Isolation of Reovirus T3D Mutants Capable of Infecting Human Tumor Cells Independent of Junction Adhesion Molecule-A

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Abstract

Mammalian Reovirus is a double-stranded RNA virus with a distinctive preference to replicate in and lyse transformed cells. On that account, Reovirus type 3 Dearing (T3D) is clinically evaluated as oncolytic agent. The therapeutic efficacy of this approach depends in part on the accessibility of the reovirus receptor Junction Adhesion Molecule-A (JAM-A) on the target cells. Here, we describe the isolation and characterization of reovirus T3D mutants that can infect human tumor cells independent of JAM-A. The JAM-A-independent (jin) mutants were isolated on human U118MG glioblastoma cells, which do not express JAM-A. All jin mutants harbour mutations in the S1 segments close to the region that encodes the sialic acid-binding pocket in the shaft of the spike protein. In addition, two of the jin mutants encode spike proteins with a Q336R substitution in their head domain. The jin mutants can productively infect a wide range of cell lines that resist wt reovirus T3D infection, including chicken LMH cells, hamster CHO cells, murine endothelioma cells, human U2OS and STA-ET2.1 cells, but not primary human fibroblasts. The jin-mutants rely on the presence of sialic-acid residues on the cell surface for productive infection, as is evident from wheat germ agglutinin (WGA) inhibition experiments, and from the jin-reovirus resistance of CHO-Lec2 cells, which have a deficiency of sialic-acids on their glycoproteins. The jin mutants may be useful as oncolytic agents for use in tumors in which JAM-A is absent or inaccessible.

Introduction

The Reoviridae constitute a family of viruses with a non-enveloped icosahedral capsid and a segmented double-stranded RNA genome. Prototypes of the mammalian Orthoreoviruses were isolated from the human respiratory and enteric tracts and have not been associated with serious human disease. The human reovirus type 3 Dearing (T3D) is frequently studied and often serves as a model for the family. The reoviruses have a lytic replication cycle and preferentially induce cell death and apoptosis in tumor cells but not in diploid, non-transformed cells [1–3]. In transformed cells reovirus uncoating and replication are stimulated [4–8]. In addition, Ras signalling sensitizes the cells to reovirus-induced apoptosis [9]. Based on these observations, reovirus T3D is a promising candidate for use as oncolytic agent, and is currently evaluated in a variety of clinical cancer therapy trials [10–13].

Reovirus attachment to cells is a multi-step process. The reovirus spike protein σ1 binds with a region of its shaft domain to cell surface-bound sialic acids with low-affinity, before the head domain of σ1 engages the high affinity receptor Junction Adhesion Molecule-A (JAM-A, also known as JAM-1) [14,15]. Following receptor binding, virions become internalized by a mechanism involving the capsid protein λ2 binding to β1 integrins [16,17]. An alternative entry pathway can be employed upon proteolytic removal of the reovirus outer capsid protein σ3 and cleavage of μ1/μ1C, yielding intermediate (or infectious) subviral particles (ISVPs). The ISVPs can directly penetrate the cellular membrane independent of the presence of JAM-A [18,19]. The ISVPs are similar to the disassembly intermediates formed during cellular entry via the endocytosis pathway.

The reovirus receptor JAM-A is expressed in epithelial and endothelial cells of several tissues including lung, kidney, pancreas, heart, brain, intestine and lymph nodes [20] but some tumor cells have down-regulated the JAM-A receptors on their cell surface, thereby limiting the susceptibility to reovirus T3D. JAM-A expression was found significantly down-regulated in clear-cell renal carcinoma cells [21]. Also, cells grown from freshly isolated colorectal tumor metastases resist reovirus infection. Immunohistochemistry demonstrated that JAM-A is not accessible at the cell surface, although JAM-A is detectable intra-cellularly [22]. Furthermore, there is an inverse correlation of JAM-A expression in breast cancer cells and their ability to migrate. JAM-A is expressed in normal human mammary epithelial cells but in...
metastatic breast cancer tumors the expression is down-regulated [23].

Here we describe the isolation and characterization of reovirus T3D mutants that are adapted to propagation in JAM-A negative, reovirus-T3D resistant cell lines. The first was identified as a spontaneously occurring mutant in one of our batches genetically retargeted reovirus [24]. Subsequently two other mutants were isolated by selection on JAM-A negative human glioblastoma cells. We demonstrate that these JAM-A-independent jin-1 mutants employ an as yet unidentified, but apparently ubiquitous receptor, which is present on a wide variety of cell types. Their potential use as novel oncolytic tools against tumor cells in which JAM-A is absent or inaccessible is discussed.

Results

Isolation of a JAM-A Independent Reovirus Mutant

Previously we described a system for generating genetically modified reoviruses. The modification strategy relies on the exchange of a genome segment encoding the spike protein σ1 by a segment encoding a his-tagged spike. The modified viruses can be selected and propagated on U118scFvHis cells. This cell line is a derivative of the JAM-A negative human glioblastoma cell line U118MG and expresses a single-chain Fv (scFv) on its surface that is capable of binding the His-tag. The scFv serves as an artificial receptor for the σ1-His containing viruses [24].

In one of the batches of σ1-His modified reoviruses, we noted that a cytopathic effect (CPE) was not only induced in the U118scFvHis cells, but also in the parental U118MG cells. This suggested that this batch contains viruses that are capable of infecting cells independent of the presence of JAM-A and independent of the artificial scFv-His receptor.

