Forward and reverse genetics strategies for improving oncolytic reoviruses

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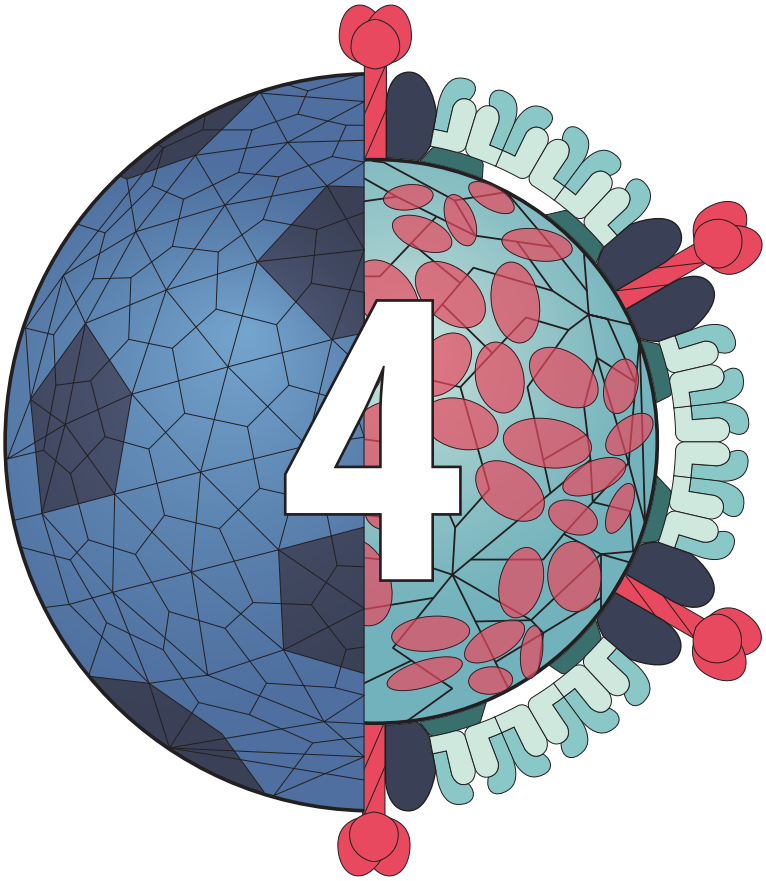
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MAMMALIAN ORTHOREOVIRUS T3D INFECTS U-118 MG CELL SPHEROIDS INDEPENDENT OF JUNCTION ADHESION MOLECULE-A

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ABSTRACT

In the canonical pathway, infection of cells by the wild-type mammalian orthoreovirus Type 3 Dearing is dependent on the interaction of the viral spike protein σ1 with the high-affinity cellular receptor Junction Adhesion Molecule-A (JAM-A). We previously demonstrated that the human glioblastoma cell line U-118 MG does not express JAM-A and resists reovirus T3D infection in standard cell culture conditions. Heterologous JAM-A expression sensitizes U-118 MG cells to reovirus T3D. Here we studied reovirus infection in U-118 MG cells grown in spheroid cultures with the premise that cells in such cultures resemble cells in tumours more faithfully than those grown under standard adherent cell-culture conditions on a plastic surface. Although the U-118 MG cells in spheroids do not express JAM-A, they are susceptible to reovirus T3D infection. We show that this can be attributed to factors secreted by cells in the spheroids. The concentration of active extracellular proteases cathepsin B and L in the medium of spheroid cultures was increased 19 and 24-fold, respectively, as compared with standard cell culture conditions. These enzymes can convert the reovirus particles into a form that can infect the U-118 MG cells independent of JAM-A. Taken together, these data demonstrate that infection of tumour cells by wild-type T3D is not strictly dependent on the expression of JAM-A on the cell surface.

INTRODUCTION

The mammalian orthoreovirus Type 3 Dearing (T3D) is a promising oncolytic agent. Currently, its use as anti-cancer therapeutic, either in stand-alone treatment or in combination with existing medication, is explored in a variety of clinical trials[1]. Reovirus T3D has been well tolerated and no dose-limiting toxicity has been reported so far. Anecdotal evidence of antitumour efficacy exists in stand-alone treatment and in combination therapies[2]. The reovirus T3D is a member of the *Reoviridae* family, which is characterized by a segmented double-stranded RNA genome. The non-enveloped virus contains two icosahedral protein shells. Reovirus has been long known to induce cell death preferentially in tumour cells, and much less in non-transformed diploid cells[3]. Various determinants underlie the virus' preferences for killing cancer cells of which active Ras signalling[4,5], receptor availability[6] viral uncoating[7], and endosomal trafficking[8] appear the most important.

Reovirus attachment protein σ1 is considered as the main determinant of the virus' tropism. Attachment of the virus to the host cell is a three-step process. The virus first scans the cell surface by high avidity binding to sialic acids with residues in the shaft domain of the spike protein[9]. This leads to the engagement of the high-affinity reovirus receptor Junction Adhesion Molecule-A (JAM-A, also named JAM-1) by a defined region in the head domain of σ1[10]. JAM-A is a transmembrane protein with two extracellular immunoglobulin domains and a short cytoplasmic tail. The protein is concentrated at the apical region of intercellular tight junctions of epithelial and endothelial cells[11]. Following JAM-A binding, β1 integrins guide internalization of the virus, most likely by clathrin-mediated endocytosis[12]. In the endosome the virus is partially uncoated by proteolytic disassembly by the host proteases such as cathepsin B and L[13]. This process is characterized by structural changes in σ1, removal of the outer-capsid protein σ3 and conformational rearrangements in outer-capsid protein μ1. In this stage the particles are designated as intermediate subviral particles (ISVPs) (reviewed by Danthi *et al*.[14]). ISVPs are able to escape the endosome, thereby delivering transcriptionally active cores into the cytoplasm[15]. In the intestines and the lungs, the initial sites of reovirus infection *in vivo*, disassembly of the virion by host proteases can take place extracellularly and the resulting ISVPs can enter cells independent of JAM-A directly at the cell membrane[16-19].

Under standard cell culture conditions (SCCC), infection of glioma cells by reovirus T3D strictly depends on expression of JAM-A. We and others demonstrated that the reovirus-resistance of the glioblastoma cell line U-118 MG, upon its growth in SCCC, can be overcome by heterologous expression of the JAM-A receptor or its extracellular

domain coupled to a heterologous transmembrane domain[20,21]. Remarkably, however, various JAM-A negative primary glioma cell cultures, which had been isolated from tumour resection material and were grown as spheroid cultures, were found to be sensitive to reovirus T3D infection[22]. These paradoxical results question the importance of JAM-A in reovirus infection. Therefore we decided to study the role of JAM-A in more detail in two *in vitro* cell culture models. These are the SCCC cultures adherent to a polystyrene surface, and the three-dimensional tumour-cell spheroids.

The spheroid cell culture models are being used in cancer research since the 1970s[23] but gained renewed interest when the limitations of two-dimensional culture methods became apparent (reviewed by Achilli *et al*.[24]). Spheroids resemble the architecture of an *in vivo* tumour (including its microenvironment) more closely than SCCC cultures. As an example, spheroids establish gradients of products that are secreted or taken up by cells. Also, cell-cell and cell-matrix interactions in spheroids better mimic tumours[25]. This also became evident from genomic profiles of adherent cell cultures and spheroids derived from resected glioma material; spheroids more closely resemble the genetic characteristics of the parental tumour[26].

