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High CD46 Receptor Density Determines Preferential Killing of Tumor Cells by Oncolytic Measles Virus

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ABSTRACT

Live attenuated Edmonston B strain of measles virus (MV-Edm) is a potent and specific oncolytic agent, but the mechanism underlying its tumor selectivity is unknown. The virus causes cytopathic effects (CPEs) of extensive syncytial formation in tumor cells but minimal damage or cell killing in normal cells. The CPE is dependent on expression of viral proteins and the presence of CD46, the major cellular receptor of MV-Edm. Using a virally encoded soluble marker peptide to provide a quantitative readout of the level of viral gene expression, we determined that tumor cells and normal cells expressed comparable levels of viral proteins. CD46 mediates virus attachment, entry, and virus-induced cell-to-cell fusion. Using engineered cells expressing a range of CD46 densities, we determined that whereas virus entry increased progressively with CD46 density, cell fusion was minimal at low receptor densities but increased dramatically above a threshold density of CD46 receptors. It is well established that tumor cells express abundant CD46 receptors on their surfaces compared with their normal counterparts. Thus, at low CD46 densities typical of normal cells, infection occurs, but intercellular fusion is negligible. At higher densities typical of tumor cells, infection leads to extensive cell fusion. Intercellular fusion also results in enhancement of viral gene expression through recruitment of neighboring uninfected cells into the syncytium, further amplifying the CPE. Discrimination between high and low CD46 receptor density provides a compelling basis for the oncolytic specificity of MV-Edm and establishes MV-Edm as a promising CD46-targeted cancer therapeutic agent.

INTRODUCTION

Live attenuated Edmonston B strain of measles virus (MV-Edm) has potent and specific oncolytic activity against a variety of human tumors, including lymphoma, multiple myeloma, epithelial ovarian cancer, and glioma (1–6). MVCEA, a recombinant MV-Edm genetically modified to express human carcinoembryonic antigen (CEA) as a biologically inert soluble marker for noninvasive monitoring of the profiles of viral gene expression, is being tested in a Phase I clinical trial for patients with recurrent epithelial ovarian cancer (7).

MV-Edm is selectively oncolytic, causing extensive syncytium formation and cell killing in a variety of human tumor cells but minimal cytopathic damage in nontransformed cells such as normal dermal fibroblasts, ovarian surface epithelium, mesothelial cells from the peritoneal cavity, and peripheral blood lymphocytes (2, 3). Until now, there have been no published studies addressing the mechanisms underlying the tumor specificity of MV-Edm. However, elucidation of these mechanisms will be pivotal to future development of MV-Edm as a cancer therapeutic agent and may provide clues that can potentially increase the efficacy or safety of this agent in the clinic.

A number of the oncolytic viruses currently being developed for cancer therapy are tumor selective because they exploit genetic defects present in tumor cells and not in normal cells (8). One of the most important defense mechanism a cell has against viral infection is induction of IFN-α/β and IFN-inducible proteins, resulting in suppression of protein synthesis and establishment of an antiviral state (9, 10). However, viruses have evolved diverse strategies to evade or antagonize the IFN antiviral response (11). Thus, measles virus (MV) encodes the V and C accessory proteins that block IFN-α/β production and/or signaling, allowing the virus to replicate in the host cell (12–15). The mechanism underlying MV-C inhibition of IFN-α/β signaling remains unclear (15), but the MV-V protein blocks the IFN response by inhibiting phosphorylation of signal transducers and activators of transcription 1 and 2 proteins (13).

MV enters cells by binding via its hemagglutinin (H) attachment protein to one of two cellular receptors, CD46 (16, 17) or signaling lymphocyte activation molecule. The pathogenic wild-type MV (which is not selectively oncolytic) uses primarily signaling lymphocyte activation molecule, expressed on activated T cells, B cells, and monocytes/macrophages, as a receptor (18, 19). In contrast, attenuated vaccine strains such as MV-Edm use predominantly CD46 (20), which is ubiquitously expressed (usually at low density) by all human cells except erythrocytes (21). In addition, CD46 is required to mediate intercellular fusion. Virally infected cells expressing the MV envelope glycoproteins, hemagglutinin (H) and fusion (F), on their cell surfaces fuse with neighboring cells through CD46 to form multinucleated syncytia, the characteristic CPE of MV-Edm infection.

CD46, also known as membrane cofactor protein, plays an important role in protecting autologous cells from complement attack by serving as a cofactor for Factor I-mediated inactivation of C3b and C4b, thus blocking the complement cascade at the C3 activation stage (22, 23). Indeed, CD46 is frequently overexpressed on cancer cells compared with their normal counterparts, possibly as a mechanism for cancer cells to overcome lysis by complement (24). Overexpression of CD46 and other membrane complement regulatory proteins, CD55 and CD59, has been documented in leukemias and gastrointestinal, hepatocellular, colorectal, endometrial, cervical, ovarian, breast, renal, and lung carcinomas and found to limit the therapeutic potential of monoclonal antibody therapy (3, 24). Overexpressed complement regulatory proteins have also been studied as potential targets for cancer therapy using bispecific antibodies and anti-idiotypic vaccination (25).

CPEs induced in MV-Edm-infected cells are dependent on virus entry, expression of MV-H and MV-F, and the CD46 cellular receptor. We first quantitated the relative expression levels of viral proteins in infected tumor cells *versus* nontransformed cells and found comparable levels of gene expression despite striking differences in CPEs. The logical hypothesis to explain these observations was that there may be a correlation between CD46 receptor density and the strikingly different CPEs observed in tumor cells *versus* nontransformed cells on MV-Edm infection. To define the role of CD46 receptor density in MV-Edm infection, a panel of Chinese hamster ovary (CHO) clones expressing a range of surface densities of CD46 was generated. Rodent cells lack CD46, but if they are engineered to express human CD46, they become infectable by MV-Edm and are susceptible to the CPEs of MV-Edm (26). Using these CHO-CD46 transfectants, MV-eGFP [a recombinant MV-Edm expressing green

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fluorescent protein (GFP)], and an adenoviral vector expressing MV-H/F proteins, we explored the relationship between CD46 receptor density, virus entry, and cell fusion. Virus entry increased progressively with CD46 receptor density and was quite efficient even at relatively low receptor densities. In contrast, syncytium formation and cell killing were minimal at low CD46 densities but increased rapidly above a threshold CD46 expression level. These findings suggest that CD46 is an interesting new cancer target and that the differential expression of CD46 in tumor cells *versus* normal cells dramatically increases the susceptibility of tumor cells to the oncolytic activity of MV-Edm, providing a mechanistic basis for tumor specificity of MV-Edm.

