New cancer therapy using genetically-engineered oncolytic Sendai virus vector

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1. ABSTRACT

We have developed a new type of Sendai virus-(SeV) based gene transfer vectors for cancer therapy. The matrix gene-, indispensable for particle formation, deficient and fusion gene-, essential for cell-fusion and deciding viral tropism, redesigned SeV vector loses vector particle formation from transduced cells and gains cell-to-cell spreading in protease-dependent, namely controllable, manner. For the selective delivery to malignant tumor cells expressing matrix metalloproteinases (MMPs) or urokinase-type plasminogen activator (uPA), we introduced MMP-cleavage (PLGMTS) or uPA-cleavage (SGRS) sequences, respectively, immediately prior to the cleavage site for activation of fusion protein with remaining essential sequences for cell-fusion. The MMP-targeted SeV vector demonstrated syncytia formation in MMP expressing HT1080 cell line in vitro, and growth inhibition of HT1080 subcutaneous xenografts in vivo. The uPA-targeted one showed the same effects in uPA expressing PC-3 cell line. Severe apoptosis occurred in fused-cells. Thus, the vector selectively spreads to tumor cells in tumor-protease dependent manner and demonstrates the antitumor effects in solid tumors, indicating the value of selective targeting and killing of tumors by recombinant SeV technology.

2. SENDAI VIRUS VECTORS FOR GENE THERAPY

The cytoplasmic RNA vector would be promising for use in gene therapy and gene vaccines to large population of patients because of its important genotoxicity-free nature. Sendai virus (SeV), a murine parainfluenza virus belongs to a family *Paramyxoviridae*, infects and multiplies its genome copy in most mammalian cells. Its replication is strictly in cytoplasm and independent of nuclear functions of host cells (1) (Figure 1), so SeV-based vectors do not need to be concerned about the transformation of cells by integration of vector materials into the host chromosomes (2,3). These properties of the vector enable us to propose the new concepts, CYTOPLASMIC GENE THERAPY (4-8) and CYTOPLASMIC VACCINATION (9-11) with ribonucleoprotein (RNP)-based treatment.

In order to establish such SeV vector system, we have taken the strategy to generate the gene(s)-deleted or modified SeV vectors. Thus, we have succeeded in the recovery in high titers of fusion (F) gene-deleted (SeV/ΔF) (12), matrix (M) gene-deleted (SeV/ΔM) (13), hemagglutinin-neuraminidase (HN) gene-deleted (SeV/ΔHN), both M and F genes-deleted (SeV/ΔMΔF)
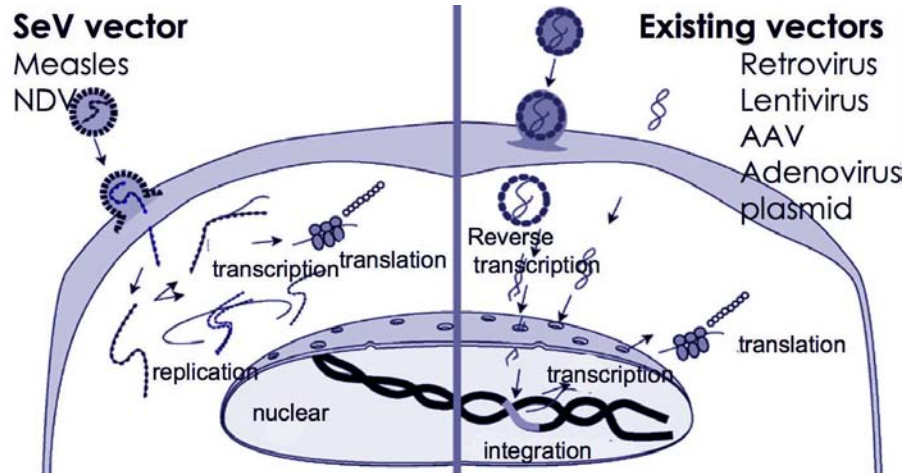
Figure 1. Cytoplasmic (genotoxicity free) RNA vector. SeV vector replicates and transcripts only in the cytoplasm, does not have DNA phase and never interacts with chromosomes. Therefore, this type vector is completely free from genotoxicity. For the broad range of use for gene therapy, such free from genotoxicity is very important. In contrast, existing vectors such as retrovirus and lentivirus are integrated into the chromosome to express the transgene. Even in the cases of adeno-associated virus, Adenovirus, and plasmid, some parts of them are known to integrate into the chromosome.
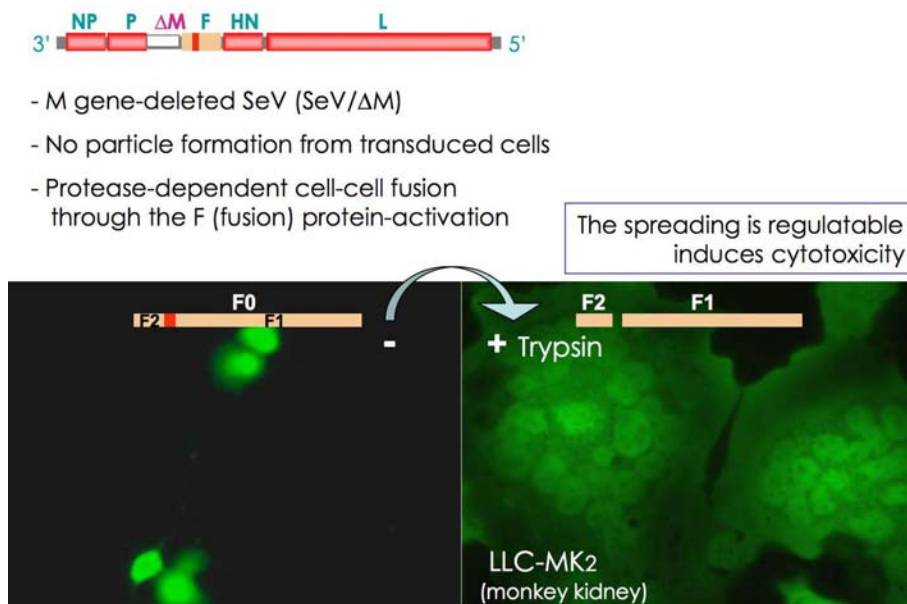