The first mutant virus isolated, which was called jin-1 (JAM-A independent), was further propagated on U118MG cells. The jin-1 mutant virus was compared to our lab reference wtT3D reovirus. In contrast to the wtT3D reovirus, the jin-1 virus induces CPE in U118MG cells as is evident from a WST-1 cell viability assay (Fig. 1A). Both viruses are equally cytolytic to 911 cells which do contain JAM-A [24]. Immunofluorescence assays using an antibody against the major capsid protein σ3 confirmed the presence of σ3 in U118MG cells infected with jin-1, but not with wtT3D (Fig. 1B). To further verify that U118MG cells support the replication of the jin-1 mutant, a metabolic labelling with [35S]-methionine was performed. As a positive control the U118-HAJAM cell line was included. This U118MG-derived cell line had been transduced with a lentivirus to overexpress an HA-tagged version of the JAM-A receptor. In U118-HAJAM and in 911 cells, exposure to the wtT3D as well as to jin-1 reoviruses established infection as is evident from the synthesis of reovirus proteins. In contrast, in U118MG cells the reoviral protein synthesis was only detectable upon infection with the jin-1 virus but not with our wtT3D (Fig. 1C). These data demonstrate that the jin-1 reovirus, in contrast to wtT3D, is capable of infecting and replicating in the JAM-A negative cell line U118MG.

Sequence Analysis of the Reovirus Mutants

The jin-1 mutant originated from the U118scFvHis cell line. Since this mutant can infect JAM-A negative U118MG cells, we speculated that the attachment protein σ1 was altered. After one round of plaque purification and further propagation for eleven passages on U118MG cells, the complete genome was sequenced. The primers used (for this) are listed in Table S1. The PCR products were purified and used for sequence analysis. In the S1 segment two mutations occurred. The mutation led to a threonine-to-methionine change at position 193 (T193M) and a glutamine-to-arginine change at position 336 of the protein (Q336R). Also in other segments mutations were found (table 1).

To expand the pool of mutants, we repeated the procedure and exposed the U118scFvHis cells with our wild-type virus before further expansion on U118MG cells. After the first selection rounds in the U118scFvHis cells, we again found the Q336R mutation in the σ1 head domain. Upon prolonged propagation (10 passages) on U118MG cells an additional mutation was found in S1, resulting in a G187R change. This mutant strain, carrying mutations resulting in a Q336R and G187R change was named jin-2. Based on the findings in jin-2 S1 in the earlier passage, we analyzed S1 of an earlier passage of jin-1 as well (prior to plaque purification) and also in this S1 segment the only mutation present was the one resulting in the Q336R change in σ1.

Another mutant reovirus (jin-3) was obtained after direct exposure of U118MG cells at very high MOI to wtT3D reovirus. This virus was blindly passaged (i.e. the cells were lysed without signs of overt CPE at the time of virus harvest) for 6 rounds on U118MG cells. After 6 rounds in U118MG cells, CPE became apparent. After plaque purification on 911 cells and 10 additional passages on U118MG the complete genome of the jin-3 mutant was sequenced. Only one mutation was found in the S1 segment, resulting in a G196R alteration. Table 1 gives a summary of all the amino acid changes found in the mutants, compared to our wtT3D. A schematic overview of the amino acid changes in σ1 is depicted in Figure 2. In all jin mutants the amino-acid alterations in the shaft of σ1 are located close to the sialic acid (SA) binding motif [6,15,25,26].

Primary Human Fibroblasts (VH10 Cells) do not Support Replication of the jin Mutants or wtT3D

To study whether the jin mutants acquired the capacity to replicate in normal, non-transformed human cells, we exposed diploid human foreskin fibroblasts (VH10 cells) to wtT3D and to the jin mutants. The skin fibroblasts were chosen because primary human fibroblasts do not express JAM-A [27]. We studied the yields of the jin viruses and compared these with the yields of wtT3D on VH10 fibroblasts and on U118MG cells. As expected U118MG cells yielded high titers of the jin reoviruses, while wtT3D virus yields were below the amounts of virus added to the cells (Fig. 3A). On VH10 fibroblasts, neither the wtT3D reovirus nor the three jin-mutants yielded significant titers (Fig. 3B). Furthermore in the VH10 cell cultures no apparent signs of cell death were observed (data not shown). These data suggest that, like wtT3D our jin mutants do not replicate in normal non-transformed diploid fibroblasts.

During the plaque assays on 911 cells for the determination of viral yields, we noted that the plaques formed by the jin-1 virus and jin-3 virus were consistently smaller than those of the wtT3D virus; the plaque surface area of the initial jin-1 virus and jin-3 virus is approximately 10-fold lower (Fig. 3C), suggesting reduced cell-to-cell spread of the mutants.