MATERIALS AND METHODS

Cell Lines. African green monkey kidney Vero cells and human ovarian carcinoma SKOV3ip.1, fibrosarcoma HT1080, and epithelial lung carcinoma A549 cells were maintained in DMEM (BioWhittaker, Walkersville, MD) supplemented with 5% (Vero), 10% (HT1080 and A549), or 20% (SKOV3ip.1) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). NHDF primary normal human dermal fibroblasts and CASMC coronary artery smooth muscle cells were purchased from Cambrex (Walkersville, MD) and maintained in media as recommended by supplier (Cambrex). CD46 expression levels in the panel of human cells were determined as described below.

Generation of CHO-CD46 Clones. Clones of CHO cells stably expressing the C1 isoform of human CD46 were generated by transfection of an expression plasmid encoding CD46 C1 (a kind gift from John Atkinson; University of Washington) into CHO cells using the calcium phosphate method. CD46-expressing CHO clones were selected by limiting dilution using 1.2 mg/ml G418 (Invitrogen). A total of 68 clones were screened for CD46 expression by flow cytometry using a 1:20-diluted phycoerythrin-labeled anti-CD46 antibody (clone E4.3; BD Biosciences PharMingen) for 1 h at 4°C. The shift in fluorescence (FL2) is expressed as mean fluorescence index. Sixteen clones expressing a range of CD46 density were used in this study and maintained in 1 mg/ml G418.

Generation of a Fiber-Modified Adenovirus Expressing MV-H and MV-F (AdK7 H/F). The K7Ad H/F vector was constructed using an *in vitro* ligation method as described previously (27). H coding sequences were cloned downstream of a human cytomegalovirus immediate early promoter/enhancer ($P_{CMV\ IE}$) in the pHM5 shuttle vector (28). The F gene was cloned downstream of the $P_{CMV\ IE}$ in the pHM11 shuttle vector (28). The plasmid pSK-Ad5F encoding for the human Ad5 fiber was obtained from a fragment of pAdHM48 (28) that was digested with *Csp*45I and *Xba*I. The DNA sequence encoding the linker region and K7 motif was generated by PCR amplification, with pSKAd5F as the template. Each generated PCR product was digested with *Stu*I/*Bam*HI or *Bam*HI/*Afl*II and subcloned into the *Stu*I/*Afl*II sites of the plasmid by a three-part ligation, resulting in pSK-Ad5F/K7. The *Csp*45I-*Xba*I fragment from pSK-Ad5F/K7 was subcloned into the pAdHM48 plasmid, resulting in pAdHM48-K7. Expression cassettes were transferred from the pHM5 or pHM11 shuttle vectors into the E1- or E3-deleted regions, respectively, of the adenoviral vector plasmid pAdHM48-K7. The resulting recombinant adenovirus genomes were transfected into 293 cells. Because expression of measles H and F proteins causes cell fusion and is toxic to 293 cells, viruses were rescued in the presence of a fusion inhibitory peptide [80 nM; *Z*-D-Phe-L-Phe-Gly-OH (FIP); Bachem, Torrance, CA], which blocks H/F-mediated fusion (29). The resulting recombinant adenoviruses were propagated in 293 cells in the presence of FIP peptide and purified by CsCl equilibrium centrifugation as described previously (30). Purified virion preparations were dialyzed against 10 mM PBS and 10% glycerol and stored at −80°C. Viral particle numbers (particles/ml) were calculated from absorbance measurements at 260 nm.

MV Production and Titration. MV stocks were generated by infecting Vero cells with MV-eGFP or MV-CEA at a multiplicity of infection (MOI) of 0.02 for 2 h at 37°C, after which the virus was removed, and cells were maintained in 5% fetal bovine serum-DMEM at 37°C. When 80–90% of the cells were in syncytia, the media were removed, and the cells were harvested into reduced serum Opti-MEM (Invitrogen) media. Cell-associated virions were released by two freeze-thaw cycles, and cell lysates were clarified by a brief centrifugation step and frozen in liquid nitrogen before storage at −80°C. Virus titers were obtained by titration on Vero cells and expressed as 50% tissue culture infectious dose (TCID50)/ml.

Infection Assays. The panel of human tumor and normal cells was infected with MV-CEA (MOI = 0.2) for 2 h at 37°C, after which the virus inoculum was removed, and the cells were maintained in standard medium for 48 h. The cells were fixed with 0.5% glutaldehyde and stained with 0.2% crystal violet solution, and the CPEs were photographed. To quantitate the relative levels of viral gene expression, the cells were infected with MV-CEA (MOI = 0.4) or MV-eGFP (MOI = 0.4) and maintained in the presence of FIP to allow analysis of single infected cells by flow cytometry. Forty-eight h later, the media were harvested, the number of viable cells per well was counted by trypan blue exclusion, and the percentage of infected GFP-positive cells was analyzed by flow cytometry. The amount of virally encoded CEA marker peptide in the medium was analyzed by the Mayo Central Clinical Laboratory.

CHO or CHO-CD46 cells were plated overnight (1×10^5 cells/well) in a 12-well plate and infected the next day with MV-eGFP (MOI = 0.5) or Ad5/35 (MOI = 100) for 2 h at 37°C. The cells were maintained in 10% fetal bovine serum-DMEM (Ad5/35) or in medium containing FIP (80 nM). Forty-eight h later, the percentage of GFP-positive cells was determined by flow cytometry. Background transduction of parental CHO cells by MV-eGFP (11.5%) or Ad5/35 (6.7%) was subtracted from the infection data presented in Fig. 3*A*.

