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RESEARCH ARTICLE

Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved

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Efficient gene transfer and regulated transgene expression in primate embryonic stem (ES) cells are highly desirable for future applications of the cells. In the present study, we have examined using the nonintegrating Sendai virus (SeV) vector to introduce the green fluorescent protein (GFP) gene into non-human primate cynomolgus ES cells. The GFP gene was vigorously and stably expressed in the cynomolgus ES cells for a year. The cells were able to form fluorescent teratomas when transplanted into immunodeficient mice. They were also able to differentiate into fluorescent embryoid bodies, neurons, and mature blood cells. In addition, the GFP expression levels were reduced dose-dependently by the addition of an anti-RNA virus drug, ribavirin, to the culture. Thus, SeV vector will be a useful tool for efficient gene transfer into primate ES cells and the method of using antiviral drugs should allow further investigation for regulated SeV-mediated gene expression.
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Introduction

Since human embryonic stem (ES) cell lines have the ability to both proliferate indefinitely and differentiate into multiple tissue cells,[1,2] they are expected to have clinical applications as well as to serve as models for basic research and drug development. Although efficient and stable gene transfer into primate ES cells would be useful for such purposes, it has been difficult and only lentiviral vectors have been successful in achieving it.[3–5] We have previously developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase.[6] SeV vectors can efficiently introduce foreign genes without toxicity into airway epithelial cells,[7] vascular tissue,[8] skeletal muscle,[9] synovial cells,[10] retinal tissue,[11] and hematopoietic progenitor cells.[12] Here we report that the SeV-mediated gene transfer into primate ES cells is very efficient and stable even after the terminal differentiation of the cells. In addition, we show that SeV-mediated transgene expression levels can be reduced by the addition of a ribonucleoside analog, ribavirin, to the culture. Ribavirin is a mutagen and inhibitor of viral RNA polymerase.[13,14] It shows antiviral activity against a variety of RNA viruses and is used to treat infections of hepatitis C virus in combination with interferon-α[15,16] and of lassa fever virus.[17] The method of using antiviral drugs might offer a novel approach for regulated SeV-mediated gene expression in primate ES cells.

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Results

SeV-mediated gene transfer into ES cells

In this study, we have used an SeV vector, which is capable of self-replication but incapable of transmitting to other cells.[6] The vector does not encode the fusion (F) protein (Figure 1a), which is essential for viral entry into cells. It can be propagated only in a packaging cell line expressing the F protein. The green fluorescent protein (GFP) gene was introduced after the leader sequence of the vector genome. Cynomolgus ES cells[18] were exposed to the SeV vector for 24 h. Flow cytometric analysis at 2 days after infection showed that 15, 38, and 61% of cells fluoresced at 2, 10, and 50 transducing units (TU) per cell, respectively (Figure 1b). The gene transfer efficiency of about 60% is comparable to or even better than that for lentiviral vectors.[3] We confirmed that the undifferentiated cell fractions remained unchanged after the infection with SeV vector, as assessed by the expression of undifferentiated markers, alkaline phosphatase and SSEA-4 (data not shown). The GFP expression after infection was stable at least for a month. On the other hand, the GFP gene transfer to cynomolgus ES cells with adenovirus- and adeno-associated virus (AAV)-based vectors resulted in much lower expression levels (<20% by flow cytometry) and the levels declined to zero within a week after infection (Figure 1c).

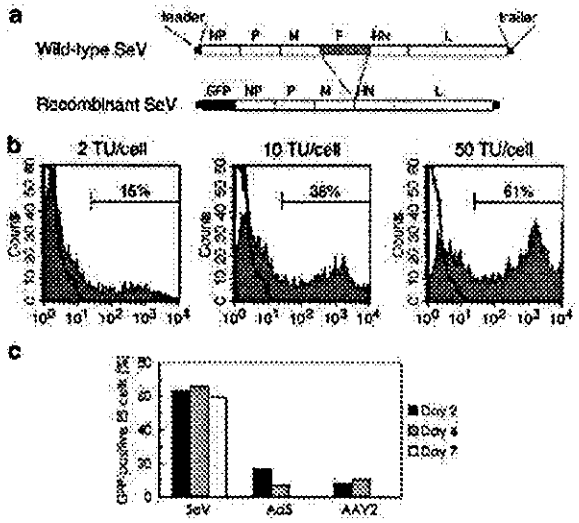

Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, and L) are in order from 3' to 5' in the negative-strand RNA. In the SeV vector, the entire fusion (F) gene was removed and the GFP gene was introduced at a unique NotI site between the leader sequence and NP gene. (b) The GFP expression by the SeV vector in cynomolgus ES cells. Cynomolgus ES cells were infected with the SeV vector at 2, 10, and 50 TU/cell. The flow cytometric profiles at day-2 postinfection are shown in gray. The white areas indicate uninfected ES cells. The fractions of GFP-positive cells are indicated. (c) The GFP expression levels in cynomolgus ES cells infected with the SeV (50 TU/cell), adenovirus serotype 5 (Ad5, 3.4×10^2 g.c./cell), and AAV serotype 2 (AAV2, 2.4×10^4 g.c./cell) vectors. The fractions of GFP-positive cells were examined by flow cytometry at 2, 4, and 7 days postinfection.

We plucked fluorescent ES cell colonies under a fluorescent microscope once at 1 month after infection and propagated them. After this selection procedure, approximately 90% of the ES cells expressed GFP (Figure 2a and b) and the high-level expression was stable for a year as assessed by flow cytometry (Figure 2c, upper). The mean fluorescence intensity per cell was also stable (Figure 2c, lower), indicating that the replicating vector genome was almost equally delivered to each cell of all progeny. The self-replication of the SeV vector in infected cells was confirmed by RNA-PCR that amplified the viral RNA genomic sequence (Figure 3a). The GFP cDNA sequence, however, could not be detected by DNA-PCR in the infected cells (Figure 3b), indicating that no DNA phase was involved in the GFP expression.

Pluripotency of infected ES cells

The SeV-infected, fluorescent cynomolgus ES cells were able to form fluorescent tumors when transplanted into immunodeficient mice (Figure 4a–c). The fluorescence was observed uniformly by fluorescent microcopy (Figure 4d and e). The tumors consisted of all three embryonic germ layer cells (Figure 4f–i). Thus, the SeV-infected ES cells were capable of forming teratomas and the SeV infection did not spoil the pluripotency of ES cells. The infected, fluorescent cynomolgus ES cells were also able to generate fluorescent embryoid bodies (Figure 5a and b), MAP-2-positive neurons (Figure 5c), clonogenic hematopoietic colonies (Figure 5d and e), and mature functional (NBT test-positive) neutrophils (Figure 5f and g), all of which fluoresced. In addition, the GFP expression levels were not decreased during the teratoma formation or differentiation, indicating that no 'silencing' of the transgene occurred.

Drug-inducible reduction of transgene expression

Next, we examined whether ribavirin inhibits the replication and transcription of the SeV vector resulting in a reduction of transgene expression. We first used a rhesus monkey kidney cell line (LLC-MK2) to test the effect of ribavirin on the replication and transcription of the SeV vector. LLC-MK2 is a standard control cell line for SeV infection. Ribavirin was added at various concentrations 2 days after the infection. The formation of viral particles quantified by the hemagglutination assay decreased drastically upon the addition of ribavirin (Figure 6a). The decrease was dependent on the dose of ribavirin. The GFP expression was also depressed dose-dependently (Figure 6b). Thus, ribavirin dose-dependently inhibits the replication and transcription of the SeV vector in LLC-MK2 cells. The toxicity associated with ribavirin was not observed in LLC-MK2 cells.

We then examined the effect of ribavirin on SeV-infected, fluorescent cynomolgus ES cells. The addition of ribavirin also resulted in a dose-dependent reduction of GFP expression in the cells (Figure 6c). Although the GFP expression was almost completely inhibited after a 3-day exposure with 4 mM of ribavirin, the cells could not be propagated thereafter. Ribavirin at high concentrations (>1 mM) hampered the proliferation of cynomolgus ES cells. With lower concentrations (0.5–0.75 mM) of ribavirin, the GFP expression level decreased by half. After the discontinuation of ribavirin treatment, the cells could be propagated and nearly regained the original level of GFP expression. The undifferentiated cell fractions were unchanged after the discontinuation as assessed by alkaline phosphatase and SSEA-4 staining (Figure 6d).

Discussion

There are several advantages in using SeV vectors over other vectors. (i) SeV vectors can infect nondividing, quiescent cells as well as dividing cells unlike oncoretroviral vectors.[7–11] Thus, they can be used to infect cells that are terminally differentiated as well as at various stages of differentiation, whether they are dividing or not. (ii) SeV vector-mediated gene transfer does not require a DNA phase. Thus, there is no concern about the unwanted integration of foreign sequences into the host genome unlike with oncoretroviral or lentiviral vectors. (iii) Transgene expression is stable even in dividing cells since the SeV vector replicates by itself in the cytoplasm of host cells. On the other hand, gene transfer using nonreplicating adenoviral and AAV vectors resulted in decreased levels of transgene expression in dividing cells over time, since the non-replicating transgene was

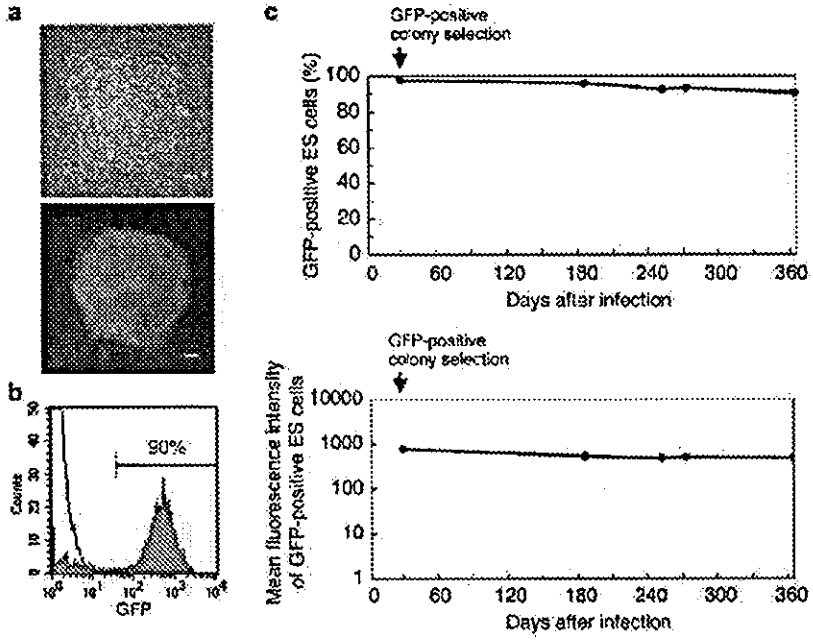

Figure 2 Stable SeV-mediated transgene expression in cynomolgus ES cells. Fluorescent ES cell colonies were plucked under a fluorescent microscope once at 1 month after infection and the cells were further propagated. (a) Phase-contrast (upper) and fluorescence (lower) images of a cynomolgus ES cell colony at day 370 after infection. Bar = 100 μm. (b) Flow cytometric analysis of SeV-infected cynomolgus ES cells at day 370 after infection (shown in green). The percentage of GFP-positive cells is indicated. Uninfected, parental cynomolgus ES cells are indicated by another line (white area). (c) The percentage of GFP-positive cells (upper) and mean fluorescence intensity per GFP-positive cell (lower) after infection with the SeV vector at 10 TU/cell are shown as a function of time (days).