The jin-2 virus also has a reduced plaque size compared with the wt virus, but the variation within the population is larger, which suggests heterogeneity in the population. Sequence analysis of the S1 segment of both the smaller and the larger jin-2 plaques revealed that the smaller plaques contained the mutations for the G187R and Q336R change, while the larger plaques only contained the mutation that yield the Q336R alteration in σ1 (data not shown).
Protease Inhibitor E64d Blocks Entry of jin-1 Virus

Reoviruses enter cells by receptor-mediated endocytosis after attachment of the σ1 protein to the JAM-A receptor [16,28]. Subsequently, the viral σ2 protein binds cellular integrins leading to endocytosis. In the endosomes the particle undergoes conformational changes by partial proteolysis, leading to intermediate subviral particles (ISVPs). The outer capsid proteins σ3 and μ1/μ1G are cleaved by cellular proteases and σ1 undergoes a conformational change. In vitro, this process can be mimicked by proteolytic treatment of complete virions. The generated ISVPs are capable to enter cells independent of the JAM-A receptor by penetration of the cytoplasmic domain [18,19,29]. One possible explanation for the JAM-A independent entry of the jin-1 virus could be a premature transition to ISVPs prior to entry into the cell. To test whether the jin-1 virus is still dependent on cellular proteases the protease inhibitor E64d was used. If cells are exposed to E64d prior to infection, intact virions are trapped in the endosome while ISVPs can complete the replication cycle [30,31]. To confirm that wt ISVPs are JAM-A independent we exposed the U118MG cells to wt T3D ISVPs and to intact T3D virions (fig. 4A). As expected, only in the cells exposed to the ISVPs, viral protein σ3 synthesis is detected, evidencing virus entry and viral protein synthesis. In 911 cells both the jin-1 virus and wt T3D virus are inhibited by E64d (Fig. 4B), while σ3 was detected in both the jin-1 and wt ISVP infected cells. Also in the U118MG cells, the jin-1 virus entry is blocked by the presence of E64d, but not the jin-1 ISVP entry. These data demonstrate that like wt T3D reovirus, the reovirus mutant jin-1 exploits the endocytotic pathway to enter U118MG cells.

jin-1 σ1 Forms Trimers

The change at amino-acid position 336 is located close to the domain that has been implicated in trimerization of σ1 [32]. Although the Q336R alteration is located at the outward surface-exposed side of every monomer in the trimeric conformation (Fig. 5A, R336 is shown in red), it is essential to confirm that the Q336R alteration does not interfere with trimer formation. To this end an in vitro trimerization assay was performed as described by Leone at al. [33]. For this wt σ1, σ1-Q336R and σ1-Y313A proteins were synthesized in vitro. The Y313A change abolishes the capacity of σ1 to form trimers [32]. The σ1 products were analysed by mild PAGE at 4 °C (Fig. 5B). Whereas the σ1-Y313A protein does not form mature trimers, both the wt T3D σ1 and σ1-Q336R do. Intermediate trimers, which consist of σ1 molecules in which only one subunit is trimerized while the head domain is in a monomeric configuration, are detectable in all σ1 variants tested. Our data show that the Q336R alteration that occurs in the jin-1 and jin-2 viruses does not affect the formation of mature σ1-trimers in vitro.

jin-1 and jin-2 have Selective Advantages Over Wild-type Reovirus in U118MG Cells

To confirm that jin-1 and jin-2 viruses have a selective advantage over wt T3D in U118MG cells, we mixed jin-1 or jin-2 with a 100-fold excess of wt T3D prior to infection of cells. Cultures of 911 cells were infected at an MOI 10 with the mixtures to allow reassortment of genome segments to take place. Two days post-infection, the virus was harvested by three freeze-thaw cycles and used to infect U118MG cells. While no CPE for jin-1/wt mixtures was observed at 7 days post-infection in the U118MG cells, the cells were freeze-thawed and the lysate was used to infect fresh U118MG cultures. In U118MG cells infected with jin-2/wt T3D mixture the virus was harvested after 4 days, with visible signs of CPE. This procedure was repeated for two more times. At passage 3 clear CPE was observed four days post-infection in both jin-1/wt and jin-2/wt selections. Sequencing of PCR products after reverse transcription PCR of the S1 segment after the third selection on U118MG cells were compared with the S1 sequences of wtT3D, jin-1 and jin-2 (Fig. 6). S1 of the jin-1/wt end population contains a T at nucleotide position 590 and a G at position 1019, identical to the jin-1 S1 segment. Sequence results for S1 of the jin-2/wt end population revealed an A at position 571 and a G at position 1019 and this is identical to jin-2 S1 sequence. From these findings we conclude that in U118MG cells σ1 proteins from jin-1 or jin-2 provide a strong selective advantage over wtT3D σ1. These data provide evidence that the amino-acid alterations in σ1

Table 1. Amino acid differences in reovirus proteins of the jin mutants and the wt T3D reovirus strain.

| RNA segment (protein) | AA position | wt | jin-1 | jin-2 | jin-3 |
|-----------------------|-------------|----|-------|-------|-------|
| S1 (σ1)               | 187         | Gly| Arg   |       |       |
|                       | 193         | Thr| Met   |       |       |
|                       | 196         | Gly| Arg   |       |       |
|                       | 336         | Gln| Arg   | Arg   |       |
| S2 (σ2)               | 254         | Ser| Phe   |       |       |
| S3 (σNS)              | No changes  | 177| Ser   | Phe   |       |
| S4 (σ3)               | 198         | Gly| Glu   |       |       |
|                       | 357         | Met| Thr   |       |       |
| M1 (σ2)               | No changes  | 388| Lys   | Arg   |       |
| M2 (σ1)               | 530         | Thr| Ala   |       |       |
| M3 (σNS)              | 705         | Ala| Val   |       |       |
|                       | 706         | Asp| Ala   |       |       |
| L1 (L3)               | 413         | Ile| Ser   |       |       |
| L2 (L2)               | 1101        | Met| Ile   |       |       |
| L3 (L1)               | 201         | Thr| Ala   |       |       |
|                       | 703         | Arg| Gly   |       |       |
|                       | 1164        | Ser| Phe   |       |       |

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provide the jin mutants with the capacity to infect and replicate in JAM-A-negative cells.