Here we grew U-118 MG glioblastoma cells as SCCC cultures and as multicellular spheroids to compare the susceptibility to reovirus in these two models. We show that, while U-118 MG cells grown in spheroid cultures are JAM-A deficient, they are highly susceptible to reovirus infection. This can be attributed to proteolytic activation of the reoviruses by proteases secreted by the cells in the spheroid cultures. This demonstrates that factors secreted by cells in the three-dimensional cultures can proteolytically activate the reoviruses which results in infection independent of JAM-A expression.

RESULTS

Growth of U-118 MG cells in SCCC and spheroid cultures

To study the effects of the culture system on the growth rates of U-118 MG cells, the cell numbers were counted at various time points after initiation of the SCCC and spheroid cultures. Both cultures started at 5,000 cells per well and cell numbers were counted regularly for five days (figure 1a). As expected, cells in SCCC cultures divide more frequently than in spheroid cultures. At five days after initiation, the cell numbers in adherent SCCC cultures increased almost ten-fold, whereas the cell number in the spheroid cultures increased only three-fold. The diameter of the spheroids increased linearly during this time (figure 1b). Ki-67 protein staining on microtome sections of spheroids revealed the presence of proliferating cells throughout the spheroid (figure

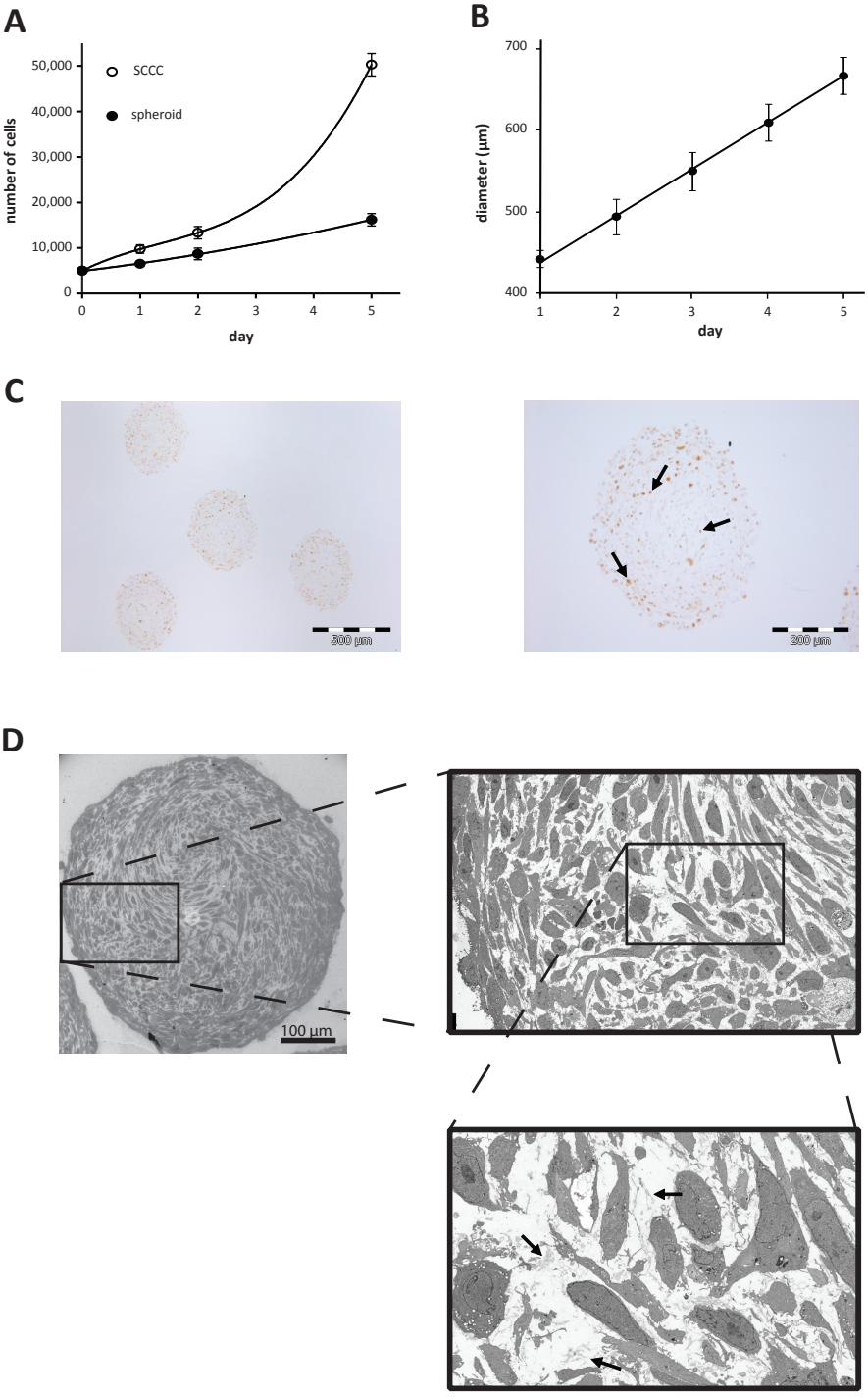
1c). These data confirm the presence of viable cells within the spheroid. Analysis using transmission electron microscopy of a section of a spheroid (figure 1d) revealed the presence of extracellular matrix (ECM). No evidence was obtained on the presence of a necrotic centre, which is often observed in multicellular spheroids[25].

U-118 MG spheroids do not express JAM-A

Cell-cell interactions of cells in spheroids inevitably differ from cells in SCCC cultures. U-118 MG cells in SCCC culture do not express reovirus' primary cellular receptor, JAM-A[20]. To study whether JAM-A expression is induced by growth in spheroid cultures, we investigated the JAM-A expression status. As a positive control, U-118HA-JAM cells were used that were generated by transduction of U-118 MG cells with a lentiviral vector encoding JAM-A[20]. Flowcytometry analyses showed that U-118 MG cells retrieved from spheroid cultures do not express JAM-A protein on their surface, whereas the U-118HA-JAM spheroids were clearly JAM-A positive (figure 2a). The absence of JAM-A expression in both U-118 MG SCCC cells and spheroids was confirmed by reverse transcription polymerase chain reaction (RT-PCR) on total cellular RNA (figure 2b).

U-118 MG cells in spheroids are more susceptible to reovirus infection than U-118 MG SCCC cultures

Results from our previous study suggested that JAM-A expression is not a prime determinant of the sensitivity of human glioma cells to reovirus infection and that significant reovirus infection can occur in cells that have only very low amounts of JAM-A at their surface[22]. Therefore, we studied the difference in sensitivity to reovirus infection of U-118 MG cells grown as SCCC- and as spheroid cultures. Spheroid cultures (consisting of 5000 cells) were prepared using methylcellulose-containing culture medium in non-adherent U-bottom plates[27]. As controls, SCCC cultures were prepared with the same number of cells. We exposed the cells in both cultures to wild-type reoviruses at varying multiplicities of infection (MOI_{911}), ranging from 0 to 100 PFU_{911} per cell. Three days post infection, the presence of the reovirus protein σ3 was visualized by immunocytochemistry. This revealed only few positive cells at both MOI_{911} of 10 and 100 in the SCCC cultures, while the outer rim of the spheroids was almost completely infected at MOI_{911} of 10. Virus infection deep into the spheroid was clearly visible at MOI_{911} of 100 (figure 3a). The number of infected cells was quantified by flow cytometry by staining for the reovirus protein σ3 with a fluorochrome R-Phycoerythrin (PE)-coupled antibody. In SCCC cultures only 0.3% and 2.4% of the cells were positive for σ3 upon exposure to MOI_{911} of 10 and 100, respectively.


