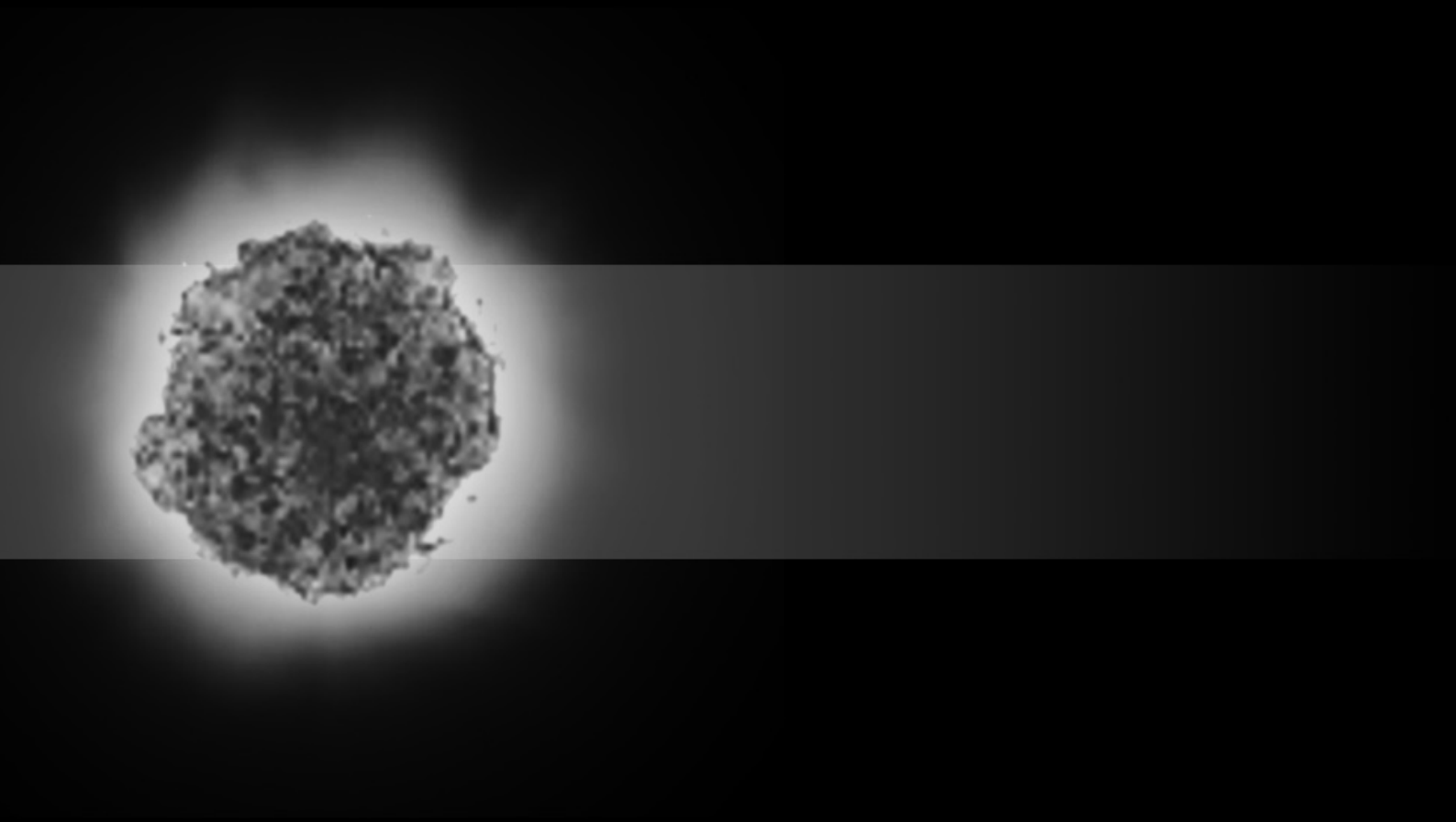
Tumor-selective coronaviruses as potential anti-cancer agents



Thomas Würdinger

Cover photo: Telescopical view of the corona formation as observed when the moon is positioned between the sun and the Earth with an OVCAR-fAPN tumor spheroid overlay.

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Tumorselectieve coronavirussen als potentiële antikankermiddelen

(met een samenvatting in het Nederlands)

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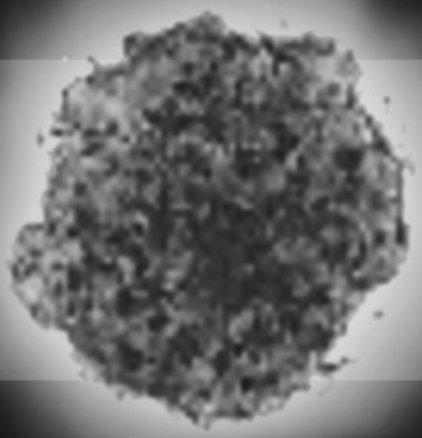
CONTENTS

CHAPTER 1 | INTRODUCTION



CHAPTER 1: GENERAL INTRODUCTION

1. ONCOLYTIC VIRUSES AND VIROTHERAPY

Cancer remains one of the most common causes of death, despite tremendous research efforts over the last decades into the nature of the disease and its causes. While significant new insights have been acquired, treatment actually still relies for the major part on classical approaches such as surgery, radio- and chemotherapy. Clearly, novel and creative methods are needed to complement the conventional treatment options. Recently, the use of viruses as potential anti-cancer agents has gained considerable interest (128).

Historically, the concept of viral therapy for cancer (oncolytic virotherapy) started to evolve when cancer patients naturally exposed to viruses in the field or to live virus vaccine preparations in certain cases revealed a conspicuous decrease in clinical signs (reviewed in (128)). The preference of many viruses to attach to and replicate within tumor cells was recognized already very early (75). Reports at the beginning of the 20th century concerning spontaneous remission of cervical cancer after immunization with live rabies virus vaccine (32, 37) provided early evidence for the phenomenon of viral oncolysis. During the 1950-60's, an increasing number of clinical observations supported the idea of oncolysis occurring in virus-infected individuals (16, 65, 91, 102, 131, 132, 149), and various mechanisms were proposed to account for the effects of viral infection on cancer, as discussed below.

The basic principle of oncolytic virotherapy - i.e. the specific targeting of viruses to tumor cells for destruction while minimizing harmful pathogenic properties - has remained unchanged since the first observations of viral oncolysis (128). However, the tremendous progress in the understanding of the molecular mechanisms of viral biology and pathogenesis has significantly advanced our ability to manipulate these agents for therapeutic purposes. New technologies permitting genetic alterations of viral agents by design have allowed us to add advantageous to, or to

eliminate unwanted properties from potential oncolytic viruses. Until now, several different viral species have entered clinical trials (Table 1) (76, 115), others may follow soon. They may offer an additional treatment option for cancer types for which conventional surgery, radio- and chemotherapy have proven not to be sufficient (6, 70, 116, 120).

Virus species	Virus name	Administration route	Cancer type	Clinical stage
Adenovirus	Onyx-015	Intratumoral injection	Head and neck cancer	Phase II (68)
		Intratumoral injection	Pancreatic cancer	Phase II (61)
		Mouthwash	Oral dysplasia	Phase I (119)
		Intratumoral injection	Oral carcinoma	Phase II (92)
		Intraperitoneal injection	Ovarian cancer	Phase I (144)
		Hepatic intraarterial infusion	Colorectal liver metastases	Phase I/II (113)
		Intravenous injection	Metastatic colorectal cancer	Phase II (57)
		Peritumoral injection	Glioma	Phase I (20)
		Intralesional injection	Hepatobiliary cancer	Phase II (82)
		Various injection routes	Liver cancer	Phase I/II (52)
		Intratumoral injection	Sarcomas	Phase I/II (47)
		Intratumoral injection	Renal cell cancer	Phase III
	CV706	Intraprostatic injection	Prostate cancer	Phase I (33)
	CV787	Intraprostatic injection	Prostate cancer	Phase I
	CG7870	Intraprostatic injection	Prostate cancer	Phase I/II (34)
	Ad5-CD/Tkrep	Intraprostatic injection	Prostate cancer	Phase I (44)
	H101	Intratumoral injection	Head and neck cancer	Phase III (153)
		Intratumoral injection	Esophagus cancer	Phase III (153)
Herpes simplex virus	HF10	Intratumoral injection	Metastatic breast cancer	Phase I (100)
	G207	Intratumoral injection	Glioma	Phase I/II (84)
	NV1020	Hepatic intraarterial infusion	Metastatic liver tumor	Phase I/II (8)
	Oncovex GM-CSF	Intratumoral injection	Melanoma/ Breast Head and neck cancer	Phase I/II (115)
	1716	Intratumoral injection	Glioma	Phase I (60)
		Intratumoral injection	Melanoma	Pilot (80)
Newcastle disease virus	MTH-68	Intravenous injection	Glioma	Pilot (25)
	PV701	Intravenous injection	Advanced solid cancers	Phase I (109)
Reovirus	Reolysin	Intratumoral injection	Skin metastasis	Phase I (1)
		Intratumoral injection	Prostate cancer	Phase I (1)
		Intratumoral injection	Glioma	Phase I/II (1)
		Intratumoral injection	Advanced solid cancers	Phase I (1)
Measles virus	MV-CEA	Intraperitoneal injection	Ovarian cancer	Phase I (94)
Vaccinia virus	Vaccinia-GM-CSF	Intralesional	Metastatic melanoma	Phase I (86)

Table 1. List of oncolytic viruses in clinical trials.

Strategy to achieve tumor selectivity	Advantages	Disadvantages	Virus species	Stage of development
Inherent tumor-selectivity	Viral modifications are not required No foreign DNA elements required	Specificity can not be easily modified Depends on natural lytic strength of virus Modifications are technically difficult	Reovirus NDV Measles virus Autonomous parvovirus VSV	Clinical (104) Clinical (25, 79) Clinical (94) Preclinical (24) Preclinical (134)
Attenuation by deletion 153) of viral genes or gene fragments	Broad mechanism of selectivity No foreign DNA elements required	Risk of revertants to wild-type Multiple functions of the deleted viral genes may result in loss of anti-tumor efficacy	Adenovirus HSV Vaccinia virus Poliovirus	Clinical (20, 69, Clinical (143) Clinical (86, 124) Preclinical (105)
Transcriptional targeting	Technology well established Targeting based on known tumor biology	Insertion of foreign DNA elements Tumor escape by promoter shutoff	Adenovirus HSV	Clinical (33) Preclinical (88)
Cellular targeting	Specific infection of tumor cells Less attenuation required Potential reduction in toxicity Potential reduction in dose size required	Technically difficult Detargeting and targeting required Tumor escape by mutation of receptor Insertion of foreign DNA elements	Adenovirus HSV Measles	Preclinical (150) Preclinical (156) Preclinical (96)

Table 2. Overview of strategies to achieve tumor-selective viruses (partially adapted from (115)).

2. MECHANISM OF ONCOLYTIC VIRUSES TO DESTROY TUMOR CELLS

Oncolytic viruses can mediate the destruction of tumor cells by several potential mechanisms (38, 71, 93). First of all the virus itself may be capable of directly lysing the cell as a result of virus infection and replication. This cycle can then be repeated by progeny viruses infecting adjacent cells and destroying them by lethal effects of replication. In addition, certain oncolytic viruses generate proteins during their replicative cycle that are directly cytotoxic to the tumor cells, like the adenovirus 11.6 kD death protein and the E4ORF4 protein (83, 126, 138). Another mode is the capacity of certain viruses to induce cell-cell fusion (syncytia formation); upon replication of measles virus (94, 97), VSV (41), and certain HSV mutants (45, 95), the infected cell fuses with its neighboring cells, resulting in a bystander effect.

Another mechanism by which oncolytic viruses can mediate tumor cell destruction is via induction of non-specific and specific anti-tumor immunity. Tumor cells are inherently weakly immunogenic, in part because they exhibit decreased expression of major histocompatibility complex (MHC) antigens and stimulatory signals, including cytokines that activate local immune responses (51). More importantly, virus-mediated induction of specific anti-tumor immunity may result in the possibility of long-term protection against tumor recurrence (122, 137, 146). In addition, oncolytic viruses can cause an increased sensitivity of tumor cells to chemotherapy and radiation therapy (34, 61, 68, 99, 121). This phenomenon is particularly attractive in the context of combination therapy.

A final mechanism by which oncolytic viruses mediate anti-cancer activity is by the expression of therapeutic transgenes inserted into the viral genome. This principle is being explored in the field of gene therapy, in which oncolytic viruses may be used as transfer vectors for therapeutic genes. As the virus amplifies itself through several rounds of replication and infection of neighboring cells, there is a concomitant amplification of transgene expression, which produces an amplified anti-tumor effect. Well known examples are genes encoding prodrug-converting

enzymes that can be incorporated into oncolytic viruses to augment tumor cell killing (19, 106, 112, 151). Alternatively, introduction of various immunostimulatory genes can support the anti-tumor immune response of the host (4, 107, 152). Also other anti-tumor proteins such as p53 and TRAIL were shown to enhance the oncolytic capacity of viruses (78, 141).

3. MECHANISMS OF TUMOR SELECTIVITY OF ONCOLYTIC VIRUSES

Besides surgery, chemotherapy and radiation therapy are currently the main treatment options for advanced cancers, but are limited by tumor cell resistance and a relatively narrow therapeutic index. Oncolytic viral therapy, on the other hand, is capable of increasing the therapeutic index between tumor cells and normal cells when viral replication occurs preferentially in tumor cells. Tumor selectivity can be achieved by selectivity either at the level of virus entry or at virus replication (Table 2) (115).

3.1 Tumor-selective replication

The first group of viruses that selectively replicate in tumor cells are the naturally occurring tumor-selective viruses. There are a number of examples of naturally occurring viruses that are tumor-selective in their replication and cytolysis. Autonomously replicating parvoviruses (23, 24, 111), human reovirus (21, 103, 104, 135), and VSV (5, 133, 134) have been shown to replicate more efficiently in transformed cells relative to non-transformed cells. Also, naturally occurring viruses of veterinary importance have been adapted by serial passage to tumor-selective replication (58, 154). Newcastle disease virus (NDV) strains adapted to Ehrlich ascites carcinoma cells (17) or to human melanoma cells (2) are two examples (reviewed in (129)). The precise mechanisms for their tumor selectivity are poorly understood and are likely to differ with each virus.

Tumor-selective replication can also be achieved by deleting genes that are critical for viral replication in normal cells but not in tumor cells. Examples of this strategy are the HSV deletion mutants (85), and conditional replicating oncolytic adenoviruses. In the process of adenovirus replication, expression of the viral E1A and E1B gene products leads to inactivation of certain cellular pRB- and p53-mediated defences. Tumor cells very frequently express mutant, non-functional forms of pRB and p53. Thus, the E1A and E1B gene products become dispensable in these tumor cells, as E1A- or E1B-defective adenoviruses can still replicate preferentially in pRb- or p53-defective tumor cells, respectively. In contrast, replication of an E1A- or E1B-defective adenovirus is impaired in normal cells with intact pRb and p53 pathways (3, 10, 46).

Another strategy commonly employed to achieve tumor-selective viral replication involves insertion of tumor-specific or tissue-specific promoters into the viral

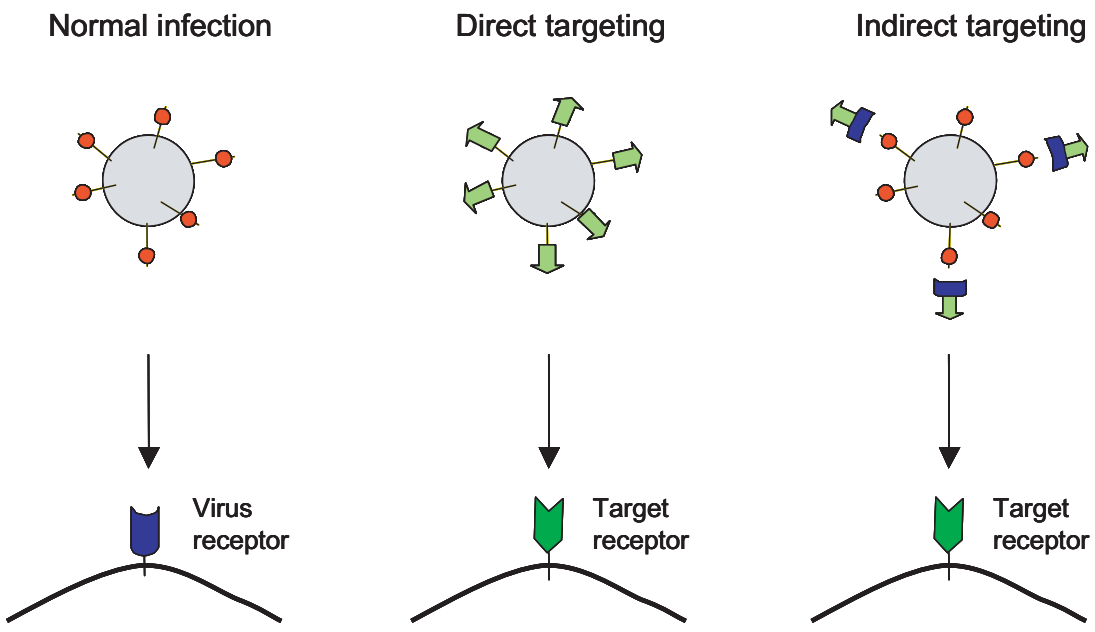


Fig. 1 Schematic representation of different approaches to target viruses to tumor cells. Ligands binding to a target receptor can be introduced in a virus protein (direct targeting), or fused to a virus-binding moiety resulting in a bispecific adapter protein (indirect targeting).

genome to regulate the expression of viral genes that are necessary for an effective replication cycle. The proof of this principle was provided by the development of an oncolytic HSV mutant in which the ICP4 gene, essential for HSV replication, was put under the control of an albumin enhancer/promoter (89). Also for the adenovirus tumor-selective replication was achieved via transcriptional targeting (49, 56). This was initially demonstrated by driving the adenovirus E1A expression from a prostate-specific antigen promoter, resulting in selective replication within cells positive for the prostate-specific antigen (117).

3.2 Tumor-selective entry

Tumor cell-selective binding and internalization of viruses is dependent upon the particular virus and its natural tropism. For example, viruses that naturally infect only a few cell types may just need a new binding specificity. However, viruses that naturally infect numerous cell types may need a new binding specificity in addition to the abolishment of the natural binding interaction. To date, this has proved to be particularly difficult, although shown to be possible for the measles virus (53). Several strategies have been examined to retarget oncolytic viruses to cancer cells (Fig. 1). These include direct modification of the virus protein responsible for cellular attachment (direct targeting), or the use of bispecific adapters (indirect targeting; reviewed in (15, 116)).

Direct targeting of viruses is based on the physical incorporation of ligands into the virus particle during biosynthesis of viral proteins in vivo. Incorporation of a ligand into a capsid (non-enveloped viruses) or envelope (enveloped viruses) protein should not change the configuration of this protein in order to not significantly affect its natural functioning. Several types of ligands may theoretically be used in the context of direct tumor targeting (15, 77, 116). Obviously, the most promising candidate ligands are short peptides that recognize specific receptors with high affinity. Preferably these receptors should be unique to or overexpressed on tumor cells. Examples of peptides include RGD-4C (36, 125) and NGR (90, 108), which target to integrins

and aminopeptidase N, respectively. Also growth factors have been introduced as ligands, e.g. EGF (123) and SDF-1α (67). An advantage of most of these molecules is the high affinity for their specific receptor. Furthermore, the growth factor receptors are commonly overexpressed on tumor cells, making them attractive targets. However, binding of the growth factor domain to the receptor may result in biological activity that interferes with the infection process. Finally, also single-chain antibodies (scFvs) have been introduced into viral proteins (14, 59, 110). ScFvs are the variable domains of antibodies responsible for antigen binding, the gene sequences of which can be readily obtained from hybridoma cells.

For the indirect targeting strategy, bifunctional bridging agents recognizing both the virus and a specific cell surface molecule can be used to direct the virus to the targeted cell population. The first approaches made use of chemically conjugated bifunctional adapters (39, 87). More recently, examples included adapter-expression constructs comprising the virus-binding part of the cellular receptor fused to growth factors and scFvs (9, 35, 66, 98, 130, 147). Also bispecific scFvs were shown to have the ability to redirect viruses to novel cell surface receptors (55, 81, 101). A limitation of the bifunctional crosslinker approach in the context of oncolytic virotherapy may be that the adapter is not covalently linked to the vector particle and is lost upon infection, leaving the progeny virus untargeted. Incorporation of the genes encoding bispecific adapter proteins into the virus genome, thereby providing the progeny virus with newly produced adapter protein, would overcome this limitation (63, 140).

4. CORONAVIRUSES

Coronaviruses belong to the order of the Nidovirales, which consists of the families Arteriviridae and Coronaviridae. Coronaviruses were named after their crown(corona)-like appearance caused by the petal-shaped spikes protruding from the coronavirus envelope (Fig. 2). They can be divided into 3 clusters on the basis

of serological and genetic properties (Table 3), and are found in a variety of species, generally causing respiratory and/or enteric infections (reviewed in (30, 127)). Coronaviruses are enveloped viruses that contain an approximately 30 kb positive strand RNA genome encapsidated by the nucleocapsid (N) protein (74) (Fig. 2). The coronavirus envelope carries three membrane proteins, the spike (S) (18), envelope (E) (42, 145), and membrane (M) (118) proteins. Furthermore, several coronaviruses from group 2 (Table 3), but not the well-studied MHV-A59, also contain the hemagglutinin-esterase (HE) protein (13).

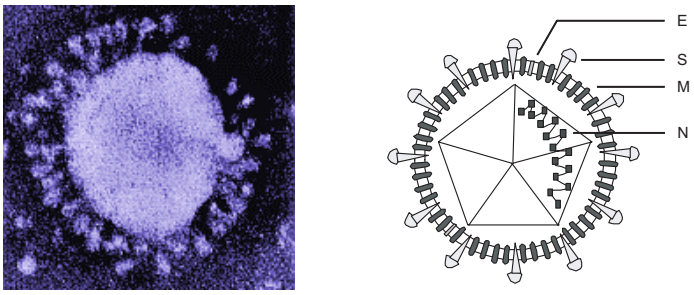


Fig. 2 Electron micrograph and schematic representation of a coronavirus. The genomic RNA together with the nucleocapsid protein (N) forms a helical nucleocapsid in an icosahedral core that is surrounded by a lipid bilayer containing the membrane protein (M), the envelope protein (E), and the spike protein (S).

Group	Virus	Host	Disease
1	feline infectious peritonitis virus (FIPV)	cat	enteritis/peritonitis
	transmissible gastroenteritis virus (TGEV)	pig	enteritis
	porcine respiratory coronavirus (PRCoV)	pig	respiratory infection
	canine coronavirus (CCoV)	dog	enteritis
	human coronavirus (HCoV)-229E	man	respiratory infection
2	mouse hepatitis virus (MHV)	mouse	respiratory infection/ hepatitis/encephalitis/enteritis
	bovine coronavirus (BCoV)	cow	enteritis
	human coronavirus (HCoV)-OC43	man	respiratory infection
	human coronavirus (HCoV)-NL63	man	respiratory infection
	hemagglutinating encephalomyelitis virus (HEV)	pig	respiratory infection
3	infectious bronchitis virus (IBV)	chicken	respiratory infection
	turkey coronavirus (TCoV)	turkey	enteritis
a)	human coronavirus (HCoV)-SARS	man	respiratory infection

Table 3. The coronavirus groups, their main representatives, host range, and associated diseases. a) The taxonomic position of the SARS coronavirus has not been formally assigned yet.

The S protein determines the coronavirus tropism by binding to the cell and by the subsequent induction of fusion of the virus envelope and the cellular membrane. The S protein is a type I glycoprotein, is synthesized as a 180-220 kDa glycosylated precursor that can be post-translationally cleaved into two subunits, S1 and S2 (18, 43), and is probably assembled into trimers (31). The N-terminal S1 subunit - representing the globular head - is responsible for binding to the cellular receptor. Binding to the receptor results in a conformational change of the S2 subunit - forming the stalk-like region - mediating the fusion of the virus envelope and the cell membrane via insertion of a yet unidentified fusion peptide into the cell membrane, and interaction of the heptad repeat regions (HR), HR1 and HR2 (12, 18, 26, 48). Prevention of this interaction by peptides corresponding to those HR1 and HR2 regions can block membrane fusion, by binding to their respective counterparts (12, 136, 155). Besides virus-cell fusion the S protein also mediates cell-cell fusion or syncytia formation (11, 27, 50). During virus replication the spike protein is transported to the plasma membrane of the infected cell where it mediates fusion with the surrounding neighboring cells leading to intercellular spread of the virus.

4.1 Coronaviruses: potential tumor specificity

The non-human coronaviruses MHV and FIPV exhibit strict species specificity, determined by their spike-receptor interaction (7, 22, 40, 139, 148). The coronavirus strain FIPV79-1146 selectively infects feline cells via its receptor feline amino peptidase N (fAPN) (139), whereas MHV-A59 selectively infects murine cells by the interaction of its spike protein with its cellular receptor, called murine CEACAM (22). Consequently, while MHV and FIPV can cause severe pathogenicity in their natural host (mouse and cat, respectively), they are not capable of infecting human cells and are thus non-pathogenic in humans. To this end, fully tumor-targeted coronaviruses would not require the ablation of their natural tropism, as is the case with therapeutic human viruses. Importantly, it was previously shown that the murine coronavirus

MHV and the feline coronavirus FIPV can be genetically modified via targeted RNA recombination (54, 72) (fig. 3). This was shown by exchanging the spike protein ectodomains of MHV and FIPV, resulting in the chimeric viruses fMHV (72) and mFIPV (54), which acquired a reciprocal tropism as a result of a switch of receptor specificity.

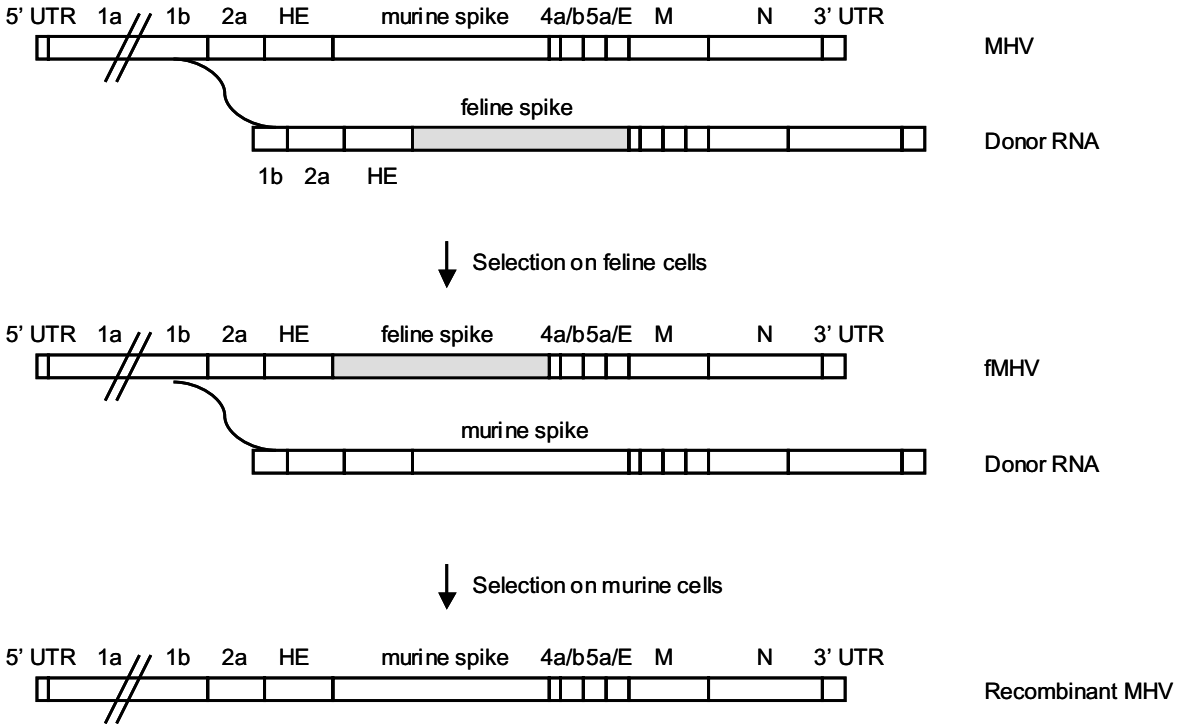


Fig. 3 Scheme for the construction of recombinant MHV mutants by targeted RNA recombination between MHV and donor RNA. The latter is an in vitro RNA transcript composed of sequences derived from MHV and FIPV. A single crossover event anywhere upstream of the S gene of the donor RNA will generate a recombinant virus, fMHV, containing the ectodomain-encoding region of the FIPV S gene (gray). Thus, the recombinant fMHV has lost the ability to infect murine cells but gained the ability to infect feline cells. The recombinant fMHV can be used in turn to produce recombinant MHV, which can then be selected on murine cells. Any additional mutations, deletions, and insertions in the donor RNA will be recombined into the recombinant virus genome (72).

4.2 Coronaviruses: potential oncolytic characteristics

Besides their host specificity and the presence of a genetic modification system, the coronaviruses MHV and FIPV also offer some other appealing characteristics in the context of oncolytic virotherapy. First, these positive strand enveloped RNA viruses replicate in the cytoplasm of cells (30, 73, 142). Their bypassing the nucleus contributes to fast virus replication, rapid production of progeny viruses, and rapid cell death and reduces the chances of cellular transformation. Second, coronavirus-induced cell death involves fusion of the plasma membranes of infected cells with those of uninfected neighbouring cells (Fig. 4). This syncytia formation causes an attractive bystander effect (11, 27, 50, 64), as was described for other viruses (41, 45, 94, 95, 97). Another potential oncolytic mechanism that might be achieved is the induction of non-specific and specific anti-tumor immunity, as coronaviruses are known to be potently immunogenic (28, 62). Finally, the potential to introduce (therapeutic) genes into the coronavirus genomes via targeted RNA recombination offers additional possibilities to enhance the oncolytic potential (29, 54, 72).

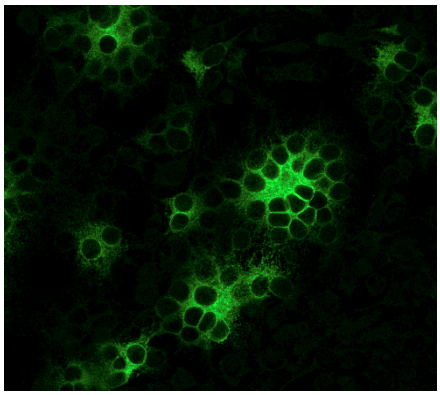


Fig. 4 Immunofluorescence analysis of MHV infected murine LR7 cells. Infections were visualized at 6 hours after inoculation by immunofluorescence microscopy on permeabilized cells using anti-MHV antibodies. A clear cytoplasmic staining can be observed, as well as multinucleated areas of fused cells, typical for coronavirus replication (adapted from (54)).

5. AIM AND OUTLINE OF THE THESIS

Despite much progress with the treatment of certain types of cancer, the successes are generally still largely insufficient and new treatment options are urgently required. Oncolytic virotherapy may offer an unconventional approach to selectively eradicate cancer cells, leaving the normal tissues largely unaffected. Several viruses are currently being analysed for their capacity to kill cancer cells. So far, coronaviruses have not been explored for their use in cancer virotherapy, despite the several features that make them attractive for this purpose. The aim of the research described in this thesis was to explore whether the non-human coronaviruses FIPV and MHV have the potential to function as oncolytic agents.

Because FIPV and MHV cannot infect human cells, due to the absence of the natural virus receptors, we first determined whether these coronaviruses have the potential to kill human cancer cells once the entry barrier is alleviated by artificial expression of the appropriate receptor (Chapter 2). The results show that the FIPV and fMHV efficiently kill cancer cells, once they gain access to these cells.

In the subsequent chapters we focus on the targeting of the non-human coronaviruses to receptors present on human cancer cells. We aimed at indirect targeting and constructed bispecific adapter proteins to direct the viruses to specific receptors expressed on cultured human cancer cells. In chapter 3 we generated bispecific single-chain antibodies, binding on the one hand to the FIPV spike protein, and on the other hand to the human epidermal growth factor receptor (EGFR). Chapter 4 comprises the analysis of adapters based on the ectodomain of the MHV receptor fused to a cancer cell receptor-binding ligand. Altogether chapters 3 and 4 show that FIPV, fMHV, and MHV can be targeted to the human EGFR via different bispecific adapter proteins, resulting in infection of human cancer cells.

A limitation of the indirect targeting approach in the context of oncolytic virotherapy is that the adapter is lost upon infection leaving the progeny virus untargeted. To this end, the adapter genes were incorporated into the MHV genome to obtain genetically targeted coronaviruses (Chapter 5). Indeed, MHV-mediated expression

of a His-tag-adapter protein resulted in efficient killing of target cells expressing a His-tag receptor.

In Chapter 6 We explored the use of antibodies to redirect viruses to Fc receptors expressed on certain types of acute myeloid leukemia. Upon combining with certain antibodies the mouse coronavirus MHV gained access to the otherwise refractory Fc-receptor expressing leukemic THP-1 cells. This novel example of indirect targeting may have major implications for the development of selective viral vector-mediated gene transfer approaches for the treatment of AML.

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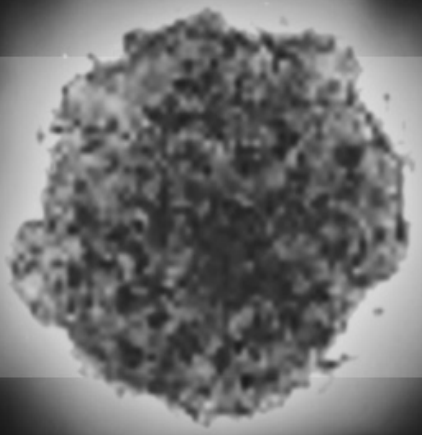
CHAPTER 2 | NON-HUMAN CORONAVIRUSES EFFICIENTLY ERADICATE HUMAN CANCER CELLS EXPRESSING THE APPROPRIATE VIRUS RECEPTOR



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ABSTRACT

The aim of this study was to explore whether non-human coronaviruses can be employed as oncolytic agents for the treatment of human cancer. To this end, the feline infectious peritonitis virus (FIPV) and a felinized murine hepatitis coronavirus (fMHV), both normally unable to infect human cells, were assessed for their ability to infect human cancer cells artificially expressing the feline coronavirus receptor aminopeptidase N (fAPN), and whether this would also lead to the killing of these cells. Here we show that various human adenocarcinoma cell lines were rendered susceptible to FIPV and fMHV infection by transient expression of fAPN and that the cell cultures were eradicated by the infection. Furthermore, infection of HeLa and OVCAR-3 cells - human cervical and ovarial cancer cells, respectively - stably expressing fAPN resulted in efficient FIPV and fMHV replication, causing cell death within 24 h. Infected cultures exhibited cell-cell fusion typical of coronavirus infection. Finally, we demonstrated that, 3-D multilayer tumor spheroids grown with OVCAR-3-fAPN cells were eradicated by fMHV with high efficacy. These findings suggest that by altering their tropism non-human coronaviruses might be converted into potent oncolytic agents.

INTRODUCTION

Replication-competent oncolytic agents derived from viruses are currently being developed for anti-cancer virotherapy. Key property of an oncolytic virus is that it should effectively eradicate tumor cells without causing severe pathogenicity to the patient. Several different DNA and RNA viruses are currently under investigation to serve as oncolytic agents (15, 17). So far, coronaviruses have not been considered for use in cancer virotherapy, despite the fact that they appear to exhibit several features that make them potentially attractive for this purpose. First, these positive strand enveloped RNA viruses replicate in the cytoplasm of cells. Their bypassing the nucleus contributes to fast virus replication and rapid cell death. Second, coronavirus induced cell death involves fusion of the plasma membranes of infected cells with those of uninfected neighbouring cells. This syncytia formation probably creates an attractive 'bystander effect' with extended cell death (11). Third, most coronaviruses display strong species specificity, determined by the specific interaction between the virus spike protein and its cellular receptor. Two examples of coronaviruses with strong species specificity are the murine hepatitis virus (MHV) and the feline infectious peritonitis virus (FIPV) that enter cells via the murine carcinoembryonic antigen-related cell adhesion molecule (mCEACAM) (4), and feline aminopeptidase N (fAPN) (18), respectively. Consequently, while MHV and FIPV can cause severe pathogenicity to their natural host they are not capable of infecting human cells and are thus non-pathogenic in humans. Importantly, we have shown previously that by modifying the spike protein the tropism of coronaviruses can be changed. Recombinant MHV containing chimeric spike proteins consisting of the FIPV spike ectodomain linked to the MHV spike membrane anchor (fMHV) was capable of killing feline cells only, via the feline coronavirus receptor fAPN (12). Conversely, the reciprocal recombinant mFIPV was only capable of infecting murine cells via the MHV receptor (9). Thus, MHV and FIPV could potentially be converted into specific oncolytic

agents for the treatment of human cancer if their spike protein would recognize a receptor on human tumor cells.

Towards developing recombinant coronaviruses with a selective human tumor tropism we set out to investigate the effectiveness of MHV and FIPV in eradicating human cancer cells expressing the virus receptor. To compare the two viruses in the same cells, we assessed FIPV and, instead of MHV, the chimeric coronavirus fMHV for their ability to kill human cancer cells artificially expressing fAPN. Here we show using a range of cells that FIPV and fMHV infect and rapidly kill fAPN expressing human cancer cells and that fMHV efficiently eradicates fAPN expressing 3-D tumor spheroids *in vitro*.

MATERIALS AND METHODS

Viruses, cells, and antibodies.