**jin-1** Reoviruses Infect Cells that are Non-permissive for wt T3D

To study whether the jin-1 mutant has expanded its tropism beyond the U118MG cell line, we evaluated whether this virus can replicate in a panel of cell lines that resists infection with wt T3D virus. These cell lines include chicken hepatoma cell line LMH [34], murine endothelium cell line Eoma [35], human bone osteosarcoma cell line U2OS [36] and human Ewing sarcoma cell line STA-ET2.1 [37]. In parallel the cell lines 911 and U118-HAJAM were included as positive controls for infection. Each of the cell lines were exposed to wt T3D or jin-1 viruses with an amount of virus corresponding to 8 PFU/cell as determined in replicate in a panel of cell lines that resists infection with wt T3D or jin-1 viruses. These cell lines include chicken hepatoma cell line LMH [34], murine endothelium cell line Eoma [35], human bone osteosarcoma cell line U2OS [36] and human Ewing sarcoma cell line STA-ET2.1 [37]. In parallel the cell lines 911 and U118-HAJAM were included as positive controls for infection. Each of the cell lines were exposed to wt T3D or jin-1 viruses with an amount of virus corresponding to 8 PFU/cell as determined in 911 cells. While no major capsid protein s3 was detected in the wt T3D-resistant cell lines exposed to wt T3D, exposure of these cells to jin-1 resulted in the detection of the s3 protein at 36 hr post infection (Fig. 7A). In 911 and U118-HAJAM cells, the s3 protein is present in wt T3D infected cells as well as in the jin-1 infected cells.

To verify that wt T3D resistant cells could support replication of the jin-1 virus, the virus yields were determined in some of these cell lines (Fig. 7B). In 911 cells both viruses give a similar yield, but in three other cell lines (U118MG, U2OS and LMH) more progeny virus was produced with the jin-1 virus than with wt virus.

The amount of wt T3D produced per cell did not rise above the amount added to the cells (MOI of 10, dashed lines). From these data we conclude that the jin-1 virus is able to productively infect our panel of wt T3D resistant cells.

**Cell Entry of Reovirus jin-1 and jin-3 Relies on Sialic Acids**

Apart from the Q336R mutation, the other mutations found in the S1 segments of the jin mutants are located close to the region involved in SA binding [6,15]. Recently the crystal structure of the sialic acid – σ1 complex was elucidated [26]. There is a remarkable heterogeneity in the amino acid sequence of the SA-binding domains of different T3D and T1L strains. Some isolates cannot bind SA as determined on JAM-A negative murine erythroleukemia (MEL) cells. The forced selection of such strains yielded mutants that could infect MEL cells probably via the interaction with SA [38].

The wt T3D that was used in our studies has an amino-acid sequence of the sialic-acid binding pocket that is identical to strains capable of binding sialic acid. The S1 mutations found in the jin mutants are not located in the region coding for the SA-binding pocket of σ1 (viz. amino acids 198–204; ref [15,26]), but are located in close proximity of this region. Nevertheless, it is conceivable that the amino acid alterations in the jin mutants affect the affinity or avidity of SA binding. To investigate the involvement of SA in binding of our mutant viruses we used Lec2 cells. Lec2 cells have a strongly reduced (by about 90%) amount of sialic acids on their cell surface [39,40]. Lec2 cells are mutants derived from Chinese Hamster ovary (CHO) cells, which are poorly infected by wt T3D reovirus [41,42]. In contrast, both the jin-1 and jin-3 mutants efficiently infect CHO cells (Fig. 8A). The wt T3D nor jin-1 and jin-3 infect Lec2 cells, as is evidenced by lack of detectable s3 in the cells exposed to these viruses (Fig. 8A). Also the replication of jin-1 in the Lec2 cells is markedly reduced compared with the yields obtained in the parental CHO cells (Fig. 8B). This suggests that the expanded tropism of jin is dependent on the presence of SA on the cell surface. To support the utilization of SA by the jin-1 mutant, we shielded the SA on the surface of the cells by pre-incubating the cells with wheat germ agglutinin (WGA). WGA is a lectin with a strong affinity to a broad range of sialoconjugates. To confirm that WGA effectively binds to the cell lines 911, U118MG and CHO, but not to Lec2, we employed FITC-labeled WGA on fixed cells grown on cover slips (Fig. 9A). For the competition experiments we blocked the sialic acids with WGA prior to the binding of jin-1 to the cells. The addition of WGA to the cells inhibited entry of jin-1 in U118MG and CHO cells (Fig. 9B). Also in 911 cells, jin-1 and wt T3D infection are inhibited (Fig. 9C). This confirms the dependency of wt T3D on SA for cell binding and entry. Our data demonstrate that also the jin-1 mutant relies on SA binding for cellular entry.
Discussion

The use of tumor-selective oncolytic viruses for killing tumor cells that resist conventional therapeutic approaches is conceptually attractive. Human reoviruses are one of the promising candidates for use as replicating oncolytic agent [7,43,44]. Reoviruses preferentially induce cell death and apoptosis in tumor cells, but not in diploid, non-transformed cells [1–3]. However, in some tumor cells expression of the JAM-A receptors is down-regulated and absent on the cell surface, thereby limiting the susceptibility of the cells to reovirus T3D infection.

Here we report the isolation of JAM-A independent T3D reoviruses with an expanded tropism. These mutants, designated as jin mutants, may be considered as oncolytic agents in those tumor types that lack accessible JAM-A on their surface [21–23,45]. Although we encountered the first jin mutant in a batch of S1-His modified reovirus after selection in the U118MG-seFvHis cell line [24], the jin mutants are not genetically modified viruses in the formal sense since they resulted from spontaneous mutations in T3D viruses.

