation, all animals had intrahepatic tumor within a few weeks, irrespective of the duration of the period between injection and surgery. Similarly, several studies demonstrated increased growth of previously injected tumor cells in animals following multiple exploratory laparotomies when compared to non-operated animals.[44,45]

Likewise, an angiogenic response appears to be involved in the accelerated outgrowth of residual microscopic tumor deposits in the liver remnant following major partial liver resections for hepatic tumors. This has particularly been proven in models of colorectal cancer metastatic to the liver (**Figure 1**).[46-50] Clinical series have shown that only 25–40% of patients treated with surgical resection of colorectal metastases are alive after 5 years, indicating growth of residual disease in those patients.[51] Many factors that are produced following major hepatic resection, including pro-angiogenic factors, elicit proliferative responses along a variety of auto- and paracrine pathways. In this respect, surgery may kickstart micrometastases to uncontrolled growth at both local and distant sites.

Growth factors in surgery-induced angiogenesis

Surgery promotes a cascade of local and systemic reactions, all leading to repair of injured structures. Irrespective of the wounded tissue type, the continuous process of wound healing can be divided into three phases: inflammation, proliferation and maturation. Neovascularization is an important byproduct of the inflammatory responses in the vascular bed. Endothelial cells play an important role in the proliferative phase as they contribute to angiogenesis. The formation of granulation tissue in secondary wound healing, as opposed to primary wound healing in sutured wounds, involves the migration of fibroblasts and the formation of new vessels to bridge the cutaneous defect.[52] Of the multiple inflammatory and growth factors induced by injury, many are known to have angiogenic effects, and are thought to be a prerequisite for tumor cells to progress into local tumor recurrence (**Table 1**).[8,53-56]

In analogy with wound repair, liver regeneration following major hepatic resection is accompanied by a similar peak in local growth factor production, resulting in the regenerative outgrowth of the liver remnant until the full original liver weight has been regained.[57,58] Again, many of the growth factors up-regulated in the regenerating liver are known for their angiogenic properties in vivo.[59,60] For instance, vascular endothelial growth factor (VEGF) is up-regulated after partial hepatectomy, and is thought to improve sinusoid reconstruction during the regeneration process.[61] Hepatocyte growth factor

Table 1. Factors involved in physiological and tumor angiogenesis

Fibroblast growth factors (FGF)
Vascular endothelial growth factor (VEGF)
Transforming growth factors (TGF-α & -β)
Platelet-derived endothelial cell growth factor (Pd-ECGF)
Interleukins (Il-1, 6 & 8)
Tumor necrosis factor (TNF-α)
Hepatocyte growth factor (HGF/SF)
Histamine
Granulocyte macrophage colony stimulating factor (GM-CSF)
Substance P

Many pro-angiogenic factors that are produced following injury are known for their angiogenic properties in cancer biology.[8,53-56]

(HGF) is dramatically increased following partial hepatectomy,[62,63] but is also a potent angiogenic factor in vivo and stimulates endothelial cell protease production, motility, proliferation, and differentiation in vitro.[64,65] Studies in mice deficient for modulators of the plasmin system show that plasminogen is essential for optimal revascularization after surgery-induced liver regeneration.[66,67] Corresponding to the increase in pro-angiogenic factors, we found increased microvessel density in the liver remnant following 70% hepatectomy in mice.[68] Studies using in vivo microscopy showed changes in vessel length and hepatic cell plate within the early regenerating liver,[69] pointing towards an important role of angiogenesis in liver regeneration.[68]

As mentioned above, the release of pro-angiogenic factors into the circulation can promote the development of distant preexisting micrometastases. Circumstantial evidence is available showing a correlation between surgery-induced angiogenic growth factor release and accelerated tumor growth. It has been demonstrated in recent studies that serum VEGF levels in patients are markedly elevated during the first postoperative days, predominantly after major surgery,[70-73] for instance after resection of lung metastases.[74] Increased tumor load and histological microvessel infiltration after exogenous administration of VEGF, and its suppression by an angiogenesis inhibitor, has provided firm evidence of the importance of circulating VEGF in the postoperative outgrowth of dormant pulmonary micrometastases.[74]

Surgery-induced hypoxia and angiogenesis

Oxygen tension has been shown to be a regulator of local growth factor production during tissue repair. VEGF-induced angiogenesis in response to tissue hypoxia has been convincingly shown to be increased in cardiac and cerebral ischemia,[75] and during the healing of ischemic wounds.[76] In addition, recent major advances have provided insight into the role of hypoxia in cancer biology.[77] Hypoxia inducible factor-1alpha (HIF-1α), a transcriptional factor that is stabilized and transported to the nucleus under hypoxic circumstances,[78] is involved in cancer progression in different organs.[79-82] One of the most important downstream effects of HIF-1α activation is gene transcription of VEGF, which is regarded as the principal angiogenic factor under ischemic and hypoxic conditions.[83]

Clinical and experimental studies from our group support the concept that surgery-induced hypoxic stress unfavorably affects the prognosis of cancer patients by inducing angiogenesis. Temporary occlusion of the inflow of the liver is applied worldwide during liver surgery to reduce perioperative blood loss during liver resections,[84,85] and to increase destruction volume by local ablative techniques.[86-90] We found that vascular clamping during hepatic surgery for colorectal liver metastases increased intrahepatic HIF-1α and serum VEGF levels (**Figure 2A**).[91] In a retrospective analysis, vascular clamping appeared to unfavorably affect the disease-related prognosis of cancer patients, as demonstrated by increased tumor recurrence and decreased survival. Moreover, increased outgrowth of experimental colorectal liver metastases was observed as a result of clamping the liver's blood supply (**Figure 2B**) (unpublished data). These putative adverse effects of surgery induced hypoxia on tumor growth need substantiating in large clinical trials.

Another inevitable phenomenon provoked by surgery is hemorrhage, especially in major surgery, leading to anemia and decreased oxygen delivery to organs. The prognostic impact of decreased hemoglobin levels on the survival of cancer patients has been well established, and has been related to hypoxia and angiogenesis.[92-94]

Despite the rather suggestive nature of the reports on adverse effects of surgery on the growth of residual tumor cells, these effects might theoretically be overcome by perioperative use of anti-angiogenic agents. Angiostatin has been shown to effectively prevent the development of metachronous liver metastases in an animal model of colorectal cancer when administered as adjuvant therapy after curative resection.[95] Endostatin has been found to inhibit the seeding of tumor cells.[37]

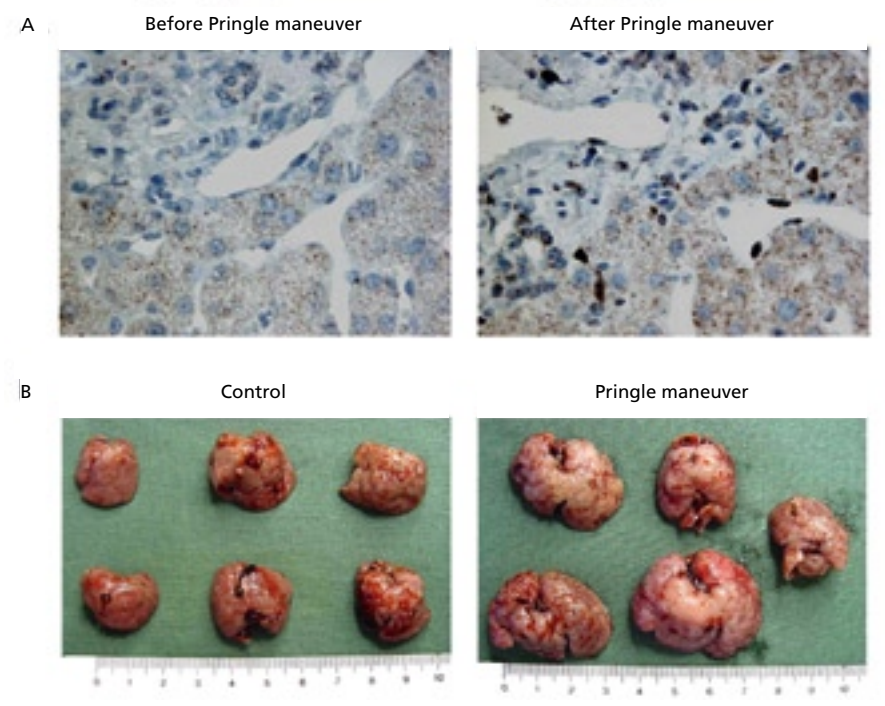

Figure 2. Effects of vascular clamping on intrahepatic HIF-1α and tumor load. **(A)** Sections of liver biopsies in patients undergoing liver surgery for colorectal metastases before and after temporary vascular clamping of the liver's inflow (Pringle maneuver). Immunohistochemically, HIF-1α was not detectable before Pringle maneuver. Following 20 min of vascular clamping and 5 min of reperfusion, HIF-1α was clearly overexpressed (shown by the brown staining) in the nuclei of hepatocytes, endothelial cells and Kupffer cells as a response to tissue hypoxia.[91] **(B)** Effects of Pringle maneuver on experimental hepatic tumor growth as shown by macroscopical appearance. In mice that underwent Pringle maneuver on day 5 following intrasplenic tumor injection, a significant increase in tumor load was observed as compared to control mice.

Surgery and anti-angiogenic treatment: should the surgeon worry?

Inhibition of tumor-associated angiogenesis has become one of the most promising novel developments in cancer treatment, which has resulted in the evaluation of a large number of angiogenesis inhibitors in clinical trials.[96-98] Ultimately, the clinical application of anti-angiogenic agents will most likely involve long-term, perioperative and neo-adjuvant treatment schedules to sustain tumor dormancy.[97,99] Consequently, surgeons will encounter more patients undergoing surgery for primary or metastatic disease during anti-angiogenic therapy, including its side-effects, e.g. thrombo-embolic events. Based on the mechanistic similarities of physiological and pathological angiogenesis, patients under surgery might be subjected to problems with angiogenesis-dependent processes like wound healing, anastomotic healing and liver regeneration, thereby increasing the risk of postoperative complications.

Thrombo-embolic side effects

Although coagulopathy in patients with malignant tumors is well recognized, the surgeon should be extra aware of postoperative thrombo-embolic events and pulmonary embolism in cancer patients under anti-angiogenic treatment. Phase I studies regarding a number of anti-angiogenic drugs, including PNU-145156E, Thalidomide and SU5416 when combined with cytotoxic agents, have reported thrombo-embolic side effects.[100-102] The mechanism by which anti-angiogenic agents act as procoagulants is unclear. Nonetheless, several hypotheses have been put forward. First, many angiogenic growth factors promote the synthesis of various clotting factors. Inhibition of those angiogenic growth factors, for instance by the VEGF receptor blocker SU5416, may cause an imbalance in coagulatory factors eventually leading to thrombo-embolic events.[103] Second, various angiogenesis inhibitors are modulated coagulatory factors that exhibit anti-angiogenic activity after transformation. For instance, after removal of the carboxyl-terminal loop responsible for its anticoagulant activity, anti-thrombin acts as an angiogenesis inhibitor, additionally resulting in impaired inactivation of thrombin.[104] Also, angiostatin is a plasminogen-derived anti-angiogenic agent.[35] Despite their origin, no thrombo-embolic side effects in the latter agents have thus far been reported. Close monitoring of the coagulation profile in patients under anti-angiogenic treatment is warranted.

Cutaneous wound healing

A variety of anti-angiogenic agents, shown to effectively inhibit tumor angiogenesis, have been evaluated in animal studies on physiological angiogenesis in cutaneous wound healing. Studies concerning the effects of TNP-470, an angio-suppressive agent derived from fumagillin, have been contradictory. TNP-470 markedly delayed wound closure as measured by macroscopic wound area and histologic appearance in mice,[105] whereas it did not impair wound healing in rabbits.[106] The vascularity in murine cutaneous wounds markedly decreased in several studies investigating anti-angiogenic agents like vasostatin,[107] Thrombospondin-1,[108] and VEGF-receptor kinase inhibitors.[109,110] Despite a reduced microvessel density in these wounds, no impairment of wound healing was observed.[107,108,111] Likewise, endostatin was shown to have no adverse effects on wound breaking strength in both low-dose and high-dose treatment schedules.[112,113] And although endostatin did not decrease vascular density in cutaneous wounds, severe blood vessel abnormalities were found following endostatin treatment, implying impaired blood vessel maturation.[113] Anti-angiogenic agents affecting the formation of the extracellular matrix like matrix metalloproteinase inhibitors do not impair cutaneous wound healing, but even

Table 2. Anastomotic healing under angiostatin treatment

	Control	Angiostatin
n	13	13
Mortality	0	1
Weight loss % (mean ± sem)	3.6 ± 1.0	8.3 ± 2.0
Paralytical ileus	0	5
Bacterial growth	0	3
Local peritonitis	0	6 *
Diameter distal colon (2 cm from anastomosis)	3.1 ± 0.1	4.7 ± 0.3 *
Adhesions	1.1 ± 0.1	3.3 ± 0.3 *
Bursting pressure (mm Hg)	175 ± 12	135 ± 20 *
Newly formed vessels	16.0 ± 2.0	6,6 ± 1.0 *

Clinical scores regarding anastomotic healing in mice. Angiostatin was administered from the time of operation until termination of the experiment (day 7 after surgery). Angiostatin treatment impaired colonic wound healing. *p<0.05

enhance wound strength.[114,115] In conclusion, anti-angiogenic agents have been shown to affect elements of physiological processes in wound healing similar to their therapeutic effect on tumor angiogenesis. Although wound healing appears not to be impaired in most animal models, the effects of anti-angiogenic therapy on wound healing in humans should be further investigated. As wound healing is a complex process involving many steps, one might expect that anti-angiogenic agents will delay, rather than entirely block, wound healing.

Healing of intestinal anastomoses

A form of wound healing that can be life-threatening if impaired is the healing of intestinal anastomoses, as anastomotic leakage in the week following colonic surgery is a disastrous event leading to high morbidity and even mortality.[116] In contrast to the healing of cutaneous wounds, the healing of colonic anastomoses is considered more dependent on angiogenesis and less on diffusion of oxygen through pre-existing vasculature.[52,117] Studies regarding the effects of anti-angiogenic strategies on intestinal anastomotic healing have provided contradictory results. Data concerning short-term administration of TNP-470 and suramin have suggested adverse effects during the early phase of anastomotic healing.[118,119] However, recent data appeared to indicate that tamoxifen does not adversely affect gut anastomotic healing in the rat.[120] Similar to wound healing, administration of matrix metalloproteinases enhanced, rather than impaired, anastomotic strength.[114,121-123] Work by te Velde et al. has demonstrated angiostatin to adversely affect healing of colon anastomoses in mice when administered continuously during the first postoperative week (**Table 2**).[124] Histological examination revealed a decreased number of newly formed vessels in the granulation tissue around the anastomosis. However, upon discontinuation of anti-angiogenic therapy, normal anastomotic healing was promptly restored. These results in mice may lead one to speculate that surgical patients who are treated with angiostatin can be safely operated on as soon as the treatment is discontinued. Assuming anti-angiogenic treatment is used perioperatively in patients undergoing colon resection, it should preferably be discontinued as briefly as possible, in an attempt to keep any

metastatic tumor cell deposits in a state of dormancy. In animal models the strength of the colonic wall was restored approximately one week following anastomosis.[124,125] Nevertheless, in individual patients sub-clinical leakage can occur as late as two to three weeks after surgery. In daily practice, the surgeon will decide based on the postoperative clinical course when the anastomosis may be considered healed and administration of the anti-angiogenic agents can be safely resumed.

Liver regeneration

As described above, liver regeneration is a tissue repair response following the loss of hepatic tissue, enabling the restoration of lost liver mass within days.[126] Based on animal studies, it has been concluded that liver regeneration is an angiogenesis-associated phenomenon.[68] Thus, application of anti-angiogenic medicine after partial hepatectomy may impair liver regeneration, with subsequent increased risk of postoperative liver failure. Evidence regarding this matter is scarce. We found in a mouse model of 70% partial hepatectomy that angiostatin inhibited liver regeneration by 60%, a phenomenon that was sustained until 14 days after surgery.[50] TNP-470 was shown to exhibit comparable inhibitory effects on murine liver regeneration in a recent study.[127] On the contrary, in a study in rabbits, TNP-470 inhibited the formation of liver metastases after partial hepatectomy without impairing liver regeneration.[106]

To confirm the relevance of the data generated in pre-clinical studies, all matters described above should obviously be investigated in large clinical trials in the near future. Sufficient knowledge of the mechanistic background of anti-angiogenic drugs and appropriate timing of administration are required to improve antitumor efficacy of (disseminated) cancer in a safe combination with surgery.

Conclusions

A certain overlap exists between physiological angiogenesis (including wound healing, liver regeneration and response to surgery-induced hypoxia) and tumor angiogenesis. Surgery may be regarded as an angiogenic factor since it has been shown to evoke the release of many angiogenic factors along with an increase in growth of residual dormant tumor cells. When given in the perioperative phase, anti-angiogenic agents in the treatment of cancer may have deleterious effects on phenomena that involve physiological angiogenesis, such as wound healing. Until the results of large clinical trials become available, such treatment should be administered with caution in the perioperative period.

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Chapter 3

European survey on the application of vascular occlusion in liver surgery

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Abstract

Background This study evaluated the frequency, the indications and techniques of vascular clamping during liver resection and during thermal destruction therapies, as currently used by hepatic surgeons throughout Europe.
Methods A web-based questionnaire was distributed among 621 physicians, including all members of the European Hepato-Pancreato-Biliary Association and the European Surgical Association.
Results The overall response rate was 50%. During liver resection vascular clamping is never applied by 10%, on indication by 71%, and routinely by 19%. Routine clamping is particularly performed by high volume and senior surgeons and appears to be associated with longer ischemia times. Intermittent inflow occlusion is the clamping method of choice for more than 65% of surgeons and total ischemia times are usually limited to 15-30 minutes. During thermal ablation vascular clamping is never used by 57%, on indication by 37% and routinely by 7%; it is particularly applied for large tumors and for tumors close to large vessels, and ischemia times are shorter.
Conclusion Vascular clamping during liver resection is frequently used; during thermal ablation it is preserved for larger tumors or tumors in the vicinity of large vessels. Complete inflow occlusion is the most frequently used technique, with a distinct preference for intermittent clamping.

Introduction

Surgical removal is the treatment of choice for many primary and secondary liver tumors, providing a potentially curative treatment.[1-3] During hepatectomy intra-operative hemorrhage is common, often necessitating blood transfusion, which is associated with unfavorable short and longterm postoperative outcome.[4-6] Therefore, approaches to control intra-operative bleeding are warranted and include vascular clamping methods. For nonresectable liver tumors thermal destruction techniques, such as radiofrequency ablation, laser-induced thermotherapy and microwave therapy, provide local tumor control and improve life-expectancy.[7-9] During thermal ablation, vascular clamping is advised to reduce dissipation of the generated heat, which creates larger destruction volumes, resulting in greater tumor free margins.[10-12]
The disadvantage of vascular clamping is ischemia/reperfusion injury to the remaining liver which may contribute to postoperative liver dysfunction and morbidity.[13] In addition, ischemic damage resulting from prolonged vascular inflow occlusion may adversely affect oncological outcome by accelerating the outgrowth of colorectal micrometastases.[14] In the background of these previously unrecognised adverse effects of vascular clamping on outcome, it is of great importance to know how often and to what extent vascular clamping methods are currently used in daily practice.
Several different clamping techniques have been described, each with its own advantages and disadvantages with respect to hemodynamic stability, the duration of the procedure, blood loss, the degree of ischemia/reperfusion damage and tumor growth stimulation.[14-23] However, the application of vascular clamping in daily practice during partial liver resection and thermal destruction therapy depends on the individual surgeon's judgement and preference. The aim of this survey was to gain insight into the frequency, the techniques and the indications for vascular clamping, as used by surgeons throughout Europe.

Materials and Methods

Invitations to participate in the survey were sent to 429 active members of the European Hepato-Pancreato-Biliary Association (EHPBA) and to 202 members of the European Surgical Association (ESA). In addition, the questionnaire was forwarded to 28 Dutch liver surgeons known from personal networks, including all members of the Dutch Liver Surgery Working Group. Participants also had the possibility to invite colleagues in the field to participate in the survey, which occurred in 15 cases. Due to 53 overlapping memberships, a total of 621 invitations were sent across 39 countries in and around Europe. The questionnaire was available online and could be entered with a username and password that was provided by e-mail. After four weeks a reminder was sent to all nonresponders and another six weeks later, a final reminder was send by postal mail. The survey was closed on September 1st 2006.

As the application of vascular clamping depends on the surgeon's individual preference, each surgeon was asked to fill out the questionnaire separately. The questionnaire consisted of four parts: I) demographic data, II) vascular clamping during liver resection, III) vascular clamping during thermal ablation and IV) suggestions and comments. For both liver resection and for thermal ablation the personal case volume per year was asked as well as the frequency, indications and contra-indications for vascular clamping. Participants were asked what clamping technique was used with regard to the extent, the technique and total ischemia times. For most questions a 4-points scale 'never-sometimes-usually-always' was used. Part II also included questions on transection techniques and additional measures to control intra and postoperative blood loss. Part III included questions on the type of ablative technique as well as the approach. Answers from returned questionnaires were extracted from the online database and were evaluated on a personal basis. Subanalysis was performed per country including countries with more than ten responders to search for any geographical preferences. Moreover, subanalyses were performed according to the personal case volume per year, per function and per type of hospital.

Statistical analysis was performed when appropriate. Pearson's Chi-Square test was used for frequency analysis in 2x2 tables and the Kendal correlation for tables with ordinal variables. Mann-Whitney U and Kruskal-Wallis statistics were used for analysis of nonparametric data. A P-value of less then 0.05 was considered statistically significant.

Results

Part I. Response rates

We received 311 responses from 31 countries, yielding an overall response rate of 50%. The geographic distribution and response rates per membership are shown in **Table 1**. Of all responders, 39 indicated not to actively practice hepatic surgery. Of the remaining, 269 completed part II and 227 completed part III, corresponding to specific response rates of 43% and 37% respectively.

Table 1. Response rates per country and membership

Country*	Invited	Participated	Response rate
The Netherlands	64	52	81%
United Kingdom	100	41	41%
Italy	70	37	53%
Germany	66	34	52%
France	43	18	42%
Greece	38	16	42%
Sweden	20	12	60%
Norway	24	12	50%
Switzerland	19	11	58%
Belgium	15	9	60%
Spain	18	9	50%
Poland	14	7	50%
Turkey	16	6	38%
Denmark	10	5	50%
Austria	13	5	38%
Czech republic	6	5	83%
Membership			
Multiple membership	51	40	74%
EHPBA members	429	239	56%
ESA members	202	73	36%
Dutch non-members	28	23	92%
Referred	15	14	93%
Overall	621	311	50%

*Countries with less than 4 responders include: Portugal (4), South Africa (4), Israel (4), Russia (4), Slovenia (3), Lithuania (3), Lebanon (2), Finland (1), Egypt (1), Ireland (1), Romania (1), Cyprus (1), Tunisia (1), Slovak Republic (1), Luxembourg (1).

Part II. Vascular clamping during liver resection

Frequencies

Personal case volumes per year, function and type of hospital of all 269 responders practicing liver resection are shown in **Table 2**. Vascular clamping during liver resection is never applied by 10%, on indication by 71% and routinely by 19% of surgeons. Interestingly, routine clamping appears to be more frequently applied by surgeons with a high personal case volume per year (p=0.033) and senior surgeons (p=0.089) (**Figures 1A and B**). Based on the minimum and maximum case volumes for each individual surgeon, it can be calculated that an estimated 24% of all patients are clamped routinely each year. Furthermore, routine clamping is more common in Norway and France, whereas 18% of respondents from the United Kingdom never clamp during hepatectomy (**Figure 1C**). The clamping preferences of surgeons from university hospitals were similar to those from local hospitals.

Indications and contra-indications

The prime indication for vascular clamping used by 69% of surgeons is excessive blood loss (**Figure 2A**). The median cut-off point for applying vascular clamping is 500 ml, but ranges from 100 to 5000 ml. Other common indications for vascular clamping include: major hepatectomy of a median of 3 (range 1-6) segments, nonanatomical resections or proximity to large vessels or bile ducts (**Figure 2A**). Rare indications (3%) include central hepatectomy, segmental resection, anatomical variations, clinical trials, chemo-perfusion, Jehovah's witness, cirrhosis, very precise dissection, "depends on localization or individual situation" and hepatic trauma.

Table 2. Personal case volumes, function and type of hospital of responders performing liver resection and local ablation techniques

	Liver resection		Local ablation	
	#	(%)	#	(%)
Personal case volume/year				
< 10	38	(14)	88	(39)
10-25	106	(39)	87	(38)
25-50	81	(31)	34	(15)
50-100	36	(13)	14	(6)
>100	8	(3)	4	(2)
Function				
Senior	220	(82)	184	(81)
Regular/fellow	36	(13)	31	(14)
Other/unknown	13	(5)	12	(5)
Type of hospital				
University hospital	214	(79)	178	(78)
Regional/local hospital	50	(19)	45	(20)
Other/unknown	5	(2)	4	(2)
Overall	269	(43)	227	(36)

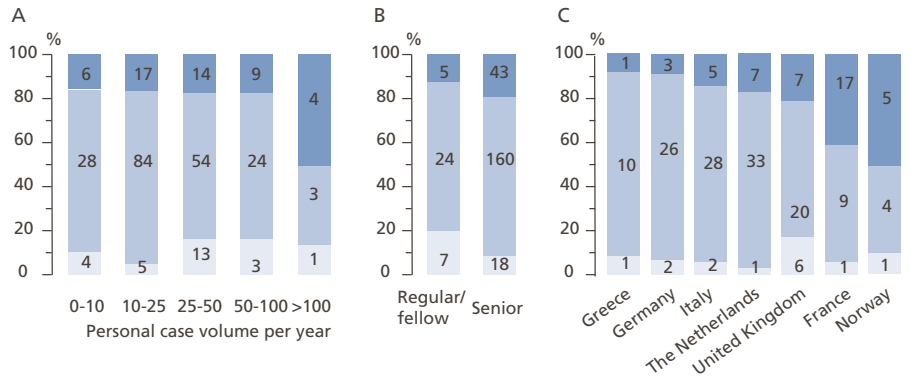

Figure 1. Application of vascular clamping during liver resection according to **(A)** personal case volume per year, **(B)** function and **(C)** country (■ routinely; ■ on indication; ■ never).

We also searched for contra-indications by asking "would you consider applying vascular occlusion in cirrhotic, steatotic and post-chemotherapy livers". All three circumstances appear to be relative contra-indications as 72-76% of surgeons would never or only sometimes apply vascular clamping in these situations. For surgeons who apply vascular clamping on a routine basis, these situations are regarded as contra-indications less frequently; vascular clamping would never or sometimes be applied in cirrhotic livers by 46%, in steatotic livers by 22% and in post-chemotherapy livers by 24% of surgeons (p<0.01). Other mentioned contra-indications are cholestasis and a likely poor functional reserve.

Technique (extent, type and duration)

Complete inflow occlusion (i.e. the Pringle maneuver) is the most frequently applied method followed by hemihepatic inflow occlusion (**Figure 2B**). The more selective clamping techniques such as segmental inflow occlusion, selective clamping of the portal vein or hepatic artery, total vascular exclusion and selective vascular exclusion (with preservation of the caval vein) are less commonly used (**Figure 2B**). Interestingly, the Pringle maneuver is more frequently used by senior surgeons when compared to regular/fellow surgeons (63% versus 21%, p<0.001), whereas regular/fellow surgeons use hemihepatic clamping more often (79% versus 23%, p<0.001). The vascular exclusion techniques are predominantly, but not exclusively, performed by high volume experts (sometimes, usually and always: 55% of surgeons with a case volume > 25 per year versus 35% of surgeons with a case volume of < 25 per year, p<0.01).

Intermittent clamping is the most frequently applied method (63%) with a typical clamping strategy of 2-3 cycles of 15-20 minutes ischemia and 5-10 minutes of reperfusion (**Figure 2C**). Ischemic preconditioning and continuous clamping are less commonly used (14% and 21% respectively). The distinct preference for intermittent clamping was irrespective of whether clamping was performed routinely or on indication, the personal case volume per year, function, type of hospital or country.

Ischemia times are usually limited to 15-30 minutes and clamping of more than 60 minutes is only used scarcely (**Figure 2D**). This is irrespective of whether clamping is performed intermittently, with preconditioning or continuously. Ischemia times tend to be slightly

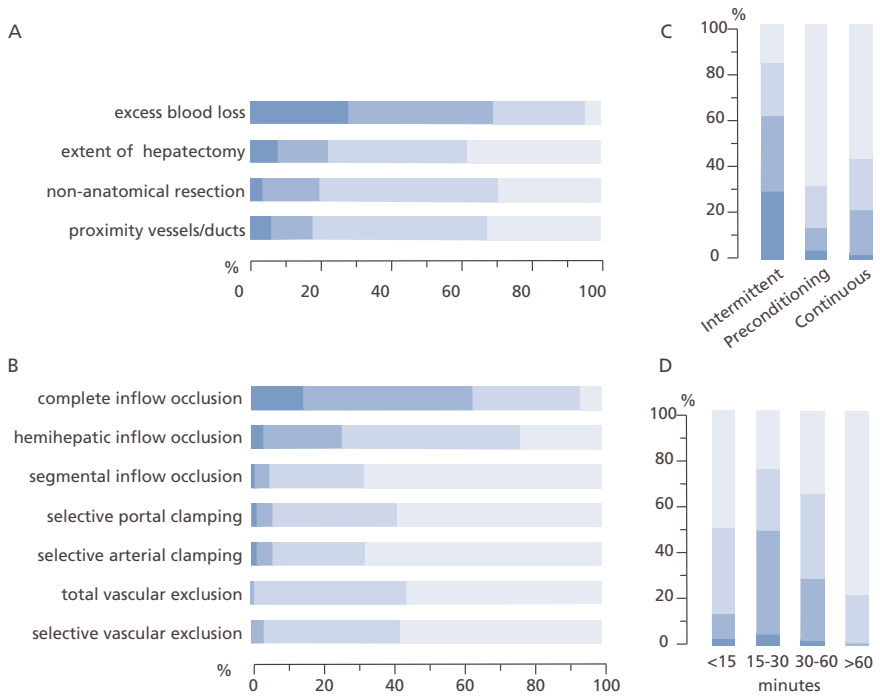

Figure 2. Indications and techniques for vascular clamping during liver resection. **(A)** Indications specified by 193 surgeons. The extent **(B)**, type **(C)** and total ischemia times **(D)** of vascular occlusion for all surgeons performing vascular clamping during liver resection, either on indication or routinely (n=243).(■always; ■usually; ■sometimes; ■never)

longer during routine clamping and by high volume experts (data not shown). With ischemia times exceeding 30 minutes in 40% of the patients who are clamped on a routine basis (by 24% of surgeons), it can be calculated that an estimated 10% of patients are routinely clamped for longer than half an hour by this cohort of surgeons every year.

Other methods to control intra-operative blood loss

Of the transection devices aimed at controlling blood loss, the CUSA is most frequently used (56%; sometimes by 16%). Precoagulation devices are usually or always used by 23% and sometimes by 34% of surgeons. The use of other transection devices, indicated by 22% of surgeons, include ultrasonic dissector, harmonic scalpel, bipolar, ligasure, diathermia, staplers, finger fracture and Kelly fracture. The use of precoagulation devices correlated to the clamping preference; 15% of surgeons who prefer precoagulation never clamp during resection versus 8% of surgeons who sometimes or never use these devices (p=0.014). Correspondingly, routine clamping is less frequently used by surgeons who prefer precoagulation (10% versus 21%).

The maintenance of a low central venous pressure is a standard procedure for the majority of surgeons (always 55%; usually 32%; sometimes 7%) and only a few never use it (6%). The application of a low central venous pressure does not correlate to the clamping preference, but is inversely related to the personal case volume per year, as it is more

frequently omitted by less experienced surgeons (p<0.01). The median accepted pressure is 5 cm H_2O, but varies from 1 to 10 cm H_2O. Additional strategies to control blood loss include: clips (71%), argon beamer (50%) and biological products, such as glues and patches (43%). Other measures (9%) involve: sutures, staplers, tissue compression, omentum and high pressure pneumoperitoneum during laparoscopic liver resection.

Part III. Vascular clamping during local ablation

Frequencies

Of the 227 responders practicing thermal destruction techniques the majority perform radiofrequency ablation (90%) through the open (44%) or percutaneous (34%) approach. Personal case volumes, function and type of hospital are shown in **Table 2**. Vascular clamping to increase lesion size during thermal ablation is never applied by 55%, on indication by 40% and routinely by 6% of surgeons. As expected, routine clamping is not applied during the percutaneous approach, unless chemo-embolization is performed. Clamping is more frequently omitted by low volume surgeons (p<0.01, **Figure 3A**) and regular/fellow surgeons (p=0.04, **Figure 3B**). It is more routinely applied in The Netherlands and France and is most frequently omitted in the United Kingdom and Greece (**Figure 3C**).

Indications and technique (extent, type, duration)

Indications for vascular clamping during local ablation include increasing size and location near large vessels or bile ducts (**Figure 4A**). The majority of surgeons who clamp during local ablation use the Pringle maneuver (46%, **Figure 4B**); other methods are used scarcely (hemihepatic 13%, segmental 3%, portal vein only 4%). In contrast to liver resection, continuous clamping is more frequently used (**Figure 4C**) and ischemia times are often within 15 minutes (**Figure 4D**). Surgeons who clamp on a routine basis use intermittent clamping (39%) more often than continuous clamping (8%), which is associated with longer ischemia times (15-30 minutes in 46%). The preferences for the clamping technique are not related to the personal case volume, function or the type of hospital.

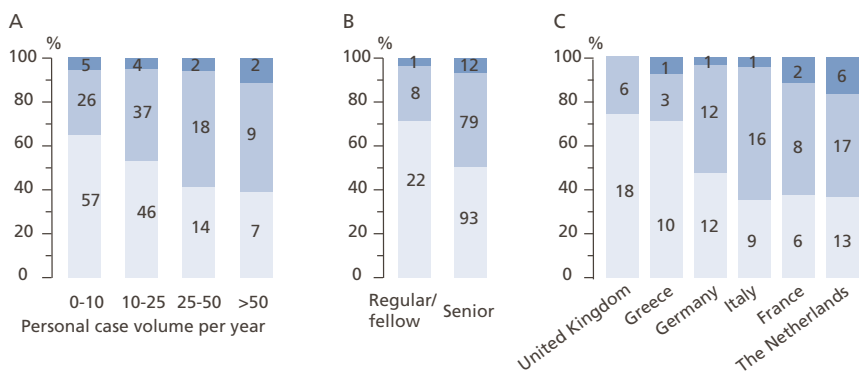

Figure 3. Application of vascular clamping during thermal ablation according to **(A)** personal case volume per year, **(B)** function and **(C)** country (■routinely; ■on indication; ■never).

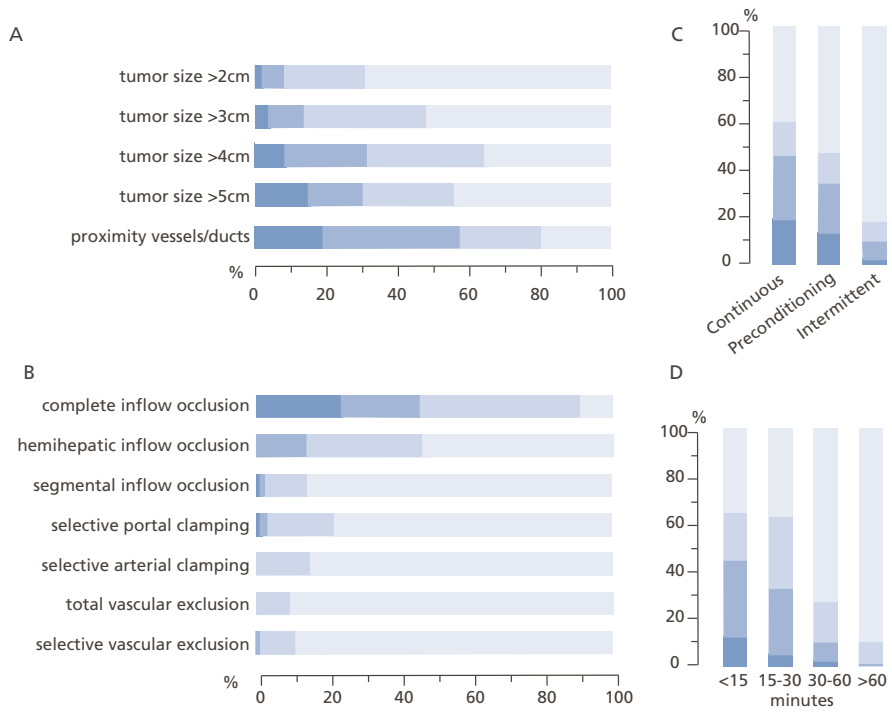

Figure 4. Indications and techniques for vascular clamping during thermal ablation. **(A)** Indications specified by 89 surgeons. The extent **(B)**, type **(C)** and total ischemia times **(D)** of vascular occlusion for all surgeons performing vascular clamping during thermal ablation, either on indication or routinely (n=102).(always; usually; sometimes; never)

Part IV. Additional commentaries

Additional remarks were made by several surgeons, which partly overlapped the results as described above. Most importantly, many surgeons underscore that there is no standard policy for clamping, but that the choice for the different techniques is highly individualised. Several surgeons only use inflow and/or outflow occlusion for atypical, difficult or central hepatectomies or for tumors involving the porta hepatis. It was also indicated by several surgeons that selective clamping techniques are preferred for diseased livers. Moreover, continuous clamping seems to be particularly used for selective clamping methods, whereas during complete inflow occlusion and vascular exclusion, intermittent clamping is preferred. Another important aspect of bloodless liver surgery indicated is the ligation of afferent and efferent vessels of the involved lobes prior to splitting of the parenchyma. Finally, the use of intra-operative ultrasound and good cooperation with expert anaesthetists is mandatory for performing bloodless hepatic surgery.

Discussion

The findings of this survey provide a comprehensive insight into the frequencies, indications and techniques of vascular clamping as currently used by hepatic surgeons throughout Europe, both during hepatic resection and thermal destruction techniques. With an overall response rate of 50%, including the majority of surgeons associated to the EHPBA and ESA, the results are likely to be representative for the global practice of particularly experienced hepatic surgeons.

The results primarily demonstrate that vascular clamping is commonly applied during liver resection: approximately one out of five surgeons clamp on a routine basis. This is consistent with a Japanese survey on control of intra-operative bleeding, showing that 25% of surgeons routinely clamp during resection.[24] Interestingly, routine clamping is most frequently applied by surgeons with a high personal case volume and by senior surgeons, which may reflect the more complex operations they perform. This is substantiated by the more frequent use of total vascular exclusion techniques and longer total ischemia times by high volume experts. Nonetheless, based on the notion that senior surgeons use a standard Pringle maneuver more often, whereas regular/fellow surgeons use more selective clamping techniques, it may also be challenged that senior surgeons apply their vascular clamping methods more habitually.