Recombinant fMHV (12), and FIPV 79-1146 (6) stocks were produced and titrated in parallel on feline FCWF-4 cells to determine the virus titer. Wild-type adenovirus type 5 (Ad5) was produced and titrated on 293 human embryonic kidney cells. HeLa cervix carcinoma cells, OVCAR-3 ovary carcinoma cells, HCT-8, Caco-2 and WiDr colon carcinoma cells, HepG2 hepatoma cells (all from the American Type Culture Collection, Manassis, VA), and feline FCWF-4 cells (obtained from Dr. N. C. Pedersen, University of California, Davis, CA) were grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Ltd., Paisley, United Kingdom). HeLa-fAPN and OVCAR-fAPN were maintained in the same medium supplemented with 0.5 and 0.25 mg/ml G418 (Life Technologies), respectively. The rabbit polyclonal MHV strain A59 antiserum (K134) and the R-G-4 monoclonal antibody directed against the fAPN receptor were described previously (10, 16). C428, ascites from a FIPV-infected cat, was obtained from Dr. B. J. Haijema (Utrecht University, Utrecht, the Netherlands).

Production and characterization of fAPN expressing cell lines.

The expression plasmid pCR3-fAPN, containing the fAPN cDNA under the control of the cytomegalovirus promoter (5), was used to transiently express fAPN on different cancer cell lines following transfection with Lipofectamine PLUS reagent (Life Technologies, Ltd., Paisley, United Kingdom). HeLa and OVCAR-3 transfected cells were also cultured in G418 containing cell culture medium to select for stable transfectants. HeLa and OVCAR-3 G418-resistant cells were cloned by two and four rounds of limiting dilution, respectively. After each round of limiting dilution the clones were propagated, tested for their susceptibility to FIPV infection and for fAPN expression by fluorescent activated cell sorter (FACS) analysis.

Furthermore, FIPV infection was blocked by the anti-fAPN antibody R-G-4, confirming that the infections occurred via fAPN.

FACS analysis.

To determine the expression of fAPN, cells were trypsinized, washed with phosphate buffered saline (PBS) (10.9 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 8.2 g/l NaCl), and fixed in PBS containing 3.7 % paraformaldehyde. The cells were washed three times and incubated for 30 min at room temperature (RT) in PBS containing 5 % FBS to block aspecific antibody binding. Next, the cells were incubated for 1 h at RT in R-G-4 containing hybridoma medium diluted 1:10 in PBS containing 5 % FBS. The cells were washed three times with PBS containing 5 % FBS, incubated in Goat-anti-Mouse FITC (DAKO, Glostrup, Denmark) diluted 1:200 in PBS containing 5 % FBS for 1 h at RT, and subsequently washed three times with PBS. Analysis by FACScan (Beckton Dickinson, Erembodegem-Aalst, Belgium) was performed according to standard procedures.

Immunohistochemical staining.

Cells were fixed in 3.7 % paraformaldehyde in PBS, permeabilized with PBS containing 1 % Triton X-100, and washed three times with PBS containing 0.5 % FBS. To block non-specific antibody binding the cells were incubated for 30 min at RT in PBS containing 5 % FBS, after which the cells were incubated with anti-MHV K134 diluted 1:200, or anti-FIPV C428 diluted 1:500, in PBS containing 5 % FBS for 1 h at RT. The cells were washed three times with PBS containing 5 % FBS and incubated with Swine-anti-Rabbit peroxidase (DAKO, Glostrup, Denmark) diluted 1:300, or Goat-anti-Cat peroxidase (DAKO) diluted 1:400, in PBS containing 5 % FBS for 1 h at RT. The cells were washed three times with PBS and stained with AEC (Brunschwig, Amsterdam, The Netherlands) according to the manufacturer's protocol.

Monitoring of virus growth.

An amount of 5 x 10^5 cells per 10 cm^2 well was seeded and inoculated the next day with virus at a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell for 1 h in serum free culture medium. The cells were washed three times with PBS, and cultured for up to 48 h. At several time-points post-inoculation (p.i.) the medium was harvested, centrifuged for 10 min at 3000 rpm, and stored at – 80 °C until analysis. The amount of virus produced at each time-point p.i. was determined by endpoint dilution titration on feline FCWF-4 cells.

Production of syncytia inhibiting peptides.

For the production of the fHR1 peptide corresponding to amino-acid 1047 - 1156 of the FIPV spike protein (acc.nr.: VGIH79), a PCR fragment was prepared using as a template the FIPV spike gene sequence from cDNA clone B1 (3). Primers were designed to introduce into the amplified fragment an upstream *Bam*HI site (5'-GTG GAT CCC AGG CTA GAC TTA ATT ATG-3'), and a downstream *Eco*RI site, as well as a stop codon preceding the *Eco*RI site (5'-GGA ATT CAT GTA ATC AGC CTA TCA AC-3'). Fragments were

cloned into the *Bam*HI/*Eco*RI sites of the pGEX-2T bacterial expression vector (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) in frame with the glutathione *S*-transferase (GST) gene just downstream of the thrombin cleavage site. Freshly transformed BL21 cells (Novagen, Madison, United States) were grown to log phase and subsequently induced by adding isopropyl-β-D-thiogalactopyranoside (Life Technologies, Ltd., Paisley, United Kingdom) to a final concentration of 0.4 mM. After 3 h the cells were pelleted and resuspended in 10 mM Tris (pH 8.5), 1 mM EDTA, 5 mM DTT. Cell homogenates were prepared by 15 min incubation in 1 mM phenylmethylsulfonyl fluoride, 100 µg/ml lysozyme on ice, a freeze-thaw step, sonification on ice, and 30 min incubation in 1% Triton X-100 on ice. Homogenates were centrifuged at 25000 rpm for 1 h at 4 °C and pellets were dissolved in 8 M urea, 20 mM NaH_2PO_4, 100 mM β-mercaptoethanol (pH 7.0). Protein refolding was performed by 10 x dilution in 20 mM Tris-Cl (pH 9.0), 1 mM EDTA, 5 mM DTT overnight (O/N) at 4 °C. Glutathione-Sepharose 4B (Amersham Pharmacia) in PBS was added to a final 1% vol concentration and the mixtures were incubated O/N under rotation at 4 °C. The beads were washed three times with PBS and resuspended in PBS. Peptides were cleaved from the GST moiety on the beads using thrombin (Amersham Pharmacia) by incubation under rotation for 4 h at RT. Peptides in the supernatant were purified by high-pressure liquid chromatography using a Phenyl-5PW reversed-phase column (Tosoh, Tokyo, Japan) with a linear gradient of acetonitrile containing 0.1 % trifluoroacetic acid. The peptide-containing fractions were vacuum dried O/N and dissolved in water. The molecular weight of the peptide was confirmed by mass spectometry (2, 19). The fHR1 concentration as determined by measuring the absorbance at 280 nm was 0.2 mM. A final concentration of 0.4 µM was used to block fMHV mediated formation of OVCAR-fAPN syncytia.

Monolayer cytotoxicity analysis.

An amount of 5 x 10^4 HeLa-fAPN or OVCAR-fAPN cells was seeded per 0.32 cm^2 well and infected in triplicate with various amounts of FIPV or fMHV. At several time-points after inoculation the cells were cultured for 1 h in 10 % WST-1 (Roche Diagnostics GmbH, Mannheim, Germany), after which the OD_{450} was measured. Viability was expressed relative to uninfected control cells, after subtraction of background values of WST-1 incubated in the absence of cells.

Spheroid cytotoxicity analysis.

Three-dimensional multilayer spheroids were produced by incubating 5 x 10^4 OVCAR-fAPN cells in 0.32 cm^2 wells coated with 100 µl 2 % Multi Purpose agarose (Roche Diagnostics GmbH, Mannheim, Germany) in PBS for 24 h on a spinner-platform set at 125 rpm, at 37 °C and 5 % CO_2. Subsequently, the spheroids were cultured for 7 days at 37 °C and 5 % CO_2, reaching a diameter of approximately 600 µm, before they were used for infection experiments. Spheroids were infected in a total volume of 100 µl and cultured for 2, 5, or 7 days, after which they were incubated for 5 h in 10 % WST-1. The OD_{450} was measured directly on spheroid-containing wells. Viability was expressed relative to uninfected control spheroids, after subtraction of background values of WST-1 incubated in the absence of spheroids. Statistical significance between different groups was determined by the *t* test.

RESULTS

FIPV and fMHV can be redirected to human cancer cells transiently expressing fAPN.

To determine whether non-human coronaviruses can infect and kill human cancer cells when the host species barrier determined by specific receptor recognition is alleviated, FIPV and fMHV were redirected to human cancer cells via the virus receptor fAPN. To this end, the cancer tumor cell lines Caco-2, OVCAR-3, and HCT-8 (Fig. 1), HeLa, HepG2, and WiDr (data not shown), of colon, ovary, cervix, and liver tissue origins, were transfected with an fAPN expression plasmid and inoculated with FIPV or fMHV 24 h later. An immunostaining for coronavirus proteins was performed at 8 h after infection. All fAPN transfected cancer cell lines stained positive, indicating susceptibility to infection and expression of FIPV and fMHV. In contrast, control infections on cells not transfected with the fAPN expression construct did not show any FIPV or fMHV expression (data not shown), confirming that infection required appropriate receptor expression.

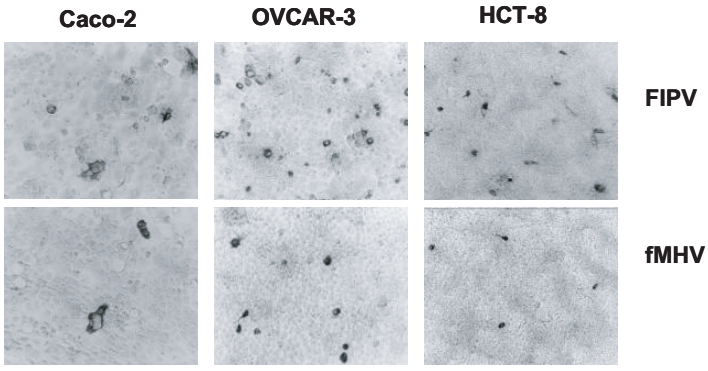

Fig. 1 Transient fAPN expression allows FIPV and fMHV replication in human cancer cells. The cancer cell lines Caco-2, OVCAR-3 and HCT-8 were transfected with pCR3-fAPN and inoculated with FIPV or fMHV 24 h later. An immunostaining for coronavirus proteins was performed at 8 h p.i.

FIPV and fMHV replicate and produce progeny virus in human cancer cells expressing fAPN.

Studies of FIPV and fMHV propagation, cytotoxicity and syncytia formation require host cells with stable receptor expression. Therefore, HeLa and OVCAR-3 derivative cell lines stably expressing fAPN were produced (Fig. 2A). FIPV and fMHV were tested for their growth characteristics in HeLa-fAPN and OVCAR-fAPN cells (Fig. 2B). In both cell lines, FIPV and fMHV progeny virus secretion started at 6-12 h p.i. and the virus continued to accumulate in the medium until 30-36 h p.i. No striking differences in replication capacity between FIPV and fMHV were observed. Evidently, infections of fAPN expressing human cancer cells with the viruses FIPV and fMHV resulted in rapid production of progeny virus.

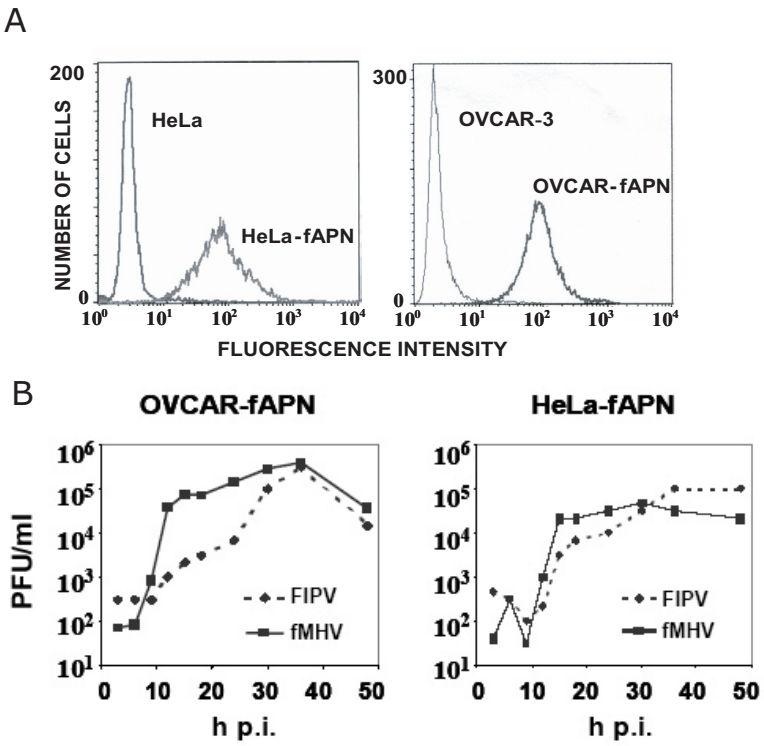

Fig. 2 Propagation of FIPV and fMHV on HeLa-fAPN and OVCAR-fAPN cells. (A) fAPN expression on HeLa and HeLa-fAPN [left panel] and OVCAR-3 and OVCAR-fAPN [right panel] as determined by FACS analysis. (B) Cells were inoculated with 5 PFU/cell FIPV or fMHV for 1 h as indicated and cultured for up to 48 h. At several time points p.i. the virus titer in the culture medium was determined by endpoint dilution titration on FCWF-4 cells. The data are from a representative experiment.

FIPV and fMHV efficiently eradicate human cancer cell monolayers.

To determine the potency of FIPV and fMHV to kill human cancer cells, HeLa-fAPN and OVCAR-fAPN cells were inoculated with various concentrations of FIPV or fMHV and the cell viability was measured at several time points after infection. FIPV and fMHV efficiently killed both cell lines in a dose-dependent manner (Fig. 3A). The onset of cell death was found to occur as soon as 12-24 h p.i. Following infection at MOI 10, both viruses completely eliminated the OVCAR-fAPN and HeLa-fAPN monolayers within 24 h and 36 h, respectively. Infections at lower MOI apparently required multiple rounds of infection resulting in the death of all or nearly all cells within 60 h. Furthermore, infected cells clearly showed membrane fusion typical of coronavirus replication (Fig. 3B). To determine the impact of syncytia formation on cancer cell death, we compared fMHV infections resulting in cell-cell fusion and infections in which the cell-cell fusion was blocked. The formation of syncytia is caused by the interaction of the coronavirus spike protein, presented on the plasma membrane of the infected cell, and its cognate receptor expressed on neighbouring cells. This interaction can be blocked by peptides derived from the heptad repeat domains of the spike protein, as was shown for MHV (1). By using similar techniques a fHR1 peptide corresponding to the heptad repeat 1 region of the FIPV and fMHV spike was produced, and used to block the formation of syncytia. OVCAR-fAPN cells were infected with fMHV at an MOI of 5 for 2 h, and washed three times before the cells were incubated in medium with or without fHR1 peptide. At several time-points after incubation cell viability was measured by WST-1 conversion. The formation of syncytia appeared to considerably accelerate coronavirus-mediated cancer cell killing (Fig. 3C).

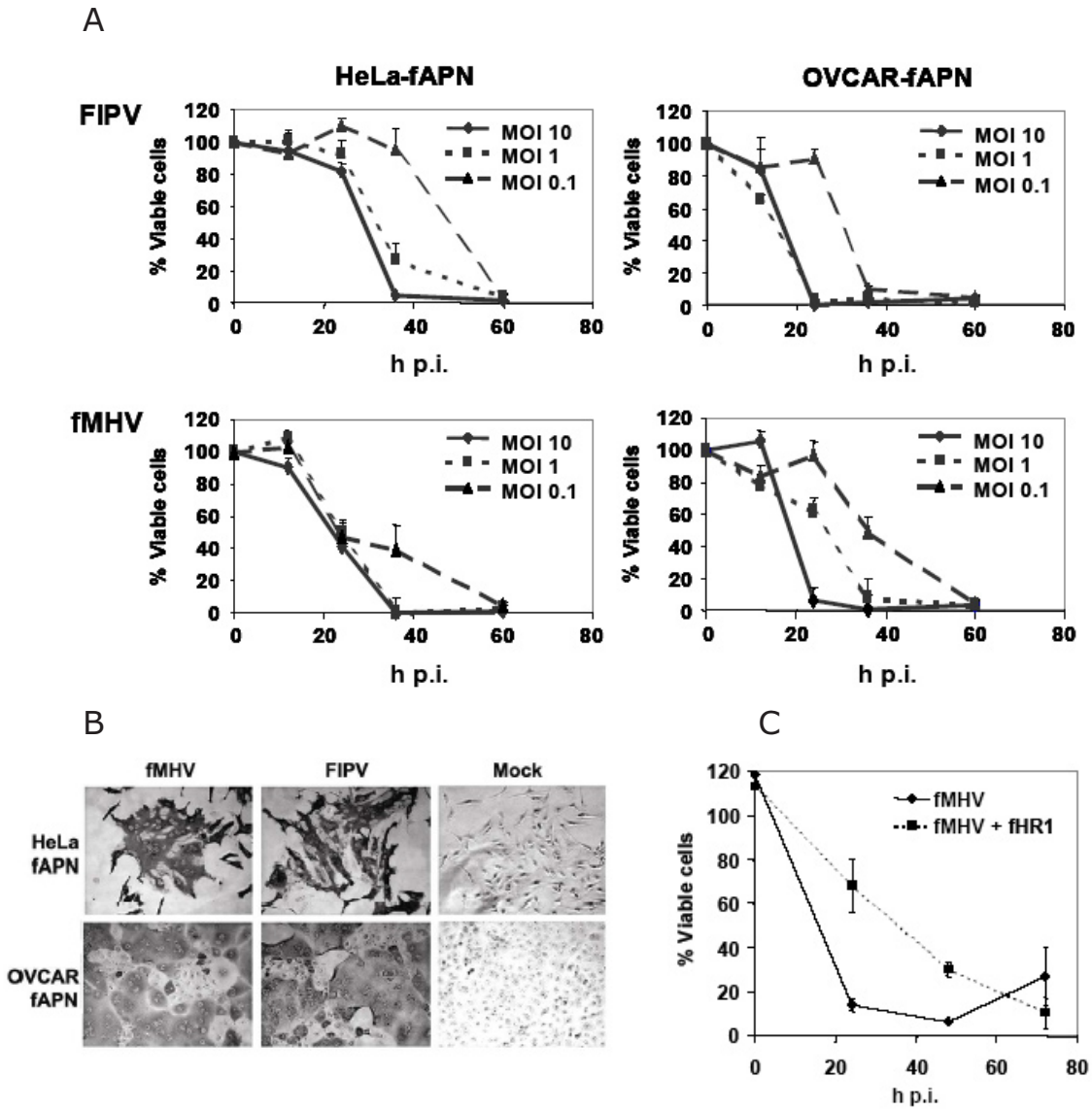

Fig. 3 FIPV and fMHV rapidly kill HeLa-fAPN and OVCAR-fAPN cells. (A) HeLa-fAPN and OVCAR-fAPN cells were infected with FIPV or fMHV at the indicated MOI and at several time points p.i. the cell viability was measured by WST-1 conversion assay. The results are depicted as the percentage viability compared to untreated control cells. The data shown are the means + standard deviations of an experiment performed in triplicate. (B) Immunohistochemical staining for coronavirus proteins on HeLa-fAPN and OVCAR-fAPN cells 15 h after infection with fMHV or FIPV at MOI 1. (C) OVCAR-fAPN cells were infected with fMHV at an MOI of 5. After 2 h, the cells were washed 3 x in PBS and incubated in medium with or without the fusion inhibitor fHR1. At several time points p.i. cell viability was measured by WST-1 conversion assay. The results are depicted as in A.

fMHV efficiently eradicates human cancer cells expressing fAPN in an *in vitro* multilayer tumor model.

Finally, we determined whether non-human coronaviruses were able to eradicate human cancer cells in an *in vitro* solid tumor model. Multilayer tumor spheroids offer a useful 3-D model for assessing virus-mediated eradication of tumor tissue. This model has already been employed to study the potency of oncolytic adenoviruses and adeno-associated viruses (5, 8). OVCAR-fAPN spheroids were established and infected with fMHV at 5 x 10^4 PFU/spheroid. For reference with other oncolytic viruses the coronavirus mediated cell death was compared to the oncolytic effect of Ad5 at 5 x 10^8 PFU/spheroid. At several days p.i. the cell viability was determined (Fig. 4A). At day 5 p.i. a clear decrease in viability was observed for spheroids infected with either virus. Two days later, the OVCAR-fAPN spheroids infected with 5 x 10^4

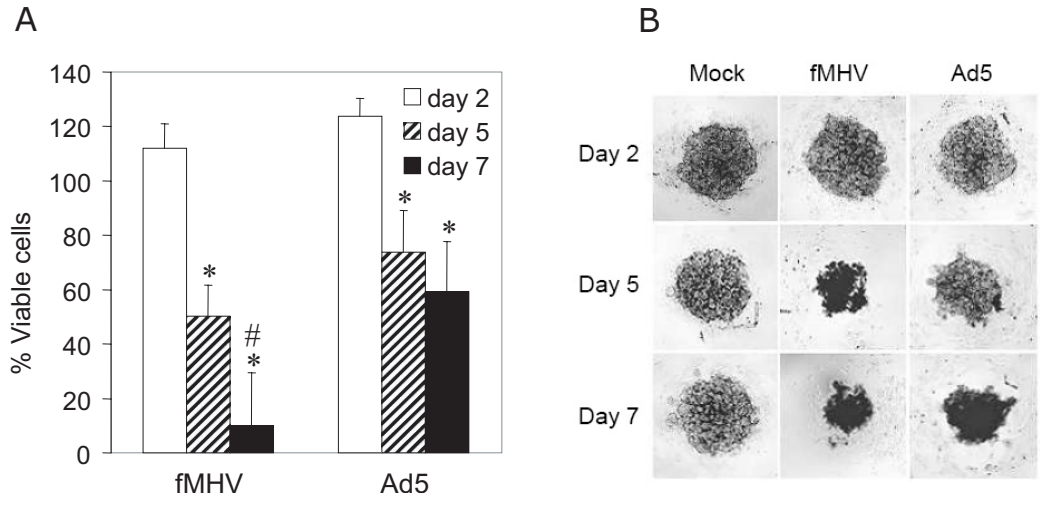

Fig. 4 fMHV efficiently kills OVCAR-fAPN multilayer tumor spheroids. (A) Spheroids were inoculated with fMHV (5 x10^4 PFU/spheroid) or Ad5 (5 x10^8 PFU/spheroid) or were mock inoculated and cultured for 2, 5, and 7 days, after which the cell viability was measured by WST-1 conversion assay. Results are depicted as the percentage of viable cells compared to mock-infected control spheroids. The data shown are the means + standard deviations of a representative experiment performed in triplicate. * Indicates a significant difference between day 2 and day 5 or 7, $P < 0.01$. # Indicates a significant difference between the cell death on day 7 mediated by fMHV compared to the cell death on day 7 mediated by Ad5, $P < 0.05$. (B) Representative images of OVCAR-fAPN multilayer spheroids inoculated with fMHV, Ad5, or mock-inoculated, and cultured for 2, 5, or 7 days.

PFU fMHV were essentially wiped out, whereas spheroids infected with 5 x 10^8 PFU Ad5 were only partially eradicated. This result was confirmed by light microscopic analysis (Fig. 4B).

DISCUSSION

The search for viruses that can be used as tools for anti-cancer therapy involves assessment of the efficacy and safety of these viruses. Here, we evaluated the anti-cancer efficacy of non-human coronaviruses. Our findings demonstrate that FIPV and fMHV possess a strong capacity to kill human cancer cells, once they are able to enter these cells through an artificially expressed receptor. This extends previous observations supporting the notion that coronavirus host cell specificity is determined at the cell entry level (14, 18). The very rapid and efficient eradication of cancer cells that we observed can be attributed to two coronavirus features. First, coronaviruses are positive strand RNA viruses that exhibit a fast replication and transcription process leading to rapid virus protein synthesis and progeny virus production. Second, the ability of coronaviruses to induce syncytia between infected and non-infected neighbouring cells amplifies their cytotoxicity. We found that FIPV and fMHV retained these properties in human cancer cells and tumor spheroids expressing the virus receptor, thus enabling a quick cytotoxic spread of the virus to surrounding non-infected cancer cells.

Coronaviruses share some of these attractive characteristics with several other viruses, including a fusogenic mutant of herpes simplex virus (7) and the live attenuated Edmonston B vaccine strain of measles virus (13). In contrast to these viruses, the non-human coronaviruses FIPV and fMHV are normally incapable of infecting human cells, due to their strict species-specific tropism. Thus, their native tropism does not need to be abolished in order to specifically restrict coronavirus entry, hence cytotoxicity, to human cancer cells.

Obviously, directing coronaviruses to cells bearing specific receptors will now

be the major challenge. There are several options to tackle this challenge. The most straightforward approach would probably be the modification of the spike protein in such a way that it would recognize the tumor cell receptor of choice. This would involve the incorporation of a receptor ligand (or fragment thereof) at an appropriate location in the spike protein without affecting the protein entry functions, a difficult task in the absence of knowledge of the 3-dimensional structure of the spike. A more feasible option might be to generate bispecific linker molecules able to bind to the coronavirus spike as well as to a target receptor. There are several ways to design such linkers. In the following chapters we describe the different approaches that we took to successfully achieve targeting of animal coronaviruses to human cancer cells.

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The authors are grateful to Dr. T. Hohdatsu (Kitasato University, Towada, Aomori, Japan) for providing anti-fAPN antibody R-G-4 and to Dr. K. Holmes (University of Colorado Health Sciences Center, Denver, CO) for supplying the fAPN expression vector pCR3-fAPN. This work was supported by the Dutch Cancer Society (UU 2001-2430). V. W. van Beusechem is supported by a research fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

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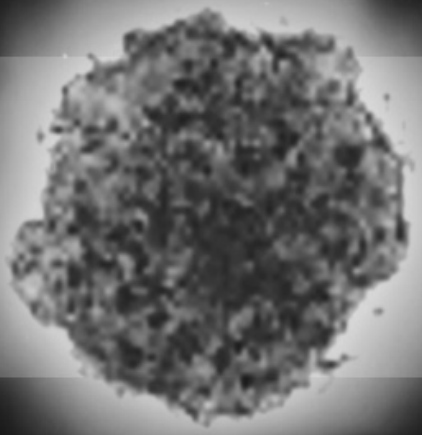
CHAPTER 3 | TARGETING NON-HUMAN CORONAVIRUSES TO HUMAN CANCER CELLS USING A BISPECIFIC SINGLE-CHAIN ANTIBODY



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ABSTRACT

To explore the potential of using non-human coronaviruses for cancer therapy we investigated whether the feline infectious peritonitis virus (FIPV) and a felinized mouse hepatitis virus (fMHV) could be targeted to human cancer cells by constructing a bispecific single-chain antibody directed on the one hand against the feline coronavirus spike protein - responsible for receptor binding and subsequent cell entry through virus-cell membrane fusion - and on the other hand against the human epidermal growth factor receptor (EGFR). When added to the inoculum the targeting antibody appeared to mediate specific infection of EGFR-expressing human cancer cells by both coronaviruses. Furthermore, in the presence of the targeting antibody, infected cancer cells formed syncytia typical of productive coronavirus infection. By their potent cytotoxicity the selective targeting of non-human coronaviruses to human cancer cells provides a rationale for further investigations into the use of these viruses as anti-cancer agents.

INTRODUCTION

Replicative oncolytic viruses represent new agents with potential utility in cancer therapy. They are aimed to effectively eradicate tumor cells without affecting normal tissues. Several different DNA and RNA viruses are currently being evaluated for their efficacy and selectivity towards cancer cells (29, 31). So far, there are no reports describing the potential use of coronavirus-based oncolytic agents in cancer virotherapy, despite a number of features that make coronaviruses potentially attractive for this purpose.

Coronaviruses are positive strand RNA viruses consisting of a nucleocapsid, that contains the approximately 30 kb genome and the nucleocapsid (N) protein, and which is surrounded by an envelope carrying three membrane proteins, spike (S), envelope (E), and matrix (M). Of these, the spike glycoprotein S is responsible for virus entry and syncytium formation, as it binds to the cellular receptor and induces membrane fusion (5, 22, 37). The specific interaction between the amino-terminal spike protein domain S1 and its cognate receptor (13, 34, 35) induces conformational changes in the spike protein domain S2 that trigger the membrane fusion process. These conformational changes require physical interaction between two heptad repeat (HR) regions, HR1 and HR2 that occur in the S2 domain. Prevention of this interaction by using peptides that correspond to these HR1 and HR2 regions can block membrane fusion, by binding of the peptides to their respective counterparts (3, 33, 41).

Most coronaviruses exhibit strict species specificity, as determined by the spike-receptor interaction (2, 7, 39). The coronavirus feline infectious peritonitis virus (FIPV), for instance, selectively infects and induces syncytium formation of feline cells via its receptor feline aminopeptidase N (fAPN) (35). Likewise, the recombinant felinized mouse hepatitis virus (fMHV) (24), a derivative of mouse hepatitis virus (MHV) carrying a chimeric spike of which the ectodomain is from the FIPV spike protein, also infects and fuses only feline cells through the fAPN molecule. As a

consequence of their species restricted tropism, FIPV and MHV are non-pathogenic to human cells. However, once the tropism barrier is alleviated coronaviruses can replicate in cells of different species (19, 24, 40). Thus, FIPV and MHV may potentially be converted into specific oncolytic agents for the treatment of human cancer if their spike protein would recognize a receptor on human tumor cells.

Towards developing recombinant coronaviruses with a selective human tumor tropism we investigated whether FIPV and fMHV could be targeted to human cancer cells expressing the epidermal growth factor receptor (EGFR), a molecule commonly overexpressed on many types of cancer cells (23) and associated with poor prognosis and response to cancer therapy (6, 26). For this purpose, we prepared a bispecific single-chain variable fragment (scFv) antibody, binding on one side to the FIPV and fMHV spike protein and on the other side to the human EGFR. The antibody indeed functioned as a specific targeting device. Our findings justify the further development of coronaviruses as oncolytic agents.

MATERIALS AND METHODS

Viruses, cells, and antibodies.

Recombinant fMHV (24) and FIPV strain 79-1146 (16) stocks were produced and titrated in parallel on feline FCWF-4 cells, yielding titers of 5 x 10^6 and 1 x 10^7 $TCID_{50}$/ml, respectively. Wild-type adenovirus type 5 (Ad5) was produced and titrated on 293 human embryonic kidney cells. The recombinant vaccinia virus vTF7-3 containing the bacteriophage T7 RNA polymerase gene was used as a T7 RNA polymerase source for the T7 promoter driven production of bispecific scFv in OST7-1 cells (15).

OST7-1 (obtained from B. Moss), NIH 3T3, HeLa, OVCAR-3, HCT-8, Caco-2, WiDr, HepG2, A431 (American Type Culture Collection) and FCWF-4 cells (obtained from N. C. Pedersen) were grown in Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, Verviers, Belgium) containing 10 % fetal bovine serum (FBS), 100 IU of penicillin/ml, and 100 µg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). NIH 3T3-EGFR cells (NIH 3T3-her14, obtained from P. M. P. van Bergen en Henegouwen) (32) were maintained in DMEM containing 10 % FBS, 100 IU of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 mg/ml G418 (Life Technologies, Ltd., Paisley, United Kingdom). The hybridoma cell line producing the 23F8.1 monoclonal antibody (MAb) against the FIPV spike protein (8) was cultured in CD Hybridoma medium supplemented with 2 mM Glutamax (all from Life Technologies, Ltd., Paisley, United Kingdom).

C428, ascitic fluid from a FIPV-infected cat (kindly provided by B. J. Haijema), was used as a source of polyclonal antibodies to FIPV. The rabbit antiserum K134 raised against purified MHV (30), and

the MAb R-G-4 directed against the fAPN receptor have been described previously (21, 30). The culture supernatant of the hybridoma cell line 23F8.1 (8) was used as a source of the MAb 23F8.1 directed against the FIPV and fMHV spike. For EGFR detection, the MAb 425 was used (culture supernatant from the hybridoma cell line 425, ATCC). To detect Myc-tagged scFv 23F-425, the anti-Myc antibody Myc was used (culture supernatant from the hybridoma cell line Myc 9E10, ATCC).

Construction of the bispecific scFv 23F-425.

The hybridoma cell line 23F8.1 was used to isolate mRNA by using the Quickprep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The Mouse ScFv Module/Recombinant Phage Antibody System (Amersham) was used to generate the scFv 23F. The mRNA was isolated from 1 x 10^7 23F8.1 hybridoma cells and the cDNA was produced by RT-PCR, according to the Mouse ScFv Module protocol. The variable domain of both the heavy chain (V_H) and the light chain (V_L) of the 23F8.1 antibody cDNA were isolated by PCR using V_H and V_L primers of the scFv isolation system. The fragments were cloned separately into the pGemTeasy cloning vector (Promega, Madison, USA) and sequenced. To introduce a 16 amino acid residue middle linker, a fusion PCR was performed joining the 23F8.1-V_H and 23F8.1-V_L fragments in a V_H-V_L configuration via a 48-nucleotide linker DNA. The linker DNA consisted of 2 primers, each comprising a part of the linker sequence of scFv 425 (20, 25) (underlined), and flanking sequences overlapping the 3' *23F8.1-V_H* (5'-<u>CAGAGCCACCTCCGCCTGAACCGCCTCCACCTGA</u>*GGAGACGGTGACCGT*-3') and the 5' *23F8.1-V_L* (5'-<u>CAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGG</u>*ACATCCAGATGACCCA*-3'), resulting in a Ser-(Gly_4-Ser)$_3$ linker. In a subsequent PCR, the assembled scFv DNA was amplified and restriction sites were added by using the RS primers of the scFv isolation system. The resulting DNA fragment contained a 5' *Sfi*I site and a 3' *Not*I site, and was subsequently cloned into pGemTeasy and sequenced. The sequence was compared to the sequences of the independent 23F8.1-V_H and 23F8.1-V_L fragments, and the resulting correct clone was named pGemTeasy-23FV_HV_L. The pGemTeasy-23FV_HV_L plasmid was digested by *Sfi*I and *Not*I to isolate the 754 bp scFv 23F, which was ligated into the pCANTAB derivative pSTCF (20) that had been digested by *Sfi*I and *Not*I, resulting in pSTCF23F. The scFv 425 directed against the EGFR was isolated from pSTCFS11-425 (20, 25) by *Not*I digestion and ligated in a V_H-V_L configuration into the *Not*I site downstream of the scFv 23F in pSTCF23F creating a 3 Ala linker between the 2 scFv fragments. This resulted in the expression vector pSTCF23F-425 which contains the 1587 bp bispecific cDNA construct encoding the anti-spike scFv 23F linked to the anti-EGFR scFv 425 in fusion with an amino-terminal Igκ signal sequence and a carboxy-terminal Myc-tag under the control of CMV and T7 promoters.

Metabolic labeling and immunoprecipitation.

To determine whether scFv 23F-425 was produced and secreted into the culture medium, subconfluent monolayers of OST7-1 cells in 2-cm^2 tissue culture dishes were inoculated with vTF7-3 at 5 PFU/cell (*t* = 0 h) and subsequently transfected (*t* = 1 h) without DNA, with pSTCF23F-425, or with pSTCFS11-425 plasmid DNA by using lipofectin (Life Technologies, Ltd., Paisley, United Kingdom) (20, 25). At *t* = 5 h, the cells were starved for 30 min cysteine- and methionine-free modified Eagle's medium containing 10 mM HEPES, pH 7.2, and 5% FBS. The medium was then replaced by 200 µl of similar medium containing 100 µCi of ^{35}S *in vitro* cell-labeling mixture (Amersham Pharmacia Biotech Europe GmbH, Germany). After 1 h the cells were either lysed or the labeling medium on the cells was replaced by 240 µl normal culture medium and incubation continued for 0, 4 or 16 h. The cells were lysed in 300 µl TESV lysis buffer

(20 mM Tris-HCl (pH 7.3), 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1 % Triton X-100). To the cleared medium 60 µl 5 x TESV lysis buffer was added. Proteins were immunoprecipitated from the medium or the lysed cells by using the anti-Myc antibody diluted 1:10. The immune complexes were adsorbed to Pansorbin cells (Calbiochem, La Jolla, United States) as described previously (11). Equal volumes of the immunoprecipitates were analyzed by SDS-PAGE containing 10 % polyacrylamide.

Production of the bispecific scFv 23F-425.

For the production of scFv 23F-425, subconfluent monolayers of OST7-1 cells were inoculated at an MOI of 5 with vTF7-3 (t = 0 h) and transfected (t = 1 h) with pSTCF23F-425, or mock transfected (no plasmid DNA), by using lipofectin (Life Technologies, Ltd., Paisley, United Kingdom). The medium was refreshed at t = 4.5 h, harvested at t = 20 h, and centrifuged for 10' at 3,000 rpm to clear it from cell debris. The mock supernatant and the supernatant containing the bispecific scFv were loaded onto a 20 % sucrose cushion, centrifuged for 30' at 13,000 rpm to clear the supernatant from vTF7-3 virus, and stored at - 20 °C in aliquots until use. The supernatant from OST7-1 cells infected with vTF7-3, but not transfected with plasmid DNA, was used as a control supernatant. A single batch of scFv 23F-425 and control supernatant was used for all experiments described.

Determination of optimal amount of 23F-425 to be used in targeted infections.