Figure 2. Trypsin-dependent cell-cell spreading with the transduction of M gene-deleted SeV vector. LLC-MK$_2$ cells were transduced with SeV/ΔM-GFP at an MOI of 0.01. Expression of GFP protein was detected 2 days after the transduction under fluorescence microscopy.

(14), all of the envelop-related genes-deleted (SeV/ΔMΔFΔHN) (15) SeV vectors by using the packaging cell lines which express respective proteins of those deleted gene(s). Among them, SeV/ΔM vector was selected in generating an oncolytic SeV vector targeting a solid tumor tissue.

3. CELL-CELL SPREADING OF M GENE-DELETED SeV VECTOR

M protein plays central role in virus assembly and budding. Therefore, deletion of the M gene from SeV almost completely abolished virus maturation into

infectious particles from transduced cells and instead caused cell-to-cell vector spreading via membrane fusion and formed large syncytia (Figure 2)(13). The addition of trypsin to the culture medium was indispensable for the spreading in this case. That is, the significant matter for this vector is that the spreading is regulatable by the presence of selected F protein-activating proteases.

4. SeV VECTOR TROPISM : POSSIBLE CONVERSION TO ECM DEGRADATION ENZYME-DEPENDENT SPREADING

The initiation of infection with SeV involves virus attachment to the cell surface via HN protein and the

subsequent envelope-plasma membrane fusion mediated by F protein, resulting in entry of the viral genome into the cytoplasm. The F protein is synthesized as a biologically inactive precursor Fo and is converted to the active F1 (and F2) by posttranslational proteolytic processing. SeV displays a narrow spectrum of tissue tropism, its multiplication being restricted to the respiratory tract of mice, because the expression of the virus activating proteases, tryptase Clara in this case, are highly specific for particular tissues, such as respiratory tracts (16,17). These facts brought the assumption that the tropism of SeV could be converted by modifying the tryptic cleavage site of F0.

Invasive metastatic, that is malignant, tumors are known to express high levels of matrix metalloprotease (MMP) and plasminogen activators (uPA, tPA) (18,19). Since extracellular matrix (ECM) around tumor cells serves as a barrier and blocks tumor cell migration for infiltration and metastasis, tumors are considered to express ECM-degrading enzymes. We also confirmed the both types of proteases are over-expressed in many kinds of tumor cell lines such as prostate, esophagus and breast cancers. Therefore, we selected these proteases for the targeting, and the cleavage sequence of F protein of SeV/ΔM was changed to that susceptible to MMP or uPA.