Although the decision process for the appropriate clamping technique depends on the combination of several individual patient characteristics and technical aspects, blood loss remains the prime indication for temporary blood flow occlusion. Excessive blood loss may necessitate blood transfusion, which is associated with unfavorable short and longterm postoperative outcome.[4-6] Remarkably, the maximally allowed amount of blood loss varied greatly between surgeons, representing a difference in risk-assessment, which warrants a universal recommendation. Whereas the cirrhotic liver is an indication for some responders because of a higher bleeding tendency, others find it a relative contra-indication due to concerns about decreased tolerance to ischemia.[25,26] Steatotic and post-chemotherapy livers also have increased susceptibility to ischemic damage,[17,19,27,28] which is reflected by a general reluctance to apply clamping in such livers. The clamping method of choice in post-chemotherapy livers is not yet well-defined, which is becoming even more relevant, as pathophysiologic changes in the liver after chemotherapy are described in 19-92% of patients.[28-32]

Among a variety of vascular clamping techniques, the Pringle maneuver is the most popular form of vascular occlusion, followed by hemihepatic clamping. Selective clamping techniques and the total vascular exclusion techniques are rarely used on a routine basis, but about one third of surgeons applies these techniques when necessary or appropriate. Good knowledge of all the benefits and drawbacks of the different techniques available is a prerequisite for appropriate individualized application of vascular clamping.[20-22,33]

The attention in recent years for ischemia/reperfusion injury resulting from prolonged continuous clamping, has clearly led to a distinct preference for techniques that protect the liver from ischemic damage, such as intermittent clamping or ischemic preconditioning.[17,19,34] Overall, ischemia times are usually within 30 minutes, nonetheless, we calculated that one out of ten patients are routinely clamped for longer than half an hour. Although the normal human liver seems to tolerate continuous normothermic ischemia of up to 60 minutes and intermittent ischemia of up to 120 minutes relatively well,[35,36] the upper safe limit of vascular clamping without adverse effects on oncological outcome needs to be defined.

Recent reports have indicated that major liver resection can be safely performed without vascular clamping,[37,38] and ten per cent of responders from this survey confirm this idea. The tendency to withhold from clamping may partly be the result of advances in parenchymal transaction,[39,40] including the use of precoagulation devices.[41,42] In fact, the frequent use of precoagulation correlated with less frequent use of clamping. The maintenance of a low central venous pressure did not correlate with the application of vascular clamping, but owing to the proven reduction in blood loss resulting from this procedure,[43-47] it has become a standard procedure for the vast majority of surgeons. We found a considerable variation in the maximally allowed central venous pressure, which deserves further attention. During laparoscopic hepatectomy, vascular occlusion may also be omitted more frequently[48], but this was not questioned in this survey. In general, in light of the previously unrecognized putative adverse effects of vascular clamping on oncological outcome, vascular clamping may be omitted more frequently in the future.

Given the evidence that vascular clamping during thermal destruction therapy is an essential part of the procedure for obtaining a safer margin around the ablated tumor,[49] particularly in larger tumors and tumors located near large vessels,[49,50] it is surprising that more than half of all responders never clamp during ablation. This may partly be explained by the fact that some surgeons do not treat patients with tumors larger than four centimeters. On the contrary, these numbers may actually represent a relative overestimation, because vascular clamping is seldomly used during the percutaneous approach and nowadays many patients are treated percutaneously by interventional radiologists, whom were not included in this survey. These findings emphasize the need for uniform guidelines for vascular clamping during ablative therapies. During local ablation, the Pringle maneuver is chosen by the majority of surgeons. Portal clamping, which is applied by 4% of surgeons, may be advantageous in thermal destruction techniques, as it provides an increase in lesion size, but minimizes ischemic damage.[10,23] For those who clamp routinely during local ablation, intermittent clamping is favored, corresponding to ischemia times of 15-30 minutes, whereas for the majority of surgeons who clamp on indication, a shorter continuous Pringle maneuver seems to be preferred.

In conclusion, the major findings of this survey demonstrate that vascular clamping during liver resection is commonly applied, whereas during local ablation techniques it is preserved for larger tumors or tumors in vicinity of large vessels. Among a variety of vascular clamping techniques, the Pringle maneuver is the most popular technique with a distinct preference for intermittent clamping. The finding that one out of ten patients is clamped routinely for more than 30 minutes underscores the need for further clinical investigation of protective strategies against the adverse effects of prolonged vascular clamping on longterm outcome. Variations in the maximally accepted amount of blood loss and central venous pressure demonstrate the importance of uniform recommendations for these issues. Finally, universal guidelines for vascular clamping during local ablation are needed.

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Chapter 4

Ischemia/reperfusion accelerates the outgrowth of hepatic micrometastases in a highly standardized murine model

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Abstract

Background Mortality in colorectal cancer is associated with the development of liver metastases. Surgical removal of these tumors is the only hope for cure, but recurrence is common. During liver surgery, ischemia/reperfusion (I/R) often occurs as a result of hemorrhage or vascular clamping. Although the adverse effects of I/R on postoperative liver function are well documented, the influence of I/R on the outgrowth of residual micrometastases is unknown.
Methods We used a highly standardized mouse model of partial hepatic I/R to study the effects of I/R on the outgrowth of pre-established colorectal micrometastases. Five days following intrasplenic injection of C26 colon carcinoma cells, the vascular structures of the left lobe were clamped for 45 minutes under hemodynamically stable conditions. Tissue glutathione, plasma liver enzymes, hepatocellular necrosis, and tumor growth were assessed over time.
Results I/R caused oxidative stress and early liver tissue damage. The outgrowth of micrometastases in occluded liver lobes was accelerated five to sixfold compared with nonoccluded lobes and was associated with areas of necrotic liver tissue surrounded by inflammatory cells and apoptotic hepatocytes. Accelerated tumor growth and tissue necrosis were completely prevented by occluding blood flow intermittently. In contrast, ischemic preconditioning or treatment with the antioxidants α-tocopherol or ascorbic acid failed to protect against late tissue necrosis and tumor growth, although early hepatocellular damage was largely prevented by these methods.
Conclusion I/R is a strong stimulus of recurrent intrahepatic tumor growth. Measures to prevent I/R-induced late tissue necrosis cross-protect against this phenomenon.

Introduction

The liver is the prime target organ for the development of metastases from colorectal cancer. Mortality is almost invariably attributable to complications associated with tumor growth in the liver.[1] Although surgical removal of hepatic tumors is as yet the only hope for cure, the vast majority of patients ultimately present with recurrent disease, predominantly in the liver.[2] During hepatic surgery, intra-operative hemorrhage is common, often necessitating blood transfusion, the latter being suggested as a predictor of poor postoperative and long-term outcome.[3,4] Therefore, approaches to control intra-operative bleeding are presently applied worldwide and include vascular clamping methods.[5,6] However, such measures cause hepatic ischemia/reperfusion (I/R) to the liver tissue, which may contribute to postoperative liver failure. The adverse effects of I/R on hepatocellular function have been well documented.[7,8] In contrast, the influence of I/R on intrahepatic tumor growth has remained underexposed. The few studies available show that I/R, when applied prior to a challenge with tumor cells, stimulates tumor cell adhesion and promotes the incidence of metastases formation.[9,10] These studies are likely to be relevant for the implantation of tumor cells that are shed into the blood circulation during surgical manipulation.[11] However, in the majority of patients undergoing liver surgery, microscopic tumor cell deposits are already present at the time of surgery, and their detection in the liver parenchyma is associated with early tumor recurrence and poor life expectancy.[12,13] At present, it is unknown how I/R in general, and vascular clamping in particular, affects the behavior of these micrometastases. We hypothesized that I/R, such as that frequently encountered during liver surgery, accelerates the outgrowth of preexistent hepatic micrometastases, thereby worsening prognosis. I/R-induced tissue damage may be affected by many confounding systemic parameters. Most importantly, hemodynamic stability is crucial, because hypotension and systemic hypo-oxygenation may induce temporary tissue ischemia.[14,15] In addition, hypothermia affects hemodynamic stability and also reduces I/R-induced injury.[16] Therefore, we established a highly standardized murine model of partial hepatic I/R by blood flow occlusion in which special attention was paid to anesthetic management, hemodynamic stability, and body temperature. Before studying the effects of hepatic I/R on the outgrowth of micrometastases, the model was validated by measuring parameters of oxidative stress and hepatocellular injury.
Several therapeutic strategies have been successfully developed to prevent liver tissue damage following I/R.[17-19] These include alternative clamping techniques such as ischemic preconditioning[8,20-23] and intermittent clamping[24-26] as well as pharmacological intervention with antioxidants such as α-tocopherol and ascorbic acid.[27-32] We evaluated whether those strategies cross-protect the liver against (accelerated) outgrowth of micrometastases in this model.

Materials and methods

Animals

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) and were housed under standard laboratory conditions.

Murine model of hepatic I/R

Partial hepatic I/R was induced by occluding the vascular inflow of the left lateral liver lobe for 45 minutes, corresponding to approximately 40% of the liver mass.[20] All surgical procedures were performed under inhalation anesthesia with a 1.5% to 2% isoflurane/oxygen mixture using a mask. Buprenorfine (3 µg/mouse) was administered intramuscularly before surgery to provide sufficient intra and postoperative analgesia. Surgical procedures were performed under aseptic conditions and surgical foil was placed over the laparotomy wound to avoid dehydration. Heparin was not administered. Body temperature was maintained at 36.5˚C to 37.5˚C by placing the animals on a heated table and covering them with aluminium foil. After all procedures, a small amount of saline was left in the abdominal cavity and the peritoneum and skin were separately closed with 5.0 vicryl. Sham-operated mice underwent laparotomy with exposure of the liver but without interruption of hepatic flow.

Blood pressure was measured by placing a 26-gauge catheter in the carotid artery in sham-operated mice and in mice subjected to 45 minutes of ischemia followed by 40 minutes of reperfusion. Mean arterial blood pressure was continuously measured for at least 120 minutes from the onset of anesthesia. Blood perfusion of the left liver lobe during clamping was measured via laser doppler (Oxyflo; Oxford Optronix, Oxford, UK), and tissue pO_2 analyses were performed using a fine needle probe (Oxylite; Oxford Optronix). To demonstrate local oxidative stress tissue levels of glutathione (GSH) and GSH disulfide (GSSG) were measured after 1 and 6 hours of reperfusion. Concurrently, the degree of hepatocellular injury was assessed according to plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Animals in the latter group were allowed to recover after surgery and were reanesthetized for blood withdrawal and liver harvesting following 6 hours of reperfusion.

Cell culture and induction of liver micrometastases

The murine colon carcinoma cell line C26 was cultured in Dulbecco's Modified Eagle Medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 µg/mL) and streptomycin (100 U/mL) in a 5% carbon dioxide environment. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% EDTA) and after centrifugation, single cell suspensions were prepared in phosphate-buffered saline to a final concentration of $5x10^4$ cells/100 µL. Cell viability was determined by trypan blue staining, and was always 98% or more. Colorectal liver metastases were induced in mice as previously described.[33,34] In brief, through a left lateral flank incision, $5x10^4$ C26 colorectal carcinoma cells were injected into the splenic parenchyma. After ten minutes, the spleen was removed to prevent intrasplenic tumor growth. Micrometastases were allowed to develop throughout the liver for 5 days. At that time point, animals were subjected to the different I/R protocols as described and morphological assessment of tumor growth, hepatocellular necrosis, and apoptosis was performed on nonischemic and ischemic lobes harvested 4, 5, and 7 days later.

Preconditioning and intermittent clamping protocol

Ischemic preconditioning was applied by occluding the blood supply to the left liver lobe for 10 minutes followed by a 15-minute reperfusion period before 45 minutes of ischemia. In pilot experiments, this preconditioning protocol proved to be the most optimal schedule in preventing elevation of liver enzymes (data not shown), as has also been recognized in other studies.[20,21] Intermittent clamping was performed by occluding the blood supply to the left liver lobe in three cycles of 15 minutes separated by 5 minutes of reperfusion,[35] leaving the total time of ischemia unaltered (45 minutes). Tissue levels of GSH and plasma levels of ALT and AST were assessed after 6 hours of reperfusion. The percentage of tissue necrosis, the presence of apoptosis, and tumor load were evaluated 5 days after ischemia.

Antioxidant treatment protocol using α-tocopherol and ascorbic acid

α-Tocopherol was prepared for intraperitoneal injection by compounding (+)-α-tocopherol acetate (Sigma-Aldrich Chemie, Schnelldorf, Germany) with ethyl alcohol (20%), benzyl alcohol (1%), and pure vegetable oil (79%). The solvent without α-tocopherol served as a control vehicle. α-Tocopherol was administered via intraperitoneal injection at a dose of 300 mg/kg/d for 3 days before surgery and a fourth injection just before I/R or sham operation.

L-ascorbic acid sodium salt was dissolved in saline (control vehicle) to a final concentration of 100 mg/kg and was administered by intravenous injection 5 minutes before I/R or sham operation. In pilot experiments, the anti-oxidative effect of both treatments was confirmed by prevention of a rise in GSSG levels after 1 hour of reperfusion (data not shown). Tissue GSH levels and plasma liver enzymes were analyzed following 6 hours of reperfusion. Late hepatocellular necrosis, apoptosis, and tumor growth were measured 4 days after ischemia.

GSSG and GSH assay

After liver harvesting, pieces of the ischemic and nonischemic lobes were snap frozen and stored at -80˚C. Liver contents of GSSG and GSH were measured in whole tissue extracts as previously described.[36] Briefly, the liver samples were homogenized in 5-sulfosalicylic acid, diluted, and added to the assay reagent containing 5,5-dithiobis(2-nitrobenzoic acid) and nicotinamide adenine dinucleotide phosphate (both purchased from ICN Biomedicals BV, Zoetermeer, The Netherlands). For GSSG measurements, 2-vinylpyridine and triethanolamine (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were added to undiluted extracts to conjugate GSH and neutralize the acidic constitution before adding the assay reagent. The reaction was initiated by adding GSH reductase (Sigma-Aldrich Chemie BV). GSSG and GSH were determined via kinetic measurements of the absorbance change of 5,5-dithiobis(2-nitrobenzoic acid) at 412 nm by comparing it with known standards. Liver contents of GSSG and GSH were expressed as nanomoles per milligram of protein. The liver protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Liver enzymes

Heparin plasma samples (500 µL) were obtained via cardiac puncture and were centrifuged at 14.000 rpm for 10 minutes. Plasma levels of ALT and AST served as indicators of liver tissue damage and were analyzed using commercially available diagnostic kits (Instruchemie BV, Delfzijl, The Netherlands).

Tumor analysis

Intrahepatic tumor load was scored as the hepatic replacement area (HRA),[34] the percentage of hepatic tissue that has been replaced by tumor cells. On two nonsequential hematoxylin and eosin stained sections per liver lobe, 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 magnification of x40. 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. Observers were blinded to treatment. Using this method, we obtained less than 5% interobserver and intraobserver variability. Finally, HRA ratios between ischemic and nonischemic lobes were calculated for each animal to express the proportional increase in HRA in the ischemic (left) lobes versus the nonischemic (right plus median) lobes.

Quantification of hepatocellular necrosis

The percentage of hepatocellular necrosis was scored simultaneously with tumor HRA analysis on nonsequential hematoxylin and eosin stained sections. The ratio of necrotic cells versus healthy hepatocytes plus tumor cells was determined for each field. The percentage of hepatocellular necrosis was expressed as the average area ratio of all fields.

Evaluation of apoptosis

Activated caspase-3 was analyzed via immunohistochemistry on tissue sections of clamped and nonclamped liver lobes. Non-tumor-bearing sections served as controls. After deparaffinization and rehydration, sections were stained with an anti-active caspase-3 antibody (C92-605, BD Biosciences PharMingen, Alphen aan den Rijn, The Netherlands) followed by a rabbit anti-mouse horseradish peroxidase (Pierce) antibody. The reaction was developed using diaminobenzidine/H_2O_2 as a chromogen substrate.

Statistical analysis

Statistical differences between groups were analyzed using the Mann-Whitney U-test for nonparametric data. Data are expressed as mean ± sem.

Results

Validation of a standardized murine model of partial hepatic I/R

In our standardized model of left lobar I/R, blood pressure remained stable for at least 120 minutes before and during I/R (**Figure 1A**); (n=3 each group) without overt changes in blood pH, pO_2, or pCO_2 (data not shown). Local hypoperfusion and hypoxia of the left liver lobe during clamping were confirmed by laser doppler and tissue pO_2 measurements. Following application of the clamp, the blood flow in the left lobe was obstructed by 85% to 90% (**Figure 1B**), followed by a dramatic drop in pO_2 to approximately 1 to 2 mmHg (**Figure 1C**). To demonstrate local oxidative stress, we measured consumption of the endogenous antioxidant GSH and production of its oxidized form, GSSG. Tissue levels of GSSG in the ischemic lobes were elevated after 1 hour of reperfusion but were not significantly different after 6 hours of reperfusion (**Figure 1D**) (n=7 each group). GSH

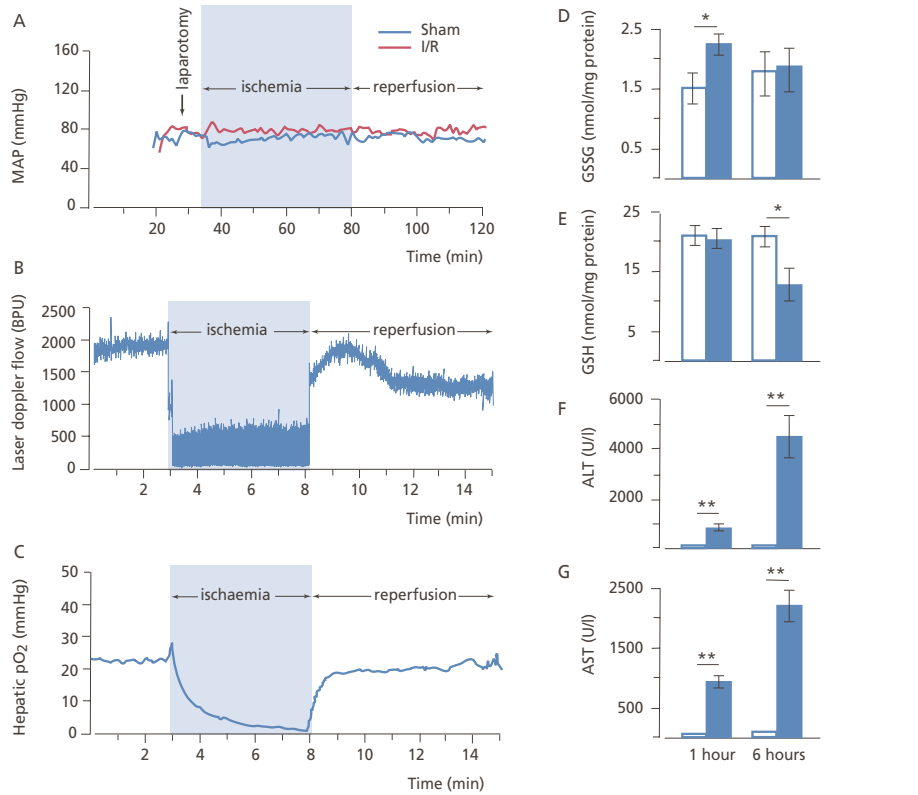

Figure 1. Validation of a standardized murine model of partial hepatic I/R. **(A)** Mean arterial blood pressure remained stable for at least 120 minutes in sham-operated mice (blue line) (n=3) and in mice subjected to 45 minutes of partial lobar I/R (red line) (n=3). Local hypoperfusion and hypoxia of the left liver lobe during clamping were confirmed by **(B)** laser doppler and **(C)** tissue pO_2 measurements. Local oxidative stress was confirmed by **(D)** a rise in GSSG levels following 1 hour of reperfusion and **(E)** consumption of GSH after 6 hours of reperfusion (□represent sham operation; ■represent I/R) (n=7 each group). I/R induced hepatocellular injury as indicated by an increase in plasma **(F)** ALT and **(G)** AST levels following 1 and 6 hours of reperfusion (n=7 each group). *p<0.05; **p<0.01; MAP, mean arterial blood pressure; GSSG, glutathione disulfide; ALT, alanine aminotransferase; GSH, glutathione; AST, aspartate aminotransferase; BPU, blood perfusion unit.

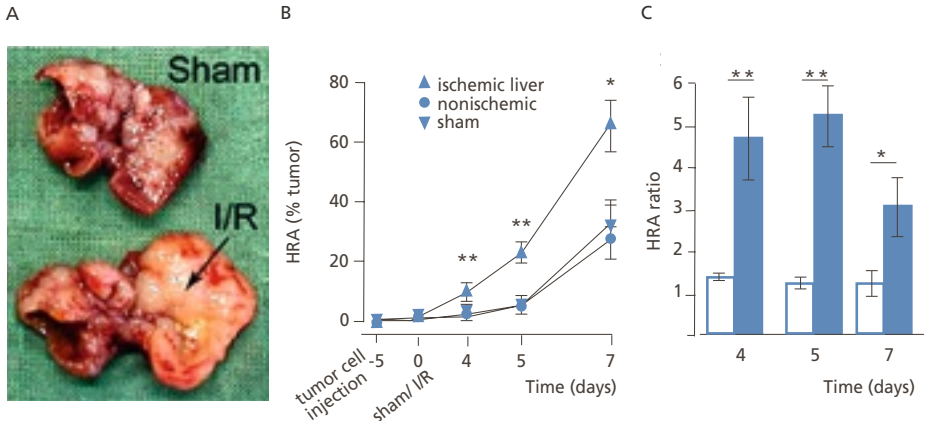

Figure 2. Accelerated outgrowth of established liver metastases following I/R. Five days after the induction of liver metastases, the left liver lobe was subjected to 45 minutes of ischemia, followed by reperfusion. The livers were harvested 4, 5, and 7 days after ischemia, and tumor growth was analyzed on hematoxylin-eosin-stained tissue sections. **(A)** Liver harvested seven days after partial ischemia clearly shows an increase in macroscopic tumor growth in the clamped lobe (arrow). **(B)** Time course of tumor growth, expressed as the hepatic replacement area (HRA). In ischemic liver lobes HRA was significantly increased compared with nonischemic liver lobes and with the left and right plus median lobes of sham-operated mice at 4 (n=5), 5 (n=6), and 7 (n=6) days after I/R. **(C)** Time course of the proportional increase in HRA in the ischemic (left) lobes versus the nonischemic (right plus median) lobes, expressed as HRA ratio. Tumor growth was stimulated over four- to fivefold on the fourth and fifth day after ischemia (represent sham operation; represent I/R). *p<0.05; **p<0.01. I/R, ischemia/reperfusion; HRA, hepatic replacement area.

content was not altered after 1 hour of reperfusion but had decreased by 50% after 6 hours of reperfusion in liver tissue isolated from the clamped liver lobes, but not in that from the nonclamped liver lobes (**Figure 1E**); (n=7 each group). Plasma ALT and AST levels were markedly increased after 1 and 6 hours of reperfusion (**Figure 1F-G**); (n=7 each group), as shown in similar I/R models.[21] Mortality was not observed in this model of left lobar I/R. In conclusion, this partial I/R model induces hepatic oxidative stress and tissue injury in a background of stable hemodynamic parameters.

Equal tumor loads in all liver lobes provide an internal control for local effects of I/R

Metastatic tumor growth was initiated by injecting C26 colorectal carcinoma cells into the splenic parenchyma. Single tumor cells reach the liver through the portal vein, where a subset grows out to form intrahepatic micrometastases.[33,34] Tumor growth was assessed in control animals by determining the percentage of liver tissue that was replaced by tumor cells (HRA) 12 days after tumor cell injection. The tumor load in the right plus median lobes was similar to that in the left liver lobe (33.3% versus 32.8%). Consequently, the ratio between the HRA values was approximately one. Based on this result, we conclude that the right plus median liver lobes may serve as an internal control for tumor growth after selective clamping of the left lobe.

I/R accelerates the local outgrowth of pre-established liver metastases

We next examined how I/R affects the growth rate of pre-established liver micrometastases. After intrasplenic tumor cell injection, micrometastases were allowed to develop throughout the liver for 5 days. Subsequently, the left liver lobes were selectively subjected to 45 minutes of ischemia, followed by reperfusion. Tumor growth was analyzed 4 (n=5), 5 (n=6), and 7 (n=6) days after I/R. The rate of tumor growth in the I/R-subjected liver lobes was markedly stimulated when compared with that in the control lobes of the same mice, or to that in any lobe of the sham-operated mice (**Figure 2A-B**). The relative increase of tumor growth in the ischemic lobes over the nonischemic lobes (expressed as HRA ratio) was maximal 5 days after I/R when tumor growth was stimulated over fivefold (**Figure 2C**). This relative increase was somewhat more modest after 7 days (approximately threefold stimulation) because of a saturation of tumor load in the left (clamped) lobes (**Figure 2B-C**). Because tumor growth was equal in all lobes of sham-operated mice and in nonclamped lobes of mice subjected to I/R, the relative increase in tumor load is due to increased tumor growth in the postischemic lobes, and not to reduced tumor growth in the nonischemic lobes. The finding that I/R in the left liver lobe had no effect on tumor growth in the right and median lobes indicates that the stimulating effect on tumor growth is not due to systemically released factors. These results unequivocally demonstrate that hepatic I/R has a dramatic and local stimulatory effect on the outgrowth of established micrometastases.

Accelerated tumor growth is associated with necrosis of the liver parenchyma

Microscopic examination of hematoxylin- and eosin stained liver sections revealed that tumor growth in the clamped liver lobes was especially dramatic around necrotic tissue areas (**Figure 3A**). These necrotic areas covered 25.8 ± 6.6% (n=5) of the total tissue area in the clamped lobes 4 days after I/R. The areas were characterized by strongly eosinophilic, amorphic cellular debris and ghost cells with nuclear dust surrounded by an area of inflammatory cells and macrophages (foam cells) (see **Figure 3A**). Staining of the hepatocellular cytoskeleton with reticulin revealed that the necrotic areas were of hepatic rather than tumoral origin (data not shown).

Because apoptosis has also been described as an important I/R-induced mechanism of liver cell death, we next assessed whether apoptosis was present via immunohistochemistry for activated caspase-3. Apoptotic hepatocytes were observed very infrequently in unclamped liver lobes. However, in clamped liver lobes, a strong caspase-3 staining in hepatocytes was detected in a zone surrounding the necrotic tissue areas. These cells were found to be closely associated with the presence of infiltrating lymphocytes (see **Figure 3A**). Hepatocyte apoptosis may be induced by inflammatory cells through stimulation of Fas, which may facilitate the invasion and outgrowth of tumor cells.[37] During the analysis of these slides, we also noted that apoptosis of tumor cells within metastases in nonischemic liver lobes occurs very infrequently (<1% of cells). In perinecrotic tumor tissue, however, caspase-3-positive cells were more frequently detected. Because apoptotic hepatocytes surround necrotic areas in tumor-bearing as well as non-tumor-bearing livers (see **Figure 3A**), the caspase-3-positive cells in perinecrotic tumor tissue most likely represent apoptotic hepatocytes rather than apoptotic tumor cells. These observations suggest that tumor cells preferably grow into the zones surrounding necrotic tissue areas that are characterized by infiltrating lymphocytes and apoptotic hepatocytes. Tissue necrosis, lymphocyte infiltration, and apoptosis were only sporadically observed in the nonclamped lobes or in the liver lobes from sham-operated animals, involving less than 1% of the liver tissue. At day 5 and day 7 after I/R, the area of tissue necrosis was reduced to 17.7 ± 4.4% (n=6) and 10.6 ± 5.4% (n=6), respectively, of the total tissue area. This reduction was attributed to invasion of tumor cells, overgrowing the necrotic areas (**Figure 3B**).

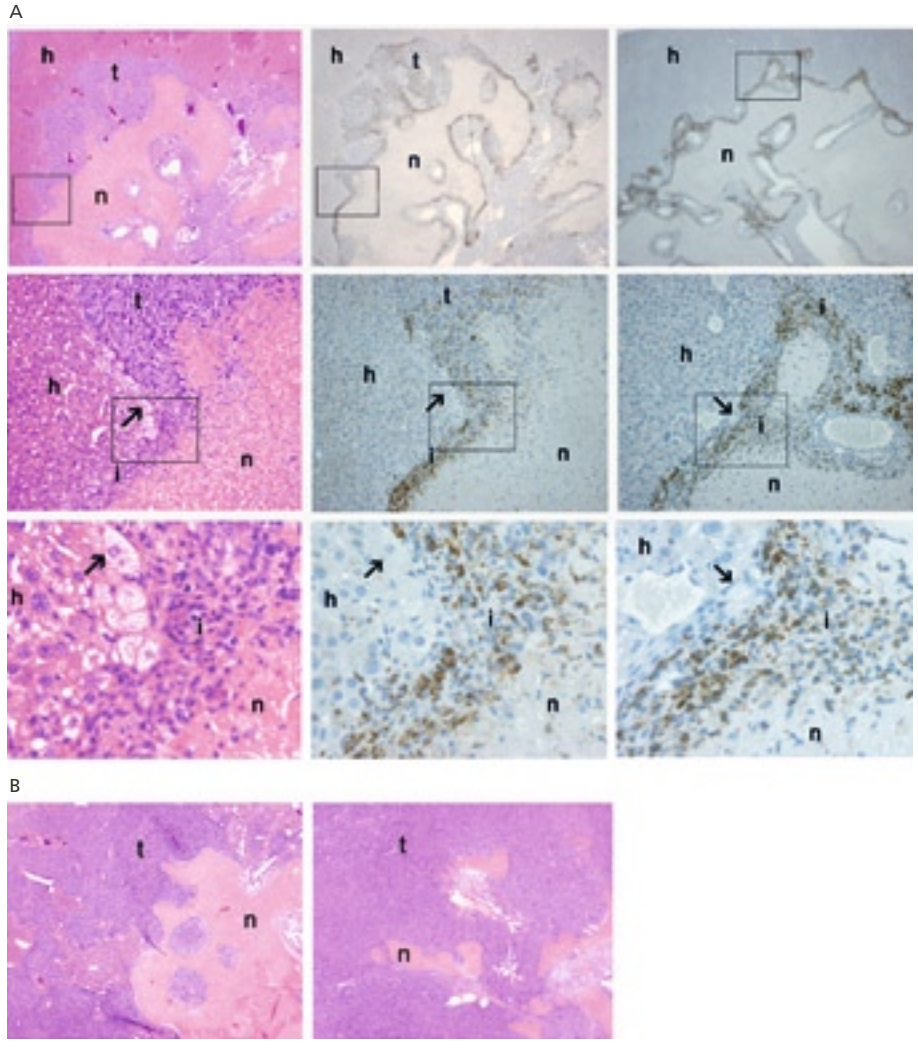

Figure 3. Accelerated tumor growth localizes around necrotic tissue areas. **(A)** Microscopic localization of I/R-accelerated tumor growth around necrotic areas is shown on hematoxylin-eosin-stained left liver lobe sections harvested four days after I/R (left upper panel; original magnification x2). The necrotic areas were characterized by strongly eosinophilic, amorphic cellular debris and ghost cells with nuclear dust surrounded by an area of inflammatory cells and macrophages (arrow) (middle and lower left panels; original magnification x10 and x20, respectively). Surrounding the necrotic areas, a zone of apoptotic hepatocytes was detected via activated caspase-3 immunohistochemistry in clamped liver lobes (upper middle panel; original magnification x2). Apoptotic hepatocytes were closely associated with the presence of infiltrating lymphocytes (i) (lower panels; original magnification x20). Tumor cells preferentially grew into these subvital zones (middle panel; original magnification x10). Identical staining patterns were observed in non-tumor-bearing ischemic livers (right panels). **(B)** At day five and day seven after I/R, the area of tissue necrosis was reduced by the invasion of tumor cells overgrowing the necrotic areas (original magnification x2). h, hepatocytes; t, C26 tumor cells; n, necrosis; i, inflammatory cells.

Intermittent clamping but not ischemic preconditioning protects the liver against accelerated tumor growth

Because I/R-accelerated tumor growth is associated with liver tissue necrosis, we examined whether alternative clamping methods that protect against hepatocellular damage would cross-protect the liver against accelerated tumor growth. The application of a short period of ischemia before prolonged clamping, called ischemic preconditioning, can render liver tissue less vulnerable to a sustained ischemic insult by triggering hepatocellular defense mechanisms.[8,22] In addition, several clinical and experimental studies have demonstrated that intermittent clamping reduces hepatocellular injury.[24-26] We measured the effects of both clamping methods on GSH tissue levels, on early and late hepatic damage, and on tumor growth. Both preconditioning and intermittent clamping prevented the depletion of GSH (**Figure 4A**; n=6 each group). In addition, both clamping methods largely prevented early hepatocellular damage (by 85% and 93%, respectively) as judged by plasma ALT and AST levels (**Figure 4B-C**; n=6 each group). Late hepatocellular damage (ie, liver tissue necrosis) was modestly (twofold) reduced by ischemic preconditioning but was virtually abolished by intermittent clamping (**Figure 4D**; n=8 each group). The detection of infiltrating lymphocytes and closely associated apoptotic hepatocytes was strictly dependent on the presence of hepatic tissue necrosis. Thus, these phenomena were modestly reduced by ischemic preconditioning and were undetectable following intermittent clamping (data not shown). Next, we determined how both clamping methods affected the outgrowth of pre-established micrometastases. Intermittent clamping completely prevented the stimulatory effect of I/R on tumor growth (**Figure 4E-F**; n=8 each group). In marked contrast, ischemic preconditioning had no protective effect on I/R-accelerated tumor growth, despite the fact that it prevented early tissue damage (see **Figure 4E-F**; n=8 each group). Tumor loads in the nonclamped lobes in all groups were not significantly different. Apoptotic tumor cells were detected very infrequently (<1%) in all treatment groups. These results suggest that the extent of late local tissue necrosis with associated inflammatory cells and apoptotic hepatocytes, rather than initial hepatocellular damage, is correlated with I/R-induced acceleration of tumor growth.

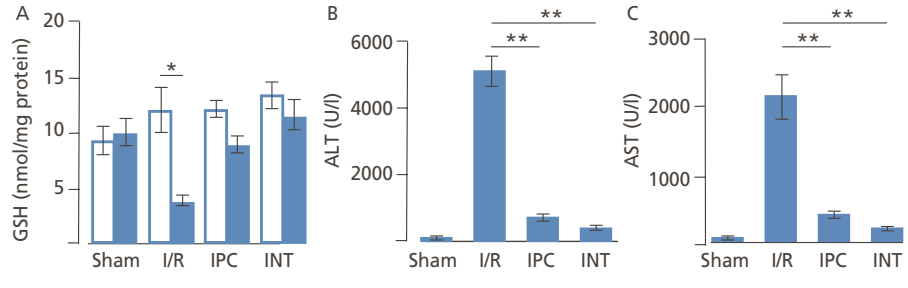

Figure 4. Ischemic preconditioning and intermittent clamping as protective strategies against early and late hepatocellular injury and tumor growth acceleration. Following induction of liver metastases, left liver lobes were subsequently subjected to sham treatment, I/R, ischemic preconditioning, or intermittent clamping. Ischemic preconditioning and intermittent clamping both **(A)** prevented the reduction in GSH tissue levels (represent nonischemic lobes; represent ischemic lobes) (n=6 each group) and **(B,C)** reduced early hepatocellular damage, as assessed by plasma ALT and AST levels following 6 hours of reperfusion (n=6 each group). *p<0.05; **p<0.01; GSH, glutathione; IPC, ischemic preconditioning; INT, intermittent clamping; I/R, ischemia/reperfusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

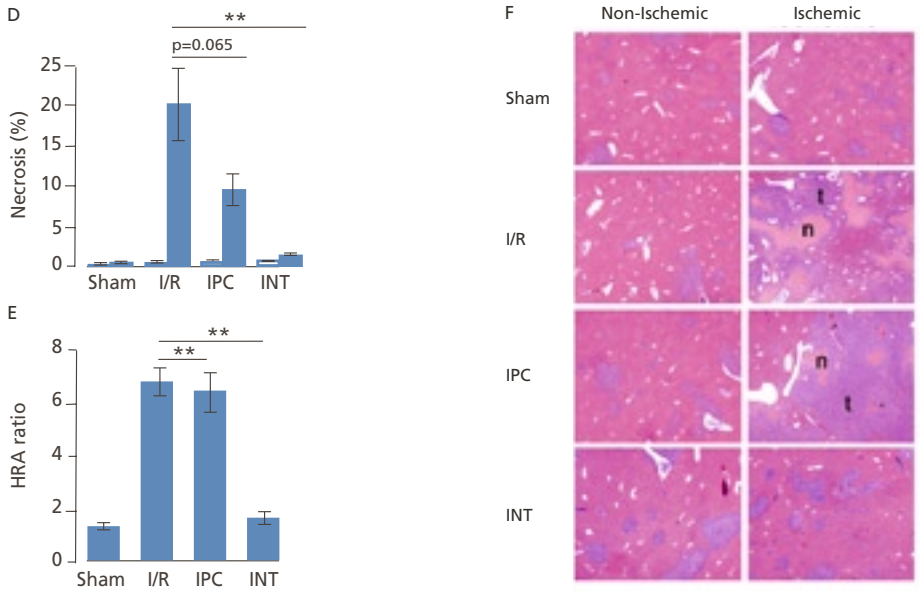

Figure 4. Ischemic preconditioning and intermittent clamping as protective strategies against early and late hepatocellular injury and tumor growth acceleration. Late hepatocellular damage (i.e., liver tissue necrosis) was quantified via morphometric analysis of hematoxylin-eosin-stained tissue sections. **(D)** Tissue necrosis was modestly (twofold) reduced by ischemic preconditioning 5 days after ischemia but was virtually abolished by intermittent clamping (□represent nonischemic lobes; ■represent ischemic lobes) (n=8 each group). **(E)** Tumor growth was quantified via morphometric analysis of the hepatic replacement areas 5 days after ischemia. Intermittent clamping, but not ischemic preconditioning, prevented the stimulatory effect of I/R on tumor growth. **(F)** Hematoxylin-eosin-stained sections of nonischemic and ischemic liver lobes of each group (original magnification x2). **p<0.01; IPC, ischemic preconditioning; INT, intermittent clamping; I/R, ischemia/reperfusion; HRA, hepatic replacement area; t, C26 tumor cells; n, necrosis.