To determine the optimal amount of scFv 23F-425 to be used in targeted infections, FIPV (MOI 5) was preincubated for 1 h with various amounts of scFv 23F-425 and inoculated in a total volume of 500 µl on A431 cells in a 2-cm^2 well. After 1 h at 37 °C the inoculum was replaced by regular culture medium and incubation of the cells was continued for 15 h. An immunostaining with serum directed against FIPV proteins was performed, after which the stained cells were counted and their numbers plotted against the amount of scFv 23F-425 used. The titration results revealed the optimal amount of bispecific antibody needed for maximal targeting efficiency under these standard conditions to be 200 µl scFv 23F-425. More antibody did not increase but, rather, decreased the level of infection, presumably because excess antibodies in the inoculum may bind to EGFR on cells thereby blocking their use by the virus. Cells inoculated with FIPV preincubated with mock control supernatant remained negative (data not shown).

Immunostaining.

Cells were incubated with C428 anti-FIPV ascites fluid diluted 1:500, or K134 anti-MHV serum diluted 1:300, followed by Goat-anti-Cat peroxidase (DAKO, Glostrup, Denmark) diluted 1:400, or Swine-anti-Rabbit peroxidase (DAKO, Glostrup, Denmark) diluted 1:300, respectively. The cells were stained by AEC (Brunschwig, Amsterdam, the Netherlands) according to the manufacturer's protocol, and analyzed by light microscopy.

Antibody blocking experiments.

To determine whether scFv 23F-425 interacts with the EGFR, 2 x 10^5 HeLa cells per 2-cm^2 well were incubated with or without 500 µl hybridoma supernatant containing MAb 425 for 30' at 4 °C in order to block the interaction of EGFR and scFv 23F-425. Next, the cells were inoculated for 15 h at 37 °C with 1 x 10^5 PFU fMHV preincubated with 200 µl scFv 23F-425 for 1 h at 4 °C in a total volume of 500 µl. Hereafter, the cells were fixed, permeabilized, and immunostained for the presence of fMHV. The number of infected

cells was counted by using light microscopy.

The interaction of scFv 23F-425 and the spike protein was analyzed by incubating 1 x 10^5 PFU fMHV with or without 200 µl hybridoma supernatant containing anti-fMHV-S antibody 23F8.1. After 1 h of incubation at 4 °C, 200 µl scFv 23F-425 was added for 1 h of incubation at 4 °C in a total volume of 500 µl. Next, the infection mixes were inoculated on 2 x 10^5 HeLa cells per 2-cm^2 well for 15 h at 37 °C. The cells were fixed, permeabilized, and immunostained for the presence of fMHV. Again, the number of infected cells was determined by using light microscopy.

Analysis of scFv 23F-425 mediated fusion.

An amount of 5 x 10^4 A431 cells per 0.32-cm^2 well was inoculated at an MOI of 5 for 2 h with fMHV preincubated with 50 µl scFv 23F-425 for 1 h at 4 °C. The cells were washed 3 times with PBS and incubated further in the presence or absence of 50 µl scFv 23F-425. An immunostaining was performed 24 h after infection, and the number of nuclei per syncytium was determined by light microscopy.

Production of the fHR1 peptide.

For the production of the fHR1 peptide corresponding to amino acids 1047 - 1156 of the FIPV spike protein (acc. nr.: VGIH79), a PCR fragment was prepared using as a template the sequence from cDNA clone B1, which contains the FIPV spike gene (9). The fHR1 peptide was expressed in E. coli, purified, and quantified as described elsewhere (3, 38, 40).

Analysis of the fusion mechanisms of coronavirus infection.

Sensitivity of the normal virus-cell fusion process to fHR1 was studied by inoculating feline FCWF-4 with fMHV at an MOI of 0.5 in the presence or absence of 0.5 µM fHR1 peptide for 8 h. The effect of fHR1 on the targeted fusion process was analyzed by preparing in parallel two inoculation mixtures by preincubating fMHV with scFv 23F-425 for 1 h at 4 °C after which 0.5 µM fHR1 peptide was added to one mixture. Two cultures of human A431 cells were washed with PBS and inoculated at an MOI of 5 for 16 h at 37 °C with the infection mixes. The cells were fixed, permeabilized, and stained for the presence of fMHV using anti-MHV rabbit antiserum K134. The number of infected cells was counted by using light microscopy.

The sensitivity of the cell-cell fusion process during normal infection of feline FCWF-4 cells was analyzed by inoculating cells with fMHV at an MOI of 0.5 for 1 h at 37 °C, washing them 3 times with PBS, and incubating for 7 h in culture medium with or without 0.5 µM fHR1 peptide. For a similar analysis of cell-cell fusion after targeted infection, cultures of A431 cells were inoculated at an MOI of 5 with fMHV preincubated with scFv 23F-425 for 1 h at 4 °C. Next, the cells were washed 3 times with PBS, and incubated for 22 h in culture medium with or without 0.5 µM fHR1 peptide. The cells were fixed, permeabilized, and immunostained for the presence of fMHV proteins. The number of nuclei per syncytium was counted under the light microscope.

RESULTS

Bispecific antibody-mediated coronavirus infection of human cancer cells.
Having previously established that FIPV and fMHV can infect and destroy cancer cells once the entry barrier has been overcome (chapter 2), we wanted to develop a general method to target these viruses to a suitable antigen expressed on such cells. To this end we constructed the bispecific scFv 23F-425 (Fig. 1a), which combines the antigen binding domains from antibodies 23F8.1 and 425, recognizing the FIPV S protein and EGFR, respectively. The protein was produced by expression in eukaryotic OST7-1 cells. Its synthesis and secretion were verified by radiolabeling followed by immunoprecipitation from the cell lysate and culture medium using an anti-myc antibody (Fig. 1b). The results clearly show the synthesis and secretion of the approx. 58 kD bispecific single-chain molecules.

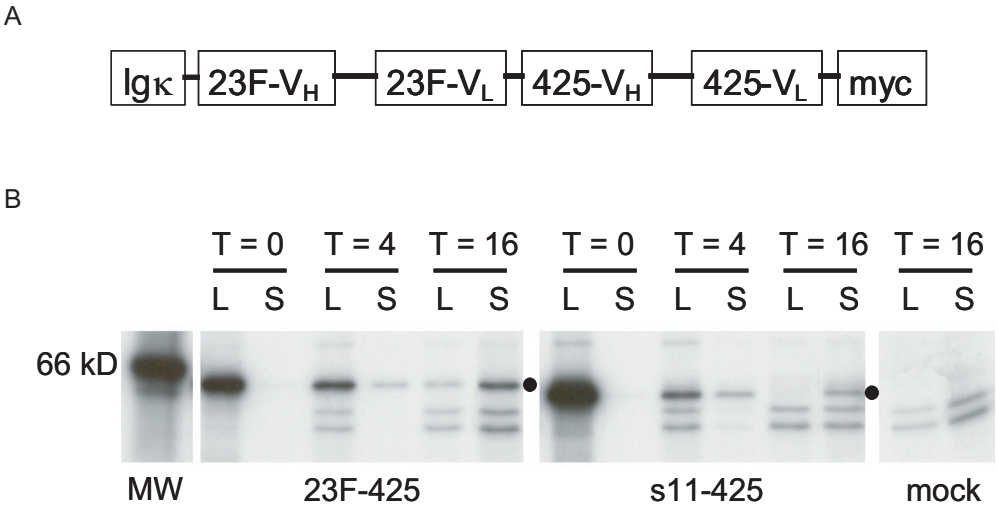

Fig. 1 Analysis of synthesis and secretion of expressed scFv 23F-425. The myc-tagged bispecific protein 23F-425 was radiolabelled in OST7-1 producer cells and analyzed by immunoprecipitation using an anti-myc antibody. At 0, 4, and 16 h after labeling equal volume fractions of cell lysate and culture medium were analyzed. The myc-tagged bispecific scFv s11-425 (20, 25) was included in the experiment for comparison. The position of the scFv's is indicated by a dot. At the right a control analysis of cells transfected without DNA indicates the non-specific nature of some proteins.

To investigate whether scFv 23F-425 could serve as an adapter molecule for FIPV and fMHV infection via human EGFR, cultures of human cancer cell lines of different tissue origin with confirmed expression of EGFR (Fig. 2) were inoculated with similar amounts of FIPV or fMHV in the presence or absence of the bispecific antibody (i.e. OST7-1 culture supernatant). After 1 h at 37 °C the inoculum was replaced by regular culture medium and incubation of the cells was continued for 15 h. The cells were immunostained for coronavirus protein expression. As can be seen in Fig. 2, all cell lines tested had become infected with FIPV and fMHV in the presence of scFv 23F-425. In contrast, none of the cells stained positive after inoculation with FIPV or fMHV that had been preincubated with mock control supernatant (data not shown). Similarly, no positive staining was observed when human cancer cells had been inoculated with the control virus MHV in the presence of scFv 23F-425 (data not shown).

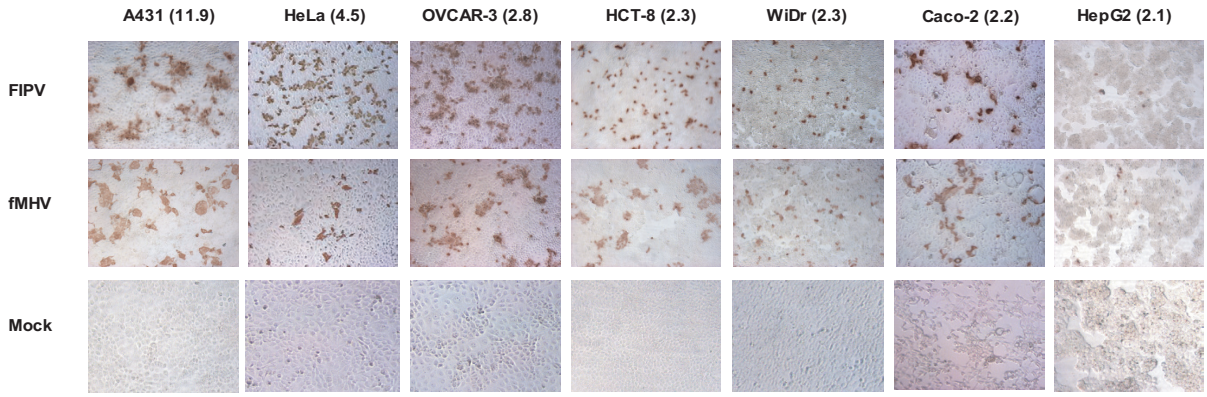

Fig. 2 Targeted coronavirus infection of human cancer cells. Cells were inoculated (MOI of 5) with preincubated mixtures of FIPV or fMHV (control: no virus) and the bispecific adapter protein scFv 23F-425. The cells were stained for coronavirus proteins 24 h after inoculation. All cell lines tested showed susceptibility to EGFR-targeted FIPV or fMHV. Values in parentheses indicate the relative EGFR expression (relative geometric mean fluorescence [GMF]) as determined for each cell line by FACS analysis and expressed as the ratio of the GMF values obtained in the presence and absence of the anti-EGFR antibody.

Differences in infection efficiency were observed between different cell lines, with the EGFR-high A431 cells showing the highest susceptibility to EGFR-targeted coronavirus infection and the EGFR-low HepG2 cells being the most poorly infected. On most cell lines, HeLa cells being the exception, EGFR-targeted FIPV exhibited a similar infection efficiency as EGFR-targeted fMHV. Interestingly, infected cells formed syncytia typical for productive coronavirus infection. The formation of infectious progeny virus was confirmed by monitoring - through titration on FCWF-4 cells - the increase in viral titers in the medium of A431 cells after inoculation with FIPV or fMHV. Typical growth curves were obtained, but only after the A431 cells had been inoculated in the presence of bispecific scFv 23F-425 (Fig. 3).

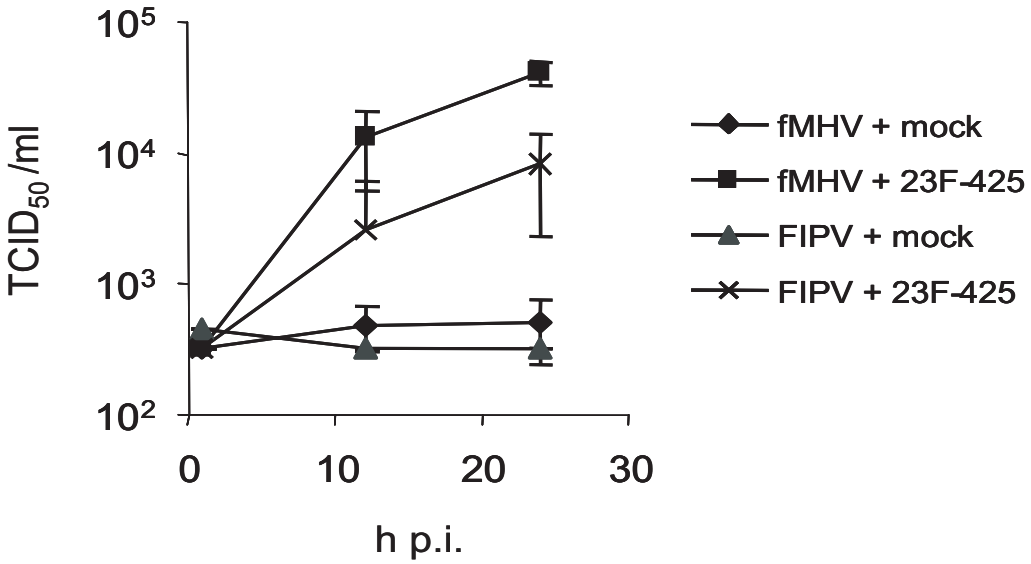

Fig. 3 Bispecific scFv 23F-425 mediated infections of A431 cells by FIPV and fMHV result in production of infectious progeny virus. FIPV and fMHV were preincubated in the presence or absence of scFv 23F-425 and inoculated on EGFR-expressing A431 cells. After 1 h the cells were washed 3 times and incubation was continued. At several time points samples were taken from the cell culture medium, which were subsequently titrated on feline FCWF-4 cells to determine the amount of virus produced. Productive infection of A431 cells was evident with both viruses but only after inoculation in the presence of the bispecific antibody. All data shown represent the average and standard deviation of an experiment performed in triplicate.

Bispecific antibody-mediated coronavirus infection is human EGFR specific.

To confirm that the infections of FIPV and fMHV established by scFv 23F-425 were indeed mediated by the human EGFR protein, we tested the mouse fibroblast cell line NIH 3T3 and its human EGFR-expressing derivative NIH 3T3-hEGFR (32) for their susceptibility to FIPV and fMHV in the presence and absence of scFv 23F-425. As illustrated by Fig. 4A, positive immunostaining for coronavirus proteins was only obtained with NIH 3T3-hEGFR cells inoculated in the presence of scFv 23F-425. This result demonstrated that FIPV and fMHV infection of these otherwise refractory NIH 3T3 cells required both the adapter molecule and expression of human EGFR. Further evidence for a specific interaction between the bispecific scFv 23F-425 and human EGFR was obtained by studying the effect of the EGFR antibody 425 on the scFv 23F-425-mediated coronavirus infection. HeLa cells were incubated with the 425 monoclonal antibody prior to inoculation of fMHV in the presence of scFv 23F-425. Fig. 4B shows that infection was blocked almost completely, confirming that a direct interaction with the EGFR is required. Similarly, the necessity of an interaction between the FIPV spike protein and scFv 23F-425 was confirmed. We incubated fMHV with and without anti-FIPV spike monoclonal antibody 23F8.1 before adding scFv 23F-425 and inoculating HeLa cells. Again, the anti-S antibody inhibited infection, demonstrating that scFv 23F-425 has to bind to the virus spike protein in order to function as a targeting adaptor (Fig. 4B).

Bispecific antibody-mediated syncytia formation of coronavirus-infected cancer cells.

Several human cancer cell lines that were infected with EGFR-targeted FIPV or fMHV showed cell-cell fusion typical for coronaviruses (see Fig. 4). Syncytium formation is an important determinant for cytotoxicity and spread of coronaviruses. Therefore, it was important to investigate if syncytium formation between infected and neighboring cells resulted from undefined interactions or from specific bridg-

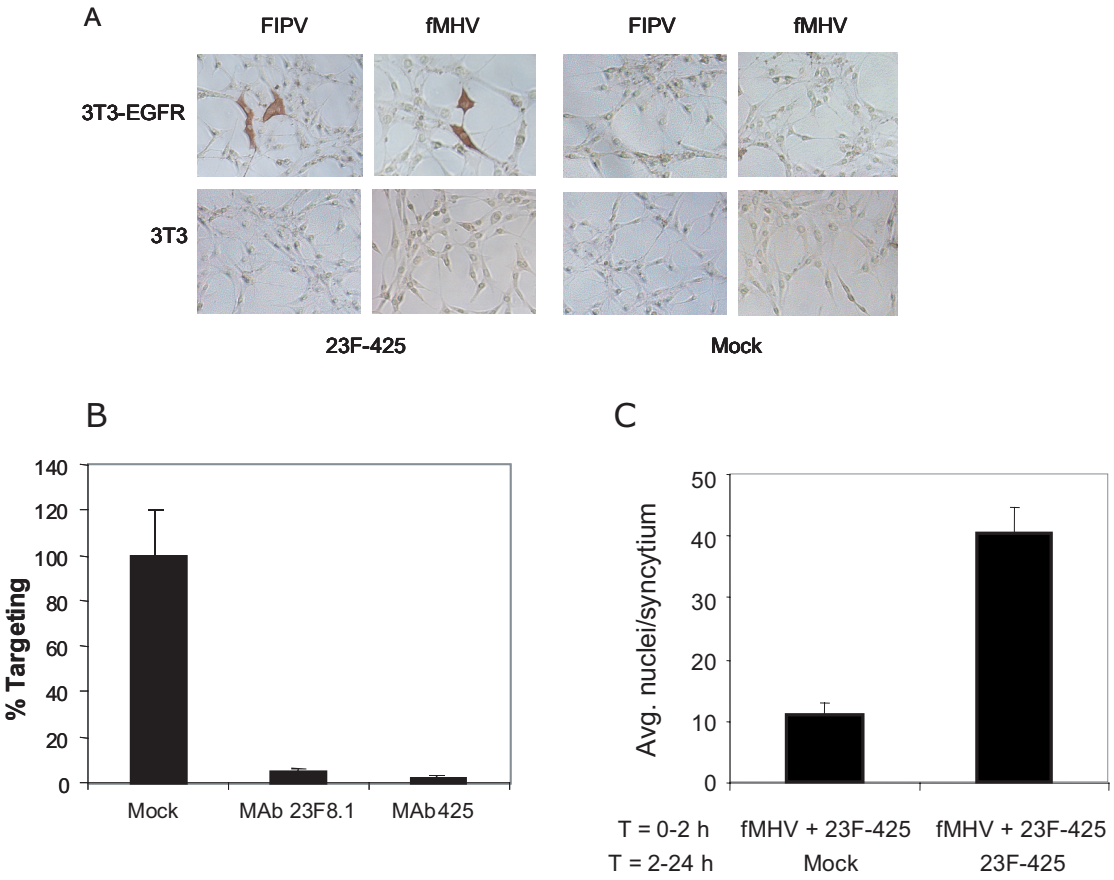

Fig. 4 EGFR specific coronavirus targeting. (A) NIH 3T3-hEGFR and NIH 3T3 cells were inoculated with FIPV or fMHV, in the presence or absence of the bispecific adapter protein scFv 23F-425. After 1 h at 37 °C the inoculum was replaced by regular culture medium and incubation of the cells was continued. The cells were stained for coronavirus proteins 24 h after inoculation. Only NIH 3T3-hEGFR cells inoculated with FIPV or fMHV in the presence of the bispecific scFv 23F-425 stained positive. (B) Compared to EGFR-targeted infections of HeLa cells in the absence of blocking antibody, virus preincubation with anti-spike MAb 23F8.1, or preincubation of the cells with anti-EGFR MAb 425, decreased the amount of infected cells. The data represent the average percentage of infected cells relative to cells inoculated in the absence of blocking antibody. The error bars show the standard deviation of an experiment performed in triplicate. (C) A431 cells were inoculated with fMHV in scFv 23F-425 containing medium for 2 h and subsequently cultured for another 22 h in the presence or absence of scFv 23F-425. The data represent the average amount of nuclei per syncytium measured in three independent experiments; ± indicates the standard deviation.

ing by scFv 23F-425. To this end, A431 cells were inoculated with fMHV in scFv 23F-425 containing medium for 2 h and subsequently cultured for another 22 h in the presence or absence of scFv 23F-425. Thus, scFv 23F-425 was either present continuously to support infection and syncytium formation or only briefy to mediate targeted infection. Removal of scFv 23F-425 after 2 h significantly reduced syncytium formation by approximately 4-fold from 40.8 ± 4.1 to 11.2 ± 2.2 nuclei per syncytium (the data represent the average amount of nuclei per syncytium measured in three independent experiments; ± indicates the standard deviation) (Fig. 4c). Hence, scFv 23F-425 did not only promote fusion between the fMHV envelope and target cells, but also fusion between fMHV infected and neighboring cells.

Targeted coronavirus infection and syncytium formation require the native spike membrane fusion function.

Coronaviruses use a class I membrane fusion mechanism (3), common to a number of enveloped virus families (14), in which heptad repeat regions occurring in the spike protein play an instrumental role. The mechanism involves conformational changes in the spike protein subsequent to receptor binding, resulting in an interaction of the HR1 and HR2 domains, which is necessary to drive the membrane fusion process. This process can be inhibited specifically using peptides corresponding to the HR domains as we showed recently for MHV (3). In order to investigate if the bispecific antibody-targeted FIPV and fMHV infections and their induction of syncytia also depend on those conformational rearrangements in the viral spike protein we tested the sensitivity of these processes to HR derived peptide. To this end we prepared a peptide corresponding to the HR1 region of the FIPV S protein and initially studied this fHR1 peptide for its ability to block FIPV and fMHV infection of feline FCWF-4 cells, which it did. As illustrated for fMHV (Fig. 5A and 5B), addition of fHR1 during or after inoculation of feline FCWF-4 cells abrogated infection and syncytium formation, respectively. Next we determined whether scFv 23F-425-mediated infection and syncytium formation are also sensitive to the fHR1 peptide. To this end, fMHV

was targeted towards EGFR on A431 cells in the presence or absence of fHR1. As can be seen in Fig. 5C and D, addition of the peptide reduced both scFv 23F-425 mediated infection and syncytium formation. Thus, both processes seem to utilize a membrane fusion process similar to that of the native coronavirus.

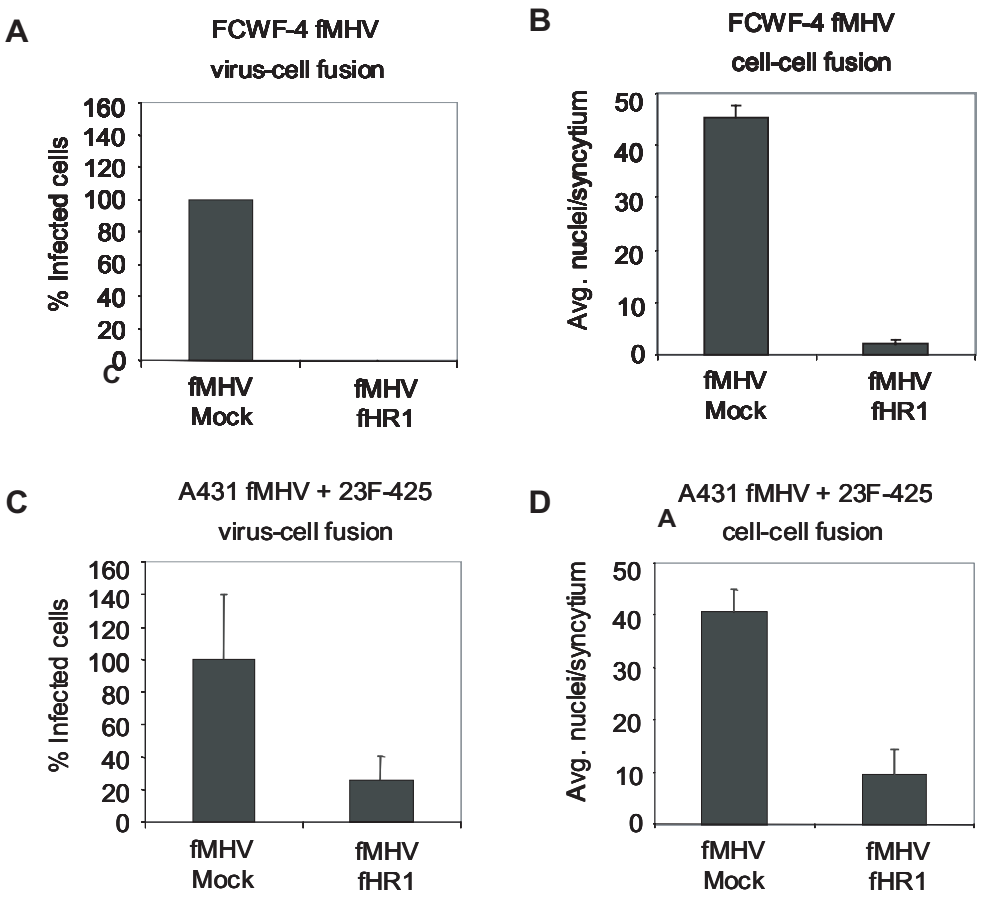

Fig. 5 Effect of fHR1 on fMHV-mediated fusion processes. (A) Preincubation of fMHV with fHR1 blocked the infection of feline FCWF-4 cells. (B) In addition, the formation of syncytia was inhibited when fHR1 peptide was added to the culture medium 1 h after infection of FCWF-4 cells. (C) fHR1 also caused a significant reduction in the number of infected human A431 cancer cells when inoculated with a preincubated mixture of fMHV and scFv 23F-425 in the presence of 0.5 μM fHR1. (D) Also the cell-cell fusion was inhibited by fHR1 when peptide and scFv 23F-425 were added to the medium of A431 cells that had been infected with a preincubated mixture of fMHV and scFv 23F-425. All data shown represent the average and standard deviation of an experiment performed in triplicate.

DISCUSSION

Despite tremendous research efforts over the last decades into the nature of the disease and its causes, and despite the significant new insights acquired, cancer remains one of the most common causes of death. Actually, treatment still relies for a major part on classical approaches such as surgery, radio- and chemotherapy. Clearly, novel and creative methods are needed to complement the conventional treatment options. Recently, the use of viruses as potential tools for anti-cancer therapy has gained considerable interest (29, 31). In this first exploratory study we demonstrate two important features that make coronaviruses attractive for this purpose, i.e. the ability to target these viruses to human tumor cells and their subsequent infection and eradication of these cells.

We previously showed that the non-human coronaviruses FIPV and fMHV possess a strong capacity to kill human cancer cells once they are able to enter these cells through an artificially expressed receptor (40), consistent with earlier observations with MHV by Baric and co-workers (28, 35). The observed rapid and efficient eradication of cancer cells can probably be attributed to two coronaviral features. First, coronaviruses are positive-strand RNA viruses that exhibit a fast, cytoplasmic transcription and replication process leading to rapid virus protein synthesis and progeny virus production. Second, the ability of coronaviruses to induce syncytia between infected and non-infected neighboring cells amplifies their cytotoxicity. We found that FIPV and fMHV retained these properties in human cancer cells expressing the virus receptor, thus enabling a rapid cytotoxic spread of the virus to surrounding non-infected cancer cells. Coronaviruses share some of these attractive characteristics with other enveloped viruses such as a fusogenic mutant of herpes simplex virus (17) and the live attenuated Edmonston B vaccine strain of measles virus (27). However, in contrast to these viruses the coronaviruses FIPV and fMHV are normally incapable of infecting human cells, due to their restricted tropism. Thus, their native tropism does not need to be abolished in order to specifi-

cally limit their access, hence cytotoxicity, to human cancer cells.

The ability to deliberately target viruses to preselected cells is a tremendous challenge with far-reaching implications for all kinds of therapeutic applications. Though we were able to demonstrate the principle of retargeting of coronaviruses by exchanging spike ectodomains (19, 24), neither the detailed structural information nor the knowledge and technology required to purposely redesign the spike for binding to any given antigen are presently available. As an alternative and potentially also versatile tool we therefore embarked on the development of a bispecific adapter. Thus, to redirect the non-human coronaviruses FIPV and fMHV to EGFR expressing cells we constructed the bispecific antibody molecule scFv 23F-425 that binds to both the feline spike and EGFR. The latter was chosen for its frequent overexpression on human cancer cells. Inoculation of FIPV and fMHV onto a number of different EGFR-expressing human cancer cell lines of various tissue origins in the presence of scFv 23F-425 resulted in infection, replication, and subsequent formation of syncytia. The targeted infection was completely dependent on the presence of the antibody and its efficiency generally correlated with the levels of EGFR expression on the cancer cells. These observations are similar to native coronavirus infections where virus-cell and cell-cell fusion efficiencies also correlate with host cell receptor density (28). The results imply that the bispecific antibody-mediated targeting approach can in principle be applied to direct coronaviruses to any cell surface antigen for which an appropriate antibody (i.e. hybridoma cell line) is available.

The successful application of bispecific adapters for viral tumor therapy will depend, amongst others, on cell surface antigens that are - preferably - unique to the tumor. Useful tumor specific markers have not yet been identified and future work should reveal whether they occur or can be specifically induced in tumor cells. Interestingly, for the attenuated measles virus it was recently described that receptor density could also be a determinant of preferential tumor killing (1). This indicates that even over-expression of certain receptors on tumor cells may result in tumor selective infection and subsequent cell killing. This may also be of importance for

the targeting of coronaviruses, since similar receptor density dependence has been described to be important for coronavirus infection and syncytia formation (28).

The application also depends on the sufficient, local presence of the adapter. This might be achieved by incorporating the adapter gene sequence into the viral genome to have the virus produce its own targeting device. We have demonstrated this principle recently for the targeting of conditionally replicating adenoviruses. Thus, adenoviruses expressing a bispecific scFv for targeting to EGFR showed enhanced oncolytic replication in EGFR-positive, adenovirus receptor-negative cancer cells (36). Foreign gene expression can also be achieved with coronaviruses as we showed recently after inserting different reporter genes at various positions in the MHV genome (11).

Coronaviruses exhibit high mutation rates and are prone to recombination. Their application in adapter-mediated targeting to tumors will thus raise serious safety questions, particularly regarding the possibility of generating viruses that acquired the capacity to infect human cells independent of targeting devices. These questions will have to be addressed. Several options to reduce the risks already exist. One is the use of coronaviruses lacking specific virulence genes; as we showed recently for MHV (10) and FIPV (18), such viruses are strongly attenuated in their natural host. Another option is to combine deletion of non-essential virulence genes with genomic rearrangement; reorganization of the order of the structural protein genes, found for MHV to be tolerated without loss of viability (12), will reduce the risk of generating viable viruses by recombination with circulating field viruses.

Entry of coronaviruses into cells normally requires binding of the spike to the receptor followed by a series of structural rearrangements in the S protein that eventually lead to the merging of viral and cellular membranes. It appeared that the bispecific antibody-mediated entry, as well as the antibody-mediated induction of syncytia, uses this same fusion mechanism as was illustrated most clearly by the inhibitory effect of the HR1 derived peptide. It will be interesting to find out how this fusion process actually takes place and whether, for instance, the necessary confor-

mational changes in the S protein are induced by its interaction with the antibody or triggered by the binding to the EGFR. Alternatively, fusion may not be mediated by the spikes that effect the binding but, rather, by the conformational changes induced in the "free" spikes upon interaction with undefined molecules on the target cells or by the particular conditions experienced when the virus is taken up in endosomes. Evidence for the latter mechanism might be obtained by studying whether infection can also be achieved when using a bispecific antibody not binding the virus through the S protein but through one of the other envelope proteins, M or E, as these are not involved in the fusion process.

An elegant application of the targeting principle has recently been described for another enveloped virus. In measles virus the receptor binding and membrane fusion functions are divided over two different envelope proteins, the heamagglutinin (H) and the fusion (F) protein, respectively. When a scFv was carboxy-terminally appended to the type II membrane glycoprotein H, the virus was successfully targeted to cells expressing the distinguishing receptor (4, 27). Obviously, appending the EGFR binding moiety of scFv 23F-425 to the amino terminus of the coronavirus S protein might also expand the targeting possibilities of these viruses.

ACKNOWLEDGEMENTS

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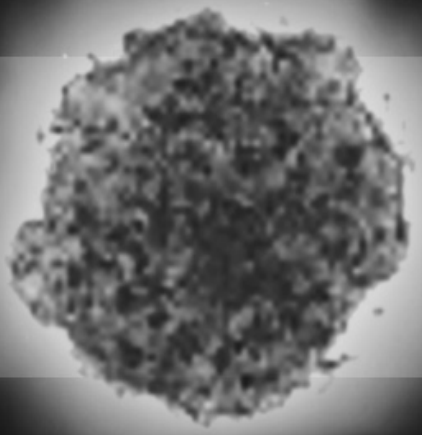
CHAPTER 4 | SOLUBLE RECEPTOR-MEDIATED TARGETING OF MOUSE HEPATITIS CORONAVIRUS TO THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR



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ABSTRACT

The mouse hepatitis coronavirus (MHV) infects murine cells by binding of its spike (S) protein to murine CEACAM1a. The N-terminal part of this cellular receptor (soR) is sufficient for S binding and for subsequent induction of the conformational changes required for virus-cell membrane fusion. Here we analyzed whether these characteristics can be used to redirect MHV to human cancer cells. To this end, the soR domain was coupled to single-chain antibody 425, which is directed against the human epidermal growth factor receptor (EGFR), resulting in a bispecific adapter protein (soR-425). SoR and soR-425 proteins, both produced using the vaccinia virus system, were able to neutralize MHV infection of murine LR7 cells. However, only soR-425 was able to target MHV to human EGFR-expressing cancer cells. Interestingly, the targeted infections induced syncytia formation. Furthermore, the soR-425-mediated infections were blocked by heptad repeat mimicking peptides, indicating that virus entry required the regular S protein fusion process. We conclude that the specific spike binding property of the CEACAM1a N-terminal fragment can be exploited to direct the virus to selected cells, by linking it to a moiety able to bind a receptor on those cells. The approach might be useful in the development of tumor-targeted coronaviruses.

INTRODUCTION

The search for additional cancer therapies continues as many different non-conventional approaches are being explored. The use of viruses in the fight against cancer started already in the 1950s and several oncolytic vectors are now being evaluated in clinical trials (28, 30). The mouse hepatitis coronavirus (MHV) possesses several attractive features to consider its use as an oncolytic virus. These include fast replication, syncytia formation and cell killing, as well as species specificity. However, in order to exploit the oncolytic potential, the ability to redirect the virus to human cancer cells is a prerequisite. In order to achieve tumor-specific virus entry, different targeting approaches have been explored for a variety of viruses. These include pseudotyping, modification of viral surface proteins, and the use of bispecific adapters (14, 28, 37).

MHV belongs to the *Coronaviridae*, a family of large (approximately 30 kb) positive strand RNA viruses. MHV displays high species specificity, determined by the interaction between the virus spike (S) protein and the virus receptor (3). The S protein is a type I glycoprotein, synthesized as a 180 kDa glycosylated precursor that is post-translationally cleaved into two 90-kDa subunits, S1 and S2 (13). The spike N-terminal S1 subunit is responsible for binding to the MHV receptor, which results in a conformational change of the S2 subunit, mediating the fusion of the virus envelope and the cell membrane. Key events in this fusion process are the insertion of an as yet unidentified fusion peptide present in the S protein into the cell membrane, and the coalescence of the heptad repeat regions, HR1 and HR2, into a tightly associated six-helix bundle (2, 31).

MHV infects murine cells via receptors belonging to the carcinoembryonic antigen family of glycoproteins in the immunoglobulin (Ig) superfamily, called murine CEACAMs (1, 7, 8, 38). Results from infection studies in mice have shown that mCEACAM1a is the major receptor for MHV strain A59 (18). Through analysis of recombinant forms of CEACAM1a it was established that the N-terminal domain is

responsible for virus binding (9, 36). Crystallization studies revealed that this domain resembles an immunoglobulin-like fold and identified the amino acids responsible for interaction with the S protein (33). Furthermore, it was reported that incubation of virions with anchorless soluble receptor proteins caused a marked increase in the hydrophobicity of the virions, which was associated with a conformational change in S2 and neutralization of infection of murine cells (24, 34, 40).

The aim of our studies is to investigate the possibilities of using animal coronaviruses as oncolytic vectors for human use. This requires the specific targeting of the viruses to antigens (over)expressed on tumor cells. Our approach is to engineer bifunctional adapters of which, when functioning, the sequence will be incorporated genomically into the virus to create a self-targeted vector. In a previous study we showed that the feline infectious peritonitis coronavirus (FIPV) could be redirected to human cancer cells expressing the epidermal growth factor receptor (EGFR) via a bispecific single-chain antibody capable of binding simultaneously to the FIPV spike protein and to the human EGFR. The same adapter was also able to target fMHV, an MHV-derivative carrying the FIPV spike protein, to the EGFR (39). Unfortunately, subsequent attempts to genomically incorporate the adapter sequence failed; the virus appeared to be unstable and rapidly lost the inserted sequence (unpublished observations). In the present study we therefore took a fundamentally different approach by designing an adapter of a different nature. We hypothesized that the N-terminal domain of CEACAM1a (soR), when fused to a cancer cell binding ligand, could also be used as a bridging adapter. To demonstrate this principle, the soR part was fused to the single-chain antibody 425 directed against the epidermal growth factor receptor (EGFR), and analyzed for its capacity to mediate EGFR specific entry of MHV into human cancer cells. The results show that, although the binding of the soR moiety to the S protein presumably triggers in the viral spike the conformational changes that normally initiate the membrane fusion process, these events do not inactivate the particles but allow the fusion process to ensue as soon as the virus-adapter complex has docked to the cell receptor.

MATERIALS AND METHODS

Viruses, cells, and antibodies.