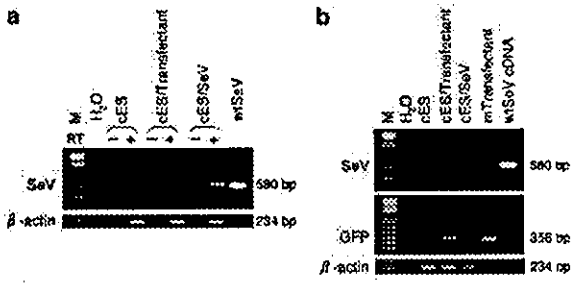

Figure 3 DNA-independent replication and transcription of SeV vector. Total cellular RNA and DNA were extracted from cynomolgus ES cells at day 284 after infection with the SeV vector. RNA-PCR (a) and DNA-PCR (b) for the SeV RNA genome or GFP sequence were conducted. The cynomolgus β-actin sequence was used as an internal control. In the RNA-PCR (a), negative results obtained without reverse transcriptase (designated RT−) confirmed that the amplified products were not derived from cellular DNA. M, 100-kb DNA ladder; cES, naive cynomolgus ES cells; cES/Transfectant, cynomolgus ES cells stably expressing the GFP gene after transfection;[33] cES/SeV, cynomolgus ES cells infected with the SeV vector; wtSeV, wild-type SeV genome; mTransfectant, a GFP-positive mouse cell line after transfection.

diluted out. (iv) The SeV vector is much less unlikely to generate wild-type virus *in vitro* or *in vivo* than oncoretroviral and lentiviral vectors, since homologous recombination between RNA genomes is very rare indeed in negative-strand RNA viruses.[19] (v) The SeV genome is not subject to cellular epigenetic modifications such as methylation, and thus it is unlikely that methylation-based silencing of transgene expression occurs.

No cytotoxic or differentiating effect on ES cells associated with the SeV infection was observed in our study. However, the wild-type SeV contains immunogenic surface proteins, hemagglutinin-neuraminidase (HN) and F proteins, which potentially induce antibody responses.[20,21] For future clinical applications, it would be desired that as many viral genes as possible are deleted from the vector backbone to permit reapplication, improve the safety, and lessen the possible toxicity of SeV vectors. To this end, we have developed a series of attenuated SeV vectors that are F gene-deleted,[6] F gene-deleted with preferable mutations,[22] M gene-deleted,[23] or have deletions of both F and M genes.[24] The modified vectors would be safer for *in vivo* use.

Ribavirin at high concentrations seems toxic to ES cells; presumably, it directly hampers viability and proliferation potential of ES cells. However, we cannot tell whether the observed toxicity is simply due to its toxicity to ES cells, as feeder cells are more highly sensitive to ribavirin than ES cells. In fact, while feeder cells died at 1 mM of ribavirin, cocultured ES cells were alive at this concentration for some time. Cynomolgus ES cells lose pluripotency and proliferation potential without feeder cells. Thus, the observed toxicity to ES cells may also be a secondary event following the injury of feeder cells. Whether the cytotoxicity is primary or secondary, it will be necessary to find modified compounds of less cytotoxicity.

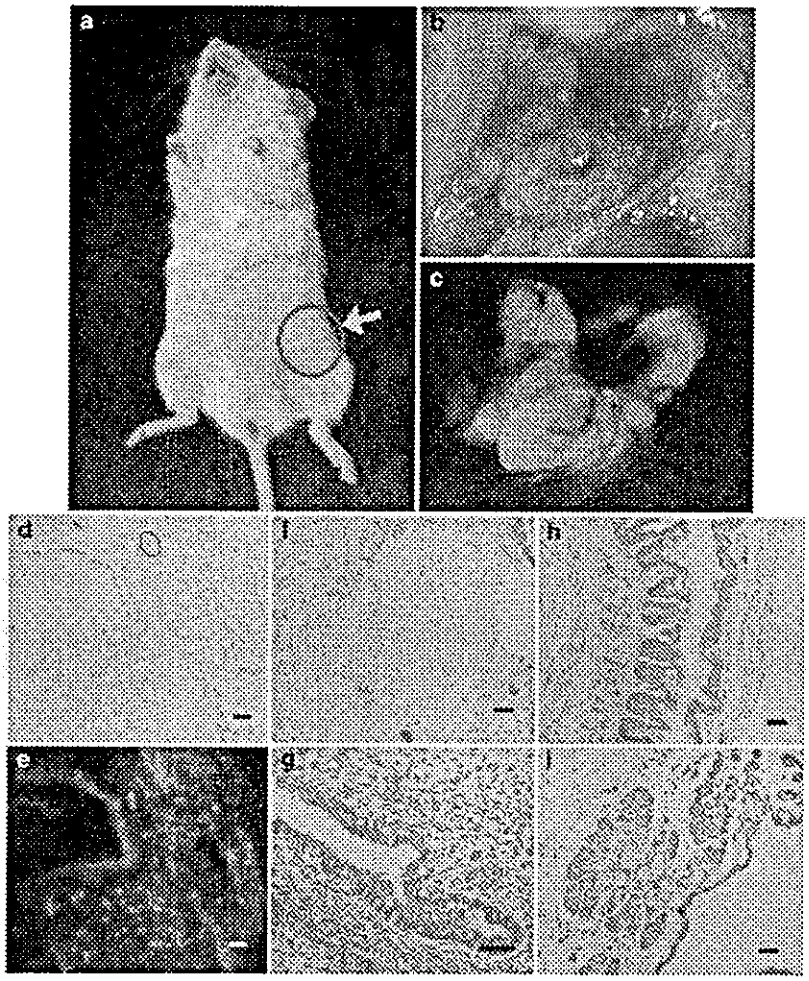

Figure 4 Pluripotency of SeV-infected cynomlgus ES cells. Tumors formed in NOD-SCID mice after inoculation of the SeV-infected cynomolgus ES cells (a). The tumor was fluorescing ((b), bright field; (c), dark field). Fluorescence was observed uniformly in the tumor under a fluorescent microscope ((d), bright field; (e), dark field). The tumor contained all three embryonic germ layer cells; cartilage (f), ciliated columnar epithelium (g), skin (h), and sebaceous gland (i) (stained with hematoxylin and eosin). Bar = 100 μm.

Materials and methods

Cell culture

Cynomolgus ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c) embryonic fibroblasts as described previously.[18] The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 15% ES cell-qualified fetal calf serum (FCS; Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Irvine Scientific, Santa Ana, CA, USA). The ES cell colonies were routinely passaged every 3–4 days after dissociation with a combined approach of 0.25% trypsin (Invitrogen) digestion and mechanical cutting. Alkaline phosphatase staining was conducted with an Alkaline Phosphatase Chromogen Kit (Biomeda, Foster City, CA, USA). Embryoid bodies were produced by culturing ES cell aggregates in Petri dishes. LLC-MK2 cells (1×10^6) were grown in six-well plates and cultured in Eagle's minimal essential medium (Invitrogen) supplemented with 10% FCS.

Vectors

The F-defective SeV vector carrying the GFP gene was constructed as previously described.[6] The vector titer was 1.8×10^9 TU/ml determined by counting fluorescent cells after the infection of LLC-MK2 cells. Gene transfer was conducted by adding various concentrations of the SeV vector solution to culture media. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added. In some experiments, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Sigma) was added at various concentrations to the culture media after infection. The

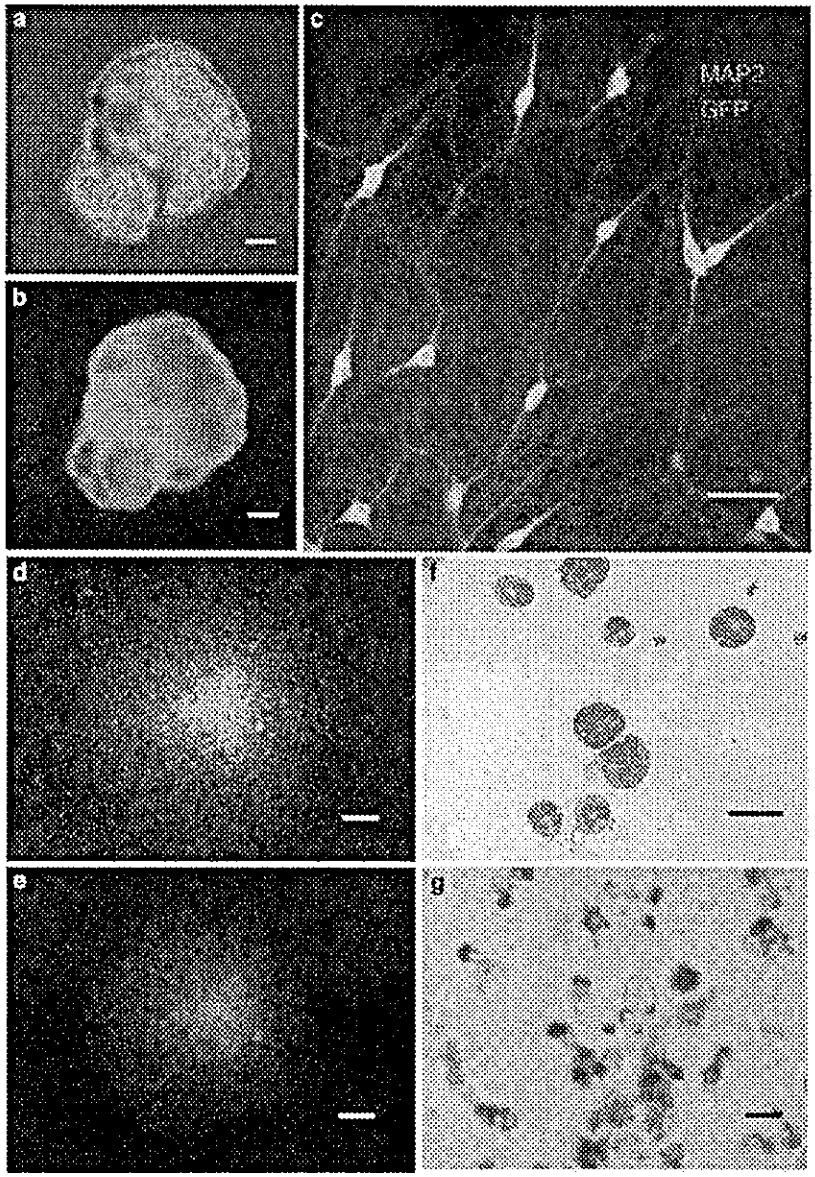

Figure 5 Stable transgene expression during differentiation. A day-20 cystic embryoid body was observed under a fluorescent phase-contrast microscope, confirming that the embryoid body was fluorescing ((a), bright field; (b), dark field). After infection with the SeV vector, fluorescent cynomolgus ES cells differentiated into neural cells. Double immunostaining with anti-GFP (green) and ant-MAP-2 (red) confirmed that differentiated neural cells expressed GFP (c). Yellow cells indicate GFP-expressing neurons. SeV-infected, fluorescent cynomolgus ES cells also differentiated into fluorescent hematopoietic cells. A clonogenic hematopoietic colony was fluorescing ((d) bright field; (e), dark field). A cytospin specimen of hematopoietic colony cells (Wright–Giemsa staining) showed that the cells were mature granulocytes (f). The infected ES cell-derived, fluorescent neutrophils were positive for NBT (stained in black (g)). Bar = 100 μm (a, b, g); 50 μm (c, f); 500 μm (d, e).

viral particles in infected cells were quantified by a hemagglutination assay as described previously.[25]

An adenovirus serotype 5-based vector carrying the GFP gene was constructed as reported.[26] It contained the cytomegalovirus (CMV) promoter, simian virus (SV)-40 intron, and SV-40 polyadenylation signal. An AAV serotype 2-based vector expressing the GFP gene under the control of the chicken β-actin promoter with the CMV immediate-early enhancer (a gift from Dr J Miyazaki) was prepared as described previously.[27] Gene transfer experiments were performed using 3.4×10^2 genome copies (g.c.)/cell of the adenoviral vector or 2.4×10^4 g.c./cell of the AAV vector. The period of exposure was 48 h.