α-Tocopherol and ascorbic acid fail to protect against I/R-stimulated tumor growth

Oxygen radicals are generated predominantly during the early phases of reperfusion and contribute to early hepatocellular cell damage.[38] α-Tocopherol is the most effective lipid-soluble antioxidant in biological systems and has been proven effective in reducing oxidative stress and hepatocellular damage in both clinical and preclinical trials in doses from 10 to 300 mg/kg.[27-29] Ascorbic acid is a water-soluble anti-oxidant known to reduce oxidative stress with maximal hepatoprotective effects at a dose of 100 mg/kg.[30-32] Therefore, we examined the effects of α-tocopherol (300 mg/kg) and ascorbic acid (100 mg/kg) on GSH levels, early hepatocellular damage, late tissue necrosis, apoptosis, and accelerated tumor growth. Both α-tocopherol and ascorbic acid pretreatment reduced consumption of GSH (**Figures 5A, 6A**; n=6 each group) and prevented early hepatocellular damage by 50%, as indicated by plasma ALT and AST levels (6 hours post-I/R) (**Figures 5B-C, 6B-C**; n=6 each group). In contrast, neither treatment had a discernable effect on I/R-induced tissue necrosis measured 4 days post-I/R (**Figures 5D, 6D**). Furthermore, tissue necrosis in antioxidant-treated groups was associated with a zone of inflammatory cells and apoptotic hepatocytes, just as in the nontreated groups (data not shown). Most importantly, we found that both α-tocopherol and ascorbic acid pretreatment failed to reduce the stimulatory effect of I/R on intrahepatic tumor growth (**Figures 5E-F, 6E-F**)

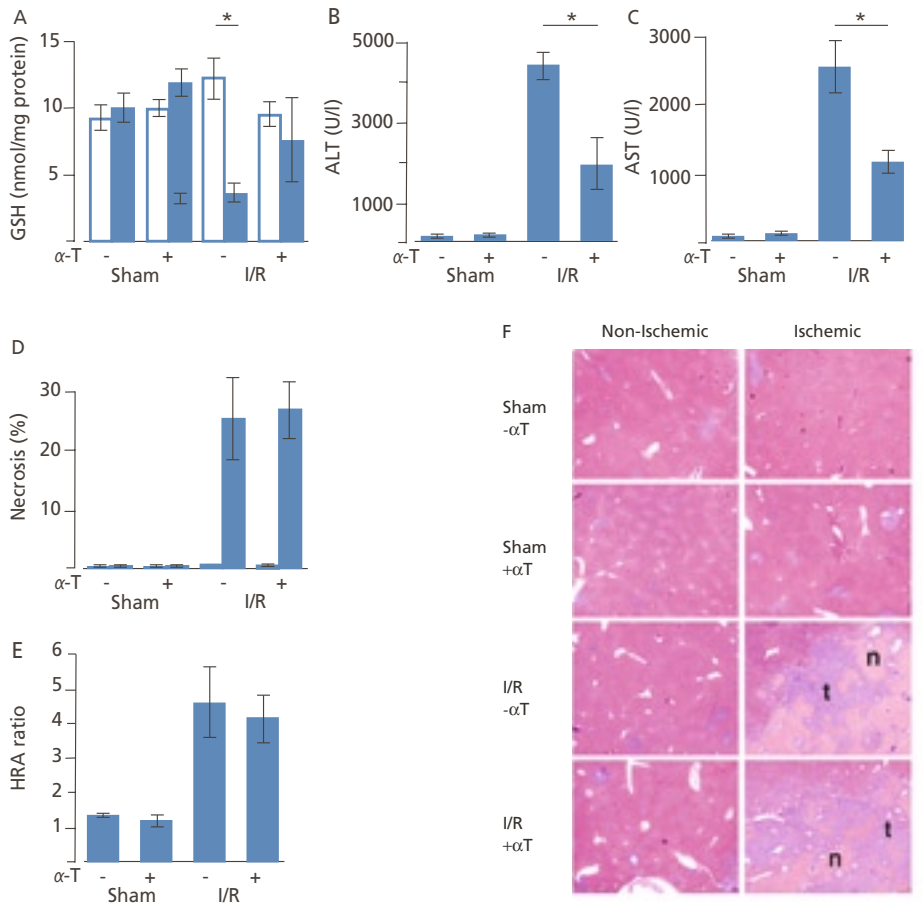

Figure 5. Effect of α-tocopherol on I/R-induced liver tissue injury and tumor growth. **(A)** α-Tocopherol, administered for three consecutive days before I/R as well as just before I/R, prevented GSH consumption by approximately 70% (□represent nonischemic lobes; ■represent ischemic lobes) (n=6 each group) and reduced early hepatocellular damage by 50%, as indicated by plasma **(B)** ALT and **(C)** AST levels (6 hours post-I/R) (n=6 each group). Tissue necrosis and tumor growth were measured 4 days after ischemia. **(D)** α-Tocopherol had no discernable effect on I/R-induced tissue necrosis and **(E)** failed to reduce the stimulatory effect of I/R on intrahepatic tumor growth. **(F)** Hematoxylin-eosin-stained sections of nonischemic and ischemic liver lobes of each group (original magnification x2). *p<0.05; **p<0.01; GSH, glutathione; I/R, ischemia/reperfusion; α-T, α-tocopherol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HRA, hepatic replacement area; t, C26 tumor cells; n, necrosis.

(n=8 each group). Neither α-tocopherol nor ascorbic acid affected tumor growth in sham-operated mice, and absolute tumor load in all nonclamped lobes of all groups was equal. Immunohistochemistry for activated caspase 3 showed that neither α-tocopherol nor ascorbic acid affected tumor cell apoptosis. These findings strengthen the notion that enhanced I/R-induced outgrowth of micrometastases is correlated with late hepatocellular necrosis rather than with early events, including the production of oxygen radicals that contribute to the induction of early tissue damage.

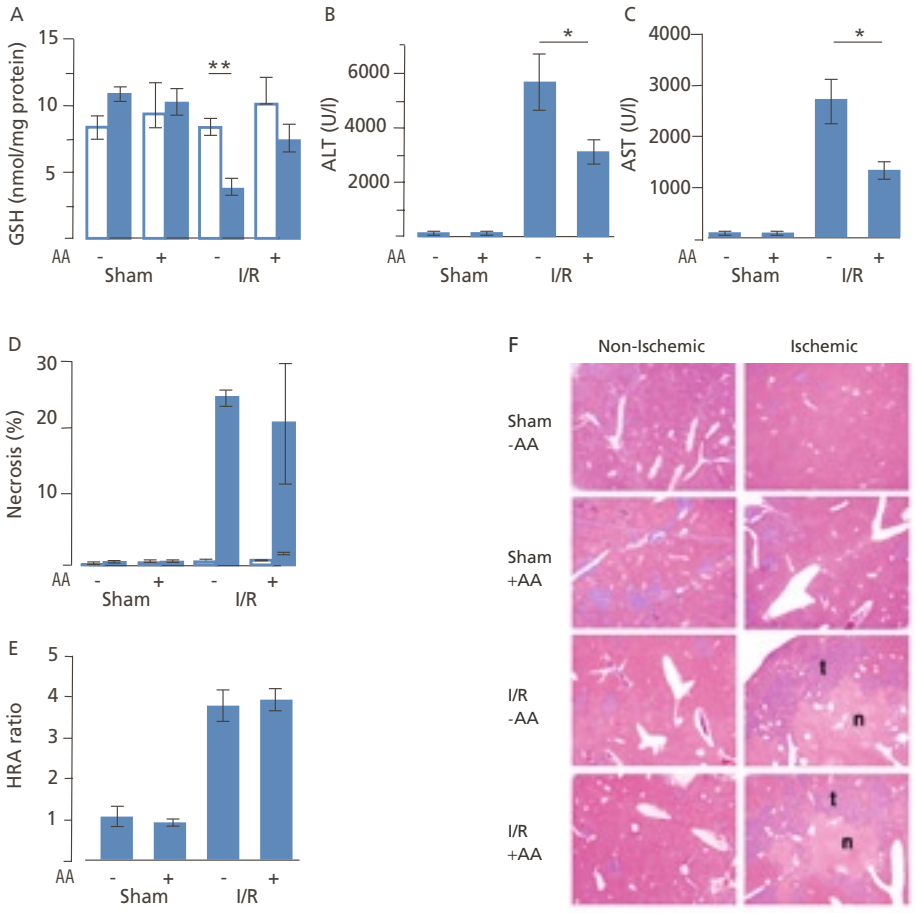

Figure 6. Effect of ascorbic acid on I/R-induced cell injury and tumor growth. **(A)** Ascorbic acid, administered five minutes before I/R, prevented GSH depression (□represent nonischemic lobes; ■represent ischemic lobes) (n=6 each group) and reduced early hepatocellular damage by 50%, as indicated by plasma **(B)** ALT and **(C)** AST levels (six hours post-I/R) (n=6 each group). **(D)** Ascorbic acid had no discernable effect on I/R-induced tissue necrosis measured four days after I/R. **(E)** In addition, ascorbic acid failed to reduce the stimulatory effect of I/R on intrahepatic tumor growth. **(F)** Hematoxylin-eosin-stained sections of nonischemic and ischemic liver lobes of each group (original magnification x2). *p<0.05; **p<0.01; GSH, glutathione; I/R, ischemia/reperfusion; AA, ascorbic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HRA, hepatic replacement area.

Discussion

The outgrowth of pre-existing micrometastases in the liver may be accelerated through various mechanisms. First, the outgrowth of distant micrometastases may be promoted by removal of the primary tumor, because the production of anti-angiogenic factors by theprimary tumor suppresses distant tumor growth.[39] Second, dormant tumor cells may be stimulated by proangiogenic factors that are released during surgery-induced tissue injury and hypoxia.[40-42] Third, partial hepatectomy may augment the growth of micrometastases by paracrine proliferative stimuli.[33,43] In the present study, we show that local I/R induced by vascular clamping is an additional and strong stimulus that promotes the outgrowth of micrometastases in the liver.

Our finding that tumor growth is stimulated in clamped but not nonclamped lobes suggests that the mechanism operates locally and does not involve systemically released factors. Local responses to I/R are complex,[7,8] but can be divided into two distinct phases. Acute hepatocellular injury is caused by reactive oxygen species and cytotoxic cytokines released by Kupffer and endothelial cells,[38,44] and is reflected by a rise in plasma liver enzymes. The late phase is characterized by neutrophil infiltration causing further damage to the parenchyma, mainly through a protease-dependent pathway.[18,45] Moreover, endothelial cell swelling, a local imbalance in vasoconstrictors and vasodilators, and activation of the coagulation system, together with the influx of neutrophils, will lead to microcirculatory disturbances, a phenomenon known as no-reflow.[46] This no-reflow further aggravates the damage and is reflected by microscopic tissue necrosis. Thus, distinct mechanisms underlie I/R-induced early liver tissue damage and late tissue necrosis.

I/R-accelerated tumor growth was shown to be associated with late tissue necrosis but not early hepatocellular injury. These results are based on our finding that ischemic preconditioning, α-tocopherol, and ascorbic acid reduced the I/R-stimulated consumption of GSH as well as the rise in liver enzyme levels but could not prevent late tissue necrosis nor I/R-stimulated tumor growth. Intermittent clamping prevented both early and late hepatocellular damage and I/R-accelerated tumor growth, confirming a relationship with late events in I/R-induced injury when necrotic tissue areas are apparent. Neutrophils that infiltrate the parenchyma during the late phase may contribute to tumor growth by producing proliferation- and angiogenesis-stimulating factors and cytokines. Moreover, microcirculatory disturbances following reperfusion will prolong intrahepatic hypoxia. Prolonged exposure of micrometastases in the liver to hypoxia could lead to the activation of several distinct pathways that contribute to I/R-accelerated tumor growth. These include pathways that stimulate tumor cell proliferation and tumor angiogenesis.[47,48] Intermittent clamping appears to be superior to ischemic preconditioning in maintaining hepatic microcirculation, especially after prolonged periods of ischemia.[49] This outcome might explain the remarkable difference between ischemic preconditioning and intermittent clamping in preventing hepatocellular necrosis[50] and accelerated outgrowth of micrometastases (present study). Finally, a change in the structure of the liver parenchyma may facilitate the outgrowth of invading tumor cells. Infiltrating lymphocytes may induce apoptosis in surrounding hepatocytes through stimulation of Fas, which may facilitate tumor outgrowth as a result of an altered tissue structure.[37] Our observation that the areas of enhanced tumor outgrowth were closely associated with zones of inflammatory cells and apoptotic hepatocytes surrounding the necrotic tissue areas supports this hypothesis.

Obviously, the above-mentioned possibilities are not mutually exclusive, and the mechanism of I/R-accelerated tumor growth is likely to be multifactorial. Finally, we have also found increased tumor outgrowth of pre-established CC531 colon carcinoma micrometastases following partial hepatic I/R in Wag/Rij rats (data not shown). Therefore, I/R-stimulated tumor growth in the liver is not restricted to the C26 cell line and presumably represents a general phenomenon.

In conclusion, our results identify hepatic ischemia and reperfusion as a result of vascular clamping as a major cause of accelerated tumor growth in a standardized mouse model of colorectal liver metastases. Clamping-induced acceleration of tumor growth may be prevented by simply interrupting blood flow intermittently, without losing the benefit of decreasing blood loss.[6] We found that intermittent clamping was superior to ischemic preconditioning, both with respect to preventing late tissue necrosis and preventing accelerated tumor growth. Therefore, we consider intermittent clamping as the clamping method of choice. Finally, these results may also be relevant for other circumstances that are associated with intrahepatic I/R, such as low-flow states following hemorrhagic or circulatory shock and resuscitation.[44] In those cases, anti-inflammatory agents or pharmacological interventions that preserve microcirculation might protect against accelerated tumor growth.

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Chapter 5

Prognostic significance of vascular inflow occlusion during liver surgery for colorectal liver metastases

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Abstract

Aims During liver surgery, temporary vascular clamping is frequently applied to minimize blood loss, but causes ischemia/reperfusion injury to the liver parenchyma. We recently found in a preclinical model that ischemia/reperfusion accelerates the outgrowth of residual micrometastases. The aim of this study was to evaluate the prognostic significance of vascular inflow occlusion during hepatectomy.

Methods Hundred and twenty eight patients who underwent intentionally curative hepatectomy between 1995 and 2005 for colorectal liver metastases were collected from a prospective database and analyzed for liver free survival (LFS) and disease free survival (DFS). The prognostic significance of vascular clamping of any type and severe ischemia due to prolonged vascular clamping was determined by logistic Cox-regression models.

Results LFS was reduced after clamping of any type ($p<0.05$), without affecting DFS. Both LFS and DFS were decreased when ischemia times exceeded 20 minutes in case of continuous clamping or when intermittent clamping was performed in more than 2 cycles of 10-15 minutes ($p<0.05$). Severe ischemia independently predicted DFS in the multivariate analysis ($p<0.05$). Stratification revealed a more pronounced adverse effect of severe ischemia for males, older patients and for patients with hepatic steatosis.

Conclusion Severe ischemia due to prolonged vascular inflow occlusion during hepatic resection for colorectal liver metastases may be well associated with early intrahepatic recurrence and decreased disease free survival, which appears to be most prominent in males, older patients and in hepatic steatosis. In case of inevitable clamping, total ischemia time should be kept to a minimum.

Introduction

Mortality due to colorectal cancer is almost invariably due to metastatic tumor growth in the liver. Surgical removal of hepatic tumors is yet the only hope for cure, offering 5-year survival rates of 30-40%.[1] However, even after an apparently complete resection, the vast majority (about two-thirds) of patients ultimately present with recurrent disease.[2] Metastases usually recur within the first two years and are located predominantly in the remaining liver. The most likely source for recurrences are microscopic tumor residues that were undetected at the time of surgery,[3,4] but may also develop from circulating tumor cells that are shed into the circulation during surgery.[5,6] In the past decades, many prognostic factors of recurrence and survival after hepatectomy for metastatic colorectal cancer have been documented. Prognostic factors preferably include preoperative criteria for selecting candidates that benefit from hepatic resection.[7] On the other hand, peri-operative variables have been reported to adversely affect outcome by altering the biological behavior of the metastases.[8-12]

Temporary clamping of the vascular inflow of the liver, i.e. Pringle Maneuver, is frequently applied in liver surgery. It is predominantly used to reduce peri-operative bloodloss during hepatic resections, in selected cases requiring additional clamping of the suprahepatic and infrahepatic caval vein, resulting in total hepatic exclusion.[13,14] Although vascular clamping has proven its usefulness worldwide, it causes ischemia/reperfusion injury to the liver, which may lead to postoperative liver dysfunction. The adverse effects of ischemia/reperfusion on hepatocellular damage and liver function have been well documented.[15,16] However, it is striking that the influence of intra-operative vascular clamping methods as a predictor of recurrence has been underexposed. An exhaustive systematic review performed prior to this study among all studies comparing different clamping techniques and all papers studying prognostic factors of recurrence and survival after partial liver resection for colorectal liver metastases revealed only four studies evaluating long-term outcome after vascular clamping.[12,17-19] Nonetheless, the patients and clamping techniques studied were highly heterogeneous and the studies lack a sufficiently detailed evaluation to draw any firm conclusion.

We have recently suggested that vascular clamping may adversely affect oncological outcome by accelerating the outgrowth of pre-existent hepatic micrometastases. In a standardized preclinical murine model of partial hepatic ischemia/reperfusion by temporary blood flow occlusion, the outgrowth of pre-existing colorectal micrometastases in occluded liver lobes was accelerated over five to sixfold compared to non-occluded lobes.[20]

The aim of the present study was to evaluate the prognostic significance of vascular clamping on long-term outcome in the human situation. Therefore, we analyzed whether vascular inflow occlusion affected outcome in patients treated by partial liver resection for colorectal liver metastases in our institution.

Patients and methods

Patients

Hundred and sixty three consecutive patients who underwent partial hepatectomy for colorectal liver metastases (1995-2005) at the University Medical Center Utrecht in The Netherlands were selected from a prospective liver database. Selection criteria for entering the study were: no sign of extrahepatic disease on preoperative imaging by routine liver contrast-enhanced four phase computed tomography; no positive hepatoligamentary lymph nodes or gross residual disease (R2) at laparotomy, no untreatable lesions as controlled by intra-operative ultrasonography and no simultaneous focal heat destruction by radiofrequency ablation, laser-induced thermotherapy or any other local ablative treatment. In addition, patients who had died within 30 days after the hepatic resection were excluded from further analysis. Following these selection criteria, 128 patients were identified from the database and form the basis of this study.

Surgical management

Fifteen patients with initially nonresectable metastases were down-staged by neo-adjuvant chemotherapy. Three patients had preoperative portal vein embolization to allow regeneration of the future remnant liver. One patient had a two-staged resection because of excessive intra-operative blood loss and was evaluated for recurrence and survival from the second operation. Blood loss was minimized by maintaining central venous pressure below 5 cm H_2O. Vascular inflow occlusion is not a standard procedure in our institution, but is only performed in cases of (expected) excessive blood loss. The attention for ischemia/reperfusion injury resulting from prolonged continuous clamping, had resulted in a preference for intermittent clamping in recent years. Intermittent clamping was performed in cycles of 10-15 minutes ischemia and 5 minutes of reperfusion. Operations were done by three expert hepatic surgeons and the criteria and preference for vascular clamping were equal for each surgeon.

Prognostic factors

Various factors were extracted from the database for each patient, including age at the time of liver resection, gender, location of the primary, nodal status of the primary, disease free interval between resection of the primary tumor and detection of the liver metastases, the number, maximal size and distribution of the hepatic metastases, preoperative carcinoembryonic antigen (CEA), extent of the resection (major defined as three segments or more), type of the resection (anatomical or non-anatomical), resection margins, blood loss and the amount of units red blood cells transfused. First, we analyzed whether clamping of any type would affect outcome when compared to patients who had no vascular clamping. Second, as the magnitude of ischemic damage is largely determined by the duration and type of vascular clamping, we performed an additional comparison by classifying patients according to the type and duration of vascular clamping during hepatectomy. This classification was based partly on our preclinical observations and partly on practical surgical considerations (e.g., the number of cycles in intermittent clamping). Severe ischemia was defined as continuous clamping for more than 20 minutes or intermittent clamping for more than 2 cycles of 10-15 minutes each. Minor ischemia was defined as no clamping, continuous clamping with a maximum of 20 minutes ischemia time or intermittent clamping of not more than 2 cycles of 10-15 minutes of ischemia.

Follow-up

Routine follow-up included at least liver contrast-enhanced four phase computed tomography of the liver, chest X-ray, and CEA levels every six months. Follow-up data were updated by personal contacts with patients during follow-up consultations, letters and telephone calls to referring physicians and general practitioners. The duration of the follow-up and the time between hepatectomy and the detection of recurrence were obtained from the database, as well as the site of recurrence and survival data. Based on our preclinical findings we hypothesized that ischemia would accelerate the outgrowth of primarily local and possibly also distant micrometastases. Therefore, the primary end-points were the time to develop liver recurrence, i.e. liver free survival (LFS) and the time to develop overall recurrence, i.e. disease free survival (DFS). Overall survival (OS) was also evaluated.

Statistical analysis

Comparison between groups for the different variables was done by independent sample T-test, Mann-Whitney U- test, Fisher exact test or Pearson Chi-square test when appropriate. Hazard ratios (HR) of liver recurrence, overall recurrence and mortality were computed for all variables to estimate relative risks and 95% confidence intervals using Cox proportional hazards regression analysis. Kaplan-Meier survival curves were compared by log rank test statistics. Multivariate analysis was performed to determine the independent prognostic impact on (liver) recurrence and overall survival while adjusting for possible confounders simultaneously. For this purpose, both covariates that significantly affected outcome by univariate analysis and variables that correlated with clamping or the severity of ischemia were entered into a logistic multivariate regression analysis. In addition, the following variables were evaluated for possible confounding: age, gender, extent and type of resection, and blood transfusion. Sub-analyses were performed to investigate the relationship between age, gender or hepatic steatosis and the effect of severe ischemia on liver recurrence, overall recurrence and overall survival. A P-value of less than 0.05 was taken to indicate significance.

Results

Patient characteristics

One hundred and twenty eight patients who underwent intentionally curative surgical resection of colorectal liver metastases during the study period were identified from the liver database. Vascular clamping was used in 67 of the procedures (52%), whereas the remaining patients (n=61) had no vascular occlusion. All patients had an inflow occlusion (Pringle Maneuver). In two patients inflow occlusion was combined with a brief occlusion of the hepatic veins for 5 and 7 minutes respectively, with preservation of caval flow resulting in selective hepatic exclusion. These patients had a tumor located in the convexity of the hepatic veins. Inflow occlusion was performed continuously in 42 patients or intermittently in 25 patients. Ischemic preconditioning was not performed. Median total ischemia time was 21 (range 2 - 45) minutes for continuous clamping and 36 (range 20-90) minutes for intermittent clamping. Forty seven patients underwent continuous clamping of more than 20 minutes or intermittent clamping of more than 2 cycles of 10-15 minutes and formed the "severe ischemia" group. The remaining 81 patients comprised the "minor ischemia" group. A detailed description of the patients' baseline characteristics according to clamping yes/no and minor/severe ischemia are provided in **Table 1 and 2** respectively. As the need for prolonged vascular clamping may be related to the extent and complexity of the operation, we investigated whether a relationship existed between both clamping of any type or the severity of ischemia and all other pre-and intra-operative characteristics. Clamping of any type as well as severe ischemia were associated with a longer duration of the operation ($p<0.05$) and increased intraoperative blood loss ($p<0.05$), as more patients in the clamped and severe ischemia group had lost more than 2 liters of blood. All other characteristics were comparable between the groups (**Table 1 and 2**).

Table 1. Base-line characteristics of patients according to clamping Yes/No

Variable	No clamping (n=67)		Clamping of any type (n=61)		
	#	(%)	#	(%)	P-value
Age (years)					
Mean (SD)	62.7	(9.4)	58.7	(10.0)	0.076*
< 62[1]	25	(41)	39	(58)	
≥ 62	36	(59)	28	(42)	0.076#
Gender					
Female	17	(28)	23	(34)	0.452#
Male	44	(72)	44	(66)	
Location of primary					
Colon	38	(62)	43	(64)	0.856#
Rectum	23	(38)	24	36	
Nodal status of primary					
Negative	28	(46)	25	(37)	0.371#
Positive	33	(54)	42	(63)	
Disease free interval (months)					
Median (range)	10.0	(0.0-109.0)	6.0	(0.0-76.0)	0.263†
≥ 12	25	(41)	22	(33)	
< 12	36	(59)	45	(67)	0.364#

Table 1. Base-line characteristics of patients according to clamping Yes/No

Variable	No clamping (n=67)		Clamping of any type (n=61)		
	#	(%)	#	(%)	P-value
Preoperative CEA (ng/mL)[2]					
Median (range)	16.5	(1.1-1992.0)	6.4	(0.5-500.0)	0.160†
< 50	21	(75)	23	(79)	
≥ 50	7	(25)	6	(21)	0.760#
Tumor number					
Solitary	28	(46)	31	(46)	1.000#
Multiple	33	(54)	36	(54)	
Maximum tumor size (cm)					
Median (range)	4.3	(0.4-18.0)	3.5	(0.8-19.5)	0.511†
< 3.0	17	(28)	26	(39)	
3.0-5.0	23	(38)	24	(36)	
≥ 5.0	21	(34)	17	(25)	0.359‡
Tumor distribution					
Unilobar	36	(59)	37	(55)	0.474#
Bilobar	25	(41)	30	(45)	
Duration of operation (min)					
Median (range)	242	(80-390)	270	(108-534)	0.020†
< 250[3]	36	(59)	28	(42)	
≥ 250	25	(41)	39	(58)	0.076#
Extent of resection					
Minor (< 3 segments)	20	(33)	20	(30)	0.849#
Major (≥ 3 segments)	41	(67)	47	(70)	
Type of resection					
Anatomical	33	(54)	40	(60)	0.590#
Non-anatomical	28	(46)	27	(40)	
Resection margin					
R0	53	(87)	59	(88)	1.000#
R1	8	(13)	8	(12)	
Blood loss (ml)					
Median (range)	1000	(70-7500)	1800	(100-11000)	0.011†
< 2000	47	(77)	38	(57)	
≥ 2000	14	(23)	29	(43)	0.011#
Red blood cell transfusion					
No	21	(34)	24	(36)	
Yes	40	(66)	43	(64)	1.000#
< 2 units	35	(57)	45	(67)	
≥ 2 units	26	(43)	22	(33)	0.349#

[1] Median age of the whole population was used as a cut-off point; [2] Pre-operative carcinoembryonic antigen (CEA) 71 missing values; [3] Median operating time was used as a cut-off point; * T-test; # Fisher exact test; † Mann-Whitney U-test; ‡ Pearson Chi square test.

Table 2. Base-line characteristics of patients according to the severity of ischemia

Variable	Minor ischemia (n=47)		Severe Ischemia (n=81)		
	#	(%)	#	(%)	P-value
Age (years)					
Mean (SD)	62.3	(9.6)	59.2	(9.9)	0.091*
< 62[1]	35	(43)	29	(62)	
≥ 62	46	(57)	18	(38)	0.066#
Gender					
Female	23	(28)	17	(36)	
Male	58	(72)	30	(64)	0.430#
Location of primary					
Colon	50	(62)	31	(66)	
Rectum	31	(38)	16	(34)	0.705#
Nodal status of primary					
Negative	38	(47)	15	(32)	
Positive	43	(53)	32	(68)	0.136#
Disease free interval (months)					
Median (range)	9.0	(0.0-109.0)	6.0	(0.0-76.0)	0.733†
≥ 12	31	(38)	16	(34)	
< 12	50	(62)	31	(66)	0.705#
Preoperative CEA (ng/mL)[2]					
Median (range)	14.0	(0.5-1992.0)	5.9	(0.8-363.0)	0.160†
< 50	29	(76)	15	(79)	
≥ 50	9	(24)	4	(21)	1.000#
Tumor number					
Solitary	38	(47)	21	(45)	
Multiple	43	(53)	26	(55)	0.855#
Maximum tumor size (cm)					
Median (range)	4.2	(0.4-18.0)	3.5	(1.2-19.5)	0.482†
< 3.0	24	(30)	19	(40)	
3.0-5.0	30	(37)	17	(36)	
≥ 5.0	27	(33)	11	(23)	0.363‡
Tumor distribution					
Unilobar	46	(57)	27	(57)	
Bilobar	35	(43)	20	(43)	0.582#
Duration of operation (min)					
Median (range)	245	(80-440)	285	(108-534)	0.049†
< 250[3]	45	(56)	19	(40)	
≥ 250	36	(44)	28	(60)	0.142#
Extent of resection					
Minor (< 3 segments)	25	(31)	15	(32)	
Major (≥ 3 segments)	56	(69)	32	(68)	1.000#
Type of resection					
Anatomical	46	(57)	27	(57)	
Non-anatomical	35	(43)	20	(43)	1.000#

Table 2. Base-line characteristics of patients according to the severity of ischemia

Variable	Minor ischemia (n=47)		Severe Ischemia (n=81)		
	#	(%)	#	(%)	P-value
Resection margin					
R0	70	(86)	42	(89)	
R1	11	(14)	5	(11)	0.784#
Blood loss (ml)					
Median (range)	1067	(70-11000)	1900	(300-10600)	0.067†
< 2000	60	(74)	25	(53)	
≥ 2000	21	(26)	22	(47)	0.020#
Red blood cell transfusion					
No	27	(33)	18	(38)	
Yes	54	(67)	29	(62)	0.572#
< 2 units	48	(59)	32	(68)	
≥ 2 units	33	(41)	15	(32)	0.349#

[1] Median age of the whole population was used as a cut-off point; [2] Pre-operative carcinoembryonic antigen (CEA) 71 missing values; [3] Median operating time was used as a cut-off point; *T-test; # Fisher exact test; † Mann-Whitney U-test; ‡ Pearson Chi square test.

Long-term outcome and factors influencing patterns of recurrence and survival

Median follow-up was 3.5 years. Altogether, 91 (71.1%) patients had a recurrence within the study period of which 47 patients (36.7% of the whole population) had a liver recurrence. Median DFS was 1.3 years, with 37.8% and 19.9% of patients being disease free after 2 and 5 years respectively. Four patients underwent repeat hepatectomy for liver recurrence, eight underwent local ablation of hepatic recurrent disease and two had lung resection for pulmonary metastases. Adjuvant chemotherapy (5-fluorouracil, leucovorin and oxaliplatin) was given to nine patients after hepatic resection. Seventy six (59.3%) patients died during follow-up with a median follow-up of 3.6 years. Overall actuarial 5- and 10-year survival was 36.3% and 21.6%.

Univariate analysis among all variables examined (**Table 3**) revealed that positive lymph nodes of the primary, disease free interval between primary and metastases of less than 12 months, preoperative CEA values of more than 50 ng/mL, multiple metastases and bilobar metastases inversely affected LFS ($p<0.05$). DFS was inversely affected by positive lymph nodes of the primary, disease free interval between primary and metastases of less than 12 months and preoperative CEA values of more than 50 ng/mL ($p<0.05$). Overall survival was not affected by any of the variables. Subsequent multivariate analysis revealed that positive lymph nodes and preoperative CEA levels of > 50 ng/mL were independent predictors for both liver recurrence and overall recurrence (**Table 4**).

Table 3. Univariate analysis for liver recurrence, overall recurrence and overall survival

Variable	#	Hazard Ratio (95% Confidence Interval) Liver recurrence	Overall currence	Mortality
Age (years)				
< 62[1]	64	1	1	1
≥ 62	64	0.70 (0.39-1.26)	0.95 (0.63-1.44)	1.41 (0.87-2.28)
Gender				
Female	40	1	1	1
Male	88	0.69 (0.38-1.26)	0.82 (0.53-1.27)	0.72 (0.53-1.36)
Location				
Colon	81	1	1	1
Rectal	47	0.93 (0.51-1.69)	0.84(0.55-1.29)	0.83 (0.50-1.37)
N-stadium				
N0	53	1	1	1
N1 or N2	75	2.44 (1.28-4.66) *	1.88 (1.22-2.92) *	1.49 (0.90-2.46)
Disease free interval (m)				
≥ 12	47	1	1	1
< 12	81	1.97 (1.02-3.81) *	1.61 (1.04-2.51) *	1.41 (0.84-2.37)
Preoperative CEA (ng/mL)[2]				
< 50	44	1	1	1
≥ 50	13	2.83 (1.10-7.27) *	2.25 (1.15-4.42) *	2.00 (0.93-4.30)
Number				
Solitary	59	1	1	1
Multiple	69	2.25 (1.19-4.23) *	1.02 (0.68-1.55)	1.05 (0.65-1.71)
Maximum size (cm)				
< 3.0	43	1	1	1
3.0-5.0	47	0.75 (0.38-1.47)	0.84 (0.51-1.37)	0.91 (0.50-1.66)
≥ 5.0	38	0.87 (0.42-1.81)	1.05 (0.63-1.76)	1.67 (0.93-3.00)
Distribution				
Unilobar	73	1	1	1
Bilobar	55	1.79 (1.00-3.20) *	1.17 (0.77-1.78)	0.89 (0.54-1.46)
Duration of operation (min)				
< 250[3]	64	1	1	1
≥ 250	64	1.11 (0.62-1.99)	0.93 (0.62-1.41)	0.72 (0.44-1.17)
Extent				
Minor (< 3 segments)	40	1	1	1
Major (≥ 3 segments)	88	1.79 (0.89-3.6)	1.00 (0.64-1.56)	1.09 (0.64-1.84)
Type				
Anatomical	73	1	1	1
Non-anatomical	55	1.08 (0.60-1.94)	1.29 (0.85-1.95)	0.75 (0.46-1.23)
Resection margin				
R0	112	1	1	1
R1	16	1.50 (0.66-3.37)	1.60 (0.90-2.83)	1.64 (0.81-3.33)
Blood loss (ml)				
< 2000	85	1	1	1
≥ 2000	43	1.00 (0.50-2.00)	0.95 (0.58-1.56)	1.06 (0.59-1.89)
Blood transfusion				
No	45	1	1	1
Yes	83	1.04 (0.55-1.96)	0.72 (0.47-1.10)	1.14 (0.67-1.93)
< 2 units	80	1	1	1
≥ 2 units	48	0.94 (0.51-1.72)	0.71 (0.40-1.13)	0.99 (0.61-1.63)
Vascular clamping				
no	61	1	1	1
yes	67	1.83 (1.00-3.49) *	1.16 (0.77-1.75)	0.83 (0.51-1.34)
minor	81	1	1	1
severe	47	1.78 (1.00-3.17) *	1.61 (1.06-2.46) *	1.22 (0.74-2.00)

* significant prognostic factor in univariate regression model (p<0.050) [1] Median age was used as a cut-off point; [2] CEA 71 (55%) missing values; [3] Median operating time was used as a cut-off point.

Effect of vascular clamping and severe ischemia on patterns of recurrence and survival

First, we analyzed whether vascular clamping of any type (i.e., irrespective of the type or duration) would affect liver free survival, disease free survival and overall survival by univariate analysis (**Table 3**). The time to develop a liver recurrence was significantly reduced when patients were subjected to vascular clamping of any type (**Figure 1A**). Vascular clamping of any type did not affect disease free survival or overall survival. Second, we investigated whether outcome would be affected by prolonged vascular clamping, defined as severe ischemia (**Table 3**). Now, not only liver free survival was significantly shorter (**Figure 1B**), also disease free survival was significantly decreased after severe ischemia (**Figure 1C**). Overall survival was not significantly different in patients who had undergone severe ischemia versus patients who did not.

Multivariate regression analysis was performed with correction for possible confounders, including both significant variables in the univariate analysis and variables that correlated with the severity of ischemia (**Table 4**). Although the trend towards decreased time to liver recurrence after both clamping of any type and severe ischemia in the multivariate analysis was maintained, it did not reach statistical significance, presumably due to the relative small number of patients with a liver recurrence in this population. Severe ischemia was maintained as an independent predictor for disease free survival in the multivariate analysis. Additional correction for preoperative CEA > 50 ng/mL in 57 patients where it was available, did not noticeably change the likelihoods for liver recurrence, overall recurrence and mortality. Likewise, additional correction for age, gender, extent and type of resection and blood transfusion did not alter the association between severe ischemia and time to liver recurrence, overall recurrence and mortality.

Effects of severe ischemia on long-term outcome according to age, gender and the presence of hepatic steatosis.

Finally, we investigated whether the adverse effect of severe ischemia on long term outcome might depend, in part, on age, gender or the presence of hepatic steatosis (**Table 5**). Surprisingly, stratification for age in the multivariate analysis revealed a more pronounced adverse effect of severe ischemia on both LFS and DFS for patients older than 62 years. Moreover, stratification for gender revealed a pronounced adverse effect of severe ischemia on LFS and DFS in males, but not in females (**Figure 2**). Forty six patients (35.9%) had mild to severe steatotic changes in their livers, as observed from pathological reports. The adverse effect of severe ischemia on LFS, but not DFS, was enhanced in hepatic steatotic livers.

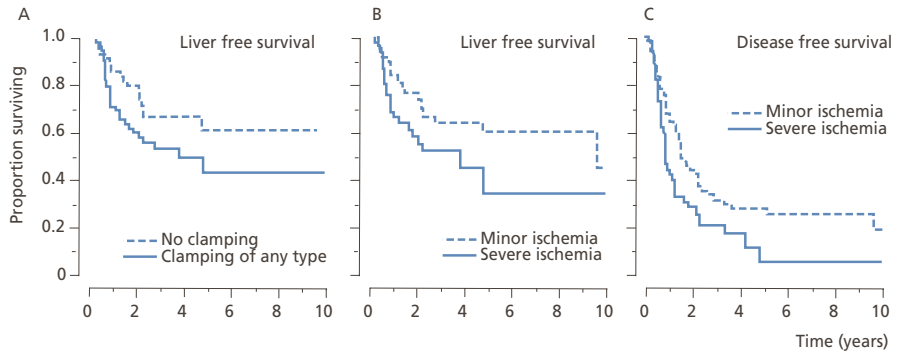

Figure 1. Kaplan-Meier curves illustrating the effects of clamping of any type on liver free survival (**A**, p=0.049, log rank test) the effect of severe ischemia on liver free survival (**B**, p=0.046, log rank test) and the effect of severe ischemia on disease free survival (**C**, p=0.023, log rank test).

Table 4. Multi variate analysis for liver recurrence, overall recurrence and mortality

	Liver recurrence[1]		Overall recurrence[2]		Mortality[3]	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Positive nodal stadium primary	**2.07 (1.0 6-4.06)**	**0.033**	**1.71 (1.08-2.69)**	**0.021**	-	-
Preoperative CEA ≥50 ng/mL	**5.83 (1.93-17.60)**	**0.002**	**2.58 (1.24-5.40)**	**0.012**	-	-
Clamping of any type	1.71 (0.90-3.23)	0.0991	1.14 (0.74-1.75)	0.5652	0.83 (0.51-1.34)	0.4433
Severe ischemia	1.76 (0.96-3.23)	0.0671	**1.65 (1.07-2.53)**	**0.0252**	1.22 (0.76-2.04)	0.4383

[1] Corrected for nodal status of the primary, disease free interval between primary and metastases, number and distribution of the metastases, duration of the operation and blood loss; [2] corrected for nodal status of the primary, disease free interval between primary and metastases, duration of the operation and blood loss; [3] corrected for duration of the operation and blood loss; additional correction for CEA (71 missing values) did not alter the association between severe ischemia and time to liver recurrence, overall recurrence or mortality.