CHO-CD46 cells were plated as described above and infected with K7Ad H/F for 6 h at 37°C, and the cells were maintained in 10% fetal bovine serum-DMEM. Forty-eight h later, the cells were fixed and stained with 2% crystal violet. The syncytia sizes were analyzed using NIH Image J software.

To determine the importance of CD46 density for bystander recruitment of neighboring cells into a H/F-expressing focus, CHO-CD46 clone 5 was infected with MV-eGFP (MOI = 1.0) and plated overnight (1×10^3 cells/well) in the presence of FIP to block fusion. The next day, 1×10^5 uninfected CHO, clone 60 (low CD46), clone 78 (medium CD46), or clone 5 cells (high CD46) were overlaid on the MV-eGFP-infected CHO-CD46 clone 5 cells, and FIP was removed. Forty-eight h later, the cocultures were fixed, and syncytia sizes were determined using NIH Image J software.

RESULTS

MV Gene Expression in Tumor Cells and Nontransformed Cells Is Comparable. A panel of human cells (SKOV3ip.1, HT1080, A549, NHDF, and CASMC) was infected with MV-eGFP or MV-CEA. Expression of GFP by the infected cells facilitates quantitation of the number of MV-infected cells by flow cytometry. The virally encoded soluble CEA marker peptide provides a convenient and precise way to quantitate and monitor MV gene expression (7). As shown in Fig. 1*A*, MV-Edm induced extensive CPEs of syncytial formation in MV-infected tumor cells but minimal damage in nontransformed NHDF or CASMC cells even at 6 days postinfection (data not shown). Using MV-eGFP, we determined that the striking difference in CPEs was not due to lack of virus infection or gene expression in these nontransformed cells because, for a given MOI, there were comparable numbers of GFP-positive infected tumor cells and normal cells (Fig. 1*B*). To compare MV viral protein synthesis between tumor cells and nontransformed cells and to precisely determine the level of viral gene expression per infected cell, the panel of cells was infected with MV-eGFP or MV-CEA and maintained in the presence of FIP, which prevents syncytium formation, allowing quantitation of the number of infected cells by flow cytometry. It is apparent from Fig. 1*C* that at day 2 or day 4 postinfection, the amount of viral protein synthesis per infected cell is similar between tumor cells and nontransformed cells. Thus, the absence of CPEs in nontransformed cells is not due to a significant difference in viral gene expression. We therefore turned our attention to cellular factors that might cause tumor cells to fuse more readily at a given level of viral gene expression.

CD46 was a logical choice because syncytium formation is dependent on the presence of the CD46 receptor, and tumor cells are known