Flow cytometry

GFP and SSEA-4 expression was analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) using the

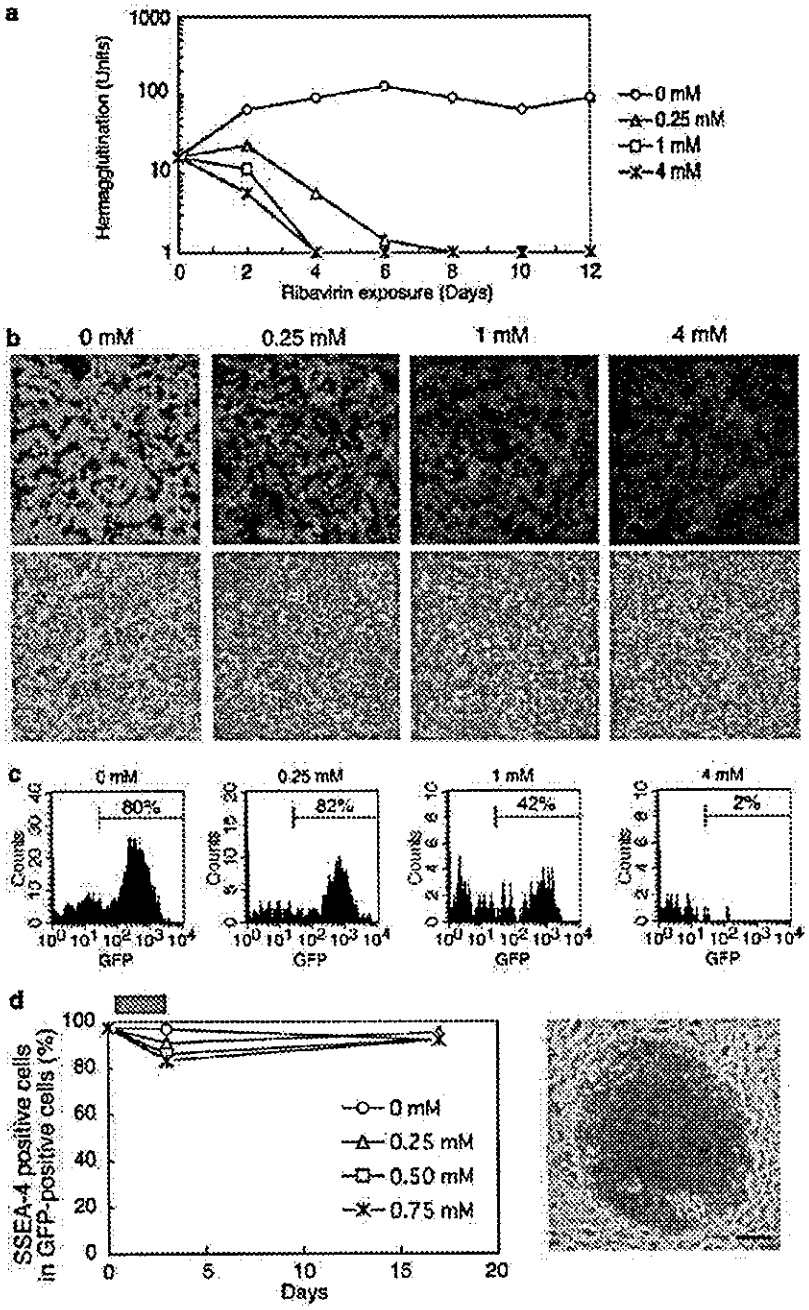

Figure 6 Ribavirin-regulated transgene expression. (a) A rhesus kidney cell line (LLC-MK2) was infected with the SeV vector at 3 TU/cell. Ribavirin was started at various concentrations on day 2 after the infection. The formation of viral particles in the infected LLC-MK2 cells was examined by the hemagglutination assay. (b) The ribavirin-treated LLC-MK2 cells were observed under a fluorescent microscope after an 8-day exposure of ribavirin (upper, dark field; lower, bright field). (c) Ribavirin was added at various concentrations to the SeV-infected, fluorescent cynomolgus ES cells. The GFP expression was assessed by flow cytometry after a 3-day exposure of ribavirin. (d) The fractions of SSEA-4-positive ES cells were assessed by flow cytometry with anti-SSEA-4 before and after a 3-day exposure of ribavirin and are shown as a function of time (days) in the left panel. A gray bar indicates ribavirin treatment. ES cells were stained for alkaline phosphatase (in red) at day 21 after a 3-day exposure of 0.75 mM ribavirin and are shown in the right panel. Bar = 100 μm.

CellQuest software (Becton Dickinson). For SSEA-4 staining, cells were incubated with a primary antibody, anti-SSEA-4 (MC-813-70; Chemicon, Temecula, CA, USA), and then a secondary antibody, PE-conjugated F(ab')2 fragment of rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark). Cocultured BALB/c feeder cells could be distinguished from cynomolgus ES cells by using PE-conjugated anti-mouse

H-2d (SF1-1.1; PharMingen, San Diego, CA, USA), which does not react to cynomolgus cells but does react to BALB/c cells.

Teratoma formation

Cynomolgus ES cells (approximately 10^6 cells per site) were injected subcutaneously into the hind leg of 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (Jackson Laboratory, Bar Harbor, ME, USA). The resulting tumors (usually 9–12 weeks after the injection) were dissected and fixed in 4% paraformaldehyde. For histological analysis, samples from the tumors were embedded in paraffin and stained with hematoxylin and eosin. To observe GFP fluorescence, samples were embedded in OTC compound (Sakura, Zoeterwoude, Netherlands), frozen, sectioned, and examined under a fluorescence microscope.

Hematopoietic differentiation

The mouse bone marrow stromal cell line OP9 was maintained in α-modified minimum essential medium (Invitrogen) supplemented with 20% FCS as described previously.[28] For induction of hematopoietic differentiation, ES cells were seeded onto a mitomycin C-treated confluent OP9 cell layer in six-well plates. Medium to support the differentiation was described elsewhere.[29] Cells at day 18 were placed in Methocult GF+ media (StemCell Technologies, Vancouver, Canada) at 1×10^4 and 1×10^5 cells per plate and clonogenic hematopoietic colonies were produced. After 14 days, individual colonies were removed and spun onto glass slides. Cells were stained with the Wright–Giemsa method. The nitro blue tetrazolium (NBT, Sigma) reduction test was performed on the cells as a granulocyte functional assay according to a previously described method.[30]

Neural differentiation

The induction of neural differentiation was carried out as described previously.[31] Day-4 embryoid bodies were plated onto tissue culture dishes and nestin-positive cells were selected in DMEM/F12 medium supplemented with 5 μg/ml of insulin (Sigma), 50 μg/ml of transferrin (Sigma), 30 nM selenium chloride (Sigma), and 5 μg/ml of fibronectin (Sigma) for 5 days. Cells were then trypsinized and plated in polyornithine-coated dishes (15 μg/ml) and expanded in N2 medium[32] supplemented with 1 μg/ml of laminin (Sigma) and 10 μg/ml of basic fibroblast growth factor (bFGF; Roche, Basel, Switzerland) for 6 days. Differentiation was induced by removal of bFGF. To confirm the neural differentiation, cells were stained with anti-human MAP-2. Briefly, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-human MAP-2 (HM-2; Sigma; diluted 1:4000) and then by Alexa Fluor 594-labeled antibody (diluted 1:500; Molecular Probe, Eugene, OR, USA). The samples were examined under a fluorescence microscope.

DNA-PCR

DNA-PCR for the SeV genome and GFP sequences was carried out as follows. DNA was extracted using the QIAamp DNA mini kits (Qiagen, Hilden, Germany) and 250 ng was used for each PCR with ExTaq (Takara, Shiga, Japan). Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted below) for 1 min, and 72°C for 1 min. The amplified products were run on 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures and product sizes were as follows: the SeV vector genome sequence: 5′-AGA GAA CAA GAC TAA GGC TAC C-3′ and 5′-ACC TTG ACA ATC CTG ATG TGG-3′ (55°C, 580 bp); the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′ (60°C, 356 bp). the cynomolgus β-actin sequence: 5′-CAT TGT CAT GGA CTC TGG CGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG GGC-3′ (60°C, 234 bp).

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Reverse transcription was conducted by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The product (250 ng) after the reverse transcription was used for the subsequent PCR as described above.

Acknowledgements

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Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases

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Malignant tumor cells often express matrix metalloproteinases (MMPs) at a high level to enable their dissemination and metastasis. Sendai virus (SeV), a nonsegmented negative strand RNA virus, spreads in the target tissues in vivo via cleavage activation of the viral fusion glycoprotein by a tissue-specific, trypsin-like enzyme. By deleting the viral matrix protein, we previously generated a recombinant SeV that does not bud to mature virions, but is highly fusogenic and spreads extensively from cell to cell in a trypsin-dependent manner. Here, we changed the tryptic cleavage site of the fusion glycoprotein of this virus to a site susceptible to MMPs. The resulting recombinant virus was no longer activated by trypsin but spread efficiently in cultured cells supplemented with MMP2 or MMP9 and in human tumor cell lines expressing these MMPs. Furthermore, the virus spread extensively in tumor cells xenotransplanted to nude mice without disseminating to the surrounding normal cells, leading to the inhibition of the tumor growth in the mice. These results demonstrate the selective targeting and killing of human tumor cells by recombinant SeV technology and greatly advance the reemerging concept of oncolytic virotherapy, which currently appears to rely largely upon a natural preference of certain viruses for cancer cells.
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Introduction

The idea of using infectious viruses as antitumor agents is again attracting attention in part because of progress in understanding virus–host interactions and because currently available chemotherapy is not completely satisfactory for many reasons, including the development of drug resistance.[1–3] The oncolytic virotherapy strategies proposed to date rely largely upon the natural preference of certain viruses for cancer cells, as exemplified by Newcastle disease virus (NDV) and vesicular stomatitis virus, which belongs to the family Paramyxoviridae and the family Rhabdoviridae, respectively. Both of these families of viruses are characterized by a nonsegmented negative-strand RNA genome, and thus are also called mononegaviruses. Preclinical studies using viruses in other families have involved extensive efforts to increase selectivity for tumor cells by manipulation of the viruses by recombinant DNA technology. Examples of such efforts include attempts to change adenovirus attachment protein so that it recognizes tumor-specific cell surface molecules and deletion of herpesvirus accessory genes that are indispensable for the viral life cycle in normal cells but likely dispensable in tumor cells.[4,5]

Sendai virus (SeV), also a member of the Paramyxoviridae, infects most mammalian cells and directs high-level expression not only in cultured cells but also in experimental animals of the genes within its genome that have been exploited for therapeutic use and vaccination.[6–10] A strong potential of NDV as a vaccine vector was also demonstrated.[11] SeV and most of the other mononegaviruses replicate independent of cellular nuclear functions and do not have a DNA phase in their lifecycle, and therefore are not considered to present a high risk of cell transformation by integration of the viral genetic information into the cellular genome. Thus, the feasibility of using SeV and other mononegaviruses as a novel class of vector is now increasing.