Table 5. Multivariate analyses for severe ischemia and the risk for liver recurrence, overall recurrence and mortality according to age, gender and the presence of liver steatosis

	Liver recurrence[1]		Overall recurrence[2]		Mortality[3]	
Severe ischemia	HR (95%CI)	P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
Age < 62.5	1.33 (0.60-2.95)	0.481	1.37 (0.75-2.51)	0.308	1.13 (0.55-2.32)	0.736
Age > 62.5	**4.03 (1.49-10.93)**	**0.006**	**2.36 (1.22-4.56)**	**0.010**	1.49 (0.73-3.06)	0.423
Female	0.91 (0.33-2.54)	0.860	1.27 (0.59-2.76)	0.543	0.93 (0.41-2.13)	0.867
Male	**2.61 (1.18-5.76)**	**0.018**	**1.94 (1.11-3.37)**	**0.019**	1.27 (0.68-2.38)	0.455
Normal liver	1.45 (0.73-2.89)	0.289	1.60 (0.68-2.64)	0.230	1.13 (0.65-1.96)	0.665
Steatotic liver	**9.56 (1.31-70.00)**	**0.026**	1.84 (0.98-4.98)	0.061	2.47 (0.67-9.09)	0.174

[1] Corrected for nodal status of the primary, disease free interval between primary and metastases, number and distribution of the metastases, duration of the operation and blood loss; [2] corrected for nodal status of the primary, disease free interval between primary and metastases, duration of the operation and blood loss; [3] corrected for duration of the operation and blood loss; *P-values indicate significance of severe ischemia compared to minor ischemia in each subgroup;

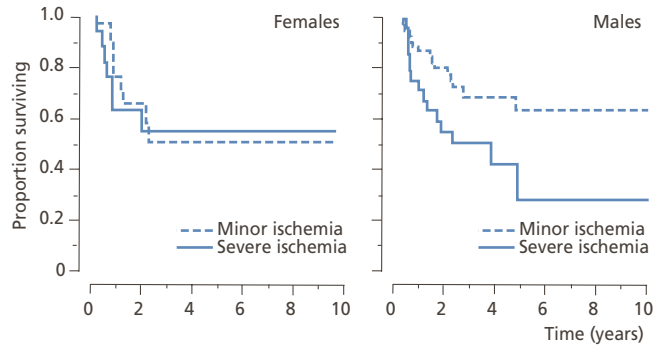

Figure 2. Kaplan-Meier curves illustrating the effect of severe ischemia on liver free survival in females (p=0.784, log rank test) and males (p=0.020, log rank test).

Discussion

Approaches to control intra-operative bleeding have been long recognized as a primary goal in liver surgery and include vascular clamping methods. In our patient analysis, we found that the time to develop liver recurrence was significantly shorter in patients who had been subjected to vascular clamping of any type. When ischemia times were prolonged, as in the severe ischemia group, disease free survival was decreased as well. From these findings it may be suggested that vascular clamping, irrespective of the total ischemia time, affects the local outgrowth of microscopic tumor deposits, whereas the systemic acceleration of tumor growth seems only present when ischemia times are long. These adverse effects were most prominent in males, older patients and in steatotic livers and appear to be irrespective of the clamping method used. Despite the retrospective nature of this study, we believe that the observed adverse effects of vascular clamping on long-term outcome may well represent clinical significance. To appreciate these findings, some strengths and limitations of our study are discussed.

In the past decades, many prognostic factors of recurrence and survival after hepatectomy for metastatic colorectal cancer have been documented.[1,7] Several experimental and human studies have pointed towards surgery-induced acceleration of tumor growth by various intra- and postoperative factors,[21] including blood transfusion,[9,11] hypotension,[8] portal vein embolisation,[22] and morbidity.[12] Surprisingly, very little has been described about the putative adverse effects of vascular occlusion on recurrence and survival, as revealed by a previously performed extensive systematic review of the literature. Among all studies describing prognostic factors in patients undergoing partial liver resection for colorectal liver metastases, three studies from two different centers had included vascular clamping in their analysis.[12,18,19] Although no significant adverse effect of vascular clamping was observed in these studies, the studies lack a detailed evaluation of the techniques or ischemia times used and for these reasons should be considered inconclusive.

Vascular clamping methods have been traditionally analyzed as a predictor of postoperative morbidity and mortality. Among studies investigating the impact of different occlusion techniques on short-term results, only one had addressed long-term outcome.[17] In this study, the groups were highly heterogeneous, making a good comparison impossible. Two other studies comparing different clamping methods in patients with hepatocellular carcinoma, demonstrated a shorter disease free and overall survival with increasing magnitude of ischemia.[23,24]

The results obtained from the present study are supported by our recent observations in a standardized murine model of hepatic ischemia/reperfusion induced by vascular clamping.[20] In this model, we found that the outgrowth of pre-established micrometastases was accelerated five to sixfold in clamped liver lobes versus non-clamped lobes. Accelerated tumor growth could be completely prevented when blood flow was occluded intermittently or by selective clamping of the portal vein,[25] whereas ischemic preconditioning could not protect against this phenomenon.[20] We have observed increased tumor outgrowth in different tumor cell lines using different animal models, suggesting that it represents a general phenomenon.

Preferably, these issues should obviously be corroborated in randomized prospective clinical trials, however this will likely be unfeasible due to ethical considerations. In most liver surgery units around the world vascular clamping during liver resection is not a standard procedure (as in our institution), but is applied in case of excessive blood loss only. Randomization between clamping and no clamping implies that some patients would be subjected to a procedure which is potentially harmful and on the other hand some patients with accelerating blood loss would be withheld from a maneuver which may be needed to perform a safe resection. Therefore, at present, we can solely rely on pre-clinical models and the present retrospective patient analysis, in which both the type and duration of vascular occlusion were taken into consideration. In combination with the dramatic effects observed in our experimental models, we believe that the adverse effects of vascular clamping on long-term outcome well represent clinical significance.

As a matter of course, a retrospective analysis harbors the risk of an inevitable indication bias, as vascular clamping is performed only in case of excessive blood loss. As a result, blood loss in the clamping group was significantly increased, which is different from other reported series in which vascular clamping was chosen in a prospective manner. One might be inclined to think that the observed poorer prognosis in patients subjected to prolonged vascular clamping would be associated with the complexity of the operation. No differences between the groups could be found for the number of lobes resected, type of resection, number, size and distribution of the metastases, but severe ischemia was associated with increased operating times and with increased blood loss. Nonetheless, neither prolonged operating times nor increased blood loss adversely affected outcome and after correction of these and other possible confounders in the multivariate analysis, severe ischemia was still maintained as an independent prognostic factor of overall recurrence. Severe ischemia did not influence overall survival, which may be explained by the aggressive approach to treat recurrent disease and the application of adjuvant chemotherapy in several patients.

Interestingly, the adverse effect of severe ischemia on outcome was most significant in males, older patients and patients with hepatic steatosis. It has been recently shown in animal models of hepatic ischemia/reperfusion that age largely determines the magnitude of hepatic injury.[26,27] Moreover, recent data provide evidence for a protective role of estrogens in females for developing ischemia/reperfusion injury,[28,29] and ischemic injury is believed to be more severe in diseased livers.[30,31]

Ischemia/reperfusion may increase the growth rate of tumor cell deposits by mechanisms yet unidentified. Hypothetically, I/R-induced microcirculatory disturbances and the subsequent prolonged tissue hypoxia may favor intrahepatic tumor growth by upregulating several hypoxia-dependant cell programs that stimulate proliferation or tumor angiogenesis.[32,33] Alternatively, the massive inflammatory response as observed after ischemia/reperfusion may enhance metastasis outgrowth by secretion of numerous cell stimulating cytokines and growth factors.[34] These issues are not only important in case of vascular clamping, but they are also relevant for other peri-operative events that may cause ischemia/reperfusion injury, such as surgical manipulation, hemorrhagic shock and sepsis.

Taken together, if prolonged vascular clamping indeed stimulates the growth of residual hepatic and systemic micrometastases, it should be omitted in patients where it is not mandatory. With the introduction of approaches that contribute to a more blood less hepatic resection, such as the maintenance of low venous pressure,[35] intra-operative ultrasonographic guidance, precoagulation devices,[36] and haemostatic biologicals,[37] vascular clamping can be omitted more often.[38] Nonetheless, in case of excessive hemorrhage, the risk of blood loss should outweigh the risk of vascular clamping. When vascular control is needed, intermittent clamping is preferred and total occlusion times should be kept to a minimum. When ischemia times are inevitably long, future strategies that counteract the adverse effects of surgery-induced ischemia/reperfusion on tumor growth may include drugs that improve post-ischemic microcirculation, anti-angiogenic therapies or anti-inflammatory agents. Adjuvant chemotherapy destructing possible

residual tumor deposits combined with strategies that protect against accelerated tumor growth may hopefully result in further improvement in long term outcome.
In conclusion, severe ischemia resulting from prolonged vascular clamping during hepatic resection for colorectal liver metastases may well be associated with decreased time to hepatic and overall recurrence. The adverse effect of prolonged vascular clamping on outcome is most prominent in males, older patients and in hepatic steatosis and appears to occur irrespective of the clamping method used. In case of inevitable clamping, total ischemia time should be kept to a minimum.

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Chapter 6

Ageing and hepatic steatosis exacerbate ischemia/ reperfusion-accelerated outgrowth of colorectal micrometastases

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Abstract

Background Ischemia/reperfusion (I/R) injury is frequently encountered during hepatic surgery and the severity is influenced by several (patho)physiological parameters. We recently showed that I/R accelerates the outgrowth of pre-established colorectal micrometastases. The aim of this study was to assess the influence of ischemia time, gender, age and liver steatosis on the accelerated outgrowth of colorectal metastases following I/R.

Methods Five days after tumor cell inoculation, mice were subjected to 20, 30 or 45 minutes of left lobar I/R. To assess the influence of age, gender, and liver steatosis on I/R accelerated tumor growth, we compared old with young mice, male with female mice and mice with healthy livers with mice with steatotic livers. Endpoints were extent of tissue necrosis and tumor growth.

Results With increasing ischemia times, tissue necrosis and I/R-accelerated tumor growth increased, with a significant stimulatory effect at 30 and 45 minutes of ischemia. I/R-stimulated outgrowth of micrometastases was further increased by 33% in aged mice and by 42% in steatotic livers and was associated with increased tissue necrosis. In female mice tissue necrosis had decreased by 47% and tumor growth was reduced in both control and clamped liver lobes. The stimulatory effect of I/R on metastasis outgrowth was similar in male and female mice.

Conclusion I/R-accelerated outgrowth of colorectal micrometastases largely depends on the duration of the ischemic period, with a safe upper limit of 20 minutes in mice. The stimulatory effects of I/R on tumor growth are exacerbated in aged mice and in steatotic livers.

Introduction

Colorectal cancer is one of the worlds' leading causes of cancer-related deaths. Mortality is strongly associated with the development of liver metastases, which eventually occurs in 50-70% of colorectal cancer patients. In selected patients, partial liver resection remains the standard treatment of choice, as it still offers the only hope for cure.[1,2] However, despite the curative intent of partial liver resection, the majority of patients ultimately develop recurrent disease from previously undetected micrometastases.[2-4]

During hepatectomy, the prime concern of the surgeon is to safely perform a radical resection, without excessive blood loss. Therefore, vascular clamping techniques are frequently applied.[5,6] The disadvantage of vascular clamping is ischemia/reperfusion (I/R) injury to the liver parenchyma, which may contribute to postoperative hepatic dysfunction and morbidity. Moreover, we recently found that I/R resulting from vascular clamping accelerated the outgrowth of pre-established colorectal micrometastases in a preclinical model.[7] Likewise, prolonged vascular clamping was associated with decreased time to develop liver recurrence and with decreased disease free survival in patients undergoing an R0 resection for colorectal liver metastases.[8] Interestingly, in this population, the adverse effect of prolonged vascular clamping on outcome appeared to be most pronounced in older patients, in males, and in patients with hepatic steatosis.

The adverse effects of I/R on hepatocellular injury have been well studied in rodent and clinical models and the magnitude of tissue damage depends on various (patho) physiological parameters. First, the degree of hepatocellular damage largely depends on the duration of vascular occlusion.[9-11] Second, I/R injury is enhanced in aged animals and patients.[12-14] Third, recent data provide evidence for a protective role of estrogens in developing I/R injury in females.[15-17] Finally, susceptibility to I/R damage largely depends on the presence of underlying liver disease, including hepatic steatosis.[18-21] Whereas the contribution of these parameters to hepatocellular damage have been well described, their effects on the outgrowth of micrometastases needs to be further evaluated. The aim of this study was to identify the conditions that modulate the stimulatory effect of vascular clamping on metastasis outgrowth.

Materials and methods

Animals

All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Male (10-12 weeks or 12-13 months) and female BALB/c mice (10-12 weeks) were purchased from Charles River (Sulzfeld, Germany). Male BALB/c young mice (10-12 weeks) served as standard controls and were used in all experiments, unless otherwise stated. For age and gender comparison, aged mice (12-13 months) and female mice (10-12 weeks) were used. Steatosis was induced in young BALB/c mice by feeding the animals a high fat diet for six weeks. The high fat diet was obtained from Arie Blok Diervoeding (Woerden, The Netherlands, cat.no. 4031.05) and consisted (in % of total energy) of 44% bovine fat, 19% protein and 37% carbohydrate. All animals were housed under standard laboratory conditions and had free access to water and chow.

Induction of hepatic micrometastases and hepatic I/R

Standardized surgical procedures were performed as previously described.[7,22] In brief, animals were operated under isoflurane inhalation anesthesia and buprenorfine (3µg/mouse) was administered intramuscularly prior to surgery to provide sufficient intra- and postoperative analgesia. Body temperature was maintained at 36.5-37.0˚C during the entire experiment.

We used the previously described liver metastasis model using C26 colon carcinoma cells in BALB/c mice.[7] In brief, through a left lateral flank incision, $5x10^4$ routinely cultured cells were injected into the splenic parenchyma. After ten minutes, the spleen was removed to prevent intrasplenic tumor growth. Intrahepatic micrometastases were allowed to grow out for five days.

Partial hepatic ischemia was induced five days after tumor cell inoculation.[7] In brief, after laparotomy, the liver hilus was exposed and the vascular inflow to the left lateral liver lobe was clamped for 20, 30 or 45 minutes. The influence of gender, age and hepatic steatosis was investigated by subjecting young male, aged male, young female and young male mice with steatotic livers to 30 minutes of left lobar I/R. Sham-operated animals underwent laparotomy with exposure of the liver and dissection of the vascular structures, but without interruption of hepatic blood flow. Five days later, all clamped and nonclamped liver lobes were harvested, fixed in 4% neutral buffered formalin and embedded in paraffin for morphological assessment of tissue necrosis and tumor load (n=8 each group).

Quantification of hepatocellular necrosis

The percentage of hepatocellular necrosis was scored on two nonsequential hematoxylin and eosin stained sections using an interactive video overlay system including an automated microscope (Q-Prodit, Leica Microsystems, Rijswijk, The Netherlands) at a magnification of x40. Using a four-points grid overlay the ratio of necrotic cells versus healthy hepatocytes plus tumor cells was determined on at least 100 fields per animal. The percentage of hepatocellular necrosis was expressed as the mean area ratio of all fields.

Quantification of intrahepatic tumor load

Intrahepatic tumor load was scored as the hepatic replacement area (HRA).[7] For each liver lobe at least 100 fields were selected on two nonsequential hematoxylin and eosin stained sections using an interactive video overlay system including an automated microscope (Q-Prodit) at a magnification of x40. 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 mean area ratio of all fields. Analyses were performed by two independent observers, blinded to the experimental protocol. Finally, HRA ratios were calculated for each animal to express the proportional increase in HRA in the clamped (left) lobes versus the nonclamped (right plus median) lobes.

Statistical analysis

Statistical differences between groups were analyzed by the Mann-Whitney U-test and ANOVA for nonparametric data. Data are expressed as mean ± sem.

Results

The influence of ischemia time on I/R-accelerated tumor growth

In sham-operated mice, tumor growth in the left liver lobes was similar to tumor growth in median plus right liver lobes (**Figure 1A**), resulting in a HRA ratio of approximately one (**Figure 1B**). Similar to our previous findings, the outgrowth of C26 coloncarcinoma cells was accelerated over fivefold in clamped liver lobes when compared with nonischemic lobes after 45 minutes of ischemia (**Figures 1A and B**). Microscopically, multiple diffuse confluent metastases were localized around areas of liver tissue necrosis, covering 59.4 ± 7.5% and 16.9 ± 3.4% of the liver parenchyma respectively (**Figures 1C and D**). With progressively shorter ischemia times, tumor growth in the clamped liver lobes gradually decreased (p<0.001) (**Figure 1A**). The outgrowth of micrometastases was still significantly accelerated after 30 minutes of ischemia, but not after 20 minutes of ischemia (**Figure 1B**). Accelerated tumor growth was associated with the presence of liver tissue necrosis, which also decreased with shorter ischemia times (**Figures 1C and D**).

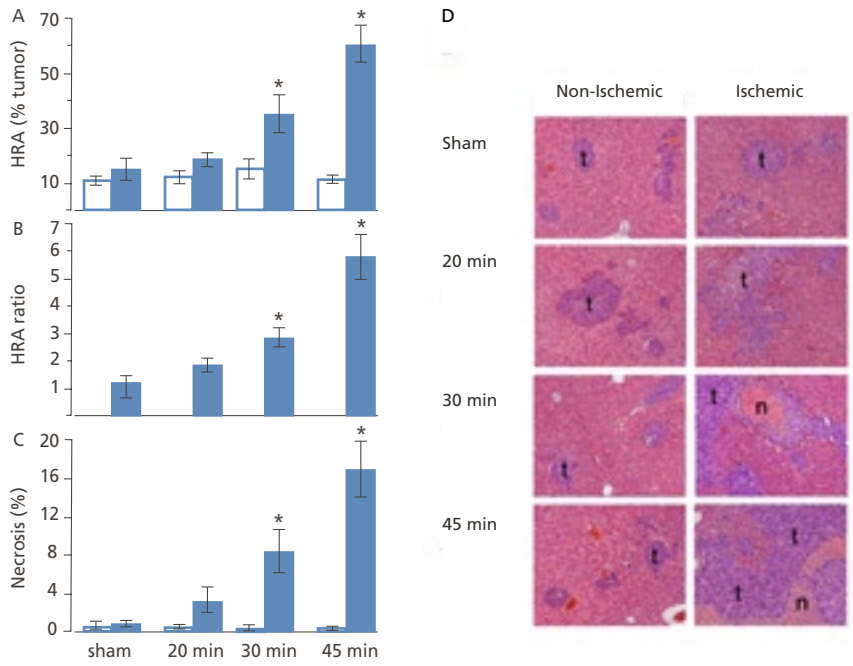

Figure 1. The influence of ischemia time on I/R-accelerated tumor growth. **(A)** Tumor growth, expressed as the hepatic replacement area (HRA), in the clamped and nonclamped liver lobes five days after sham operation and 20, 30 or 45 minutes of left lobar I/R. □, nonclamped liver lobes; ■, clamped liver lobes; *p<0.05 versus tumor growth in nonclamped liver lobes. **(B)** Stimulated tumor growth, expressed as the HRA ratio, the relative increase of tumor growth in the clamped liver lobes compared with the nonclamped liver lobes five days after sham operation and 20, 30 or 45 minutes of left lobar I/R. *p<0.05 versus tumor growth in sham-operated mice. **(C)** Tissue necrosis, quantified via morphometric analysis of clamped and nonclamped liver lobes at five days after sham operation and 20, 30 or 45 minutes of left lobar I/R. □, nonclamped liver lobes; ■, clamped liver lobes; *p<0.05 versus nonclamped liver lobes. **(D)** Microscopic appearance of I/R-accelerated outgrowth of micrometastases five days after sham operation and 20, 30 or 45 minutes of left lobar I/R, showing massive tumor (t) growth surrounding necrotic tissue areas (n). Original magnification x10.

The influence of age on I/R-accelerated tumor growth

In young control mice, the outgrowth of micrometastases was accelerated threefold after 30 minutes of I/R, similar to the above experiment (**Figures 2A and B**). In aged mice, tumor growth in the clamped liver lobes had increased to 47.6 ± 3.9% compared with 26.6 ± 4.3% in the clamped liver lobes of control mice (p=0.004) (**Figure 2A**). In nonclamped liver lobes, a nonsignificant increase in tumor growth was observed when compared with control mice (**Figure 2A**). When compared with young mice, the net acceleration of tumor growth in the clamped lobes of old mice had increased by 33% (p=0.048) (**Figure 2B**). Tissue necrosis did not significantly increase in aged mice (**Figure 2C**), but this was attributed to a massive invasion of tumor cells, overgrowing the necrotic areas (**Figure 2D**).

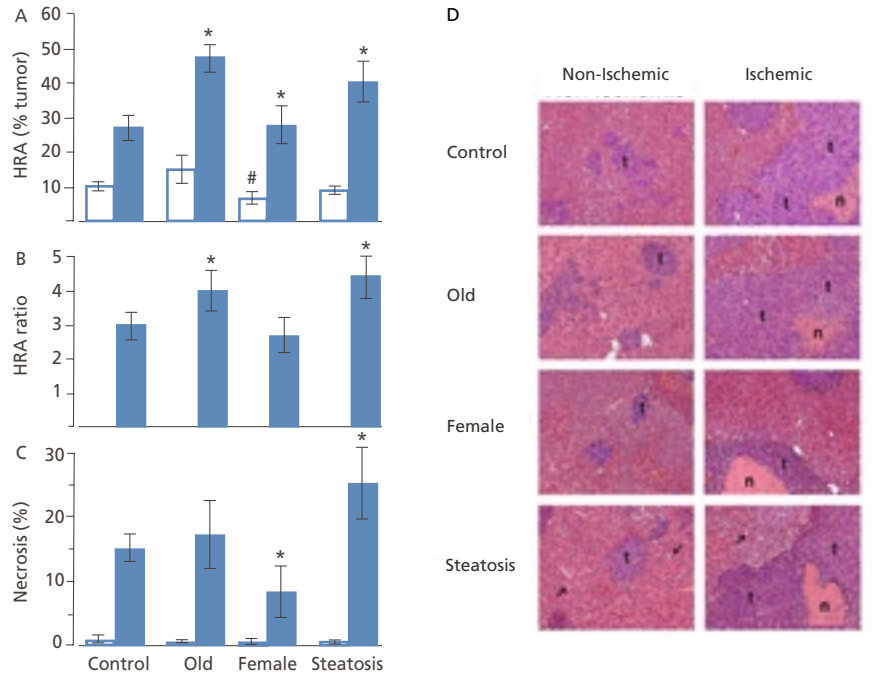

Figure 2. The influence of age, gender and hepatic steatosis on I/R-accelerated tumor growth. **(A)** Tumor growth, expressed as the hepatic replacement area (HRA), in the clamped and nonclamped liver lobes five days after 30 minutes of left lobar I/R. □, nonclamped liver lobes; ■, clamped liver lobes; *p<0.05 versus tumor growth in clamped liver lobes; #p<0.05 versus tumor growth in nonclamped liver lobes. **(B)** Stimulated micrometastasis outgrowth, expressed as the HRA ratio, the relative increase of tumor growth in the clamped liver lobes compared with the nonclamped liver lobes at five days post-clamping; *p<0.05 versus control. **(C)** Tissue necrosis, quantified via morphometric analysis of clamped and nonclamped liver lobes five days after I/R. □, nonclamped liver lobes; ■, clamped liver lobes; *p<0.05 versus tumor growth in nonclamped liver lobes. **(D)** Microscopic appearance of metastases in nonischemic and ischemic liver lobes in normal control mice, in aged mice, female mice and in mice with hepatic steatosis, showing massive tumor (t) growth surrounding necrotic tissue areas (n). Arrows indicate vacuolization in steatotic livers. Original magnification x10.

The influence of gender on I/R-accelerated tumor growth

In female mice, ischemic tissue damage was dramatically reduced, as shown by a 47% reduction in tissue necrosis when compared with male controls (p=0.040) (**Figures 2C and D**). Moreover, tumor growth in clamped liver lobes had reduced from 26.6 ± 4.3% in male mice to 13.0 ± 3.2% in female mice (p=0.030) (**Figure 2A**). However, tumor growth in the nonclamped liver lobes was also significantly reduced in female mice when compared their male littermates (10.7 ± 0.9% versus 6.5 ± 1.9%, p=0.030) (**Figure 2A**). This resulted in a relative two to threefold increase in tumor growth in clamped lobes compared with nonclamped liver lobes in female mice, which was not significantly different from that in male mice (**Figure 2B**).

The influence of hepatic steatosis on I/R-accelerated tumor growth

Six weeks of high fat diet induced mild to severe steatosis in all mice, as indicated by vacuolization in the liver parenchyma (**Figure 2D**, arrows). In steatotic mice, tissue damage was pronounced, as shown by a significant 64% increase in liver tissue necrosis when compared with control mice with healthy livers (p=0.036) (**Figure 2C**). Concomitantly, tumor growth in the clamped liver lobes increased by 51% when compared with tumor growth in clamped lobes of control mice (p=0.048) (**Figure 2A**). Tumor growth in nonclamped liver lobes was not affected in steatotic livers, resulting in a significant enhancement of the stimulatory effect of I/R on the outgrowth of micrometastases in steatotic livers (p=0.026) (**Figure 2B**). Tumor growth was invariably localized around areas of tissue necrosis (**Figure 2D**).

Discussion

In this study we assessed the influence of several (patho)physiological parameters on the accelerated outgrowth of pre-established micrometastases in a murine model of hepatic I/R. The stimulatory effects of I/R on tumor growth correlated with the duration of the ischemic period. The stimulated outgrowth of micrometastases was more pronounced in aged mice and in steatotic livers. In this experimental model, female mice were relatively resistant to tumor growth in general, but this did not protect them against accelerated outgrowth of colorectal micrometastases.

In the past decades the knowledge on I/R-induced tissue damage has dramatically increased and several (patho)physiological conditions that influence the degree of ischemic damage have been identified. The contribution of ischemia time to hepatocellular damage has been thoroughly evaluated in animal models, showing that prolonged ischemic periods induce time-dependent hepatocellular damage, liver failure and mortality.[9-11] The normal human liver seems to tolerate continuous normothermic ischemia of up to 60 minutes and intermittent ischemia of up to 120 minutes relatively well,[23-25] but even longer cumulative ischemia times have been applied with relatively mild and transient hepatic dysfunction and low complication rates.[26-28] Although prolonged vascular clamping may be performed without any severe postoperative morbidity or mortality, it may induce severe long-term adverse effects by accelerating the outgrowth of residual disease. In our animal study 45 minutes of ischemia induced transient liver dysfunction without any morbidity or mortality, but induced a five to sixfold local acceleration of tumor growth.[7] In the present study we found that the safe upper limit of vascular clamping is 20 minutes. Interestingly, in patients undergoing hepatic resection for colorectal liver metastases, continuous clamping of more than 20 minutes or intermittent clamping of more than 30 minutes significantly decreased liver recurrence free and disease free survival.[8] A European survey on vascular clamping showed that, in daily practice, vascular clamping is primarily performed intermittently and ischemic periods of more that 30 minutes are needed in approximately one out of ten patients undergoing hepatic resection.[6] The safe upper limit of intermittent clamping without adverse effects on prognosis still needs to be defined.

Age has been associated with an increased susceptibility to I/R damage, which may contribute to increased morbidity and postoperative mortality following vascular clamping.[12-14] Although major liver resection can be safely performed in selected elderly patients with minimal mortality, providing survival rates similar to younger patients,[29-31] it has also been suggested that older patients have a poorer long-term prognosis after hepatectomy.[32,33] In the present study we found that the stimulatory effects of I/R on the outgrowth of micrometastases was increased in aged mice when compared with younger controls, which is consistent with our clinical data.[8] With increasing age, the protective capacity of the liver cells against I/R-damage is diminished due to decreased production of endogenous anti-oxidants and an increased inflammatory response,[14,34] which may contribute to the increased stimulation of tumor growth. These findings imply that younger patients are relatively protected against the adverse effects of vascular clamping on outcome and thus protective strategies in older patients who need vascular clamping during hepatic surgery are warranted.

Moreover, gender has been implicated as a prognostic factor after hepatectomy by several authors,[35,36] whereas others did not show a correlation between gender and prognosis.[8,37] In addition, females are protected against ischemic damage, which was confirmed by the present study.[15-17] Although tumor growth was generally slower in female mice, the

stimulatory effect of I/R on the outgrowth of micrometastases was not reduced, which is different from our observation in patients. Possibly, recurrent intrahepatic tumor growth after clamping in female patients may become clinically manifest at a later stage due to the general slower growing rate of hepatic metastases. The correlation between gender, vascular clamping and outcome needs further evaluation in clinical trials, as this may help to further select candidates for pharmacological intervention to protect against the adverse effect of I/R on metastasis outgrowth.

Finally, patients with underlying liver disease, including hepatic steatosis, have increased susceptibility to ischemic damage,[19,21] which is reflected by a general reluctance to apply clamping in patients with such livers.[6] In the present study we clearly show that I/R induces an increase in hepatic tissue necrosis and the adverse effect of I/R on the outgrowth of micrometastases was concomitantly aggravated. Again, this is consistent with the findings in patients, as liver free survival and disease free survival were both significantly reduced in patients with hepatic steatosis.[8] It has been shown that microcirculatory disturbances are worsened in fatty livers, which may induce prolonged hypoxia.[20] Hypoxia is one of the most potent stimulators to tumor growth,[38,39] and may contribute to the observed exacerbated metastasis outgrowth in steatotic livers. Importantly, neo-adjuvant chemotherapy induces steatohepatitis in 19-92% of patients, which is associated with increased susceptibility to ischemic damage.[40-42] This implies that prolonged vascular clamping in patients who underwent neo-adjuvant chemotherapy may induce an increased risk for recurrent tumor growth after hepatectomy. This is a clinically relevant issue as, at present, an increasing number of patients receive neo-adjuvant chemotherapy.

In conclusion, in this experimental model of partial hepatic I/R, vascular clamping accelerated the outgrowth of pre-established micrometastases, with safe ischemia times of up to 20 minutes in mice. The stimulatory effects of I/R on tumor growth are most pronounced in old mice and in mice with hepatic steatosis, whereas females did not appear to be protected against the stimulatory effects of I/R on tumor growth. Further knowledge on the underlying mechanisms of I/R-stimulated tumor growth may lead to the development of therapeutic strategies for selected patients to minimize accelerated outgrowth of micrometastatic disease after I/R during liver surgery.

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Chapter 7

Selective portal clamping to minimize hepatic ischemia/reperfusion damage and avoid accelerated outgrowth of experimental colorectal liver metastases

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Abstract

Background Temporary vascular clamping during local ablation for colorectal liver metastases increases destruction volumes. However, it also causes ischemia/reperfusion (I/R) injury to the liver parenchyma and accelerates the outgrowth of microscopic tumor deposits. The aim of this study was to investigate the effects of selective portal clamping on hepatocellular damage and tumor growth.

Methods Mice carrying pre-established hepatic colorectal micrometastases underwent either simultaneous clamping of both the portal vein and the hepatic artery or selective clamping of the portal vein to the median and left liver lobes for 45 minutes. Sham-operated mice served as controls. Hepatic injury and tumor growth were assessed over time.

Results Standard inflow occlusion resulted in a rise in liver enzymes, a local inflammatory response and hepatocellular necrosis. The outgrowth of pre-established micrometastases was accelerated three to fourfold in clamped compared with non clamped liver lobes (27.4 versus 7.8%, $p<0.01$). Conversely, selective portal clamping induced minimal liver injury, tissue inflammation or hepatocellular necrosis, and completely stopped the accelerated outgrowth of micrometastases.

Conclusion Selective portal clamping does not induce liver tissue damage or accelerate micrometastasis outgrowth and may therefore be the preferable clamping method during local ablative treatment of hepatic metastases.

Introduction

Colorectal cancer is one of the most prevalent malignancies in the western world and is one of the world's leading causes of cancer-related deaths.[1] Mortality is strongly associated with the development of liver metastases, which eventually occurs in 50-70% of patients with colorectal cancer. Once liver metastases have developed, the natural course of the disease is associated with poor survival rates.[2-4] Partial liver resection remains the only hope for cure, offering 5-year survival rates of 30-40%, however, only 15-20% of patients are eligible for curative resection.[5-8] For non resectable liver metastases, local ablative techniques such as radiofrequency ablation and laser-induced thermotherapy may provide local control and increase life expectancy.[9-11] In these patients, the tumor is destroyed by heat from energy-transmitting sources, resulting in coagulative necrosis.[12] Unfortunately, even after an apparently complete destruction, most patients will have tumor recurrence in the liver within two years.[13,14] Several mechanisms may underlie tumor recurrence.[12-14] First, new regional intrahepatic recurrences may develop from previously undetected micrometastases, as reported in 60-90 % of patients.[10,13,15] Second, local recurrences in and around the primary lesion occur in 10-68% of cases and are the result of incomplete heat-destruction of tumor tissue, or from the outgrowth of previously undetected microsatellite lesions.[16] Insufficient heat diffusion at the tumor periphery may cause unsuccessful treatment, especially in tumors greater than 4 cm.[16,17] Finally, local recurrences may develop from viable tumor cells that survive around blood vessels due to the cooling effect of the bloodstream, i.e. the heat sink effect.[18,19] To overcome these obstacles, vascular inflow occlusion, or the Pringle manoeuvre, is advisable to reduce dissipation of the generated heat, providing increased destruction volumes and greater tumor-free margins.[14,20-23]

As 75% of hepatic blood flow is carried by the portal vein, the heat sink effect is determined mainly by portal flow. Indeed, selective clamping of the portal vein results in increases in lesion size similar to simultaneous clamping of the hepatic artery and the portal vein.[20,24-27]

The main drawback of interrupting the hepatic blood supply is ischemic injury to the liver parenchyma. On restoration of the blood flow, reperfusion injury will aggravate the ischemic damage, which may contribute to postoperative liver dysfunction. The adverse effects of ischemia/reperfusion (I/R) on the liver parenchyma have been well documented.[28-30] In addition, we have recently shown that the outgrowth of pre-established micrometastases was strongly stimulated following temporary vascular clamping.[31] We have hypothesized that selective clamping of the portal vein may not only prevent ischemic damage to the liver parenchyma, but may also protect against accelerated outgrowth of residual tumor cell deposits. The aim of this study was to assess the effects of selective clamping of the portal vein on hepatic tissue damage and micrometastasis outgrowth in a highly standardized murine model.

Materials and methods

Animals

All experiments were carried out 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 bought from Charles River (Sulzfeld, Germany) and were housed under standard laboratory conditions.

Murine model of hepatic ischemia/reperfusion

Animals were operated under inhalation anesthesia with a 1.5-2.0% isoflurane/oxygen mixture using a mask. Buprenorfine (3 µg/mouse) was given intramuscularly before surgery for intra- and postoperative analgesia. Surgical procedures were performed under aseptic conditions and surgical foil was placed over the laparotomy wound to avoid dehydration. Heparin was not administered. Body temperature was maintained at 36.5-37.5˚C by placing the animals on a heated table and covering them with aluminium foil. After all procedures, a small amount of saline was left in the abdominal cavity and the peritoneum and skin were separately closed with 5.0 vicryl. A model of partial I/R to the left plus median lobes was used, corresponding to approximately 70% of the liver mass. After laparotomy, the liver hilus was exposed and the portal vein was carefully freed from the hepatic artery by microsurgical dissection. By randomization, either both the portal vein and the hepatic artery were clamped simultaneously for 45 minutes or the portal vein to the left and median liver lobes was clamped selectively (n=8 each group). Sham-operated mice underwent laparotomy with exposure of the liver and dissection of the vascular structures, but without interruption of hepatic blood flow.

Blood pressure measurements

Blood pressure was measured in a separate set of mice by placing a 26 gauge catheter in the carotid artery in sham-operated mice and in mice subjected to 45 minutes of ischemia to the left and median lobes followed by 40 minutes of reperfusion (n=3 each group). Mean arterial blood pressure was continuously measured for at least 120 minutes from the onset of anesthesia.

Liver enzymes

The degree of early liver tissue damage was assessed in separate groups by plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Animals were allowed to recover after surgery and were reanesthetized for blood withdrawal following 6 hours of reperfusion (n=8 each group). Heparin plasma samples (500 µl) were obtained by cardiac puncture and centrifuged at 14.000 rpm for 10 minutes. Plasma levels of ALT and AST were analyzed using commercially available diagnostic kits (Instruchemie BV, Delfzijl, The Netherlands).

Cell culture and induction of liver micrometastases

The murine colon carcinoma cell line C26 was cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% carbon dioxide environment. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% ethylenediamine tetra-acetic acid) and, after centrifugation, single-cell suspensions were prepared in phosphate buffered saline to a final concentration of $5x10^4$ cells/100µl. Cell viability was determined by trypan blue staining, and was always at least 98%. Colorectal liver metastases were induced in the mice as follows: through a left lateral flank incision, $5x10^4$ C26 colorectal carcinoma cells were injected into the splenic parenchyma.[31-33] Single tumor cells reach the liver through the portal vein where a subset grows out to form intrahepatic micrometastases. After ten minutes, the spleen was removed to prevent intrasplenic tumor growth. Micrometastases were allowed to develop throughout the liver for 5 days. At that time, animals were subjected to clamping of the portal vein plus hepatic artery, selective clamping of the portal vein or sham operation. Morphological assessment of tumor growth was performed on clamped and non clamped lobes harvested 5 days later.

Tumor analysis

Intrahepatic tumor load was scored as the hepatic replacement area (HRA), the percentage of hepatic tissue replaced by tumor cells.[31,33] For each liver lobe, at least 100 fields were selected on two non sequential sections stained with hematoxylin and eosin using an interactive video overlay system including an automated microscope (Leica-Q-Prodit, Leica Microsystems, Rijswijk, The Netherlands) at a magnification of x40. Using a four-points grid overlay, the ratio of tumor cells to normal hepatocytes plus necrotic cells was determined for each field. Tumor load (HRA) was expressed as the mean area ratio of all fields. The two observers were blinded to treatment. Finally, HRA ratios between clamped and non clamped lobes were calculated for each animal to express the proportional increase in HRA in the clamped (left plus median) lobes versus the non clamped (right) lobes.

Quantification of hepatocellular necrosis

The percentage of hepatocellular necrosis was scored simultaneously with tumor HRA analysis on two non sequential hematoxylin and eosin stained sections. The ratio of necrotic cells to healthy hepatocytes plus tumor cells was determined for each field. The percentage of hepatocellular necrosis was expressed as the mean area ratio of all fields.

Statistical analysis

Statistical differences between groups were analyzed by the Mann-Whitney U-test for non parametric data. Values are expressed as mean ± sem, $p<0.05$ was considered statistically significant.

Results

The model of partial hepatic I/R induced by occluding the vascular inflow of the left lateral liver lobe has been described previously.[31] However, in mice, selective clamping of the portal vein at the level of the left lateral liver lobe is technically complicated. Therefore, a model of partial I/R to the left plus median lobes was used, corresponding to approximately 70% of the liver mass. Selective occlusion of the portal flow (**Figure 1A**) was confirmed by injection of methylene blue into the portal system, showing a lack of staining of the left and median lobes (not shown).

Hemodynamic stability in models of I/R is crucial, as hypotension and systemic hypo-oxygenation may induce temporary tissue ischemia.[34,35] In addition, hypothermia affects hemodynamic stability and reduces I/R-induced injury.[36] Therefore, special attention was paid to anesthesia, hemodynamic stability and body temperature. After clamping both the portal vein and hepatic artery to the left plus median lobes, blood pressure remained stable for at least 120 minutes before and during I/R (**Figure 1B**) without overt changes in blood pH, pO_2 or pCO_2 (not shown). Vascular inflow obstruction to 70% of the liver mass did not adversely affect hemodynamic stability in this model.