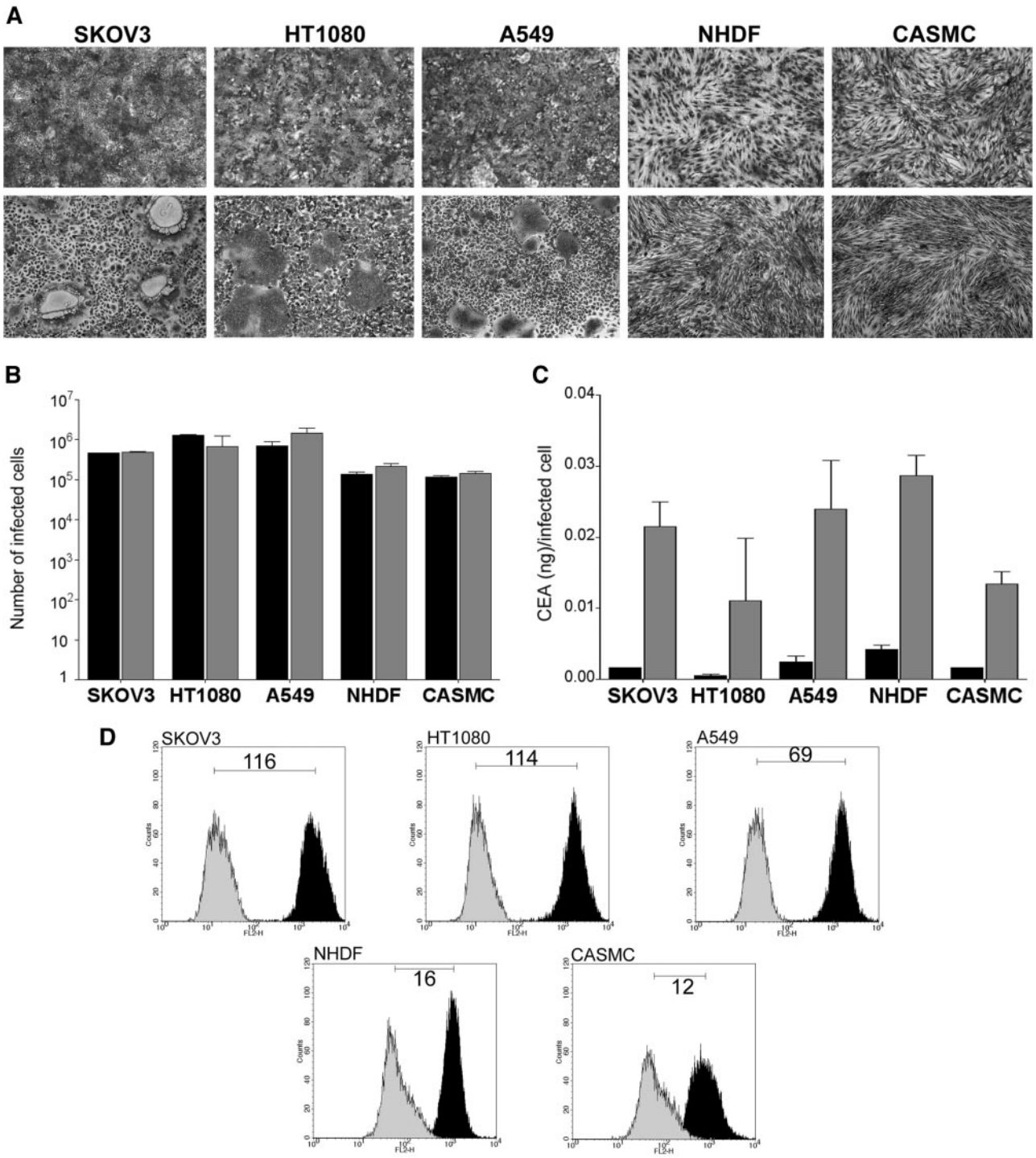

Fig. 1. The difference in cytopathic effects in MV-Edm-infected tumor cells and normal cells is not due to a lack of virus infection or viral gene expression in normal cells. *A*, MV-Edm is selectively oncolytic. A panel of human tumor cell lines (SKOV3ip.1, HT1080, and A549) and primary normal human dermal fibroblasts (NHDF) and coronary artery smooth muscle cells (CASMC) were infected with MV-CEA [multiplicity of infection (MOI) = 0.2] for 2 h and photographed 48 h later. *B*, the absence of cytopathic effects in NHDF and CASMC cells is not due to lack of viral infection. Cells were infected with MV-eGFP (MOI = 0.4), maintained in the presence of *Z*-D-Phe-L-Phe-Gly-OH, and analyzed by flow cytometry 48 h (■) or 96 h (▨) later. There were comparable numbers of green fluorescent protein-positive infected cells in the cultures. *C*, viral protein expression per infected tumor cell and normal cell is comparable. Cells were infected with MV-CEA (MOI = 0.4) or MV-eGFP (MOI = 0.4) and maintained in the presence of *Z*-D-Phe-L-Phe-Gly-OH. The number of infected cells was quantitated by cell counting and flow cytometry. CEA levels in the media were quantitated and expressed as the amount of CEA produced per infected cell at 48 h (■) and 96 h (▨). *D*, tumor cells express higher levels of CD46 receptors on their surfaces compared with normal cells. Cells were stained with anti-CD46 PE antibody (*black histogram*) or an isotype control (*gray histogram*) and analyzed by flow cytometry. The *number* is the ratio of the mean fluorescence index of the *black histogram:gray histogram* and indicates the CD46 receptor expression levels on the cells.

to overexpress CD46 receptors compared with their normal counterparts (3, 24). We therefore measured CD46 expression levels on the surfaces of this panel of cells and determined that CD46 expression levels were considerably higher in SKOV3ip.1, HT1080, and A549 tumor cells than in the NHDF or CASMC cells (Fig. 1*D*).

Correlation between the Oncolytic Activity of MV-Edm and CD46 Receptor Density. To define the role of CD46 receptor density in MV-Edm entry and CPEs, we used CHO cells and transfected them with a vector encoding the C1 isoform of human CD46. CD46-expressing CHO cells are infectable by MV-Edm and express viral

Table 1 *Relative CD46 expression levels on surfaces of the panel of CHO[a]-CD46 clones*

Clone designation	MFI
CHO	14.97
75	14.28
64	14.59
61	23.50
60	24.52
41	24.69
78	51.53
25	74.67
21	84.53
42	89.21
46	93.85
62	346.1
22	476.3
5	509.2
31	562.2
63	1000.4
80	1213.9

[a] CHO, Chinese hamster ovary; MFI, mean fluorescence index.

proteins, whereupon they fuse with each other to form syncytia (26). A total of 68 clones of CHO-CD46 cells were screened for CD46 expression by flow cytometry, and 16 clones expressing a range of CD46 densities (Table 1) were selected for the studies presented below.

To first determine the relationship between CD46 receptor density and the oncolytic activity of MV-Edm, the panel of CHO-CD46 clones was infected with MV-eGFP (MOI = 0.5 and 5.0), and the extent of CPEs and cell killing in the infected cultures was evaluated. CPEs (MOI = 0.5) were quantitated by determining the syncytial index, which is a multiple of the number of syncytia in the culture and the average syncytium size (measured as pixels/syncytium using NIH Image J software). Interestingly, we found that the panel of CHO-CD46 clones could be divided into two distinct groups based on their syncytial indices (Fig. 2*A*). CPEs were minimal in clones (clones 75, 64, 61, 60, and 41) that express a lower density of CD46 receptors