A
number of cells
50,000
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1
2
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day
B
diameter (µm)
700
600
500
400
1
2
3
4
5
day
C
500 µm
200 µm
D
100 µm

Figure 1. Growth of U-118 MG SCCC and spheroid cultures. (a) Growth curves of U-118 MG cells in SCCC and spheroid cultures, showing the number of cells per culture, which were initiated with 5000 cells per culture. (b) The diameter of U-118 MG spheroids was measured at five consecutive days. (c) Viability assay by Ki-67 protein staining in cross-sections of U-118 MG spheroids, two days after preparation. The right panel is an enlargement of the lower right spheroid. Examples of Ki-67-positive cells are indicated by an arrow. To enhance the visibility of weak signals, no counterstain was applied in these sections. (d) Electron microscopy image of a U-118 MG spheroid, two days after preparation. The boxed sections are enlarged in the right, lower panel. The extracellular matrix (ECM) between cells is indicated by arrows.

The cells in the spheroids cultures were more efficiently infected, with 13.0% and 39.0% of the cells positive for σ3 expression upon exposure to MOI_{911} of 10 and 100, respectively (figure 3b). Finally, the viability of SCCC cells and spheroids after reovirus infection was assessed eight days post infection (figure 3c). Although the viability of SCCC cultures only slightly decreased at increasing virus titer, spheroids showed a distinct decrease in viability especially at MOI_{911} of 10 and 100. We considered the possibility that the methylcellulose used in formation of the cultures may increase the sensitivity of the cells to reovirus infection. To investigate this, we compared the viability of reovirus infected spheroids generated in culture medium containing methylcellulose with those generated on a solid agarose matrix. In both spheroids systems cells were equally susceptible to reovirus (data not shown), demonstrating that the methyl-cellulose treatment does not increase the cells' susceptibility to reovirus infection.

As compared with cells in monolayer cultures, cells in a spheroid grow at higher concentrations. This allows the cells of a spheroid to affect their local environment to a greater extent than cells in monolayer cultures. To test whether a high local concentration of cells in SCCC cultures can mimic the spheroid environment and stimulated JAM-A-independent transduction, we generated SCCC cultures at low, normal, and high density and exposed them to reovirus at MOI_{911} of 100. This assay revealed higher infection efficiency (as concluded from σ3 protein staining) for the high density cultures (figure 4a). This was confirmed by flow cytometry to quantify the number of infected cells. Cell cultures with low and normal density exhibited only 0.9% and 3.3% reovirus-infected cells, respectively, whereas 20.8% of cells in the high density culture were infected (figure 4b). These results demonstrate that, as in spheroid cultures, JAM-A is dispensable for reovirus infection in dense SCCC cultures.

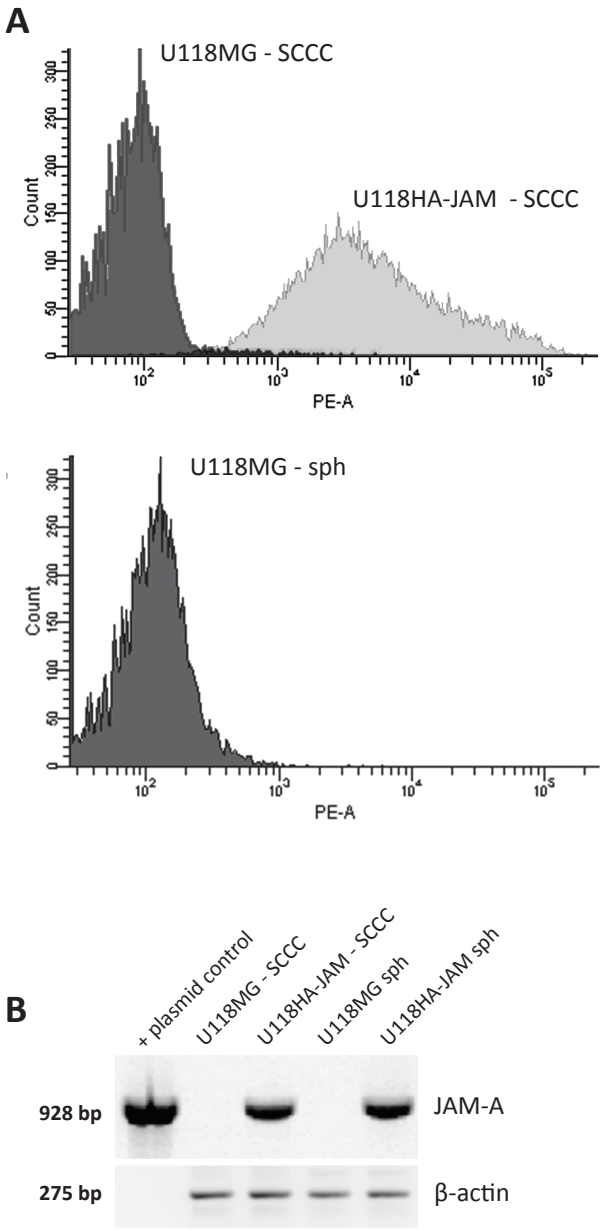
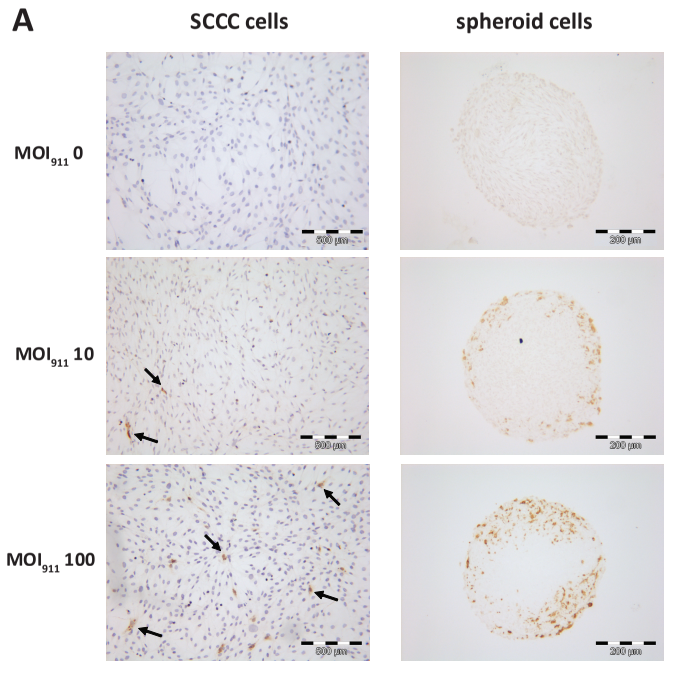


Figure 2. JAM-A expression in U-118 MG cells. (a) Flow cytometry analysis of JAM-A expression on U-118 MG and U-118HA-JAM SCCC cells and U-118 MG spheroid cells using a JAM-A specific antibody. (b) RT–PCR assay on total cellular RNA of U-118 MG and U-118HAJAM SCCC and spheroid cultures using JAM-A-specific primers. β-actin analysis was performed to confirm the integrity of the RNA.