In three independent virus batches we identified jin mutants. Two of these (jin-1 and jin-2) carried an identical mutation in the head domain of the spike protein σ1. The mutation replaces the glutamine (Q) residue at position 336 by an arginine (R). The amino acid 336 is located at a surface exposed position, close to the region involved in the trimerization of the σ1 spikes [32]. The alteration renders the area more positively charged. This could potentially result in conformational changes that may disturb the formation of σ1-trimers. However, the results of a trimerization assay (depicted in Fig. 3) revealed that altered σ1 of jin-1 still forms mature trimers, showing that the Q336R alteration in S1 of the jin-1 and jin-2 viruses does not affect trimer formation of this domain.

While the jin mutants were isolated on human glioblastoma cell line U118MG, we found that the jin-1 mutant efficiently infects a variety of reovirus T3D resistant cell lines, including the chicken hepatoma cell line LMH, but not non-transformed primary...
human skin fibroblasts (VH10). This expanded tropism, together with the small-plaque phenotype observed in the JAM-A positive cell line 911, is reminiscent of changes observed in other virus families. Adaptation to cell culture conditions can result in selection of viruses that acquired the capacity to bind heparan sulfates [46–48]. Variants of foot-and-mouth disease virus (FMDV) which bind strongly to heparan sulfate in vitro, show small plaques on BHK cells and these variants are attenuated in cattle. Furthermore, they have a decreased ability to spread from the site of inoculation [48]. Alternatively, mutants of Sindbis Virus that exhibit a reduced binding to heparan sulfate give rise to larger plaques in vitro and are more virulent in vivo with slower clearance from the circulation [46]. However, the Q336R change in jin-1 and jin-2 does not yield a typical linear heparin-binding domain consensus -X-B-B-X-B-X- and -X-B-B-B-X-X-B-X- in which B is a basic residue (mainly K or R) and X a hydropathic residue [49–51]. It should be noted that the presence of a linear consensus sequence is not a strict prerequisite for glycosaminoglycan binding. Some of Venezuelan equine encephalitis virus (VEE) mutants bind heparan sulfates (HS) through a conformational domain and do not contain the linear HS-binding domain [52]. Our observation that reovirus jin-1 entry into U118MG cells cannot be inhibited by incubation with heparin or heparan sulphate (data not shown) suggests that binding to glycosaminoglycans is not responsible for the broadened tropism of jin-1 and jin-2. It therefore remains to be established if and how the Q336R change contributes to the expanded tropism. Also with respect to sialic acid binding, in some DNA viruses mutations in the SA-binding pocket resulted in changes in plaque morphology [53,54].

Upon continued serial passaging of jin mutants in U118MG cells, additional amino acid alterations accumulated in spike protein σ1, T193M in jin-1 and G187R in jin-2. For the jin-3 mutant, a single mutation in S1 resulted in G196R substitution in S1. Those changes are located in close proximity of the region implicated in sialic acid binding [6,15,25,26]. This is in line with the previous observation that passaging of reoviruses incapable of binding SA on mouse erythroleukemia cells yielded mutants capable of binding sialic acids [38]. In co-crystallization experiments of σ1 in complex with SA it was shown that the changes were mapped in the σ1 region between amino acids 198 and 204 [26].

Also outer capsid protein σ3 plays an important role in the process of reoviral entry [16,18,55,56]. A mutation found in so-
called persistent-infection reoviruses leads to an amino acid change Y354H in the σ3 protein. This alteration has been linked to the reoviral resistance to the protease inhibitor E64d [57]. A mutation in jin-1 results in M357T in σ3, which is in close proximity of position 354. However, no such mutations are found in jin-2 and jin-3. Moreover, jin-1 is sensitive to E64d, demonstrating that the jin-1 virus still depends on cysteine proteases for uncoating and infection. This suggests that the jin-1 virus enters JAM-A negative cells via the endocytic pathway, like wt T3D in JAM-A expressing cells.

So far, we have evaluated the jin mutants in in vitro studies only and it remains to be elucidated what the effect will be in vivo, both with regards to safety, as well as to their oncolytic efficacy.

While we cannot exclude the possibility of the recruitment of secondary receptors, our data suggest that the jin mutants rely on sialic acid binding for internalization. It is tempting to speculate that a changed affinity for sialic acids underlies the changed tropism of our jin viruses, since they show a decreased ability to spread in cultured cells, exhibit a small plaque phenotype, and shielding SA moieties with WGA prevents the jin viruses to enter cells. It remains to be established if the mutation in S1 affects reovirus pathology in mice. In this respect, it is noteworthy that the pathology of reoviruses is, in part, dependent on the σ1 protein [58].