Stocks of MHV-EFLM (5) and fMHV (21) were produced and titrated as before; MHV-EFLM is a derivative of MHV strain A59 that contains a firefly luciferase gene between the E and M genes (5). The recombinant vaccinia virus vTF7-3 containing the bacteriophage T7 RNA polymerase gene was used as a T7 RNA polymerase source for the T7 promoter driven production of the soR adapter proteins in OST7-1 cells (10). OST7-1 (10) (obtained from B. Moss), BHK-21 (ATCC), A431 (ATCC), and LR7 (21) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, Verviers, Belgium) containing 10 % fetal bovine serum (FBS), 100 IU of penicillin/ml, and 100 µg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). The rabbit antiserum k134 raised against purified MHV was described previously (29). The polyclonal antibody anti-N-CEACAM-Fc, directed against the mCEACAM1a N domain was kindly provided by T. Gallagher (15). The anti-Myc antibody 9E10 (culture supernatant from the hybridoma cell line 9E10, ATCC) was used to detect Myc-tagged adapter proteins.

Construction of the soR adapters.

In order to obtain the gene encoding the N-terminal domain of the mCEACAM1a receptor (further denoted as soR), a PCR was performed on plasmid pCEP4:sMHVR-Ig (kindly provided by T. Gallagher (15)), using forward primer 2296 (5'-catgggcccagccggccgagctggcctcagcacat-3') and reverse primer 2297 (5'-catg-gcggccgcggggtgtacatgaaatcg-3'). In addition, PCRs were performed on the same plasmid using primers 2296 and 2298 (5'-tgtcacaagatttgggctggggtgtacatgaaatcg-3'), 2296 and 2299 (5'-cggtgggcatgtgtgagtttt-gtcacaagatttg-3'), and 2296 and 2300 (5'-catggcggccgctgggcacggtgggcatgtgt-3') to generate a similar soR fragment with a 3'-extension encoding a hinge linker region derived from human IgG1 (15). The resulting DNA fragments soR (429 nt) and soR-h (472 nt) contained a 5' *Sfi*I site and a 3' *Not*I site (underlined in the primers), and were subsequently cloned using these restriction enzymes into the expression vector pSecTag2 (Invitrogen, Breda, The Netherlands). The resulting expression vectors pSTsoR and pSTsoR-h encode the N domain of mCEACAM1a in fusion with an amino-terminal Igκ signal sequence and a carboxy-terminal myc- and his-tag under the control of a CMV and a T7 promoter. The hinge region in pSTsoR-h is located directly downstream of the N domain of mCEACAM1a. The single-chain antibody 425, directed against EGFR, was isolated from pSTCFS11-425 (17, 26) by *Not*I digestion and ligated in a V_H-V_L configuration into the *Not*I site downstream of the soR and soR-h sequences in pSTsoR and pSTsoR-h creating a 3 Ala linker between the soR and single-chain fragments, resulting in the expression vectors pSTsoR-425 and pSTsoR-h-425. The composition of the adapter genes was confirmed by sequencing.

Production and analysis of the soR constructs.

For the production of the soR adapter proteins, subconfluent monolayers of OST7-1 cells were inoculated at a multiplicity of infection (MOI) of 5 with vTF7-3 (t = 0 h) and transfected (t = 1 h) with pST-soR, pST-soR-h, pST-soR-425, pST-soR-h-425, or were mock transfected, by using lipofectin (Life Technologies, Ltd., Paisley, United Kingdom). The medium was refreshed at t = 4.5 h, harvested at t = 20 h, and centrifuged for 10' at 3,000 rpm to clear it from cell debris. The supernatants containing the soR proteins

were loaded onto a 20 % sucrose cushion and centrifuged for 30' at 13,000 rpm to clear them from vTF7-3 virus prior to 20-fold concentration using Vivaspin columns. The protein batches were aliquoted, and stored at - 20 °C.

Western blot assay.

The concentrated soR adapter protein preparations were analyzed by SDS-PAGE and subsequent blotting on a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). To block aspecific antibody binding the blot was incubated in blocking buffer (PBS containing 5 % Protivar and 0.05 % Tween-20) for 30 min at room temperature. Incubation was continued using anti-N-CEACAM-Fc antibodies diluted 1:3000 in blocking buffer for 1 h at room temperature, followed by Swine-anti-Rabbit peroxidase (DAKO, Glostrup, Denmark) diluted 1:3000 in blocking buffer for 1 h at room temperature. Finally the blot was incubated with a 1:1 mixture of luminal and enhancer (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), and analyzed using Hyperfilms (Amersham Pharmacia Biotech Europe GmbH), according to the manufacturer's protocol.

Anti-N-CEACAM-Fc dot blot assay.

The concentration of the soR proteins in our concentrated preparations was determined by dot blot analysis. Serial dilutions in a total volume of 50 µl were blotted onto a nitrocellulose membrane using a vacuum pump. A standard curve of purified N-CEACAM-Fc protein (100 nM) was prepared in parallel to estimate the concentration of the soR adapter proteins in our preparations. The immunoassay using antibodies directed against N-CEACAM-Fc was performed as described above for the Western blot. The density x mm^2 of each dot was measured using a densitometer (Bio-Rad Laboratories), and plotted against the corresponding dilution factor after which the soR protein concentrations were calculated. The different preparations were brought to the same concentration with culture medium before use.

Determination of luciferase expression.

At the indicated time points the culture media were removed and the cells were lysed using the appropriate buffer provided with the firefly assay system (Promega). Intracellular luciferase expression was measured according to the manufacturer's instructions, and the relative light units (RLUs) were determined with a LUMAC biocounter M2500.

Anti-MHV immunostaining .

Cells inoculated with MHV or fMHV in the presence or absence of adapter protein were fixed using PBS containing 3.7 % paraformaldehyde, permeabilized with PBS containing 1 % Triton-X100, and subsequently incubated with K134 anti-MHV serum diluted 1:300, followed by Swine-anti-Rabbit peroxidase (DAKO, Glostrup, Denmark) diluted 1:300, both in PBS containing 5 % FBS. The cells were stained using AEC (Brunschwig, Amsterdam, the Netherlands) according to the manufacturer's protocol, and analyzed by light microscopy.

Antibody blocking experiments.

To determine whether soR-425 and soR-h-425 interact with the EGFR, 1 x 10^5 A431 cells per 0.32-cm^2 well were incubated with or without 100 µl hybridoma supernatant containing MAb 425, for 30 min at 4 °C. Next, the cells were inoculated for 1 h at 37 °C with 1 x 10^4 $TCID_{50}$ units of MHV-EFLM preincubated for 1 h at 4 °C with 0.5 nM soR-425 or soR-h-425. The cells were washed and incubation was continued for 20 h, after which they were lysed and luciferase expression was measured.

Analysis of the effect of mHR2 on coronavirus infection.

The effect of the fusion-inhibitory peptide mHR2 on the targeted infection process was analyzed by preparing in parallel three sets of inoculation mixtures. An amount of 1 x 10^4 $TCID_{50}$ units MHV-EFLM was preincubated in duplo with 5 nM soR-425, soR-h-425, or mock supernatant for 1 h at 4 °C. To one of each set mHR2 peptide had been added to a concentration of 20 µM. Cultures of 1 x 10^5 human A431 cells were washed with PBS and inoculated with the infection mixes for 1 h at 37 °C. The cells were then washed and incubation continued for 20 h. Finally, the cells were lysed and luciferase activity was measured.

Monitoring of productive infection.

An amount of 5 x 10^5 A431 cells per 10-cm^2 well was seeded and inoculated the next day with 2.5 x 10^6 $TCID_{50}$ units MHV-EFLM in the presence or absence of adapter protein for 1 h. The cells were washed three times with PBS, and cultured for up to 48 h. At several time points after inoculation a small sample of the medium was harvested, centrifuged for 10 min at 3,000 rpm and stored at – 80 °C until analysis. The amount of virus produced at each time point after inoculation was determined by endpoint dilution on murine LR7 cells.

Measurement of soR-425-mediated cell-cell fusion.

5 x 10^4 A431 cells per 0.32-cm^2 well were inoculated for 2 h with 5 x 10^4 $TCID_{50}$ units MHV-EFLM preincubated with 5 nM soR-425 for 1 h at 4 °C. The cells were washed 3 times with PBS and incubated further in the presence or absence of 5 nM soR-425, soR-h-425, or mock control supernatant. An immunostaining was performed at 24 h after inoculation, and the number of nuclei per syncytium was determined by counting under the light microscope.

RESULTS

Construction and analysis of the soR adapter proteins.

In order to redirect MHV to receptors overexpressed on human cancer cells, different adapter proteins were designed composed of the N domain of CEACAM1a (soR) fused to the anti-EGFR single-chain antibody 425 (soR-425). In addition, adapter proteins were constructed in which an IgG Fc hinge region - responsible for disulfide linkage of IgG heavy chains - was present between the soR and the single-chain 425 (soR-h-425). Similar constructs composed of the CEACAM1a N domain only, named soR and soR-h, functioned as control proteins for the soR-425 and soR-h-425 adapters (Fig. 1). The proteins were expressed in OST7-1 cells by vTF7-3 infection and subsequent transfection with the expression constructs. Their expression was confirmed by immunostaining of the cells with anti-Myc and anti-N-CEACAM-Fc antibodies (data not shown). Next, the release of the soluble receptor proteins into the culture medium was demonstrated by Western blot (Fig. 2A, left panel). The apparent molecular masses of soR, soR-h, soR-425, and soR-h-425 were slightly larger than calculated (18.9, 20.6, 46.0, and 47.6 kDa, respectively), due to their glycosylation (data not shown). To determine whether the presence of a hinge region resulted in protein dimerization, the proteins were analyzed by SDS-PAGE under non-reducing conditions, followed by Western blotting using anti-N-CEACAM-Fc antibodies (Fig. 2A, right panel). Dimerization was indeed observed for soR-h and soR-h-425, but not for soR and soR-425, which lack the hinge region. Dimerization was, however, not complete as also monomers of the hinge-containing proteins were detected. The different soR adapter batches were titrated using the anti-N-CEACAM-Fc dot blot assay and diluted to the same concentration of anti-N-CEACAM antibody-reacting protein (Fig. 2B, left panel). The concentration of the soR adapter batches as used was between 25-50 nM, as determined by comparing to a standard of N-CEACAM-Fc protein (Fig. 2B, right panel).

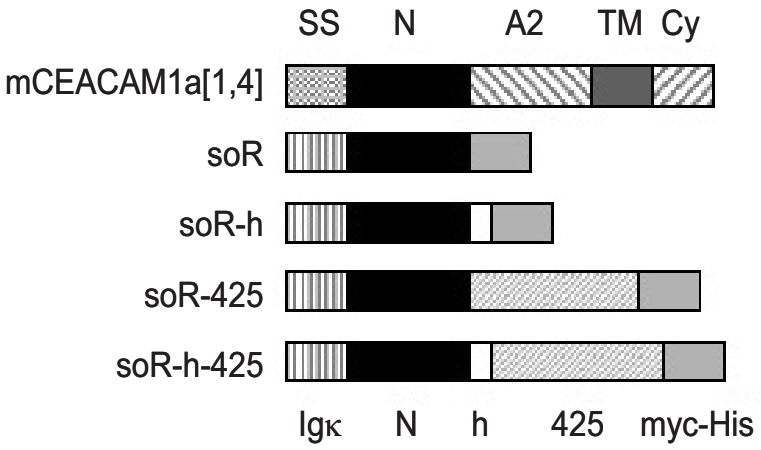

Fig. 1. Schematic diagram of the soR constructs. The native mCEACAM1a[1,4] is composed of two ectodomains (N and A2) and contains a signal sequence (SS). The N and A2 domains are linked to the transmembrane (TM) and cytoplasmic (Cy) domain. The soluble receptor constructs are depicted below mCEACAM1a[1,4]; (soR) CEACAM1a N domain, (h) Ig hinge region, (425) single-chain antibody 425. The different soR proteins contain an Igκ leader sequence for secretion, and a myc-His tag for detection.

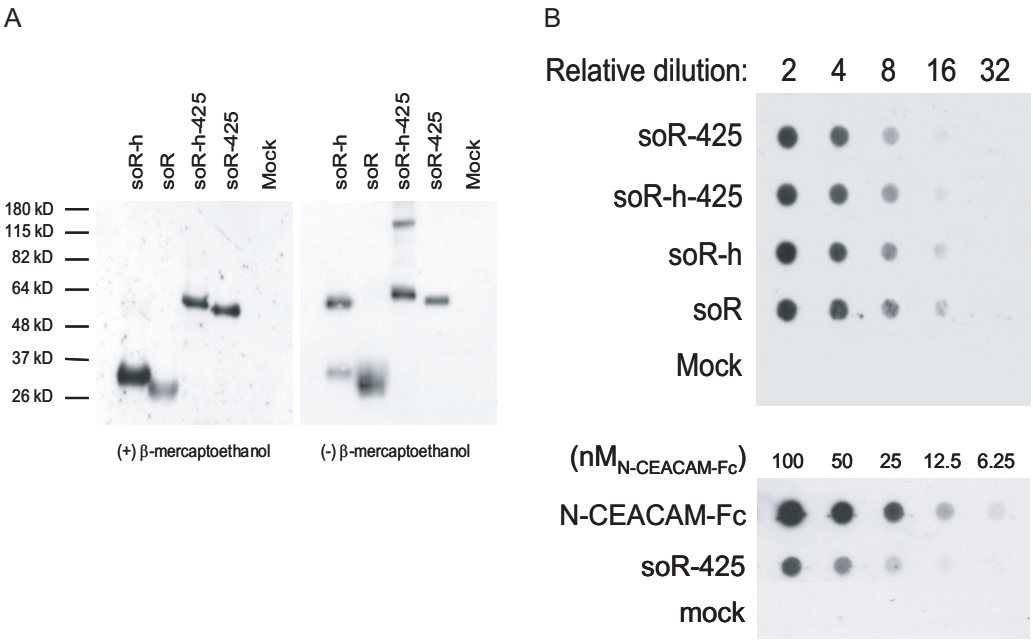

Fig. 2. Immunoblot analysis of the soR adapter proteins. (A) Western blot analysis of the soR adapter proteins in the presence (left panel) and absence of β-mercaptoethanol (right panel) (B) Anti-N-CEACAM-Fc dot blot analysis was used to equalize the soR protein concentrations in the different batches. Two-fold dilutions were made and analyzed for their reactivity with anti-N-CEACAM-Fc antibody. In addition, the adapter concentration in the soR preparation was determined by an anti-N-CEACAM-Fc dot blot assay in which a standard curve of purified N-CEACAM-Fc protein (100 nM) was used to estimate the concentration of the soR adapter batches, as shown here for soR-425.

Soluble receptor-mediated neutralization of MHV infection on murine cells.

Various forms of the CEACAM1a ectodomains can neutralize MHV by binding to the MHV spike protein (24, 34, 40). To evaluate the binding of the soR proteins to the virus, neutralization experiments were carried out. MHV-EFLM (5), a recombinant MHV-A59 containing a firefly luciferase reporter gene, was preincubated with various amounts of soR or its derivatives. After inoculation for 1 h the LR7 cells were washed and incubation continued for 7 h, after which luciferase activity was measured (Fig. 3). All adapter proteins appeared to have neutralized viral infectivity, as opposed to infections carried out in the presence of mock control supernatant. Strikingly, the presence of a hinge region in the proteins resulted in an increased neutralizing activity as compared to the proteins lacking this region. Moreover, the fusion of single-chain antibody 425 to the soR domain resulted in decreased neutralization efficiency compared to soR alone. This effect, however, was completely normalized to soR neutralization levels by the presence of the hinge region in soR-h-425.

Soluble receptor-mediated MHV infection of cells expressing human EGFR.

To determine whether MHV can be redirected via the N domain of mCEACAM1a to cells lacking the MHV receptor, MHV-EFLM was preincubated with 0.5 nM soR-425, or with mock supernatant. The viruses were inoculated for 1 h onto EGFR overexpressing human A431 cancer cells, after which the cells were washed and incubated further. At several time points after inoculation, intracellular luciferase expression was monitored to determine whether MHV infection was taking place. As shown in Fig. 4A, significant luciferase expression became measurable at 9 h after inoculation and reached a maximum at 20 h after inoculation, indicating that MHV-EFLM had successfully entered the human cancer cells in the presence of soR-425. That this infection was dependent on the activity of the adapter protein was evident from the lack of luciferase expression in its absence.

Next the efficiencies of the targeted infections established by soR-425 and soR-h-425 were compared. To this end, MHV-EFLM was inoculated for 1 h on human A431 cells in the presence of different amounts of the soR adapter proteins. The cells were washed and incubation was continued under normal culture medium. After 20 h the cells were lysed and luciferase expression was measured. Fig. 4B shows that the expression of luciferase was dependent on the dose of soR-425 or soR-h-425. In contrast, MHV-EFLM preincubated with soR, soR-h, or with mock supernatant did not induce detectable luciferase expression (not shown). Interestingly, whereas the presence of the hinge region appeared to enhance the efficiency of virus neutralization by the adapter protein (Fig. 3), it resulted in a significant, approximately 10-fold decrease in targeting efficiency.

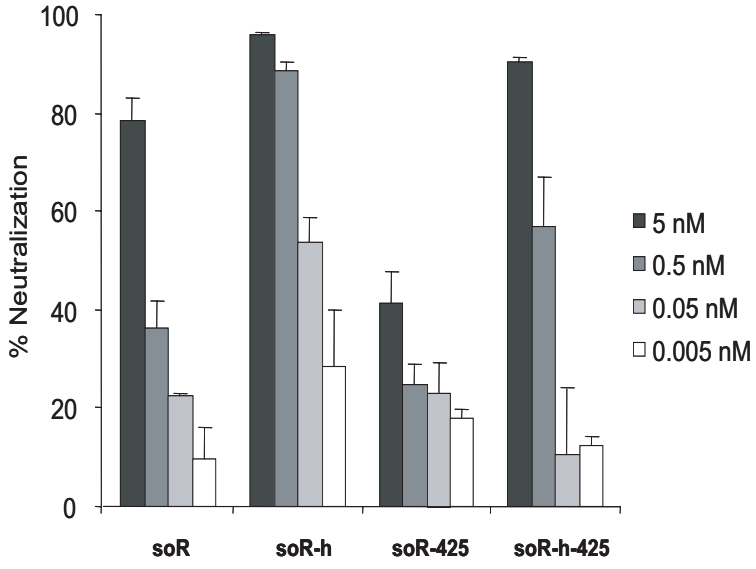

Fig. 3. Virus neutralization by the soR adapter proteins. MHV-EFLM (MOI 0.1) was preincubated for 1 h at 4°C with various concentrations of the different soR adapter proteins, and inoculated onto 1 x 10^5 murine LR7 cells for 1 h. The cells were washed, incubation continued, and after 7 h luciferase expression was measured. Shown is the percentage neutralization, calculated as the decrease in luciferase expression relative to infections in the absence of adapter protein. The data represent the average of a representative experiment performed in triplicate. The error bars indicate the standard deviation.

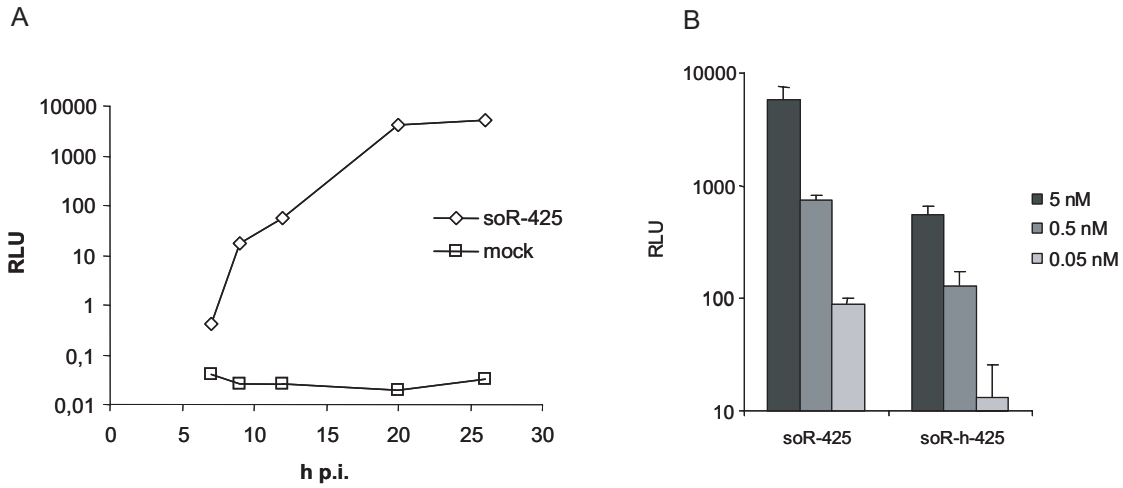

Fig. 4. Targeting of MHV-EFLM to human A431 cells. (A) To determine whether MHV-EFLM was able to infect human A431 cells, 1 x 10^4 $TCID_{50}$ units of MHV-EFLM were preincubated in the presence or absence of 0.5 nM soR-425. After inoculation onto 1 x 10^5 A431 cells for 1 h at 4°C, the cells were washed and incubated further. At several time points after inoculation intracellular luciferase expression was measured. Shown are the data from a representative experiment. (B) MHV-EFLM (1 x 10^4 $TCID_{50}$ units) was preincubated with the different soR adapter proteins at various concentrations, and inoculated onto A431 cells for 1 h. At 20 h after inoculation the luciferase expression was determined. All data shown represent the average and standard deviation of an experiment performed in triplicate.

Soluble receptor-mediated MHV neutralization, but not targeting, is affected by soR dimer formation.

To further investigate the effect of the hinge region in the soR proteins on MHV neutralization and targeting, we disrupted the soR-h and soR-h-425 dimers by treatment with a minimal concentration of DTT required for complete reduction of the hinge disulfide bonds. Thus, all soR proteins were treated with 1.56 µM DTT for 20' at 4 ºC. Dimer dissociation was confirmed by Western blot analysis performed under non-reducing conditions (Fig. 5A). Incubation of soR-h and soR-h-425 in the presence of DTT resulted in full reduction to monomers, whereas in the absence of DTT protein bands corresponding to the size of dimers were detected. No effects of the DTT treatment on soR and soR-425 migration were observed.

Next we analyzed whether the reduced soR-h and soR-h-425 proteins displayed similar neutralization and targeting efficiencies as their counterparts

lacking the hinge region. MHV-EFLM was inoculated onto murine LR7 cells in the presence of the different soR proteins, each pre-treated in the presence or absence of DTT. After 7 h of incubation, the cells were lysed and luciferase expression was measured. As shown in Fig. 5B, after reduction the soR-h and soR-h-425 proteins lost their enhanced neutralization capacities relative to the soR and soR-425 proteins, which both lack the hinge dimerization region. These results indicate that dimerization of the soR proteins has a beneficial effect on MHV neutralization. We also determined the effect of reduction of the soR-h-425 dimers on MHV targeting. MHV-EFLM was inoculated onto A431 cells in the presence of 5 nM of soR-425 or soR-h-425 protein, both pre-treated in the presence or absence of DTT. After 1 h the

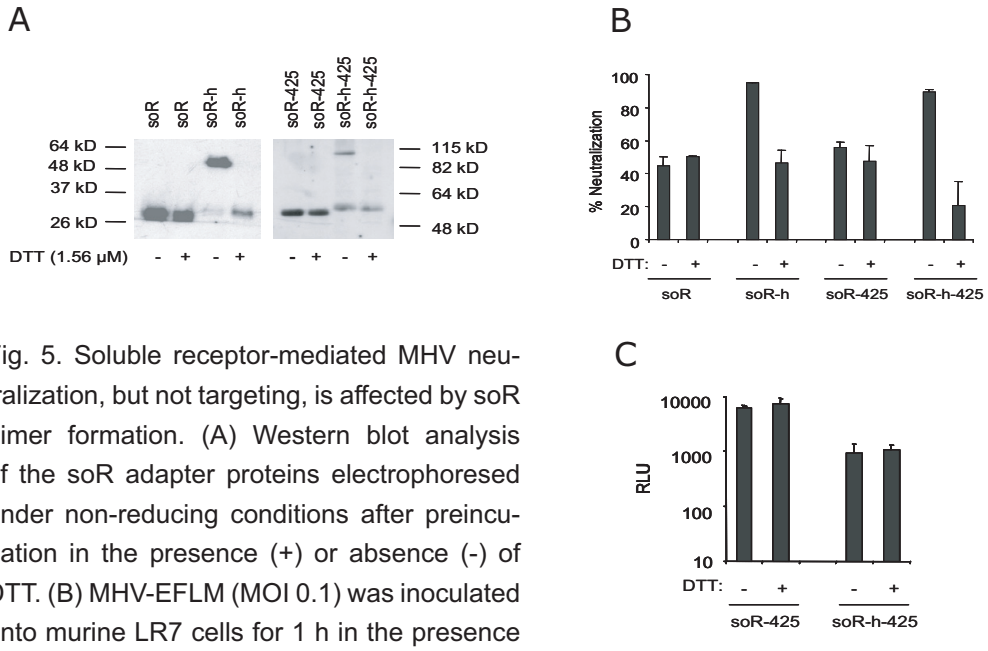

Fig. 5. Soluble receptor-mediated MHV neutralization, but not targeting, is affected by soR dimer formation. (A) Western blot analysis of the soR adapter proteins electrophoresed under non-reducing conditions after preincubation in the presence (+) or absence (-) of DTT. (B) MHV-EFLM (MOI 0.1) was inoculated onto murine LR7 cells for 1 h in the presence or absence of various amounts of the different soR adapter proteins, pre-treated in the presence (+) or absence (-) of DTT. At 7 h after inoculation the luciferase expression was determined. Shown is the percentage neutralization, calculated as the decrease in luciferase expression relative to infections in the absence of adapter proteins. The data represent the average of a representative experiment performed in triplicate. The error bars indicate the standard deviation. (C) An amount of 1 x 10^4 $TCID_{50}$ units of MHV-EFLM was inoculated onto 1 x 10^5 A431 cells in the presence of 5 nM of soR-425 or soR-h-425 protein, both pre-treated in the presence (+) or absence (-) of 1.56 µM DTT. After 1 h the cells were washed and incubation was continued for 20 h, after which the cells were lysed and luciferase expression measured. All data shown represent the average and standard deviation of an experiment performed in triplicate.

cells were washed and incubation was continued for 20 h, after which the cells were lysed and luciferase expression measured. Interestingly, whereas dimer reduction had a profound effect on the neutralization capacity of soR-h-425, no significant DTT-mediated effects on the targeting efficiency were observed (Fig. 5C).

Soluble receptor-mediated coronavirus infection is EGFR and S protein specific.

To further confirm that the infection of MHV-EFLM established by soR-425 or soR-h-425 was indeed mediated by the EGFR expressed on A431 cells, we studied the effect of the anti-EGFR MAb 425. A431 cells were incubated with the antibody prior to inoculation with MHV-EFLM in the presence of 0.5 nM soR-425 or soR-h-425 for 1 h. The cells were then washed and incubation continued for 20 h. Fig. 6A shows that infection was blocked almost completely by MAb 425, confirming that a direct interaction with the EGFR is required for adapter-mediated infection.

To verify that the interaction of the soR adapters with MHV was S protein specific, MHV-EFLM or fMHV was preincubated with the soR-425 or soR-h-425 proteins and inoculated onto A431 cells. The chimeric virus fMHV carries S proteins the ectodomain of which is derived from the feline infectious peritonitis virus (FIPV). It infects cells through the feline aminopeptidase N molecule rather than using murine CEACAM1a (21). An immunostaining for coronavirus proteins was performed at 20 h after inoculation to determine whether the A431 cells had been infected. Only the cells inoculated with MHV-EFLM stained positive, whereas cells inoculated with fMHV had remained negative (Fig. 6B). These results demonstrated that the MHV spike ectodomain is essential for CEACAM1a N domain-mediated infection. In addition, the immunostaining revealed the presence of large spots of fused cells, typical for MHV infections of murine cells. The results collectively indicate that the targeted infections of MHV in the presence of soR-425 or soR-h-425 are dependent on interactions of the adapter proteins both with the MHV spike and with human EGFR.

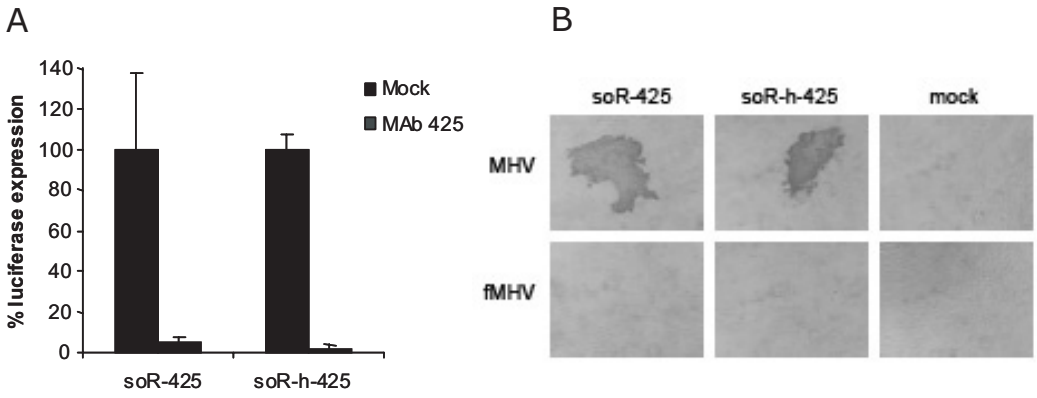

Fig. 6. EGFR and spike specific coronavirus targeting. (A) MHV-EFLM was preincubated with soR-425 or soR-h-425, and inoculated onto A431 cells preincubated for 1 h at 4 °C in the presence or absence of anti-EGFR MAb 425. The data represent the average luciferase expression relative to cells inoculated in the absence of blocking antibody. The error bars show the standard deviation of an experiment performed in triplicate. (B) The specificity of the interaction of the soR adapters with the MHV spike was determined by preincubating 1 x 10^4 $TCID_{50}$ units of MHV, or fMHV, in the presence or absence 0.5 nM of the soR adapters. After 1 h the inoculum was replaced by normal culture medium and incubation continued for 20 h. Polyclonal MHV antibodies were used to detect the presence of coronavirus proteins in the A431 cells at 20 h after inoculation. Representative images are shown.

Targeted coronavirus infections require S protein mediated membrane fusion.

Addition of a heptad repeat-mimicking peptide, mHR2, during inoculation of murine LR7 cells abrogates MHV infection by inhibition of the coalescence of the heptad repeat regions, HR1 and HR2, required for fusion (2). In order to investigate whether the targeted MHV infections also depend on those conformational rearrangements in the viral spike protein we tested their sensitivity to the mHR2 peptide. To this end, MHV-EFLM was targeted towards EGFR expressed on A431 cells using 5 nM of adapter proteins soR-425 and soR-h-425, in the presence or absence of 20 μM mHR2. As can be seen in Fig. 7, addition of the peptide efficiently blocked both soR-425 and soR-h-425-mediated infection. Thus, the EGFR-mediated MHV infections appear to depend on conformational changes in the S protein, similar to the ones occurring during normal infection.

Soluble receptor-mediated syncytia formation and production of progeny virus.

Interestingly, A431 cells infected by EGFR-targeted MHV showed cell-cell fusion typical for coronaviruses (Fig. 6B). To investigate whether syncytium formation between infected and neighboring cells resulted from undefined interactions or from specific bridging by the adapter protein, A431 cells were inoculated with MHV-EFLM in 5 nM soR-425 containing medium for 2 h. The cells were washed and subsequently cultured for another 22 h in the presence or absence of 5 nM soR-425 or soR. Thus, soR-425 was either present continuously to mediate both infection and syncytium formation or only briefly to mediate the targeted infection. Removal of soR-425 after 2 h did not have a significant effect on the number of syncytia observed for each inoculated cell culture (data not shown); however, we did observe a reduction of the extent of syncytium formation by approximately 3-fold (Fig. 8A). The addition of soR proteins, which lack the scFv 425 domain, did not result in increased cell-cell fusion, indicating an EGFR-related effect. Hence, soR-425 may not only promote fusion between the MHV envelope and target cells during virus entry, but also between MHV-infected and neighboring cells in the course of the infection process.

Finally we determined whether the soR-425-mediated infection was productive and yielded infectious virus. A431 cells were inoculated for 1 h with MHV-EFLM (MOI 5) that had been preincubated in the presence or absence of 5 nM soR-425 or soR-h-425. They were then washed and incubated further during which samples were taken from the culture medium at different time points after inoculation for titration on murine LR7 cells. As depicted in Fig. 8B, virus preincubated in the presence of soR-425 or soR-h-425 produced progeny virus reaching titers up to 10^5-10^6 $TCID_{50}$ units/ml. In contrast, inoculation with MHV-EFLM in the absence of adapter protein did not lead to detectable virus production. Thus, the adapter-mediated redirection of MHV to human cells results in the formation and secretion of progeny virus.

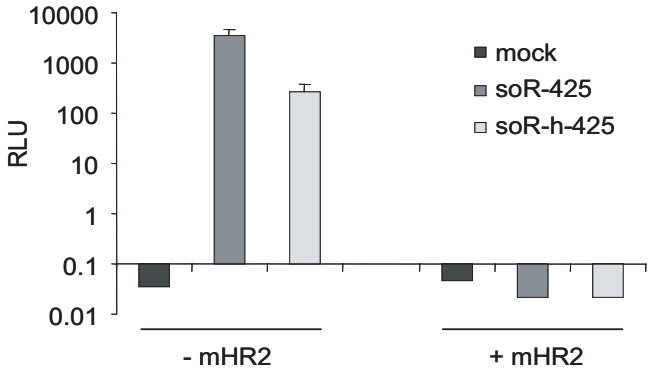

Fig. 7. Effect of mHR2 on the bispecific adapter-mediated entry process. A431 cells were inoculated with a preincubated mixture of MHV-EFLM (1 x 10^4 $TCID_{50}$ units) and 5 nM soR-425 or soR-h-425 in the presence or absence of 20 µM mHR2. After 1 h the cells were washed and incubation continued for 20 h. Finally, the cells were lysed and luciferase activity was measured. The error bars show the standard deviation of an experiment performed in triplicate.

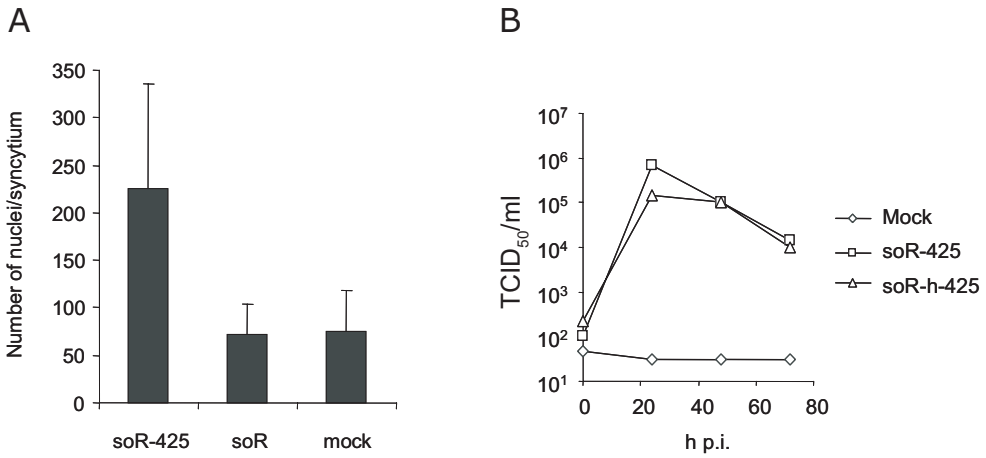

Fig. 8. Soluble receptor-mediated syncytia formation and production of progeny virus after targeted infection. (A) A431 cells were inoculated with MHV-EFLM in soR-425 containing medium for 2 h and subsequently cultured for another 22 h in the presence or absence of soR-425, or soR. The cells were fixed and stained for the presence of coronavirus proteins using polyclonal MHV antibodies. The number of nuclei of 8 randomly selected syncytia per inoculated, stained cell culture was counted under the light microscope and this was carried out in parallel in triplicate. The data represent the average number of nuclei per syncytium based in each case on the counting of 24 syncytia. The error bars show the standard deviation of such an experiment. (B) MHV-EFLM was preincubated in the presence or absence of soR-425, or soR-h-425, and inoculated onto A431 cells. After 1 h the cells were washed and incubation was continued. At several time points thereafter, samples were taken from the cell culture medium and subsequently titrated on murine LR7 cells to determine the amount of virus produced. The results shown are from a representative experiment.

DISCUSSION

Recently, the use of viruses as potential tools to complement the conventional treatment options for cancer therapy has gained considerable interest (28, 30). In this study we demonstrate that the mouse coronavirus MHV may be an attractive candidate for this purpose. MHV has a narrow species tropism and a strong capacity to kill cells. These features might be used to advantage by the controlled targeting of the virus to human tumor cells. Using the specificity of the mCEACAM1a MHV receptor we constructed bispecific adapter molecules composed of the S protein binding fragment of this receptor coupled to an antibody fragment directed to the human EGF receptor, known to be overexpressed on cells of many human cancers. The adapter proteins were found to effectively mediate infection of EGFR expressing human A431 cancer cells. Cell entry appeared to be effected through the normal spike protein-driven membrane fusion mechanism and the infection resulted in the efficient formation of syncytia, which we observed earlier to ultimately lead to the efficient eradication of the culture (39).