5. ALTERATION OF F PROTEIN TO RENDER IT SUSCEPTIBLE TO MMP OR uPA

The sequence encoding the activation site was redesigned to acquire MMP- or uPA-dependent cleavage. There have been many reports of the sequences of cleavable substrates of MMP, and it is possible to apply them. However, the N-terminal region of F1 of paramyxovirus F protein is important for fusion activity and amino acid mutation in the region may lose its fusion activity (20). Therefore, we left the sequence of the N-terminal region of F1 unmodified. Although the N-terminal was not modified, insertion of the common cleavable substrate of MMP consisting of six residues adds three residues to the N-terminal of F1 upon cleavage by MMP (Figure 3), which possibly affects the fusion activity of F1, although F protein is cleaved by MMP. Accordingly, to design an F protein that is activated by MMP-dependent cleavage, it is necessary to consider the following two points: MMP substrate specificity and retention of fusion activity of F protein after cleavage.

We selected the substrate sequence MMP-subII (PLG-MTS) that was modified from the original substrate sequence of MMP2 and 9, PLGMWS (21), according to the consensus sequence in MMP9, Pro-X-X-Hy-(Ser/Thr), which has been clarified by phage display (22). The newly designed sequence, PLG-MTS, fulfills the two requirements, MMP substrate specificity and retention of fusion activity of F protein after cleavage. The uPA-subII (SGR-S) was also designed for the sequence that is susceptible to uPA. Thus, the two types of F gene-modified M gene-deleted SeV vectors, SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subI)ΔM-GFP, were constructed and recovered using M protein-expressing packaging cell line (13). For the propagation of SeV/F(MMP-subII)ΔM-GFP

and SeV/F(uPA-subI)ΔM-GFP, collagenase type IV and trypsin were added to culture medium, respectively. Both vectors were successfully recovered in high titers (up to 10^8 cell infectious unit (CIU)/ml) without any concentration procedure (23).

6. CONFIRMATION OF ALTERATION OF TROPISM OF F-MODIFIED SeV/ΔM

Alteration of tropism, infection causing protease-dependent cell fusion, was confirmed using tumor cells. As shown in Figure 3, several ten-fold or more spread of SeV/F(MMP-subII)ΔM-GFP transduction was observed only in HT1080 cells (human sarcoma) that highly express MMPs[24] and not in the SW620 cells (human colon carcinoma) expressing MMPs at very low level. Spread of cell fusion type transduction of SeV/F(uPA-subI)ΔM-GFP was observed only in PC-3 cells (human prostate cancer) that highly express uPA and not in the LNCaP cells (human prostate cancer) expressing uPA at very low level (data not shown). Similar transduction experiments in other tumor cells were performed, and SeV/F(MMP-subII)ΔM-GFP caused syncytium formation in high MMP-expressing lines, U87MG, A172, and U251, and SeV/F(uPA-subI)ΔM-GFP caused syncytium formation in uPA-expressing LS174 cell line (data not shown).

7. ANTITUMOR EFFECT OF F-MODIFIED SeV/ΔM IN TUMOR-BEARING NUDE MICE

HT1080 tumor-bearing nude mice were prepared and the effect of F-modified SeV/ΔM was evaluated *in vivo*. To confirm spreading of the modified SeV vector, 5 x10^7 CIU of the vector was injected once into the subcutaneously transplanted HT1080 tumor. Two days later, the skin was externally irradiated and observed by fluorescence microscopy. When the parent vectors, wild type SeV carrying GFP gene (SeV-GFP) and SeV/ΔM-GFP, were administered, fluorescence was detected only around the administration site. In contrast, when SeV/(MMP-subII)ΔM-GFP was administered, GFP was spread over the tumor (Figure 4). In the magnified figure on the right, fluorescence was detected in each cell in animals treated with SeV-GFP and SeV/ΔM-GFP, while the cell boundaries were not clear in animals treated with SeV/F(MMP-subII)ΔM-GFP, strongly suggesting cell fusion.