Materials and Methods

Cell Lines

Cell lines 911 (generated previously in our lab, see reference [55]), U118MG (obtained from ATCC), U2OS (obtained from ATCC, see reference [29]), CHO (obtained from ATCC), Eoma (obtained from ATCC, see reference [28]) and VH10 (primary human foreskin fibroblasts, provided by B. Klein [59]) were cultured in Dulbeco’s Modified Eagle Medium (DMEM) containing high glucose (Invitrogen, Breda, The Netherlands), supplemented with penicillin, streptomycin (pen-strep) and 8% fetal bovine serum (FBS) (Invitrogen, Breda, The Netherlands). The U118-HAJAM cells were cultured in DMEM plus 8% FBS and pen-strep, supplemented with 200 μg/ml G418. LMH cells (obtained from ATCC, see reference [27]) were grown on collagen

Figure 6. S1-sequence analysis of the jin-1/wt T3D and jin-2/wt T3D selection assay on U118 MG cells. The jin-1 or jin-2 viruses were mixed with a 100-fold excess of wt T3D virus with regards to MOI. 911 cells were exposed to the mixtures first, before propagation on U118MG cells for three more passages. Reovirus RNA was isolated from the virus derived from the third passage on U118MG cells and subjected to RT-PCR to obtain the S1 products from the total population (jin-1/wt end or jin-2/wt end). Sequence histograms of the indicated regions were compared to the S1 sequences of the input reoviruses. Arrows indicate the nucleotide differences between the wt T3D and jin-1 or jin-2. (S1 nucleotide positions 571, 590 and 1019).

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Figure 7. Reovirus mutant jin-1 can infect cell lines that resist wt T3D reovirus infection. (A) Several cell lines were infected with wt T3D or jin-1 and 32 hr post-infection cells were lysed. Protein samples (30 µg) were analyzed by 10% SDS-polyacrylamide gel electrophoresis. For the immunodetection anti-reovirus s3 (4F2) was used. The cell lines 911 and U118-HAJAM are included to serve as positive controls for the infectivity of wt T3D. The cells were mock-infected (m); wt T3D infected, or jin-1 infected. (B) Virus production of wt T3D and jin-1 in the different cell lines. Cells were exposed to virus at MOI of 10 for one hour, washed with PBS and immediately lysed (1 hour time point) or left for 48 or 72 hours. For 911 cells an additional harvest point at 32 hours post-infection was included. The viral titers in the samples were determined by plaque assays on 911 cells. The graph shows a representative example of the assay. Open circles: wt T3D(o), crosses: jin-1(x). The dashed line represents the input amount of the initial infection (10 PFU/cell).

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I coated dishes (Rat Tail collagen, 2.5 μg/cm², Invitrogen, Breda, The Netherlands) in DMEM plus 8% FBS and pen-strep. STA-ET2.1 cells were grown on collagen I coated dishes (5.0 μg/cm²) in RPMI 1640 medium (Invitrogen, Breda, The Netherlands), supplemented with pen-strep and 10% FBS. Lec2 cells (derived from ATCC, see reference [33]) were cultured in alpha-MEM (Invitrogen, Breda, The Netherlands) with 8% FBS. All cells are cultured in an atmosphere of 5% CO₂ at 37°C.

Reovirus Propagation

The wild-type T3D virus strain R124 (see accession numbers) was isolated from a reovirus T3D stock obtained from the American Type Culture Collection (stock VR-824) by two rounds of plaque purification on 911 cells. The 911 cells were used to propagate R124 (referred to as wt T3D in the text) as described previously [60]. Briefly, cells were exposed to reovirus in DMEM plus 2% FBS for 2 hours at 37°C. Subsequently, the inoculum was replaced by DMEM containing 8% FBS. The virus was harvested 48 hours post infection by resuspending the cells in phosphate-buffered saline (PBS) with 2% FBS and subjecting the suspension to three cycles of freezing and thawing. The sample was cleared by centrifugation for 10 minutes at 300 g. For the mutant jin viruses, U118MG cells were used to propagate the viruses after one round of plaque purification on 911 cells. The jin viruses were routinely harvested from U118MG cells 72 hours post infection. The experiments were done with virus-containing freeze-thaw lysates, unless otherwise indicated. The infectious reovirus titers of the strains were determined by plaque assay on 911 cells.

Origin of the jin-mutants

jin-1 is derived from U118scFvHis cells during our experiments on genetically modifying reovirus [24]. The jin-1 virus was first grown on U118scFvHis cells for two propagations, before three passages on U118MG cells (first point of S1-sequence analysis). The virus was subjected to one round of plaque purification on 911 cells to obtain a homogenous population. This was further propagated on U118MG cells for 11 rounds before analysis of the complete genome sequence (Table 1).

jin-2 was isolated from U118scFvHis cells infected with wt T3D reovirus and passaged twice in these cells. The S1 segment was sequenced from this passage. Subsequently it was passaged 10 times on U118MG cells. After plaque purification, the complete genome sequence was determined (Table 1).

jin-3 was isolated from U118MG cells exposed to wt T3D virus followed by blind passaging the virus for 6 rounds. From the resulted preparation a virus was isolated by plaque purification and following by 10 additional passages on the U118MG cells. (Table 1).

Yield Determinations

To determine the replication of wtT3D, jin-1, jin-2 and jin-3 in U118MG and VH10 cells (Fig. 3), cells were seeded in 24-well plate with a cell density of 1×10⁵ cells/well. Viruses (in DMEM plus 2% FBS) were added to the cells with an MOI of 10, two wells per virus. After an exposure of one hour in incubator (37°C, 5% CO₂) the inoculum was removed and the cells were washed once with PBS and fed by fresh DMEM plus 2% FBS. Reoviruses were harvested from medium and cells by 3 cycles of freeze-thawing, 72 hours after infection. Yields were determined by plaque assays on 911 cells.