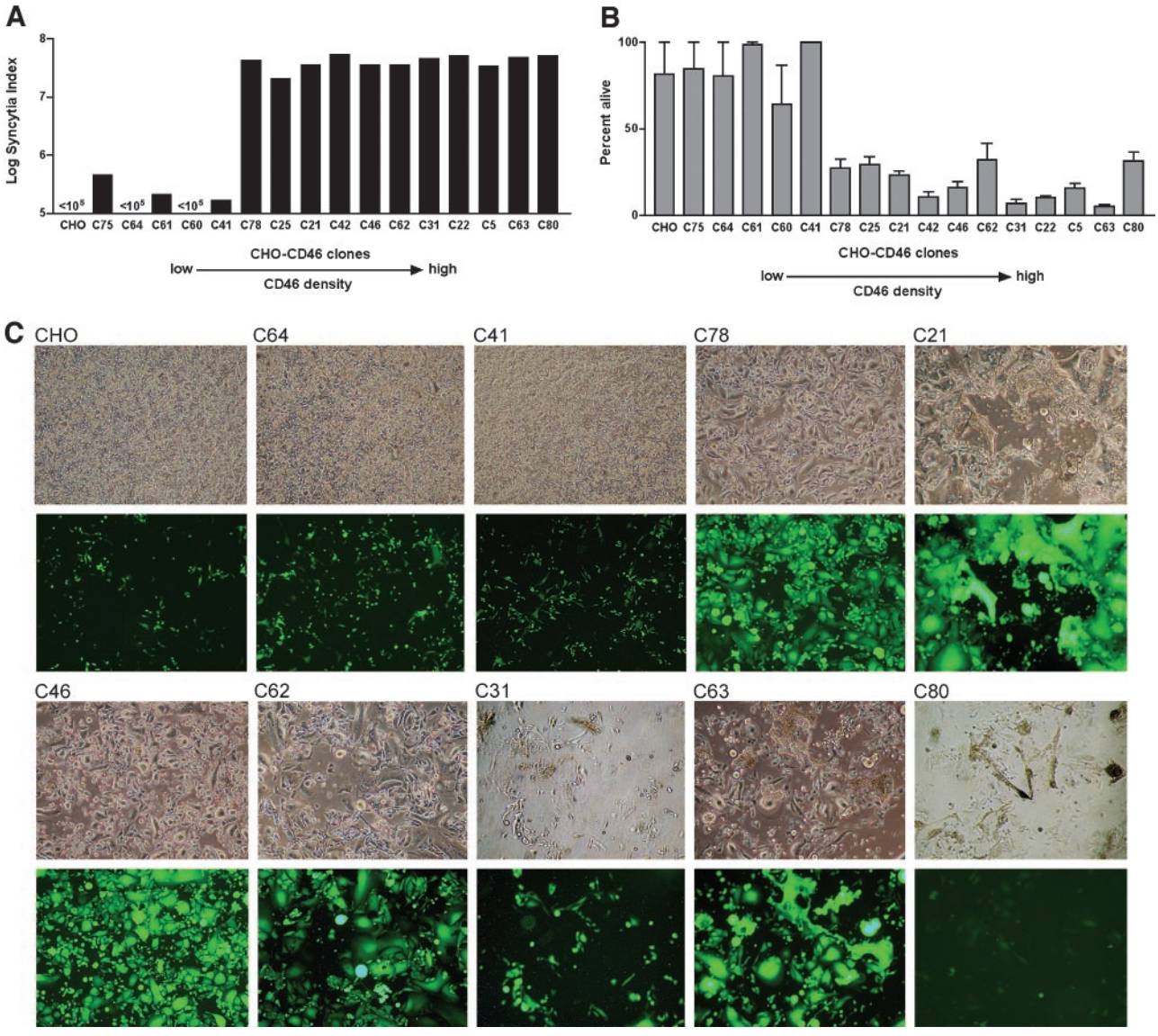

Fig. 2. The extent of MV-Edm-induced cytopathic effects and cell killing is correlated with CD46 receptor density. *A*, cells were infected with MV-eGFP [multiplicity of infection (MOI) = 0.5], and the extent of cytopathic effects (syncytial index) was quantitated at 48 h postinfection by counting the number of syncytia in the culture and multiplying it by the average syncytium size (pixels) as determined using NIH Image J software. *B*, cells were infected with MV-eGFP (MOI = 5.0), and cell viability at 48 h postinfection was quantitated by trypan blue exclusion assay. *C*, representative photographs of MV-eGFP-infected cells (MOI = 5.0) taken at 48 h postinfection.

than clone 78. In contrast, the syncytial indices increased sharply by 2 logs in clones that express higher levels of CD46 receptors (Fig. 2*A*). Cell viability at 48 h postinfection (MOI = 5.0) was determined by trypan blue exclusion assay, and in line with the minimal CPEs observed in clones 75, 64, 61, 60, and 41 (Fig. 2*A*), cell death in these infected cultures was minimal compared with significant cell death in the remaining clones (Fig. 2*B*). Microscopic examination of the MV-eGFP-infected cultures (MOI = 5.0) shows that despite a good level of infection, most of the infected cells remain as unfused single cells in the low CD46- expressing clones (Fig. 2*C*). In contrast, there was extensive syncytium formation in clones expressing more than the "threshold number" of CD46 receptors. In clone 80, most of the syncytia in the culture have detached from the plate or were no longer viable (loss of GFP fluorescence). These results demonstrate that MV-Edm-induced CPE correlates closely with CD46 receptor density and that the target cells need to express enough CD46 receptors before significant CPE and cell killing will occur.

The Relationship between CD46 Receptor Density, Virus Entry, and Virus-Induced Cell Fusion. To determine whether the dramatic differences in CPEs and cell death between low CD46- and high CD46-expressing CHO clones were modulated predominantly by virus entry or by intercellular fusion, we studied the two processes separately. First, we investigated virus entry in CHO or CHO-CD46 clones that were infected with MV-eGFP (MOI = 0.5) and maintained in medium containing FIP. As shown in Fig. 3*A*, the number of cells infected by MV-eGFP increased progressively as the cell surface density of CD46 receptors increased. For comparison, the panel of CHO-CD46 cells was infected with another CD46 tropic virus, a GFP-expressing chimeric Ad5 adenoviral vector pseudotyped with Ad35 coat (a kind gift from Dr. Andre Lieber; Ref. 31), and a similar relationship between virus infection and CD46 receptor density was observed (Fig. 3*B*).

To investigate the role of CD46 in intercellular fusion, we used an adenoviral vector to express the H and F proteins in our cell panel. In this way, we were able to exclude differences in MV-Edm entry from the equation and achieve high levels of MV-H and MV-F proteins in the CHO-CD46 clones (28). A type 5 serotype adenoviral vector expressing the MV-H and MV-F proteins inserted, respectively, in the E1 and E3 positions (AdH/F) under control of a cytomegalovirus promoter was used (28). The fiber protein of the vector was also modified to display a seven-lysine (K7) COOH-terminal peptide for enhanced CHO cell transduction. CHO and CHO-CD46 cells were transduced with K7Ad H/F at various MOIs, and 2 days later, the cells were fixed and stained with crystal violet for visualization of syncytia (Fig. 4*A*, MOI = 300). At low CD46 receptor densities, syncytium formation was minimal and small, but as the number of CD46 receptors per cell increased, syncytia formation became increasingly evident. In clones expressing the highest levels of CD46, large syncytia merged with each other, causing extensive destruction of the cell monolayers (Fig. 4*A*). Syncytia sizes in the cell monolayers (MOI = 30, 50, and 100) were also measured quantitatively using NIH Image J software (the number of pixels per CHO cell is 7000–9000) and are shown in Fig. 4*B*. From Fig. 4, it is evident that extensive cell fusion requires "sufficiently" high levels of CD46 receptors.