Secreted products enhance reovirus infection of JAM-A negative cells

Our data show that U-118 MG cells in spheroid cultures are more susceptible to reovirus infection than their counterparts in SCCC cultures, despite the absence of JAM-A. As tumours are known to secrete proteases into their microenvironment[28], which may result in reovirus disassembly outside the cell, we speculated that a similar process may occur in the medium surrounding the U-118 MG cells in spheroid cultures. To test this hypothesis, we collected medium from U-118 MG cells in SCCC and in spheroid cultures 5 days after establishing these cultures. Reoviruses were added to the collected media, which was subsequently exposed to semi-confluent U-118 MG SCCC cultures at MOI_{911} of 100. Three days post infection, the number of infected cells was analysed by flow cytometry. While U-118 MG cells infected with reovirus in presence of fresh medium and medium from adherent cells show only 2.8% and 2.3% infected cells, respectively, the spheroid medium with reovirus yielded an infection in 16.2 % of the cells (figure 5a). This increase in infection was supported by a viability assay on semi-confluent U-118 MG cells. As semi-confluent U-118 MG cells do not readily exhibit a cytopathic effect upon reovirus infection, cells were replated in fresh medium three days post infection and allowed to attach and proliferate. The next day cell viability was assessed. While infection (at MOI_{911} of 10) in the context of fresh medium or SCCC-derived medium showed a marginal decrease in viability, the viability was reduced to 70% after infection in the context of spheroid-derived medium (figure 5b). At MOI_{911} of 100, a more pronounced decrease in viability was observed for cells infected in presence of fresh medium or SCCC cell medium. However, the viability of cells infected in spheroid-derived medium decreased even more, up to 40%.

We next investigated whether the medium derived from spheroid cultures indeed has a direct effect on the virions, thereby promoting the infection of JAM-A-deficient cells. We dissolved reovirus into medium from SCCC cells, medium from spheroids, and non-conditioned control medium, and subsequently purified the virus by CsCl banding. Infection of semi-confluent U-118 MG cells in monolayer cultures revealed that the virus particles incubated with SCCC-derived medium do not enhance transduction of U-118 MG cells (figure 5c). In contrast, infection with virus that had been pre-exposed with spheroid-derived medium was significantly increased. These data demonstrate that one or more factors secreted by the U-118 MG cells in spheroid cultures enhance the reovirus infection of JAM-A negative cells.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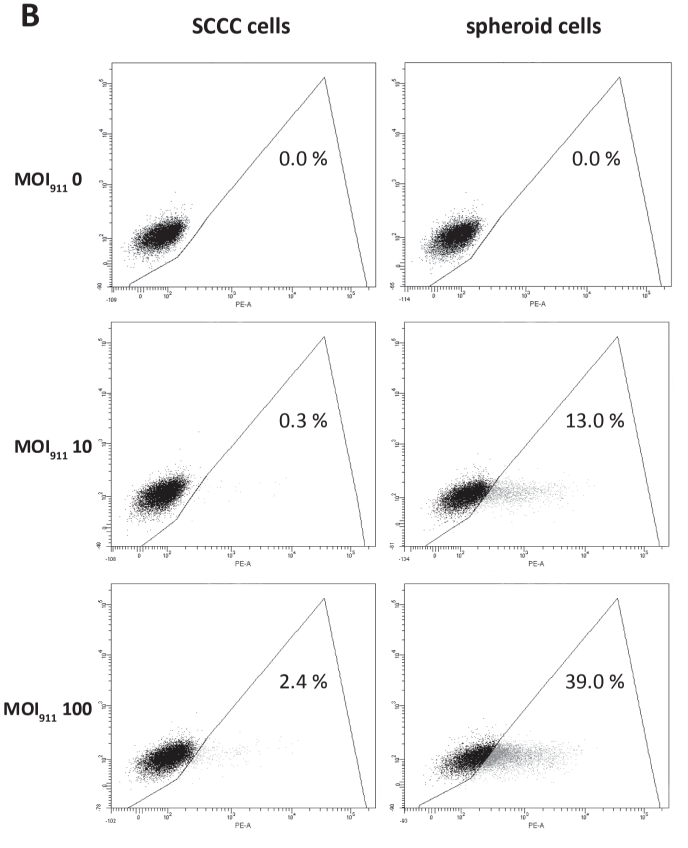
B
SCCC cells
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MOI911 0
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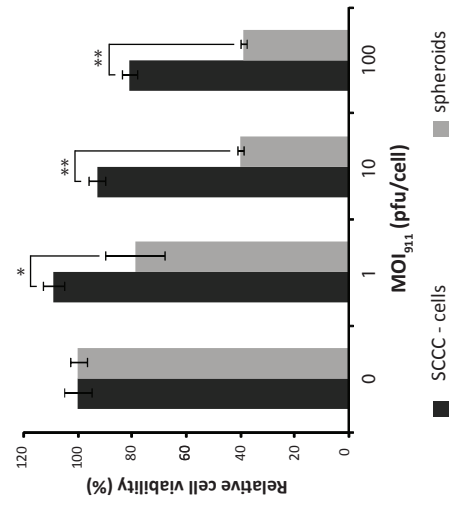

Figure 3. Reovirus infection of U-118 MG SCCC and spheroid cultures. (a) Detection of reovirus protein σ3 by DAB staining in reovirus-infected U-118 MG SCCC and spheroid cultures, at 72 hours post infection. The arrows indicate σ3-positive cells in the SCCC culture. (b) Flow cytometry analysis of reovirus-infected U-118 MG SCCC and spheroid cells by staining for reovirus protein σ3 at 72 hours post infection. The figure is representative for three independent samples. (c) Viability assay of reovirus-infected U-118 MG SCCC and spheroid cultures, at eight days post infection. The viability of uninfected samples is set to 100%. The error bars represent the s.e.m. (n= 12 samples), P-values: *P < 1.0E – 2, **P < 5.5E – 9.