Next, the size of the tumor was measured every other day. When saline and the parent vectors, SeV-GFP and SeV/ΔM-GFP, were administered, tumors grew rapidly. In contrast, when SeV/F(MMP-subII)ΔM-GFP was administered, tumor cell growth was markedly inhibited (Figure 4). As a part of elucidation of the tumor cell growth inhibition mechanism, TUNNEL staining was performed. Since each vector contained the GFP gene, staining with anti-GFP antibody was also performed to identify transduced cells. When SeV/ΔM-GFP was administered, transduction was localized to the administration site and there were only a few TUNNEL-positive cells. When SeV/F(MMP-subII)ΔM-GFP was
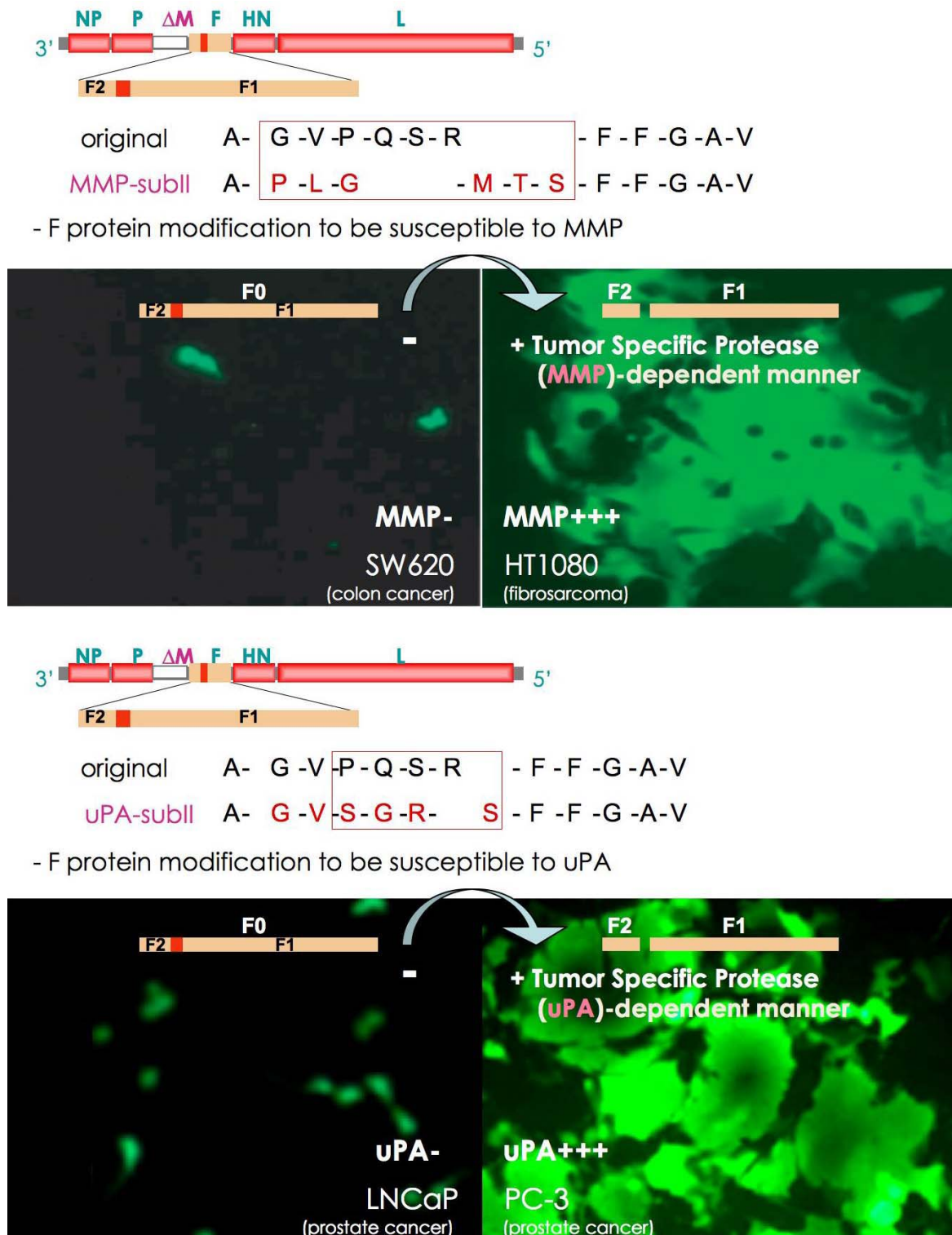
Figure 3. Cell-cell spreading of F-modified M gene-deleted SeV vector dependent on endogenous protease from tumor cells. MMP-expressing HT1080 and SW620 expressing low levels of MMP and uPA-expressing PC-3 and LNCaP expressing low levels of uPA were transduced with SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subII)ΔM-GFP, respectively, at