Syncytium Formation Amplifies Viral Gene Expression. The importance of syncytium formation in amplifying viral gene expression in the cell culture was evaluated by infecting the panel of clones with MV-CEA (MOI = 0.5) and maintaining the cells in the presence or absence of FIP. In these experiments, CEA concentration in the conditioned medium provides a quantitative measurement of overall level of viral gene expression in the infected culture (7). When the infected cells were not allowed to fuse and form syncytia (+FIP),

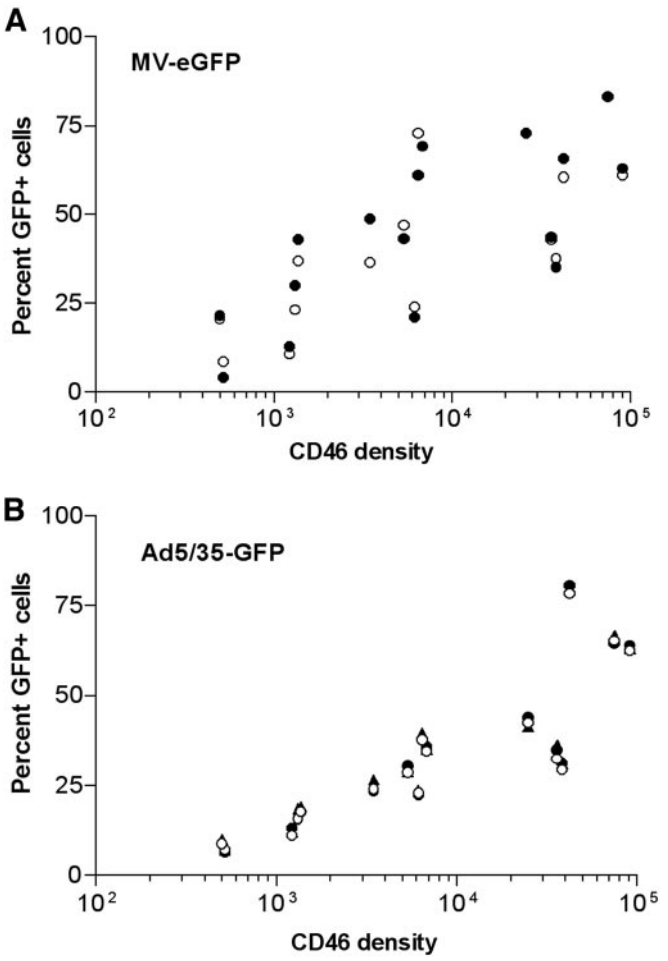

Fig. 3. MV-Edm entry increases progressively as a function of CD46 receptor density. *A*, Chinese hamster ovary-CD46 cells were infected with MV-eGFP (multiplicity of infection = 0.5; $n = 2$) and maintained in medium containing a fusion-inhibitory peptide to prevent intercellular fusion. The percentage of green fluorescent protein-positive cells in the infected cultures was analyzed by flow cytometry at 48 h postinfection and plotted against CD46 receptor density. *B*, a chimeric Ad5/35-GFP vector (Ad5 capsid and Ad35 fiber) was used to infect the panel of Chinese hamster ovary-CD46 cells (multiplicity of infection = 100; $n = 3$), and the percentage of green fluorescent protein-positive cells was analyzed by flow cytometry 48 h later. ○, ●, ▲, individual data points.

CEA gene expression increased in parallel with the number of transduced cells (compare Figs. 5*A* and 3*A*). However, in the absence of FIP, CEA expression levels were little affected in low CD46-expressing CHO clones, where there was little intercellular fusion, but were greatly amplified in CHO clones expressing higher densities of CD46 (Fig. 5*B*). In these high CD46-expressing clones, there is a large bystander effect resulting from intercellular fusion that recruits neighboring uninfected receptor-rich cells into each syncytium, leading to an amplification of the overall level of viral gene expression, which in turn expands the CPE.

To confirm the importance of CD46 density in bystander recruitment of neighboring cells by the MV-H/F-expressing cell, CHO or CD46-expressing clones (low, medium, and high) were overlaid on a small number of MV-eGFP-infected CHO-CD46 cells (clone 5), and the syncytia sizes ($n = 10$/clone) were determined using NIH Image J software. We determined that bystander recruitment, reflected by syncytium size, increases as a function of CD46 density. Average syncytia sizes (mean + SD $\times$ 10^4) in CHO, clone 60, clone 78, or clone 5 were 0.22 + 0.37, 0.44 + 0.52, 3.86 + 2.25, and 6.20 + 2.55, respectively, which corresponds approximately to 4, 8, 77, and 124 cells per syncytium.