We have shown recently that targeted entry of the feline coronavirus FIPV and the chimeric fMHV could also be accomplished by making use of an adapter composed of two single-chain antibodies, one directed against the feline viral spike, the other to the EGFR (39). Like the soR-425-mediated MHV infections, the bispecific antibody-mediated fMHV infections were blocked by an HR-mimicking peptide derived in this case from the feline S protein (39). These observations suggest that binding of the adaptors to particular regions of the S protein - i.e. the receptor-binding domain or a certain epitope - results in conformational changes that, upon binding of the virus-adapter complex to a cellular target receptor, facilitate the insertion of the fusion peptide into the cell membrane. Binding of these adapters apparently does not inactivate the virus, as is also indicated by studies in which MHV, purified by sedimentation through a sucrose cushion after preincubation of the virus with the soR-425 adapter protein, had maintained its infectivity for EGFR

positive cells (our unpublished data). These results suggest that the adapter proteins, once bound to the virus, may freeze the S protein in a stable pre-fusion conformation ready to proceed its structural rearrangements upon contacting the target cell. What the actual trigger is that activates the fusion process at this stage is unknown, but it is quite likely that the insertion of the fusion peptide into the cellular membrane is a critical event.

An interesting paradox that we noticed in our studies was the opposite efficiency of hinge-containing and hinge-less adapter molecules in neutralization and targeting. Thus, the virus neutralizing capacities of the adapters soR-h and soR-h-425 were significantly higher than those of the soR and soR-425 molecules, respectively, while the reverse was true for the relative targeting efficiencies of soR-h-425 and soR-425 (Fig. 3-5). The reasons for these differences appear to be unrelated. This can be concluded from the differential effects of reducing agent treatment of the adapters on the two activities. Whereas this treatment did not affect the targeting efficiencies of the adapters it reduced the virus neutralizing capacities of the hinge-containing adapters to about the level of their hinge-less counterparts, the activity of which did not significantly change by the treatment. Dimeric forms of the adapters apparently neutralize the infectivity of the virus more efficiently than monomers, possibly by their stronger interaction with the spikes or their ability to cross-link spikes on the viral particle. Remarkable as it may seem, these effects apparently have no bearing on the efficiency of the targeting of such viral particles or on the subsequent steps leading to membrane fusion.

Previous studies have shown that the N domain of the mCEACAM1a receptor is sufficient by itself to induce conformational changes in the MHV spike protein (22, 24, 34, 40). Remarkably, however, this same N domain when linked directly to the transmembrane domain, thus lacking the intermediate CEACAM1a A2 domain (Fig. 1), failed to function as a receptor for MHV (9). A possible explanation for this observation might be that the functional structure of the N domain is affected by linking it to the transmembrane region. This appeared, however, not to be the case

when the N domain was replacing the corresponding domain in the mouse poliovirus receptor homolog (25), which also belongs to the family of immunoglobulin-like viral receptors (23). The resulting chimeric receptor molecule functioned as a receptor for MHV (6). It is conceivable that the CEACAM1a N domain needs a certain distance from the cellular membrane to allow interaction with the S protein to lead to effective membrane fusion. Interestingly, the efficiency of the targeted infections described here was significantly higher with the soR-425 than with the soR-h-425 adapter, and this difference was not accounted for by the dimeric state of a fraction of the latter, as the targeting efficiency of the hinge-containing adapter was unaffected by dissociation through DTT treatment. These observations indeed suggest that also the dimensions of our adapters may affect the efficiency of their targeting. Whether this is really the case, and how, remains to be investigated.

The ability to target viruses by design to pre-selected cells is a tremendous challenge with far-reaching implications for the development of virus-based therapies. We previously established the principle of retargeting of coronaviruses by exchanging spike ectodomains (16, 21). Subsequent attempts to modify the tropism of these viruses further by incorporating tumor-binding ligands into different parts of the spike protein were unsuccessful as no viable viruses could be rescued (M. H. Verheije, T. Würdinger and P. J. M. Rottier, unpublished data). As an alternative and potentially also versatile targeting approach we therefore aimed at the development of bispecific adapters. The use of soluble receptor-based adapters to expand virus tropism has been described before for retroviruses (32), and adenoviruses (20). Recently, also the herpes simplex virus was redirected to a new receptor via the variable domain of its cellular receptor, nectin-1, fused to a single-chain antibody (27). However, in contrast to these viruses the coronavirus MHV is normally incapable of infecting human cells, due to its restricted tropism. Hence, its native tropism does not need to be eliminated to obtain a truly targeted virus, which avoids possible reduction of its cytotoxicity to the specific target cells. In order to fully exploit the oncolytic properties of MHV, multi-round genetic targeting will be

essential. As we described earlier, incorporation of the gene encoding the bispecific single-chain antibody s11-425 into the adenovirus genome leads to the persistent self-targeting of the virus to a specific receptor (35). Also others showed virus retargeting by genomically expressed bispecific adapters, though this resulted in a reduction of the oncolytic potency of the adenovirus in the case of the sCAR-EGF adapter (19). The same approach seems also feasible for the development of tumor-targeted coronaviruses. As we and others showed recently, MHV can tolerate and express foreign genes from various insertion sites of its genome (4, 5, 11, 12). Therefore, our next step in the development of oncolytic coronaviruses will be to introduce the gene encoding a bispecific adapter molecule into the virus genome.

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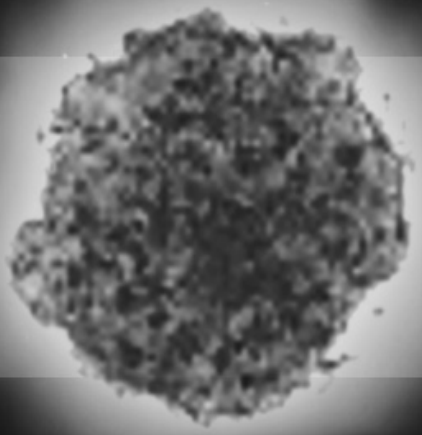
CHAPTER 5 | REDIRECTING CORONAVIRUS TO A NON-NATIVE RECEPTOR THROUGH A VIRUS-ENCODED TARGETING ADAPTER



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ABSTRACT

Murine hepatitis coronavirus (MHV)-A59 infection depends on the interaction of its spike protein S with the cellular receptor mCEACAM1a present on murine cells. Human cells lack this receptor and are therefore not susceptible to MHV. Specific alleviation of the tropism barrier by redirecting MHV to a tumor-specific receptor could lead to a virus with appealing properties for tumor therapy. To demonstrate that MHV can be retargeted to a non-native receptor on human cells, we produced bispecific adapter proteins composed of the N-terminal D1 domain of mCEACAM1a linked to a short targeting peptide, the 6-amino-acid His-tag. Pre-incubation of MHV with the adapter proteins and subsequent inoculation of human cells expressing an artificial His receptor resulted in infection of these otherwise non-susceptible cells and to subsequent production of progeny virus. To generate a self-targeted virus able to establish multi-round infection of the target cells, we subsequently incorporated the gene encoding the bispecific adapter protein as an additional expression cassette into the MHV genome through targeted RNA recombination. When inoculated onto murine LR7 cells, the resulting recombinant virus indeed expressed the adapter protein. Furthermore, inoculation of human target cells with the virus resulted in a His receptor-specific infection that was multi-round. Extensive cell-cell fusion and rapid cell killing of infected target cells was observed. Our results show that MHV can be genetically redirected via adapters composed of the S protein binding part of mCEACAM1a and a targeting peptide recognizing a non-native receptor expressed on human cells, consequently leading to rapid cell death. The results provide interesting leads for further investigations on the use of coronaviruses as anti-tumor agents.

INTRODUCTION

The ability to genetically modify viral genomes has greatly increased the opportunities of developing viruses for tumor therapy. The main aim of designing tumor-selective viruses is to generate agents with destructive potency toward cancer cells but without, or with only limited, pathogenicity to normal tissues. This goal can either be achieved by engineering viruses with a preference for replication in cancer cells or by constructing viruses with a tumor-selective tropism (reviewed by (40, 44). To achieve the latter requires viruses to be redirected to tumor-specific antigens. For viruses naturally infecting human cells, redirection additionally requires ablation of their natural tropism in order to prevent infection of normal tissue as is, for instance, the case with human adenoviruses (reviewed by (33). Ultimately, the combination of virus-specific features and the applied genetic modifications will determine their potential as anti-tumor agents.

Coronaviruses, a family of positive strand enveloped RNA viruses in the order *Nidovirales*, have several appealing characteristics that might make them suitable as oncolytic viruses. First, they have a very narrow host range, determined by the interaction of their spike (S) glycoprotein - a class I fusion protein - and their cellular receptor. For instance, the host cell tropism of murine hepatitis coronavirus (MHV) strain A59 is determined by the specific interaction between its S protein and the murine carcinoembryonic antigen cell adhesion protein 1a (mCEACAM1a) (1, 11, 13, 35, 56, 59). The presence of this receptor on the cell is a major prerequisite for infection as cells that are normally non-susceptible can be efficiently infected and killed once made to express the mCEACAM1a molecule (13, 16, 41, 42). Since mCEACAM1a is not expressed on human cells, MHV-A59 cannot establish an infection of such cells, either normal or cancer cells. Therefore, redirecting the virus to an antigen on human cancer cells is likely to be sufficient for establishing a specific infection of these cells. To change the tissue tropism of MHV, a convenient recombination system (28) is available for the genetic modification of the viral

genome. A second feature that makes MHV attractive as an oncolytic virus is that, upon infection of susceptible cells, the cells form large multinucleated syncytia, recruiting surrounding uninfected cells (54). This characteristic property, in combination with its short replication cycle (6-9 h), makes that MHV rapidly kills cells once they are infected. We therefore hypothesize that MHV-A59, once redirected to human cancer cells, might be a potential new anti-tumor virus.

The MHV-A59 spike protein is a type I transmembrane glycoprotein of 180-kDa. It is proteolytically cleaved by furin resulting in an amino (N)-terminal S1 protein and a membrane-anchored S2 protein. Upon binding of the N-terminal 330 amino acids of S1 (50) to residues in the N-terminal immunoglobulin-like domain (D1) of mCEACAM1a (14, 27, 43, 52, 53, 55) a conformational change in the spike protein is induced that leads to the exposure of the fusion peptide and its insertion into the target cell membrane (30, 32, 53, 60). Subsequent changes in the S protein, driven also by interactions between the heptad repeat (HR) regions HR1 and HR2, result in the fusion of the viral and the target cell membrane (2, 4). Upon binding of the soluble form of the mCEACAM1a molecule to the spike protein, a similar conformational change in the spike protein is induced (32, 53, 60), with concomitant reduction of virus infectivity. Here we hypothesize that a soluble receptor domain of mCEACAM1a linked to a targeting peptide that can bind to a non-native receptor can be used to redirect MHV to normally non-susceptible target cells.

Recently we have demonstrated that the feline coronavirus FIPV can be redirected to the non-native receptor EGFR by applying a bispecific single chain antibody during inoculation (57). To generate a virus able to produce this device and grow on EGFR-positive cells independently, we attempted to incorporate the gene encoding the antibody into the FIPV genome. This failed, probably because of high instability of the foreign gene in the genome due to its specific nucleotide composition. In the present study, we therefore circumvented the use of a bispecific antibody and designed a targeting adapter to redirect MHV composed of the virus-binding N-terminal domain of the mCEACAM1a MHV receptor (17) fused to a short

targeting peptide, the 6-amino-acid His-tag. We assumed that a C-terminal His tag would probably not affect the folding of the receptor protein while likely remaining accessible for interaction with the corresponding sFvHis, a surface-displayed single-chain variable fragment (sFv) with specificity for a C-terminal His. Our results show that the adapter proteins produced by expression using the vaccinia virus T7 expression system, were able to redirect MHV to sFvHis-expressing target cells and that subsequent incorporation of the adapter gene into the MHV genome as an additional expression cassette, to allow multi-round infection, indeed resulted in extensive cell-cell fusion and efficient cell killing of the target cells. The observations indicate that the soluble receptor-based targeting moiety can be the basis for coupling to a range of targeting ligands to further explore the use of genetically redirected MHV for tumor therapy.

MATERIALS AND METHODS

Viruses and cells.

Recombinant felinized MHV (fMHV) (28) stocks were produced and titrated in parallel on *Felis catus* whole fetus (FCWF) cells (obtained from N.C. Pedersen). Mouse LR7 cells (28) were used to analyze and propagate the recombinant MHV-A59-derived viruses.

Murine LR7 cells, feline FCWF-4 cells, murine Ost-7 cells (obtained from B. Moss), human U118MG cells, His-receptor expressing U118MG cells (U118MG-HissFv.rec, kindly provided by J. Douglas (10)), human 293 cells and His-receptor expressing 293 cells (293-HissFv.rec, kindly provided by J. Douglas (10)) were grown in Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, Verviers, Belgium) containing 10 % fetal calf serum (FCS), 100 IU of penicillin/ml, and 100 µg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). U118MG-HissFv.rec and 293-HissFv.rec culture medium contained 0.25 mg/ml G418 (Life Technologies, Ltd., Paisley, United Kingdom).

Construction of the genes encoding the adapter proteins soR-His and soR-h-His.

In order to obtain the gene fragment encoding the amino-terminal D1 domain of the mCEACAM1a receptor (further denoted as soR), a PCR was performed on pCEP4:sMHVR-Ig (kindly provided by Thomas Gallagher (17)) using forward primer 2296 (5'-catgggcccagccggccgagctggcctcagcacat-3', nt 4-22 of mCEACAM1a) and reverse primer 2297 (5'-catggcggccgcggggtgtacatgaaatcg-3', nt. 409-426 of mCEACAM1a). In addition, three subsequent PCRs were performed on the same plasmid using primers 2296 and 2298 (5'-tgtcacaagatttgggctggggtgtacatgaaatcg-3', nt. 409-426 of mCEACAM1a), 2296

and 2299 (5'-cggtgggcatgtgtgagttttgtcacaagatttg-3'), and 2296 and 2300 (5'-catggcggccgctgggcacggtgggcatgtgt-3') to generate a similar soR fragment but carrying a 3'-extension encoding a hinge linker region derived from IgG1 (17), as the authentic mCEACAM1a occurs as a dimer (12). The resulting DNA fragments soR (429 nt) and soR-h (472 nt) contained a 5' *Sfi*I site and a 3' *Not*I site (underlined in the primers), and were subsequently cloned using these restriction enzymes into the expression vector pSecTag2 (Invitrogen, Breda, The Netherlands). The resulting expression vectors pSTsoR-His and pSTsoR-h-His encode the N-terminal D1 domain of mCEACAM1a in fusion with an amino-terminal Igκ signal sequence and a carboxy-terminal myc- and His-tag under the control of a CMV and a T7 promoter. The hinge region in pSTsoR-h-His is located directly downstream of the D1 domain of mCEACAM1a. The composition of the genes encoding the adapter proteins was confirmed by sequencing.

Production of the adapter proteins soR-His and soR-h-His.

The adapter proteins soR-His and soR-h-His were produced in a similar way as described previously for bispecific single chain variable fragments (57). Briefly, vTF7-3 (15)-infected Ost7 cells were transfected with pSTsoR-His and pSTsoR-h-His using lipofectin (Life Technologies, Ltd., Paisley, United Kingdom). The culture supernatant was harvested at 20 hours post infection (h p.i.), the proteins were separated from vTF7-3 virus by pelleting the latter by centrifugation on a 20% sucrose cushion and the adapter proteins in the culture supernatant were concentrated on a Vivaspin20 filter column (VivascienceAG, Hannover, Germany). The concentration of the proteins was determined by dot blot analysis of serial dilutions of the adapter proteins, with reference to a standard curve similarly prepared with purified N-CEACAM-Fc protein (100 nM). Quantifications were done by immunoassay using antibodies directed against N-CEACAM-Fc, performed as described below for the Western blot. The density x mm^2 of each dot was measured using a densitometer (Bio-Rad Laboratories), and plotted against the corresponding dilution factor after which the adapter protein concentrations were calculated. The different adapter preparations were brought to the same concentration with culture medium before use.

Western blot.

Equal volumes of the concentrated culture supernatant, containing the adapter proteins soR-His and soR-h-His, were run on a 15% SDS-PAGE in the presence or absence of β-mercaptoethanol. The proteins were blotted onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) and the blot was further processed using antibodies directed against N-CEACAM-Fc (1:3,000; kindly provided by Thomas Gallagher (17)) and swine-anti-rabbit peroxidase (1:3,000, DakoCytomation, Glostrup, Denmark). The proteins were visualized by ECL (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

determination of firefly expression

Monolayers of $1x10^5$ 293-HissFv.rec and U118MG-HissFv.rec cells were inoculated with $1x10^4$ $TCID_{50}$ (as determined by end point dilution on LR7 cells) of the firefly luciferase-expressing MHV-EFLM (6) pre-incubated at 4º C for 1 h with the indicated amounts of adapter proteins. At the indicated time points, the cells were lysed using the appropriate buffer provided with the firefly luciferase assay system (Promega Corporation, Madison, WI, USA). Intracellular luciferase expression was measured according to the manufacturer's instructions, and the relative light units (RLU) were determined with a luminometer (Turner

Biosystems, Sunnyvale, CA).

FACS.

The cell lines 293-HissFv.rec and U118MG-HissFv.rec were analyzed for their relative sFvHis receptor expression by exploiting the presence of an extracellular HA-tag C-terminal of sFvHis (10). To this end, U118MG, U118MG-HissFv.rec, 293, and 293-HissFv.rec cells were collected, and incubated either in the presence or absence of anti-HA high affinity antibody (1:250; Roche Diagnostics GmbH, Mannheim, Germany) and subsequently incubated with goat-anti-rat FITC (1:200; DakoCytomation, Glostrup, Denmark). Flow cytometry was performed using FACScan analysis (Beckton Dickinson, Erembodegem-Aalst, Belgium). The relative geometric mean (Gm) fluorescence of each cell line in the presence or absence of anti-HA high affinity antibody was determined by using WinMDI 2.8 (http://facs.scripps.edu/). The Gm ratio indicates the sFvHis expression level of sFvHis-expressing cell lines relative to that of their parental cell lines.

Construction of the vectors for targeted recombination.

To allow expression of the adapter proteins from an additional expression cassette in the viral genome of MHV-A59, a transcription regulation sequence (TRS, underlined in the primers) was first introduced into pSTsoR-His and pSTsoR-h-His directly upstream of the soR-encoding region, as described before for pXH1bMS (7). To this end, the plasmids were digested with *Nhe*I and subsequently the oligonucleotides 1838 (5'-ctagcgatatctaatctaaactttag-3') and 1839 (5'-ctagctaaagtttagattagatatcg-3'), containing *Nhe*I-site extensions, were ligated into this site, resulting in pST-T-soR-His and pST-T-soR-h-His, respectively. The presence and orientation of the TRS was confirmed by sequencing. Next, the fragments TRS-soR-His and TRS-soR-h-His were obtained from pST-T-soR-His and pST-T-soR-h-His by digestion of the vectors with *Eco*RV and *Pme*I and the purifiedfragments were cloned into the Klenow-treated *Hind*III site of pXH1802 (5), containing approximately 1200 bp of the 3'end of the replicase gene 1b fused to the S gene of MHV-A59. Then, the resulting plasmids were digested with *Rsr*II and *Avr*II and the obtained fragments were cloned into pMH54 (28), treated with the same enzymes. This resulted in the transcription vectors pMHsoR-His and pMHsoR-h-His suitable for targeted recombination. The transcription vector in which the *Rsr*II-*Avr*II fragment from pXH1802 was cloned into the corresponding sites of pMH54 was used as a control vector for targeted recombination (further denoted as pXHd2aHE (5)).

Targeted recombination.

The adapter genes soR-His and soR-h-His were introduced as additional expression cassettes into the MHV genome by targeted RNA recombination as described previously (5, 28). Briefly, donor RNAs transcribed *in vitro* from *Pac*I-linearized plasmids pXHd2aHE, pMHsoR-His and pMHsoR-h-His were transfected by electroporation into feline FCWF-4 cells that had been infected with fMHV at a multiplicity of infection (moi) of 0.5 4 h earlier. These cells were then plated in culture flasks and the culture supernatant was harvest 24 h later. Progeny virus was plaque purified and virus stocks were grown on LR7 cells. After confirmation of the presence of the additional expression cassettes by reverse transcription-polymerase chain reaction (RT-PCR) on purified viral RNA from these virus stocks, the virus titer of the stocks was determined by end point dilution on LR7 cells. These passage 2 virus stocks were subsequently used

in the experiments. For each virus, two independent recombinants were generated to control for effects caused by unintended mutations in other parts of the viral genome.

Viral RNA isolation and RT-PCR.

First, from 140 μl virus-containing culture supernatant, viral RNA was isolated using the Qiagen Viral RNA Isolation Kit (according to the manufacturer). Reverse transcription on the isolated RNA was then performed using reverse primer 1127 (5'-ccagtaagcaataatgtgg-3'), located at nt 24,110-24,128 of the MHV genome (Accession nr. NC 001846). PCR was performed using primers 1173 (5'-gacttagtcctctcctt-gattg-3', nt. 21650-21671) and 1260 (5'-cttcaacggtctcagtgc-3', nt. 24,041-24,058), overlapping the region that contains the inserted expression cassette. The resulting fragments were subsequently sequenced to confirm the sequence of the inserts.

Analysis of viral growth kinetics.

An amount of 1 x 10^5 cells per 2-cm^2 well of the indicated cells were inoculated with 0.5 x 10^5 $TCID_{50}$ (as determined by end point dilution on LR7 cells). After 1 h the culture supernatant was removed, the cells were washed and fresh culture medium was added. At several time points p.i. the medium was harvested, and stored at - 80 °C until analysis. The amount of virus produced at each time point was determined by end point dilution on LR7 cells and $TCID_{50}$ values were calculated.

Immunofluorescence assay.

For indirect immunofluorescence, LR7 cells were grown on 10-mm coverslips and infected with the recombinant MHV viruses at an moi of 0.5. The cells were fixed at 5 h p.i., permeabilized, and processed for double labeling. Incubations were subsequently performed with rabbit polyclonal MHV antiserum K134 (1:400; (45), goat-anti-rabbit-FITC (1:200; ICN-Cappel, Aurora, OH, USA), monoclonal antibody directed against the C-terminal His_6-tag (1:100; Invitrogen, Woerden, The Netherlands), and donkey-anti-mouse-Cy5 (1:100; Jackson ImmunoResearch, West Grove, PA, USA). The staining was visualized using a Leica TCS SP confocal laser scanning microscope and Leica software (http://www.confocal-microscopy.com/website/sc_llt.nsf).

Immunoperoxidase staining of cells.

An amount of 1 x 10^5 293-HissFv.rec and U118MG-HissFv.rec cells were inoculated with 0.5 x 10^5 $TCID_{50}$. At 16 h p.i. the cells were fixed and stained to analyze the expression of viral proteins. The cells were incubated with rabbit polyclonal MHV antiserum K134 (1:300; (45) followed by incubation with swine-anti-rabbit peroxidase (1:300; DakoCytomation, Glostrup, Denmark) and staining using AEC (Brunschwig, Amsterdam, the Netherlands) according to the manufacturer's protocol.

Metabolic labeling and radio-immunoprecipitation.

Subconfluent monolayers of LR7 cells were infected with the recombinant viruses at an moi of 1. At 5.5 h p.i. the cells were washed and starved for 30 min in cysteine- and methionine-free DMEM medium,

containing 5% FCS, 1 mM glutamax and 10mM Hepes, pH=7.3 (all from Life Technologies, Ltd., Paisley, United Kingdom). The cells were labeled for 1 h with ^{35}S Translabel (Amersham Pharmacia Biotech Europe GmbH, Germany), and chased with non-radioactive culture medium for 3 h. Culture supernatants were used for immunoprecipitation using antibodies directed against N-CEACAM-Fc.

Monolayer cytotoxicity analysis.

An amount of 5 x 10^4 293-HissFv.rec cells per 0.32-cm^2 well was seeded and infected in triplicate with various amounts of MHVsoR-h-His. At several time points after inoculation, the culture medium was replaced by DMEM containing 10% WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) and the cells were cultured for 2 h. Hereafter, the OD_{450} was measured and the viability of the infected cells was calculated relative to uninfected control cells, after subtraction of background values of WST-1 incubated in the absence of cells.

RESULTS

Generation of soR-His and soR-h-His adapter proteins.

To explore the possibilities of redirecting MHV to a non-native receptor on human cells (Fig. 1A), we generated adapter proteins composed of the N-terminal D1 domain of the MHV receptor CEACAM1a linked via a stretch of three alanine residues and a myc-tag to a 6-amino-acid His-tag. The genes encoding these adapter proteins were preceded by a sequence encoding the Igκ signal peptide and constructed either without or with a hinge-encoding region (-h-) of an IgG molecule directly downstream of the soR, resulting in soR-His and soR-h-His, respectively (Fig. 1B). The Igκ signal sequence would direct adapter protein secretion, the soR would provide for binding to and induction of a conformational change in the spike protein, and the His-tag would effect the binding to a non-native sFvHis receptor on target cells. Furthermore, the hinge region would allow dimerization of the adapter protein soR-h-His, as occurs with the natural mCEACAM1a receptor (12, 17), whereas the adapter protein soR-His would remain in a monomeric state.

Both adapter proteins were expressed using a vaccinia virus T7 expression system in Ost-7 cells. Western blot analysis (Fig. 1C) of the culture supernatants

electrophoresed under reducing conditions using antibodies directed against N-CEACAM-Fc (17) showed that the adapter proteins soR-h-His and soR-His were produced (Fig. 1C, left panel), whereas no protein was detected in culture supernatant from mock-transfected cells. The apparent sizes of soR-His and soR-h-His proteins were somewhat larger than their predicted sizes of 18.4 and 20.0 kDa, respectively. Treatment of the resulting proteins with endoglycosidase H reduced their apparent size, confirming that both soR-His and soR-h-His were N-glycosylated (data not shown). Analysis of the adapter proteins under non-reducing conditions (Fig. 1C, right panel) demonstrated that soR-h-His migrated at 55-60 kDa, which is consistent with the size of a soR-h-His dimer. Hence, the presence of a hinge region in the protein indeed induced dimerization of soR-h-His. In contrast, the size of the soR-His protein analyzed under non-reducing condition was similar to that analyzed under reducing conditions, indicating that it was produced as a monomer.

The adapter proteins soR-His and soR-h-His can redirect MHV to the non-native sFvHis receptor on human cells.

To study the ability of the adapter proteins to redirect MHV to the corresponding non-native receptor, we selected two human cell lines, 293-HissFv.rec and U118MG-HissFv.rec (10) that express a surface-displayed single-chain variable fragment (sFv) with specificity for a C-terminal His-tag (sFvHis)(29). FACS analysis (Fig. 2A) was performed to determine the relative sFvHis expression on these cells. Gm ratios of 16.5 (293-HissFv.rec cells; Fig. 2A, left panel) and 1.8 (U118MG-HissFv.rec cells; Fig. 2A, right panel) were observed, respectively. This confirmed that both cell lines expressed sFvHis, but indicated that the expression level was much higher on the 293-HissFv.rec cells than on the U118MG-HissFv.rec cells.

Next, we analyzed whether soR-His and soR-h-His were able to redirect MHV-EFLM, an MHV-A59 derivative expressing firefly luciferase (6), to sFvHis on 293-HissFv.rec and U118MG-HissFv.rec cells. To this end, 1 x 10^4 $TCID_{50}$ units of MHV-EFLM (as determined by end point dilution on LR7 cells) were incubated

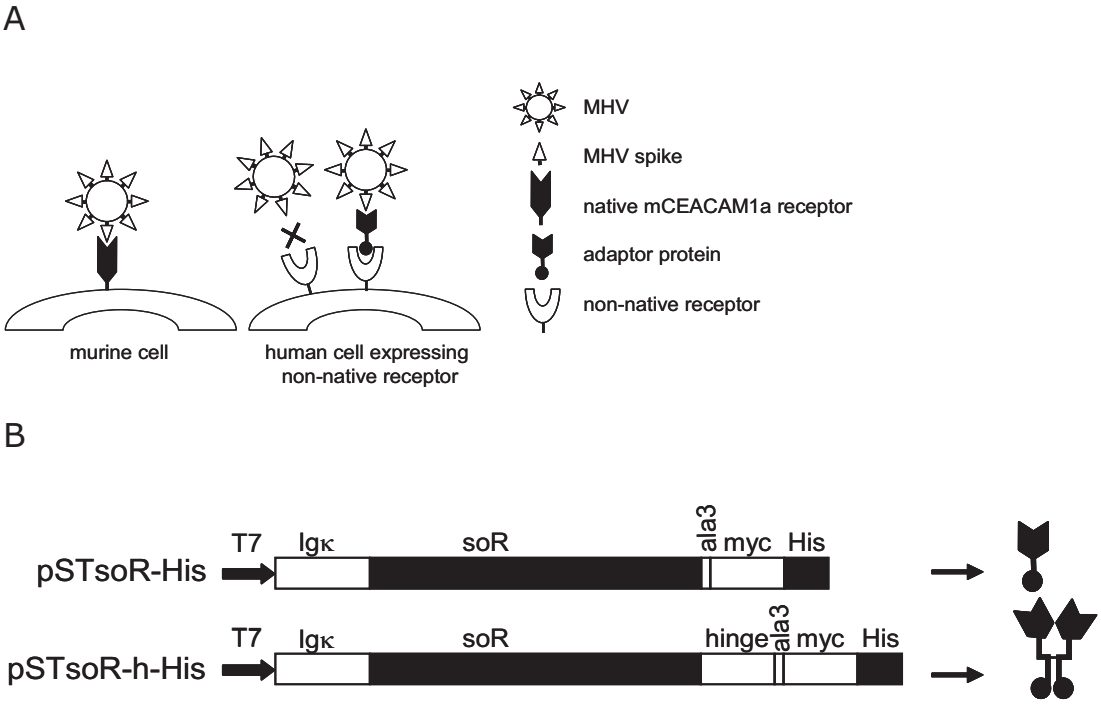


Fig. 1 Approach to redirect MHV to a non-native receptor. (A) Rationale for using adapter proteins to redirect MHV to a non-native receptor present on human cells. (B) Schematic diagram of the expression cassettes of pSTsoR-His and pSTsoR-h-His and of the design of the adapter proteins soR-His and soR-h-His. The Igκ signal sequence directs adapter protein secretion, the N-terminal D1 domain of mCEACAM1a (soR) provides for binding to and induction of conformational changes in the MHV spike protein, and the 6-amino-acid His-tag (His) provides for binding to the artificial sFvHis receptor. The hinge (-h-) linker region present in soR-h-His should allow formation of disulfide-linked dimers of the resulting protein (T7: T7 promoter, myc: myc-tag, ala3: three alanine residues). The resulting adapter proteins are depicted on the right. (C) Western blot analysis of soR-h-His and soR-His adapter proteins. Ost-7 cells were infected with vaccinia vTF7-3 and transfected with the plasmids pSTsoR-h-His and pSTsoR-His. Mock transfection was taken along as a control. The culture supernatants were analyzed by Western blot using antibodies directed against N-CEACAM-Fc (17) both under reducing (left panel) and under non-reducing (right panel) conditions. In each case the positions of marker proteins run in the same gel are shown at the left.

with the indicated amounts of soR-His or soR-h-His (Fig. 2B) and subsequently inoculated onto 1 x 10^5 293-HissFv.rec or U118MG-HissFv.rec cells. Luciferase activity was measured to determine whether inoculation had resulted in successful infection of the target cells. The data show that both soR-His and soR-h-His were able to redirect MHV-EFLM to 293-HissFv.rec and U118MG-HissFv.rec cells, whereas target cells inoculated with mock-preincubated MHV-EFLM remained uninfected, indicating that the infection was specifically mediated by the adapter proteins. This was confirmed by similar inoculations of the parental cell lines 293 and U118MG, which resulted only in background levels of luciferase activity (data not shown).

Consistently, the infection efficiencies were clearly dependent on the amount of adapter protein present. In both cell lines luciferase expression increased upon inoculation of similar amounts of MHV-EFLM in the presence of increasing amounts of soR-His and soR-h-His (Fig. 2B). Strikingly, luciferase expression was consistently much higher for soR-h-His-mediated infection than when using soR-His, as can be observed by comparing the potency of similar amounts of adapter proteins. In addition, luciferase activity was invariably higher when targeting MHV-EFLM to 293-HissFv.rec cells than to U118MG-HissFv.rec cells. This can be explained, at least in part, by the different levels of sFvHis receptor expressed on these cells. However, we cannot exclude that MHV replication and, thus, protein expression in U118MG-HissFv.rec cells was inherently lower than in 293-HissFv.rec cells.

Targeted MHV infection requires the native spike membrane fusion function.

To investigate whether the adapter-mediated entry of MHV into human cells occurs by the same membrane fusion mechanism used by the virus to naturally infect murine cells, we applied an inhibitor known to specifically block this process. 293-HissFv.rec cells were inoculated with MHV-EFLM preincubated with 0.5 nM soR-His or soR-h-

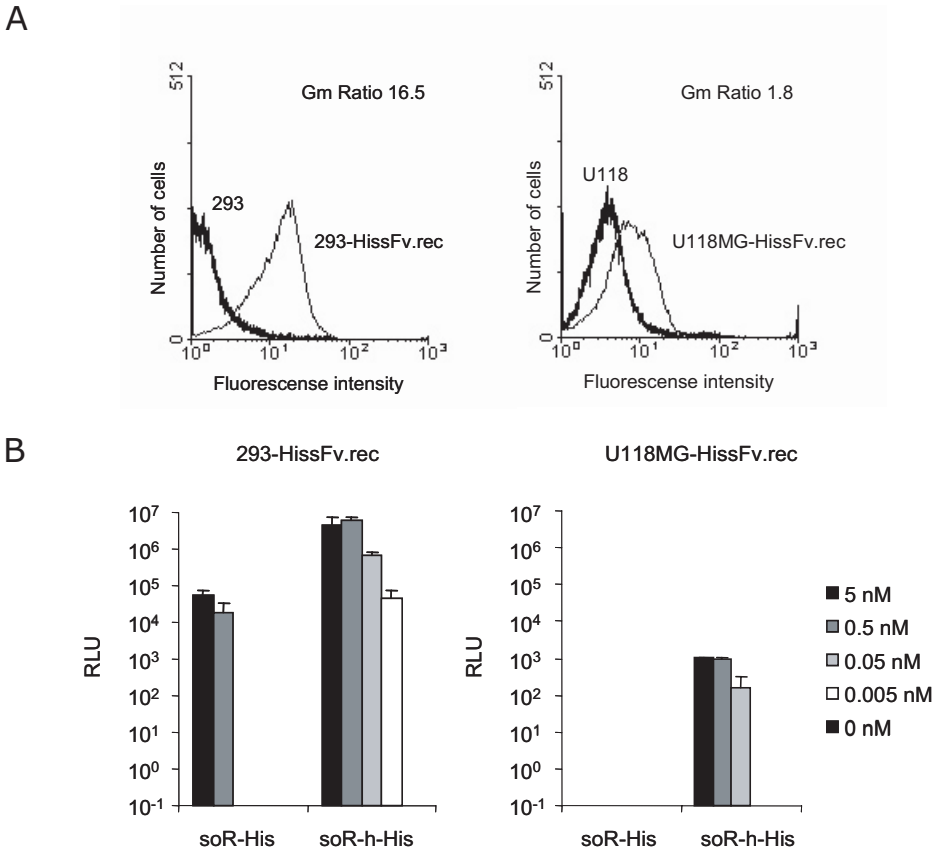


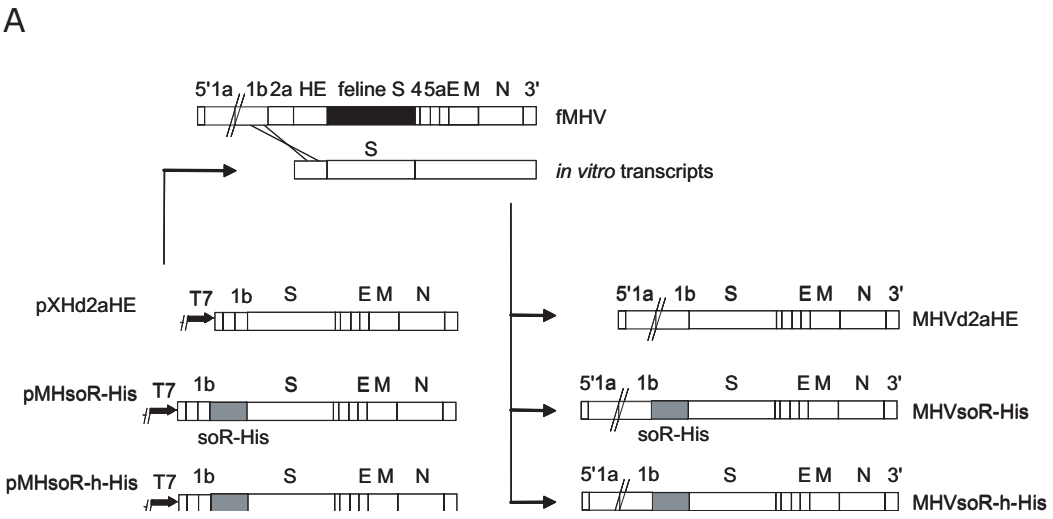
Fig. 2 Redirection of MHV to an artificial sFvHis receptor expressed on human cells using the adapter proteins soR-His and soR-h-His. (A) FACS analysis of the target cell lines 293-HissFv.rec and U118MG-HissFv.rec and their parental cell lines 293 and U118MG. Flow cytometry was performed using the anti-HA high affinity antibody to determine the expression of sFvHis for each cell line. The indicated Gm ratio's relate to the ratio between the relative geometric mean fluorescence between 293-HissFv.rec and 293 cells (left panel) and between U118MG-HissFv.rec and U118MG cells (right panel), and are an indication of the relative amount of sFvHis expression on the target cell lines. (B) Inoculation of human sFvHis-expressing cells with MHV-EFLM in the presence or absence of the adapter proteins soR-His and soR-h-His. Monolayers of 1 x 10^5 293-HissFv.rec and U118MG-HissFv.rec cells were inoculated with firefly luciferase-expressing MHV virus MHV-EFLM (6) pre-incubated with the indicated amounts of soR-His, soR-h-His or mock culture supernatant, as described in Materials and Methods. The cells were lysed 20 h p.i. and the luciferase activity (RLU) was measured using a luminometer. The values depicted are the means of an experiment performed in triplicate; bars show standard deviations. (C) Redirection of MHV-EFLM using soR-His and soR-h-His proteins can be blocked with the MHV-specific fusion inhibitor mHR2 (4). 293-HissFv.rec cells were inoculated with MHV-EFLM pre-incubated with either 0.5 nM soR-His or soR-h-His in the absence (-mHR2) or presence (+mHR2) of 20 μM mHR2 peptide. Intracellular luciferase activity (in RLU) was determined at 18 h p.i. The values depicted are the means of an experiment performed in triplicate; bars show standard deviations.