an MOI of 0.02. Those cells were cultured for four days with the culture medium containing 1% fetal bovine serum, and cell fusion was observed.
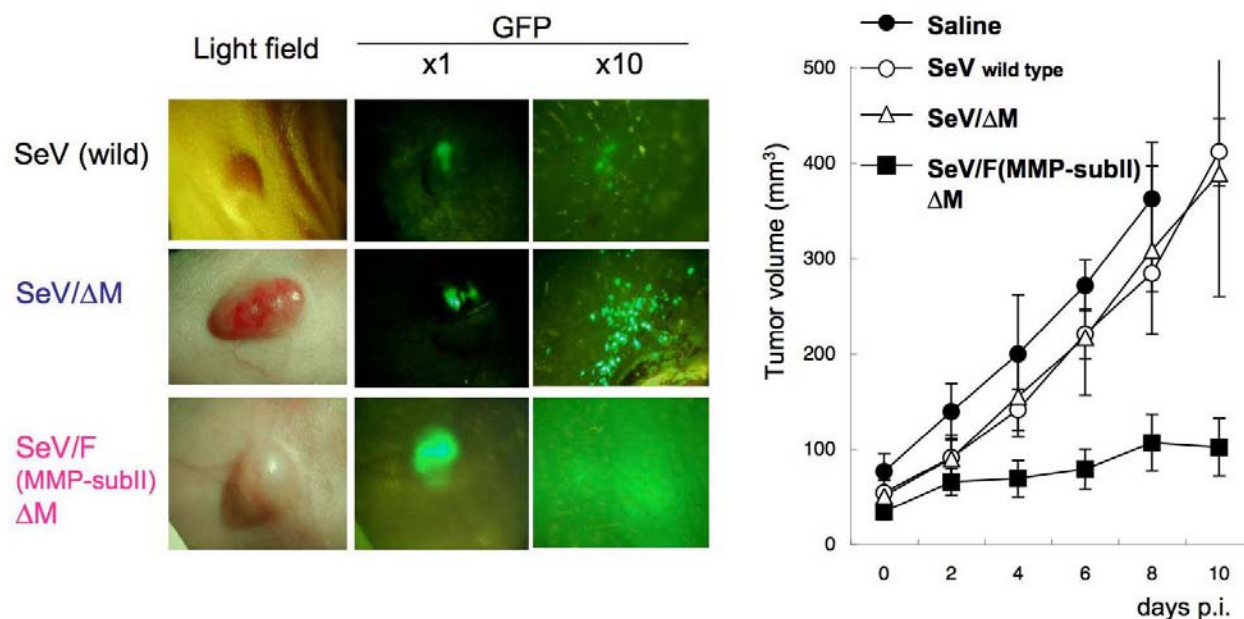
Figure 4. Antitumor effect of F-modified SeV/ΔM *in vivo*. Cell-cell spreading of F-modified SeV/ΔM. 5 x 10^7 CIU of viral vectors, SeV-GFP, SeV/ΔM-GFP, and SeV/F(MMP-subII)ΔM-GFP, were injected once directly into the subcutaneously transplanted HT1080 tumors. Two days later, GFP expression was observed from outside the body under fluorescence microscopy. F-modified SeV/ΔM inhibits the tumor growth. After the vector injection, tumor length, width and height were measured with time. Tumor volume was calculated with the formula (Volume = p/6 x abc :length(a), width(b), height (c)) and expressed by an average (n=7). Statistical differences were observed between the group administered SeV/F(MMP-subII)ΔM-GFP and all other groups ($P < 0.05$, Student's t-test) at 8 and 10 days after injection.