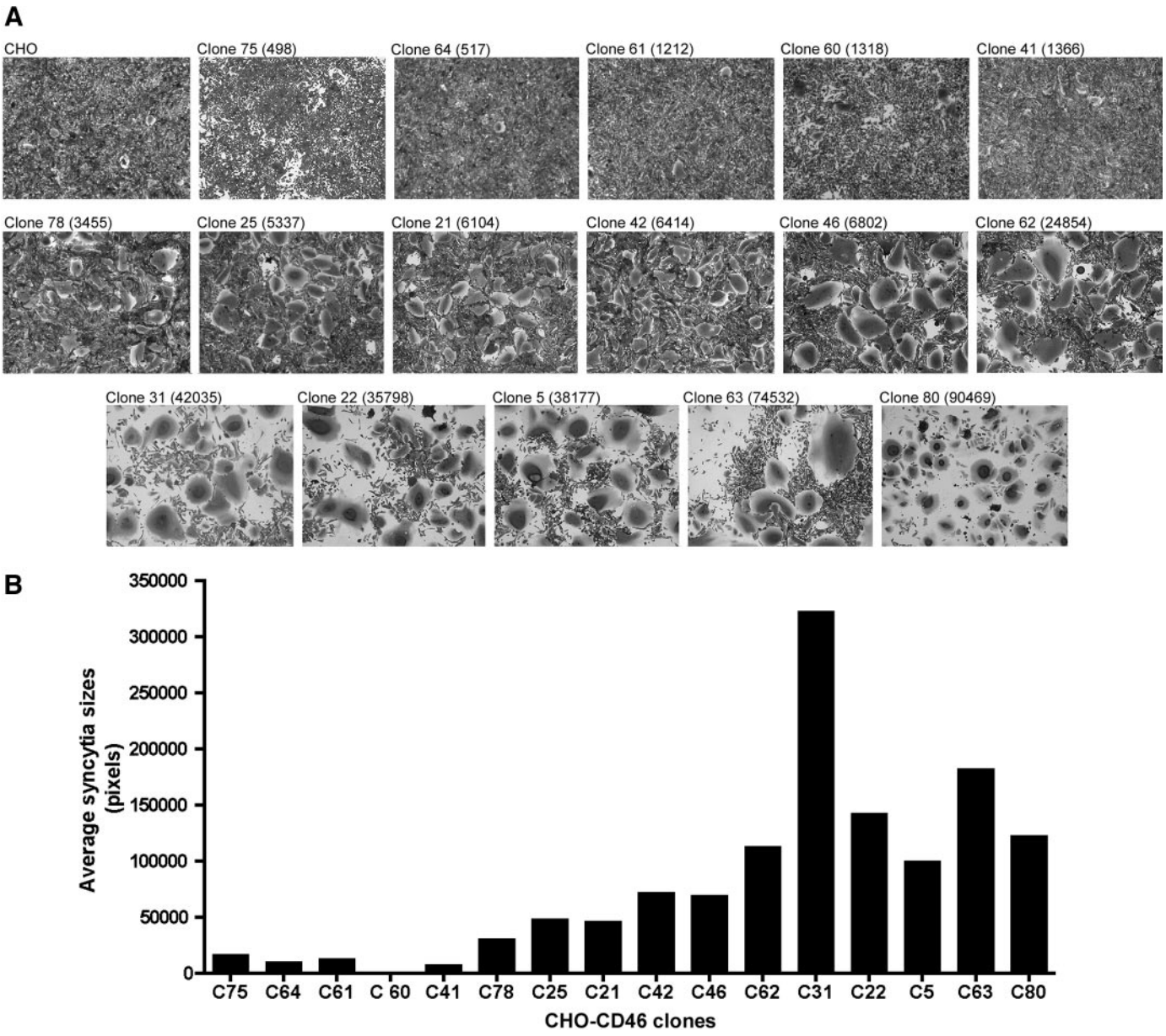

Fig. 4. Sufficiently high levels of CD46 receptors are required for extensive cell fusion in MV-Edm-infected cultures; below these levels, cell fusion is minimal. *A*, photographs of Chinese hamster ovary-CD46 cells transduced by an Ad5 adenoviral vector expressing MV-H/F proteins and displaying a K7 peptide on the Ad fiber (K7Ad5 H/F; multiplicity of infection = 300). The cells were fixed and stained with crystal violet at 48 h posttransduction. Photographs of the Chinese hamster ovary-CD46 clones are ranked in order according to the density of CD46 receptors (shown in *parentheses*) on the cells. *B*, plot of MV-H/F-induced syncytium size of the respective clones. Digital photographs of K7Ad5 H/F-transduced cells (multiplicity of infection = 30, 50, and 100) were taken, and syncytia sizes (pixels) were determined using NIH Image J software.

DISCUSSION

Attenuated MV-Edm has potent and selective antineoplastic activity, but the mechanism underlying its oncolytic specificity has not been previously understood (2, 3). Here, we demonstrate that the striking difference in CPEs between three tumor cell lines and two primary normal cells is not due to lack of virus infection or a large difference in viral gene expression levels. We therefore focused on CD46 because it is required for virus entry and cell fusion. We hypothesized that there may be a correlation between CD46 receptor density and the strikingly different CPEs observed in MV-Edm-infected tumor cells *versus* nontransformed cells. Mean fluorescence indices reflecting CD46 receptor density on the tumor cells were about 7–10-fold higher compared with the normal cells. This observation is in agreement with the vast literature showing that tumor cells generally express higher levels of complement regulatory proteins (*e.g.*, CD46, CD55, and CD59) compared with their normal counterparts (3, 24). To define the role of CD46 receptor density in MV-Edm-induced CPEs, we used a panel of CHO cells expressing different levels of CD46 receptors and showed that MV-Edm selectively fuses cells that express a high density of CD46 receptors. Both virus entry and intercellular fusion induced by MV-H and MV-F proteins are dependent on CD46 density. However, syncytium formation is affected much more significantly by the same change in CD46 receptor density. Cell fusion also amplifies the spread of the initial infection event by recruiting neighboring uninfected cells into the syncytium and usurping their host cell machinery for production of viral proteins and virus progeny. Indeed, viral gene expression was at least 5-fold higher in high CD46-expressing clones if syncytia were allowed to form. The syncytia remain viable for some time, producing viral proteins and expanding in size, but they eventually die by apoptosis

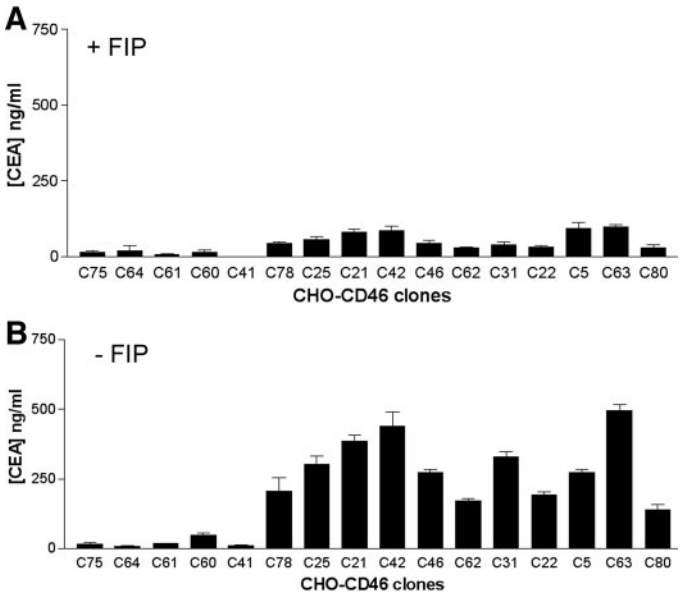

Fig. 5. Viral gene expression is amplified by syncytium formation. Carcinoembryonic antigen levels in the culture medium of MV-CEA-infected cells (multiplicity of infection = 0.5) at 48 h postinfection are shown in (*A*) medium containing 80 nM *Z*-D-Phe-L-Phe-Gly-OH (+ *FIP*) or (*B*) lacking of *Z*-D-Phe-L-Phe-Gly-OH (− *FIP*).