His in the presence or absence of 20 μM of mHR2, a peptide fusion inhibitor derived from the C-terminal heptad repeat region (HR2) of the MHV S protein (4). As is clear from Fig. 2C, luciferase expression was efficiently blocked by the peptide, indicating that adapter-targeted MHV infection is dependent on S mediated membrane fusion.

Generation and growth characteristics of recombinant MHVsoR-His and MHVsoR-h-His viruses.

Having demonstrated that MHV can be retargeted by an exogenously added adapter protein, our next aim was to incorporate this information into the viral genome to generate a virus producing its own targeting device, thereby acquiring the ability to be independently propagated on the target cells. To this end, the soR-His and soR-h-His expression cassettes were provided with a transcription regulation sequence (TRS) to allow transcription of the foreign gene from the viral genome (6), and inserted into the recombination vector pMH54, thereby replacing the genes 2a/HE from MHV-A59 (Fig. 3A). To obtain the recombinant MHV viruses, targeted recombination was performed as described earlier (28), resulting in the viruses MHVsoR-His and MHVsoR-h-His (Fig. 3A). In parallel, a control recombinant MHV was generated that lacks the genes 2a/HE, resulting in MHVd2aHE. The genotypes of the recombinant passage 2 viruses were confirmed by RT-PCR on viral RNA isolated after plaque purification and stock production on murine LR7 cells (data not shown).

The *in vitro* growth kinetics of the recombinant MHVsoR-His and MHVsoR-h-His viruses were compared with that of MHVd2aHE by comparing their one-step growth curves made on murine LR7 cells (Fig. 3B). Pairs of independently generated recombinant viruses (marked A and B) were taken to verify that the observed phenotypes were not a consequence of accidental, unintended mutations. The recombinant MHVsoR-His viruses appeared to replicate very similar to MHVd2aHE, whereas MHVsoR-h-His viruses grew with similar kinetics but to considerably lower titers. The yields of MHVsoR-h-His were almost two log units lower than that of MHVsoR-His and MHVd2aHE.



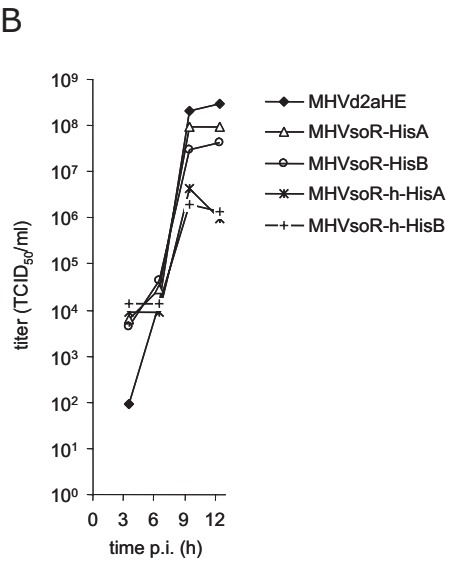


Fig. 3 Recombinant MHVsoR-His and MHVsoR-h-His viruses and their growth kinetics on murine cells. (A) Targeted recombination vectors, recombination procedure and resulting viral genomes. The transcription vectors from which the RNAs are transcribed *in vitro* by T7 RNA replicase are depicted on the left side. The inserted additional expression cassettes encoding soR-His and soR-h-His are represented by a grey box. The recombinant viruses generated by targeted recombination are depicted at the right. (B) Single-step growth kinetics of the MHV recombinants on LR7 cells. LR7 cells were infected with at a moi of 0.5, the production of progeny virus in the culture supernatant at different times p.i. was determined by end point dilution on LR7 cells and $TCID_{50}$ values were calculated. Viruses marked A and B represent independently generated recombinants. In each case the growth curve shown is a representative example of two independent experiments.

MHV recombinant viruses are able to express and secrete the adapter proteins from infected murine LR7 cells.

To demonstrate that the adapter proteins soR-His and soR-h-His were produced from their respective expression cassettes in the recombinant MHVsoR-His and MHVsoR-h-His viruses, murine LR7 cells were inoculated with the recombinant MHV viruses and processed for a double immunofluorescence labeling. The following antibodies were used in successive order: anti-MHV serum, FITC-labeled secondary antibody, anti-His_6 (C-terminal) monoclonal antibody and Cy-5 secondary antibody. Confocal scanning laser microscopy performed on the processed cells showed His staining in cells infected with either virus (Fig. 4A, middle and lower row), but not in uninfected cells (not shown) or cells infected with the control virus MHVd2aHE-infected LR7 cells (upper row).

To investigate the excretion of the adapter proteins from MHVsoR-His and MHVsoR-h-His infected cells into the culture supernatant, LR7 cells were infected with the different recombinant viruses at an moi of 1 and labeled for 3 h with ^{35}S-labeled amino acids starting at 6 h p.i. Subsequently, the culture supernatants were processed for immunoprecipitation with anti-N CEACAM-Fc antibodies (17) and analyzed by SDS-PAGE under reducing conditions (Fig. 4B). Diffuse bands representing the proteins soR-His and soR-h-His were observed. The presence of the hinge region in soR-h-His accounts for the difference in size between the two proteins. Their electrophoretic mobilities are similar to those of the proteins expressed from pSTsoR-His and pSTsoR-h-His (Fig. 1C). Analysis of the immunoprecipitates under non-reducing conditions showed that only a small fraction of the soR-h-His adapter protein occurred as dimers (data not shown). In conclusion, MHVsoR-His and MHVsoR-h-His recombinant viruses expressed and excreted the adapter proteins from infected LR7 cells.

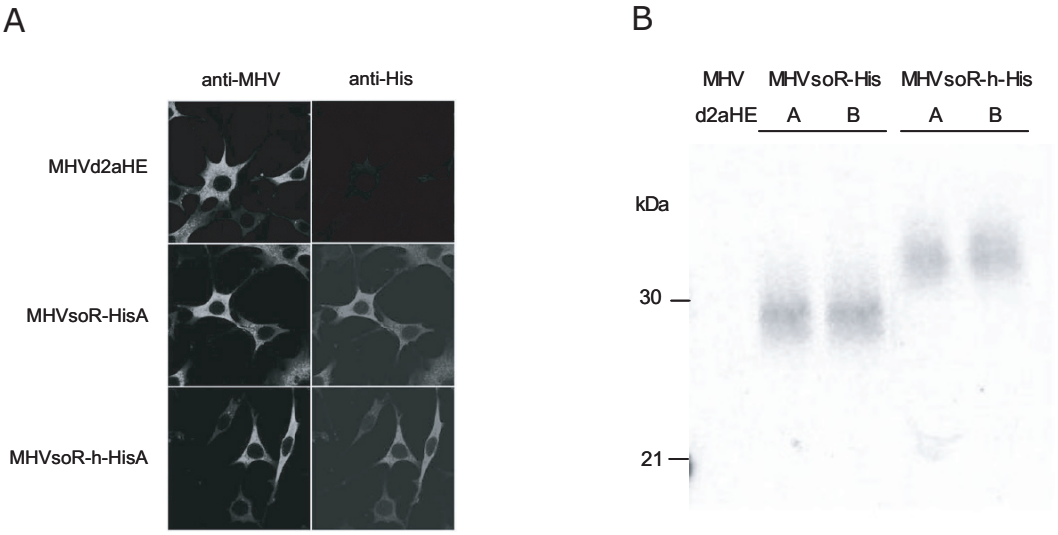


Fig. 4 Expression of soR-His and soR-h-His by MHV recombinant viruses. (A) Confocal laser scanning microscopy of LR7 cells infected with MHVd2aHE, MHVsoR-His and MHVsoR-h-His recombinant viruses. LR7 cells were infected with recombinant viruses at a moi of 0.5, fixed at 6 h p.i., permeabilized and processed for immunofluorescence by subsequently applying the anti-MHV serum K134, a green fluorescent FITC secondary antibody, the anti-His_6 (C-terminal) antibody and a red fluorescent Cy5 secondary antibody (×100 magnification). The staining was visualized using a Leica TCS SP confocal laser scanning microscope by excitation at 488 nm (FITC; left column) or 568 nm (Cy5; right column). For each recombinant MHV virus, one representative example of the independently generated viruses is shown. (B) Radio-immunoprecipitation of the secreted soR-His and soR-h-His proteins. LR7 cells were inoculated with MHVd2aHE, MHVsoR-His and MHVsoR-h-His at a moi of 1, and subsequently labeled with ^{35}S-labeled Met/Cys from 6 to 7 h p.i. and chased with cold medium for 3 h. Immunoprecipitation was performed on the culture medium with anti-N-CEACAM-Fc antibodies (17) and the precipitates were analyzed by SDS-PAGE under reducing conditions. The positions of molecular mass marker proteins are indicated on the left of the gel.

MHVsoR-h-His, but not MHVsoR-His, infects sFvHis-expressing human target cells.

To determine whether the adapter protein-expressing recombinant MHV viruses were able to infect human cells displaying the non-native sFvHis receptor, 293-HissFv.rec and U118MG-HissFv.rec cells ($1x10^5$ each) were inoculated with $0.5x10^5$ $TCID_{50}$ units of virus grown in and titrated on LR7 cells. The cells were fixed 16 h after inoculation, permeabilized, and stained using polyclonal MHV antiserum (Fig. 5). For both 293-HissFv.rec and U118MG-HissFv.rec cells, positive staining was

only observed after inoculation with MHVsoR-h-His recombinant viruses, not with MHVsoR-His recombinant viruses. Neither of the two cell lines could be infected with MHVd2aHE, indicating that infection was critically dependent on expressed soR-h-His. Moreover, much more cells became infected after inoculation of 293-HissFv.rec cells than of U118MG-HissFv.rec cells. These results are in agreement with the differences in targeting efficiencies observed when redirecting MHV-EFLM by means of exogenous soR-h-His protein to these cells (Fig. 2B). Subsequent cell-cell fusion was only observed in 293-HissFv.rec cells, and was very obvious 24 h p.i. (Fig. 5, upper two panels).

To confirm the specificity of the targeted infection, similar infectious amounts of MHVsoR-h-His viruses were used to inoculate the parental cell lines 293 and U118MG, which lack sFvHis-receptor expression. No infected cells could be detected by immunoperoxidase staining, indicating that the infection by MHVsoR-h-His was sFvHis-specific (data not shown).

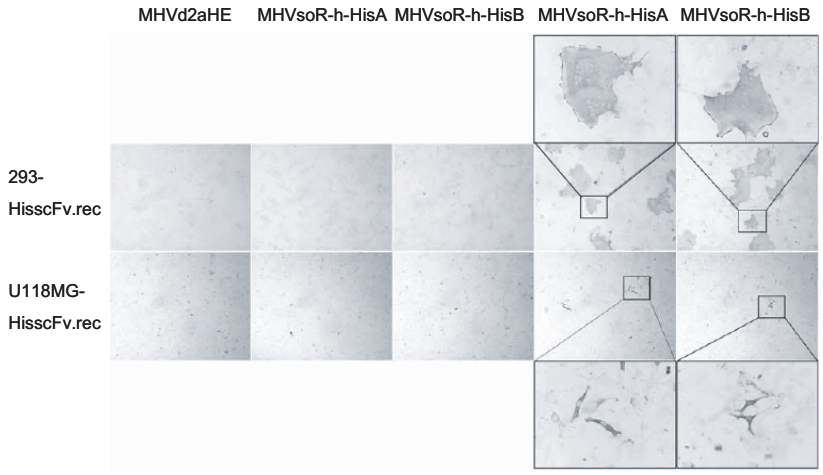


Fig. 5 Immunoperoxidase staining of sFvHis-expressing human cells inoculated with MHVsoR-His and MHVsoR-h-His recombinant viruses. 293-HissFv.rec and U118MG-HissFv.rec cells were inoculated with MHVd2aHE, MHVsoR-His and MHVsoR-h-His, as described in Materials and Methods. Cells were fixed 16 h after inoculation, permeabilized and subsequently processed using anti-MHV serum K134. The infection of the target cells was analyzed by AEC staining. Enlargements of pictures taken at 4x magnification (non-boxed) are shown in a box (10x magnification). In the enlargements, syncytia formation is clear in 293-HissFv.rec cells infected with MHVsoR-h-His , but absent in similarly infected U118MG-HissFv.rec cells.

Analysis of the growth kinetics of and cell killing by the MHVsoR-h-His recombinant viruses in human target cells.

In order to establish whether infection of susceptible cells by MHVsoR-h-His was productive and to determine the kinetics of progeny virus release, we prepared an *in vitro* growth curve of the virus in 293-HissFv.rec cells. An amount of $1x10^5$ 293-HissFv.rec cells was inoculated with $0.5x10^5$ $TCID_{50}$ of MHVsoR-h-His virus grown in and titrated on LR7 cells. At several time points samples were taken from the culture supernatant and the infectivity was determined by end point dilution on LR7 cells. MHVd2aHE was taken along as a negative control. Figure 6A shows the results of a representative experiment demonstrating that recombinant MHVsoR-h-His viruses replicate with normal kinetics and with reasonable yields, with maximal virus titers of 10^6-10^7TCID_{50}/ml as determined by end point dilution on LR7 cells.

To confirm that MHVsoR-h-His virus could indeed establish a multi-round infection of the target cells, we first produced an MHVsoR-h-His virus stock on 293-HissFv.rec cells and determined the viral titer on murine LR7 cells. Of this virus stock, $5x10^4$ (1:1) or $5x10^3$ (1:10) $TCID_{50}$ units were inoculated onto $5x10^4$ fresh 293-HissFv.rec cells. The cell viability was measured by WST-1 assay at different time points after inoculation (Fig. 6B). The results indicate that the infection spreads through the 293-HissFv.rec cell culture, killing increasing numbers of cells, eventually eradicating the entire culture. The spread of the infection was confirmed by immunoperoxidase staining of the infected cells at different times p.i., in which an increasing amount of positive cells was observed over time (data not shown). The amount of virus used to inoculate 293-HissFv.rec cells determined the rate at which cell killing occurred; when inoculated at an moi of 1 destruction of the cell culture took about 24 h, whereas after inoculation at an moi of 0.1 the process was slower, being essentially complete after about 48 h.

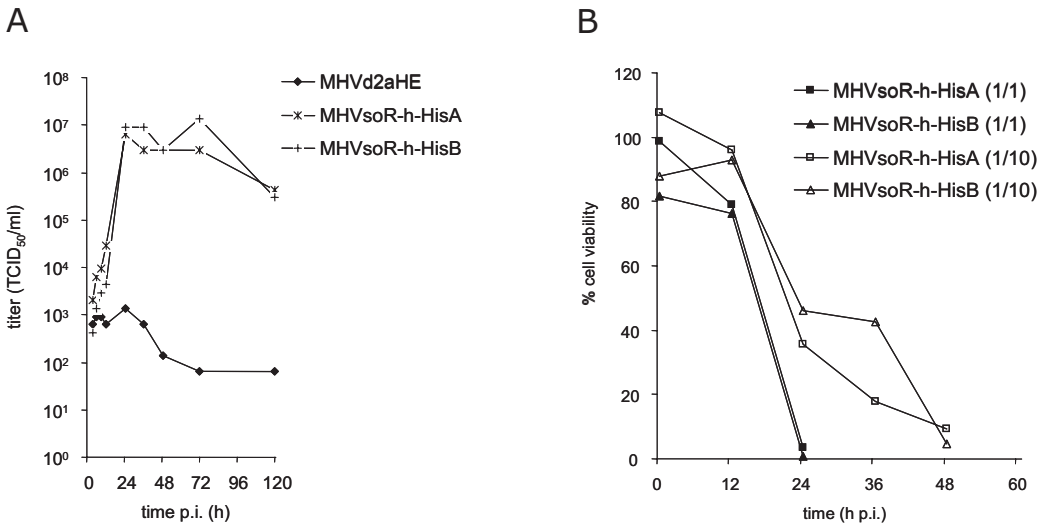


Fig. 6 MHVsoR-h-His progeny virus production and cell killing of 293-HissFv.rec target cells. (A) Growth kinetics of MHVsoR-h-His on 293-HissFv.rec cells. 293-HissFv.rec cells were inoculated with MHVsoR-h-His as described in Materials and Methods. The appearance of progeny virus in the culture media at different times p.i. was determined by end point dilution on murine LR7 cells and $TCID_{50}$ values were calculated. The viruses marked A and B indicate the independently generated recombinants. (B) Cell viability of 293-HissFv.rec cells after inoculation with MHVsoR-h-His. MHVsoR-h-His virus stocks grown on 293-HissFv.rec cells were used to inoculate fresh 293-HissFv.rec cells. After inoculation of $5x10^4$ target cells with $5x10^4$ (1:1) and $5x10^3$ (1:10) $TCID_{50}$ (as determined by end point dilution on LR7 cells), the cell viability was measured at different time points after inoculation. The indicated OD_{450} values represent the WST-1 values relative to uninfected control cells, after subtraction of background values of WST-1 incubated in the absence of cells, and are the results of an experiment performed in triplicate. The viruses marked A and B indicate the independently generated recombinants.

DISCUSSION

The results described in this paper demonstrate the feasibility of redirecting recombinant murine coronavirus MHV to a non-native receptor on human cells. To achieve this, we introduced bidirectional adapter protein encoding genes, composed of the spike-binding domain of mCEACAM1a fused to a His-tag targeting peptide into the MHV genome. This yielded a recombinant MHV that could establish a receptor-specific infection of sFvHis-receptor expressing human cells, resulting in

extensive cell-cell fusion and efficient killing of the target cells.

Many viruses have been explored in different ways for use as oncolytic tumor agents. Several strategies successfully applied genetic modification of virus, for instance by altering the receptor binding specificities through direct incorporation of targeting molecules into the viral surface proteins (9, 19-21, 23, 25, 26, 31, 36, 39, 47-49). Similar strategies were not effective for MHV and the felinized MHV (fMHV) as viable spike-modified viruses could not be rescued (our unpublished data). This is most probably due to the complex nature and dual function of the S protein, changes in which may easily lead to impairment of the conformational rearrangements in the spike essential for its functioning during cell entry. Soluble receptor proteins have been used to redirect replication-deficient adenoviruses (8, 24) and very recently herpes simplex virus (34) to cancer cells. Strikingly, replication-competent adenoviruses were impaired in their oncolytic potential when expressing a soluble receptor targeting molecule (22). In contrast to adenoviruses and herpes simplex virus, MHV does not infect human cells, so no ablation of the natural tropism is needed. This species-specificity of MHV combined with its fast replication and its ability to recruit uninfected neighboring cells by cell-cell fusion is a very appealing feature for its application in anti-tumor therapy. Our results indeed demonstrate that, once the species-barrier is alleviated by insertion of adapter proteins into the MHV viral genome, human cells can be infected with subsequent extensive cell destruction.

In general, soluble receptors - i.e. the functional fragments from otherwise membrane-anchored cell surface antigens acting as receptors - bind to viruses and neutralize virus infectivity. These features have been explored in quite some detail for murine coronaviruses (18, 32, 37, 38, 46, 51, 60-62). It has been shown that the N-terminal domain of the mCEACAM1a receptor is sufficient for MHV-A59 binding and for induction of a conformational change in the S protein. This N-terminal domain can block virus infectivity on susceptible cells. Its expression on non-susceptible cells is only sufficient to render cells susceptible to MHV once a second

domain of the mCEACAM1a is also introduced (14) or when it interacts with another unidentified molecule on the plasma membrane (12). Here we demonstrated that when the N-terminal domain of mCEACAM1a is linked to a suitable peptide ligand, the fusion protein can function as an adapter protein that targets MHV to a corresponding receptor on a non-susceptible cell. The subsequent establishment of infection functionally demonstrates that the adapter protein is sufficient to bind to and induce the conformational changes in the spike essential for MHV infection (17, 52, 60-62). Subsequent binding of the His tag to the sFvHis receptor brings virus- and cell membrane in close proximity, allowing the viral fusion peptide to be inserted into the latter, thereby triggering the membrane fusion reaction, as in a normal MHV infection (3, 58). Although only target cells expressing the sFvHis protein could be infected, we cannot rule out that the close proximity of virus and cell membranes effected by the adapter protein subsequently results in MHV entering the cells via another unidentified receptor. Furthermore, previous results have shown that a bispecific adapter protein directed with one arm against the feline coronavirus spike and with the other arm against the EGF receptor can redirect the feline coronavirus FIPV and fMHV to EGFR-expressing cells (57), but that a similar bispecific adapter protein with the one arm directed against the M protein of MHV did not direct fMHV or MHV to EGFR-expressing cells (our unpublished results). This suggests that induction of the conformational change in the spike protein and subsequent binding to an alternative receptor by the targeting peptide is required for efficient infection of non-susceptible target cells.

The efficiency of MHV targeting to a non-native receptor was critically dependent on the presence of a hinge-region in the adapter protein. Adapter proteins containing such a region established a more effective infection of sFvHis-expressing target cells than similar adapter proteins lacking this region. Actually, the recombinant MHVsoR-His viruses were unable to establish an infection of target cells. The hinge region might function as an extended linker between the spike-binding domain soR and the target cell receptor, thereby affecting the conformation of the adapter protein

or the spacing between the virus- and cell membrane. That the distance between these membranes may be important was also suggested by the results of Dveksler *et al* (14), who found that, in contrast to expression of the N-terminal domain of mCEACAM1a solely, extension of this domain with a second immunoglobulin-like motif rendered cells susceptible to MHV infection. Alternatively, the formation of disulfide-linked adapter protein dimers might account for the difference in targeting efficiency. Indeed, reduction of the dimer into a monomeric adapter protein using DTT led to a drastic decrease in infected target cells (data not shown). Further research will be needed to conclusively determine the role of the hinge region in the adapter protein.

The presence of the hinge region also had a pronounced effect on the yields of adapter protein-expressing recombinant MHV viruses from murine LR7 cells. While MHVsoR-His replicated to titers similar to the control virus MHVd2aHE, a severe reduction in yield was observed for MHVsoR-h-His (Fig. 3B). It is unlikely that the merely 51 nucleotides larger gene of soR-h-His caused this difference. Since the amounts of the adapter proteins produced by the MHV recombinant viruses were approximately similar (Fig. 4B), it is more likely that the effect is the result of interactions between the adapter proteins and the spike proteins within the infected cell, which might lead to an impairment of virus release. Differences in intracellular retention of the spike protein are not anticipated, consistent with the plaque sizes of MHVsoR-His and MHVsoR-h-His being similar when culturing infected LR7 cells under soft agar (data not shown). Accordingly we observed that soR-h-His neutralized MHV infection on murine LR7 cells more efficiently than did soR-His (T. Wurdinger *et al.,* Chapter 4 of this thesis). Apparently, the disulfide-linked dimer of soR has a higher affinity for the spike protein than the monomeric form and it thereby acts as a stronger competitor for binding of MHV to cellular mCEACAM1a. The subsequent reduction in entry of MHVsoR-h-His would explain the lower yield of this virus from mCEACAM1a-expressing cells.

The efficiencies with which our self-targeted MHVs could infect human target cells differed between the two HissFv-expressing cell lines; 293-HissFv.rec cells were the most susceptible, giving rise to extensive cell-cell fusion and ultimately eradication of cell cultures, which was not observed with U118MG-HissFv.rec cells. The significantly higher sFvHis receptor expression on 293-HissFv.rec cells probably accounts for this difference. As a high level of mCEACAM1a expression is also essential for an effective native infection of MHV (11, 13, 41), a similar receptor concentration dependent infection might apply as well to non-native receptors. We can, however, not exclude that other inherent differences between 293-HissFv.rec and U118MG-HissFv.rec cells are responsible for the observed different outcomes. A receptor-density dependence of infection might actually be beneficial when applying MHV as a virotherapeutic agent, as candidate receptors for this purpose are antigens that are highly over-expressed on cancer cells, but these are often also present in low amounts on normal tissues. Inspired by our successful self-targeting of MHV to an artificial receptor our current work hence aims to achieve similar targeting to a physiologically relevant tumor-specific antigen.

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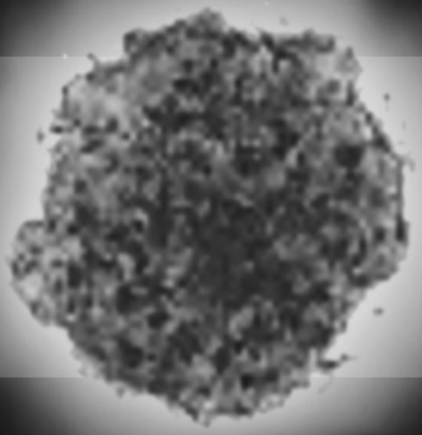
CHAPTER 6 | ANTIBODY-MEDIATED TARGETING OF CORONAVIRAL VECTORS TO THE FC RECEPTOR EXPRESSED ON ACUTE MYELOID LEUKEMIA CELLS



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ABSTRACT

To increase the survival rate of acute myeloid leukemia (AML) patients, there is a serious demand for additional treatment modalities. One of the experimental anti-cancer approaches currently explored involves the use of viral vector-mediated gene transfer. Here we report on a novel IgG-mediated targeting approach to redirect viral vectors to the high affinity Fc receptor (FcγRI; CD64) expressed on AML cells. As a model vector we made use of a mouse hepatitis coronavirus (MHV) encoding a firefly luciferase gene (MHV-EFLM), which normally only infects murine cells via interaction of its spike (S) protein with the cellular receptor murine CEACAM1a. First, we showed that the fusion protein N-CEACAM-Fc composed of the S protein binding domain of CEACAM1a linked to the Fc tail of a human IgG1 antibody, can be used to redirect MHV-EFLM to cells of the FcγRI-positive AML cell line THP-1. Next, we determined whether also IgG antibodies could target MHV-EFLM infection to FcγRI-positive cells of AML origin. Of the different antibodies tested, a murine monoclonal antibody of IgG subclass 2a directed against the S protein, A3.10, was able to successfully mediate selective MHV-EFLM infection of THP-1 cells *in vitro*, resulting in profound luciferase expression. Since many different viruses have been described to exploit antibody-mediated entry mechanisms, the results described here may have major implications for the development of selective viral vector-mediated gene transfer approaches for the treatment of AML.

INTRODUCTION

Despite advances in chemotherapy and radiotherapy, only in 20–30% of patients with acute myeloid leukemia (AML) long-term disease-free survival is achieved after first-line therapy (35, 43). Selective approaches targeting cytotoxic agents to these cells might offer a promising avenue for the specific elimination of residual disease following such therapy (1, 11, 21, 52). One of the currently explored experimental approaches involves the use of viral vector-mediated gene transfer of immunomodulatory genes, which may be capable of acting on residual cancer cells after first-line therapy (27, 31, 44, 47). However, specific targeting of the leukemic cells remains a significant obstacle (16, 21, 25, 30, 41). Several strategies have been examined to target viral vectors to specific receptors expressed on cancer cells. These include pseudotyping, direct modification of the virus protein responsible for cellular attachment, or the use of bispecific adapters (reviewed in (9, 42)).

Circulating virus-specific antibodies normally act to neutralize virus infections. Such antibodies are obviously a complication in the context of virus-based therapies. However, in addition to using the common receptor-dependent virus entry mechanisms, some viruses can, under certain usually pathological conditions, also use the antiviral antibodies for their efficient entry into other target cells. This mechanism, known as Fc receptor-mediated antibody-dependent enhancement (ADE) of viral infection, was proposed by Halstead and Porterfield and colleagues (26, 39, 40). The concept is that virus–antibody complexes can bind to cells expressing Fc receptors (such as cells of the immune system, including macrophages, monocytes, B-cells, neutrophils and granulocytes) through interaction between the Fc portion of the antibody and the Fc receptor on the cell surface, providing a bridge that mediates the attachment of viruses to those cells. Several human Fc receptors exist, each exhibiting a different Ig Fc tail specificity. The high affinity receptor FcγRI efficiently binds to human IgG1; in addition, it cross-reacts with mouse IgG2a (reviewed in (48, 50, 54)). There is a significant correlation of the expression of the FcγRI

and AML cells of type M4 and M5 morphology (according the French-American-British classification system (6)). In addition, the FcγRI is absent on pluripotent stem cells and on CD34+ hematopoietic progenitor cells (4, 36). Thus, the FcγRI may represent an appropriate AML target receptor for cytotoxic agents (52) considering that the normal FcγRI-positive immune effector cells repopulate after termination of the FcγRI-targeted therapy.

We have recently explored various strategies for retargeting animal coronaviruses to human cells. These studies were aimed at developing these viruses into therapeutic agents, particularly against cancer cells. We generated bispecific adapter molecules that could mediate infection of appropriate human cancer cells when combined with the virus before or during inoculation. Such molecules also appeared to function when their coding sequence was incorporated into the viral genome, creating thereby self-targeted viruses. One of the adapters consisted of a single-chain variable antibody fragment (scFv) binding to the viral spike (S) protein and a moiety binding to the particular target cell receptor (58). The successful targeting by these adapters revealed the potential of viral antibodies as mediators for targeting of viruses to cells expressing Fc receptors.

Here, we wanted to further explore the use of antibody-mediated entry mechanisms for viral vector-mediated gene transfer into AML cells. Because the bispecific single chain adapter mentioned above was composed of antibody fragments directed against the feline infectious peritonitis virus (FIPV), the only coronavirus so far shown to exhibit ADE (10, 29), we used as a model vector a mouse hepatitis coronavirus (MHV) encoding firefly luciferase (MHV-EFLM) (13). This virus normally infects only murine cells via interaction of S protein with the cellular receptor murine CEACAM1a. To determine whether MHV infection could be redirected to the human FcγRI expressed on AML cells, we first made use of a chimeric protein consisting of the N-domain of mCEACAM1a fused to a human IgG1 Fc tail (N-CEACAM-Fc) (22). This protein was tested for its ability to redirect MHV-EFLM-mediated luciferase expression to THP-1 cells, which lack mCEACAM1a expression but express

high amounts of FcγRI (16, 18). Next, we analyzed several anti-MHV monoclonal antibodies for their ability to redirect MHV-EFLM infection to the FcγRI-positive THP-1 cells. The results open new perspectives for the development of targeted viral vector-based therapies for the treatment of AML.

MATERIALS AND METHODS

Viruses, cells, and antibodies.

A recombinant MHV-EFLM (MHV-A59 containing the firefly luciferase gene between the E and M genes) stock was produced as described before (13). MHV-A59 and MHV-EFLM stocks were produced and titrated on murine LR7 cells (32). The recombinant vaccinia virus vTF7-3 containing the bacteriophage T7 RNA polymerase gene was used as a T7 RNA polymerase source for the T7 promoter driven production of the adapter proteins in OST7-1 cells (17). OST7-1 (17) (obtained from B. Moss), THP-1 (ATCC), LR7 (32), and BHK-21 cells (ATCC) were all grown in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, Verviers, Belgium), containing 10% FCS, 100 IU of penicillin/ml, and 100 µg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). The rabbit antiserum K135 raised against purified MHV was described previously (45). The murine monoclonal antibody (MAb) directed against the MHV M protein, J1.3 (20), and the anti-MHV S MAbs A1.3, A1.4, A1.9, J1.16, and A3.10 (24), were all obtained from the corresponding hybridoma cell lines. For each antibody the culture medium was harvested and centrifuged to clear it from cells and debris, and stored in aliquots at – 20 °C for further use. Anti-human CD64 (clone 10.1; Biolegend, San Diego, Ca, USA) is a mouse MAb directed against the human FcγRI.

Production and analysis of N-CEACAM-Fc and HE^0-Fc proteins.

N-CEACAM-Fc is a disulfide-linked dimer consisting of the N-terminal domain of mCEACAM1a linked to the hinge, CH2, and CH3 regions of human IgG1. HE^0-Fc is a similar protein construct, containing instead of the N-CEACAM domain, the ectodomain of the hemagglutinin-esterase (HE) of MHV strain DVIM in which the enzymatic site was inactivated by mutation (S40 → T40). To produce these proteins 293E cells (1000 ml) were transfected with 1 mg pCEP4-N-CEACAM-Fc or pCEP4-HE^0-Fc using polyethylenimine (PEI). The medium was harvested 5 days after transfection, centrifuged to remove cell debris, incubated overnight with protein A sepharose beads, and subjected to elution by incubation with 0.2 M glycine, pH 2.8 for 5 min. After centrifugation the supernatant was neutralized by adding Tris-HCl, pH 9.0, aliquoted, and stored at – 20 °C for further use. Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequent staining of the gel with Commassie Brilliant Blue staining showed that the proteins were essentially pure (data not shown). Protein concentrations were determined by measuring the OD280.

Analysis of MHV-EFLM neutralization on murine LR7 cells.

MHV-EFLM (MOI 1) was preincubated for 1 h at 4 °C with a series of dilutions of N-CEACAM-Fc or with the different anti-MHV MAbs diluted 20 times in serum-free culture medium and inoculated on LR7 cells in triplicate. At 7 h after inoculation the cells were lysed and luciferase expression measured.

Determination of the optimal amounts of N-CEACAM-Fc and MAb for the targeting of MHV to leukemic THP-1 cells.

An amount of 1 x 10^5 THP-1 cells per 0.32-cm^2 well were inoculated with 1 x 10^4, 1 x 10^5, or 1 x 10^6 $TCID_{50}$ units of MHV-EFLM mixed with dilution series of N-CEACAM-Fc or of the different anti-MHV MAbs. At several time points after inoculation luciferase lysis buffer was added to the cell culture and luciferase expression was measured. Based on the results, in all further targeting experiments we used N-CEACAM-Fc protein and MAb A3.10 at a concentration of 5 nM and diluted 20 times in serum-free culture medium, respectively.

Determination of luciferase expression.

At the indicated time points the LR7 culture media were removed, and the cells were lysed using the appropriate buffer provided with the firefly assay system (Promega). Intracellular luciferase expression was measured according to the manufacturer's instructions, and the relative light units (RLUs) were determined with a LUMAC biocounter M2500. For the THP-1 cells, 100 µl of a 2 x lysis buffer was added to 100 µl of the THP-1 cell suspension, after which total luciferase expression was measured.

MAb-Spike binding assay.

In order to determine whether the anti-MHV MAbs were able to bind to the MHV-A59 S protein, radio-immunoprecipitations were performed. Briefly, the MHV-A59 S protein was expressed in OST7-1 cells using the vaccinia virus vTF7-3 expression system (17) and metabolically labeled with ^{35}S amino acids, as described previously (12). Subsequently, the cells were lysed and prepared for immunoprecipitation using equal volumes of the different anti-MHV MAbs. The immunoprecipitates were analyzed by SDS-PAGE containing 10 % polyacrylamide, as described before (12).

FcγRI blocking experiments.

To determine whether N-CEACAM-Fc and MAb A3.10 interacted directly with the human FcγRI, 1 x 10^5 THP-1 cells per 0.32-cm^2 well were incubated with or without 50 µl serum-free culture medium containing 2 µg of MAb anti-human CD64, for 30 min at 4 °C. Next, a total volume of 50 µl was added, containing 1 x 10^6 $TCID_{50}$ units MHV-EFLM in the presence or absence of N-CEACAM-Fc or of MAb A3.10. As controls we used 5 nM HE^0-Fc or MAb A1.4 diluted 20 times. After an incubation of 24 h the cells were lysed and luciferase expression was measured.

FcγRI transient transfection.

The expression plasmid pRc/CMV-FcγRIa was a kind gift of Dr. Janet Allen (Physiological Laboratory, Cambridge, U.K.). It was constructed by inserting the FcγRIa cDNA from clone p135 (2) as HindIII/NotI fragment into pRc/CMV (Invitrogen) and was used to transiently express the FcγRI on BHK-21 cells following transfection with Lipofectamine PLUS reagent (Life Technologies, Ltd., Paisley, United Kingdom), according to the manufacturer's protocol. The FcγRI expression was confirmed by immunostaining using the anti-human CD64 Mab 10.1 diluted 1:100 in PBS containing 5 % FBS, followed by Goat-anti-Mouse peroxidase diluted 1:200 in PBS containing 5 % FBS (data not shown). At 48 h after transfection the BHK-21 cells were inoculated with MHV-EFLM (MOI 10) in the presence or absence of N-CEACAM-Fc or of MAb A3.10. At 24 h after inoculation the inoculum was removed and the cells were lysed and luciferase expression measured.

Anti-MHV immunofluorescence assay.

1 x 10^5 THP-1 cells were inoculated with 1 x 10^6 $TCID_{50}$ units of MHV-A59 mixed with N-CEACAM-Fc or MAb A3.10, in the presence or absence of 10 μM MHV infection-inhibiting peptide mHR2. The inoculation was continued for 24 h, after which the cells were fixed using PBS containing 3.7 % paraformaldehyde, permeabilized by PBS containing 1 % triton-X100, and subsequently incubated with K135 anti-MHV serum diluted 1:300 in PBS containing 5 % FBS, followed by Swine-anti-Rabbit FITC (DAKO, Glostrup, Denmark) diluted 1:200 in PBS containing 5 % FBS. The cells were analyzed using a fluorescence microscope.

RESULTS

N-CEACAM-Fc binds to MHV and targets the virus to human THP-1 cells.