SeV displays a narrow spectrum of tissue tropism in susceptible hosts, growing in the respiratory tract of mice or in the allantoic cells of embryonated chicken eggs with little appreciable spreading to other tissues in these host organisms, even though its receptor is sialic acid residues, which are ubiquitous throughout the body. This restricted tropism is primarily due to the fact that the specific tissue proteases required for cleavage activation of viral fusion (F) glycoprotein, and thus for infectivity of progeny (the capacity to penetrate into and initiate infection of the next cell), are available only on the surface of those limited types of tissue.[12] This concept

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of protease-dependent tropism of SeV was substantiated by the identification of responsible tissue proteases: the blood clotting factor Xa ectopically expressed by allantoic cells *in ovo* and secreted into the allantoic fluid,[13] and tryptase Clara secreted into the airway epithelium in the lungs of rodents.[14] The cleavage motif for these proteases in the fusion glycoprotein precursor F_0 is the sequence Q–S–R. Most tissue culture cells so far tested do not express SeV-activating proteases, and hence require a low concentration of exogenously added trypsin to allow SeV spreading.[15] The receptor recognition is mediated by another envelope glycoprotein called hemagglutinin-neuraminidase (HN).

The extracellular matrix (ECM) surrounding a tumor cell serves as a barrier that blocks tumor cell migration, infiltration and metastasis. Highly invasive, metastatic cancer cells express high levels of ECM-degrading enzymes such as matrix metalloproteinase (MMPs) and urokinase-type plasminogen activator (uPA).[16,17] We have expanded the plasmid-based reverse genetics technology originally developed to manipulate the full-length SeV genome[18] in various directions, including deletion of certain viral genes, to generate nontransmissible, safer versions of SeV vector for gene therapy.[19–21] Among them, M (matrix protein) gene-deleted SeV (SeV/ΔM) is unique in that it does not bud into a mature particle in infected cells but rapidly spreads from cell to cell in the presence of trypsin, with the induction of massive syncytia followed by rapid cell death throughout the monolayer.[22] We therefore considered it likely that SeV/ΔM would be more potent in killing solid tumor tissue than the wild-type SeV or any other deletion mutants. Here, we used the SeV/ΔM plasmid as the starting material and applied the concept of 'protease-dependent viral tropism' in order to generate a recombinant SeV/ΔM capable of 'tumor-specific protease-dependent oncolysis' at high efficiency.

Results

Cell-to-cell spreading of M gene-deleted SeV

Deletion of the viral M gene from SeV (Figure 1a) almost completely abolished virus maturation into particles and instead greatly facilitated cell-to-cell virus spreading via membrane fusion in the presence of F protein-activating trypsin[22] (also see Figure 2a). Thus, the M gene-deleted SeV (SeV/ΔM) appeared to be more promising for killing the cells in a solid tumor than the wild-type SeV, because the facilitated cell fusion itself is cytotoxic in many cases. In addition, the absence of free infectious particles indicated that the mutant should undergo little undesired spreading to tissues distant from the initially infected cells. Therefore, the SeV/ΔM cDNA plasmid was used as the starting material for all subsequent manipulations to generate oncolytic SeVs. Virus growth and spreading were monitored by the expression of enhanced green fluorescent protein (GFP), whose open-reading frame (ORF) had been inserted into the viral genome in place of the M ORF (see Figure 1a).

Attempt to alter the tryptic cleavage site in the SeV F protein to render it susceptible to MMPs

We initially had to know whether or not it would be possible to alter the tryptic cleavage site of SeV F

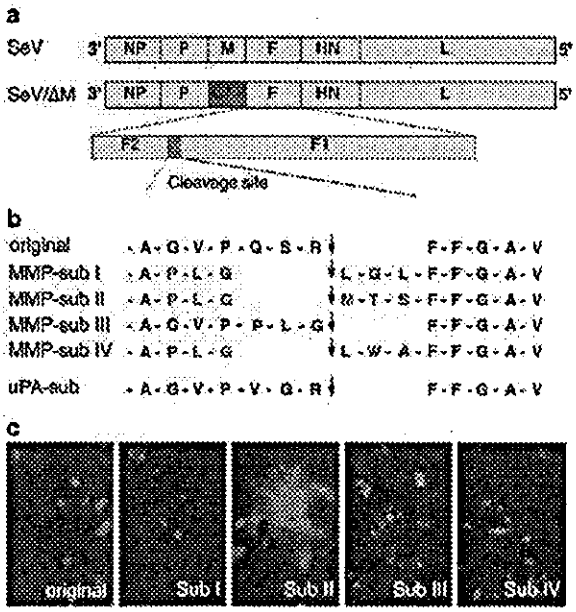

Figure 1 Gene maps of the wild-type SeV and SeV/ΔM (a), amino-acid changes at the F protein cleavage site (b) and the capacity of the resulting F protein mutants to induce cell fusion (c). In SeV/ΔM, the ORF of the M protein was replaced by the GFP ORF. The amino-acid changes made to render the F protein an MMP substrate (subI–subIV) and those made to render it an uPA substrate are shown in red and blue, respectively. Cell fusion was analyzed by cotransfection into MMP-expressing HT1080 cells of the chimeric F/HN genes with the modified cleavage sequences and the plasmid-expressing GFP. Syncytia formation was observed 48 h after transfection under a fluorescence microscope.

glycoprotein to a site susceptible to MMPs. There are a number of known MMPs, and the cleavage sites recognized by the MMPs appear to consist of the tripeptide PLG followed by an additional tripeptide LGL or LWA, according to the findings of studies using synthetic substrates for MMP assays.[23–25] Cleavage takes place between the two (upstream and downstream) tripeptides (Figure 1b). On the other hand, the natural cleavage of SeV F protein into F2 and F1 occurs between the R and F in Figure 1b, and the resulting N-terminal sequence of the F1 begins with FFG. A previous study demonstrated that the carbobenzoxy-FFG oligopeptide could specifically inhibit SeV fusion activity and infectivity in cultured cells,[26] suggesting that the N-terminal FFG was specifically required for fusion capacity. Thus, the addition of LGL or LWA to the natural N-terminal FFG might impair fusion activity. Under these circumstances, the alteration of the tryptic cleavage site to an MMP-specific site without impairing fusion activity would not be easy. We therefore designed numerous sequences and show some of them (designated MMP-subI to MMP-subIV) in Figure 1b. These include MTS (subII), which was newly designed here by changing the MMP2- and MMP9-specific sequence MWS[27] to MTS to accord with the favorable sequence for MMP9 substrates, Pro-X-X-Hy-(Ser/Thr), which was identified by phage display.[28] MTS is less bulky and less hydrophobic than LGL or LWA, and may thus be less obstructive for fusion when it is attached to the N-terminus of F1

after cleavage. Total omission of the downstream tripeptide specific for MMPs was also attempted so that FFG would appear at the N terminus. Designing uPA-specific substrate was relatively easy, because uPA and trypsin generally cleave after an R. Thus, only the two residues Q and S before the R were changed to V and G, respectively, to make the substrate more favorable for uPA (Figure 1b).

These and other modifications of the cleavage site were introduced into a plasmid encoding the F and HN proteins as a fusion polypeptide. Each of the resulting plasmids was transfected, together with a GFP-expressing plasmid, pCAGGS/GFP, into human fibrosarcoma cell line HT1080 and human stomach cancer line MKN28, which expressed and secreted MMP and tissue-type plasminogen activator (tPA), respectively, at high levels.[29,30] tPA has the same cleavage specificity as uPA. As shown in Figure 1c, the F protein with the cleavage motif compatible with MMP-subII was fully fusion competent in the relevant target cell line, HT1080, whereas the other three substrates (subI, subIII and subIV) were not. While the subII mutant was fusion incompetent in the MKN28 cell line, the uPA-sub mutant was highly fusion inducing in this cell line but not in HT1080 (not shown).

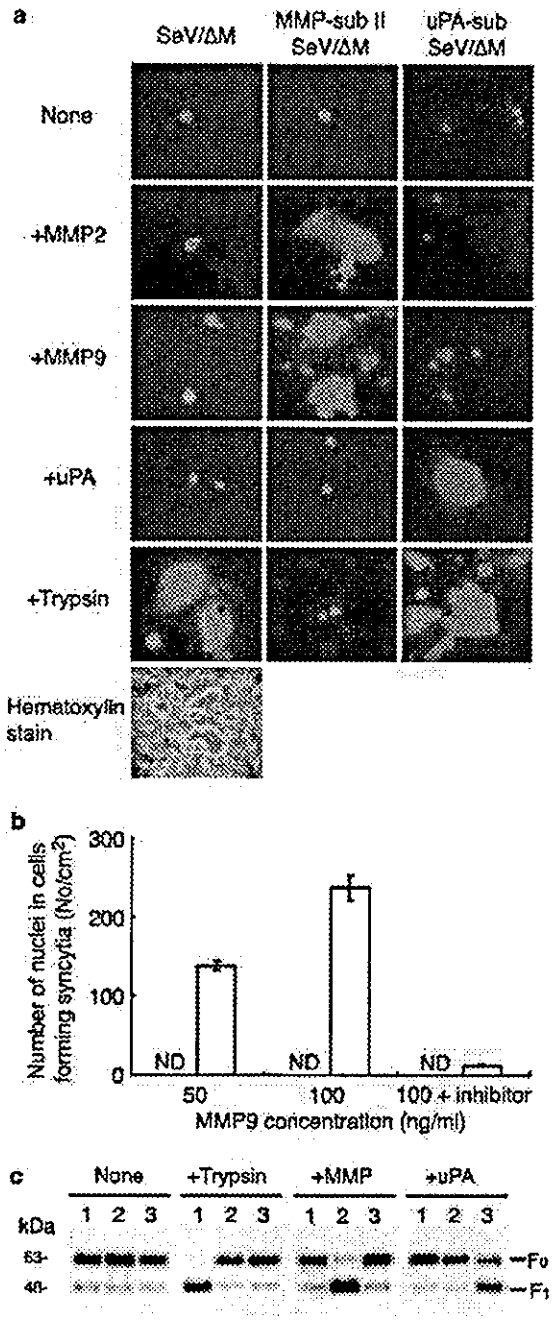

Figure 2 Confirmation of alterations of protease requirement and cell tropism of SeV/ΔM by exogenously added proteases. (a) LLC-MK₂ cells infected with parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 were cultured with MMP2, MMP9, uPA (0.1 μg/ml each) or trypsin (7.5 μg/ml). The induction of cell fusion and cell-to-cell viral spreading was monitored by assessing the GFP expressed from each virus under a fluorescence microscope 4 days later. Cells infected with parental SeV/ΔM and cultured with trypsin were also observed after hematoxylin staining. (b) Quantitative analysis of cell fusion of LLC-MK2 cells infected with MMP-subII SeV/ΔM in response to added MMP9. MMP9 was added to the medium (serum-free MEM) immediately after the infection with MMP-subII SeV/ΔM (open bars) or parental SeV/ΔM (closed bars, ND: not detected) at an MOI of 0.1. The number of nuclei in the cells forming syncytia was counted under a microscope 2 days after the infection. (c) Specific cleavage of the modified F proteins by treatment of the virions with proteases in vitro. Virus particles of the parental SeV/ΔM (lanes 1), MMP-subII SeV/ΔM (lanes 2) and uPA-sub SeV/ΔM (lanes 3) were prepared from the culture supernatants of an M protein-expressing helper cell line after infection at an MOI of 3 (CIU/cell) and subsequent incubation for 2 days in the absence of any protease. The virus particles produced were pelleted by centrifugation at 18 500 g for 3 h, resuspended in PBS and incubated with trypsin (7.5 μg/ml), MMP-9 (0.1 μg/ml) or uPA (0.1 μg/ml) for 30 min at 37°C. The controls (none) were incubated similarly in the absence of any protease. The viral proteins were then analyzed by Western blotting using an anti-F_1 antibody that recognized both F_0 and F_1 proteins.