(4, 32). Discrimination between high and low densities of CD46 receptors on target cells provides a novel strategy for tumor selectivity, in addition to the targeting mechanisms used by other oncolytic agents, for example, adenovirus discrimination of p53 status, IFN sensitivity of vesicular stomatitis virus, or exploitation of the ras-activated pathways by reovirus and herpes simplex virus (8, 33).

It is well established that CD46 is expressed abundantly on tumor cells, and this has been interpreted as a possible mechanism by which they resist lysis by complement. In particular, CD46 expression levels were found to be considerably higher in malignant ovarian cancer, cervical cancer, breast cancer, endometrial cancer, lung cancer, hepatoma, and leukemia than in corresponding normal tissues (3, 24). However, CD46 has not been previously exploited as a cancer target. In 2001, Durrant and Spendlove (25) proposed using cancer vaccines to target CD46 and other membrane regulatory proteins overexpressed on tumor cells. They reasoned that the remaining CD46-dim tumor cells would be more highly susceptible to complement-mediated lysis and therefore easy to eliminate with monoclonal antibody therapy. Anti-idiotypic vaccination or bispecific antibodies that recognize both tumor antigens and CD55 or CD59 have been tested for cancer therapy, although none that recognize CD46 have yet been tested (24, 25, 34). However, whatever the therapeutic agent may be, it must be able to discriminate the relative receptor density on tumor cells and normal cells and induce potent damage to the tumor cells (25). Our study shows that MV-Edm efficiently discriminates between cells with higher and lower surface densities of CD46 and therefore qualifies as an appropriate agent to target CD46 on tumor cells. There remains a possibility that CD46 expression among the tumor cells may be heterogenous due to loss or down-modulation of the receptor on a minority of tumor cell surfaces. However, down-modulation of CD46 can potentially make these cells more susceptible to complement lysis and the effects of monoclonal antibody therapy.

CD46 is used as a receptor by other biological agents besides MV such as *Streptococcus pyogenes* (35), *Neisseria gonorrhoeae*, and *Neisseria meningitides* (36), human herpesvirus 6 (37), and group B adenoviruses (31, 38). With the exception of the group B adenoviruses (39), the oncolytic potential (if any) of these pathogens has not yet been tested. Adenoviruses based on the type 5 (Ad5) serotype (group C) have shown promising oncolytic activity and are being tested in the clinic for antitumor efficacy (40). These group C adenoviruses do not use CD46 but interact with the coxsackie adenovirus receptor for attachment and entry into host cells. Coxsackie adenovirus receptor is often down-regulated and expressed at very low levels on tumor cells, and various strategies are therefore currently under investigation to enhance Ad5 infection of tumor cells by redirecting virus entry through cancer-associated receptors such as integrins and epidermal growth factor receptor (41, 42). In contrast to coxsackie adenovirus receptor, CD46 is frequently expressed at high levels on human tumor cells, and pseudotyping the Ad5 capsid with fibers from group B adenoviruses (*e.g.*, with Ad35) can redirect virus entry through CD46 and enhance infectivity of the chimeric virus on tumor cells. Indeed, we found that Ad5/35 infectivity on the panel of CHO-CD46 cells increased progressively with CD46 receptor density. However, in contrast to Ad35, which exploits CD46 for entry only, MV-Edm uses CD46 to mediate both entry and cell-to-cell fusion, leading to destruction of the MV-H/F-expressing cells if they express high levels of CD46 receptors. This unique relationship between the CPE of cell-to-cell fusion (which greatly enhances bystander killing of the infected cells) and CD46 expression levels makes MV-Edm or the fusogenic MV-H/F proteins appealing for use in cytoreductive cancer therapy.

There are likely to be additional factors contributing to the tumor specificity of attenuated MV besides differences in CD46 receptor density between tumor cells and normal cells (for example, intrinsic fusogenicity). This difference in membrane fusogenicity would mean that different levels of CD46 would be required to trigger efficient fusion in different cell types. In our model, the CHO clones have similar fusogenicity, and the only difference was the density of CD46 receptors. The "threshold" receptor density required for extensive fusion will not necessarily be at the same level for human cells. Clearly, additional studies are needed to investigate a wider panel of human cells, preferably derived from primary sources, to precisely evaluate the range of CD46 expression levels. The primary innate response of a virally infected cell serves to inhibit viral protein synthesis and is coordinated through IFN-α/β, double-stranded RNA-dependent protein kinase, 2′,5′-oligoadenylate synthetase, and the Mx proteins (9–11). The IFN-α/β or RNA-dependent protein kinase response pathways are often impaired in tumor cells, but not in normal cells, and this is the mechanism underlying the tumor selectivity of a number of RNA viruses currently being tested for cancer therapy, for example, vesicular stomatitis virus (43, 44), reovirus (45, 46), and certain herpes simplex virus mutants (47, 48). If these antiviral mechanisms are induced by MV-Edm infection, they will serve to amplify the difference in selectivity conferred by the role of CD46 density in regulating CPEs. However, MV-Edm encodes V and C viral proteins that can respond to the host defense by antagonizing IFN-α/β production and signaling (11–15). We also determined that viral protein synthesis in infected normal cells was only 2–4-fold lower compared with infected tumor cells, and the striking difference in CPEs cannot therefore be explained by a preferential shutdown of viral protein synthesis in these normal cells as part of their antiviral response.

In conclusion, we have shown that high CD46 receptor density on tumor cells is a key determinant of the oncolytic specificity of attenuated MV. Whereas virus entry increases progressively with CD46 density, there is a threshold number of CD46 receptors required for cell-to-cell fusion, which leads to death of all of the cells incorporated into synyctia. This study establishes attenuated MV-Edm as a targeted oncolytic agent that can discriminate between high CD46 receptor densities typical of tumor cells and low CD46 receptor densities typical of nontransformed cells.

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