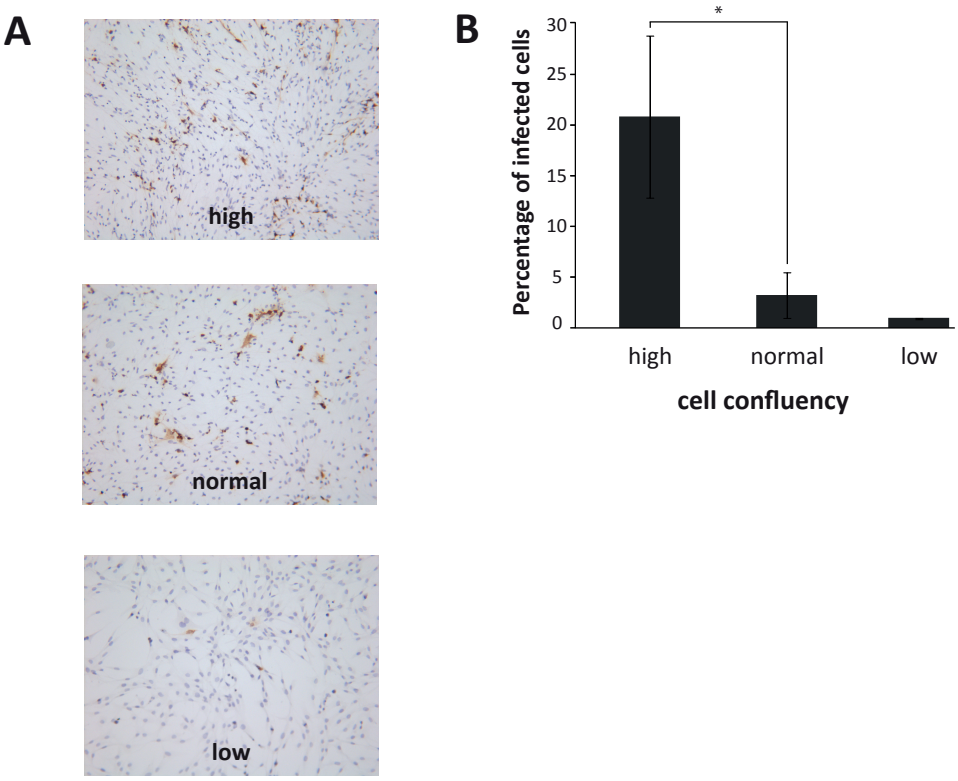

Figure 4. Reovirus infection of U-118 MG SCCC cultures at different densities. (a) Detection of reovirus protein σ3 by DAB staining in SCCC cultures at different densities infected with reovirus at MOI_{911} of 100, at 4 days post infection. (b) Flow cytometry analyses of reovirus-infected (MOI_{911} of 100) SCCC cultures at different densities. The error bars represent the s.d. (n=3), *P < 3.0E − 2.

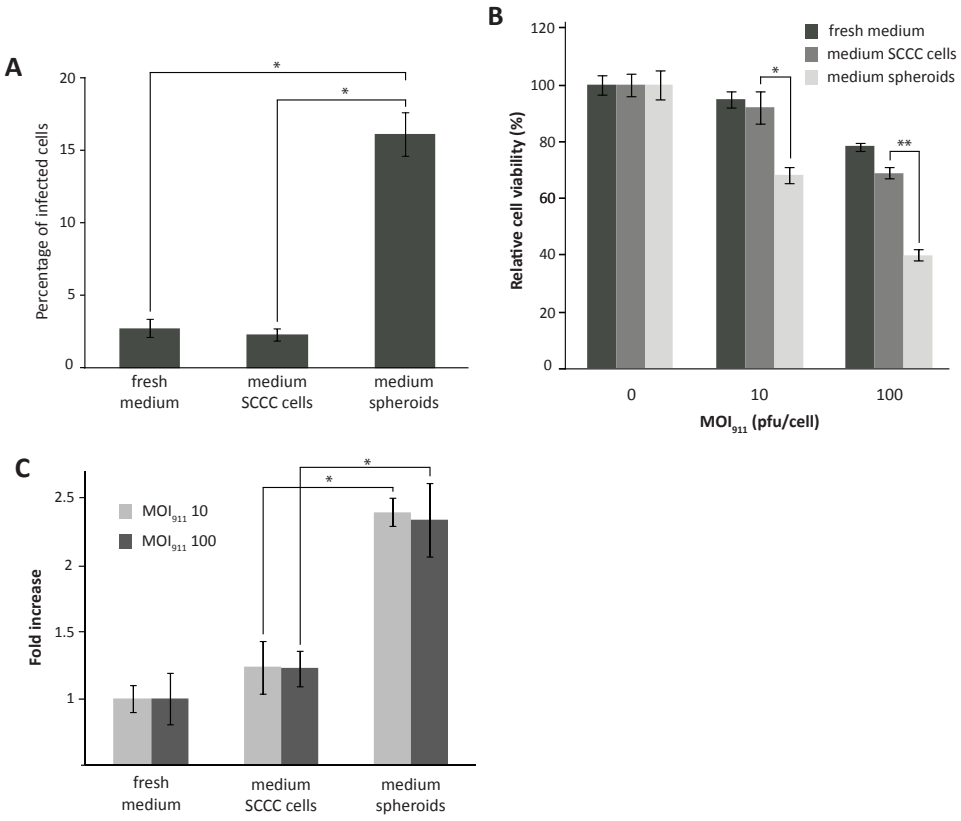

Figure 5. Assessment of the effects of culture-derived factors on reovirus infection efficiency. (a) Flow cytometry assay on reovirus-infected U-118 MG cells (MOI_{911} of 100) in the presence of medium derived from U-118 MG SCCC cultures, U-118 MG spheroid cultures or fresh medium, at 3 days post infection. Error bars indicate the s.d. (n=3), *P < 2.5E − 4. (b) Viability analysis of U-118 MG cells exposed to reovirus at MOI_{911} of 100 in the presence of medium derived from U-118 MG SCCC cultures, spheroid cultures or fresh medium. Error bars represent the s.e.m. (n=12), *P < 3.6E − 3, **P < 1.2E − 6. (c) Flow cytometry analyses of U-118 MG cells infected with reovirus that were previously incubated with medium derived from U-118 MG SCCC cultures, spheroid cultures or fresh medium. The error bars indicate the s.d. (n=3), *P < 5.0E − 3.