For the replication of wtT3D or jin-1 in 911, U118MG, LMH, U2OS, CHO and Lec2 cells (Fig. 7B and 8B), cells were seeded in 6 well plates with a density of 1.5×10⁵ cells/well. Wild-type T3D or jin-1 was added to 4 wells in case of 911 cells and 3 wells for the other cell lines with an MOI of 10 (in DMEM plus 2% FBS). After one hour of exposure in incubator, the viruses were washed from...
the cells and medium was replaced. From one well, immediately after washing once with PBS, cells in medium were collected and subjected to freeze-thaw cycles (1 hour time point). 32 Hours (911 cells only), 48 and 72 hours after infection cells and medium were collected and subjected to freeze-thaw cycles. Viral yields were determined by plaque assays on 911 cells.

**Cell Viability Assay**

WST-1 reagent (Roche, Almere, The Netherlands) was used to assay the viability of cells after reovirus infections. U118MG and 911 cells in 96-well plate were mock-infected or infected with wt T3D or jin-1 with an MOI of 10, in triplo. Six days post infection WST-1 reagent was added, according to the manufacturer’s manual. The viability measurements in mock-infected cell cultures, were set to 100%.

**35S Methionine Labelling**

Infected cells (911 cells infected at MOI = 1; U118MG and U118-HAJAM with MOI = 5) or mock-infected cells were incubated with TRAN35S - LABEL™ (10mCi/ml; MP Biomedicals, Eindhoven, The Netherlands) for 4 h; one day (911 cells) or two days (U118MG and U118-HAJAM cells) post infection. Cells were washed once with phosphate-buffered saline and lysed in Giordano Lysis Buffer (50 mM Tris HCl pH 7.4, 250 mM NaCl, 0.1% Triton, 5 mM EDTA) containing protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). The labelling assays were performed in 24-well plates with 5 μl (50 μCi) TRAN35S - LABEL™ per well. The cells were lysed with 100 μl lysis buffer. After addition of sample buffer, 50 microliters per lystate was loaded in the wells of a 10% SDS-polyacrylamide gel. Gels were dried and exposed to a radiographic film to visualize the labeled proteins.

**Immunofluorescence Assay**

For immunofluorescence assays, U118MG and 911 cells were grown on glass coverslips in 24-well plates before infection with wt T3D or jin-1 with an MOI of 5 or no virus. One day post infection the cells were fixed with cold methanol (15 minutes, 4°C), washed with PBS containing 0.05% Tween-20, and incubated with antibody 4F2 directed against reovirus σ3 (monoclonal antibody developed by T.S. Dermody [61]; obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242), diluted in PBS containing 3% BSA. After incubation at room temperature the cells were washed (PBS, 0.05% Tween-20) and incubated with secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse serum for 30 minutes at room temperature. The mounting solution consisted of glycerol containing 0.02 M Tris HCl pH9.0, 2.3% 1,4-diazabicyclo-[2.2.2]-octane and 0.5 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.
RT-PCR and Sequencing

Total RNA was isolated (Absolutely RNA miniprep kit; Stratagene, Agilent Technologies, Amstelveen, The Netherlands) from U118MG cells infected with the different reovirus mutants (%) one day post infection. For wt T3D total RNA was isolated from infected 911 cells. Primers used for the RT-PCR procedures are listed in Table S1. DNA synthesis of all the segments started with the unique endR primer designed for every segment, using SuperScript III (Invitrogen, Woerden, The Netherlands) for the reverse transcription process. For the PCR, Pfu polymerase (Promega, Leiden, The Netherlands) was used with the primer combinations unique for every segment. PCR products were first cleaned with S1 (Bioline, London, UK), according to the manual, before direct analysis of the sequence. In some cases the resulting PCR products were cloned into plasmid pJE1 (GeneJet, PCR cloning kit; Stratagene, Agilent Technologies, Amstelveen, The Netherlands) and their DNA sequences were determined. All sequence data were generated by The Leiden Genome Center (LGTC, Leiden, The Netherlands).

In vitro Transcription-Translation and Trimerization Assay

All primer sequences can be found in table S1. With DualSHFor and DualSHRev primers, S1 PCR product was generated from plasmid pCDNART3S1 [24], according to manual of the pDual-GC vector (Stratagene, Agilent Technologies, Amstelveen, The Netherlands). The resulting construct (pDualS1His) contained no stop codon, meaning that the Myc-His tag was present behind S1. To introduce a stop codon behind the S1 ORF in pDualS1His, the DualSHist for and rev primers were used in a mutation PCR, with EXL polymerase (Stratagene, Agilent Technologies, Amstelveen, The Netherlands). This resulted in the plasmid pDGC-S1delTag, which was used for the trimerization assay and as start to generate the pDGC-S1QR and pDGC-S1Y313A (with S1-QRmut2Rev and For combi or S1- Y313AmRev and For combi, respectively) also with EXL polymerase. For the in vitro transcription-translation (ITT) part, TNT® T7 Quick Coupled Transcription/Translation kit (Promega, Leiden, The Netherlands) was used. Input for the ITT assays were the plasmids pDGC-S1delTag, pDGC-S1QR and pDGC-S1Y313A. The total reaction volume was 15 μl, scaled according to the manual (in the presence of 6 μCi TRAN35S - LABEL™; MP Biomedicals, Eindhoven, The Netherlands). For the trimerization Assay, one fifth of every ITT reaction per construct was used and incubated with Sample buffer (final concentrations: 10% glycerol, 2% SDS, 60 mM Tris HCl pH 6.7, 2.5% β-mercaptoethanol and 2.5% β-mercaptoethanol and 0.025% bromophenol blue) for 30 minutes at 37°C. Gels were dried and exposed to a radiographic film to visualize the labeled proteins.