Recovery of SeV/ΔM viruses with modified cleavage site and confirmation of their protease requirements

According to the above results obtained by plasmid-based expression, the SeV/ΔM plasmid was engineered to possess the MMP-subII or uPA-sub cleavage motif, and infectious progeny were rescued in an M protein-expressing helper cell line.[22] The rescued viruses were examined for the protease requirement for the induction of cell fusion and virus spreading in LLC-MK₂ cells (Figure 2a). SeV/ΔM with the MMP-subII cleavage motif in the F gene (MMP-subII SeV/ΔM) spread extensively via cell–cell fusion in the presence of MMP2 or MMP9. The number of nuclei per syncytium reached as high as about 100–300, and syncytium formation was almost completely inhibited by the addition of the MMP-inhibitor 1, 10 phenanthroline (Aldrich, Milwaukee, WI, USA) (Figure 2b). The same mutant could neither spread nor induce cell fusion in the presence of trypsin or uPA or in the absence of any protease (Figure 2a). The substrates of uPA and trypsin share an arginine at position −1 relative to the cleavage site, whereas the substrates of the MMPs do not have an arginine at this position. Thus, SeV/ΔM with uPA cleavage motif

(uPA-sub SeV/ΔM) spread in the presence of not only uPA but also trypsin, but not in the presence of the MMPs (Figure 2a). Spreading of the parental SeV/ΔM was possible only in the presence of trypsin. Trypsin might be able to cleave both the natural QSRFFG and artificial VGRFFG motifs (see Figure 1b) because it is a digestive enzyme with a broad specificity, whereas uPA might act only at the latter because it generally causes limited proteolysis with strict substrate specificity.

The highly selective protease dependency of virus-induced cell fusion and virus spreading nicely paralleled the cleavability of the F_0 precursor on the virions into F_1 (Figure 2c) and F_2 (not shown) only by the relevant proteases but not by any irrelevant ones. The F_0 of uPA-sub SeV/ΔM virions was not well cleaved by trypsin under the conditions employed, including 30 min of incubation with the enzyme, but incubation for a longer period (4 h) resulted in complete cleavage (not shown). This may account for the remarkable spreading of this mutant in the presence of trypsin (Figure 2a).

In perfect correlation with the above results obtained with exogenously added proteases, MMP-subII SeV/ΔM spread extensively in a human fibrosarcoma cell line, HT1080, endogenously producing and secreting MMPs, but not in a human stomach cancer line, MKN28, producing tPA, whereas uPA-sub SeV/ΔM did the opposite, namely, spread in MKN28 but not in HT1080 (Figure 3a). Neither of the cell lines was permissive at all for the parental SeV/ΔM.

A human pancreatic epithelioid carcinoma cell line, Panc I, which expresses MMP2 and MMP9 but at lower levels than HT1080 (Figure 3c), was unable to activate MMP-subII SeV/ΔM (Figure 3a). However, Panc I cells became permissive for this mutant (Figure 3a and b) when MMP9 production was enhanced by treating the cells with a phorbol ester, phorbol 12-myristate 13-acetate (PMA) (Figure 3c). In this experiment, SW620 (a human lymph node colorectal adenocarcinoma cell line) served as a control showing no stimulation of MMP production with PMA and no enhancement of virus spreading. Production of both MMP2 and MMP9 in HT1080 cells was enhanced by PMA (Figure 3c), but this enhancement resulted in only slight augmentation of MMP-sub II SeV/ΔM spreading beyond the levels seen in the cells not treated with PMA (Figure 3b). SeV activation by the MMPs thus appeared to be nearly an 'all or none' phenomenon with a critical threshold of the enzyme level. In addition, it should be emphasized that MMP2 and MMP9, together with MMP7, are likely more

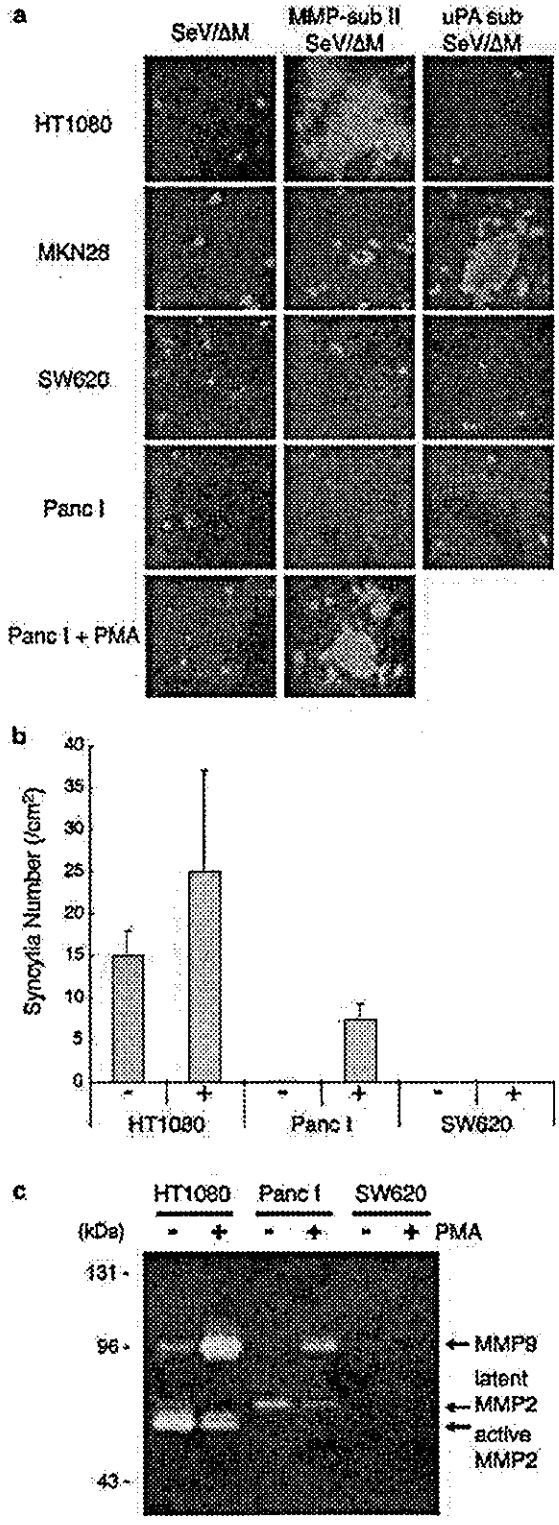

Figure 3 Confirmation of alterations of protease requirement and cell tropism of SeV/ΔM by endogenous proteases. (a) Cell-to-cell spreading dependent on endogenous proteases expressed by tumor cells. Four different tumor cell lines, HT1080, Panc I, SW620 and MKN28, were infected with the parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 and cultured in the medium containing 1% heat-inactivated fetal bovine serum, and cell fusion was observed 4 days later. Panc I cells were additionally analyzed to assess cell fusion following culturing with 20 nM PMA for 1 day. (b) Cell fusion of HT1080, Panc I and SW620 cells was quantified by counting the number of cells forming syncytia as observed under a microscope after infection and incubation for 4 days following culturing with (+) or without (−) 20 nM PMA for 1 day. (c) Gelatin zymography[47] of HT1080, Panc I and SW620 cells incubated with (+) or without (−) 20 nM PMA for 1 day and cultured in serum-free medium for 4 days. Gelatin zymography is a sensitive technique for the estimation of both active and latent types of MMP-2 and MMP-9 content in biological samples. PMA enhanced the levels of MMP9 in HT1080 and Panc I but not in SW620 cells.

important for tumor metastasis than some 20 other known MMP species, since only the former three have the capacity to disrupt basement membranes.[27,28]

Inhibition of HT1080 tumor growth in vivo by MMP-subII SeV/ΔM

MMP-subII SeV/ΔM was injected directly into the center of the mass of a HT1080 tumor that had been subcutaneously transplanted into nude mice and grown to an approximate diameter of 3–8 mm. As control viruses we used not only the immediate parent, SeV/ΔM, of the mutant but also a standard SeV with a full-length genome that harbored the GFP gene before its NP gene. After 2 days, light emission from the GFP protein in tumor cells was observed in the mice placed under a fluorescence microscope. GFP expressed from both of the control viruses was detected as small foci, likely at the injection site. In contrast, GFP expressed from MMP-subII SeV/ΔM spread throughout the entire tumor (×1 magnification in Figure 4a). Differences between MMP-subII SeV/ΔM and the control viruses were also remarkable at higher magnification (×10). The boundaries between the tumor cells were not seen in the former, giving a highly amorphous impression, whereas in the controls infection foci were much smaller in size and separated from one another, and cell boundaries were clearly seen (Figure 4a). This strongly suggested vigorous viral spreading of the MMP-dependent mutant in the tumor, leading to efficient tumor lysis. Staining with anti-GFP was much less marked in the central area including the site of inoculation (arrow) than in the peripheral region in the MMP-subII SeV/ΔM-infected tumor, whereas the central area was strongly positive for TUNEL staining and the peripheral region was negative or only poorly positive (Figure 4b). The TUNEL-positive cells are apoptotic ones characterized by internucleosomal DNA fragmentation. This result suggested that the cells in the peripheral area were actively supporting virus growth and viral gene expression, whereas those in the central area, which were probably infected earlier, were now dying via the apoptotic pathway. It should be noted that viral gene expression appeared to remain in the central area following infection by SeV/ΔM, with no strong TUNEL staining in any area (Figure 4b). Furthermore, we quantitatively showed that MMP-subII SeV/ΔM strongly inhibited HT1080 tumor growth in the mice, whereas neither of the control viruses caused marked inhibition (Figure 4c).