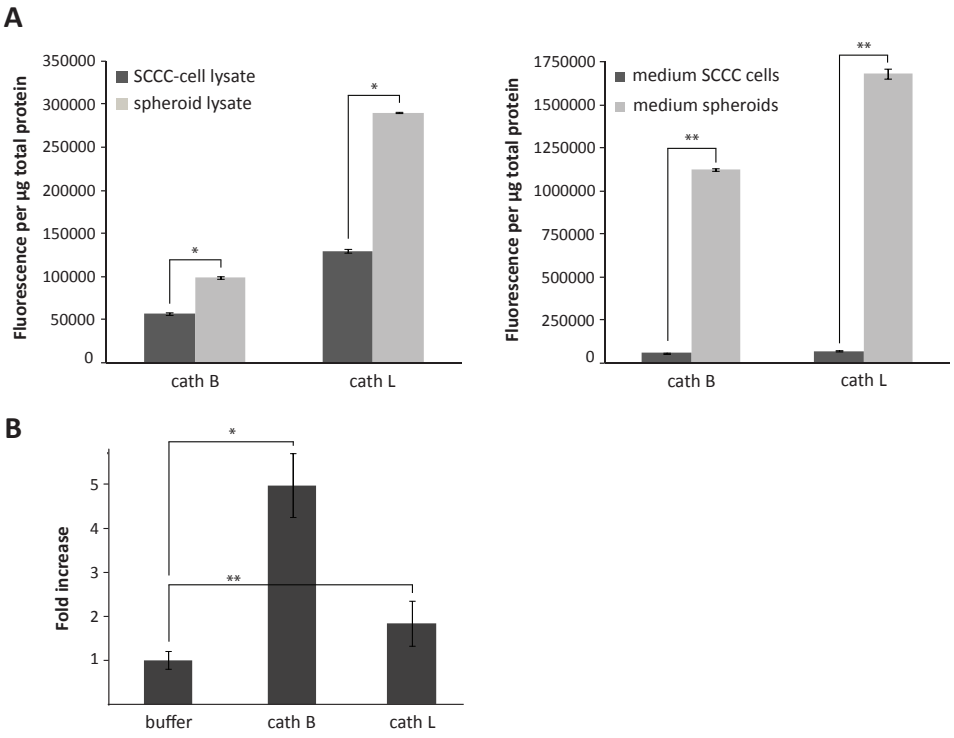

Figure 6. Cathepsin B and L activity in U-118 MG cultures and culture medium, and its effect on reovirus infection. (a) Cathepsin B and L activity in the cells and conditioned media of U-118 MG SCCC cultures and spheroids. Error bars indicate the s.d. (n=3), *P < 2.0E− 6, **P < 9.0E − 10. (b) Increase in reovirus infectivity in semi-confluent SCCC cultures of U-118 MG cells upon exposure of the virus to Cathepsin B, Cathepsin L or buffer. After treatment, the virus preparations were purified by CsCl centrifugation before the infectivity assay. The number of infected cells was assayed by flow cytometry at 3 days post infection. The graph shows the fold increase in infectivity over buffer-treated virus. The error bars indicate the s.d. (n =3), *P < 5.0E − 3; **P < 5.0E − 2.

Cathepsin B and L secretion of spheroids is upregulated and enhances reovirus infection of cells

Many tumours produce high amounts of cathepsins that are secreted into the extracellular microenvironment[28,29]. Cathepsin B and L are known to be involved in reovirus disassembly in the endosome[13]. To test whether cathepsin B and L are secreted by U-118 MG spheroids and could activate the reovirus entry, we examined the cathepsin B and L activity in cells and medium of U-118 MG SCCC and spheroid

cultures. This demonstrated a two-fold increase in intracellular cathepsin B and L activity in the U-118 MGspheroid cultures compared with SCCC cultures (figure 6a). In the media, this difference was even more pronounced, with a 19 and 24-fold increase, respectively. To investigate whether cathepsin B and L can modify reovirus particles and enhance infection of semi-confluent SCCC cultures of U-118 MG cells, we incubated reovirus stocks with either cathepsin B or L. After virus purification, semi-confluent SCCC cultures of U-118 MG cells were exposed to these particles and analysed 3 days post infection by flow cytometry. This revealed a five-fold increase in the percentage of infected cells incubated with cathepsin B-treated virus, as compared with virus incubated in the cleavage buffer alone. Cathepsin L treatment rendered the virus two-fold more infectious in U-118 MG cells (figure 6b). These data demonstrate that cathepsins secreted by U-118 MG cells grown in spheroid cultures can modify reovirus particles allowing them to infect JAM-A deficient U-118 MG glioma cells.

DISCUSSION

On the basis of *in vitro* studies, JAM-A has originally been characterized as the major cellular receptor of reovirus[10]. The function of JAM-A in reovirus T3D infection *in vivo* became evident by studies using JAM-A-deficient animals[30]. While the virus' distribution towards the central nervous system, which is the major tropism in mice, can occur via both neural- and bloodstream routing, only the bloodstream routing appears to be JAM-A dependent[30]. Also, JAM-A is not required for the initial *in vivo* infection with reovirus T3D in the intestines[16,17] and in the lungs[18]. Here the reovirus can infect as ISVPs upon proteolytic conversion by extracellular proteases.

For the use of reovirus as an oncolytic agent, the importance of JAM-A is unclear. Whereas reovirus is able to induce apoptosis after JAM-A independent cell entry[31], the presence of JAM-A on the cell membrane of tumour cell lines has been reported to be important for the cells' susceptibility to reovirus infection *in vitro*[32,33]. JAM-A positive tumours *in vivo* are generally well infected by reovirus[33,34] and resistance to reovirus is observed when JAM-A is absent, or not located on the tumour cell surface[6,33]. Remarkably, some cell lines that are JAM-A negative *in vitro*, can form reovirus susceptible tumours upon implantation as xenografts in mice. Alain *et al*. demonstrated that U-118 MG tumours in mice are susceptible to reovirus infection, in marked contrast to the reovirus resistance of U-118 MG cells grown in adherent cell monolayer cultures[7]. Here we demonstrated that also *in vitro* JAM-A can be dispensable for reovirus infection. Despite high resistance

to reovirus of U-118 MG cells in monolayer cultures, U-118 MG spheroids can be readily infected and killed. Similar observations have been made in glioblastoma stem-like cell (GSC) cultures, isolated from tumour resections of glioma patients[22].

We demonstrate that the increased sensitivity to reovirus infection of U-118 MG cells in spheroid cultures is mediated by components secreted into the medium. Infection of JAM-A deficient adherent cells in presence of conditioned medium from spheroid cultures significantly enhances the capacity of the reovirus to infect these cells. In many tumour types, such as mammary carcinoma, prostate cancer and glioma, the secretion of proteases is upregulated[28,29]. In a tumour and its microenvironment these proteases are part of complex, multidirectional pathways interacting to modulate key processes as angiogenesis, metastasis and ECM composition, ultimately promoting tumour progression[28,35]. The main factors in this network are cathepsins (predominantly cathepsin B), metalloproteinases (MMPs) and urokinase plasminogen activator[28,29,36]. For the reovirus infectious pathway, cathepsins B, L and S are important in the disassembly process of the virion[13,37,38], creating ISVPs that are able to escape the endosome. In the acidic environment of tumours and spheroids[25,39,40], reovirus ISVPs may be generated extracellularly. This could provide the reoviruses with an entry mechanism that bypasses JAM-A for cell entry[15]. There is evidence that cathepsin B expression is upregulated in U251N glioma spheroids compared to monolayer cells[41] and that cathepsin B and L expression is higher in reovirus sensitive U-118 MG tumours in mice than in resistant adherent cells[7]. We extend these observations and show that cathepsin B and L expression and secretion were strongly enhanced in U-118 MG cells grown in spheroids compared with cells in adherent monolayer cultures. Approximately 20-25 fold enhanced secretion of active cathepsins B and L was found in the medium of spheroids, reminiscent of the enhanced secretion of cathepsins by tumours *in vivo*. The cathepsins may be involved in the deposition and organisation of ECM components, such as fibronectin and proteoglycans, as observed in U-118 MG- and other glioma spheroids[42]. The depositions of ECM in our spheroid cultures, as readily observed by EM analysis, may have been the result of increased cathepsin secretion.

We also demonstrated that the infectivity of reovirus particles in U-118 MG cells can be affected by components in the spheroid-conditioned medium. The amount of activating proteases is limiting activation, as is evident from the observation that 5- and 25-fold dilutions of the conditioned media in fresh medium were incapable of generating reoviruses capable of infecting U118MG cell in SCCC cultures (data not shown). It should be noted, however, that the effective concentrations of activating proteases at the cell surface may be higher than the amounts that are present in the conditioned media. Purified cathepsins could convert reoviruses to particles that are infectious

for U118 cells in SCCC cultures. Moreover, we demonstrated that JAM-A independent infection by reoviruses could be enhanced when the U-118 MG cells were grown at very high densities (figure 4a). These conditions may mimic the high cell density seen in spheroid cultures.