Plaque Assay and Size Measurements

Plaque assays were performed in a standard assay as previously described for adenoviruses [62] with minor modifications. Briefly, virus stocks were serial diluted in DMEM containing 2% FBS. The dilutions were added to near-confluent 911 cells in six-well plates. Four hours after infection, medium was replaced with agar-medium. Agar-medium consists of (final concentrations) 0.5% agarose (Ultrapure™, Invitrogen, The Netherlands), 1 × minimal essential medium (MEM), 2% FBS, 12.5 mM MgCl2, 2 mM GlutaMAX™ (Invitrogen, Almere, The Netherlands) and 1 × pen-strep antibiotic mixture (Invitrogen, Almere, The Netherlands). Plaques are counted six days post infection. Plaques sizes were measured four days post infection. For the measurements a CKX41 Olympus microscope was used and the plaque area was measured with the software of Olympus: Olympus DP-soft.

Western Analysis

Cell lysates were made in Giordano Lysis Buffer supplemented with protease inhibitors (Complete mini tablets, Roche Diagnostics, Almere, The Netherlands). Total amount of protein in the lysates was measured (Bradford, Bio-rad, Venendaal, The Netherlands) and the same amount of lysate (30 μg) was loaded into the wells of a 10% SDS-polyacrylamide gel after addition of western sample buffer (final concentrations: 10% glycerol, 2% SDS, 50 mM Tris HCl pH 6.8, 2.5% β-mercaptoethanol and 0.025% bromophenol blue). The proteins were transferred to Immobilon-P (Millipore, Etten-Leur, The Netherlands) and visualized using standard protocols. Antibodies used in this study: 4F2 directed against reovirus σ3; β-Actin antibody: Immuno anti-Actin clone C4 (MP Biomedicals, Eindhoven, The Netherlands).

E64d Inhibition

E64d (Sigma Aldrich, Zwijndrecht The Netherlands) was dissolved in DMSO before use. U118MG and 911 cells were seeded in 24 well plates; half of the cells were exposed to 100 μM E64d at 37°C, 5%CO2 for one hour. Purified wt T3D or jin-1 virus and ISVPs (approximately 2×10^10 particles per cell) were added to the cells and left for one hour at 4°C; cells were washed with PBS and transferred back to 37°C, 5%CO2 in the absence or presence of E64d, for 36 hours. Lysates were made as described in Western analysis. For the immunodetection the anti-σ3 antibody (4F2) and β-Actin antibody were used.

Generation of ISVPs

wt T3D or jin-1 virus ISVPs are freshly prepared by treating CsCl purified virions [60] with chymotrypsin. Purified viruses were diluted to a concentration of 10^{-1} PFU/ml in Reovirus Storage Buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM MgCl2) and treated with 200 μg/ml Chymotrypsin (TLCK treated, Sigma Aldrich, Zwijndrecht The Netherlands; C3142) at 37°C for 1 hour. Reaction was stopped by adding 5 mM phenylmethylsulfonyl fluoride (Sigma Aldrich, Zwijndrecht The Netherlands).

Wheat Germ Agglutinin (WGA) Binding and Competition Experiment

For the detection of sialic acids in the different cell lines, FITC-labeled WGA (Sigma Aldrich, Zwijndrecht The Netherlands) was used. Cells (grown on round glass coverslips in 24-well plate) were fixed with ice-cold Methanol (15 minutes, 4°C) before lysates were made as described in Western analysis. For the immunodetection the anti-σ3 antibody (4F2) and β-Actin antibody were used.

WGA competition experiment was done by exposing cells (in 24-well plate wells) to WGA at a concentration of 100 μg/ml in culture medium for one hour in CO2 incubator at 37°C. The pre-incubation medium was removed and wt T3D or jin-1 virus was added to the cells with an MOI of 10 in DMEM containing 2% FBS at 4°C for one hour. Cells were washed with ice-cold PBS and normal culture medium was added to the cells. Cells were left for 32 hr in CO2 incubator (37°C) before lysates were made as described in Western analysis. For the immunodetection the anti-σ3 antibody (4F2) and β-Actin antibody were used.
Supporting Information
Accession Numbers
GenBank ID’s of wt T3D (R124) and jin-1 segments: R124 T3D-L1 GU991659; R124 T3D-L2 GU991660; R124 T3D-L3 GU991661; R124 T3D-M1 GU991662; R124 T3D-M2GU991663; R124 T3D-M3 GU991664; R124 T3D-S1 GU991665; R124 T3D-S2 GU991666; R124 T3D-S3 GU991667; R124 T3D-S4 GU991668. jin-1-L1 GU991669; jin-1-L2 GU991670; jin-1-L3 GU991671; jin-1-M1 GU991672; jin-1-M2GU991673; jin-1-M3 GU991674; jin-1-S1 GU991675; jin-1-S2 GU991676; jin-1-S3 GU991677; jin-1-S4 GU991678.

Table S1  List of primers used in this study. First part of the table contains the primers used for the Reverse Transcription PCR experiments. Middle part contains the additional primers used to sequence the different segments. The last part contains the primers used to clone the S1 and S1mutants in the Dual-GC system for the trimerization experiments.

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Author Contributions
Conceived and designed the experiments: DJMW IJCD SKH. Performed the experiments: DJMW IJCD SKH SJC. Analyzed the data: DJMW IJCD SKH RJG SJR CJC. Wrote the paper: DJMW IJCD SKH RJG SJC.
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