well as in three other families (*Rhabdo-*, *Filo-*, and *Bornaviridae*) in the *Mononegavirales*.

Competition to be the first and best

The competition to be the first in rescuing a mononegavirus from cDNA lasted over a decade because it required a complex procedure with delicate fine tuning. In case of positive-strand RNA viruses such as poliovirus, recovery of virus from cDNA is relatively simple because the RNA genome, intracellularly expressed from the transfected cDNA or *in vitro* synthesized and transfected into cells, immediately acts as the mRNA that is translated into the proteins capable of immediately initiating virus replication cycle. The naked DNA genomes of many DNA viruses can also initiate the viral lifecycle upon transfection to cells; they are transcribed by the cellular DNA-dependent RNA polymerase to generate mRNAs encoding the viral proteins to initiate viral replication. In contrast to these two cases in which the genome, either RNA or DNA, is infectious, in the natural lifecycle of paramyxoviruses and other mononegaviruses, the negative-strand (-)RNA genome, after entering the cytoplasm, does not become naked but rather has to remain as the (-)RNP complex to be transcribed. This transcription is mediated by the virion-associated, virus-specific RNA polymerase comprising the L and P proteins. The same polymerase complex replicates (+)RNP using a (-)RNP template and replicates in turn the (-)RNP using the (+)RNP template. Thus, nothing happens when the entire genomic RNA, either (+) or (-) sense, is intracellularly expressed or transfected to cells. Effective virus recovery requires careful reconstitution of functional (-)RNP complexed with the L and P proteins in cells by expressing genome RNA and the three proteins N, P and L from the respective expression plasmids.

Most research groups constructed a plasmid carrying the full-length (+)RNA genome flanked by a T7 promoter (T7) and hepatitis delta virus ribozyme and three T7-driven plasmids carrying the genes encoding the N, P, and L proteins (Fig. 2a). The T7 bacteriophage RNA polymerase was expressed from a recombinant vaccinia virus, vTF7-3. It is plausible to have a (+)RNA genome expressed to bypass the (+)RNP synthesis step.

In 1994, at the end of a decade of trial and error experimentation, Conzelmann and his colleagues in Germany eventually succeeded in rescue of a rhabdovirus, rabies virus, from a cDNA (approx. 12 kb) plasmid (4). Shortly thereafter, six laboratory groups across the globe, including ours, achieved rescue of SeV, measles virus, or respiratory syncytial virus in the *Paramyxoviridae* and another rhabdovirus, vesicular stomatitis virus (reviewed in ref. 5).

While the other systems appeared to require as many as 10^7 transfected cells to rescue an infectious virus, our SeV system yielded a virus from as few as 10^5 transfected cells, thus exhibiting an efficiency 100 times greater than that

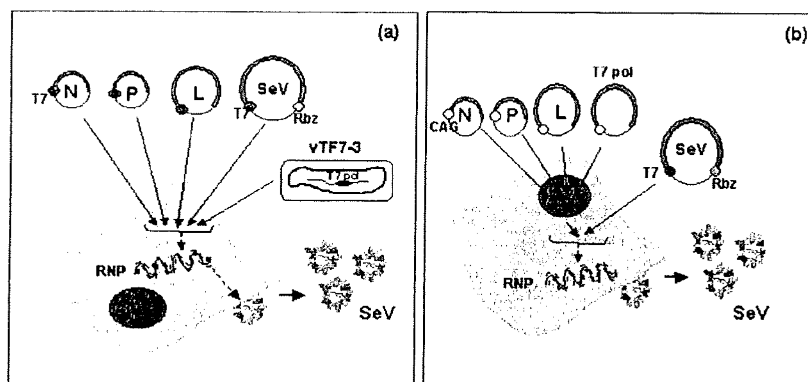

Figure 2. SeV rescue from cDNA. (a) Method with T7 RNA polymerase (T7 pol)-expressing recombinant vaccinia virus (vTF7-3). (b) Method without vaccinia virus. T7: T7 promoter; Rbz: ribozyme derived from delta hepatitis virus; CAG: CAG promoter.

of the other systems (6). Moreover, in our system, viruses were even effectively recovered from transfected, naked 15.3-kb, full-length SeV genome RNA synthesized *in vitro* from cDNA, as long as appropriate amounts of N, P, and L proteins were expressed and supplied to the transfected RNA, irrespective of whether the transfected RNA was plus or minus-stranded (6). These results, while suggesting the high efficiency of our rescue system, might surprise the majority of researchers in this field, who generally had the idea that RNA replication was a very sophisticated and refined process in which the elongation of a *de novo* synthesized RNA strand is conjugated with encapsidation by *de novo* synthesized N-protein molecules.