Previous results described by Gallagher showed that N-CEACAM-Fc has the ability to bind to the MHV spike protein (22). To confirm this we analyzed whether the N-CEACAM-Fc protein can block MHV-A59 infection of murine cells. Murine LR7 cells were inoculated at an MOI of 1 with MHV-EFLM (MHV strain A59 expressing a firefly luciferase gene) in the presence of various concentrations of N-CEACAM-Fc protein. After 7 h the cells were lysed and luciferase activity was measured. As shown in Fig. 1A, N-CEACAM-Fc efficiently neutralized MHV-EFLM infection of murine LR7 cells in a concentration-dependent manner, indicating that the N-CEACAM-Fc protein blocked the MHV-EFLM entry into murine cells through interaction with the

spike protein.

Our next step was to determine whether N-CEACAM-Fc when bound to the virus could mediate targeting of the virus to cells carrying an appropriate Fc receptor molecule. To this end, human THP-1 cells, which express high levels of FcγRI, were inoculated at different MOI's with MHV-EFLM in the absence or presence of different concentrations of N-CEACAM-Fc protein. After 20 h the cells were lysed and luciferase activity was measured. Fig. 1b shows that N-CEACAM-Fc has the ability to redirect MHV-EFLM-mediated luciferase expression to the otherwise refractory THP-1 cells. Furthermore, a clear dose-dependent luciferase expression was observed, which depended both on the amount of virus used and on the added amount of N-CEACAM-Fc. Interestingly, the maximum luciferase expression measured in THP-1 cells was achieved at a subneutralizing concentration of N-CEACAM-Fc as can be inferred by comparing the results of the targeting and neutralization carried out at an MOI of 1 (Fig. 1A and 1B).

To determine the time range of the targeted luciferase expression, THP-1 cells were inoculated at an MOI of 10 with MHV-EFLM in the presence of 5 nM N-CEACAM-Fc. At several time points after inoculation the cells were lysed and luciferase expression was measured. Fig. 1C shows that the MHV-EFLM infection mediated by N-CEACAM-Fc resulted in a rapid onset of luciferase expression, reaching a maximum at 30 h after inoculation and gradually decreasing thereafter, which may be explained by loss of cell viability due to virus replication.

Antibody-mediated targeting of MHV to THP-1 cells.

We used a set of available monoclonal antibodies to test their capacity in targeting MHV to THP-1 cells. The set consisted of antibodies with known MHV protein specificity and IgG subclass (20, 24), as indicated in Fig. 2A. To determine their binding to virions we analyzed their neutralization capacity. The antibodies were preincubated with MHV-EFLM and inoculated onto murine LR7 cells, after which the expression of luciferase was measured. It appeared that all antibodies inhibited

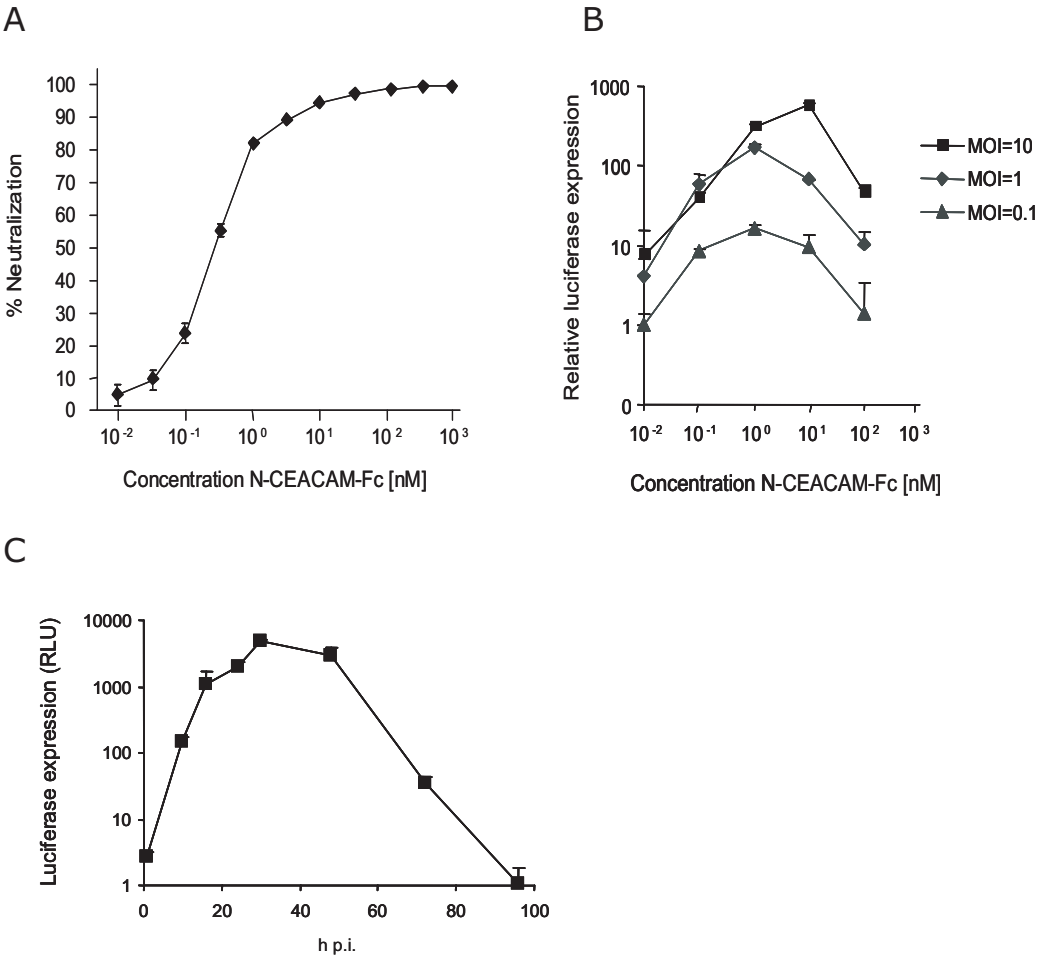


Fig. 1 N-CEACAM-Fc neutralizes MHV-EFLM infection of murine LR7 cells and targets MHV-EFLM to human THP-1 cells. (A) MHV-EFLM was inoculated at an MOI of 1 onto murine LR7 cells in the absence or presence of various concentrations of N-CEACAM-Fc protein. At 7 h after inoculation the cells were lysed and luciferase expression measured. Shown is the percentage neutralization, calculated as the decrease in luciferase expression relative to inoculations in the absence of N-CEACAM-Fc protein. The data represent the average of an experiment performed in triplicate. The error bars indicate the standard deviation. (B) THP-1 cells were inoculated with MHV-EFLM at different MOIs in the absence or presence of various concentrations of N-CEACAM-Fc protein. At 20 h after inoculation the luciferase expression was determined. Shown is the relative luciferase expression, calculated as the increase in luciferase activity relative to inoculations carried out in the absence of N-CEACAM-Fc protein. All data shown represent the average and standard deviation of an experiment performed in triplicate. (C) To determine the time range of MHV-EFLM-mediated luciferase expression 1 x 10^6 $TCID_{50}$ units of MHV-EFLM were inoculated onto 1 x 10^5 THP-1 cells in the presence or absence of 5 nM N-CEACAM-Fc. At several time points after inoculation intracellular luciferase expression was measured. Shown are the average data from an experiment performed in triplicate, and error bars indicating the standard deviation.

the luciferase expression, except for the M-specific MAb J1.3, known to require complement for neutralization (19), and the S-specific MAbs A1.3, A1.9, and J1.16, which have previously been described to be incapable of neutralizing MHV-A59 infection (55).

In order to determine whether the antibodies were able to bind to the S protein, their capacity to precipitate the MHV-A59 S protein was analyzed. The protein was expressed and radiolabeled using the vaccinia virus expression system after which the OST7-1 cells were lysed and the cell lysate was used for immunoprecipitation with the different MAbs. The immune complexes were analyzed by SDS-PAGE (Fig. 2B), which showed that all S-specific antibodies were able to precipitate the MHV-A59 S protein, in contrast to the M protein specific MAb J1.3. The efficiencies with which the antibodies precipitated the S protein varied considerably, which might - at least in part - be accounted for by variations in the concentrations of the MAb in the hybridoma culture supernatants.

The anti-MHV antibodies were finally analyzed for their capacity to redirect MHV to human THP-1 cells. Serial dilutions of the different anti-MHV MAbs were mixed with 1 x 10^5 $TCID_{50}$ units MHV-EFLM and inoculated onto 1 x 10^5 THP-1 cells. At 16 h after inoculation luciferase expression was measured. Interestingly, of all the antibodies tested, only MAb A3.10 was able to successfully target MHV-EFLM to THP-1 cells; none of the other MAbs could mediate infection resulting in luciferase expression (Fig. 2C).

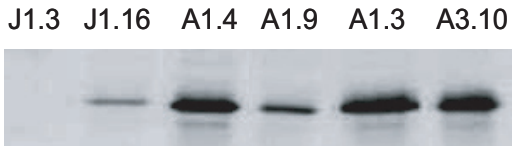
Analysis of the targeted MHV infection specificity.

The N-CEACAM-Fc fusion protein contains a human IgG1 Fc tail, that of monoclonal antibody A3.10 is of mouse IgG2a origin. Both Fc tails have been described to bind to human FcγRI (54). To further confirm their binding to this receptor, we analyzed whether the targeted infections mediated by N-CEACAM-Fc and MAb A3.10 could be blocked by preincubating the THP-1 cells with the MAb 10.1, which is directed against CD64/FcγRI. As specific controls we included MAb 1.4 (murine IgG2a isotype)

A

Antibody	Immunizing antigen	IgG subclass	Entry inhibition of MHV-EFLM in murine LR7 cells
J1.3	MHV-M	IgG2b	-
J1.16	MHV-S2	IgG2b	-
A1.4	MHV-S1	IgG2a	+
A1.9	MHV-S1	IgG3	-
A1.3	MHV-S1	IgG3	-
A3.10	MHV-S1	IgG2a	+

B



C

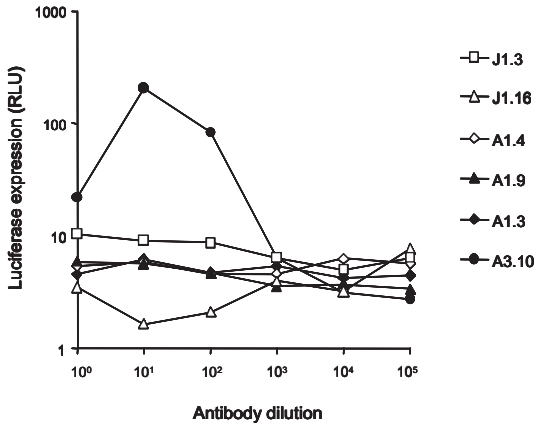


Fig. 2 Analysis of antibody-mediated targeting of MHV to THP-1 cells. (A) Features of the MAbs used: protein specificity, murine IgG subclass, and MHV-EFLM neutralization capacity as determined on murine LR7 cells (+: > 50 % decrease in luciferase expression relative to inoculations in the absence of antibody). (B) The S protein was expressed and radiolabeled in OST7-1 cells. Equal portions of the cell lysate were used for immunoprecipitation using equal volumes of the MAb hybridoma supernatants. Shown is the radiograph with the bands corresponding to the MHV S protein. (C) MHV-EFLM (1 x 10^5 $TCID_{50}$ units) was inoculated onto 1 x 10^5 human THP-1 cells in the presence of the different anti-MHV MAbs at various dilutions. At 16 h after inoculation luciferase lysis buffer was added to the cell suspension and total luciferase expression was measured. The data are from a representative experiment.

and HE^0-Fc, a human IgG1-Fc tailed hemagglutinin-esterase (HE) protein derived from MHV strain DVIM. After pretreatment of the cells in the presence or absence of the anti-FcγRI MAb 10.1, they were inoculated and incubated for 24 h, after which the cells were lysed and luciferase expression was measured. As Fig. 3A shows, preincubation of the cells with the anti-FcγRI antibody reduced the N-CEACAM-Fc and MAb A3.10 mediated luciferase expression by 100-1000 times, indicating that these infections are, solely or primarily, targeted towards the FcγRI. No infection was established when using the HE^0-Fc protein while MAb A1.4 appeared to mediate a very low but significant luciferase expression. The results indicate that specific interactions with the S protein are essential.

To further confirm the FcγRI specificity, BHK-21 cells were transfected with

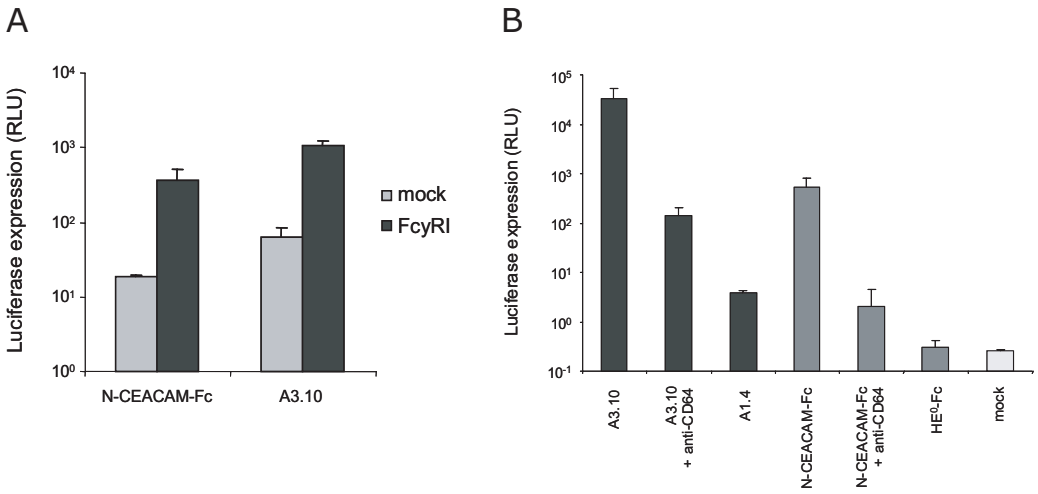


Fig. 3 Analysis of the antibody-mediated infection specificity. (A) MHV-EFLM was mixed with N-CEACAM-Fc or MAb A3.10, and inoculated onto THP-1 cells that had been pre-incubated for 30 min at 4 °C in the presence or absence of anti-FcγRI MAb. In parallel, inocula were prepared by mixing MHV-EFLM with HE^0-Fc or MAb A1.4 and inoculated onto THP-1 cells. At 24 h after inoculation luciferase lysis buffer was added and total luciferase expression measured. The data represent the average luciferase expression of an experiment performed in triplicate. The error bars show the standard deviation. (B) BHK-21 cells were transfected with an FcγRI expression construct or, as a control, without plasmid DNA. After 48 h, the transfected BHK-21 cells were inoculated with MHV-EFLM mixed either with N-CEACAM-Fc or with MAb A3.10 and incubated for 24 h, after which the cells were lysed and intracellular luciferase expression measured. The data represent the average luciferase expression of an experiment performed in triplicate. The error bars show the standard deviation.

an FcγRI expression construct or, as a control, without plasmid DNA. After 48 h, the transfected BHK-21 cells were inoculated with MHV-EFLM mixed either with N-CEACAM-Fc or with MAb A3.10 and incubated for 24 h, after which the cells were lysed and luciferase expression measured. The expression of the FcγRI significantly enhanced the susceptibility of the BHK-21 cells to N-CEACAM-Fc and MAb A3.10 mediated infection as measured by the luciferase expression (Fig. 3B).

Analysis of the amount of coronavirus infected THP-1 cells.

To determine the efficacy of the FcγRI-mediated infections we determined the amount of targeted cells. THP-1 cells were inoculated with MHV-A59 at an MOI of 10 in the presence or absence of N-CEACAM-Fc or MAb A3.10. After 24 h of incubation the cells were analyzed for the presence of coronavirus proteins by immunofluorescence using MHV-specific antibodies. As shown in Fig. 4, infected cells (> 20 %) were observed only in the presence of N-CEACAM-Fc protein or MAb A3.10. In addition, we determined whether the infection could be blocked by the fusion-inhibiting peptide mHR2 (8). To this end, THP-1 cells were inoculated in the presence or absence of 10 μM of mHR2 peptide. As shown in Fig. 4, the FcγRI-mediated infection of the THP-1 cells was efficiently blocked, indicating that normal coronavirus entry mechanisms are involved in the FcγRI targeted entry of MHV-A59 into THP-1 cells.

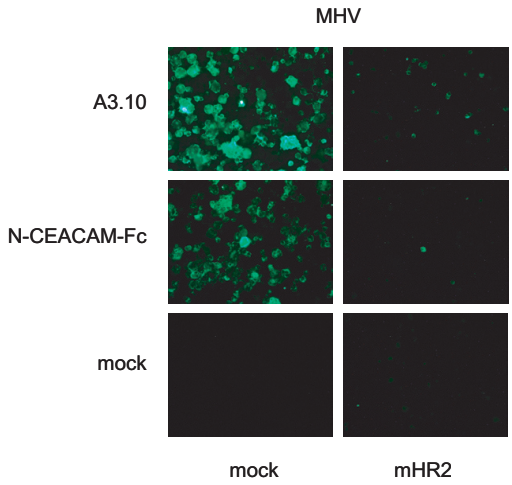


Fig. 4 Visualization of the FcγRI-mediated MHV infection of leukemic THP-1 cells. An amount of 1 x 10^6 $TCID_{50}$ units MHV-A59 was mixed with N-CEACAM-Fc or MAb A3.10 and inoculated onto 1 x 10^5 THP-1 cells in the presence or absence of 10 μM MHV entry-inhibiting peptide mHR2. After 24 h the cells were analyzed for the presence of coronavirus proteins by immunofluorescence using MHV-specific antibodies.

DISCUSSION

Adult acute myeloid leukemia (AML) is a cancer of the blood and bone marrow. It is the most common type of acute leukemia in adults. AML is also known as acute myelogenous leukemia, acute myeloblastic leukemia, acute granulocytic leukemia, and as acute nonlymphocytic leukemia. AML is not a single disease, but refers to a number of related diseases each distinguished by unique cytogenetic markers, which in turn help determine the most appropriate treatment. However, despite advances in chemotherapy, radiotherapy and stem cell therapy, the majority of patients still die from their disease (35, 43). Failure of standard therapy to induce sustained remission in the majority of patients with acute myeloid leukemia (AML) is related in part to the tumor's ability to evade destruction by the immune system. One strategy to enhance the immunogenicity of malignant hematopoietic cells has been to increase the expression of stimulatory molecules. Various groups have explored the use of gene transfer to increase the immunostimulatory protein expression in AML cells (14, 15, 27, 47). Clearly, for the delivery of immunostimulatory genes specific transduction of AML cells is of importance. For most viral gene transfer vectors AML cells do not express a proper specific receptor, resulting in either inefficient or aspecific gene transfer (25, 38, 44, 53).

The FcγRI is highly expressed on AML cells of type M4 and M5 morphology (according the French-American-British classification system (6)). Acute myelomonocytic leukemia (AMML; FAB Classification M4) is characterized by the proliferation of neutrophil and monocyte precursors. Patients usually present with anemia and thrombocytopenia. This type of AML comprises approximately 15-25 % of cases of AML. Acute monoblastic and acute monocytic leukemia (FAB classifications M5a and M5b, respectively) are AMLs in which ≥ 80 % of the leukemic cells are of a monocytic lineage. Acute monoblastic leukemia comprises 5-8 % of cases of AML and occurs most commonly in young individuals. Acute monocytic leukemia comprises 3-6 % of cases and is more common in adults.

Common clinical features for these acute leukemias include bleeding disorders, extramedullary masses, cutaneous and gingival infiltration, and central nervous system involvement. Median actuarial disease-free survival for acute monocytic leukemia has been reported to be in the order of 21 months (5, 6, 34).

Here we analyzed whether the characteristic expression of the FcγRI on cells of certain types of AML might be exploited for the specific targeting of viral gene transfer vectors to these cells. As a model vector we used the coronavirus MHV-EFLM, which is normally unable to infect human AML cells due to lack of the appropriate cellular receptor, but which we showed in previous studies to be efficiently targeted towards cancer cells via adapter proteins consisting of scFv antibody fragments (Chapter 4). Here we investigated whether proteins that bind on the one hand to the MHV S protein and on the other hand to the FcγRI protein could function as bridging molecules redirecting the virus to cells of the AML cell line THP-1. We found that the murine IgG2a MAb A3.10 enabled efficient MHV-EFLM infection of THP-1 cells in a dose-dependent way. Furthermore, the infection was FcγRI specific and resulted in MHV-EFLM-mediated luciferase expression.

Interestingly, not all anti-MHV MAbs tested were able to redirect MHV-EFLM to the FcγRI expressed on THP-1 cells. It appeared that successful antibody-mediated targeting of the coronaviral vector to the FcγRI has two different kinds of requirements involving the interactions of the antibody with the virus S protein and with the FcγRI protein. Binding of the antibody to the virus S protein per se appeared not to be the sole requirement for successful targeting. We found that of all S-binding antibodies only MAb A3.10 mediated efficient transduction of THP-1 cells. Results from our previous studies suggest that for successful coronavirus-mediated transduction to occur a specific conformational change of the S protein is required (58). Thus, only certain antibodies capable of inducing such selective conformational changes in the S protein would be suited for the antibody-mediated targeting approach. In addition, the binding of these antibodies to the S protein should not interfere with subsequent steps in the virus entry process. It seems that

MAb A3.10 meets all of these requirements. It would be of interest to determine whether other MHV S protein-specific antibodies can compete with MAb A3.10 for the binding to the virus S protein and whether these antibodies are also able to mediate coronavirus entry in THP-1 cells. It also became apparent that binding of the antibody to the S1 domain is also not the only prerequisite, since MAb A1.4 (S1-binding, IgG2a subtype), as opposed to A3.10 (S1-binding, IgG2a subtype), was not able to mediate antibody-targeted gene transfer. Mapping of the exact S protein binding sites of MAb A3.10 - and relating this to the CEACAM1a receptor-binding domain - may give more insight in the binding requisites for the induction of the subsequent conformational changes essential for successful entry of the viral vector into the cell.

In addition to the interaction with the virus S protein, an interaction of the antibody with the FcγRI expressed on the AML cells is required. We showed that antibody A3.10 of murine subclass IgG2a was able to redirect MHV-EFLM-mediated luciferase expression to the THP-1 cells, in contrast to MAbs of other subclasses. In addition to the high-affinity FcγRI, THP-1 cells also express the low-affinity FcγRII (16), although this did not result in efficient transduction mediated by other subclasses than that of human IgG1 or murine IgG2a. Additional specificity studies showed that the expression of the FcγRI on BHK-21 cells was sufficient to allow antibody-mediated gene transfer in these otherwise refractory cells. In conclusion, the antibody seems to require the capacity to induce the proper conformational changes in the S protein and to efficiently bind to the high-affinity FcγRI, as is the case for antibodies of human subclass IgG1 and murine subclass IgG2a (48, 50, 54).

The use of gene transfer to achieve eradication of leukemia has been described for different viral vector systems, i.e. lentiviruses (31, 47), retroviruses (27, 59), adenoviruses (25), AAV (56, 57), and HSV-1 amplicons (51). One of the major advantages in the context of immuno-modulatory gene transfer is the pre-clinical observation of anti-leukemic bystander effects suggesting that not every cell needs to be transduced, as was shown for e.g. B7.1 (27, 47), IL-12 (15), TNF-α and GM-CSF (14, 47). In addition, many viral vectors may themselves have

immuno-stimulatory properties, which upon infection of the cancer cell may lead to increased antigen presentation and stimulation of an anti-cancer immune response (reviewed in (7)). In addition, various viruses have been described to use Fc receptors as an alternative entry pathway; examples include lentivirus (37), herpes simplex virus-1 (23), poliovirus (3), yellow fever virus (46), influenza A virus (49), alphavirus (33), and coronavirus (28). Therefore, the use of antibodies to target viral vectors to AML cells may be applied to other viral vector systems. Interestingly, for several of these viruses specific Fc receptor targeting antibodies have already been identified. Compared to other viral vector targeting strategies, the use of IgG antibodies may offer easy of-the-shelf targeting potential for the systemic delivery of these viral vectors. Further studies on the antibody-mediated targeting of viral vectors to Fc receptors expressed on cells of certain types of AML are warranted.

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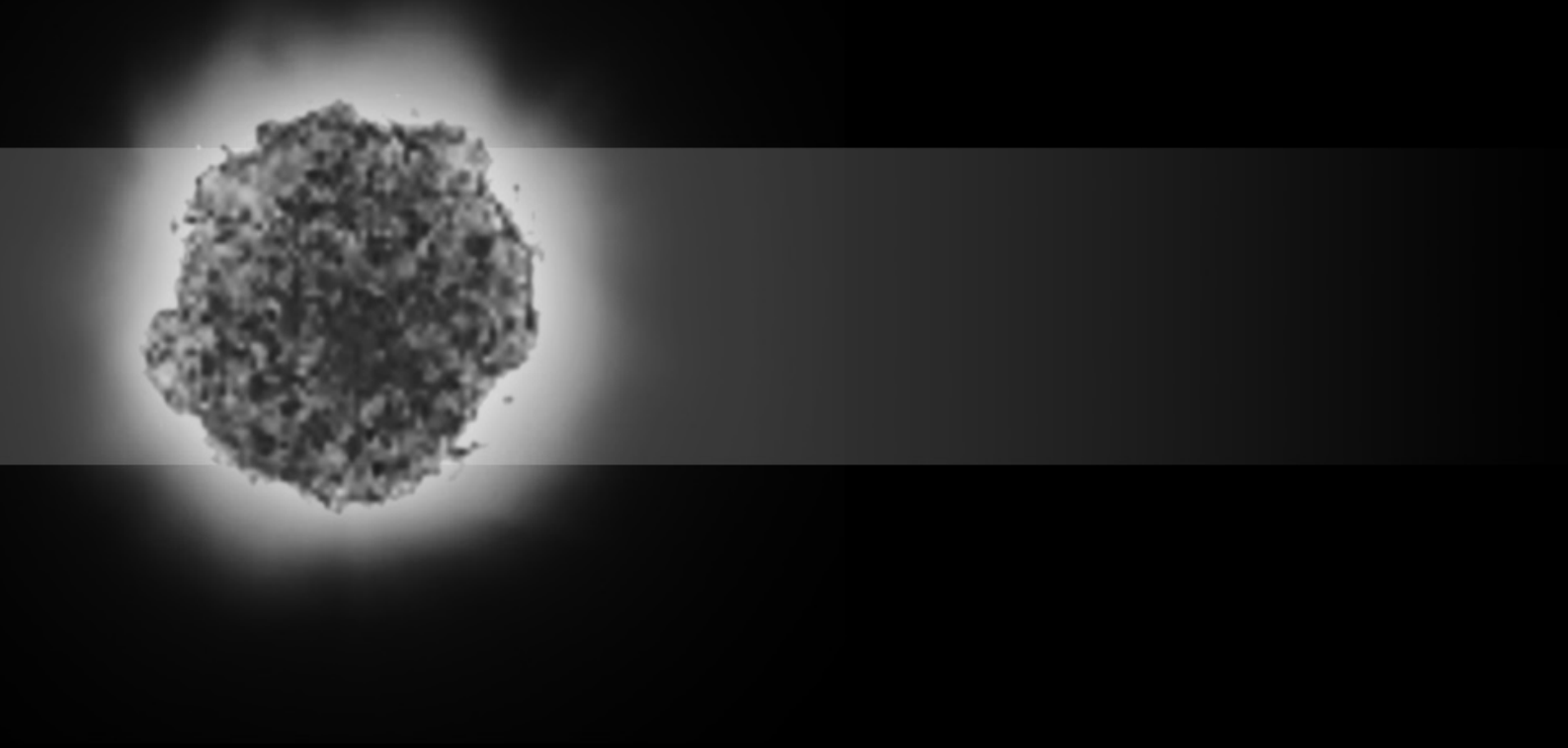
CHAPTER 7 | SUMMARIZING DISCUSSION



CHAPTER 7: SUMMARIZING DISCUSSION

Cancer is still one of the most common causes of death. Various unconventional strategies are currently being explored to back-up the standard therapies such as surgery, chemo-, and radiotherapy. One of these strategies involves the use of viruses to kill cancer cells. Viruses may be used in various ways to eradicate cancer cells. They may be used either as anti-cancer gene transfer vehicles, or as immunostimulatory anti-cancer vaccines, or as direct oncolytic agents. Currently various viruses are under investigation worldwide. Apart obviously from the safety aspects related to using viruses as therapeutic agents, the most important considerations are the anti-tumor efficacy and tumor-selectivity (Chapter 1).

In this thesis project we investigated whether non-human coronaviruses can be developed into oncolytic viruses. The coronaviruses MHV-A59 and FIPV79-1146 do not have the ability to infect and kill human cells, simply because the virus receptors are only expressed on certain cells of their native hosts, the mouse (16) and the cat (34), respectively. We showed that once the appropriate receptor is expressed on human cancer cells, they are rendered susceptible to the non-human coronaviruses and are rapidly destroyed through virus replication and the subsequent formation of syncytia. These results show that at least *in vitro* the tropism is determined by the specific spike-receptor interactions (Chapter 2).

This prompted us to analyze whether the coronavirus spikes can be modified by insertion of ligands binding to antigens over-expressed on human cancer cells. This direct targeting approach was started with the insertion of RGD-4C peptides at various positions in the MHV or FIPV spike S1 domain, responsible for receptor binding. It was previously described that insertion of the RGD-4C peptide in the adenovirus HI loop, resulted in adenovirus targeting to $\alpha V\beta 3$ integrins, over-expressed on a variety of cancer cells (10). For the non-human coronaviruses we were able to show that it is possible to insert RGD-4C peptides into certain locations of the MHV or FIPV spike S1 domain. In addition, we were able to grow

some of these viruses on murine LR7 or feline FCWF-4 cells, suggesting that the functioning of the spike S2 domain, responsible for fusion, was not dramatically affected by some of these insertions. However, no infection was achieved when inoculating these viruses on human cancer cells expressing $\alpha V\beta 3$ integrins (unpublished data). It remains to be investigated whether the viruses containing the RGD-4C motif in their spike proteins lack the ability to bind to integrins, or whether a subsequent post integrin-binding process is affected.

As an alternative virus targeting method, we therefore first took the indirect targeting approach, making use of adapters binding on the one hand to the coronavirus and on the other hand to a receptor over-expressed on human cancer cells. Several bispecific adapters were produced and enabled redirection of both FIPV and MHV to human cancer cell receptors. In order to redirect FIPV to human cancer cells we produced the bispecific single-chain antibody 23F-425 binding to the FIPV spike protein and to the human EGFR. We showed that the bispecific single-chain interacts with the EGFR expressed on human cancer cells, and that a combined interaction with the FIPV spike ectodomain was essential for infection. This was further shown by the ability to redirect fMHV (21) - an MHV mutant carrying the FIPV spike ectodomain - but not MHV to the EGFR (Chapter 3). Interestingly, whereas the anti-spike bispecific single-chain 23F-425 was able to redirect fMHV to human cancer cells, a bispecific single-chain directed against the MHV M protein ectodomain - J1.3-425 produced under similar conditions as 23F-425 - could not redirect fMHV to human cancer cells (unpublished data). These results suggest that for the indirect coronavirus targeting approach binding to the spike protein is essential for the induction of the subsequent fusion process resulting in infection.

These observations also suggest that for adapter-mediated coronavirus infections similar conformational changes occur in the spike protein upon binding of the adapter protein as after its binding to the receptor during natural infections (Fig. 1). Binding of the virus-adapter complex to a cellular target receptor may further facilitate the insertion of the fusion peptide into the cell membrane. Interestingly,

inoculation of the coronavirus after preincubation with the adapters and subsequent purification of the virus-adapter complex by sucrose cushion centrifugation, still resulted in infection of the target cells, indicating that the adapter binding did not inactivate the virus (unpublished results). Therefore, adapters bound to the virus seem to “freeze” the spike in a stable fusion-active conformation, ready to proceed its structural rearrangement upon binding of the virus-adapter complex to a cellular target receptor (Fig. 1).

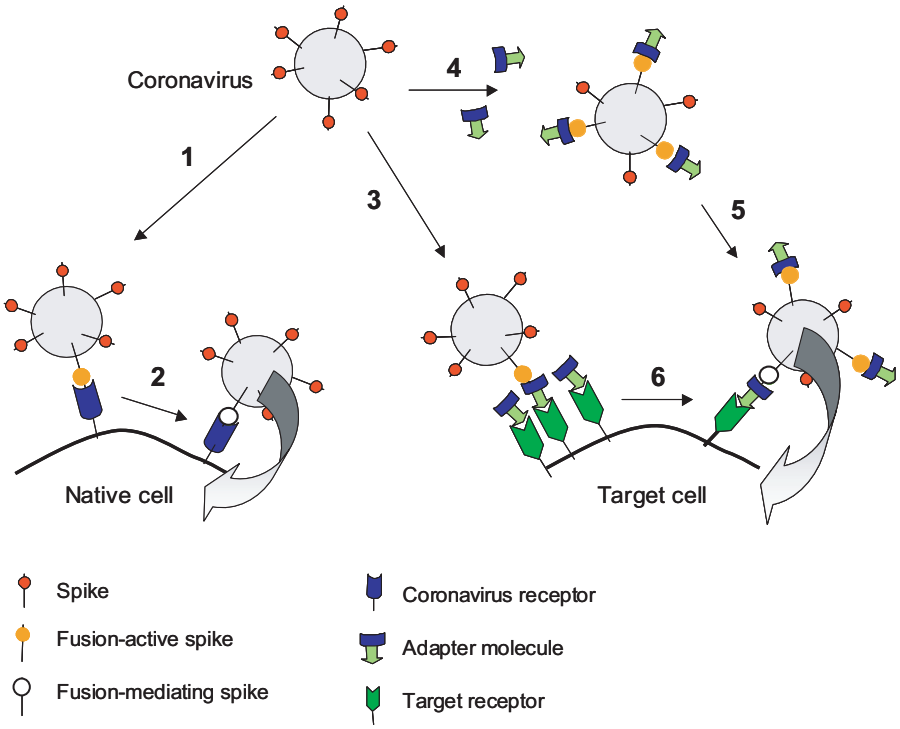


Fig. 1 Schematic overview of the different stages of adapter-mediated coronavirus entry. (1) Under normal conditions the coronavirus spike binds to the cellular receptor, after which conformational changes in the S2 domain are induced leading to a fusion-active spike. (2) The fusion peptide interacts with the cellular membrane and subsequently the S protein undergoes conformational changes leading to the coalescence of the heptad repeat regions and the merging of the virus envelope and the cellular membrane. (3) In the context of the indirect targeting approach, the virus spike may bind to adapters anchored on target receptors, (6) resulting in a similar entry process as described for the native entry process. (4) Alternatively, the coronavirus spike protein binds free adapter proteins, (5) resulting in fusion-active spike proteins, which mediate the fusion process upon binding of the virus-adapter complex to a cellular receptor.

To enable more practical use of the oncolytic capacity of the non-human coronaviruses, establishment of multi-round targeted infection is a prerequisite. Therefore the genes encoding the adapter proteins were introduced into the viral genomes, thereby providing the progeny virus with newly produced adapter protein during every round of replication. As a safety measure the adapter genes were introduced in attenuated coronaviruses lacking certain virulence genes. First, it was determined whether the bispecific single-chain 23F-425 gene could be introduced at various locations in the FIPVdel3abc, fMHVdel2aHE, and MHVdel2aHE genomes. Unfortunately, we observed that the 23F-425 adapter sequence was not tolerated in these virus genomes. Rapid loss of the adapter insert during viral growth was observed for all the recombinant viruses produced. This intolerance did not appear to result from an interaction of the adapter protein with the virus spike proteins during replication, since the FIPV adapter 23F-425 gene construct was also unstable in the background of the MHVdel2aHE genome (unpublished data). Studies on the stability of foreign genes in coronavirus genomes have indicated that the stable maintenance of foreign genes is dependent on the nature of the heterologous gene, on the particular coronavirus vector used, and on the particular site of gene insertion (6). Since we analyzed both MHV and FIPV vectors, and inserted the 23F-425 gene at various locations, the loss of the adapter sequence seems to be due to specific features of the 23F-425 adapter sequence itself.

The observations prompted us to develop bispecific adapters of a different nature. This time, the adapters were composed of the MHV spike-binding receptor domain (soR) of mCEACAM1a fused to various cancer cell-binding ligands. These ligands included the synthetic peptides 6His (Chapter 5), RGD-4C and NGR (unpublished data), the single-chain 425 (Chapter 4), or the biological factors EGF and SDF1a (unpublished data). Of these adapters the soR-RGD-4C and soR-NGR adapters appeared unable to redirect MHV to cancer cells. All others showed efficient targeting of MHV to specific receptors expressed on human cancer cells. These results suggest that RGD-4C (10, 31) and NGR (25, 29) - targeting

integrins and aminopeptidase N, respectively - are no useful ligands for coronavirus targeting and may explain the inability to target coronaviruses through insertion of RGD-4C peptides into the spike protein. Therefore, as a proof of principle of the direct targeting approach, it might be of considerable interest to introduce the 6His peptide into the spike protein at locations that tolerated the RGD-4C peptide.

Furthermore, the specific composition of the adapter protein appeared to be important for the targeting efficiency observed. For some of the adapters the presence of an Ig hinge (-h-) region affected the adapter-mediated targeting. It depended on the target receptor whether the hinge region had a beneficial or an adverse effect. The differences in the targeting efficiency attributed to the presence of the Ig hinge region may be due to a dimerization effect mediated by the hinge region, or might be explained by the spacer effect of the hinge region. Others showed for the adenovirus that multimerization of targeting adapter proteins could also affect the adapter-mediated targeting efficiency (20). In addition, for some of the adapters that we produced the location of the myc/6His tag in the adapter protein influenced the outcome of the coronavirus targeting efficiency. In conclusion, to optimize the adapter-mediated targeting of viruses to certain receptors expressed on human cancer cells it is of importance to determine the effects of multimerization and composition of the specific adapter, which may vary for different target receptors. Moreover, the biological function of the target receptor itself may be of importance too. The use of receptor-activating ligands has been described to negatively influence the oncolytic capacity of EGFR-targeted adenoviruses (17), in contrast to non-activating ligands (36). Similar results were obtained for the EGFR-targeted HSV-1 vectors (27). On the other hand, for the EGFR-targeted measles virus no negative effects on receptor activation were described (30).