Standard clamping of the median and left liver lobes induced severe early hepatocellular damage, as shown by elevated plasma ALT and AST levels after 6 hours of reperfusion (**Figures 2A and B**). Areas of severe early hepatocellular damage were seen 6 hours after standard clamping in all clamped liver lobes, characterized by hemorrhage, eosinophilic hepatocytes, signs of nuclear pyknosis and loss of cell-cell contact (**Figure 2C**). Five days after standard clamping, necrotic areas were observed in all animals, covering 14% of the clamped liver tissue (**Figure 2D**). Thus, 45 minutes of 70% hepatic ischemia caused considerable liver tissue damage without any mortality, allowing long-term evaluation of hepatocellular damage and tumor growth.

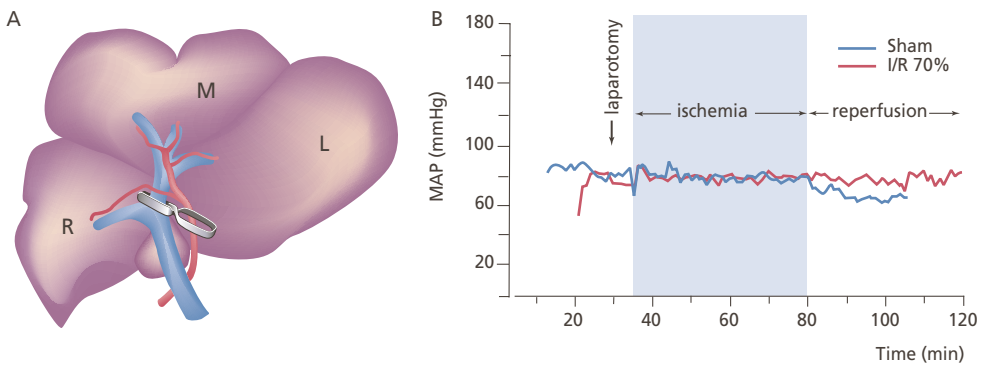

Figure 1. Standardized murine model of left and median lobar ischemia/reperfusion (I/R) of the liver. **(A)** Schematic image of selective portal clamping technique. The portal vein is carefully freed from the hepatic artery by microsurgical dissection and is selectively occluded to the left **(L)** and median **(M)** liver lobes by a microvascular clamp for 45 minutes, leaving the oxygen-rich blood supply via the hepatic artery intact. **(B)** Mean arterial blood pressure (MAP) during I/R of the left and median lobes in comparison to sham-operated mice.

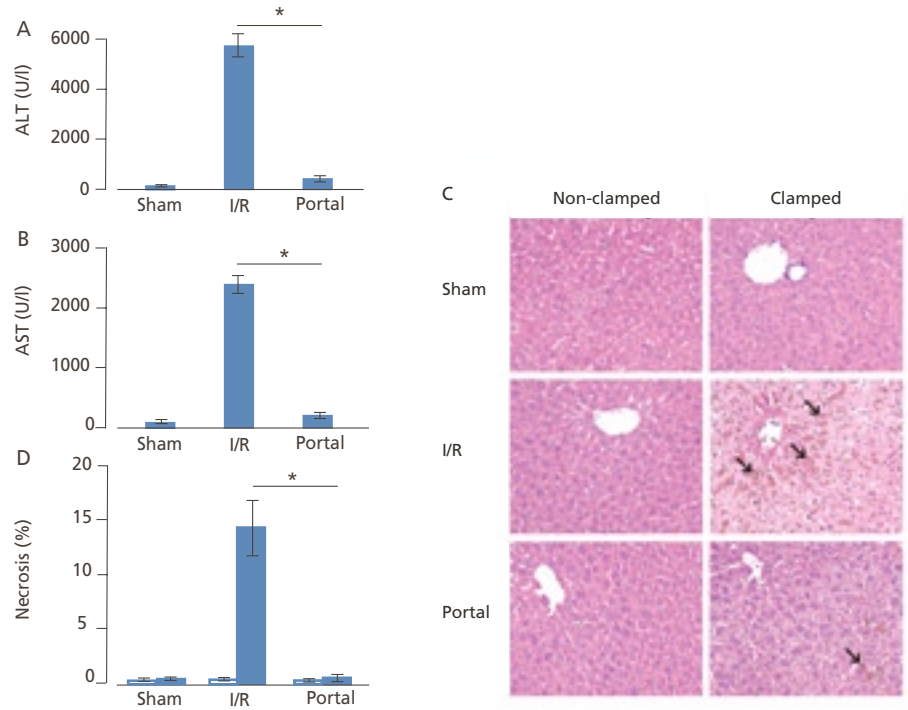

Figure 2. Hepatocellular injury after sham operation, ischemia/reperfusion (I/R) and portal clamping. **(A)** Plasma alanine aminotransferase (ALT) and **(B)** aspartate aminotransferase (AST) at 6 hours of reperfusion. **(C)** Microscopic hematoxylin and eosin stained sections of the nonclamped and clamped liver lobes, harvested 6 hours after clamping. After I/R, liver cell damage in the clamped lobes is characterized by hemorrhage (arrows), eosinophilic hepatocytes, signs of nuclear pyknosis and loss of cell-cell contact. Portal clamping induced minor haemorrhage in the clamped lobes (arrow) without signs of hepatocellular injury (original magnification x20). **(D)** The percentage of liver tissue necrosis 5 days after clamping. *p<0.01

Ten days after intrasplenic tumor cell injection, tumor load in the right lobe was similar to that in the left plus median liver lobes in sham-operated mice (**Figure 3A**, 6.9 ± 2.1 versus 5.3 ± 1.5%, p=0.571). Consequently, the ratio between the HRA values was approximately one (**Figure 3B**). Based on these results, it can be concluded that in this model the right liver lobe may serve as an internal control for tumor growth after selective clamping of the left and median lobes. Standard clamping of both the left and median lobes caused a significant increase in tumor load in occluded liver lobes (**Figure 3A**). Tumor growth was stimulated three to fourfold compared with the non occluded lobes (**Figure 3B**). Similar to our previous findings on selective left lobar pedicle clamping, accelerated tumor growth was located in peri necrotic tissue areas (**Figure 3C**), where tumor cells preferentially grew into zones of inflammation surrounding these necrotic areas.

It was reasoned that portal clamping alone would reduce ischemic damage, as the oxygen-rich blood supply through the hepatic artery would be maintained. Selective portal clamping prevented early hepatocellular damage by more than 90%, indicated by plasma ALT and AST levels 6 hours post-clamping (**Figures 2A and B**). Despite focal spots of minimal hemorrhage, no signs of severe liver cell damage were observed after portal

clamping compared with standard clamping (**Figure 2C**). In addition, late hepatocellular damage, or liver tissue necrosis, as occurred after standard clamping, did not ensue following selective portal clamping (**Figure 2D**). Next, it was determined how selective portal clamping affected the outgrowth of pre-established micrometastases. As demonstrated in **Figures 3A and B** selective clamping of the portal vein, in contrast to standard clamping, did not accelerate the outgrowth of micrometastases. Moreover, microscopic examination showed no signs of liver tissue necrosis or inflammation (**Figure 3C**).

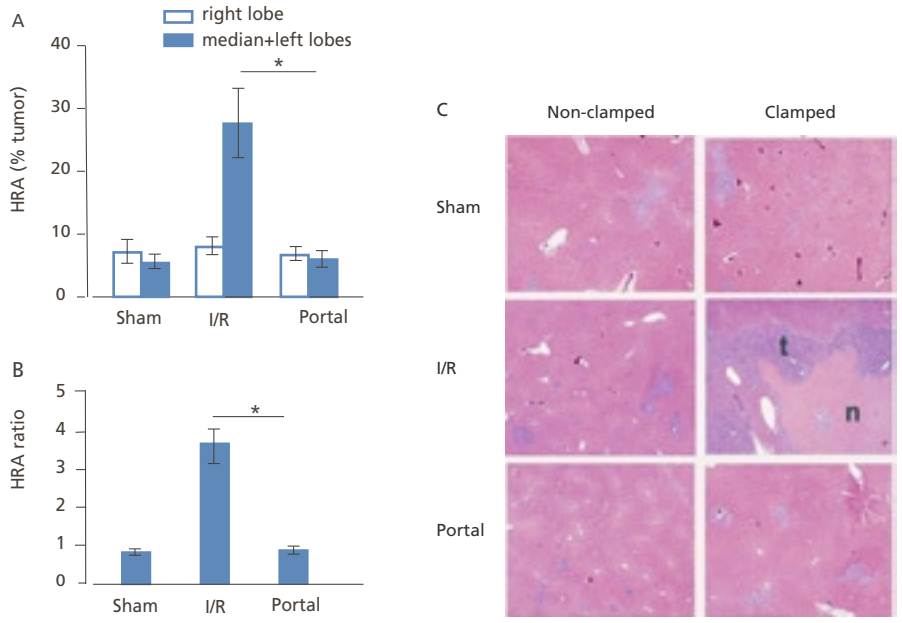

Figure 3. The outgrowth of pre-established micrometastases 5 days after sham operation, hepatic ischemia/reperfusion (I/R) and portal clamping. **(A)** Tumor growth, expressed as the hepatic replacement area (HRA). **(B)** Proportional increase in HRA in the clamped (left plus median) lobes versus the nonclamped (right) lobe, expressed as HRA ratio. *p<0.01 **(C)** Microscopic hematoxylin and eosin stained sections of the nonclamped and clamped liver lobes, harvested 5 days after clamping. I/R-induced tumor growth acceleration (t, tumor cells) is observed around necrotic areas (n) (original magnification x10).

Discussion

I/R due to standard inflow occlusion of the left plus median liver lobes for 45 minutes resulted in a three to fourfold increase in outgrowth of pre-established micrometastases. This is in concordance with our previous findings on selective left lobar clamping.[31] When the portal vein to the left and median lobes was selectively clamped, leaving the oxygen-rich blood supply via the hepatic artery intact, liver tissue damage was minimal and outgrowth of micrometastases remained unaffected. Based on these results, it appears that ischemic tissue damage due to clamping of the arterial blood supply in addition to the portal vein is crucial for stimulation of tumor growth following I/R in the liver.

Several mechanisms may underlie the stimulating effect of I/R on tumor outgrowth. First, although relatively short, the oxygen deprivation during standard clamping may directly activate several hypoxia-dependent proliferation-stimulating signaling pathways in the tumor cells.[37,38] During portal clamping, the oxygen supply to the liver tissue is maintained, which prevents tissue hypoxia and, by inference, hypoxia-stimulated tumor growth. Second, ischemic damage following standard clamping is characterized by widespread liver cell death, the influx of inflammatory cells and microcirculatory disturbances. These local responses may create an ideal milieu for tumor cell proliferation or invasion. Portal clamping did not result in severe liver tissue damage, as liver enzymes were only marginally increased and only minor histopathological changes were observed. Selective portal clamping may avoid stimulation of tumor growth by failing to induce liver tissue damage.

The acceleration of tumor growth following vascular clamping could theoretically decrease the life-expectancy of patients undergoing local tumor ablation under standard hepatic inflow occlusion, but clinical data to support this are lacking. As local ablative treatments become more accepted for nonresectable liver metastases, an increasing number of patients may be expected to undergo hepatic clamping. During local ablation, particularly of large colorectal liver metastases (larger than 4 cm) or lesions close to large vessels, it is essential to occlude intrahepatic blood flow to obtain a safe margin around the ablated tumor. Moreover, to avoid unacceptably high local recurrence rates, inflow occlusion has been shown to be mandatory in a recently published meta-analysis on this topic.[14] Here an alternative occlusion technique is presented that minimizes I/R injury, successfully reduces the heat sink effect,[20,24-26,39,40] and eliminates the risk of accelerated tumor outgrowth after standard inflow occlusion (this study).

Portal clamping has several advantages over standard inflow occlusion, but its application in surgical practice may depend on the specific clinical circumstances. Selective clamping of the portal vein may be the clamping method of choice during open local ablation and during ablation of multiple liver tumors, particularly when the expected ablation time is relatively long. In those patients, occlusion time during surgery is not restricted as oxygen supply to the liver is maintained. Moreover, the hepatoprotective effect of portal clamping may contribute to a decreased postoperative morbidity. Portal clamping may be particularly useful in high-risk patients in whom partial resection was abandoned because of poor medical condition. Nonetheless, in patients who have undergone previous liver surgery, portal clamping is technically challenging because of adhesion formation in the perihilar region, and may increase operating times.

Intermittent inflow occlusion may also be a safe alternative during local ablation, as complete absence of accelerated outgrowth of micrometastases following intermittent compared with continuous clamping has been reported.[31] For single tumors and after previous operations, intermittent clamping may be easier than portal clamping, as it

avoids dissection of the hepatoduodenal ligament. When large hepatic arteries run close to or within the tumor, the hepatic artery may be clamped in addition to the portal vein for short periods to avoid local recurrence that may develop from viable tumor cells around these arteries. For multiple tumors or complicated cases, the disadvantage of intermittent clamping is that it needs stringent time management and additional manipulation during ablation. In contrast, using continuous portal clamping, the operation is not delayed by successive periods of reperfusion between clamping periods.
Selective arterial embolization has also been reported to increase lesion size and may be easier than selective portal clamping during the laparoscopic and percutaneous approach.[27,41] However, several authors have shown that arterial clamping alone is not sufficient to produce larger coagulation diameters.[25,26]
In combination with image-guided probe positioning and monitoring,[42,43] and adjuvant chemotherapy,[44,45] selective portal clamping may improve the oncological outcome of patients with colorectal cancer.
In conclusion, selective portal clamping induces minimal post-ischemic liver tissue injury and does not promote outgrowth of resident micrometastases. Prospective studies in patients are required to assess whether selective portal clamping can help reduce hepatic tumor recurrence and improve the prognosis of patients undergoing local ablation of colorectal liver metastases.

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Chapter 8

Perinecrotic hypoxia contributes to ischemia/ reperfusion-accelerated outgrowth of colorectal micrometastases

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Abstract

Backround Ischemia/reperfusion (I/R) is often inevitable during hepatic surgery and may stimulate the outgrowth of colorectal micrometastases. Postischemic microcirculatory disturbances contribute to I/R damage and may induce prolonged tissue hypoxia and consequent stabilization of hypoxia-inducible factor (HIF)-1α. The aim of this study was to evaluate the contribution of postischemic microcirculatory disturbances, hypoxia, and HIF-1α to I/R-accelerated tumor growth.

Methods and results Partial hepatic I/R attributable to temporary clamping of the left liver lobe induced microcirculatory failure for up to 5 days. This was accompanied by profound and prolonged perinecrotic tissue hypoxia, stabilization of HIF-1α, and massive perinecrotic outgrowth of pre-established micrometastases. Restoration of the microcirculation by treatment with Atrasentan and L-Arginine minimized hypoxia and HIF-1α stabilization and reduced the accelerated outgrowth of micrometastases by 50%. Destabilization of HIF-1α by the HSP90 inhibitor 17-DMAG caused an increase in tissue necrosis but reduced I/R-stimulated tumor growth by more than 70%.

Conclusion Prevention of postischemic microcirculatory disturbances and perinecrotic hypoxia reduces the accelerated outgrowth of colorectal liver metastases after I/R. This may, at least in part, be attributed to the prevention of HIF-1α stabilization. Prevention of tissue hypoxia or inhibition of HIF-1α may represent attractive approaches to limiting recurrent tumor growth after hepatic surgery.

Introduction

The liver is the most common site for metastases, developing in more than 50% of colorectal cancer patients. In selected cases, hepatic resection is the only curative option offering 5-year survival rates of 30 to 40%.[1-4] For non-resectable metastases, focal heat-destruction therapies, such as radiofrequency ablation or laser-induced thermotherapy, have emerged as effective strategies to achieve tumor clearance and to potentially increase life-expectancy.[5-7] Unfortunately, the majority of patients ultimately develop recurrent disease from previously undetected micrometastases, predominantly in the liver. During liver resection, the blood flow to the liver is temporarily occluded to prevent excessive blood loss.[8-10] During local ablation, vascular clamping is applied to increase destruction volumes.[11,12] However, vascular clamping induces ischemia/reperfusion (I/R) injury to the liver parenchyma and may contribute to postoperative morbidity.[13-15] Moreover, we have recently demonstrated that the outgrowth of pre-established micrometastases was strongly stimulated after I/R in a murine model.[16] The mechanisms that contribute to this phenomenon are unknown.

Microcirculatory disturbances, attributable to an imbalance of vasoconstrictors (eg, endothelin-1) and vasodilators (eg, nitric oxide), play a pivotal role in the manifestation of I/R injury.[17-21] This no-reflow phenomenon has been extensively studied at the microcirculatory level, but measurements have been limited to relatively short-term reperfusion periods (0 to 120 minutes). Prolonged microcirculatory failure beyond 2 hours of reperfusion may lead to sustained hypoxia in the liver, but data to support this are currently lacking. In response to tissue hypoxia, the transcription factor hypoxia-inducible factor (HIF)-1α is stabilized and acts as a cellular survival factor.[22-28] HIF-1α is a strong stimulator of tumor cell proliferation, anaerobic metabolism, migration, and angiogenesis.[29-33] Thus, hypoxia and consequent stabilization of HIF-1α in tissue areas containing residual micrometastases may lead to enhanced tumor cell growth.

The aim of this study was to assess the role of microcirculatory disturbances and hypoxia on the outgrowth of micrometastases after I/R. Therefore, we investigated whether improvement of the microcirculation by restoring the endothelin-1/nitric oxide balance by means of Atrasentan and L-Arginine could reduce tissue hypoxia and thereby prevent the accelerated outgrowth of pre-established micrometastases after I/R. Moreover, we used the geldanamycin analogue 17-DMAG, which has been shown to induce a von Hippel-Lindau-independent degradation of HIF-1α,[34,35] and inhibit hypoxia-dependent tumor growth.[36,37] We investigated whether destabilization of HIF-1α by 17-DMAG could reduce I/R-accelerated tumor growth.

Materials and methods

Animals

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 to 12 weeks of age) were purchased from Charles River (Sulzfeld, Germany) and housed under standard laboratory conditions.

Drug characteristics

Atrasentan (ABT-627), a selective endothelin-A receptor antagonist, was kindly provided by Abbott Laboratories (Abbott Park, IL, USA). Atrasentan was dissolved in 4.2% $NaHCO_3$ and injected intravenously as a single bolus (10 mg/kg body weight) before ischemia, followed by continuous oral administration offered in the drinking water (10 mg/kg body weight per day).

The nitric oxide donor L-Arginine (Sigma-Aldrich, St. Louis, MO, USA) was administered via continuous subcutaneous infusion using Alzet osmotic minipumps (model 2001; Durect Corporation, Cupertino, CA, USA) at a dose of 30 µg/kg/minute starting before ischemia.

17-DMAG (Invivogen, San Diego, CA, USA) was dissolved in saline and administered by intraperitoneal injections at a preoperative dose of 15 mg/kg body weight, followed by three postoperative doses of 7.5 mg/kg body weight every 12 hours after ischemia.

Standardized murine model of hepatic I/R

We used an established model of partial hepatic I/R as previously described.[16] In brief, after a midline incision, temporary ischemia was induced by occluding the vascular inflow of the left lateral liver lobe for 45 minutes. Sham-operated mice underwent laparotomy with exposure of the liver but without interruption of hepatic blood flow. Surgical procedures were performed under isoflurane inhalation anesthesia. Body temperature was maintained at 36.5 to 37.0˚C. Non-tumor-bearing animals were subjected to left lobar I/R and were randomized to receive control vehicle or Atrasentan treatment combined with L-Arginine (n=8 each group). Sham-operated mice served as controls. After 2 hours, 6 hours, 24 hours, and 5 days of reperfusion, the animals were re-anesthetized for measurements of the hepatic microcirculation. At each time point, ethylenediamine tetra acetic acid plasma samples were collected via cardiac puncture for measurements of liver enzymes and livers were harvested and processed for pimonidazole and HIF-1α immunohistochemistry.

Liver metastases model

Colorectal liver metastases were induced in mice as described.[16,38,39] In brief, routinely cultured C26 colorectal carcinoma cells were injected into the splenic parenchyma ($5x10^4$ cells/100µl). After ten minutes, the spleen was removed to prevent intrasplenic tumor growth. Five days later, animals were subjected to left lobar I/R and randomized into different treatment groups (n=8 each group). Mice received either continuous administration of Atrasentan and L-Arginine or control vehicle. In a second experiment, I/R-treated mice received either 17-DMAG or control vehicle. Sham-operated mice served as controls (n=8 each group). Morphometric assessment of tumor growth and hepatic necrosis was performed on clamped and nonclamped liver lobes harvested 5 days later.

Intravital fluorescence microscopy

The hepatic microcirculation was analyzed by intravital fluorescence microscopy using a Nikon TE-300 inverted microscope (Uvikon, Bunnik, The Netherlands) equipped with a fluorescence filter for fluorescein isothiocyanate (excitation 450 to 490 nm, emission >515 nm).[40] For contrast enhancement, fluorescein isothiocyanate-labeled dextran (molecular weight 446.000, 2% dextran in 0.9% NaCl; 100 µl per 20 g of body weight) was injected intravenously and excited with blue light (450 to 490 nm). The midline incision was reopened, and the left liver lobe was exteriorized and moistened. The mice were placed on an inverted microscopic stage, using a template to minimize tension and respiratory movement. Body temperature was monitored and maintained at 36.5 to 37.0˚C during the entire experiment. Ten to fifteen fields were randomly selected per animal and recorded for ten seconds at a x40 magnification. Images were captured by a charge-coupled device camera (Exwave HAD; Sony, Badhoevedorp, The Netherlands) and relayed to a personal computer for offline analysis. Sinusoidal perfusion rates were analyzed by two independent observers, blinded to treatment. Sinusoidal perfusion rates were expressed as the percentage of the number of normally perfused sinusoids divided by the total number of sinusoids observed. Perfusion rates of sham-operated animals were set at 100%. Using this method, interobserver and intraobserver variability was less than 5%.

Liver enzymes

Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using commercially available diagnostic kits (Instruchemie BV, Delfzijl, The Netherlands) (n=8 each group).

Quantification of hepatocellular necrosis

The percentage of hepatocellular necrosis was scored on two nonsequential hematoxylin and eosin stained sections using an interactive video overlay system including an automated microscope (Q-Prodit; Leica Microsystems, Rijswijk, The Netherlands) at a magnification of x40. Using a four-points grid overlay, the ratio of necrotic cells versus healthy hepatocytes plus tumor cells was determined on at least 100 fields per animal (n=8 each group). The percentage of hepatocellular necrosis was expressed as the mean area ratio of all fields.

Pimonidazole and HIF-1α immunohistochemistry

Liver samples were harvested, fixed in 4% buffered formaldehyde, paraffin-embedded, and sectioned at 5 µm. Tissue sections of clamped and nonclamped liver lobes were hematoxylin and eosin stained for standard histology. The extent and localization of hypoxia were analyzed using the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1, 90201; Chemicon International, Temecula, CA, USA) (n=4 each group).[41,42] Under hypoxic conditions, pimonidazole binds to thiol groups in proteins, peptides, and amino acids and therefore serves as a valuable marker for measuring in vivo hypoxia at the cellular level. One hour before termination, pimonidazole was injected intravenously at a dose of 60 mg/kg. Pimonidazole adducts were visualized by immunohistochemistry by using fluorescein isothiocyanate-conjugated mouse anti-Hypoxyprobe-1 monoclonal antibody (1:100, TM 90529; Chemicon International), followed by anti-fluorescein isothiocyanate antibody labeled with horseradish peroxidase (1:50; DAKO, Glostrup, Denmark). For HIF-1α detection, a polyclonal rabbit antibody against mouse HIF-1α was used for incubation overnight (1:50, NB 100-449; Novus, Littleton, CO, USA) and goat anti-rabbit Power-Vision+ (Immunologic, Duiven, The Netherlands) with 2% mouse serum was used as

secondary antibody. Before immunolabeling, endogenous peroxidase activity was blocked using a solution of methanol and hydrogen peroxide. Antigen retrieval was performed by boiling sections for 20 minutes in 0.01 mol/L citrate buffer (pH 6.0) for pimonidazole staining and in ethylenediamine tetra acetic acid-buffered solution (pH 9.0) for HIF-1α staining. Reactions were developed using diaminobenzidine/H_2O_2 as a chromogen substrate. Primary-deleted negative controls were treated with the antibody diluent alone and were all free of nonspecific background staining. Immunostaining for HIF-1α was scored on three to four randomly selected fields at a magnification of x40 by two independent observers, blinded to treatment (n=6 each group). The nuclear and cytoplasmic staining patterns were scored as the product of the staining intensity (weak, 1+; moderate, 2+; strong, 3+) and the percentage of positive cells (1 to 10% of cells, 1+; 11 to 50% of cells, 2+; >50% of cells, 3+).

Quantification of tumor load

Intrahepatic tumor load was scored as the hepatic replacement area (HRA).[16,38,39] For each liver lobe at least 100 fields were selected on two nonsequential hematoxylin and eosin stained sections using an interactive video overlay system including an automated microscope (Q-Prodit; Leica Microsystems) at a magnification of x40. 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 mean area ratio of all fields. Observers were blinded to treatment. Finally, HRA ratios between clamped and nonclamped lobes were calculated for each animal to express the proportional increase in HRA in the clamped (left) lobes versus the nonclamped (right plus median) lobes.

Statistical analysis

Statistical differences between groups were analyzed using the Mann-Whitney U-test. Data are expressed as mean ± sem, unless otherwise stated.

Results

Microcirculatory failure after I/R is associated with prolonged perinecrotic tissue hypoxia

Intravital fluorescence microscopy revealed normal hepatic microcirculation with ~80% regularly perfused sinusoids in sham-operated animals throughout the study period. I/R induced severe sinusoidal perfusion failure, as shown by a 70% relative reduction of perfused sinusoids after 2 hours of reperfusion when compared with sham-operated mice (**Figure 1A**). Sinusoidal perfusion rates remained low during the 5-day reperfusion period demonstrating a long-term disturbance of the microcirculation. Concurrently, I/R induced severe hepatocellular injury as shown by elevated levels of plasma alanine aminotransferase (**Figure 1B**) and aspartate aminotransferase (**Figure 1C**), with a maximum increase at 6 hours of reperfusion followed by normalization after 5 days. Histopathologically, areas of severe hepatocellular damage were seen at 2 and 6 hours after ischemia in all clamped liver lobes, characterized by hemorrhage, eosinophilic hepatocytes, signs of nuclear pyknosis, and loss of cell-cell contact (**Figure 2A**). After 24 hours, areas of necrosis started to develop, characterized by a massive infiltration of neutrophils. Five days after I/R,

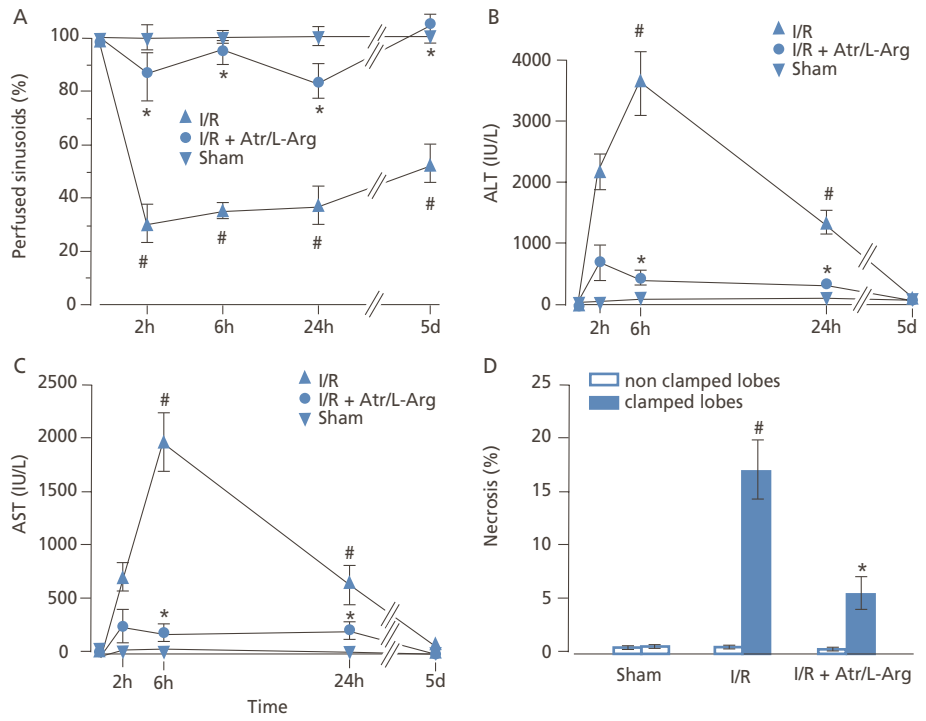

Figure 1. The effect of Atrasentan/L-Arginine therapy on I/R-induced microcirculatory disturbances and liver injury. **(A)** Hepatic sinusoidal perfusion rates 2 hours, 6 hours, 24 hours, and 5 days after sham operation, I/R, and I/R with Atrasentan/L-Arginine therapy (n=4 each group). **(B and C)** Hepatocellular damage measured 2 hours, 6 hours, 24 hours, and 5 days after I/R by plasma alanine aminotransferase (ALT) **(B)** and aspartate aminotransferase (AST) **(C)** levels (n=8 each group). **(D)** Tissue necrosis, quantified via morphometric analysis of clamped and nonclamped liver lobes at 5 days after clamping (n=8 each group). #p<0.05 versus sham; *p<0.05 versus I/R.

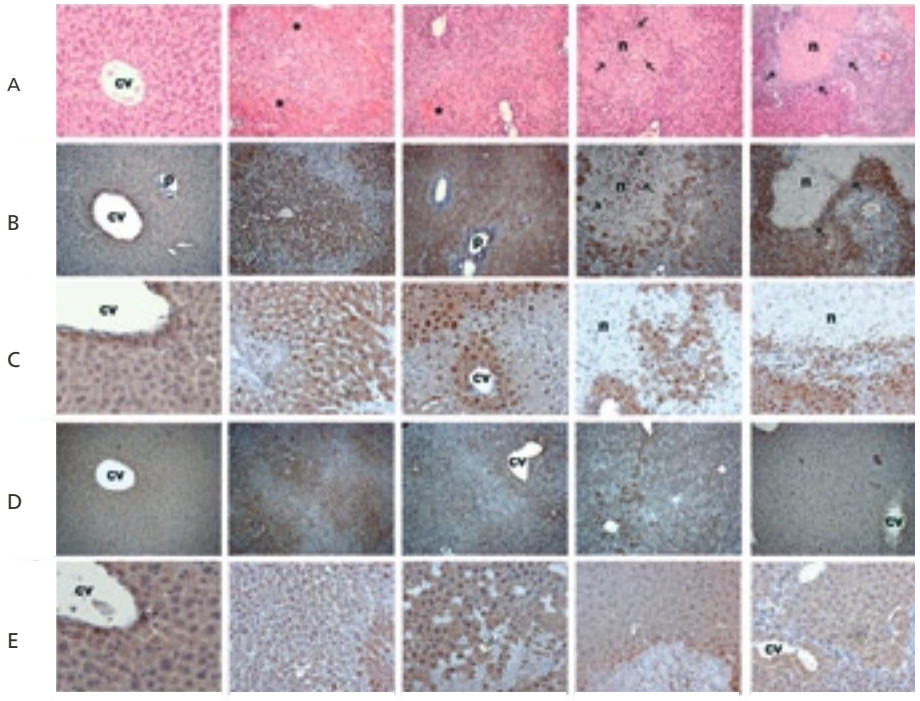

Figure 2. **(A)** Hematoxylin and eosin stained tissue sections showing hepatocellular damage characterized by hemorrhage (*), eosinophilic hepatocytes, signs of nuclear pyknosis, and loss of cell-cell contact at 2 and 6 hours of reperfusion, influx of neutrophils (arrows) after 24 hours, and areas of necrosis (n) surrounded by an inflammatory infiltrate (arrows) 5 days after I/R. **(B and D)** Tissue hypoxia as shown by pimonidazole immunohistochemistry (brown) 2 hours, 6 hours, 24 hours, and 5 days after hepatic I/R **(B)** and after hepatic I/R with Atrasentan/L-Arginine therapy **(D)**. **(C and E)** HIF-1α immunostaining (brown) 2 hours, 6 hours, 24 hours, and 5 days after hepatic I/R **(C)** and after hepatic I/R with Atrasentan/L-Arginine therapy **(E)**. n, necrosis; cv, central vein; p, portal region. Original magnifications: x10 (A, B and D); x20 (C and E).

necrotic areas were observed in all animals, covering 17 ± 3% of the clamped liver tissue **(Figure 1D)**. As reported previously, these necrotic areas were surrounded by a massive inflammatory infiltrate **(Figure 2A)**. The extent and localization of hepatic tissue hypoxia after I/R were assessed by pimonidazole immunohistochemistry. In control liver tissue of nonclamped lobes or of sham-operated mice, pimonidazole staining was exclusively observed around central venules, which are known to be characterized by relatively lower oxygen concentrations **(Figure 2B)**.[41] Two hours after I/R, profound diffuse tissue hypoxia was observed throughout the liver parenchyma of the clamped but not of the nonclamped lobes **(Figure 2B)**. During the ensuing 5 days, the liver parenchyma remained remarkably hypoxic. Whereas pimonidazole staining was diffuse after 2 hours of reperfusion, it was selectively localized around areas of hepatocellular necrosis at 5 days after clamping.

Increased expression of HIF-1α in hypoxic tissue areas

HIF-1α immunohistochemistry was performed on non-tumor-bearing tissue sections of livers from sham-operated mice and of clamped and nonclamped liver lobes 2 hours,

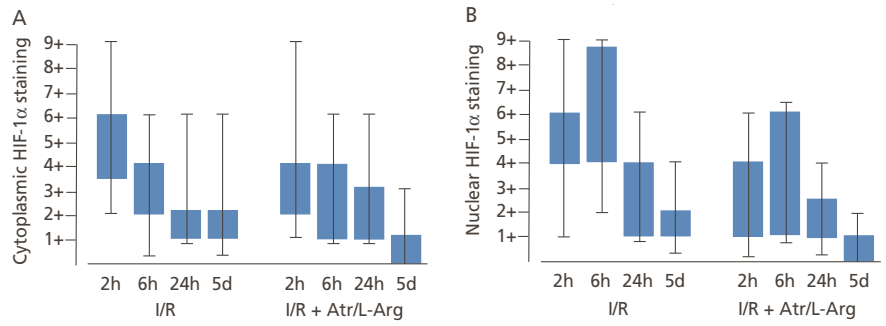

Figure 3. Cytoplasmic **(A)** and nuclear **(B)** HIF-1α immunostaining in clamped liver lobes 2 hours, 6 hours, 24 hours, and 5 days after hepatic I/R and after hepatic I/R with Atrasentan/L-Arginine therapy (n=6 each group). Immunostaining was scored as the product of the staining intensity (weak, 1+; moderate, 2+; strong, 3+) and the percentage of positive cells (1 to 10% of cells, 1+; 11 to 50% of cells, 2+; >50% of cells, 3+). Boxes indicate 25 to 75% interval, and lines indicate outer limits.

6 hours, 24 hours, and 5 days after I/R. In sham-operated mice and in the nonclamped liver lobes, HIF-1α staining was observed around central venules, similar to the pimonidazole staining **(Figure 2C)**.[43] Two hours after I/R, strong cytoplasmic and nuclear HIF-1α staining were observed in hepatocytes throughout the clamped liver lobes **(Figures 2C, 3A and 3B)**. After 6 hours of reperfusion, staining was mainly nuclear and was localized in zones surrounding tissue necrosis **(Figures 2C, 3A and 3B)**. From 24 hours onwards, both cytoplasmic and nuclear HIF-1α staining could still clearly be observed in perinecrotic zones **(Figures 3A and B)**, which is consistent with the perinecrotic pimonidazole staining **(Figure 2C)**. The nuclear staining at 5 days after clamping was partly attributable to nuclear staining of inflammatory cells surrounding the necrotic tissue areas.

Accelerated tumor growth occurs in areas of hypoxia and increased HIF-1α expression

I/R resulted in a six to sevenfold increase in micrometastasis outgrowth in occluded liver lobes when compared with non occluded liver lobes **(Figure 4A)**. Similar to our earlier observations, accelerated tumor growth was predominantly located around necrotic areas **(Figure 4B)**. We observed a strong association of I/R-stimulated tumor growth with areas of tissue hypoxia and increased parenchymal HIF-1α expression. Strikingly, high levels of HIF-1α were detected in the nuclei of tumor cells at the tumor-necrosis margin, 5 days after clamping **(Figure 4C)**. HIF-1α immunostaining in control tumor tissue in sham-operated mice is rare **(Figure 4C)**.

Attenuation of microcirculatory disturbances, hypoxia, and hepatocellular damage by Atrasentan/L-Arginine is associated with reduced tumor outgrowth

Treatment with Atrasentan/L-Arginine clearly restored the postischemic microcirculation, with a significantly higher percentage of perfused sinusoids at all time points when compared with the control vehicle-treated I/R group **(Figure 1A)**. Correspondingly, Atrasentan/L-Arginine treatment effectively reduced hepatocellular injury **(Figures 1B and C)**. In addition, tissue necrosis was reduced by 70% and covered 5 ± 2% of the

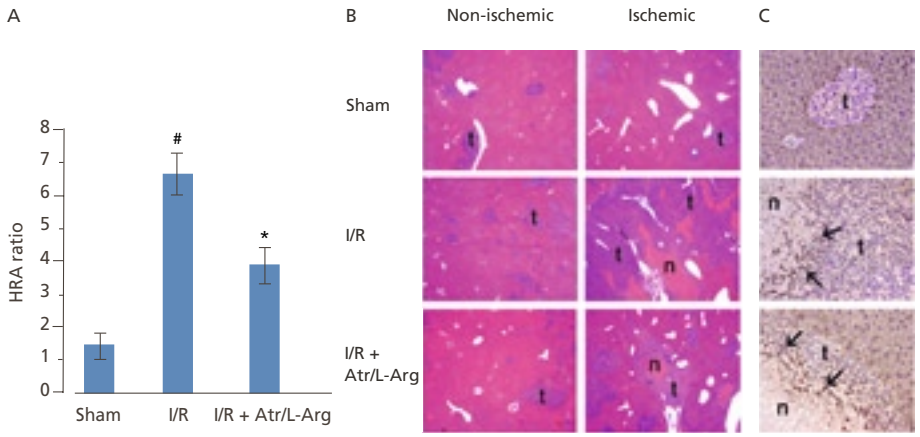

Figure 4. The effect of Atrasentan/L-Arginine on the outgrowth of pre-established micrometastases. **(A)** Tumor growth expressed as the HRA ratio, the relative increase in the percentage of liver tissue that has been replaced by tumor tissue in ischemic lobes compared with nonischemic lobes (n=8 each group). #p<0.05 versus sham; *p<0.05 versus I/R. **(B)** Microscopic appearance of I/R-accelerated outgrowth of micrometastases at 5 days after clamping, showing massive tumor (t) growth surrounding necrotic tissue areas (n). **(C)** HIF-α immunostaining in tumor tissue (t) from sham-operated mice and in ischemic clamped liver lobes. Arrows indicate HIF-α-positive tumor cells at the tumor-necrosis (n) margin. Original magnifications: x2 (B); x20 (C).

hepatic tissue (**Figure 1D**). Pimonidazole immunohistochemistry revealed minimal tissue hypoxia at 2 hours of reperfusion in mice treated with Atrasentan/L-Arginine (**Figure 2D**). At later time points, Atrasentan/L-Arginine had completely prevented detectable tissue hypoxia. HIF-1α immunohistochemistry was reminiscent of pimonidazole staining and revealed reduced immunostaining when compared with I/R at all time points (**Figure 2E**). Despite a distinct cytoplasmic immunostaining at 2 hours of reperfusion, this was not associated with exacerbated nuclear staining (**Figures 3A and B**).