There may be other cellular processes or components that enhance reovirus infection in our spheroid model. Our data do not exclude the potential involvement of other secreted proteases such as metalloproteinases. Metalloproteinase expression is upregulated in many gliomas[28] and metalloproteinases have been shown to enhance the intratumoural spread of herpes simplex viruses and adenoviruses by remodelling the ECM[43,44]. A similar mechanism might also augment reovirus spread in spheroids and tumours. It may therefore be useful to study the production of proteases by cellular explants from solid tumours that will be subjected to oncolytic reoviruses therapy.

By showing that spheroids can upregulate the secretion of proteases, and consequently display an enhanced susceptibility to reovirus, we demonstrated that spheroids are a valuable *in vitro* model, better resembling the in vivo situation than SCCC cultures. The spheroid model should therefore be considered as alternative for monolayer tumour cell cultures. It could be implemented in studies for new therapeutics before experiments in tumour-bearing animals. It could provide information about the anticancer agent's efficacy that may be more faithfully mimicking the *in vivo* effects than adherent cell monolayer cultures.

MATERIALS AND METHODS

Cell lines

The human cell lines U-118 MG (glioblastoma) and 911 (adenovirus type 5 early region 1 transformed human embryonic retinoblast)[45] were maintained at 37 °C in a humidified atmosphere of 5% CO_2, in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL) and penicillin-streptomycin (pen-strep). The generation of U-118HA-JAM cells by transducing U-118 MG cells with a lentiviral vector encoding hemagglutinin (HA)-tagged JAM-A, was described previously[20]. These cells were cultured in DMEM high glucose, supplemented with 8% FBS, penicillin, streptomycin, and 200 µg/ml G418 (Life Technologies, Carlsbad, CA, USA).

Reovirus production and purification

The wild-type T3D reovirus strain R124 (here referred to as reovirus) was isolated previously from a reovirus T3D stock (stock VR-824) obtained from the American Type Culture Collection (ATCC) by two rounds of plaque purification using 911 cells. Large-scale virus production was performed by infecting 911 cells with a MOI of 1 to 3 plaque forming units (PFU_{911}), followed by medium replacement at three hours post infection, and collection of cells and medium at 72 hours post infection. Cells and medium were separated by centrifugation for 15 minutes at 3000 g. The pellet was resuspended in 2 ml of the medium fraction and subjected to three cycles of freezing and thawing. The sample was cleared by centrifugation for 10 minutes at 3000 g and the supernatant was mixed with the medium fraction. From this suspension, the virus was further purified by a double discontinuous cesium chloride (CsCl) gradient protocol. Hereby, the virus suspension was layered onto a double-layered (1.45 and 1.20 g/cm^3) CsCl cushion in phosphate buffered saline (PBS) and centrifuged in a SW28 rotor at 95,000 g for 3 hours at 16 °C. After the first centrifugation step the lowest of the two virus bands (containing the infectious virions) was isolated with a syringe, and loaded onto a new CsCl gradient and centrifuged as before. After isolation the virus particles were desalted in an Amicon Ultra-15 Centrifugal Filter Device (molecular weight limit of 100kDa,Millipore, Billerica, MA, USA). The virus was stored in reovirus storage buffer (10 mM Tris pH 7.5, 150 mM NaCl, 10mM $MgCl_2 \cdot 6\ H_2O$, 5% sucrose) at - 80 °C. The infectious titer of the virus was determined by plaque assay on 911 cells.

Preparation and infection of SCCC cultures and spheroid cultures

To grow U-118 MG and U-118HA-JAM cells in standard cell cultures conditions (SCCC) (adherent to a plastic surface) and as spheroid cultures, the cells were harvested from semi-confluent monolayers by trypsin treatment, counted and resuspended in medium containing 2.4 mg/ml methylcellulose (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) at the concentration of 50,000 cells/ml (described in[27]). Of these suspensions, 100 µl was added into each well of a flat-bottom (for SCCC cultures) or U-bottom (for spheroids) 96-well-plate. Cells were re-inserted in the incubator for 24 hours to allow their adherence or establishment as spheroid (one spheroid per well (5,000 cells/well)). Methylcellulose was removed by repeated washes with medium. Reovirus infection was done with an MOI_{911} ranging from 0 to 100 PFU per cell. Unless stated otherwise, the virus concentrations (PFU/µl) were kept equal for SCCC cultures and spheroids. As the number of cells per well differed between the two cell culture methods, the culture medium volume in the wells was adjusted to obtain the desired titer in PFU per cell. To count the number of cells per well, cells were dissociated using

TrypLe Select (Gibco-BRL) and counted using a hemocytometer. The diameter of the spheroids was measured on an Olympus CK40 microscope, using an Olympus Camedia Digital Camera C-3030 and Olympus software; Olympus DP-soft.

Immunocytochemistry analysis

SCCC cultures. For immunocytochemistry analysis, SCCC cultures were grown on glass coverslips, fixed in 4% formaldehyde for 15 minutes at room temperature (RT), washed with PBS and further permeabilized in PBS containing 0.1% triton for 15 min. After exposure to blocking solution (10% goat serum in PBS) for 1 hour at RT, cells were incubated for 3 hours at RT with antibody 4F2 directed against reovirus σ3 protein (monoclonal antibody[46], obtained from the Developmental Studies Hybridoma Bank, maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242), 1:200 diluted in blocking solution. After washing in PBS, the samples were incubated with polyclonal goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) (P0447, Dako, Glostrup, Denmark), 1:50 in blocking solution, for 30 minutes at RT. After extensive washing, the cells were stained with a filtered 3,3'-diaminobenzidine (DAB) solution (10mg DAB dissolved in 10 ml PBS and mixed with 10 ml H_2O and 10 µl 30% H_2O_2) under the microscope and immersed in H_2O after appearance of brown colour. After dehydration by incubation in increasing concentrations of ethanol (50-100%), the samples were briefly exposed to xylene and mounted in Pertex (Pertex Mounting Medium, Leica Biosystems, Wetzlar, Germany).

Spheroids. Spheroids were fixed overnight in 4% formaldehyde at 4 °C, dehydrated and stained in an eosin solution (1% eosin in ethanol) for 10 min. After removing the excess of eosin by washing with 100% ethanol, spheroids were incubated twice in xylene for 15 minutes and embedded in paraffin. The paraffin blocks were sectioned in 6 µm thick slices on a microtome and after paraffin stretching in a 40 °C water bath, transferred to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and allowed to dry. The slides were deparaffinised in xylene, before rehydration of the samples by immersion in decreasing concentrations of ethanol (100-50%). The antigens were retrieved by heating the slides in 10 mM sodium citrate buffer (0.19 g citric acid monohydrate and 1.2 g trisodium citrate dehydrate in 500 ml H_2O) and maintaining the temperature just below the boiling point for 6 min. Samples were allowed to cool, washed twice in H_2O and incubated in 0.3% H_2O_2 for 10 min. After a brief wash in H_2O and PBS, samples were blocked and further processed as described above for the SCCC samples. Ki-67 protein staining was performed to detect proliferating cells. For

this, uninfected spheroids were treated as outlined above, though incubated with a primary purified mouse antibody against human Ki-67 (BD Biosciences), 1:100 diluted in blocking solution.