The soR-based adapters were also examined for their stable incorporation into the MHVdel2aHE genome. First, the anti-EGFR adapter soR-425 was inserted into the MHVdel2aHE backbone. Unfortunately, the viruses were not stable for many passages and, in addition, were not able to efficiently produce infectious progeny

virus in the target cells expressing human EGFR. Since both the MHVdel2aHE-23F425 and MHVdel2aHE-soR425 viruses were unstable, it seemed that the nature of the scFv425 sequence itself leads to the instability of the coronavirus genome. To avoid the further use of single-chain domains for redirecting coronaviruses to human cancer cells, we tested the insertion of the soR-h-His gene into the MHVdel2aHE genome. We were able to functionally and specifically redirect MHVsoR-h-his to the corresponding artificial His-tag receptor expressed on human cancer cells, thereby providing a proof-of-principle of multi-round coronavirus self-targeting via the indirect targeting approach (Chapter 5).

We explored the use of indirect targeting approaches one step further. In Chapter 6, targeted viral entry was accomplished by using coronavirus antibodies. We showed that MAb A3.10, directed against the MHV S protein, could mediate FcγRI-dependent MHV infection of human leukemic cells, in contrast to MAb J1.3, which is directed against the MHV M protein. It appeared that the specific binding of the MAb A3.10 to the spike protein could mediate a fusion active spike confirmation, leading to subsequent fusion of the virus envelope with the cell membrane upon interaction with the FcγRI protein. For the bispecific single-chain antibody 23F-425 redirecting FIPV and fMHV to EGFR-expressing cancer cells, the spike-binding scFv 23F domain was derived from antibody 23F8.1, which also enabled redirection of FIPV to human FcγRI-expressing cells (unpublished results). This may suggest that the spike-binding domains of antibodies like 23F8.1 and A3.10 are able to mimic the function of the normal receptor upon binding to the spike protein. Previous studies described in Chapter 1 as well as our results described in Chapters 3 and 4 show that indirect targeting of viruses to cancer cells can be achieved by making use of bispecific adapter proteins. The virus-binding domains of these adapter proteins were composed of a scFv or of a part of the ectodomain of the natural virus receptor protein. The use of complete antibodies to redirect viruses to Fc receptor-expressing cells as described in Chapter 6 is a novel way to exploit bispecific molecules for indirect virus targeting purposes and may be applied in the context of

targeted coronavirus-mediated immunostimulatory gene transfer for the treatment of acute myeloid leukaemia (Fig. 2).

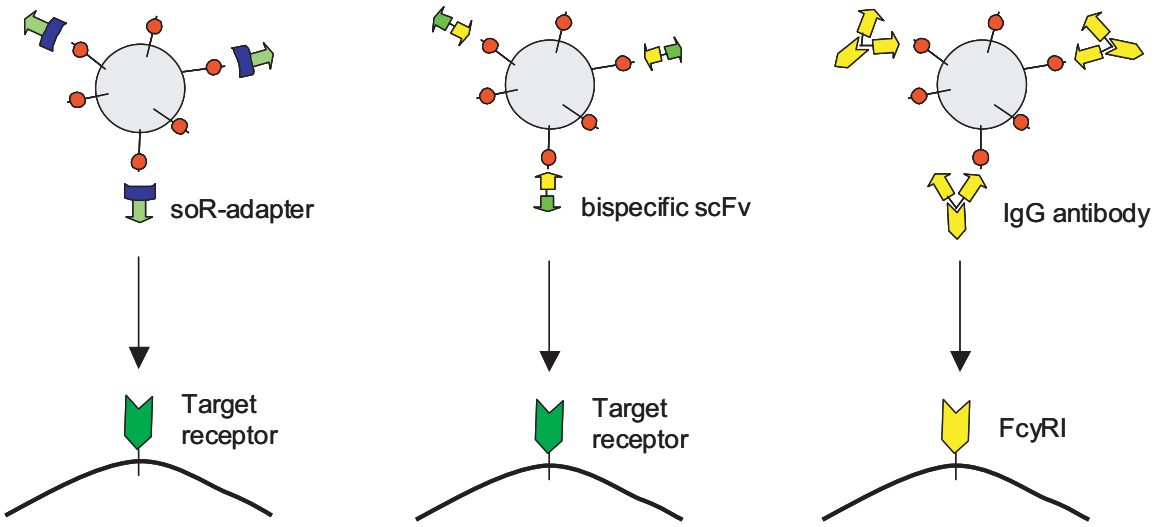


Fig. 2 Indirect targeting approaches for the redirection of coronaviruses to receptors expressed on cancer cells. Targeting of MHV to EGFR (Chapter 4), artificial His-tag receptor (Chapter 5), FcγRI (Chapter 6), and CXCR4 (unpublished data) was achieved by using adapters composed of the N-terminal domain of the MHV receptor (soR) fused to scFv 425 or EGF, 6His peptide, IgG1 Fc-tail, and SDF1a, respectively [Left]. Targeting of FIPV and fMHV to the EGFR was achieved (Chapter 3) via adapters composed of scFv 23F fused to scFv 425 [Middle]. MHV was redirected to the FcγRI expressed on leukemic cells (Chapter 6) via MAb A3.10 [Right].

Safety is an important issue in the field of virotherapy and can be achieved at different levels. However, for each type of virus different strategies are appropriate. For instance, tumor selective virus replication through control of tumor specific promoters can only be applied to DNA viruses, and does thus not apply to RNA viruses like coronaviruses. Another example is the use of inherently tumor-selective viruses. Most of these viruses are attenuated laboratory strains, or vaccine strains. For these viruses it is often difficult to pinpoint the exact mechanism that makes their replication tumor selective and these mechanisms may be different for the various viruses. When considering selectivity at the level of virus replication it should be noted that these viruses might still have the ability to enter normal cells.

Though not replicating in normal cells, the viruses entering normal cells are no longer available and may even deplete the pool of viruses applied for the infection and killing of tumor cells. Therefore, tumor specific entry has gained much interest, not only for safety reasons, but also to increase the therapeutic efficacy of these viruses. However, any modifications of the virus might reduce its fitness and result in a reduced anti-tumor efficacy. Hence, the balance of efficiency and selectivity should be carefully analyzed for all viruses developed for oncolytic virotherapy.

As an additional safety aspect besides tumor selectivity based on specific coronavirus entry we wanted to make use of viruses that are attenuated in their replication in normal cells. Therefore, we used coronaviruses with specific deletion of one of their virulence clusters. For MHV the deletion mutant lacking the gene cluster 2aHE was used (8) and for FIPV the deletion mutant lacking the gene cluster 3abc was used (14). The exact functions of these gene clusters are not yet understood. However, deletion of these clusters resulted in loss of pathogenicity of the viruses in their natural host. Interestingly, these coronaviruses were not dramatically attenuated in their replication and cell killing capacity in immortal cell cultures *in vitro* (8, 14). In addition, the MHV deletion mutant MHVdel2aHE showed enhanced replication in transformed cells as compared to untransformed cells *in vitro* (unpublished data). Furthermore, infection experiments performed on murine astrocytoma DBT cell line monolayers and 3-D tumor spheroids showed a strong oncolytic effect for the MHVdel2aHE deletion mutant, similar to the MHV-A59 wild type virus (unpublished data). However, it remains to be investigated whether these viruses exhibit tumor-selective replication *in vivo*, as is the case for instance for oncolytic HSV deletion mutants (24) and conditionally replicating oncolytic adenoviruses (2, 4, 12).

There are many different types of cancer and not every type of oncolytic virus will be able to kill all sorts of tumors to the same extent. However, for each type of cancer the ‘ideal’ oncolytic virus should at least meet some of a number of criteria. Below I discuss these criteria (partially adapted from (11)) in relation to the potential of coronaviruses for tumor therapy:

1. The virus should efficiently kill cancer cells.

For coronaviruses it generally holds that, once a cell has become infected, the fast cytoplasmic replication results in rapid cell death. This is superior to most DNA viruses such as HSV-1 and adenoviruses that first have to enter the nucleus for their replication. Furthermore, the cell killing mediated by the coronaviruses is amplified by the formation of syncytia, which can be considered as a clear advantage. We indeed observed rapid eradication of cancer cell monolayers and 3-D tumor spheroids once the cell tropism barrier was alleviated.

2. The virus should enter target cells preferentially or, rather, exclusively.

In contrast to most other oncolytic viruses, the animal coronaviruses used in this research are normally not able to infect human cells. Thus, detargeting of the normal coronavirus tropism is not needed to obtain truly targeted oncolytic agents. For other oncolytic viruses currently under investigation, this ablation of the natural tropism has been shown to be very difficult. So far, only the measles virus has been fully detargeted from its normal receptors and retargeted to specific receptors expressed on cancer cells (26). We showed that coronaviruses could be truly targeted to specific receptors, as was exemplified by the introduction of genes encoding targeting adapter proteins into the mouse hepatitis coronavirus genome using the reverse genetics modification system (Chapter 5). This gives coronaviruses a tremendous advantage in tumor selective entry as compared to oncolytic viruses that are not targeted to specific receptors expressed on tumor cells (Table 1). Furthermore, the indirect coronavirus targeting approach offers a versatile targeting system in which viruses can be directed to different target receptors overexpressed on specific tumor cell types.

TABLE 1

Virus species	Genome	Strategy to induce tumor selectivity	Stage
Adenovirus	dsDNA	Transcriptional targeting Cellular targeting Attenuation by deletion of gene fragments	Clinical
Herspes simplex virus	dsDNA	Transcriptional targeting Cellular targeting Attenuation by deletion of gene fragments	Clinical
Vaccinia virus	dsDNA	Attenuation by deletion of gene fragments	Clinical
Autonomous parvovirus	ssDNA	Inherent tumor-selectivity	Preclinical
Reovirus	dsRNA	Inherent tumor-selectivity	Clinical
Newcastle disease virus	ssRNA-	Inherent tumor-selectivity	Clinical
Measles virus	ssRNA-	Inherent tumor-selectivity Cellular targeting	Clinical
Vesicular stomatitis virus	ssRNA-	Inherent tumor-selectivity	Preclinical
Influenza virus	ssRNA-	Attenuation by deletion of gene fragments	Preclinical
Poliovirus	ssRNA+	Attenuation by deletion of gene fragments	Preclinical
Coronavirus	ssRNA+	Cellular targeting Attenuation by deletion of gene fragments	Preclinical

3. The virus should replicate preferentially or exclusively within target cells.

As described above, we based our oncolytic animal coronaviruses on viruses that are attenuated in their replication in their normal host due to specific deletion of one of their virulence clusters. However, it remains to be determined whether the attenuation of the coronaviruses is maintained in normal human cells, as is the case for several of the other oncolytic viruses which are attenuated by deletion of gene fragments (Table 1).

4. The virus should elicit an autologous anti-tumor immune response and should not be limited by the innate immune response during tumor therapy.

After initial infection the wild-type coronaviruses are known to elicit strong immune responses in the course of time, leading to profound inflammations of the target organ(s) after which significant tissue damage can be observed (18, 28). Attraction of the immune effector cells to the infected tumors can result in bystander effects in the context of autologous anti-tumor immune responses. Interestingly, reports have been made of mice bearing human tumor xenografts showing a decreased tumor growth in the presence of contaminating MHV infections that may be caused by such responses (1, 22). An additional advantage of the use of non-human coronaviruses *in vivo* would be the absence of pre-existing immunity in the form of circulating neutralizing antibodies in the cancer patients. This would give the virus additional time to kill tumor cells before being counteracted by the antiviral immune response, which was shown for other RNA (i.e. VSV) and also DNA (i.e. adenovirus and HSV) viruses to be a problematic limitation of oncolytic efficacy (5, 19, 32, 33).

5. The virus should preferably act synergistically with conventional therapies.

It has been described that MHV infection enhances the anti-tumor effect of the

chemotherapeutic drug cyclophosphamide against P388 leukemia in mice (23). These results suggest that MHV has indeed the potency to induce an anti-tumor bystander effect - even in the absence of virus replication in the tumor cells - and may have a potential synergistic effect when used in combination therapy with chemotherapeutic drugs.

6. The virus should cause only mild, self-limited or no human disease and should cause no safety concern for the contacts of the treated individual.

It should be safe to administer the oncolytic virus to the individual requiring therapy. The animal coronaviruses MHV and FIPV described in this thesis are not associated with any known human disease. In addition, in their natural host - the mouse and the cat, respectively - the deletion mutant coronaviruses do not cause disease (8, 14). In addition, no public health issues should be generated. Exposure of medical personnel, family and, ultimately, the general public to persons possibly shedding a therapeutically administered replicating virus must not present a source of concern. Shedding of the recombinant viruses during and after treatment should be very carefully analyzed, in particular in the case of the oncolytic animal coronaviruses to which the normal hosts (mouse or cat) are probably still susceptible contributing to the unwanted spread of the recombinant organisms into the environment. Furthermore, an important consideration is that the virus should be genetically stable, particularly if engineered, and not recombine with environmental organisms. Coronaviruses are RNA viruses that are in general inherently unstable. In contrast to most DNA viruses they lack a proper genetic proofreading system since they replicate outside the nucleus. Mutations may lead to target receptor-independent infections by possible acquisition of novel cell-binding properties of the spike protein (7), emphasizing the importance of additional mechanisms of tumor-selectivity. Furthermore, recombination of the oncolytic coronavirus with wild-type coronaviruses in the field, such as the human coronaviruses HCoV-229E (3) and HCoV-NL63 (37), is an unpredictable possibility that may increase the

pathogenicity of these coronaviruses - normally causing mild respiratory infections - and should thus be avoided. Therefore, an additional possible safety feature to prevent recombination would be the use of coronaviruses with rearranged gene order (9, 15). Clearly, the genetic stability of the oncolytic coronaviruses needs to be carefully examined in proper preclinical safety systems.

7. Treatment to control or eliminate viral replication should be available.

Availability of a safe and effective antiviral treatment of uncontrolled adverse effects following therapeutic exposure would be highly advantageous. In general, infections with human coronaviruses can be controlled by antiviral substances like interferons and corticosteroids (13, 35). In addition, for the tumor-targeted coronaviruses we have shown that infections *in vitro* can be efficiently blocked by heptad repeat mimicking peptides (Chapter 3, 4, and 5). Finally, treatment of patients through passive immunization with viral antibody preparations was shown to be an effective measure during the SARS epidemic (39).

8. The virus should not be able to lead to cellular transformation.

Coronaviruses replicate in the cytoplasm. They are true RNA viruses, i.e. replicate without involvement of DNA intermediates. Hence, the lack of integration of the viral genome into cellular chromosomes prevents possible cellular transformation, as was shown to occur when using retroviral vectors (38).

9. A robust virus manufacturing system should be available.

For coronaviruses a large-scale manufacturing system under GMP (good manufacturing practice) conditions has not yet been developed. However, coronaviruses can easily be grown in cell monolayers and be purified and concentrated by ultracentrifugation, resulting in sufficient virus for preclinical studies.

Nonetheless, the development of a large-scale production facility would be essential in order to translate the preclinical studies into clinic trials. Although the development of such a system is costly and demands high efforts, there do not seem to be any obvious problems regarding its feasibility.

In conclusion, the multi-round tumor-targeted coronaviruses exhibited potent cell killing kinetics *in vitro*. The coronaviruses were able to efficiently and selectively replicate in human cancer cells expressing the target receptor resulting in profound syncytia formation and the rapid production of progeny virus. The next steps will be to manufacture non-human coronaviruses targeted to relevant tumor targets and to analyze their oncolytic capacity and safety in appropriate *in vivo* tumor models.

The aim of my thesis was to explore the possibilities of coronaviruses as potential novel oncolytic agents. The work represents the first study to systematically approach this issue. The observations are certainly promising but it is obvious that it is just a start. Nevertheless I believe that the combined results justify adding the coronaviruses to the collection of candidate oncolytic viruses (Table 1).

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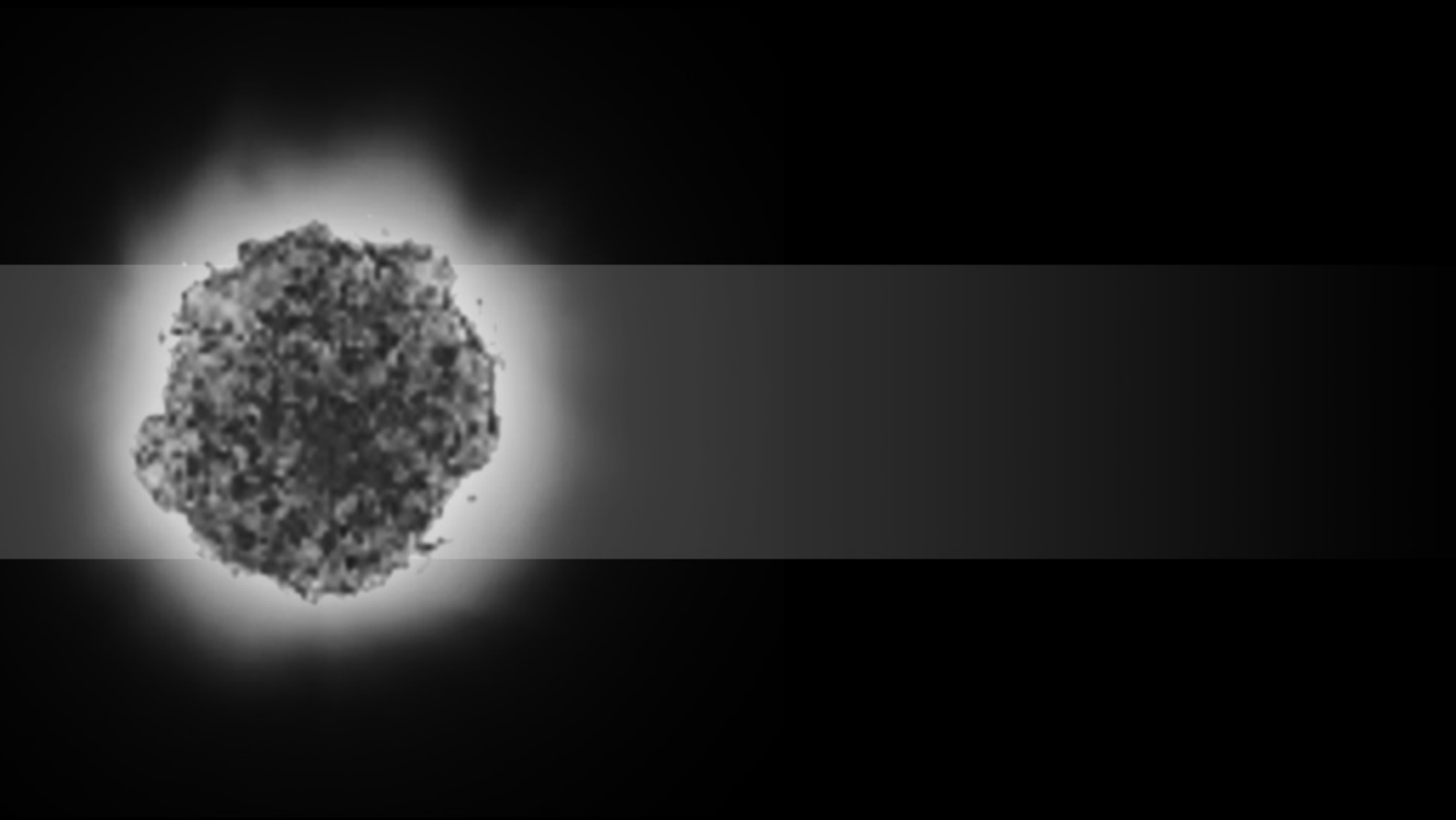
SAMENVATTING



SAMENVATTING

Kanker is een van de grootste levensbedreigingen voor de mens. Dankzij verbeteringen in de behandelmethoden kunnen steeds meer mensen genezen worden. Niettemin sterft nog steeds het merendeel van de patiënten aan de gevolgen van woekerende tumoren. Het blijft daarom van belang om de ontwikkeling van nieuwe kankertherapieën voort te zetten.

Kanker ontstaat door veranderingen in een cel waardoor de gemuteerde cel de controle over zijn normale celdeling verliest. Dit leidt tot ongeremde vermenigvuldiging van de cel en het ontstaan van tumoren die door het lichaam kunnen uitzaaien en zo de normale functies van onze weefsels verstoren. De huidige therapieën bestaan voornamelijk uit het wegsnijden van tumoren, het injecteren van chemische stoffen (chemotherapie), en het bestralen van tumoren (radiotherapie). Het wegsnijden van tumoren is niet altijd mogelijk, de locatie van de tumor en het aantal tumoren in het lichaam spelen hierbij een grote rol. Chemotherapie en radiotherapie zijn ontwikkeld om snel delende cellen kapot te maken. Helaas worden ook normale snel delende cellen kapot gemaakt wat kan leiden tot ernstige bijwerkingen. Ook kunnen kankercellen resistent worden tegen therapieën en na verloop van tijd weer uitgroeien tot grote tumoren.

Onderzoekers zijn bezig met het ontwikkelen van nieuwe therapieën als aanvulling op de huidige behandelmethoden. Een van de ontwikkelingen is het gebruik van virussen om kankercellen te doden. Virussen zijn intra-cellulaire parasieten. Ze komen de gastheer binnen via de luchtweg, het maag-darm kanaal, of via het bloed. Vervolgens binden ze aan een bepaald eiwit op de cel en dringen deze binnen. In de cel vindt virus replicatie plaats waarna de nieuw gevormde virus deeltjes uit de cel ontsnappen en waarbij de cel sterft. In het begin van de vorige eeuw zagen onderzoekers al dat wanneer sommige virussen in de tumor worden gespoten de kankercellen dood gaan. De virussen die destijds getest werden waren echter niet krachtig genoeg om hele tumoren op te ruimen en hadden ook niet de specificiteit om alleen de kankercellen kapot te maken. Gedurende de afgelopen decennia heeft de vooruitgang in de genetica en de

moleculaire biologie ervoor gezorgd dat we steeds meer weten over virussen en kankercellen. De ontwikkeling van efficiënte en selectieve anti-kanker virussen is dan ook in volle gang en heeft zelfs al geleid tot enkele veelbelovende klinische resultaten. Er zijn verscheidene criteria waaraan anti-kanker virussen moeten voldoen. De meest belangrijke zijn dat de virussen zorgen voor een snelle en robuuste celdood en dat dit alleen gebeurt in tumoren en niet in gezonde weefsels. In dit proefschrift beschrijven we hoe dierlijke coronavirussen snel en specifiek cultures van kankercellen kapot kunnen maken.

De in dit proefschrift beschreven dierlijke coronavirussen kunnen menselijke cellen normaal gesproken niet infecteren. Dit komt doordat de virussen niet aan menselijke cellen kunnen hechten omdat de receptor eiwitten voor deze virussen alleen in muizen en katten aanwezig zijn. In hoofdstuk 2 laten we zien dat wanneer het receptor eiwit voor het kattencoronavirus op menselijke kankercellen wordt aangebracht deze kanker cellen binnen 24 uur door het katten coronavirus kapot worden gemaakt. Hieruit kan geconcludeerd worden dat wanneer dierlijke coronavirussen de mogelijkheid krijgen om menselijke kanker cellen binnen te dringen zij deze snel en efficiënt kunnen doden.

Zoals gezegd zijn dierlijke coronavirussen normaal gesproken niet in staat om aan menselijke cellen te binden om ze vervolgens te infecteren. Om die simpele reden zijn deze virussen ongevaarlijk voor ons. In hoofdstukken 3, 4, en 6 wordt beschreven hoe dierlijke coronavirussen naar kankercellen gedirigeerd kunnen worden door middel van zogenoemde adapter eiwitten. Deze in het laboratorium ontwikkelde eiwitten binden aan de ene kant aan het coronavirus en aan de andere kant aan een specifiek eiwit op de kanker cel. Hierdoor ontstaat voor het coronavirus een nieuwe bindingsmogelijkheid en kan het virus de kankercel binnendringen om nieuwe virus deeltjes te vormen. Door gebruik te maken van adapter eiwitten kunnen dierlijke coronavirussen dus naar menselijke kanker cellen worden gestuurd. Gezonde cellen blijven buiten schot wanneer de coronavirussen via de adapter eiwitten naar receptor eiwitten worden gedirigeerd die alleen op kanker cellen voor komen.

Het is echter van belang dat ook de nieuw gevormde virus deeltjes de mogelijkheid hebben om nieuwe kankercellen binnen te dringen zodat de virussen in grote tumoren kunnen penetreren en de replicerende virussen zelf de strijd aan kunnen gaan met de groeiende tumoren. Hiervoor is een continue aanwezigheid van adapter eiwitten vereist. In hoofdstuk 5 wordt beschreven hoe met behulp van genetische modificatie een gen dat codeert voor een adapter eiwit in het muizencoronavirus wordt ingebracht. Hierdoor wordt gedurende elke ronde van virus replicatie ook een nieuwe dosis adapter eiwit aangemaakt waardoor de virusnakomelingen weer nieuwe kankercellen binnen kunnen dringen, net zolang totdat er geen kanker cellen meer zijn en het virus geen nakomelingen meer kan maken.

Concluderend, de dierlijke coronavirussen hebben de mogelijkheid om snel en efficiënt menselijke kankercellen te doden wanneer hen de mogelijkheid wordt geboden deze cellen binnen te dringen. Door middel van adapter eiwitten kunnen de coronavirussen selectief aan kankercellen binden waarna deze efficiënt worden gedood. Gezonde cellen waaraan het virus niet kan binden worden niet gedood. Door het gen coderend voor deze adapter in het virus in te bouwen kunnen de replicerende coronavirussen de strijd aan gaan met de delende kankercellen. De volgende stap is om coronavirussen te ontwikkelen die via adapter eiwitten aan tumor-specifieke eiwitten binden en deze verder te testen in geschikte kankermodellen. In het onderzoek beschreven in dit proefschrift is de basis gelegd voor de verdere ontwikkeling van anti-kanker coronavirussen als aanvulling op de huidige tumortherapieën.

DANKWOORD

DANKWOORD

DANKWOORD

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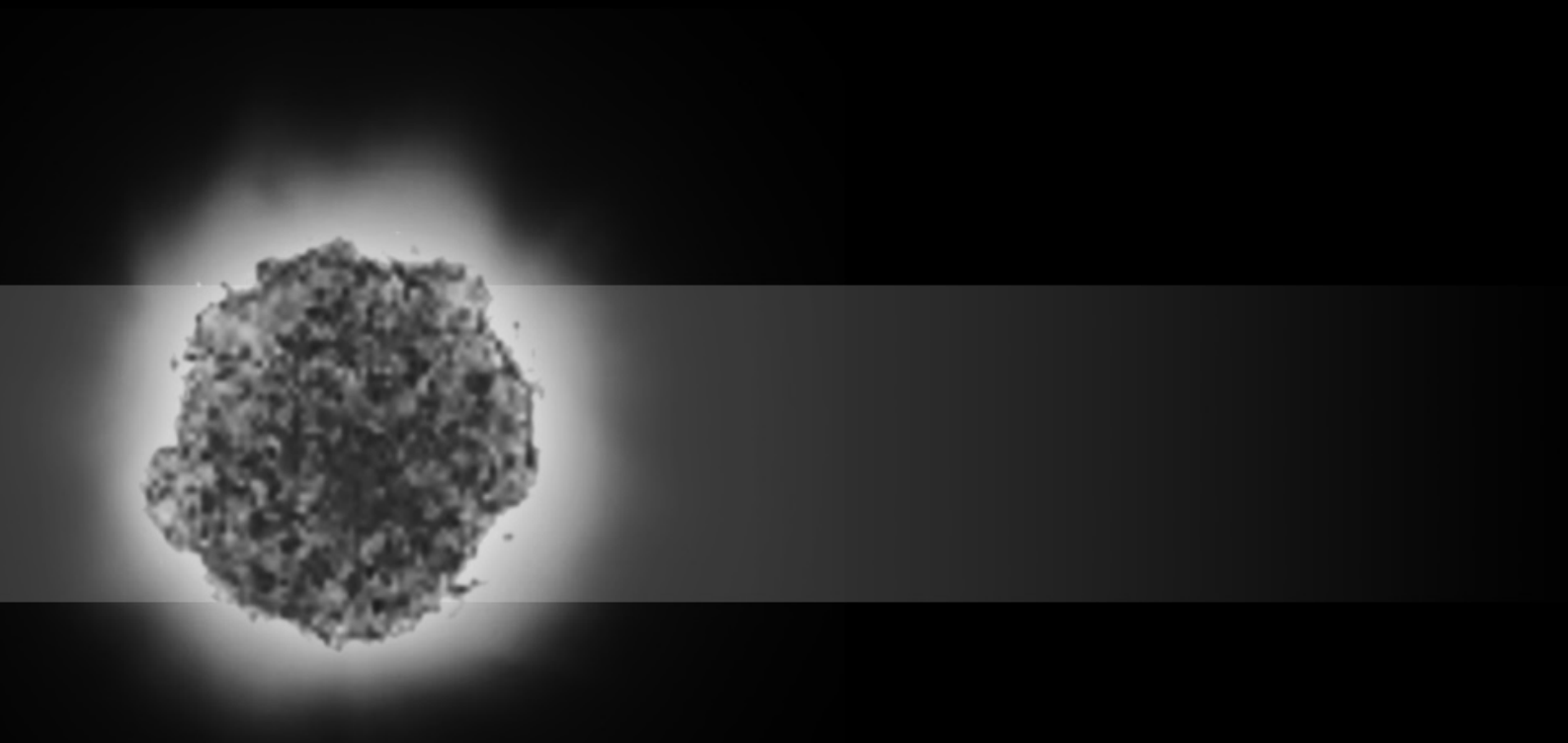
CURRICULUM VITAE AND PUBLICATIONS



CV AND PUBLICATIONS

CURRICULUM VITAE

Thomas Würdinger werd op 23 oktober 1978 geboren te München. In 1996 behaalde hij zijn diploma aan de regionale scholengemeenschap te Enkhuizen. In datzelfde jaar begon hij met de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Tijdens zijn eerste stage op de afdeling Gentherapie (Dr. V. W. van Beusechem) van het Vrije Universiteit medisch centrum deed hij onderzoek naar het redirigeren van adenovirussen naar kanker cellen. In zijn tweede stage deed hij onderzoek naar de ontwikkeling van gentransfer met behulp van HIV en AAV virale vectoren tegen hersen onstekingen bij MS patienten. Dit onderzoek was een samenwerking tussen de afdeling Medische Pharmacologie (Dr. A.-M. van Dam) van het Vrije Universiteit medisch centrum en het Nederlands Instituut voor Hersenonderzoek (Prof. Dr. J. Verhaagen). In 2001 studeerde hij af, waarna hij in september dat jaar startte met het promotieonderzoek om anti-kanker coronavirussen te ontwikkelen, zoals beschreven in dit proefschrift. Het onderzoek werd uitgevoerd op de Afdeling Virologie van de Diergeneeskunde faculteit van de Universiteit Utrecht in samenwerking met de afdeling Gentherapie van het Vrije Universiteit medisch centrum. Het onderzoek stond onder leiding van Prof. Dr. P. J. M. Rottier, Dr. W. R. Gerritsen, en Dr. V. W. van Beusechem. Sinds November 2005 is hij werkzaam op de Molecular Neurogenetics Unit van het Massachusets General Hospital, Harvard Medical School, te Boston.

CV AND PUBLICATIONS

LIST OF PUBLICATIONS

V. W. van Beusechem, D. C. Mastenbroek, P. B. van den Doel, M. L. M Lamfers, J. Grill, T. Würdinger, H. J. Haisma, H. M. Pinedo, W. R. Gerritsen. Conditionally replicative adenovirus expressing a targeting adapter molecule exhibits enhanced oncolytic potency on CAR-deficient tumors. *Gene Ther.* 2003; 10: 1982-1991.

T. Würdinger, M. H. Verheije, M. Raaben, B. J. Bosch, C. A. M. de Haan, V. W. van Beusechem, P J. M. Rottier, W. R. Gerritsen. Targeting non-human coronaviruses to human cancer cells using a bispecific single-chain antibody. *Gene Ther.* 2005; 12 (18): 1394-1404.

T. Würdinger, M. H. Verheije, K. Broen, B. J. Bosch, B. J. Haijema, C. A. M de Haan, V. W. van Beusechem, W. R. Gerritsen, P. J. M Rottier. Soluble receptor-mediated targeting of mouse hepatitis coronavirus to the human epidermal growth factor receptor. *J. Virol.* 2005; 79 (24).

M. H. Verheije, T. Würdinger, C. A. M de Haan, V. W. van Beusechem, W. R. Gerritsen, P. J. M Rottier. Recombinant mouse hepatitis coronavirus expressing an adaptor protein can be redirected to a non-native receptor on human cancer cells. *J. Virol.* 2006.

T. Würdinger, M. H. Verheije, L. van der Aa, B. J. Bosch, B. J. Haijema, C. A. M de Haan, V. W. van Beusechem, W. R. Gerritsen, P. J. M Rottier. Antibody-mediated coronavirus targeting to acute myeloid leukemia cells. *In preparation.*

CV AND PUBLICATIONS

CONFERENCE PROCEEDINGS

J. Grill, M. L. M. Lamfers, T. Würdinger, R. Alemany, P. W. Vandertop, W. R. Gerritsen, V. W. van Beusechem, C. M. F. Dirven. Oncolytic adenoviruses for the treatment of human gliomas: in vitro comparison of E1B-deleted and E1A-deleted viruses with E1-positive viruses. 1st International symposium on genetic anticancer agents. NDDO Oncology, VU, Amsterdam, 2000. Poster presentation.

D. Mercier, T. Würdinger, W. Hermens, S. P. Niclou, J. Verhaagen, A.-M. van Dam. Production of viruses expressing IL-1ra and IL-10: a way to accomplish anti-inflammatory cytokine gene transfer to the rat brain during cr-EAE? J. Neuroimmunol. 2001; 118 (1): 37. Poster presentation.

T. Würdinger. Targeting coronaviruses to human tumor cells. Spring Symposium Dutch Society of Gene Therapy (NVGT), 4 April 2003, Leiden, the Netherlands. Oral presentation.

M.H. Verheije, and T. Würdinger. Development of anti-tumor coronaviruses: receptor targeting and conditional replication. Invited speaker, 27 October 2003, RUG, Groningen, the Netherlands.

T. Würdinger, M. H. Verheije, M. Raaben, V. W. van Beusechem, C. A. M. de Haan, P. J. M. Rottier, and W. R. Gerritsen. Targeting of a non-human coronavirus to human tumor cells by using a bispecific single-chain antibody. 6th Annual Meeting of the American Society of Gene Therapy (ASGT), 4-8 June 2003, Washington, D.C., U.S.A. Poster presentation.

T. Würdinger, M. H. Verheije, V. W. van Beusechem, C. A. M. de Haan, P. J. M. Rottier, and W. R. Gerritsen. Coronaviruses are able to efficiently eradicate human tumor cells if provided with the appropriate virus receptor. 6th Annual Meeting of the American Society of Gene Therapy (ASGT), 4-8 June 2003, Washington, D.C., U.S.A. Poster presentation.

T. Würdinger, M. H. Verheije, M. Raaben, V. W. van Beusechem, C. A. M. de Haan, P. J. M. Rottier, and W. R. Gerritsen. Targeting of a non-human coronavirus to human tumor cells by using a bispecific single-chain antibody. European Society of Gene Therapy (ESGT), 14-17 November 2003, Edinburgh, U.K. Poster presentation.

T. Würdinger, M. H. Verheije, V. W. van Beusechem, C. A. M. de Haan, P. J. M. Rottier, and W. R. Gerritsen. Coronaviruses are able to efficiently eradicate human tumor cells if provided with the appropriate virus receptor. European Society of Gene Therapy (ESGT), 14-17 November 2003, Edinburgh, U.K. Poster presentation.

M. H. Verheije, T. Würdinger, M. Raaben, B. J. Bosch, V. W. van Beusechem, C. A. M. de Haan, P. J. M. Rottier, and W. R. Gerritsen. Non-human Coronaviruses as potential tool for cancer therapy: selective targeting to and effective eradication of human tumor cells in vitro. Seventh international symposium on positive strand RNA viruses, 27 May-1 June 2004, San Fransisco (U.S.A.). Poster presentation.

T. Würdinger. Targeting non-human coronaviruses to human cancer cells using bispecific single-chain antibody. 4 March 2005, Dutch Annual Virology Symposium, Amsterdam, the Netherlands. Oral presentation.

T. Würdinger. Coronaviruses: Development of novel oncolytic vectors. Dutch Society of Gene Therapy (NVGT), 18 March 2005, Groningen, the Netherlands. Oral presentation.

T. Würdinger. Coronaviruses: Development of novel oncolytic vectors. American Society of Gene Therapy (ASGT), 1-5 June 2005, St. Louis, USA. Oral presentation.

T. Würdinger. Coronaviruses: Development of novel oncolytic vectors. Invited speaker, 26 July 2005, Massachusetts General Hospital, Harvard Medical School, Boston, USA.

T. Würdinger. American Society of Gene Therapy (ASGT), 1-5 June 2005, St. Louis, USA. Travel award.

penumbra pe•num•bra (pi num,brƒ) n., pl.
), -bras. T. a. the partial or imperfect outside
the complete shadow of an opaque body,
as a planet, where the
light from the source
of illumination is only
partly cut off. b. the
grayish marginal por-

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