The same *in vivo* experiment was performed using the other tumor line, SW620, that is low in MMP expression. After the injection of MMP-subII SeV/ΔM into the SW620 tumor, GFP expressed from MMP-subII SeV/ΔM spread slightly in the tumor (Figure 5a). Also, SW620 tumor growth in the mice was very slightly inhibited by MMP-subII SeV/ΔM injection (Figure 5c). However, immunohistochemical analysis showed that neither GFP- nor TUNEL-positive cells were significantly

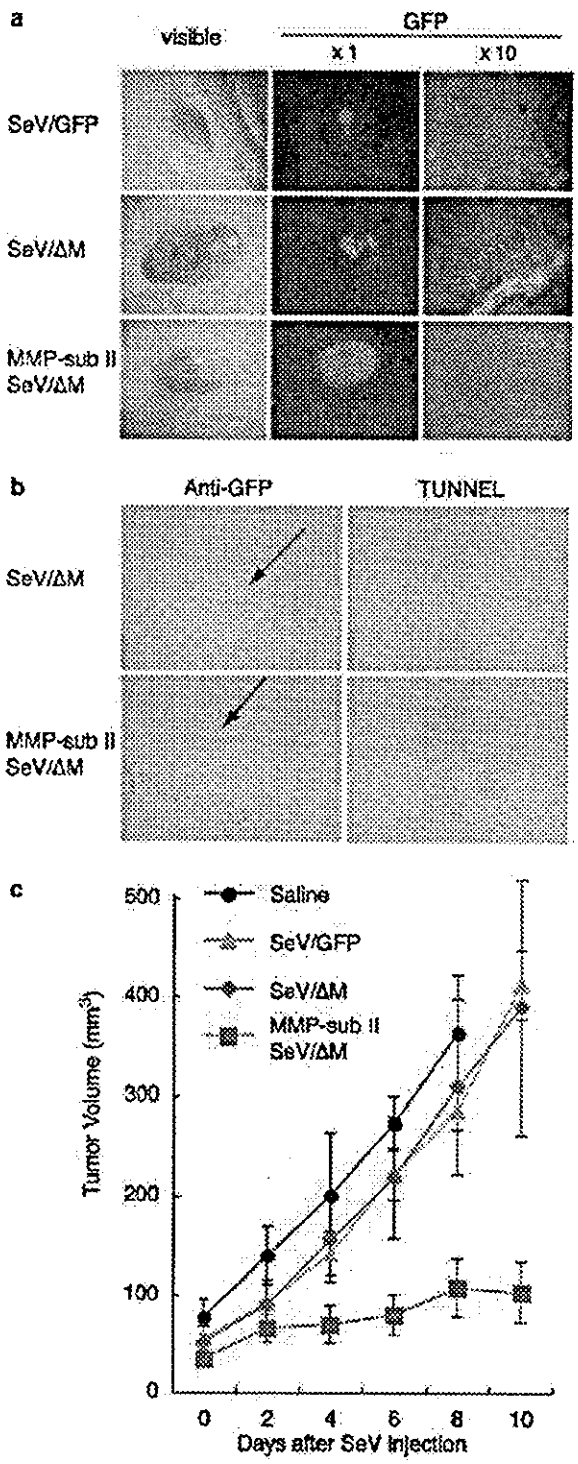

Figure 4 Inhibition of HT1080 tumor growth in nude mice by MMP-subII SeV/ΔM. (a) Wild-type SeV, parental SeV/ΔM and MMP-subII SeV/ΔM (5×10^7 CIU/50 μl, each) were injected once directly into the subcutaneously transplanted HT1080 tumors in nude mice. After 2 days, light emission from the GFP protein in tumor cells was observed externally under a fluorescence microscope. Magnification, ×1 and ×10. (b) TUNEL staining of cells. Arrows indicate the sites of virus injection. (c) Tumor volumes were calculated using the formula (volume = π/6 × abc:(length (a), width (b), height (c)) and were expressed as the average (n = 7). Significantly different volumes were observed between the group administered MMP-subII SeV/ΔM and all other groups ($P < 0.05$, Student's t-test) at 8 and 10 days after injection.

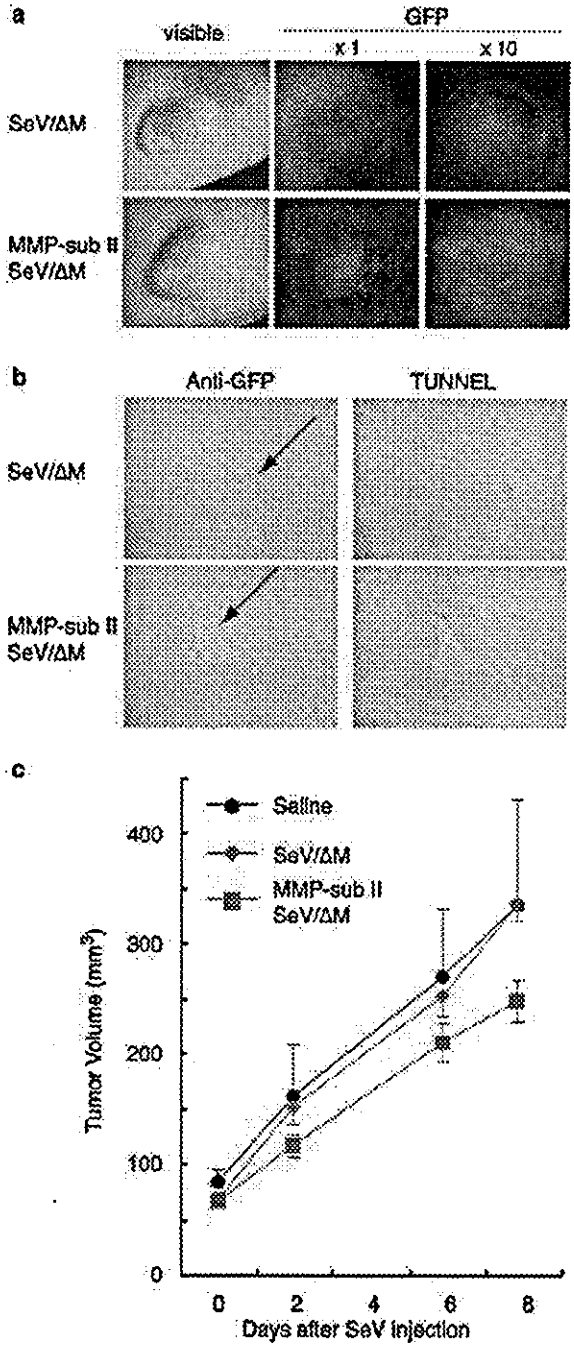

Figure 5 Restricted spreading of MMP-subII SeV/ΔM in the SW620 tumors in vivo. (a) Parental SeV/ΔM and MMP-subII SeV/ΔM (5×10^7 CIU/50 μl, each) were injected once directly into the subcutaneously transplanted SW620 tumors in nude mice. After 2 days, light emission from the GFP protein in tumor cells was observed externally under a fluorescence microscope. (b) TUNEL staining of cells. Arrows indicate the sites of virus injection. (c) Tumor volumes expressed as the average ($n = 5$). No significant difference was observed between the respective groups.

increased in either MMP-subII SeV/ΔM- or SeV/ΔM-injected SW620 tumors (Figure 5b). As the expression of MMPs of SW620 cells is enhanced after the engraftment,[31] MMP-subII SeV/ΔM would spread slightly in SW620 *in vivo*. However, viral spreading was limited in SW620 even *in vivo*, as the expression level of MMPs in SW620 was significantly lower than that in HT1080. In addition, no spreading was observed when MMP-subII SeV/ΔM was administered in normal tissues such as lung, trachea, nasal cavity and skin and muscle (data not shown). These results demonstrate the selective spreading of MMP-subII SeV/ΔM in the tumors that are high in MMP expression *in vivo*.

Discussion

We report here the generation of oncolytic recombinant SeVs that selectively target human tumor cells expressing MMP2, MMP9 and uPA by changing the tryptic cleavage-activation signal to a signal susceptible to those tumor-associated proteases. An additional alteration that we made was to delete the viral M gene because this conferred on SeV the capacity to spread extensively from cell to cell with the induction of massive syncytia, and at the same time rendered the virus nontransmissible to distant tissues and thus safer than the wild type.[22] Another M gene-deleted paramyxovirus, measles virus, was reported to propagate efficiently from cell to cell in an animal model (mouse brain).[32] The successful recovery of MMP-subII SeV/ΔM that selectively targeted and lysed MMP2- and MMP9-expressing tumor cells is of particular interest as these proteases represent two of a few proteases that have shown strong association with tumor metastasis among a large variety of MMP protease family members. Further improvement of MMP-subII SeV/ΔM is now being attempted by increasing the fusogenic activity and/or by the construction of vectors carrying therapeutic gene(s) such as immuno- and suicide genes, since MMP-subII SeV/ΔM did not succeed in complete eradication of HT1080 tumors in our experiments.

Johnson *et al*[33] succeeded in targeting the cytotoxicity of a fusogenic viral glycoprotein to an MMP-expressing glioma cell line. The strategy involved conditional inactivation and activation of the gibbon leukemia virus envelope protein. It was inactivated due to an extra peptide attached to its N-terminus with an MMP-cleavable linker and became active (fusion competent and cytotoxic) only when it encountered an MMP-expressing cell that removed the peptide. However, their studies published so far were limited to plasmid-based expression of the engineered glycoproteins and not expression in the context of the viral replicon of either a full-length or defective genome. Thus, their studies and ours are largely different from each other in both conceptual and practical details, although both depend on MMP–substrate interaction. Aside from this, the measles virus was modified for the targeting to tumor cells by linking a single-chain antibody recognizing tumor-cell-specific proteins to its envelope protein.[34,35] Although it affects only targeting and not tumor-cell-specific spreading, such modification (for targeting) could be combined with one of our vectors (for tumor-cell-specific spreading), such as MMP-subII SeV/ΔM.

Our strategy is theoretically and practically applicable to a large number of other paramyxoviruses, including NDV, which is now under extensive clinical trials based on its natural preference for cancer cells, and members of other virus families, such as influenza viruses, because they share the same cleavage-activation mechanism of the envelope proteins for initiation and spread of infection[12,36] and because recombinant DNA technologies have been established for them. The matrix gene deletion from the genome may not always be necessary but would be advantageous when targeting a tumor mass.

A key issue in the application to actual medicine of the findings obtained here using clonal tumor cell lines is to 'individualize'[37] the approach by identifying the protease species in actual tumors of individual patients. There is increasing evidence suggesting that the higher the protease level at diagnosis the worse the prognosis,[27,38] and we have shown here that there would be a critical threshold of the enzyme levels required for virus activation. Thus, not only the species but also the amounts of the proteases involved in the actual tumor will have to be included in 'individualization'. The protease species as well as their levels would be relatively easy to determine by the analysis of biopsy materials from individual patients by conventional immunohistochemistry, ELISA and other assay methods.[39–42] Once they are determined, it would also be easy to generate appropriate protease activation mutants of SeV and related envelope viruses. The safety of recombinant SeVs is another key issue. They so far do not appear to cause any serious problems or symptoms in rhesus monkeys after administration in various ways, including intramuscular injection as well as intranasal inoculation (unpublished data).