Electron microscopy analysis of the spheroids

Spheroids were fixed in 1.5% glutaraldehyde and post-fixed in 1% osmium tetroxide and subsequently en-block stained in 1% aqueous uranyl acetate. After dehydration in ethanol, the samples were embedded in Epoxy resin LX-112 (Ladd Research). Ultrathin 100-nm sections were prepared and post-stained with uranyl acetate and lead citrate for ultrastructural analyses. Electron microscopy images were obtained in an FEI Tecnai 12 BioTwin operated at 120 kV and equipped with an Eagle cooled slow-scan chargecouple device (CCD) camera (FEI Company, USA). A total of 3545 overlapping images were collected at a magnification of × 6800 and binning 2 (pixel size of 3.2 nm at the specimen level) and stitched together into a single virtual slide as previously described [47].

Flow cytometry analysis

Anti-JAM immunostaining. Adherent cell cultures of U-118 MG and U-118HA-JAM cells and spheroids of U-118 MG cells were prepared as described before. Three days post preparation of spheroids or SCCC cultures, cells were harvested by dissociating the cells using TrypLe Select. Cells originating from 24 wells were pooled and fixed by incubation in 4% formaldehyde for 15 minutes at RT. After washing cells in staining buffer (1% FBS, 0.09% w/v sodium azide in PBS, pH 7.5, filtered), cells were incubated for 1 hour at RT with a 1:200 dilution of mouse monoclonal anti-JAM-A antibody (clone M.Ab. F11, Abcam, Cambridge, UK) in staining buffer. After washing, the cells were exposed to R-phycoerythrin (PE) fluorochrome-conjugated rat anti-mouse immunoglobulins (IgG_1, BD Biosciences) for 30 minutes at RT in the dark, 1:100 diluted in staining buffer. Subsequently, the cells were washed extensively, resuspended in FACS buffer (0.5% BSA, 2mM EDTA in PBS) and assayed on a BD LSRII flow cytometer, 10,000 events per sample. Data were analysed with FACSDiva software (BD Biosciences).

Anti-reovirus immunostaining. To analyse the susceptibility to a reovirus infection, SCCC cultures and spheroids of U-118 MG cells were prepared and infected with MOI_{911} of 10 or 100 PFU per cell. Three days post infection cells were dissociated, pooled and fixed as above, washed in staining buffer. After permeabilizing the samples in Perm/Wash Buffer (BD Biosciences, San Jose, CA, USA) for 15 minutes at RT, they were incubated with primary antibody against reovirus σ3 protein, 1:200 diluted in Perm/Wash Buffer for 1 hour at RT. Subsequently cells were washed and exposed to

PE fluorochrome-conjugated rat anti-mouse antibody ($IgG2_{a+b}$, BD Biosciences) for 30 minutes at RT in the dark, 1:100 diluted in Perm/Wash buffer. After extensive washing, the samples were resuspended in FACS buffer and analyzed on the flow cytometer as described above.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from SCCC cultures or spheroids of U-118 MG and U-118HA-JAM cells was extracted using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol, upon sonification at 4 °C of the spheroids. First-strand cDNA was generated with 100 ng total RNA using SuperScript II reverse transcriptase (Life Technologies - Invitrogen, Carlsbad, CA, USA) and oligonucleotide hJAM_RT Rev (5'-CACCAGGAATGACGAGGTC-3'). The resulting cDNA was amplified in 30 cycli using oligonucleotide hJAM_RT rev and hJAM for (5'-ATGGGGACAAAGGCGCAAGTC-3') employing Pfu DNA polymerase (Thermo Fisher Scientific – Fermentas) and visualized with gel electrophoresis on a 1% agarose matrix together with plasmid pcDNA-HA-JAM as a positive control. As a loading control, 25 ng of total RNA was used to generate cDNA with oligonucleotide Hum_β-actin Rev (5'-TCCTTCTGCATCCTGTCGGGCA-3'). Amplification was performed using oligonucleotides Hum_β-actin For (5'-CAAGAGATGGCCACGGCTGCT-3') and Hum_β-actin Rev.

Cell Viability assay

Eight days post infection of SCCC cultures or spheroids, the cell viability was assessed by replacing the culture medium by fresh medium containing 10% WST-1 reagent (Roche, Penzburg, Germany) in 12 wells per condition. After 1 hour (for SCCC cultures) or overnight (for spheroids) the absorbance was measured in a microplate reader (Bio-Rad model 550, Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm. The viability measurements were normalized to the viability of uninfected cultures.

Analyzing high and low confluent adherent cells cultures

Mimicking spheroid cultures by (dense) adherent cell cultures was achieved by growing U-118 MG cells in low (split ratio 1 to 25), normal (1 to 10) or high (1 to 2.5) confluence in 6 well (high and normal confluence) or 24 well (low confluence) plates. Two days post preparation the cells were counted and infected as described before; four days post infection the cells were harvested, fixed and assayed for reovirus expression on the flow cytometer or stained for immunocytochemistry analysis as described before.

Analyzing secretion products of spheroids

Semi confluent U-118 MG monolayers were grown in 6 well plates (flowcytometry assay) or 96 well plates (viability assay) and infected with reovirus with an MOI ranging from 0 to 100 PFU per cell in fresh culture medium or culture-derived medium (isolated from SCCC cultures or spheroid cultures after 5 days of incubation). The percentage of reovirus infected cells was analyzed on the flow cytometer. For viability analysis, cells were dissociated, at four days post infection, using TrypLe Select and seeded in 48 well plates using fresh medium. The next day, the viability of the cells was examined by WST-1 assay.

Reovirus incubation with conditioned medium or cathepsins

The effect of secretion products of spheroids on the virus was studied by incubating 10^9 PFU reovirus with 1.5 ml fresh medium, or 5-day-old conditioned medium from adherent cells or spheroids. At 24 and 48 hours post incubation, 1 ml fresh or conditioned medium was added to the incubation, to ensure the presence of active secretion products. For determining the effect of cathepsins on the virus, 10^9 PFU reovirus was incubated with 12.5 µg cathepsin B or L in cathepsin cleavage buffer (100 mM NaCl, 15 mM $MgCl_2$, 50 mM sodiumacetate (pH 5.0)[48]. After 72 hours incubation, the solutions were loaded on a CsCl gradient (1.55, 1.45 and 1.20 g/cm^3 in PBS) and centrifuged as described before. The virus band was isolated and the CsCl was removed by Amicon filter purification. In the purification the volumes and conditions were standardized for all samples. The purified virus was added to semi-confluent cultures of U-118 MG cells at concentrations of 10 and 100 PFU/cell. Three days post-infection the percentage of reovirus-infected cells was analyzed by flow-cytometetry.

Measurement of cathepsin L and B activity

Enzymatic cathepsin L and B activity was examined in conditioned medium and cell lysates of SCCC and spheroid cultures with a Cathepsin B or Cathepsin L Activity Assay Kit (Abcam). After five days of culturing, cells and medium were separated, medium was stored at 4 °C and cells were counted. Cells were lysed in the kit's lysis buffer and the lysate from two 96 well plates was pooled, centrifuged and protein content was measured using a BCA protein assay (Pierce, Etten-Leur, The Netherlands). 50 µl of cell lysate or medium was assayed for cathepsin B or L activity according to the manufacturer's protocol.

Statistical analysis

The statistical significance of key data was assessed with GraphPad Prism V6 using the Student's t-test. P-values below 0.05 were considered significant.

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