administered, GFP protein flowed out from the fused cell region during preparation of sections and little GFP-positive cells were observed, but spread of GFP was observed in the tip outside there, and TUNNEL staining was positive over the cell fusion area (23), confirming that apoptosis was induced in a wide cell-cell spreading area after infection with SeV/F(MMP-subII)ΔM-GFP. Thus, the concept of the vector design of "F-modified SeV/ΔM" was proved.

8. FUSOGENIC ACTIVITY IMPROVEMENT OD F-MODIFIED SeV/ΔM VECTOR

Both SeV/F(MMP-subII)ΔM-GFP and SeV/F(uPA-subI)ΔM-GFP showed extensive spreading in the tumor and led to significant inhibition of the tumor growth in the mice (23). However, as we observed tumor re-growth in some cases even after the vector treatment, we try to increase the fusogenic and tumor-killing activity by further modifications of F protein. One modification is the partial deletion of the cytoplasmic domain of F protein (F-truncated), and the other one is the genetic fusion of the F and HN (F/HN fusion). In the latter design, F and HN genes were fused with a linker sequence. Thus generated SeV vectors, F-truncated and F/HN fusion vectors, showed drastically increased fusogenic activity (enhanced cell-fusion) even in the tumor cells that express in low level of tumor-specific proteases (data not shown). That is, both F-truncated and F/HN fusion vectors showed quite increased cell-fusion. Typical example is shown in Figure 5. The first modification of F cleavage sequence enables the tumor-specific spreading, and the second modification of the cytoplasmic domain efficiently increased the cell-killing activity of the vector. Such characteristic change was achieved only by the genetic modifications of SeV vector.

Another method to potentiate F-modified SeV/ΔM is expectative by integrating therapeutic gene(s) such as immune stimulating or suicide genes. Our results showed that the tropism-modified cell-cell spreading of F-modified SeV/ΔM exhibit a strong antitumor effect on tumor cells without carrying a therapeutic gene, and thus, the vectors may be promising for tumor therapy. Loading an effective therapeutic gene(s) on the vectors will be effective to further increase the therapeutic effect, and we are improving and evaluating the vectors in this direction.