Materials and methods

Cell lines

The cell lines HT1080 (human fibrosarcoma), Panc I (human pancreatic epithelioid carcinoma), SW620 (human lymph node colorectal adenocarcinoma) and LLC-MK_2 (monkey kidney) were purchased from ATCC (Manassas, VA, USA). The human stomach cancer cell line MKN28 was purchased from the Institute of Physical and Chemical Research (Tsukuba, Japan).

Construction of plasmids expressing SeV F and HN proteins as a fusion polypeptide with modified cleavage site sequences in the F protein

The F protein is a direct mediator of cell fusion but requires coexpression of the receptor-binding HN protein from a second plasmid for the actual expression of fusion capacity.[43,44] Alternatively, we found that the expression of the F and HN proteins as a single polypeptide connected by 30 unrelated amino acids was also fusion inducing (details will be published elsewhere). The plasmid for this F–HN-coupled expression was constructed by the amplification of the F and HN genes in the plasmid pSeV18+b[18] by polymerase chain reaction and cointroduction of the amplified genes into the mammalian expression vector pCAGGS.[45] The primer pair specific for the F gene was Fu-F (5′-ATCCGAATTCAGTTCAATGACAGCATATATCCAGAG-3′) and Fu-R (5′-ATCCGCGGCCGCCGGTCATCTGGAT TACCCATTAGC-3′), and that for the HN gene was HN-F(+linker) (5′-ATCCGCGGCCGCAATCGAGGGA AGGTGGTCTGAGTTAAAAATCAG GAGCAACGAC GGAGGTGAAGGACCAGAGGACGCCAACGACCCA CGGGGAAAGGGGTGAACACATCCATATCCAGCCA TCTCTACCTGTTTATGGACAGAGGGTTAGG-3′) and HN-R (5′-ATCCGCGGCCGCTTAAGACTCGGCCTTG CATAA-3′). The amplified F and HN genes were digested with *Not*I and *Xho*I (for the F gene) or *Not*I (for the HN gene) and ligated together with pCAGGS digested with *Not*I and *Xho*I. Conversion of amino-acid sequences around the cleavage site of F protein was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The primer pairs used for mutagenesis were pMMP #1-F (5′-CAAAATGCCGGT GCTCCCCcGTtGgGATTCTTCGGTGCTGTGATT-3′) and pMMP #1-R (5′-AATCACAGCACCGAAGAATCc CaACgGGGGAGCACCGGCATTTTG-3′) for MMP-subI; pMMP #2-F (5′-CTGTCACCAATGATACGACACAAA ATGCCccTctTggCatGaCGAGtTTCTTCGGTGCTGTGAT TGGTACTATC-3′) and pMMP #2-R (5′-GATAGTACCA ATCACAGCACCGAAGAAaCTCGtCatGccAagAggGG CATTTTGTGTCGTATCATTGGTGACAG-3′) for MMP-subII; pMMP #3-F (5′-GTCACCAATGATACGACACA AAATGCCccTctTggCctGtgGgcaTTCTTCGGTGCTGT GATTGGTACTATC-3′) and pMMP #3-R (5′-GATAG TACCAATCACAGCACCGAAGAAtgcCcaCagGccAa gAggGGCATTTTGTGTCGTATCATTGGTGAC-3′) for MMP-subIII; pMMP #4-F (5′-CTGTCACCAATGATAC GACACAAAATGCCccTctTggCCtGggGttA TTCTTCGGT GCTGTGATTGGTACTATCG-3′) and pMMP #4-R (5′-CGATAGTACCAATCACAGCACCGAAGAATaaCccCa GGccAagAggGGCATTTTGTGTCGTATCATTGGTGA CAG-3′) for MMP-subIV; and puPA-F (5′-GACACAA AATGCCGGTGCTCCCgtGggGAGATTCTTCGGTGCTG TGATTG-3′) and puPA-R (R: 5′-CAATCACAGCACC GAAGAATCTCccCacGGGAGCACCGGCATTTTGTGTC-3′) for uPA-sub. Lower case letters in the primer sequences described above represent the substituted nucleotides.

Construction and amplification of F-modified SeV/ΔM

The parental plasmid SeV/ΔM, which is pSeV18+/ΔM-GFP with a bacteriophage T7 promoter,[22] was digested with *Sal*I and *Nhe*I, and the resulting fragment (9.6 kb) was subcloned into LITMUS 38 (New England Biolabs. Beverly, MA, USA). Site-directed mutagenesis was performed on this plasmid. Recovery and amplification of F-modified SeV/ΔM from cloned cDNA were performed essentially as described,[19–21] using recombinant vaccinia virus expressing T7 RNA polymerase[46] and the SeV M protein-expressing LLC-MK_2 packaging cell line.[22] Type IV collagenase (5 U/ml) of *Clostridium histolyticum* (ICN, Aurora, OH, USA) and 7.5 μg/ml of trypsin were added to the medium to activate the modified F protein in the cases of MMP-subII SeV/ΔM and uPA-sub SeV/ΔM, respectively. The amplification of recovered viruses was also carried out using the M-expressing LLC-MK_2 packaging cell line.[22]

Cell fusion analysis by transfection of F/HN-expressing plasmids

The above-described expression vector, pCAGGS, carrying the individual F/HN fusion gene with a modified

cleavage sequence in the F protein was transfected together with pCAGGS/GFP expressing the enhanced GFP into MMP-expressing HT1080 cells using the transfection reagent FuGENE6 (Roche, Basel, Switzerland). Syncytia formation was observed 48 h after transfection under a fluorescence microscope (Leica, Wetzlar, Germany).

Cell fusion analysis following infection with F-modified SeV/ΔM

LLC-MK_2 cells infected with the parental SeV/ΔM, MMP-subII SeV/ΔM or uPA-sub SeV/ΔM at an MOI of 0.02 were cultured in serum-free MEM containing MMP2, MMP9 or uPA (0.1 μg/ml, each) or trypsin (7.5 μg/ml). For the inhibition study, the MMP-inhibitor 1, 10 phenanthroline was added to the medium at a final concentration of 12.5 μM. To quantify the fused cells, the number of nuclei in cells forming syncytia was counted after culturing in serum-free MEM containing a series of concentrations of collagenase or MMP-9. Tumor cell lines HT1080, Panc I, SW620 and MKN28, which express the proteases indicated in the text endogenously, were also infected with these viruses at an MOI of 0.02, and cultured in the medium containing 1% heat-inactivated fetal bovine serum. In both of these exogenous and endogenous protease requirement studies, cell fusion was observed under a fluorescence microscope (Leica, Wetzlar, Germany) 4 days after infection.

Western blotting

SDS-PAGE and Western blotting were carried out according to the method described.[22] A rabbit polyclonal anti-F_1 antibody was raised against three mixed synthetic peptides of SeV F protein, that is, (1) FFGAVIGT + Cys, (2) EAREAKRDIALIK and (3) CGTGRRPISQDRS, corresponding to amino acids 117–124, 143–155 and 401–413 of SeV-F, respectively. Immunization was carried out after conjugation of the peptides to keyhole limpet hemocyanin. Incubation of the Western blotting membrane with the anti-F_1 primary antibody was followed by incubation with the second antibody, anti-rabbit IgG conjugated with HRP (ICN, Aurola, OH, USA). The proteins on the membrane were detected by a chemiluminescence method (ECL Western blotting detection reagents; Amersham Biosciences, Uppsala, Sweden).

In vivo study

HT1080 or SW620 cells (5×10^6 cells/mouse) were injected subcutaneously into the right back of Balb/c nude mice (Charls River Co, Kanagawa, Japan). The engrafted tumors developed to 3–8 mm in diameter by 8–9 days after transplantation. The mice were then divided into four groups ($n = 5$ or 7/group) that received intratumoral injection of saline, wild-type SeV, parental SeV/ΔM or MMP-subII SeV/ΔM (5×10^7 cell infectious unit (CIU)) in a 50 μl volume using a 26-gauge needle.

Immunohistochemistry

For anti-GFP antibody staining (Molecular Probes, Eugene, OR, USA) and Apotag peroxidase *in situ* apoptosis detection (Intergen Co., Norcross, GA, USA), tumors were removed, fixed with 4% paraformaldehyde and embedded in paraffin 2 days after injection of each virus. In all, 5-μm-thick sections were used for stainings.

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RESEARCH ARTICLE

Recombinant Sendai virus vectors deleted in both the matrix and the fusion genes: efficient gene transfer with preferable properties

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Abstract

Background Sendai virus (SeV) is a new type of cytoplasmic RNA vector, which infects and replicates in most mammalian cells, directs high-level expression of the genes on its genome and is free from genotoxicity. In order to improve this vector, both the matrix (M) and fusion (F) genes were deleted from its genome.

Methods For the recovery of the M and F genes-deleted SeV (SeV/ΔMΔF), the packaging cell line was established by using a Cre/*loxP* induction system. SeV/ΔMΔF was characterized and compared with wild-type and F or M gene-deleted SeV vectors in terms of transduction ability, particle formation, transmissible property and cytotoxicity.

Results SeV/ΔMΔF was propagated in high titers from the packaging cell line. When this vector was administered into the lateral ventricle and the respiratory tissue, many of the ependymal and epithelial cells were transduced, respectively, as in the case of wild-type SeV. F gene-deletion made the SeV vector non-transmissible, and M gene-deletion worked well to inhibit formation of the particles from infected cells. Simultaneous deletions of these two genes in the same genome resulted in combining both advantages. That is, both virus maturation into particles and transmissible property were almost completely abolished in cells infected with SeV/ΔMΔF. Further, the cytopathic effect of SeV/ΔMΔF was significantly attenuated rather than that of wild type *in vitro* and *in vivo*.

Conclusions SeV/ΔMΔF is an advanced type of cytoplasmic RNA vector, which retains efficient gene transfer, gains non-transmissible properties and loses particle formation with less cytopathic effect. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords Sendai virus; cytoplasmic RNA vector; gene(s)-deletion; attenuation; packaging cell

Introduction

Sendai virus (SeV), that belongs to the genus *Respirovirus* of the family *Paramyxoviridae*, infects and replicates in most mammalian cells including human cells and directs high-level expression of the genes on its genome. SeV replicates independently of cellular nuclear functions and does not have a DNA phase during its life cycle so that the possible transformation of cells by integration of the genetic information of the virus into the cellular genome is not a concern [1]. These properties make SeV vectors very promising for application to gene therapy via the

expression of therapeutic genes and vaccine antigens [2–6].