Next, we investigated whether improvement of the microcirculation and reduction of tissue hypoxia by means of Atrasentan/L-Arginine treatment would also reduce tumor growth after I/R. Because Atrasentan and/or L-Arginine may influence tumor growth independent of I/R damage, we first evaluated the effect of Atrasentan/L-Arginine treatment on tumor growth in sham-operated mice. In these animals, tumor growth was unaffected (data not shown). In mice subjected to I/R, the accelerated outgrowth of micrometastases was inhibited by 50% after Atrasentan/L-Arginine treatment (**Figure 4A**). Interestingly, microscopic evaluation revealed that the few necrotic areas present were surrounded by fields of tumor cells (**Figure 4B**), which accounted for a residual 50% increase in HRA ratio when compared with sham operation. Similar to the observations in vehicle-treated animals, we found strong nuclear HIF-1α staining in cells at the tumor-necrosis margin (**Figure 4C**). These data strongly suggest that perinecrotic tissue hypoxia and, possibly, the subsequent stabilization of HIF-1α after I/R contribute to the accelerated outgrowth of micrometastases.

Inhibition of I/R-accelerated tumor growth by 17-DMAG

Finally, we used the heat shock protein-90 inhibitor 17-DMAG to promote destabilization of HIF-1α.[34,35] In control vehicle-treated mice, tumor growth was stimulated more than

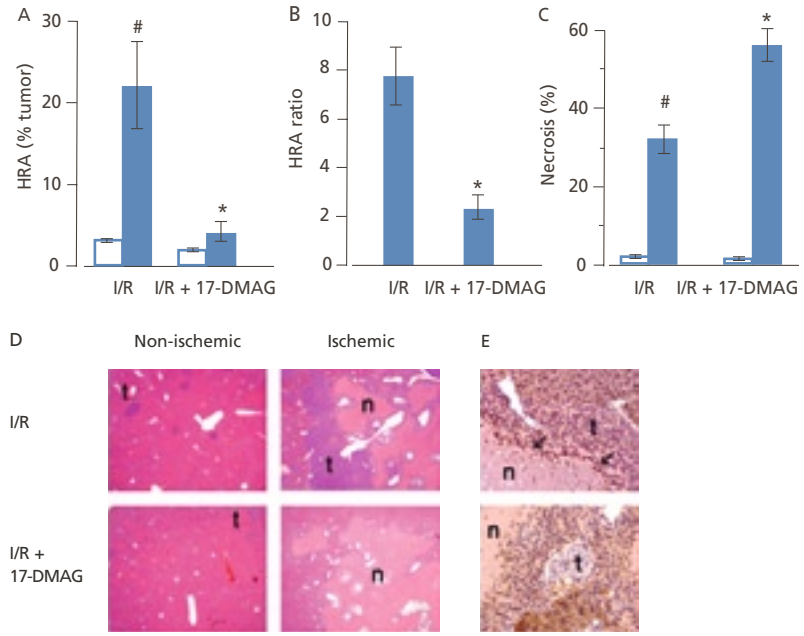

Figure 5. The effect of 17-DMAG on the outgrowth of pre-established micrometastases and tissue necrosis. Tumor growth expressed as the HRA **(A)** and as the HRA ratio **(B)**, the relative increase in the percentage of liver tissue that has been replaced by tumor tissue in ischemic lobes compared with nonischemic lobes (n=8 each group). #p<0.05 versus nonclamped liver lobes; *p<0.05 versus I/R. **(C)** Tissue necrosis, quantified via morphometric analysis of clamped and nonclamped liver lobes at 5 days after clamping (n=8 each group). **(D)** Microscopic appearance of I/R-accelerated outgrowth of micrometastases at 5 days after clamping, showing massive tumor (t) growth surrounding necrotic tissue areas (n). **(E)** HIF-α immunostaining (brown) in tumor tissue (t) in clamped liver lobes from untreated mice and mice treated with 17-DMAG. Arrows indicate HIF-1α-positive tumor cells at the tumor-necrosis (n) margin. Original magnifications: x2 (D); x20 (E).

sevenfold after I/R, similar to the first set of experiments (**Figures 5A and B**). Again, tumor growth was associated with tissue necrosis (**Figures 5C and D**) and positive nuclear HIF-1α immunostaining at the tumor-necrosis margin (**Figure 5E**). 17-DMAG had no significant inhibitory effect on tumor growth in sham-operated mice (data not shown). Strikingly, 17-DMAG induced a significant reduction in tumor growth in the clamped liver lobes (**Figure 5A**), without affecting tumor growth in nonclamped liver lobes. This resulted in a 70% decrease in HRA ratio, reflecting a selective inhibitory effect on the perinecrotic stimulation of tumor growth (**Figure 5B**). Nonetheless, the percentage of necrotic tissue after 17-DMAG had increased from 31 to 56% (**Figure 5C**), indicating that the treatment had also interfered with the tissue-protecting effect of HIF-1α. Despite this increase in tissue necrosis in the clamped liver lobes, the accelerated perinecrotic tumor growth was not observed (**Figure 5D**). This is the first time that we observed extensive tissue necrosis without associated tumor growth. Most importantly, microscopic lesions at the necrosis margin did not show significant HIF-1α staining, indicating that 17-DMAG indeed prevented HIF-1α stabilization (**Figure 5E**).

Discussion

The major findings of the work presented here are that 1) the accelerated outgrowth of micrometastases after I/R is associated with long-term microcirculatory disturbances, profound perinecrotic tissue hypoxia and stabilization of HIF-1α; 2) prevention of postischemic microcirculatory disturbances minimizes tissue hypoxia, avoids HIF-1α stabilization, and reduces the accelerated outgrowth of micrometastases; and 3) destabilization of HIF-1α by 17-DMAG reduces I/R-stimulated tumor growth. We conclude that prolonged tissue hypoxia and, possibly, subsequent stabilization of HIF-1α play an important role in the altered behavior of micrometastases in the liver after I/R.

Whereas prolonged periods of hypoxia are deleterious to most cells, tumor cells have adapted to survive under hypoxic conditions.[44] Intratumoral hypoxia has been demonstrated in a number of human cancers, and elevated expression of HIF-1α has been related to tumor aggressiveness.[33,45-49] HIF-1α activates the transcription of several genes that are implicated in cancer progression, including proliferation-promoting cytokines and growth factors, angiogenesis-promoting growth factors, glucose transporters, and serine-, aspartic-, and metalloproteases.[29-33] Thus, prolonged hypoxia as it occurs after I/R may provide a protumorigenic microenvironment through up-regulation of HIF-1α. Interestingly, pimonidazole and HIF-1α immunostaining were mainly located around necrotic tissue areas. This is consistent with the HIF-1α staining patterns at the necrosis-viable margin of several tumors.[47,50] In the clamped liver lobes, HIF-1α immunoreactivity in hepatocytes was primarily seen after 2 and 6 hours of reperfusion. The strong cytoplasmic staining may be the result of enhanced protein stabilization and accumulation.[43,46,47] It was recently suggested that in the liver, HIF-1α targets to the peroxisome rather than the nucleus after hepatic hypoxia/reoxygenation.[28] We found that HIF-1α staining was sustained up to 5 days, which is consistent with other reports.[51] We used the geldanamycin analogue 17-DMAG to promote degradation of HIF-1α.[34,35] In this study a short-term treatment of 17-DMAG clearly reduced the accelerated outgrowth of micrometastases. Because 17-DMAG inhibits the function of heat shock protein-90, other mechanisms may have co-contributed to the effectiveness of 17-DMAG in reducing postischemic tumor growth. Heat shock proteins are expressed acutely in response to hypoxia and I/R and they have numerous target genes aimed at promoting cell survival and growth.

Several other mechanisms may have indirectly or directly contributed to I/R-stimulated tumor growth. After I/R, endothelin-1 is increased in the reperfusion period and exerts its vasoconstrictive action via the endothelin-A receptor, contributing to the microcirculatory disturbances after I/R.[52-54] Endothelin-1 may also directly stimulate tumor cell proliferation,[55,56] and its expression correlates with the stabilization of HIF-1α.[57] Finally, the inflammatory response associated with I/R, including the influx of neutrophils and activation of Kupffer cells, may also contribute to the stimulation of tumor growth. Both activated neutrophils and macrophages have been associated with increased metastatic potential, proliferation, and invasion.[58-62] Interestingly, hypoxic macrophages secrete growth factors and angiogenic factors that may favor tumor progression. Furthermore, increased HIF expression has been associated with the presence of tumor-associated macrophages.[63] In addition, the influx of neutrophils contributes to microcirculatory disturbances.[64] Evidently, inflammation and hypoxia mutually influence each other and may co-activate tumor cell proliferation. Our observation that perinecrotic hypoxia was closely associated with the presence of inflammatory cells supports this notion. Thus, tampering the inflammatory response may also minimize hypoxia and thereby reduce tumor growth.

Taken together, although the effects of endogenous (i.e. intratumoral) hypoxia on tumor growth have been well documented, this study now shows that exogenous (i.e. I/R-induced) hypoxia stimulates tumor growth as well. This may possibly be, at least in part, attributed to the stabilization of HIF-1α. Clinically this is very relevant because recurrent tumor growth after an apparently complete tumor resection occurs in the majority of patients undergoing liver surgery for colorectal liver metastases. We recently found that severe ischemia as a result from prolonged vascular clamping was associated with a reduced time to develop liver recurrence and a decreased disease-free survival (unpublished data). However, these issues are not only important in case of inevitable clamping of the hepatic blood flow during liver surgery but also during other events that cause I/R injury, such as hemorrhagic shock or sepsis.[65,66] Similar phenomena may occur during surgery-induced tissue hypoxia after wounding, inflammation, and organ manipulation.[42,67-69] Thus, prevention of tissue hypoxia or inhibition of HIF-1α activity may represent attractive approaches to limiting recurrent tumor growth after hepatic surgery. First, therapeutic strategies that restore postischemic microcirculatory flow are potential candidates for reducing I/R-accelerated tumor growth. In the past, several compounds that aim at restoring the endothelin-1/nitric oxide imbalance have been successfully applied to reduce microcirculatory disturbances and liver tissue damage.[19,21,54,70,71] Atrasentan is effective in reducing postischemic microcirculatory disturbances in models of renal,[72] cardiac,[73] and cerebral I/R,[74] and we here show that, in combination with the nitric oxide donor L-Arginine, it provides maximal improvement of the microcirculation after hepatic I/R. Second, agents that target the HIF-1α pathway, including 17-DMAG, are gaining increased attention as novel anticancer drugs.[75,76] Such compounds may ideally be administered perioperatively to prevent hypoxia-induced tumor growth stimulation. However, HIF-1α is also involved in the physiological response to tissue damage, and thus, their perioperative application may hamper wound and anastomosis healing, which may contribute to increased morbidity. Indeed, we found increased necrosis in clamped liver lobes after treatment with 17-DMAG. Further studies are needed to investigate the effectiveness and safety of such compounds in the postoperative setting.

In conclusion, long-term microcirculatory disturbances, perinecrotic hypoxia, and, possibly, the stabilization of HIF-1α play an important role in the accelerated outgrowth of colorectal liver metastases after I/R. In case of inevitable ischemic damage and hypoxia during hepatic surgery, improving microcirculatory flow or targeting the HIF-1α pathway may decrease the stimulation of microscopic tumor deposits and improve prognosis in colorectal cancer patients.

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Chapter 9

Inhibition of the HIF-1α/ VEGF axis suppresses accelerated outgrowth of hepatic colorectal micrometastases after thermal ablation

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Abstract

Background Thermal destruction therapies of nonresectable colorectal liver metastases, including laser-induced thermotherapy (LITT), can provide tumor clearance, but local recurrences are common. The aim of this study was to assess the effect of thermal ablation on the outgrowth of perilesional micrometastases and the contribution of the hypoxia-inducible factor (HIF)-1α/vascular endothelial growth factor (VEGF)-axis on local tumor growth stimulation.

Methods Three days after tumor cell inoculation LITT was applied to the left liver lobe. Perilesional microcirculatory disturbances, tissue hypoxia, HIF-1α and the outgrowth of micrometastases were evaluated over time. Intervention strategies were aimed at improving microcirculatory flow (by Atrasentan/L-Arginine), preventing the stabilization of HIF-1α (by 17-DMAG), and inhibition of VEGF-receptor signaling (by PTK787/ZK-222584).

Results Following thermal ablation the outgrowth of perilesional micrometastases was stimulated over fourfold compared with tumor growth in the remaining liver. LITT induced perilesional microcirculatory disturbances, profound hypoxia and stabilization of HIF-1α. Atrasentan/L-Arginine improved the microcirculation further away from the lesion, but perinecrotic microcirculatory flow was still severely disturbed and tumor growth remained unaffected. Treatment with 17-DMAG prevented HIF-1α stabilization and reduced tumor growth in the transition zone by 34% without affecting tumor growth in sham-operated animals. PTK787/ZK-222584 reduced both LITT-stimulated tumor growth and tumor growth in the remaining liver by more than 33%.

Conclusion Thermal tissue destruction accelerates the outgrowth of perilesional micrometastases, which may be caused by prolonged tissue hypoxia, subsequent stabilization of HIF-1α and, possibly, the promotion of tumor neovascularization. Adjuvant treatment with 17-DMAG or PTK787/ZK-222584 may represent an attractive approach to reduce local recurrence after thermal destruction therapy for liver metastases.

Introduction

The liver is the most common site of metastases from colorectal carcinoma. The only curative treatment option for patients with hepatic metastases is surgical resection, leading to 5-year survival rates up to 40%.[1-3] Despite recent advances in neo-adjuvant chemotherapy, surgical resection is applicable to only 10-30% of patients.[4,5] Thermal destruction therapies, such as radiofrequency ablation (RFA) and laser-induced thermotherapy (LITT), are increasingly used for treating nonresectable colorectal liver metastases and can provide tumor clearance and improve life-expectancy.[6-10] Nonetheless, despite an apparently complete tumor destruction, local recurrences may develop from microscopic tumor deposits or satellite metastases that reside at the periphery of the lesion.[11,12] Recent reviews on this topic revealed that local perilesional recurrences from colorectal metastases occur in approximately 10-15%.[13-15] Nonetheless, with increasing tumor size and during percutaneous ablation, the risk of treatment failure increases and local recurrences are reported in up to 60% of cases.[15-19]

The biological behavior of residual tumor cell deposits largely determines the time to develop a recurrence, which eventually influences survival. Evidence is accumulating that the altered microenvironment after operative trauma may enhance the outgrowth of residual tumor cells.[20-22] Recently, several clinical case studies have described rapid local tumor progression after thermal ablation of various solid tumors.[23-28] Recently, we have shown that ischemia/reperfusion due to vascular clamping accelerates the outgrowth of pre-established micrometastases.[29] The stimulated outgrowth after ischemia/reperfusion was preferentially located around areas of tissue necrosis. Based on these observations, we hypothesized that thermal destruction, which univocally generates tissue necrosis, may similarly affect the local outgrowth of residual tumor cells at the lesion periphery.

In the present work we used a murine model with established colorectal micrometastases as a model of diffuse residual disease in the liver to study the effect of LITT on the outgrowth of tumor cell clusters at the lesion periphery. A second model using RFA in rats was developed to assess whether the phenomenon is restricted to the first model or that it represents a more general phenomenon. Furthermore, we assessed whether tumor growth in the transition zone between the central necrotic zone and the unaffected reference zone is associated with microcirculatory disturbances, hypoxia and stabilization of hypoxia-inducible factor (HIF)-1α. Finally, we tested whether intervention strategies aimed at 1) improving microcirculatory flow (by Atrasentan/L-Arginine), 2) preventing the stabilization of HIF-1α in response to tissue hypoxia (by 17-DMAG), or 3) inhibiting vascular endothelial growth factor (VEGF)-signaling (by PTK787/ZK-222584) would be beneficial in controlling accelerated tumor recurrence following thermal ablation.

Materials and methods

Animals and surgical procedures

The experiments were carried out 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, 20-25 gram) and male Wag/Rij rats (16-20 weeks, 200-250 gram) were purchased from Charles River (Sulzfeld, Germany) and Harlan (Horst, The Netherlands) respectively. Animals were housed under standard laboratory conditions. All surgical procedures were performed under isoflurane inhalation anesthesia. Buprenorfine was administered intramuscularly prior to surgery to provide sufficient peri-operative analgesia. Body temperature was maintained at 36.5-37.0 ˚C during the entire experiment.

Induction of hepatic micrometastases

Colorectal liver metastases were induced in mice as described previously.[29-31] In brief, through a left lateral flank incision, $5x10^4$ routinely cultured C26 colon carcinoma cells were injected into the splenic parenchyma. After ten minutes, the spleen was removed to prevent intrasplenic tumor growth. Diffuse intrahepatic micrometastases were allowed to grow out for three days.

For tumor inoculation in rats, we used the routinely cultured CC531 colon carcinoma cell line, which was kindly provided by Dr. PJK Kuppen (Department of Experimental Surgery, Leiden University Medical School, Leiden, The Netherlands).[32,33] Through a midline incision, $1x10^6$ CC531 cells were injected into the portal vein. To prevent bleeding after injection, the puncture hole was sealed with an absorbable fibrin-collagen-coated patch (Nycomed, Breda, The Netherlands). Micrometastases were allowed to develop in the ensuing six days.

Laser induced thermotherapy and radiofrequency ablation

A Nd:YAG laser (Medilas 4060 N, MBB, Medizin Technik, Munchen, Germany) with a wavelength of 1064 nm was used in mice. The laser light was delivered in a continuous wave mode through a 400 µm fiber with a diffuser tip applicator (outer diameter 1.2 mm, active length 10 mm) (Trumpf Medizine Systeme, Umkirch, Germany). Based on previous dose-effect relation studies,[34] LITT was applied to the left liver lobe at a power setting of 3 watt per centimeter diffuser length for 90 seconds, corresponding to a total energy output of 270 joule. In rats, thermo-ablation was performed to the left liver lobe by radiofrequency using a bipolar electrode (Celon AG, Teltow, Germany) with a noncooled diffuser tip (outer diameter 1.4 mm, active length 10 mm) at 2 watt for 150 seconds, corresponding to a total energy output of 300 joule. Sham-operated animals underwent laparotomy with insertion of the probe into the liver but without heat application.

Drug characteristics

Atrasentan (ABT-627), a selective endothelin-A receptor antagonist, was kindly provided by Abbott Laboratories (Abbott Park, IL, USA). Atrasentan was dissolved in 4.2% $NaHCO_3$ and injected intravenously as a single bolus (10 mg/kg body weight) prior to thermal ablation, followed by continuous oral administration offered in the drinking water (10 mg/kg body weight per day).[35]

The nitric oxide donor L-Arginine (Sigma-Aldrich, St. Louis, MO, USA) was administered via continuous subcutaneous infusion using Alzet osmotic minipumps (model 2001; Durect Corporation, Cupertino, CA, USA) at a dose of 30 µg/kg/min starting prior to thermal ablation.[35]

17-DMAG (Invivogen, San Diego, USA), a geldanamycin analogue known to destabilize HIF-1α,[36,37] was dissolved in saline and administered by intraperitoneal injections at a preoperative dose of 15 mg/kg body weight, followed by three postoperative doses of 7.5 mg/kg body weight every 12 hours postablation.

PTK787/ZK-222584 (PTK/ZK), a selective VEGF receptor tyrosine kinase inhibitor that blocks all known VEGF receptors,[38] was kindly provided by Schering AG (Berlin, Germany). PTK/ZK was dissolved in polyethyleenglycol 400 and administered by twice-daily intragastric injections at a dose of 50 mg/kg starting prior to thermal ablation until 48 hours after ablation.

Experimental design

The effect of thermal ablation on perinecrotic outgrowth of micrometastases was assessed in two different animal models using two different ablative techniques. Three days after intrasplenic tumor cell injection mice were subjected to either LITT or sham operation and sacrificed 7 days later (n=8 each group). In rats, micrometastases were allowed to develop for 6 days and at that time point, animals were subjected to RFA or sham operation. Tumor growth was assessed 9 days later (n=8 each group). The livers were harvested and fixed in 4% neutral buffered formaldehyde and embedded in paraffin for morphological assessment of tumor load.

The effect of LITT with and without Atrasentan/L-Arginine treatment on the perilesional hepatic microcirculation was assessed in non-tumor-bearing mice 2 hours, 24 hours and 7 days after LITT (n=4 each group) by intravital fluorescence microscopy. Sham-operated mice served as controls. The extent and localization of hypoxia were analyzed using the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1, 90201, Chemicon International, Temecula, CA, USA),[39,40] which was injected intravenously one hour prior to termination at a dose of 60 mg/kg. Livers were harvested 2 hours, 24 hours and 7 days after LITT (n=4 each group) for pimonidazole and HIF-1α immunohistochemistry.

For the intervention studies, mice with pre-established micrometastases underwent LITT and were randomized into different treatment groups (n=8 each group). Mice received either 1) Atrasentan and L-Arginine, 2) 17-DMAG, 3) PTK/ZK or 4) control vehicle. Tumor growth was assessed 7 days later. The effect of all drugs on tumor growth in sham-operated mice was evaluated separately. For these control experiments, metastases were allowed to grow out for 9 days after sham operation.

Intravital fluorescence microscopy

The hepatic microcirculation was assessed by intravital fluorescence microscopy using a Nikon TE-300 inverted microscope (Uvikon, Bunnik, The Netherlands) equipped with a fluorescence filter for fluorescein isothiocyanate (excitation 450-490 nm, emission >515 nm).[41] For contrast enhancement, fluorescein isothiocyanate-labeled dextran (MW 446.000, 2% in 0.9% NaCl; 100 µl per 20 g bodyweight) was injected intravenously and excited with blue light (450-490 nm). The midline incision was re-opened and the left liver lobe was gently exteriorized and moistened. The mice were placed on an inverted microscopic stage, using a template to minimize tension and respiratory movement. Microcirculatory flow was assessed at two distances from the lesion edge (2 mm and 6 mm). For each distance ten to fifteen fields were recorded for ten seconds per animal. Images were captured at a x40 magnification by a charge coupled device camera (Exwave HAD, Sony, Badhoevedorp, The Netherlands) and relayed to a personal computer for offline analysis. Sinusoidal perfusion rates were analyzed by two independent observers, blinded to treatment. Sinusoidal perfusion rates were calculated by dividing the number of normally perfused sinusoids by the total number of sinusoids observed.

Pimonidazole and HIF-1α immunohistochemistry

Pimonidazole adducts were visualized by immunohistochemistry by using fluorescein isothiocyanate-conjugated mouse anti-Hypoxyprobe-1 monoclonal antibody (1:100, TM 90529, Chemicon International), followed by anti-fluorescein isothiocyanate antibody labeled with horseradish peroxidase (1:50, DAKO, Glostrup, Denmark). For HIF-1α detection, a polyclonal rabbit antibody against mouse HIF-1α was used for incubation overnight (1:50, NB 100-449, Novus, Littleton, USA) and goat anti-rabbit PowerVision+ (Immunologic, Duiven, The Netherlands) with 2% mouse serum was used as secondary antibody.

Before immunolabelling, endogenous peroxidase activity was blocked using a solution of methanol and hydrogen peroxide. Antigen retrieval was performed by boiling sections for 20 minutes in 0.01 mol/L citrate buffer (pH 6.0) for pimonidazole staining and in ethylenediamine tetra acetic acid-buffered solution (pH 9.0) for HIF-1α staining. Reactions were developed using diaminobenzidine/H_2O_2 as a chromogen substrate. Primary-deleted negative controls were treated with the antibody diluent alone and were all free of nonspecific background staining.

Immunostaining for HIF-1α in tumor cells at the tumor-necrosis interface was scored in seven to eight randomly selected fields per animal by two independent observers, blinded to treatment. The nuclear staining pattern was scored as the product of the staining intensity (weak 1+, moderate 2+, strong 3+) and the percentage of positive cells (1-10% of cells 1+, 11-50% of cells 2+, >50% of cells 3+) (n=4 each group).

Quantification of tumor load

Intrahepatic tumor load was scored as the hepatic replacement area (HRA).[29] HRA was measured in the transition zone, defined as the area stretching 2 mm outside the necrotic central area, and in the reference zone, i.e. the remaining part of the liver. Analyses were performed by two independent observers, blinded to treatment, using an automated microscope with an interactive video overlay system (Q-Prodit, Leica Microsystems, Rijswijk, The Netherlands). The scar tissue induced by LITT was excluded from analysis. Based on the more expansive growth pattern of CC531 cells, the transition zone was defined as the area stretching 10 mm outside the lesion in the rat model.

Statistical analysis

Statistical differences between groups were analyzed by the Mann-Whitney U-test and ANOVA for nonparametric data. Data are expressed as mean ± sem.

Results

Thermal ablation enhances perinecrotic outgrowth of colorectal micrometastases in mice

The effect of thermal tissue ablation on the outgrowth of pre-established micrometastases was evaluated in two different animal models. First, we assessed the effect of LITT in our previously described C26-BALB/c model.[29] Metastases in sham-operated mice were distributed equally throughout the liver lobes and covered approximately 15-20% of the liver tissue (**Figures 1A-D**). Seven days after LITT, performed three days after tumor cell inoculation, a clear necrotic central zone of 7.2 ± 0.2 x 4.9 ± 0.2 mm had developed in all livers. The lesions were clearly encircled by a rim of vital tumor (**Figure 1A**). Tumor load in the transition zone surrounding the central necrotic zone had increased three to fourfold compared with tumor load in the unaffected reference zone (48.5 ± 3.9% versus 14.7 ± 1.9%, p=0.0002) and compared with tumor growth in the livers of sham-operated mice (48.5 ± 3.9% versus 17.9 ± 1.2%, p=0.00021) (**Figure 1B**). No differences were found between tumor growth in the reference zone of LITT-treated animals when compared with tumor growth in sham-operated mice (**Figure 1B**). Microscopically, massive confluent metastases were observed directly surrounding the necrotic tissue area (**Figures 1 C and D**). Tumor cells in the transition zone clearly had a remarkably disorganized and morphologically irregular phenotype and many single invading tumor cells were observed detaching from the tumor mass (**Figure E**), which was markedly different from the compact growth pattern of control tumor tissue in the reference zone.

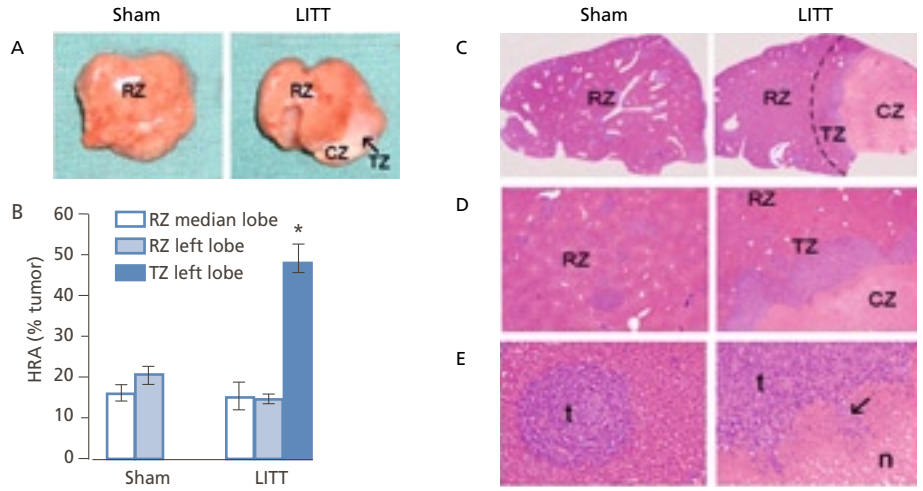


Figure 1. Accelerated perilesional outgrowth of pre-established C26 micrometastases following laser-induced thermotherapy (LITT) in BALB/c mice. Three days after intrasplenic tumor cell inoculation, LITT was applied to the left liver lobe and tumor growth was evaluated 7 days later. (**A**) Macroscopic appearance of C26 metastases in the reference zone (RZ) and in the transition zone (TZ) surrounding the necrotic central zone (CZ), 7 days after sham operation and LITT. (**B**) Tumor growth, expressed as the hepatic replacement area (HRA), in the RZ of the median and left liver lobes and in the TZ, defined as the area extending 2 mm outside the necrotic lesion (dotted line). *p<0.05 versus tumor growth in the RZ. (**C-D**) Microscopic appearance of metastases in control liver lobes and of metastases surrounding the necrotic lesion induced by LITT (Original magnifications: C : x0.5, D : x2). (**E**) Coherent appearance of tumor cells (t) in control liver and infiltrative growth pattern of tumor cells (t) around the LITT-induced necrotic (n) lesion (Original magnification x20).

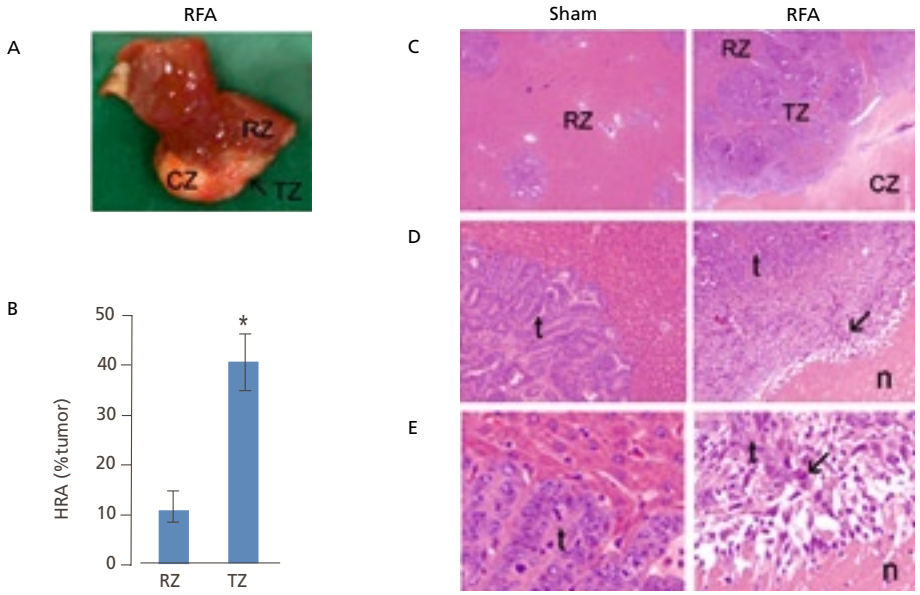


Figure 2. Accelerated perilesional outgrowth of pre-established CC531 micrometastases following radiofrequency ablation (RFA) in Wag/Rij rats. Six days after intraportal tumor cell inoculation RFA was applied to the left liver lobe and tumor growth was evaluated 9 days later. **(A)** Macroscopic appearance of CC531 metastases in the reference zone (RZ) and in the transition zone (TZ) surrounding the central zone (CZ), 9 days after RFA. **(B)** Tumor growth, expressed as the hepatic replacement area (HRA), in the reference zone and in the transition zone, defined as the area extending 10 mm outside the necrotic lesion. *p<0.05 versus tumor growth in the RZ. **(C and D)** Microscopic appearance of metastases in the RZ **(C)** and in the TZ surrounding the necrotic lesion induced by RFA **(D)** (Original magnification x2). **(E-H)** Coherent and well-differentiated appearance of tumor cells (t) in control liver (E and G) and infiltrative and poorly differentiated growth pattern of tumor cells (t) around the RFA-induced necrotic (n) lesion **(F and H)** (Original magnifications: E and F: x10, G and H; x40).

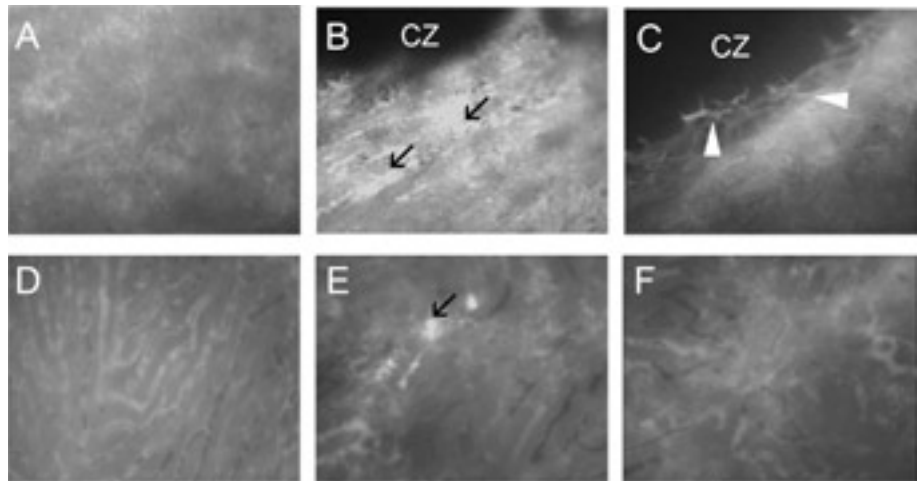
Thermal ablation enhances perinecrotic outgrowth of colorectal micrometastases in rats

Next, we assessed how RFA affected tumor growth surrounding the lesion in rats using the CC531 coloncarcinoma cell line. Similar to the murine model, RFA induced a three to fourfold increase in tumor tissue in the transition zone surrounding the ablated region when compared with the other nontreated reference areas (40.4 ± 6.1 versus 10.6 ± 3.9, p=0.007) (**Figures 2A and B**). Microscopically, control CC531 metastases appeared as well-differentiated tumors displaying glandular organized structures throughout the tumor lumps (**Figures 2C-E, left panels**). However, confluent tumor masses in the transition zone displayed a disorganized and poorly differentiated phenotype with little glandular architecture and many single invading tumor cells at the tumor-necrosis interface (**Figures 2C-E, right panels**). No extrahepatic metastases were found in any of the animals.

The above results obtained with two distinct tumor models using two different heat sources strongly suggest that thermal ablation has a profound local stimulatory effect on established microscopic tumor cell deposits in close proximity to the necrotic lesion and that this represents a general phenomenon.

LITT induces prolonged microcirculatory disturbances

We next evaluated microcirculatory flow at two distances from the lesion edge in non-tumor-bearing animals by using intravital microscopy 2 hours, 24 hours and 7 days following LITT. Different from sham-operated mice (**Figure 3A**), the central zone induced by LITT could be clearly distinguished by complete absence of contrast around the insertion site of the probe at all time points (**Figures 3B and C**). The diameter of the central zone increased over time from 6.3 ± 0.6 mm at 2 hours to 8.6 ± 1.0 mm at 24 hours and measured 7.1 ± 0.6 mm at 7 days after LITT (p=0.016) (**Figure 3G**). Two hours after



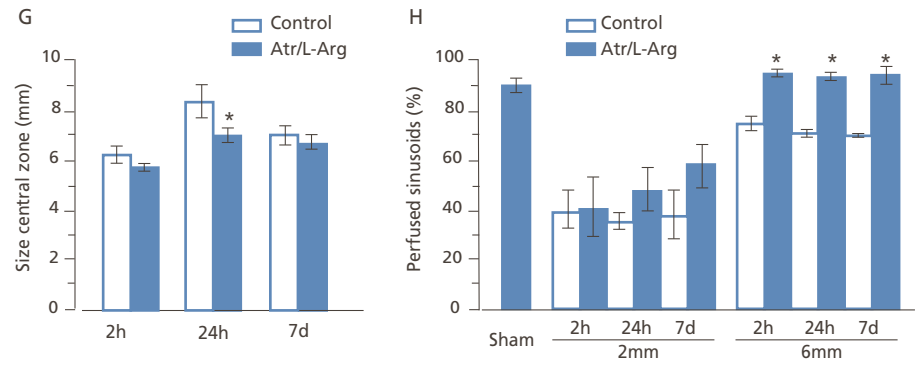


Figure 3. Perilesional microcirculatory flow 2 hours, 24 hours and 7 days after LITT as evaluated by intravital microscopy (IVM) and the effect of Atrasentan/L-Arginine treatment. **(A-C)** Microscopic appearance of the hepatic microcirculation by IVM in the reference zone **(A)**, and in the transition zone, showing extravasation of fluorescein isothiocyanate-labeled dextran (arrow) and disturbed microcirculation in the transition zone, surrounding the avascular central zone (CZ) at 2 hours **(B)** and a hypervascular rim (arrowheads) at 7 days after LITT **(C)** (Original magnification x10). **(D-F)** Microscopic appearance of the hepatic microcirculation in the reference zone **(D)**, and at the transition zone at 2 mm **(E)** and 6 mm **(F)** from the lesion edge 2 hours after LITT (Original magnification x40). **(G)** Lesion size as induced by LITT with and without Atrasentan/L-Arginine treatment. *p<0.05 versus untreated controls. **(H)** Hepatic sinusoidal perfusion rates 2 hours, 24 hours and 7 days after sham operation and LITT with and without Atrasentan/L-Arginine treatment, measured at 2 and 6 mm distance from the lesion edge. *p<0.05 versus untreated controls.

LITT, extravasation of fluorescein isothiocyanate dextran was observed directly outside the lesion, indicating loss of sinusoidal integrity shortly after laser treatment (**Figures 3B**). Extravasation of fluorescein isothiocyanate dextran decreased at 24 hours and was absent at 7 days after LITT (**Figure 3C**). Instead, at 7 days after LITT, we observed large irregular vessels protruding into the central zone (**Figure 3C**). Moreover, LITT induced severe perilesional sinusoidal perfusion failure, as shown by reduced sinusoidal perfusion rates within the first 2 mm outside the necrotic lesion when compared with sham-operated mice ($p<0.0001$) (**Figures 3D, E and H**). Interestingly, this microcirculatory failure persisted for at least 7 days following LITT. Sinusoidal perfusion rates gradually increased further away from the lesion, but were still abnormal at 6 mm from the lesion edge ($p=0.0002$) (**Figures 3F and H**). No significant changes were noted between the different time-points. These data demonstrate a longterm disturbance of the perilesional microcirculation following thermal ablation treatment of the liver. In addition, the intralesional large vessels observed 7 days after LITT suggest neovascularization at the lesion margin.