9. CONCLUSION : A NOVEL ONCOLYTIC VECTOR BASED ON AN ENGINEERED VECTOR TROPISM

We generated a new type of oncolytic SeV/ΔM vector that is activated by MMPs or uPA but no longer by trypsin like proteases (23). The virus vector spreads selectively in MMP- or uPA-expressing tumor cells and those engrafted to mice, leading to rapid death of these cells and strong inhibition of their growth (Figure 6). Malignant tumor-specific cell fusion leading to efficient cell death was produced by transducing a novel genetically-engineered oncolytic Sendai virus vector.
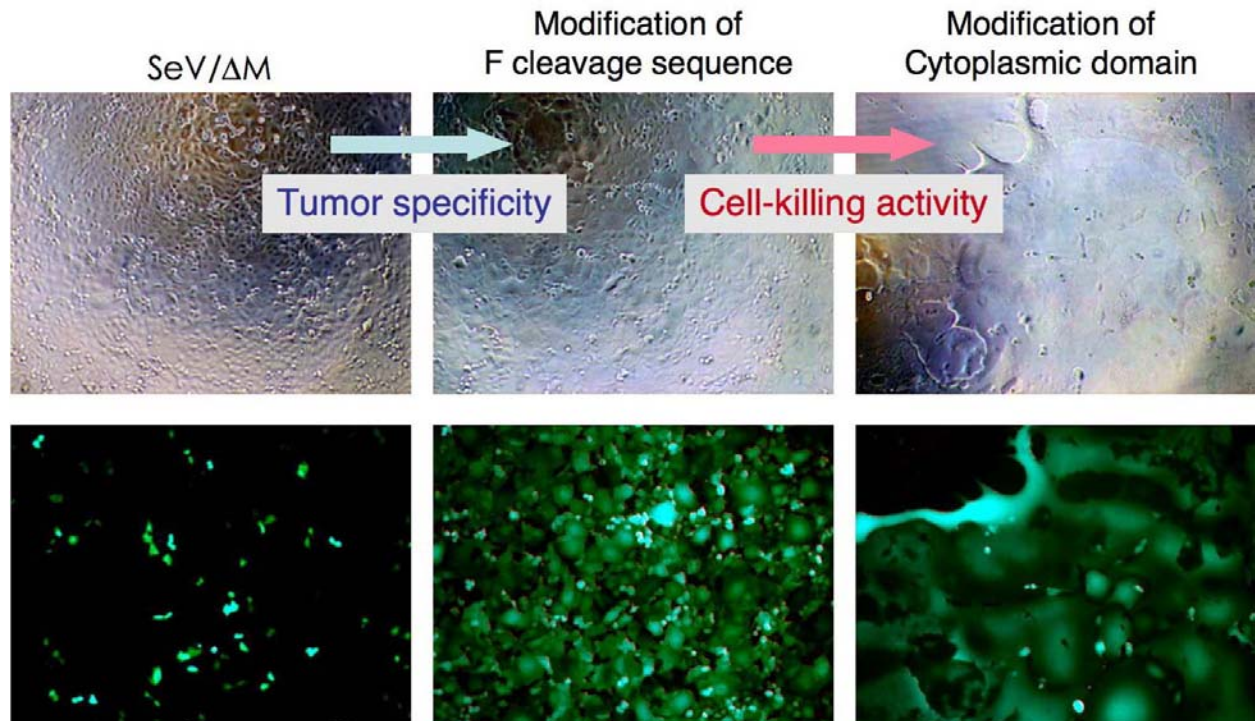
Figure 5. Typical example of synergistic effect of F-modification. The first modification of F cleavage sequence enables the tumor-specific spreading, and the second modification of the cytoplasmic domain efficiently increased the cell-killing activity of the vector.
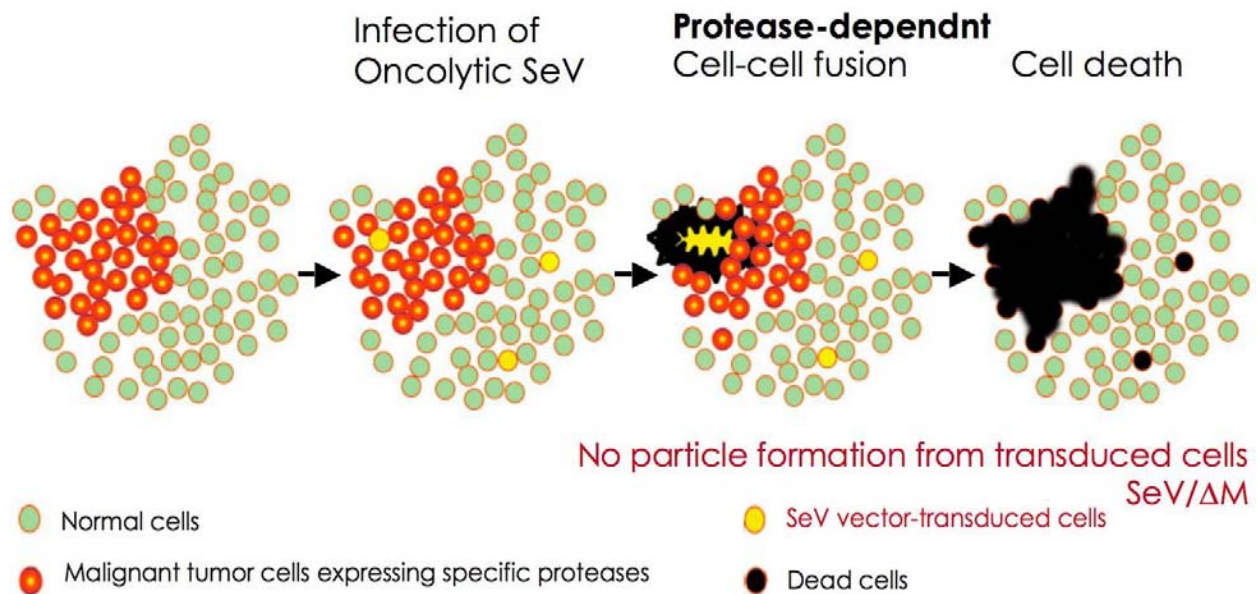

Figure 6. Oncolytic strategy using F-modified M gene-deleted SeV vector. The many types of malignant tumor cells are known to express the tumor-specific proteases such as MMP and uPA for their malignancy. F-modified SeV/ΔM transduces both tumor and normal cells, and spread only in the tumor cells through the tumor-specific protease-dependent manner and with cell-cell spreading, and lyses the malignant tumor cells. One of the important characteristics of this strategy is that there is no virion formation from transduced cells because SeV/ΔM is used, indicating shedding problem will be minimum.

10. ACKNOWLEDGMENTS

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