The genome of SeV is a linear and nonsegmented negative-strand RNA of approximately 15.4 kb. It contains six major genes, which are arranged in tandem on its genome, and is tightly encapsidated with the nucleoprotein (NP) and is further complexed to phosphoprotein (P) and large protein (L; the catalytic subunit of the polymerase) [7]. This RNP complex constitutes the internal core structure of the virion. The viral envelope contains two spike proteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), which mediate the attachment of virions and the penetration of RNPs into infected cells, respectively. M protein functions in virus assembly and budding. The establishment of technology to rescue SeV entirely from cDNA and hence enable the plasmid-based reverse genetics [8,9] has provided the possibility of using SeV as a novel class of vector to deliver and express therapeutic genes. In particular, we plan the clinical application of the SeV vector carrying human fibroblast growth factor-2 (FGF-2) for the treatment of peripheral arterial disease. Intramuscular injection of this vector strongly boosted FGF-2, resulting in significant therapeutic effects for limb salvage with increased blood perfusion associated with enhanced endogenous VEGF expression in murine models of critical limb ischemia [4]. For such use in human gene therapy, F gene-deleted SeV (SeV/ΔF) has been produced and demonstrated to be non-self-transmissible due to loss of the F gene in its genomic RNA [10]. Recently, we have found that non-transmissible virus-like particles (VLPs) are formed in cells infected with SeV/ΔF and succeeded in its reduction by introducing temperature-sensitive mutations in M (G69E, T116A, A183S) and HN (A262T, G264R, K461G) proteins to about 1/10 of parental SeV/ΔF [11]. However, complete reduction of its formation is still one goal that we are trying to achieve in the next-generation SeV vectors. For this purpose, M gene-deleted SeV (SeV/ΔM) has recovered and proved that virus maturation into a particle was almost completely abolished in cells infected with SeV/ΔM [12]. Instead, SeV/ΔM infection brought about significant increase of syncytium formation under conditions in which the fusion protein was proteolytically cleaved and activated by trypsin-like protease, indicating that SeV/ΔM may be suitable for the treatment of diseases required for cytolytic features such as in cancer. Additional gene(s)-deletion such as F and HN would be necessary to apply SeV/ΔM for the treatment of diseases required for reduced cytotoxicity.

In this study, we first succeeded in the establishment of both M and F proteins-expressing packaging cell lines and thus performed the recovery of both M and F genes-deleted SeV (SeV/ΔMΔF) in high titer. F gene-deletion made the SeV vector non-transmissible, and M gene-deletion worked well to inhibit formation of the particles from infected cells. Simultaneous deletions of these two genes in the same genome resulted in combining both advantages with efficient gene transfer and less cytopathic effects.

Materials and methods

Cells and viruses

Two monkey kidney cell lines, LLC-MK_2 and CV-1, and a human adenocarcinoma cell line, HeLa, were cultured in Eagle's minimal essential medium (MEM; Gibco-BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). All three cell lines were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. The genome of the attenuated SeV Z strain was used as the starting material for genome modifications in this study. Wild-type SeV carrying the enhanced green fluorescent protein (GFP) gene inserted before the NP ORF (SeV^{+18}GFP) [13] was grown in 10-day-old embryonated chicken eggs [9]. F and M genes-deleted SeV vectors were prepared using stable transformed LLC-MK_2 cells carrying the F gene (LLC-MK_2/F7 [10]) and M gene (LLC-MK_2/F7/M62 [12]), respectively. The SeV vector with both M and F genes deleted was prepared using a new packaging cell line (LLC-MK_2/F7/M#33; this report). An adenovirus vector, AxCANCre [14], expressing Cre recombinase was used for the induction of F protein in LLC-MK_2/F7 cells (thus-induced cells are referred to as LLC-MK_2/F7/A), M protein in LLC-MK_2/F7/M62 cells (thus-induced cells are referred to as LLC-MK_2/F7/M62/A) and both M and F proteins in LLC-MK_2/F7/M#33 cells (thus-induced cells are referred to as LLC-MK_2/F7/M#33/A), respectively. Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase [15] was inactivated with psoralen and long-wave UV irradiation and then used for RNP recovery experiments [16,17].

Antibodies

A rabbit polyclonal anti-M antibody, N-39F, was raised against three mixed synthesized peptides of SeV M protein [11]. Anti-F, γ236 [18], and Anti-HN, HN-2 [19], are mouse monoclonal antibodies. Anti-SeV rabbit polyclonal serum was described previously [9]. Goat anti-rabbit IgG and goat anti-mouse IgG + IgM conjugated with horseradish peroxidase (HRP) were purchased from ICN (Aurola, OH, USA) and Biosource Int. (Camarillo, CA, USA), respectively.

Plasmid construction

For the construction of genomic cDNA of both M and F genes-deleted SeV carrying the enhanced green fluorescent protein (GFP) gene, full-length genomic cDNA of the F gene-deleted SeV carrying the reporter GFP gene ($pSeV^{18+}$/ΔF-GFP) [10] was utilized. $pSeV^{18+}$/ΔF-GFP was constructed from $pSeV^{18+}$, in which 18 base oligonucleotides containing the NotI restriction site designed for the insertion of the gene of interest (GOI) between the leader and the NP gene was introduced [9]. The 4.9-kb

*Nae*I fragment containing the M gene of pSeV^{18+}/ΔF-GFP was cloned into pBluescript II (Stratagene, La Jolla, CA, USA) to generate pBlueNaeIfrg-ΔFGFP. Site-directed mutagenesis was conducted by using a QuikChange™ site-directed mutagenesis kit (Stratagene) and the pBlueNaeIfrg-ΔFGFP as a template. The primer pairs of 5′-AGAGTCACTGACCAACTAGAGTGCACGTGAGGCATCCT-ACCATCCTCA-3′ [sense] and 5′-TGAGGATGGTAGGATG-CCTCACGTGCACTCTAGTTGGTCAGTGACTCT-3′ [antisense] were used to introduce the *Apa*LI site just behind the P gene. The generated plasmid was partially digested (for 5 min at 37 °C) with *Apa*LI and the resulting 3.7-kb fragment was self-ligated; thus the M gene was deleted from pBlueNaeIfrg-Δ FGFP. The 1.5-kb *Sal*I- and *Apa*LI-digested fragment adjacent to the genes-deleted region was cloned into LITMUS 38 (NEB, Beverly, MA, USA) with the 6.3-kb *Apa*LI and *Nhe*I fragment of pSeV^{18+}/ΔF-GFP to generate LitmusSalINheIfrg-ΔMΔFGFP. The 7.8-kb *Sal*I- and *Nhe*I-digested fragment containing both M and F genes deleted was substituted for the corresponding fragment of pSeV^{18+}/ΔF-GFP to generate pSeV^{18+}/ΔMΔF-GFP. For another design of SeV genome, the GFP gene was deleted from pSeV^{18+}/ΔMΔF-GFP. Firstly, it was deleted from pSeV^{18+}/ΔF-GFP. PCR was conducted with the primer pair of 5′-CTGCAGGATATCTGGATCCAC-3′ and 5′-CTTTCACCTGGTACAAGCACAGATCATGGATGG-3′ on LitmusSalINheIfrg-ΔFGFP, which was generated by insertion of the 8.9-kb *Sal*I- and *Nhe*I-digested fragment of pSeV^{18+}/ΔF-GFP to LITMUS 38 as a template. Another PCR was carried out with the primer pair of 5′-GTTTACCAGGTGGAGAGTTTTGCAACCAAGCAC-3′ and 5′-CCATCAACACTCCCCAAGGACC-3′ on pSeV^{18+}/ΔF-GFP as a template. These amplified 5.3-kb and 2.9-kb fragments were digested with *Nhe*I/*Sex*AI and *Sal*I/*Sex*AI, respectively, and cloned into the 8.3-kb *Sal*I- and *Nhe*I-digested fragment of pSeV^{18+}/ΔF-GFP. Thus, pSeV18+/ΔF, in which the GFP gene was deleted from pSeV^{18+}/ΔF-GFP, was generated. The 1.6-kb *Sex*AI-digested fragment of pSeV18+/ΔF was substituted with the corresponding 0.5-kb *Sex*AI-digested PCR fragment which had been amplified with the primer pair of 5′-AGAGAACAAGACTAAGGCTACC-3′ and 5′-GTGCTTGGTTGCAAAACTCTCCACCTGGTAAAC-3′ on LitmusSalINheIfrg-ΔMΔFGFP as a template. Thus, pSeV18+/ΔMΔF, in which the GFP gene was deleted from pSeV^{18+}/ΔMΔF-GFP, was generated. To construct the cDNA of SeV/ΔMΔF possessing an insertion of the GFP gene before the viral NP gene (pSeV^{18+}GFP/ΔMΔF), the GFP gene with EIS at its 3′ end [13] was inserted into the site previously created with 18 nucleotides (GAGGGC-CCGCGGCCGCGA) containing a unique *Not*I restriction site. For the plasmids expressing M and F proteins under the control of the Cre/*loxP*-induction system, pCAL-NdLw [20] was utilized. The neomycin and hygromycin resistance genes of pCALNdLw/F [10] and pCALNdLw-hygroM [12], respectively, were replaced with the zeocin resistance gene. The 5.4-kb *Spe*I and *Eco*T22I fragment containing the F gene and the 1.8-kb *Xho*I and *Spe*I fragment, which does not contain the neomycin resistance gene, were prepared from pCALNdLw/F. The zeocin resistance gene was prepared by performing PCR with pcDNA3.1zeo(+) (Invitrogen, Groningen, The Netherlands) as the template using the pair of primers: 5′-TCTCGAGTCGCTCGGTACGATGGCCAAGTTGACCAGTG-CCGTTCCGGTGCTCAC-3′ and 5′-AATGCATGATCAGTA-AATTACAATGAACATCGAACCCCAGAGTCCCGCTCAGT-CCTGCTCCTCGGCCACGAAGTGCACGCAGTTG-3′, and then digesting the product with *Xho*I and *Eco*T22I. These three fragments were ligated together to generate pCALNdLw-zeoF. To generate pCALNdLw-zeoM, the 1.7-kb XhoI fragment of pCALNdLw-hygroM was substituted with the corresponding 1.0-kb fragment of pCALNdLw-zeoF.

Insertion of the SEAP gene upstream of the NP ORF

To quantify the expression level of a foreign gene carried in the SeV genome, the gene for the SEAP was inserted upstream of the NP ORF. The SEAP gene with EIS [11] was introduced into the *Not*I site of pSeV^{18+}/ΔMΔF-GFP to generate pSeV^{18+}SEAP/Δ MΔF-GFP.

Cloning and analysis of helper cells

LLC-MK$_2$/F7/M62 cells were transfected with pCALNdLw-zeoF and pCALNdLw-zeoM using the Superfect transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Zeocin (500 μg/ml) resistant clones that had been propagated from single cells were selected 2 or 3 weeks after the transfection and analyzed for their expression of F and M proteins. Each clone was plated in 6-well plates, and, when they reached a nearly confluent state, they were infected with AxCANCre at a multiplicity of infection (MOI) of 5 [20,21]. After culturing for 2 days at 32 °C, the cells were recovered and subjected to semi-quantitative Western blotting using the anti-M and anti-F antibodies according to the method described [11].

Both M and F genes-deleted SeV recovery from cDNA

Preparation of cell lysate containing RNPs and primary virions of SeV18+/ΔMΔF-GFP was carried out according to the method described [11,12]. Briefly, approximately 10^7LLC-MK$_2$ cells seeded in a 10-cm diameter dish were infected with psoralen- and long-wave UV-treated [17] vTF7-3 at an MOI of 2. After 1 h of incubation at room temperature, the cells were washed three times with MEM and transfected with the plasmid mixture described below. For each transfection, plasmids pGEM-NP (4 μg), pGEM-P (2 μg), pGEM-L (4 μg) [9], pGEM-M (4 μg), pGEM-FHN (4 μg) [12] and the indicated SeV cDNA plasmid (12 μg) were suspended in 200 μl of OptiMEM (Gibco-BRL) with 110 μL of Superfect