Prolonged microcirculatory disturbances are associated with perinecrotic tissue hypoxia and stabilization of HIF-1α

Histopathologically, LITT induced progressive hepatocellular injury as shown by an eosinophilic central zone with sinusoidal congestion and nuclear pyknosis at 2 hours, followed by infiltration of neutrophils at 24 hours and a leucocytic infiltrate at 7 days after treatment (**Figure 4A**). The extent and localization of tissue hypoxia after LITT were assessed by pimonidazole immunohistochemistry (**Figures 4B**). In control liver tissue of sham-operated mice, pimonidazole staining was exclusively observed around central venules, which are known to be characterized by relatively low oxygen concentrations.[39] Two hours after LITT, profound diffuse tissue hypoxia was observed throughout the transition zone. During the ensuing 7 days, the perilesional liver parenchyma remained remarkably hypoxic. Similar to the pimonidazole staining, HIF-1α was detected around central venules in control liver tissue (**Figures 4C**). At 2 hours after LITT strong nuclear staining of HIF-1α was primarily observed in the central zone, which was still present at 24 hours but had disappeared at 7 days after LITT (not shown). In the transition zone, both cytoplasmic and nuclear HIF-1α were observed at the lesion edge, with maximal staining at 24 hours.

Accelerated tumor growth is associated with high HIF-1α levels in tumor cells

As described above, the accelerated tumor growth after LITT was predominantly located in the first 2 mm surrounding the necrotic lesion and tumor cells at the lesion edge displayed an infiltrative and poorly differentiated phenotype when compared with tumor cells in the reference zone (**Figure 5A**). In control tumor tissue, minimal pimonidazole staining was observed, whereas in tumor tissue surrounding necrotic tissue areas, pimonidazole staining was strongest at the tumor-necrosis interface (**Figure 5B**). Similarly, HIF-1α staining in control tumor tissue was rare, but we observed several regions of infiltrating tumor cells at the tumor-necrosis margin with strong nuclear HIF-1α staining (**Figure 5C**). These data strongly suggest a role for hypoxia and HIF-1α in the altered growth rate and phenotypic changes of perilesional micrometastases after LITT.

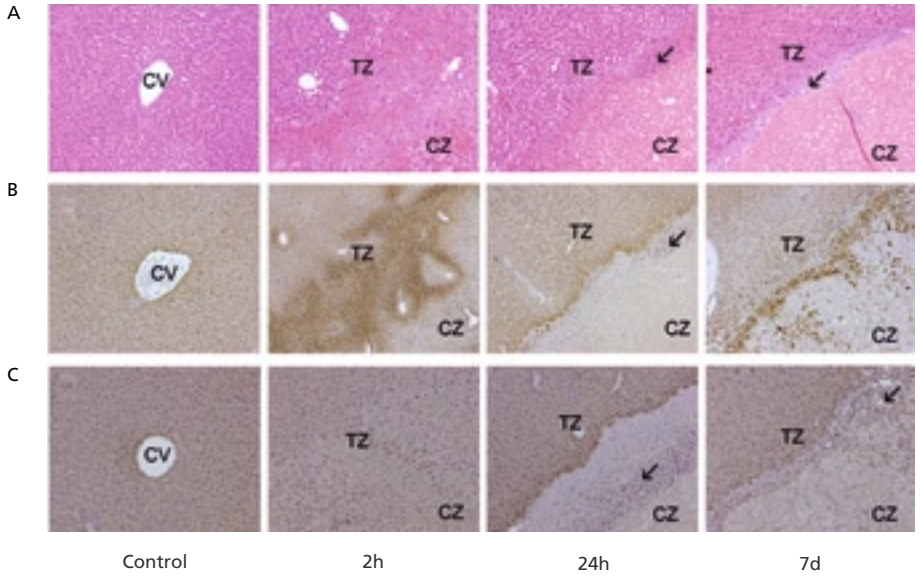


Figure 4. Hypoxia and HIF-1α in liver parenchyma after LITT. **(A)** Hematoxylin and eosin-stained sections of control liver tissue and of tissue sections after LITT, showing eosinophilic hepatocytes, signs of nuclear pyknosis and sinusoidal congestion at 2 hours, followed by infiltration of neutrophils (arrows) at 24 hours and a leucocytic infiltrate at the lesion edge at 7 days after LITT. **(B)** Tissue hypoxia as shown by pimonidazole immunohistochemistry (brown) in the transition zone (TZ) surrounding the central necrotic zone (CZ) 2 hours, 24 hours and 7 days after LITT. **(C)** HIF-1α immunostaining (brown) in the transition zone (TZ) surrounding the central necrotic zone (CZ) 2 hours, 24 hours and 7 days after LITT (Original magnification x10).

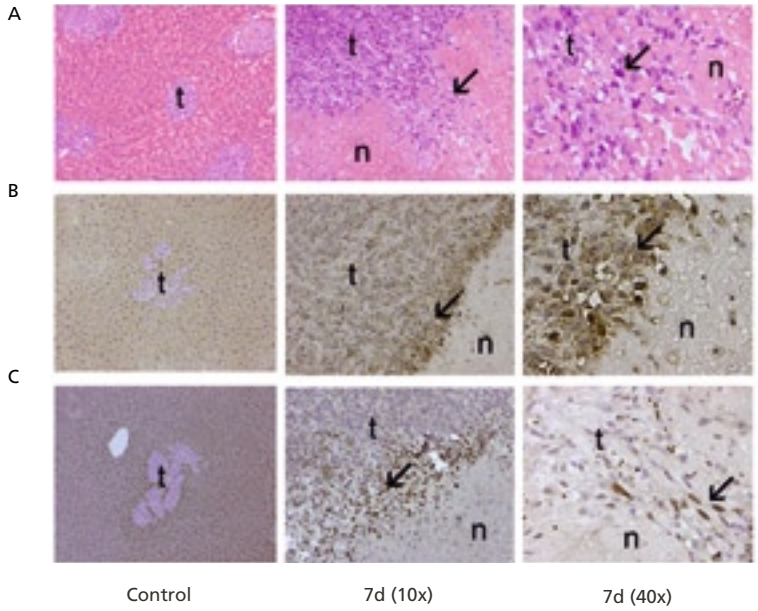


Figure 5. Hypoxia and HIF-1α in control tumor tissue in the reference zone and in stimulated tumor tissue in the transition zone (TZ) 7 days after LITT **(A)** Hematoxylin and eosin-stained tissue sections showing confluent circumscript tumor cell deposits (t) in the reference zone and perilesional infiltration of single tumor cells (arrows) into the necrotic (n) central zone. **(B)** Hypoxia as shown by pimonidazole immunohistochemistry (brown) of tumor cell deposits (t) in the reference zone and the transition zone surrounding the central necrotic zone (n) **(C)** HIF-1α immunostaining showing minimal staining in control tumor tissue (t) and strong nuclear staining in tumor tissue at the tumor-necrosis (n) interface (arrow). (Original magnifications: left panels x10; right panels x40)

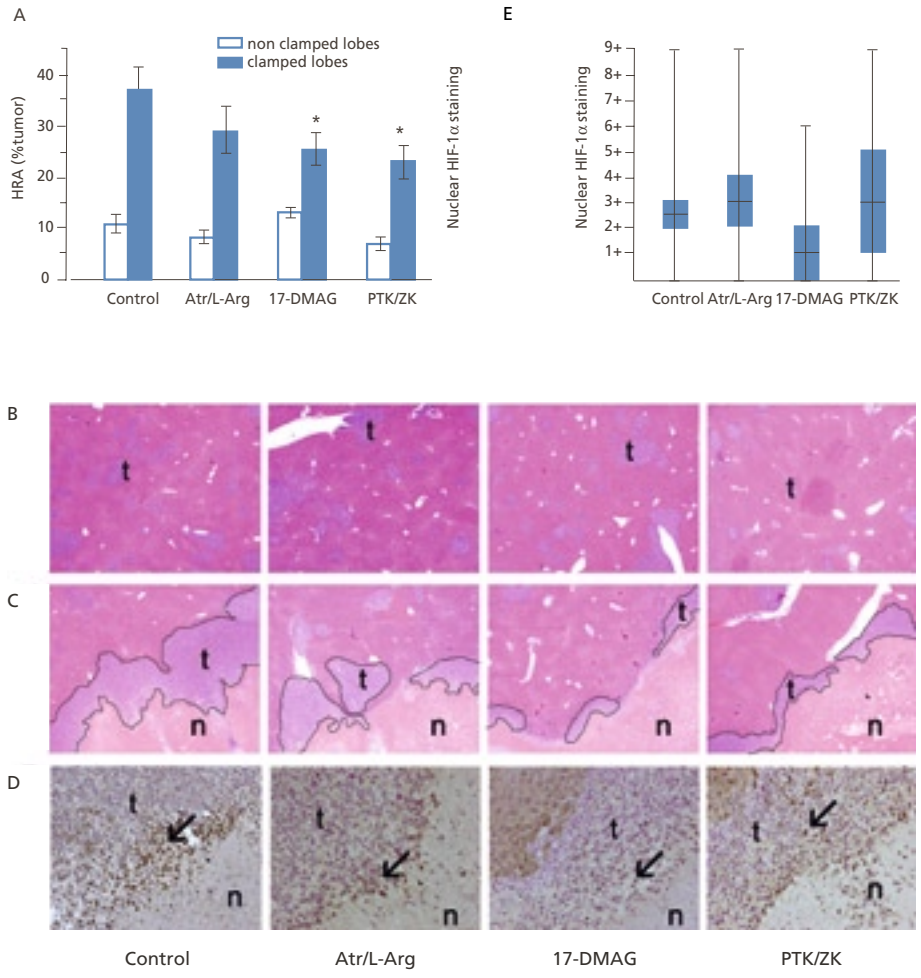


Figure 6. The effect of Atrasentan/L-Arginine, 17-DMAG and PTK/ZK on perilesional outgrowth of pre-established micrometastases evaluated 7 days after LITT. (**A**) Tumor growth, expressed as the hepatic replacement area (HRA), in the transition zone and in the reference zone. *p<0.05 versus untreated controls. (**B and C**) Microscopic appearance of tumor growth in the reference zone (**B**) and in the transition zone (**C**) (**D**) HIF-1α immunohistochemistry (brown) in tumor tissue (t) from untreated mice and mice treated with Atrasentan/L-Arginine, 17-DMAG or PTK/ZK. Arrows indicate HIF-1α positively stained tumor cells at the tumor-necrosis (n) margin (Original magnification x20). (**E**) HIF-1α immunostaining in tumor cells from untreated mice and mice treated with Atrasentan/L-Arginine, 17-DMAG or PTK/ZK, scored as the product of the staining intensity (weak 1+, moderate 2+, strong 3+) and the percentage of positive cells (1-10% 1+, 11-50% 2+, >50% 3+).

Effect of improved microcirculation on HIF-1α stabilization and perilesional tumor growth

Next we assessed whether the observed stimulation of perilesional tumor growth could be prevented by improving the microcirculation. The increase in lesion size 24 hours after LITT, as observed in untreated controls, was inhibited by Atrasentan/L-Arginine, suggesting a protective effect on microvascular injury (**Figure 3G**). Moreover, peri-operative administration of Atrasentan/L-Arginine improved the microcirculation, as shown by improved sinusoidal flow at 6 mm from the lesion edge ($p<0.0001$ compared with untreated controls) (**Figure 3H**). Nonetheless, directly adjacent to the lesion (at 2 mm), microcirculatory flow was still largely impaired at all time points ($p=0.52$ compared with untreated controls, $p<0.0001$ compared with sham-operated mice) (**Figure 3H**). Similar to untreated controls, pimonidazole and HIF-1α nuclear expression could still be observed directly around the necrotic lesion (not shown).

We next assessed how Atrasentan/L-Arginine treatment would affect perilesional tumor growth. In untreated controls, again the outgrowth of micrometastases in the transition zone was stimulated over fourfold compared with tumor growth in the reference zone in control mice subjected to LITT (36.9 ± 4.3% versus 10.5 ± 1.8%, $p=0.0011$) (**Figures 6A-C**). Atrasentan/L-Arginine treatment reduced tumor growth in the transition zone by 21%, but this was not significant when compared with perilesional tumor growth in untreated controls (**Figure 6A**). Atrasentan/L-Arginine treatment did not affect tumor growth in control lobes nor in sham-operated mice (**Figures 6A and B**). Microscopically, massive micrometastasis outgrowth was observed surrounding the necrotic lesion (**Figure 6C**) and many HIF-1α-positive invading tumor cells were observed at the tumor-necrosis interface (**Figures 6D and E**).

Thus, although distant microcirculatory flow was improved by Atrasentan/L-Arginine, perinecrotic sinusoidal flow was still impaired, hypoxia was invariably located around the necrotic lesion and accelerated tumor growth remained unaffected.

Inhibition of LITT-accelerated tumor growth by 17-DMAG and PTK/ZK

Next, we used the heat shock protein-90 inhibitor 17-DMAG to promote destabilization of HIF-1α.[36,37] After sham operation, tumor growth in animals treated with 17-DMAG was not significantly different from tumor growth in untreated animals. In sharp contrast, in LITT-treated animals, 17-DMAG reduced the outgrowth of micrometastases in the transition zone from 36.9 ± 4.3% to 24.4 ± 4.0% ($p=0.043$) without affecting tumor growth in the reference zone (12.5 ± 1.4% versus 10.5 ± 1.8%, $p=0.48$) (**Figures 6A-C**). This resulted in a 34% relative growth reduction, reflecting a selective inhibitory effect on the perilesional stimulation of tumor growth. In the majority of mice small micrometastatic lesions were observed in the transition zone (**Figure 6C**). These lesions did not show significant HIF-1α expression, indicating that 17-DMAG indeed prevented HIF-1α stabilization (**Figures 6D and E**).

Finally, we assessed whether inhibition of VEGF signaling by PTK/ZK would inhibit LITT-stimulated tumor growth. Treatment with PTK/ZK clearly reduced tumor growth in the transition zone by 39%, as measured 7 days after LITT (22.4 ± 3.6 versus 36.92 ± 4.3, $p=0.010$ (**Figure 6A**). In addition, tumor growth in the reference zone had also decreased by 33% (**Figures 6A and B**). Moreover, in sham-operated animals, PTK/ZK treatment significantly reduced tumor load by 38%, measured 9 days after surgery (28.0 ± 3.9% versus 44.8 ± 1.5%, $p=0.026$). Microscopically, tumor growth in the transition zone was similar to that in mice treated with 17-DMAG (**Figure 6C**). In those mice showing perilesional micrometastases, HIF-1α expression was observed at the tumor-necrosis margin (**Figures 6D and E**).

Discussion

In this study we show in two preclinical models that local heat-induced tissue destruction has a dramatic stimulatory effect on the outgrowth of microscopic tumor cell clusters at the lesion periphery, representing a general and clinically relevant phenomenon. Tumor cells at the lesion periphery displayed aggressive morphological features with infiltrative growth and loss of differentiation. Our data support several recent observations of highly aggressive tumor behavior in patients with various tumor types treated by thermal ablation.[23-28] Moreover, our results are in accordance with recent work from others, showing stimulatory effects of thermal ablation on recurrent tumor growth in different models.[42-44] Moreover, we show that the stimulated outgrowth of perilesional micrometastases is associated with perilesional microcirculatory disturbances, profound tissue hypoxia and stabilization of HIF-1α. This is similar to the accelerated micrometastasis outgrowth after ischemia/reperfusion.[35]

The observed sinusoidal perfusion failure and loss of sinusoidal integrity correspond to the findings of Nikfarjam et al.[45] Atrasentan/L-Arginine treatment, aimed at restoring the endothelin-1/nitric oxide imbalance, has been successfully applied to reduce microcirculatory disturbances and liver tissue damage after ischemia/reperfusion,[35] but could only partly reduce the microcirculatory disturbances after LITT. Whereas the combination treatment improved the microcirculation further away from the lesion, perinecrotic microcirculatory flow directly adjacent to the necrotic lesion was still severely disturbed and tumor growth in the transition zone remained unaffected. Although this treatment strategy may be effective in reducing ischemia/reperfusion-stimulated tumor growth, it seems ineffective in reducing LITT-stimulated perilesional tumor growth.

Prolonged microvascular disturbances at the lesion periphery resulted in a prolonged hypoxic microenvironment and subsequent stabilization of HIF-1α. Whereas prolonged periods of hypoxia are deleterious to most cells, tumor cells have adapted to survive under hypoxic conditions.[46] Intratumoral hypoxia has been demonstrated in a number of human cancers and elevated expression of HIF-1α has been related to tumor aggressiveness.[47-51] HIF-1α activates the transcription of several genes that are implicated in cancer progression, including proliferation-promoting cytokines and growth factors, glucose transporters and serine-, aspartic- and metalloproteases. Moreover, HIF-1α promotes tumor neovascularization through upregulation of VEGF.[52,53] Thus, prolonged perinecrotic hypoxia as it occurs after thermal ablation may provide a protumorigenic microenvironment through stabilization of HIF-1α and by promoting tumor angiogenesis. Interestingly, pimonidazole and HIF-1α immunostaining were mainly localized around necrotic tissue areas and strong nuclear HIF-1α staining was observed in tumor cells at the necrosis-tumor interface 7 days after LITT. Our finding that 17-DMAG markedly reduced the accelerated outgrowth of micrometastases, but did not influence tumor growth in sham-operated mice or in untreated areas suggests that the compound may be particularly effective in reducing hypoxia-stimulated tumor growth. While 17-DMAG promotes degradation of HIF-1α, it also inhibits the function of heat shock protein [90.36,37] Heat shock proteins are acutely expressed in response to hypoxia and hyperthermia and they modulate numerous targets aimed at promoting cell survival.[54,55] Thus, other mechanisms may have co-contributed to the effectiveness of 17-DMAG in reducing tumor growth in the transition zone. A possible role for heat shock proteins in the stimulation of tumor growth after hyperthermia requires further investigation.

VEGF is one of the most potent downstream effectors of HIF-1α and it plays a pivotal role in the stimulation of hypoxia-driven angiogenesis.[56,57] Angiogenesis has been long recognized as a prerequisite for metastatic progression,[58-60] and has been previously implied in surgery-induced tumor growth.[22,61] Data from this study and from others suggest that thermal ablation therapy promotes angiogenesis around the necrotic lesion. Several angiogenic factors, including VEGF, are upregulated primarily adjacent to the ablation site.[42,44,62-64] Moreover, we found increased vascularization at the lesion edge 7 days after LITT by intravital microscopy, corresponding to the perilesional hyperechogenity as often observed on computed tomography or magnetic resonance imaging in the first 3 months following RFA.[13,65] The LITT-induced angiogenic stimulus may thus contribute to the facilitated tumor growth in this region.

Currently, angiogenesis inhibitors are widely investigated as anticancer agents and are increasingly used in clinical practice.[66,67] Several antiangiogenic compounds have been successfully applied to reduce stimulated tumor growth after hepatectomy.[30,68,69] PTK/ZK is an orally active angiogenesis inhibitor blocking all known receptors of VEGF and is currently under investigation in phase III trials for metastatic colorectal cancer.[38] PTK/ZK has previously been shown to reduce neovascularization and tumor growth of solid tumors, including colorectal liver metastases.[70-72] In the present study, short postoperative administration of PTK/ZK not only reduced the perilesional outgrowth of micrometastases, it also largely reduced tumor growth in control liver tissue and sham-operated mice. Thus, PTK/ZK may represent a very potent drug in the postoperative setting, as it may delay both ablation-accelerated recurrent tumor growth as well as recurrences in the remaining liver. Compounds that target HIF-1α or VEGF, such as 17-DMAG or PTK/ZK, may best be given in conjunction with conventional chemotherapy to achieve maximal efficacy and improve survival.[31,34] Randomized clinical trials to identify the optimal treatment strategy to improve outcome after local thermo-ablation are warranted.

The HIF-1α/VEGF-axis is not necessarily the sole contributor to ablation-stimulated tumor growth, as several other mechanisms may have co-stimulated the outgrowth of micrometastases. First, the inflammatory response evoked by thermal ablation, including altered cytokine expression, neutrophil infiltration and Kupffer cell activation may help to facilitate tumor outgrowth.[73-76] Interestingly, macrophages have been implied in cancer progression and may be a source for VEGF production.[77-79] Finally, the changes in tissue structure as induced by thermal ablation may facilitate the invasion and expansion of growing tumor cells. Our observation of migratory incoherent tumor cells in the transition zone displaying a remarkably different phenotype from tumor cells in control tissue underscores this possibility.

In addition to the local protumorigenic effect as described in the present study, several authors have described antitumorigenic effects after local ablative therapy on the outgrowth of remote tumor cell deposits.[80-82] Hyperthermia may elicit an antitumor T-cell response, by presenting tumor antigens to the immune system resulting in reduced spread and tumor growth in contralateral lobes and extrahepatic locations.[83,84] In our study we have not observed any antitumor effects at distant sites. However, at the time that LITT or RFA was applied in our model, tumor load was relatively low, and therefore, the likelihood of generating tumor-derived antigens is limited.

In conclusion, the findings of this study show that thermal ablation of liver tissue accelerates the outgrowth of residual microscopic tumor cell deposits, which is accompanied by poorly differentiated phenotypic morphology and increased tumor cell invasion. We conclude that prolonged tissue hypoxia, subsequent stabilization of HIF-1α and, possibly, angiogenesis contribute to the altered behavior of colorectal micrometastases. The prime concern of surgeons and interventional radiologists remains to maintain a safety margin of healthy liver tissue surrounding the ablated tumor. Improvement in radiographic assessment of residual disease may help to select candidates for adjuvant treatment.[85] In selected patients, at risk for local recurrence, postablative treatment with 17-DMAG or PTK/ZK may represent an attractive approach to prolong disease free survival.

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Chapter 10

Summary and general discussion

Surgical injury and wound healing in general have been long associated with enhanced tumor growth and early tumor recurrence. In **chapter 2** the role of surgery-induced angiogenesis in the uncontrolled growth of dormant micrometastases is reviewed. The impact of two frequently used procedures in hepatic surgery on metastasis outgrowth have been examined in detail in this work: ischemia/reperfusion (I/R) resulting from vascular clamping (**chapters 3-8**) and thermal ablation (**chapter 9**). The findings of the studies from this thesis and the answers to the central questions as formulated in the introduction are summarized and discussed here.

I How often and to what extent are vascular clamping methods currently used by hepatic surgeons in and around Europe?

Control of intra-operative bleeding is a primary goal in liver surgery and is traditionally achieved by vascular clamping.[1-3] To gain insight into the frequencies, the indications and the techniques of vascular clamping in current daily practice, we have undertaken an extensive online survey among 621 physicians, including all members of the European Hepato-Pancreato-Biliary Association, the European Surgical Association and the Dutch Liver Surgery Working Group (**chapter 3**). With an overall response rate of 50%, this survey revealed that vascular clamping during partial liver resection is routinely used by one out of five surgeons and that the majority of surgeons (70%) apply it in case of excessive blood loss or during major hepatic resection. Complete inflow occlusion is the most frequently used technique, with a distinct preference for intermittent clamping. Although total ischemia times are usually limited to 15-30 minutes, we were able to calculate that approximately one out of ten patients are routinely clamped for longer than 30 minutes. During thermal ablation vascular clamping is preserved for larger tumors or tumors in the vicinity of large vessels. Taken together, the survey primarily demonstrates that vascular clamping is frequently applied during hepatic surgery, which is consistent with a Japanese survey on control of intra-operative bleeding.[4] These findings underscore the importance of investigating putative adverse long-term effects of vascular clamping during hepatic surgery for colorectal liver metastases.

II Does I/R resulting from vascular inflow occlusion promote the outgrowth of residual micrometastases in the liver and how does this affect prognosis?

The short-term adverse effect of I/R resulting from temporary vascular clamping on hepatocellular integrity and function have been well documented in literature. In this work we show for the first time that I/R has a dramatic adverse effect on long-term outcome as well, by accelerating the outgrowth of colorectal micrometastases. In the highly standardized murine model described in **chapter 4** using C26 colorectal cancer cells in BALB/c mice, the outgrowth of pre-established micrometastases was accelerated five to sixfold in clamped liver lobes when compared to nonclamped liver lobes following 45 minutes of left lobar I/R. Similar phenomena were found in two additional animal models, using different colorectal cancer cell lines (MC38-C57BL/6 and CC531-Wag/Rij) (unpublished data). In all murine models, I/R had no discernable effect on the growth rate of micrometastases in the nonclamped liver lobes and no extrahepatic metastases were found, suggesting that the tumor growth promoting effect of I/R operates locally and primarily affects pre-existent micrometastases in the clamped part of the liver.

As recurrences develop in the majority of patients after hepatic resection, patients are likely to have residual micrometastastic disease, even after an apparently complete tumor removal. In **chapter 5**, we investigated the effect of vascular clamping on long-term outcome in a cohort of patients undergoing an intentionally curative hepatic resection for colorectal liver metastases in our institution. Indeed, we found that vascular clamping was associated with early intrahepatic recurrence, further substantiating a local tumor growth promoting effect. In addition, when ischemia times were prolonged (more than 20 minutes in case of continuous clamping or more than 2 cycles of 10-15 minutes in case of intermittent clamping), disease free survival decreased as well. This finding suggests that severe I/R may also have systemic stimulatory effects on remote micrometastases. Remote organ damage, predominantly in the lungs, is a well known consequence of I/R and is thought to be induced by the excretion of pro-inflammatory cytokines and oxygen species into the circulation.[5,6] The effect of prolonged vascular clamping on established remote (pulmonary) metastases was not further evaluated in this thesis, but experimental studies on this topic are worthwhile to pursue.

Vascular clamping did not have any impact on overall survival in our patient cohort, which may be attributed to the aggressive approach towards treating recurrent disease and the use of adjuvant chemotherapy in several patients. Nonetheless, early hepatic recurrence may hamper further treatment and is generally associated with a poorer prognosis.[7]

Preferably, the adverse effects of I/R on outcome should be corroborated in randomized prospective clinical trials. However, several ethical considerations may hamper well-designed and thorough investigations. In most liver surgery units around the world (as in our institution) vascular clamping during liver resection is not a standard procedure, but is applied in case of excessive blood loss only. Randomization between clamping and no clamping implies that some patients would be subjected to a procedure which is potentially harmful, while on the other hand, some patients with accelerating blood loss would be withheld from a maneuver which may be needed to perform a safe resection. Therefore, at present, we can solely rely on retrospective patient data and on preclinical models. Our patient data, in combination with the dramatic effects observed in three experimental models, leads us to propose that the adverse effects of vascular clamping on long-term outcome well represent clinical significance. Therefore, we plea that vascular clamping should be omitted when it is not mandatory. Major hepatic resection may be safely performed without vascular clamping,[8,9] by using alternative approaches that contribute to a more bloodless hepatic resection, such as the maintenance of low venous pressure,[10-13] intraoperative ultrasonographic guidance,[14] precoagulation devices,[15] and hemostatic biologicals.[16]

III How does ischemia time affect the outgrowth of micrometastases after I/R?

The contribution of ischemia time to hepatocellular damage has been thoroughly evaluated in animal models, showing that prolonged ischemic periods induce time-dependent hepatocellular damage, liver failure and mortality.[17-19] The normal human liver seems to tolerate continuous normothermic ischemia of up to 60 minutes and intermittent ischemia of up to 120 minutes relatively well, but also longer cumulative ischemia times have been applied with mild and transient hepatic dysfunction and low complication rates.[20-25] Nonetheless, although prolonged vascular clamping may be performed without any severe postoperative morbidity or mortality, it may induce severe long-term adverse effects by accelerating the outgrowth of residual disease. In our animal study 45 minutes

of partial hepatic I/R induced only transient liver dysfunction without any morbidity or mortality, but induced a five to sixfold local acceleration of tumor growth. In **chapter 6** we show that the effects of I/R on metastasis outgrowth correlate with the duration of the ischemic period. The safe upper limit of vascular clamping in our murine model appeared to be only 20 minutes of continuous ischemia. Interestingly, in the patient cohort, clamping of any type was associated with early intrahepatic recurrence, which was irrespective of the duration (**chapter 5**). When ischemia times were prolonged (i.e. continuous inflow occlusion of more than 20 minutes or intermittent clamping of more than two cycles of 10-15 minutes each), both liver free survival and disease free survival were decreased. Based on these findings we suggest that in case of inevitable blood loss, ischemia times should be kept to a minimum. The safe upper limits in patients should be further defined in large cohorts.

IV How are the adverse effects of vascular clamping on tumor growth influenced by age, gender and hepatic steatosis?

The magnitude of tissue damage resulting from I/R has been reported to rely on various (patho)physiological parameters, such as age,[26-28] gender,[29-31] and underlying liver disease, including hepatic steatosis.[32-34] We aimed to define the influence of these parameters on the outgrowth of colorectal micrometastases following hepatic inflow occlusion. Interestingly, in our patient study (**chapter 5**), the adverse effect of severe ischemia resulting from prolonged vascular clamping on liver free survival and disease free survival were exacerbated in older patients, males and in patients with hepatic steatosis. In **chapter 6** we investigated whether these observations could be confirmed in our murine model. We found that the stimulated outgrowth of micrometastases following 30 minutes of left lobar ischemia had increased in aged mice and in mice with hepatic steatosis by 33% and 51% respectively. Female mice were relatively resistant to tumor growth in general, as shown by an equal decrease in tumor load in both clamped and non-clamped liver lobes, but this did not protect them against accelerated metastasis outgrowth. Thus, using this experimental model, the relative protection against the stimulatory effects of I/R on tumor growth in female patients could not be confirmed. Our data suggest that (prolonged) vascular clamping should be particularly omitted in older patients and in patients with hepatic steatosis. Importantly, neo-adjuvant chemotherapy induces steatohepatitis in up to 92% of patients, which is associated with increased susceptibility to ischemic damage.[35-37] This implies that prolonged vascular clamping in patients who have undergone neo-adjuvant chemotherapy may induce an increased risk for recurrent tumor growth after hepatectomy, which is clinically very relevant as an increasing number of patients receive neo-adjuvant chemotherapy. The correlation between gender, vascular clamping and outcome needs further evaluation in clinical trials, as this may help to further select candidates for practical or pharmacological protective strategies against the adverse effect of I/R on metastasis outgrowth. The influence of I/R in older females or in females with hepatic steatosis should also be investigated.

V What alternative clamping methods can be used to circumvene the adverse effects of vascular clamping on tumor growth?

The attention in recent years for I/R injury resulting from prolonged continuous clamping, has clearly led to a distinct preference for techniques that protect the liver from ischemic damage, such as intermittent clamping and ischemic preconditioning,[22,27,38-42] which was confirmed by our survey. In **chapter 4** we investigated how these alternative clamping techniques affect the outgrowth of pre-established micrometastases in our murine model. We found that both intermittent clamping and ischemic preconditioning largely prevented early hepatocellular damage, as judged by reduced liver enzyme levels (by 85% and 93%, respectively). Accelerated tumor growth and tissue necrosis were virtually completely prevented by occluding blood flow intermittently, whereas ischemic preconditioning could not protect against this phenomena. In our patient data (**chapter 5**), we could not confirm any evident difference between intermittent clamping and continuous clamping with regard to long-term outcome. This may be attributed to an increase in blood loss during reperfusion associated with intermittent clamping,[39] which in turn, may worsen prognosis as well.[43,44] Ischemic preconditioning was not performed in our patients. Based on our preclinical data and its proven hepatoprotective effect, we conclude that intermittent clamping should be favored over continuous clamping and ischemic preconditioning, particularly when ischemia times are long. Although in mice, intermittent clamping was safely performed up to 45 minutes, the safe upper limit of intermittent clamping without adverse oncological effects needs to be defined. The disadvantage of intermittent clamping is that it needs stringent time management and additional manipulation during resection.

In **chapter 7** we investigated how selective clamping of the portal vein affects the outgrowth of pre-established micrometastases in a model of 70% hepatic I/R. In this model we found that when the portal vein was selectively clamped, leaving the oxygen rich blood supply via the hepatic artery intact, liver tissue damage was minimal and micrometastases outgrowth was not stimulated. Based on this result, we suggest that portal clamping may be particularly useful during thermal destruction therapies, as it minimizes I/R injury, successfully reduces the heat sink effect,[45-47] and eliminates the risk of accelerated tumor outgrowth after standard inflow occlusion. In particular, portal clamping is preferred during open focal heat destruction techniques and in case of multiple tumors when the expected ablation time is relatively long. In those cases, occlusion time during surgery is not restricted as oxygen supply to the liver is maintained. For thermal ablation of single tumors and after previous operations intermittent clamping may be easier than portal clamping, as it avoids dissection of the hepatoduodenal ligament. Portal clamping may also be used during hepatic resection, however using this technique, bleeding from the hepatic artery is not prevented.

VI What mechanisms contribute to the stimulated metastasis outgrowth after I/R and how can these be mediated by pharmacological interventions?

We aimed to identify the mechanisms that play a role in the observed local stimulatory effects of I/R on tumor growth and to develop pharmacological strategies against I/R-stimulated tumor growth. An important observation in all murine studies was that, microscopically, tumor growth was mainly located around necrotic tissue areas. We reasoned that prevention of tissue necrosis would also reduce the acceleration of tumor growth after I/R. Moreover, events that take part at the necrosis-tumor interface could play a role in tumor growth stimulation.

Oxygen radicals are generated during the early phases of reperfusion and contribute to early hepatocellular damage.[48-50] The anti-oxidants α-tocopherol and ascorbic acid have both been proven to be effective in reducing oxidative stress and hepatocellular damage in both clinical and preclinical trials.[51-56] In **chapter 4** we investigated whether α-tocopherol and ascorbic acid would also protect the liver against the accelerated outgrowth of micrometastases. Although early hepatocellular damage was largely prevented by α-tocopherol and ascorbic acid, both anti-oxidants failed to protect against late tissue necrosis. Moreover, I/R-accelerated tumor growth remained unaffected, suggesting that the enhanced outgrowth of micrometastases correlates with the late events causing hepatic necrosis rather than with early events, including the production of oxygen radicals.

Microcirculatory disturbances, resulting from an imbalance of vasoconstrictors (e.g. endothelin-1) and vasodilators (e.g. nitric oxide), play a pivotal role in the late phase of I/R injury.[57-60] Prolonged microcirculatory disturbances may induce sustained tissue hypoxia and stabilization of hypoxia-inducible factor (HIF)-1α. Hypoxia, partly through upregulation of HIF-1α, is one of the most powerful stimulatory conditions to tumor growth.[61] Intratumoral hypoxia has been demonstrated in a number of human cancers and elevated expression of HIF-1α has been related to tumor aggressiveness.[62-65] HIF-1α activates the transcription of several genes that are implicated in cancer progression, including proliferation-promoting cytokines and growth factors, angiogenesis–promoting growth factors, glucose transporters and serine-, aspartic- and metallo-proteases.[66,67]

In **chapter 8**, we provide evidence that prolonged post-ischemic microcirculatory disturbances, tissue hypoxia and subsequent stabilization of HIF-1α play an important role in the altered behavior of micrometastases in the liver following I/R. We demonstrated that I/R is associated with long-term microcirculatory disturbances, profound peri-necrotic tissue hypoxia and stabilization of HIF-1α, as shown by intravital microscopy, pimonidazole immunostaining and HIF-1α immunohistochemistry respectively. Furthermore, we found that restoration of the microcirculation (by treatment with the endothelin-1 antagonist Atrasentan and the nitric oxide donor L-Arginine) minimized tissue hypoxia, avoided HIF-1α stabilization and reduced the accelerated outgrowth of micrometastases. To further substantiate the role of peri-necrotic hypoxia and HIF-1α in I/R-stimulated metastasis outgrowth, we used the geldanamycin analogue 17-DMAG, to promote degradation of HIF-1α. Despite an 80% increase in tissue necrosis, 17-DMAG reduced I/R-stimulated tumor growth by more than 70%. This is the first time that we observed extensive tissue necrosis without associated tumor growth and without tumoral HIF-1α immunostaining. In summary, these data strongly suggest that prolonged microcirculatory disturbances and hypoxia, as occur after I/R, provide a protumorigenic microenvironment through up-regulation of HIF-1α. Of note is that intermittent clamping appears to be superior to ischemic preconditioning in maintaining hepatic microcirculation, especially after prolonged periods of ischemia.[40] This may explain the remarkable difference between ischemic preconditioning and intermittent clamping in preventing metastasis outgrowth (**chapter 4**). Moreover, it has been shown that microcirculatory disturbances are worsened in fatty livers,[33] which may explain the exacerbated metastasis outgrowth in steatotic livers (see chapter 6).

In the event of severe ischemic damage due to prolonged vascular clamping, improving microcirculatory flow or targeting the HIF-1α pathway may decrease the stimulation of microscopic tumor deposits and improve prognosis in colorectal cancer patients treated by partial liver resection. We showed maximal efficacy of Atrasentan combined with L-Arginine, but other (combinations of) compounds may provide similar protective effects. Agents that target the HIF-1α pathway, including 17-DMAG, are gaining increased attention as novel anti-cancer drugs.[68,69] Such compounds may ideally be administered peri-operatively to prevent hypoxia-induced tumor growth stimulation. However, HIF-1α is also involved in the physiologic response to tissue damage and thus, their peri-operative application may hamper wound and anastomosis healing, which may contribute to increased morbidity. Indeed, we found increased necrosis in clamped liver lobes following treatment with 17-DMAG. Further studies are needed to investigate the effectiveness and safety of such compounds in the post-operative setting.

In addition to the proposed mechanism, other mechanisms may have cooperated in stimulating the outgrowth of colorectal micrometastases after I/R. One of the major downstream effects of HIF-1α stabilization is the upregulation of vascular endothelial growth factor (VEGF), which is a key modulator of tumor neovascularisation.[66,70,71] After I/R dormant micrometastases may switch into a vascularized state due the up-regulation of VEGF and other pro-angiogenic factors, allowing metastases to grow beyond 2 mm in size.[72] Moreover, the pro-inflammatory response associated with I/R, including the influx of neutrophils and activation of Kupffer cells, may also contribute to the stimulation of tumor growth. Both activated neutrophils and macrophages have been associated with increased metastatic potential, proliferation and invasion.[73-75] Interestingly, hypoxic macrophages secrete growth factors and angiogenic factors that favor tumor progression.[76] Furthermore, increased HIF-1α expression has been associated with the presence of tumor associated macrophages.[77] Evidently, inflammation and hypoxia mutually influence each other and may co-activate tumor cell proliferation. Thus, tampering the inflammatory response may also minimize hypoxia and thereby reduce tumor growth. Furthermore, the altered ultrastructure of the liver parenchyma after I/R may facilitate the invasion, migration and outgrowth of tumor cells. Infiltrating lymphocytes may induce apoptosis in surrounding hepatocytes through stimulation of Fas, which may facilitate tumor outgrowth as a result of an altered tissue structure.[78] Our observation that the areas of enhanced tumor outgrowth were closely associated with zones of inflammatory cells and apoptotic hepatocytes surrounding the necrotic tissue areas supports this hypothesis. These issues are currently under investigation.

VII Does thermal ablation, which is associated with pathophysiological events similar to I/R, also stimulate the outgrowth of residual tumor cell deposits; and how can this be inhibited?

As previously stated, surgical resection remains the only potentially curative treatment option for colorectal liver metastases, but is applicable to 10-30% of patients. Thermal destruction therapies, such as radiofrequency ablation (RFA) and laser-induced thermotherapy (LITT), are widely used to locally destroy nonresectable metastases. Despite an apparently complete tumor destruction, local perilesional recurrences occur in up to 60%. As both RFA and LITT univocally generate tissue necrosis, we hypothesized that thermal ablation may stimulate the outgrowth of residual tumor cells at the lesion periphery similar to I/R-accelerated tumor growth.

In **chapter 9** two animal models with established colorectal micrometastases were used to study the effect of RFA and LITT on the outgrowth of tumor cell clusters at the lesion periphery. We found that the outgrowth of micrometastases at the lesion periphery was stimulated over fourfold compared to tumor growth in the remaining liver. Moreover, similar to I/R, LITT induced prolonged perilesional microcirculatory disturbances, profound peri-lesional hypoxia and stabilization of HIF-1α.

We next tested whether intervention strategies aimed at improving microcirculatory flow (by Atrasentan/L-Arginine), preventing the stabilization of HIF-1α in response to tissue hypoxia (by 17-DMAG), or inhibiting VEGF-signaling (by PTK787/ZK-222584) would be beneficial in controlling accelerated tumor recurrence following thermal ablation. Atrasentan/L-Arginine improved the microcirculation further away from the lesion, but perinecrotic microcirculatory flow was still severely disturbed and tumor growth remained unaffected. Treatment with 17-DMAG prevented HIF-1α stabilization and reduced tumor growth in the transition zone by 34% without affecting tumor growth in sham-operated animals. PTK787/ZK-222584 reduced both LITT-stimulated tumor growth and tumor growth in the remaining liver. We conclude that adjuvant treatment with 17-DMAG or PTK787/ZK-222584 may represent attractive approaches to reduce local recurrence after thermal destruction therapy for liver metastases.

Our data support several recent observations of highly aggressive tumor behavior in patients with various tumor types treated by thermal ablation.[79-84] Moreover, our results are in accordance with recent work from others, showing stimulatory effects of thermal ablation on recurrent tumor growth in different models.[85-87] The mainstay of effective thermal destruction therapy remains to preserve a safety margin of healthy liver tissue surrounding the ablated tumor. Paradoxically, improved tumor clearance and safe ablation margins can be best achieved by blocking the vascular flow, which in turn induces I/R injury and accelerates the outgrowth of microscopical tumor residues. Therefore, during local ablation, the combination of portal vascular inflow occlusion and compounds that target HIF-1α or VEGF may induce maximal efficacy and improve survival after local ablation, which needs to be investigated in clinical trials.

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Chapter 11

Conclusions and future perspectives

Conclusions

Based on the findings described in this work, the following conclusions may be drawn:

I Prolonged vascular clamping is frequently applied during hepatic resection
II I/R resulting from vascular clamping worsens prognosis by accelerating the outgrowth of colorectal micrometastases
III The tumor growth promoting effect of I/R depends on ischemia time, with safe upper limits of 20 minutes of continuous ischemia and at least 45 minutes of intermittent ischemia in mice
IV Ageing and hepatic steatosis exacerbate I/R-accelerated outgrowth of colorectal micrometastases
V Intermittent clamping and portal clamping, but not ischemic preconditioning protect against vascular clamping-induced tumor growth stimulation
VI Peri-necrotic hypoxia and HIF-1α play an important role in I/R-accelerated outgrowth of colorectal micrometastases and are key targets for pharmacological intervention
VII Thermal destruction therapy accelerates the outgrowth of perilesional micrometastastic lesions, which may be suppressed by inhibition of the HIF-1α/VEGF-axis

Supported by these results we recommend that vascular clamping during hepatic resection is omitted when it is not obligatory. In case of inevitable excessive blood loss, ischemia times should be kept to a minimum. Based on its proven hepatoprotective effects and its suggested inhibitory effects on I/R-induced tumor growth stimulation, intermittent clamping is advocated during hepatic resection. Vascular clamping used to increase lesion size during thermal destruction therapies, should preferably be applied to the portal vein only. In case of expected severe I/R damage following hepatic resection, improving post-ischemic microcirculatory flow or targeting the HIF-1α pathway may decrease the stimulation of microscopic tumor deposits and improve prognosis. Protective strategies against I/R induced metastasis outgrowth should particularly be employed in older patients and in patients with pre-existent or chemotherapy-induced hepatic steatosis. During thermal destruction techniques, inhibition of the HIF-1α/VEGF- axis may overcome ablation-accelerated outgrowth of residual disease in the lesion periphery and is therefore advised as adjuvant treatment strategy.

Future perspectives

The studies as presented in this thesis may form an incentive to change the surgical management of colorectal liver metastases. Clinical and experimental studies are warranted to further investigate several aspects of I/R and ablation-induced tumor growth stimulation.
Prospective randomized clinical trials should be developed to investigate how different clamping techniques (e.g. intermittent clamping versus ischemic preconditioning) affect tissue hypoxia, stabilization of HIF-1α, pro-angiogenic cytokines and long-term outcome. We emphasize that the time to develop intrahepatic recurrence and disease free survival should be investigated as a primary end-point in these clinical trials. Ideally, these trials should include stratification according to gender, age, underlying liver disease, and neo-adjuvant chemotherapy to further select candidates for whom protective strategies against I/R accelerated tumor growth need to be employed. Given the fact that prolonged vascular clamping is frequently applied during hepatic resection, experimental studies that further unravel the mechanistic background of I/R-stimulated outgrowth are worthwhile to pursue. Although we propose several pharmacological approaches that counteract the adverse effects of I/R on tumor growth, there is room for improvement regarding the pharmacological prevention of outgrowth of residual disease after hepatic resection and thermal ablation. One of the most promising improvements in hepatic surgery may come from adjuvant peri-operative chemotherapy strategies to destroy minimal micrometastatic disease, currently under investigation in large clinical trials. Future trials should be aimed at combining chemotherapy and targeting biological agents to achieve maximal efficacy.[1,2] Our studies suggest that treatment strategies that include HIF-1α and/or VEGF-targeted therapeutics may result in further improvement in long-term outcome after hepatic resection and may offer an increasing number of patients a hope for cure.

Finally, we would like to underscore that the studies as described in this thesis are not only relevant for tumor growth stimulation after vascular clamping or local ablation, but may be taken to a broader perspective. First, similar phenomena may occur following other surgically or non-surgically induced events that cause I/R (e.g. hemorrhagic or septic shock followed by blood transfusion and resuscitation)[3,4] or tissue hypoxia (e.g wounding, inflammation, organ manipulation, laparoscopy and chemotherapy).[5] Second, it is presently unknown whether I/R also accelerates the outgrowth of other malignant tumor cells from hepatocellular carcinoma, neuroendocrine tumors, gastric carcinoma, breast cancer or melanoma. This warrants further evaluation, given that, these malignancies may be treated by hepatic resection as well.

In conclusion, this thesis identifies I/R and peri-necrotic tissue hypoxia as strong stimuli for recurrent intrahepatic tumor growth and offers several practical and pharmacological treatment strategies to protect the liver against surgery-induced tumor growth stimulation. These insights may help to improve the outcome in patients treated by partial liver resection or local ablation for colorectal liver metastases.

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Chapter 12

Nederlandse samenvatting

Achtergrond

Chirurgische behandeling van uitgezaaide darmkanker

Darmkanker of colorectaal carcinoom is een van de meest voorkomende vormen van kanker in de westerse wereld. Jaarlijks komen er in Nederland alleen al 10.000 nieuwe patiënten met darmkanker bij. De prognose van darmkanker wordt in grote mate bepaald door de uitbreiding ervan. Wanneer darmkankergezwellen zich beperken tot de darm, biedt operatieve verwijdering ervan grote kans op genezing. Echter, bij ruim de helft van de patiënten zaait de ziekte vroeg of laat uit naar andere organen, vooral naar de lever (levermetastasen). Met behulp van chemotherapie is, dankzij recente ontwikkelingen, de gemiddelde overleving van patiënten met uitgezaaide darmkanker de afgelopen jaren toegenomen van 10 naar ongeveer 20 maanden, maar toch overlijden helaas bijna alle patiënten binnen vijf jaar. Voor ongeveer een kwart van de patiënten met beperkte uitzaaiingen in de lever kan een operatie uitkomst bieden, waarbij het aangedane deel van de lever wordt verwijderd (leverresectie). Na een dergelijke operatie leeft 30-40% van de patiënten nog na vijf jaar. Om die reden wordt resectie vooralsnog beschouwd als de enige hoop op genezing. Voor patiënten met niet-operabele levermetastasen zijn technieken ontwikkeld die met behulp van hitte de tumor kunnen vernietigen (lokale ablatie). Met deze lokale ablatie technieken, zoals radiofrequente ablatie (RFA) en laser-geïnduceerde thermotherapie (LITT), worden inmiddels veelbelovende resultaten behaald.

Recidieven en het belang van micrometastasen

Ondanks deze hoopgevende operaties komen levermetastasen bij een groot deel van de patiënten terug, er is dan sprake van een recidief. De meest waarschijnlijke bron voor recidieven zijn kleine, onzichtbare uitzaaiingen (micrometastasen), die zich al ten tijde van de operatie in de lever bevonden. De groeisnelheid en het biologisch gedrag van deze micrometastasen bepalen in grote mate hoe snel een patiënt een recidief krijgt en wat zijn of haar kansen zijn op overlijden.

Chirurgie-gestimuleerde tumorgroei

Micrometastasen kunnen jaren lang in een soort slapende toestand verkeren, totdat een bepaalde situatie er voor zorgt dat deze gaan uitgroeien. Er wordt wel gedacht dat weefselschade tijdens een operatie mogelijk een groeistimulerend effect heeft op kankercellen. Zo wordt een snelle postoperatieve ziekteprogressie door patiënten wel eens toegeschreven aan het feit dat **"er lucht bij gekomen is"** tijdens de operatie. Deze gedachte lijkt niet geheel onterecht, want ook in de literatuur zijn al sinds 1959 verschillende experimentele studies en klinische casereports gepubliceerd over chirurgie-gestimuleerde tumorgroei. In **hoofdstuk 2** wordt een overzicht gegeven van beschikbare studies uit de literatuur die versnelde tumorgroei in verband brengen met wondgenezing en vaatnieuwvorming (angiogenese). In dit proefschrift hebben we het effect van twee belangrijke procedures in de leverchirurgie op de uitgroei van colorectale micrometastasen uitvoerig onderzocht: het afklemmen van de bloedtoevoer en lokale ablatie.

Afklemmen van de bloedtoevoer naar de lever tijdens leverchirurgie

Het belangrijkste doel van de chirurg tijdens een leverresectie is om alle metastasen volledig te verwijderen zonder (veel) bloedverlies. De lever is een zeer bloedrijk orgaan en bloedverlies treedt dan ook regelmatig op, hetgeen de kans op postoperatieve complicaties en sterfte op korte en lange termijn sterk kan vergroten. Om bloedverlies te beperken wordt tijdens de operatie de bloedtoevoer naar de lever tijdelijk afgeklemd.

Bij de lokale ablatie methoden is het van belang dat al het tumorweefsel door de vrijgekomen hitte vernietigd wordt. Tijdens deze techniek wordt, zoals een hete pan onder de koude kraan, de hitte door de bloedstroom weggevoerd, waardoor de volledige destructie van een tumor bemoeilijkt kan worden. Om die reden wordt door experts geadviseerd om ook tijdens lokale ablatie methoden de bloedtoevoer naar de lever af te klemmen.
Verschillende klemmethoden zijn beschreven in de literatuur. De zogenoemde Pringle Manoeuvre werd in 1909 voor het eerst beschreven en is de simpelste techniek waarbij de aanvoerende vaten naar de lever tijdelijk worden afgeklemd. Bij andere methoden worden ook de afvoerende vaten afgeklemd of alleen de vaten naar één zijde van de lever. Ook kan alleen het grootste aanvoerende vat naar de lever selectief worden afgeklemd (de poortader), waardoor het zuurstofrijke bloed door de leverslagader intact blijft. De bloedtoevoer kan continu worden afgeklemd of in intervallen (intermitterend) en de duur van het afklemmen kan worden gevarieerd.

Ischemie/reperfusie en levercelschade

Een nadeel van het tijdelijk afklemmen van de bloedvaten is dat de zuurstoftoevoer naar de lever kortdurend stagneert. Dit leidt tot zuurstoftekort (ischemie) in de levercellen. Wanneer de klem verwijderd wordt en de circulatie naar de lever is hersteld, spreekt men van reperfusie. Het is reeds lange tijd bekend dat ischemie gevolgd door reperfusie (I/R) schade aan de lever veroorzaakt. Dit kan leiden tot leverfunctiestoornissen en complicaties na de operatie.
De mate van schade hangt voornamelijk af van de methode en de duur van het klemmen en wordt beïnvloed door verschillende omstandigheden, zoals leeftijd, geslacht en de aanwezigheid van leveraandoeningen, zoals leververvetting.
In de afgelopen jaren is de kennis over de effecten van ischemie/reperfusie op levercellen enorm toegenomen. Opvallend genoeg is de invloed van ischemie/reperfusie op de uitgroei van tumorcellen nog niet eerder onderzocht.

Centrale vraagstellingen en belangrijkste bevindingen van dit proefschrift

Het thema van dit proefschrift is de invloed van chirurgie op de groeisnelheid van uitgezaaide darmkankercellen in de lever. Het effect van twee belangrijke procedures in de leverchirurgie op de uitgroei van deze colorectale micrometastasen werd uitvoerig onderzocht: ischemie/reperfusie ten gevolge van het afklemmen van de bloedtoevoer (**hoofdstukken 3-8**) en lokale ablatie (**hoofdstuk 9**). De belangrijkste resultaten van dit proefschrift en de antwoorden op de zeven centrale vraagstellingen worden hieronder besproken.

I Hoe vaak klemmen leverchirurgen in Europa de bloedtoevoer naar de lever af en welke methoden gebruiken zij hierbij?

Voordat we de invloed van het afklemmen van de bloedtoevoer op tumorgroei hebben onderzocht, vroegen we ons af hoe vaak er momenteel door leverchirurgen in Europa geklemd wordt en welke technieken daarvoor gebruikt worden (**hoofdstuk 3**). Daarvoor hebben we een vragenlijst rondgestuurd aan 621 artsen, waaronder alle leden van de European Hepato-Pancreato-Biliary Association, de European Surgical Association en de Nederlandse Werkgroep Leverchirurgie. De vragenlijst werd beantwoord door 311 leverchirurgen uit 39 landen. De uitkomsten van deze vragenlijst lieten vooral zien dat één op de vijf chirurgen routinematig (dus bij elke operatie) de bloedtoevoer naar de lever afklemt, terwijl 70% van de chirurgen alleen klemt wanneer er (te veel) bloedverlies optreedt. Meestal worden alleen de aanvoerende vaten afgeklemd (de Pringle Manoeuvre) en er bestaat een duidelijke voorkeur voor het klemmen in intervallen (intermitterend). Ondanks dat het afklemmen meestal maar 15-30 minuten duurt, hebben we berekend dat de bloedtoevoer bij een op de tien patiënten routinematig langer dan een half uur afgeklemd wordt. Deze resultaten onderstrepen het belang van het onderzoeken van mogelijke negatieve effecten van het afklemmen van de bloedtoevoer op de lange termijn.

II Wat is de invloed van ischemie/reperfusie (door het afklemmen van de bloedtoevoer) op de uitgroei van colorectale micrometastasen in de lever?

Het nadelige effect van het klemmen van de bloedtoevoer op normaal leverweefsel is uitvoerig onderzocht. In dit werk laten we voor het eerst zien dat ischemie/reperfusie ten gevolge van langdurig klemmen ook een nadelige invloed heeft op de prognose van darmkankerpatiënten, omdat het tumorgroei kan stimuleren.
In **hoofdstuk 4** laten we in een gestandaardiseerd muizenmodel zien dat de uitgroei van colorectale micrometastasen na ischemie/reperfusie wel vijf tot zes keer versneld is. Dit proces lijkt heel lokaal plaats te vinden, omdat het alleen tumorgroei in de geklemde leverhelft stimuleert en niet in de niet-geklemde leverhelft. Vervolgens hebben we in **hoofdstuk 5** onderzocht wat de invloed is van langdurig klemmen op de lange termijnuitkomsten bij patiënten die een leverresectie hebben ondergaan voor uitgezaaide darmkanker in het UMC Utrecht. We vonden inderdaad dat patiënten, bij wie de bloedtoevoer tijdelijk was afgeklemd tijdens de operatie, eerder een recidief in de lever kregen dan patiënten die niet geklemd waren. Wanneer er langdurig afgeklemd werd (gedurende 20 minuten continu of twee perioden van 10-15 minuten) vonden we dat ook de totale ziektevrije overleving verlaagd was.

Bij voorkeur zou dit fenomeen onderzocht moeten worden in zogenaamde prospectieve gerandomiseerde studies, waarbij patiënten worden geloot tussen bijvoorbeeld wel of niet klemmen. Dit type onderzoeken wordt in de medische wetenschap namelijk beschouwd als de enige harde waarheid. In dit geval is een dergelijke studie op ethische gronden echter niet uitvoerbaar. In de meeste centra in de hele wereld (zoals ook in het UMC Utrecht) wordt de bloedtoevoer alleen afgeklemd bij buitensporig bloedverlies. Loten tussen wel of niet klemmen zou betekenen dat bij een deel van de patiënten geklemd wordt terwijl dat misschien niet nodig is, terwijl aan de andere kant patiënten een dergelijke procedure wordt onthouden, terwijl zij dat wel nodig hebben. Daarom kunnen we momenteel slechts varen op retrospectieve patiëntenstudies en dierexperimentele studies. Onze patiëntendata in combinatie met de dramatische effecten in het muizenmodel suggereren dat de nadelige effecten van ischemie/reperfusie op tumorgroei klinisch zeer relevant zijn. We pleiten er daarom ook voor dat, wanneer het niet strikt noodzakelijk is, het klemmen van de bloedtoevoer naar de lever tijdens leverchirurgie achterwege gelaten moet worden. Uiteraard blijft het belangrijk om bloedverlies tijdens de operatie te voorkomen. Hiervoor wordt geadviseerd gebruik te maken van alternatieve technieken die gericht zijn op het voorkomen van bloedverlies, zoals het laag houden van de centraal veneuze druk tijdens de operatie, goede lokalisatie van de metastasen met behulp van echografie en het dichtbranden van het leverweefsel alvorens het door te nemen.

III Wat is de invloed van de duur van het klemmen op de uitgroei van micrometastasen na ischemie/reperfusie.

Door dierexperimentele studies en patiëntenstudies weten we dat schade aan levercellen na het klemmen van de bloedtoevoer erger is naarmate er langer geklemd wordt. Een mensenlever weerstaat een periode van 60 minuten continu klemmen en 120 minuten intermitterend klemmen redelijk goed en zelfs langere perioden zijn beschreven zonder ernstige leverfunctiestoornissen of complicaties. Ondanks dat de schade na ischemie/reperfusie op korte termijn relatief mild is, kan het ernstige gevolgen hebben op de lange termijn. In ons muizenmodel bijvoorbeeld, had 45 minuten ischemie milde leverfunctiestoornissen tot gevolg zonder complicaties of sterfte, maar veroorzaakte wel een vijf tot zes keer snellere tumorgroei. In **hoofdstuk 6** laten we zien dat de effecten van ischemie/reperfusie op de uitgroei van micrometastasen sterk gerelateerd is aan de duur van het klemmen. De maximale veilige duur in dit model was slechts 20 minuten. Op basis hiervan is ons advies dat wanneer klemmen onvermijdelijk is, de duur ervan zo kort mogelijk moet zijn. De maximale klemduur die in patiënten nog veilig is zou nader uitgezocht moeten worden in grotere groepen patiënten.

IV Wat is de invloed van leeftijd, geslacht en de aanwezigheid van leverziekten op de uitgroei van colorectale micrometastasen na ischemie/reperfusie?

Uit de literatuur is bekend dat de schade aan levercellen door ischemie/reperfusie beïnvloed wordt door geslacht, leeftijd en onderliggend leverlijden, zoals leververvetting (steatose). We hebben onderzocht of dit ook geldt voor de tumorgroeistimulerende effecten van ischemie/reperfusie. Interessant genoeg vonden we in onze patiënten populatie (**hoofdstuk 5**) dat de nadelige effecten van langdurig klemmen van de bloedtoevoer vooral aanwezig waren bij mannen, oudere patiënten en bij leververvetting.

In **hoofdstuk 6** beschrijven we dat het stimulerende effect van ischemie/reperfusie op de uitgroei van colorectale micrometastasen in oudere muizen en in muizen met leververvetting werd verergerd met respectievelijk 33% en 51%. De versnelde tumorgroei na ischemie/reperfusie in mannetjes muizen was gelijk aan die van vrouwtjesmuizen.
Deze gegevens laten zien dat langdurig klemmen vooral achterwege gelaten zou moeten worden bij oudere patiënten en mensen met leververvetting. Een belangrijk gegeven daarbij is dat preoperatieve chemotherapie ook een vorm van leververvetting kan veroorzaken. Ook bij deze patiënten zou daarom het liefst zo min mogelijk geklemd moeten worden. Meer onderzoek naar de invloed van verschillende omstandigheden op tumorgroeistimulering is nodig om patiëntengroepen te selecteren bij wie interventie maatregelen genomen moeten worden.

V Zijn er alternatieve klemmethoden die stimulering van tumorgroei kunnen voorkomen?

Door de toegenomen kennis bestaat er een sterke voorkeur voor alternatieve klemmethoden die het leverweefsel beschermen tegen schade. De belangrijkste en de meest gebuikte methode is het klemmen in intervallen, ook wel intermitterend klemmen. Bij ischemische preconditionering wordt er kortdurend geklemd voordat er een lange periode van klemmen volgt. Door het kortstondig zuurstoftekort komen er stoffen vrij die een weefselbeschermend effect hebben, waardoor het leverweefsel ongevoeliger wordt voor schade ten gevolge van langdurig klemmen, vergelijkbaar met het warmlopen voor de marathon.
In **hoofdstuk 4** onderzochten we wat de invloed was van deze alternatieve klemmethoden op de uitgroei van colorectale micrometastasen. We vonden inderdaad dat beide klemmethoden een beschermend effect hadden op het leverweefsel, het intermitterend klemmen meer dan ischemische preconditionering. Wanneer de bloedtoevoer intermitterend werd afgeklemd (3 keer 15 minuten met steeds 5 minuten reperfusie ertussen, in plaats van 45 minuten achtereenvolgend), trad er helemaal geen tumorgroeistimulerend effect op. Ischemische preconditionering daarentegen kon de versnelde tumorgroei niet voorkomen. Ondanks dat we in onze patiëntenpopulatie geen verschil vonden tussen intermitterend klemmen en continu klemmen blijft, op basis van onze muizenstudie en op basis van een bewezen beschermend effect op het leverweefsel, intermitterend klemmen de klemmethode van keuze.
In **hoofdstuk 7** hebben we onderzocht wat de invloed is van het selectief klemmen van de poortader op de uitgroei van colorectale micrometastasen in het muizenmodel. Er lopen twee aanvoerende vaten naar de lever: de grootste (de poortader) vervoert bloed uit de darmen naar de lever en draagt bij aan 70% van de bloeddoorstroming van de lever; de leverslagader vervoert zuurstofrijk bloed van het hart naar de lever. Wanneer de poortader selectief werd afgeklemd en de zuurstofrijke bloedtoevoer door de leverslagader dus intact bleef, was levercelschade minimaal en trad geen versnelde tumorgroei op. Portaal klemmen is vooral geschikt tijdens lokale ablatie methoden, omdat het even effectief is als een standaard Pringle Manoeuvre in het voorkomen van afkoeling door de bloedstroom en in het bereiken van maximale hittedestructie.

VI Welke mechanismen liggen ten grondslag aan het tumorgroeistimulerende effect van ischemie/reperfusie en hoe kunnen deze beïnvloed worden met medicijnen?

We hebben getracht het mechanisme achter de groeistimulerende effecten van ischemie/reperfusie te achterhalen, opdat er medicijnen zouden kunnen worden toegepast om de prognose na leverchirurgie te verbeteren.
Een belangrijk mechanisme dat bijdraagt aan vroege levercelschade is het vrijkomen van zuurstofradicalen. Vitamine E (α-tocopherol) en vitamine C (ascorbic acid) zijn zeer effectief in het neutraliseren van deze zuurstofradicalen en daarmee ook in het voorkomen van weefselschade na ischemie/reperfusie. In **hoofdstuk 4** onderzochten we of deze vitaminen ook beschermen tegen de versnelde uitgroei van micrometastasen na ischemie/reperfusie. Ondanks dat beide vitaminen inderdaad vroege levercelschade konden voorkomen, trad er toch late levercelschade op en vond er versnelde uitgroei van micrometastasen plaats. Die late schade wordt gekenmerkt door de aanwezigheid van gebieden met uitgebreid leververval (necrose). Een belangrijke observatie was dat de versnelde tumorgroei vooral plaatsvond rondom deze necrotische gebieden in de lever. Dit deed vermoeden dat het mechanisme gerelateerd moet zijn aan gebeurtenissen die late celschade veroorzaken.
In de late fase na ischemie/reperfusie treedt er een verstoring op van de microdoorstroming (microcirculatie) van de lever. Wanneer deze microcirculatiestoornissen lang duren, veroorzaakt dit een aanhoudend zuurstoftekort in het weefsel (hypoxie) en late celschade. Terwijl normale cellen niet goed bestand zijn tegen zuurstoftekort, hebben tumorcellen een soort overlevingsmechanisme ontwikkeld en kunnen tumorcellen zelfs harder gaan groeien bij zuurstoftekort. Dit doen zij door het vrijmaken van hypoxia inducible factor-1alpha (HIF-1α).
In **hoofdstuk 8** laten we met bepaalde kleuringen zien dat de versnelde tumorgroei vooral plaatsvindt in gebieden van zuurstoftekort en HIF-1α. Wanneer we met bepaalde medicijnen (Atrasentan en L-Arginine) de microcirculatie verbeterden, was er minder zuurstoftekort en minder HIF-1α productie aanwezig in de weefsels en werd zowel de late schade als de gestimuleerde tumorgroei voor 50% geremd. Als we vervolgens de muizen behandelden met een middel dat het vrijmaken van HIF-1α voorkomt (17-DMAG), werd de gestimuleerde tumorgroei voor 70% geremd. In deze muizen werd voor het eerst uitgebreide necrose waargenomen, zonder gestimuleerde tumorgroei. Deze resultaten laten zien dat microcirculatie stoornissen, zuurstoftekort en HIF-1α een belangrijke rol spelen in het veranderde biologische gedrag van colorectale micrometastasen in de lever na ischemie/reperfusie. Wanneer er tijdens leverchirurgie toch langdurig geklemd moet worden, kunnen medicijnen die de microcirculatie verbeteren of het vrijkomen van HIF-1α onderdrukken de groeistimulatie van darmkankercellen in de lever remmen. Vervolgstudies zijn nodig om de effectiviteit en veiligheid van deze middelen te testen in patiënten met uitgezaaide darmkanker.
Naast het mechanisme zoals hierboven beschreven, zijn er nog andere mechanismen die mogelijk bijdragen aan de gestimuleerde uitgroei van colorectale micrometastasen na ischemie/reperfusie. Een van de belangrijkste gevolgen van zuurstoftekort en HIF-1α is vaatnieuwvorming (angiogenese). Vaatnieuwvorming is een belangrijke gebeurtenis die tumoren in staat stelt om verder te kunnen groeien en staat momenteel erg in de belangstelling bij de behandeling van darmkanker. Daarnaast vindt er een ontstekingsreactie plaats in het leverweefsel en verschillende ontstekingscellen zijn in verband gebracht met versnelde celdeling en tumorgroei. Medicijnen die vaatnieuwvorming kunnen remmen of die ontstekingsreacties kunnen onderdrukken zouden eveneens de versnelde tumorgroei na ischemie/reperfusie kunnen beperken. Deze mogelijkheden worden momenteel in ons laboratorium onderzocht.

VII Treedt er versnelde uitgroei van colorectale micrometastasen op na lokale ablatie en hoe kan dit worden voorkomen?

Zoals eerder gezegd, blijft de chirurgische verwijdering van levermetastasen de behandeling van keuze, maar dit is slechts bij ongeveer een kwart van de patiënten met uitgezaaide darmkanker mogelijk. Lokale ablatie technieken, zoals radiofrequente ablatie (RFA) en laser-geïnduceerde thermotherapie (LITT), worden toegepast om lokale tumor vernietiging te verkrijgen. Het risico van deze methoden is, dat niet al het tumorweefsel vernietigd wordt en er levende tumorcellen achterblijven. Omdat bij deze methoden altijd een gebied met celdood (necrose) ontstaat, hebben wij ons afgevraagd of ook rondom deze gebieden (lesies) een groeistimulerend effect zou optreden, vergelijkbaar met dat na ischemie/reperfusie.
In **hoofdstuk 9** werd in twee verschillende diermodellen aangetoond dat de uitgroei van colorectale micrometastasen rondom de lesie meer dan vier keer versneld was vergeleken met tumorgroei in de rest van de lever. Vergelijkbaar met ischemie/reperfusie ging dit gepaard met lokale microcirculatiestoornissen, zuurstoftekort en HIF-1α-productie rondom de lesie. Vervolgens hebben we onderzocht of we dit fenomeen konden remmen. Behandeling met middelen die de microcirculatie verbeteren (Atrasentan en L-Arginine), hadden geen invloed op de aanwezigheid van zuurstoftekort, HIF-1α en tumorgroei rond de lesie. Wanneer we de muizen behandelden met een middel dat het vrijmaken van HIF-1α voorkomt (17-DMAG), werd de gestimuleerde tumorgroei echter wel voor 34% geremd. Zoals hierboven gemeld, is vaatnieuwvorming (angiogenese) een van de belangrijkste gevolgen van zuurstoftekort en speelt een belangrijke rol bij tumorgroei. Behandeling met een middel dat deze vaatnieuwvorming onderdrukt (PTK787/ZK-222584), had een duidelijk tumorgroeiremmend effect op zowel micrometastasen rondom de lesie als elders in de lever. Voortvloeiend uit deze resultaten kunnen we concluderen dat gelijktijdige behandeling met HIF-1α of angiogenese remmers de effectiviteit van lokale behandeling zou kunnen verbeteren, hetgeen uitgezocht dient te worden in grote klinische studies. Uiteraard is het belangrijkste bij deze lokale behandelingen dat het tumorweefsel volledig wordt vernietigd. Paradoxaal genoeg, kan maximale tumor vernietiging worden bereikt door tijdelijk de bloedtoevoer naar de lever af te klemmen. Echter, zoals we hierboven hebben laten zien, kan dit op zichzelf ook weer tumorgroei stimuleren. In dat geval kan het beste alleen de poortader worden afgeklemd (zie hoofdstuk 7).

Conclusies

Op basis van de hierboven beschreven resultaten kunnen de volgende conclusies worden getrokken:

- **I** Het afklemmen van de bloedtoevoer naar de lever wordt veelvuldig toegepast tijdens operatieve verwijdering van colorectale metastasen in de lever, met als belangrijkste doel het voorkomen van bloedverlies
- **II** Ischemie/reperfusie (veroorzaakt door het afklemmen van de bloedtoevoer) heeft een nadelige invloed op de prognose door het versnellen van de uitgroei van colorectale micrometastasen in de lever
- **III** Het tumorgroeistimulerende effect van ischemie/reperfusie hangt af van de duur van het klemmen
- **IV** Het stimulerende effect van ischemie/reperfusie op tumorgroei verergert bij hogere leeftijd en bij leververvetting
- **V** Alternatieve klemmethoden, zoals intermitterend klemmen en het selectief klemmen van de poortader kunnen de versnelde uitgroei van micrometastasen voorkomen
- **VI** Zuurstoftekort en HIF-1α spelen een belangrijke rol bij de versnelde uitgroei van micrometastasen na ischemie/reperfusie en vormen een belangrijk aanknopingspunt voor medicamenteuze interventie
- **VII** Lokale ablatie technieken versnellen de uitgroei van microscopisch kleine uitzaaiingen rondom het verhitte gebied, hetgeen kan worden tegengegaan door onderdrukking van HIF-1α en het remmen van vaatnieuwvorming

Gesteund door deze conclusies adviseren we dat het afklemmen van de bloedtoevoer naar de lever tijdens leverresectie voor colorectale metastasen achterwege gelaten moet worden, wanneer het niet noodzakelijk is. Wanneer er toch bloedverlies optreedt, wordt geadviseerd kortdurend te klemmen. Intermitterend klemmen is dan de klemmethode van keuze. Tijdens lokale ablatie dient bij voorkeur alleen de poortader te worden afgeklemd om het afkoelend effect van de bloedstroom te verminderen. Wanneer er toch ischemie/reperfusie schade wordt verwacht, zouden medicijnen, die de doorstroming van het bloed verbeteren of HIF-1α remmen, de gestimuleerde uitgroei van micrometastasen kunnen beperken en de prognose verbeteren. Interventiestrategieën dienen vooral te worden ondernomen bij oudere patiënten, patiënten met leververvetting en na preoperatieve chemotherapie. Tijdens lokale ablatie wordt gelijktijdige behandeling met middelen die HIF-1α of vaatnieuwvorming remmen geadviseerd.

Toekomst

In de hoop dat dit proefschrift aanleiding geeft voor veel chirurgen om tijdens lever operaties minder te klemmen, zijn vervolgstudies zeer de moeite waard om verschillende aspecten van chirurgie-geïnduceerde tumorgroei te onderzoeken.
Bij voorkeur worden prospectieve gerandomiseerde studies gedaan om verschillende klemmethoden tegen elkaar uit te zetten en om het effect ervan op zuurstoftekort, HIF-1α en vaatnieuwvorming in patiënten te onderzoeken. Verder onderzoek naar de invloed van verschillende omstandigheden op tumorgroei stimulering is nodig om patiëntengroepen nauwkeurig te selecteren bij wie interventiemaatregelen genomen moeten worden.
In dit proefschrift bieden we enkele aanknopingspunten voor medicamenteuze interventie om de tumorgroeistimulering na ischemie/reperfusie of lokale ablatie te kunnen remmen. Experimentele studies dienen gecontinueerd te worden om nieuwe en effectieve medicijnen te ontwikkelen die veilig toegepast kunnen worden in de kliniek. De effectiviteit van deze middelen dient te worden onderzocht in klinische studies met grote aantallen patiënten. Gezegd moet worden dat de middelen zoals geadviseerd in dit proefschrift, alleen het gedrag van eventueel achtergebleven micrometastasen kunnen beïnvloeden, maar deze niet kunnen vernietigen. Deze middelen moeten dan ook bij voorkeur gecombineerd worden met tumorceldodende medicijnen (chemotherapeutica) om maximale effectiviteit na te streven. Dergelijke behandelingsstrategieën resulteren vermoedelijk in een verdere verbetering van de lange termijnuitkomsten na leverchirurgie en bieden zo mogelijk een groeiend aantal patiënten met uitgezaaide darmkanker hoop op genezing.

Chapter 13

Dankwoord

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Jarmila van der Bilt
aan de Amstel, Amsterdam
15 april 2007

Curriculum vitae

Jarmila van der Bilt was born on February 20th 1976 in The Hague, The Netherlands. In May 1994 she graduated from the Sorghvliet Gymnasium in The Hague. After one year of studying Medical Biology, she started her medical training in 1995 at the University of Utrecht, The Netherlands. She had her first scientific experience during a studentcourse at the Department of Experimental Cardiology, Utrecht (Dr. P.F. Grundeman) and a scientific course at the Oregon Health and Science University (Dr. L.E. Davis and Dr. R. Hohimer) in Portland, Oregon, USA. During her internship she performed research at the Department of Pediatric Surgery at the University Medical Center Utrecht (UMCU), The Netherlands (Prof. dr. N.M.A. Bax) and did a vacant internship at the Department of Pediatric Surgery at the Amsterdam Medical Center (Dr. D. Aronson). After she graduated from medical school in May 2002, she was responsible for the education of thirdyear (junior) interns at the Department of Surgery at the UMCU (Prof. dr. H.G. Gooszen). One year later she started as a PhD-student with the work presented in this thesis at the Department of Surgery (Prof. dr. I.H.M. Borel Rinkes) and the Laboratory of Experimental Oncology (Prof. dr. E.E. Voest and Prof. dr. R. Medema). Several studies from this thesis were awarded with the ImagO price for 'best article 2005' at the Wetenschapsdag in Utrecht, the 'Johnson & Johnson poster price' at the annual meeting of the Dutch Surgical Society in Veldhoven and the price for 'best abstract' at the Symposium Experimental Research Surgical Specialties in Groningen, The Netherlands. In 2004 she received a scientific fund from The Netherlands Organization for Health Research and Development (NWO). With pleasure she started her surgical training in January 2007 at the Meander Medical Center, Amersfoort, The Netherlands (Dr. G.H.M. Verberne). This program will be completed in 2013 at the Department of Surgery, UMCU (Prof. Dr. I.H.M. Borel Rinkes).

Jarmila van der Bilt werd op 20 februari 1976 geboren in Den Haag. In mei 1994 legde zij haar eindexamen af aan het Sorghvliet Gymnasium in Den Haag. Na een jaar Medische Biologie, werd zij in 1995 ingeloot voor de studie Geneeskunde aan de Universiteit Utrecht. Haar eerste wetenschappelijke ervaring deed zij op tijdens een studentenstage bij de afdeling Experimentele Cardiologie (Dr. P.F. Grundeman) en een wetenschappelijke stage aan de Oregon Health and Science University (Dr. L.E. Davis en Dr. R. Hohimer) in Portland, Oregon, Verenigde Staten. Tijdens haar co-schappen verrichte zij onderzoek bij de afdeling Kinderchirurgie van het Universitair Medisch Centrum Utrecht (UMCU) (Prof. dr. N.M.A. Bax) en deed haar keuze co-schap bij de afdeling Kinderchirurgie in het Amsterdams Medisch Centrum (Dr. D. Aronson). Na het behalen van haar artsexamen in mei 2002 was zij gedurende een jaar als Arts Klinisch Onderwijs (AKO) verantwoordelijk voor het onderwijs van derdejaars co-assistenten van het CRU '99 op de afdeling Chirurgie in het UMCU (Prof. dr. H.G. Gooszen). Een jaar later startte zij als artsonderzoeker met het onderzoek zoals beschreven in dit proefschrift bij de afdeling Chirurgie (Prof. dr. I.H.M. Borel Rinkes) en het Laboratorium voor Experimentele Oncologie (Prof. dr. E.E. Voest en Prof. dr. R. Medema). De studies uit dit proefschrift werden bekroond met de ImagO-prijs voor 'best article 2005' tijdens de Wetenschapsdag in Utrecht, de 'Johnson & Johnson posterprijs' tijdens de Chirurgendagen in Veldhoven en de prijs voor 'best abstract' tijdens het Symposium Experimenteel Onderzoek Heelkundige Specialismen in Groningen. In 2004 ontving zij een onderzoeksbeurs van het Nederlands Wetenschappelijk Onderzoeksinstituut (NWO), in de constructie van AGIKO (assistent geneeskunde in opleiding tot klinisch onderzoeker).
In januari 2007 is zij met veel plezier gestart met de opleiding Heelkunde in het Meander Medisch Centrum te Amersfoort (Dr. G.H.M. Verberne), welke voltooid zal worden in 2013 bij de afdeling Chirurgie van het UMCU (Prof. dr. I.H.M. Borel Rinkes).

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