Several factors contributed to the unparalleled high performance of our rescue system, which was commended by an independent reviewer (7). First, we optimized the ratio of the plasmids respectively expressing N, P, and L proteins. Second, we successfully used suitable agents to reduce the cytotoxicity incurred by vTF7-3 proliferation without significantly hampering the expression of T7 RNA polymerase. Third and perhaps most important, because T7 promoter sequence-templated guanine residues are commonly attached to the 5' terminal of T7 promoter-dependent transcripts, we deleted a part of the promoter sequence, even at the cost of some reduced transcription ability, so that the transcription initiated at the exact point. Thus, our system might be the best, although not the first. During the subsequent decade up to the present, reverse genetics has come of age for most paramyxoviruses and several other mononegaviruses (reviewed in refs. 8, 9).

Further increase of efficiency and generation of the helper vaccinia virus-free system

Psoralen compound covalently binds to double-stranded DNA under UV (ultra violet)-irradiation. UV irradiation of vTF7-3 in the presence of psoralen under certain conditions caused further reduction of the vaccinia virus-associated cytotoxicity, while T7 RNA polymerase expression remained unchanged. As a result, the virus recovery rate was further increased by 100- to 1,000-fold (1 infectious unit from 10^2–10^3 transfected cells) (10), and SeV engineering was enormously facilitated when compared with its initial stages.

Removal of vTF7-3 from rescued SeV preparations was always necessary. In addition, vTF7-3 was provided by the US National Institutes of Health on the condition that the virus would be used solely for basic research. These terms of use eventually proved to be a problem in clinical application of SeV technology (which we refer to later) and led us to develop a vaccinia virus-free system. All three supporting plasmids to express the N, P, and L proteins, respectively, and an additional plasmid to express T7 polymerase were driven by cellular RNA polymerase II under the control of a CAG promoter (Fig. 2b). The plasmid to generate SeV genome RNA was left unchanged under the control of the T7 promoter. The resulting all-plasmid–based method without vTF7-3 demonstrated virus recovery efficiency similar to or even higher than that using UV irradiated vTF7-3 (unpublished data).

4. Accessory genes as a focus of active investigation by SeV reverse genetics

There were many unsolved questions and enigmas and also many hypotheses to be verified in paramyxovirus lifecycle and pathogenesis. Reverse genetics has proved to be powerful enough to address and settle these outstanding issues. Here, we illustrate only our studies on SeV accessory genes, as a comprehensive review is beyond the scope of this paper. For more details, see refs 5, 8, and 11.

Coding strategies of the SeV accessory genes

SeV encodes V and C proteins in addition to six major gene products (N, P, M, F, HN, and L). These two proteins are widespread among the members of *Paramyxoviridae*, although not as ubiquitous as the six major structural proteins, and therefore have been regarded as non-essential "accessory" gene products. However, the central question of how they contribute to actual viral replication and pathogenesis has remained unanswered since they were first described three decades ago (reviewed in ref. 11). Even the non-essentiality of these proteins had not been established. This is where reverse genetics started to play a prominent role.

The V and C proteins are both *P* gene products. An open reading frame (ORF) different from that of the P protein gives rise to four proteins: C', C, Y1, and Y2, collectively referred to as the C proteins. This diversity is accomplished by use of translation initiation codons at different points [nucleotide positions (nt) 81, 114, 183, and 201] and the stop codons at the same position (nt 726-728) (Fig. 3). Thus, the frames for the C proteins are accessed by "ribosomal choice". Among the four C proteins, C is the major species expressed in infected cells at a molar ratio several-fold higher than those of the other three. The number of expressed C proteins differs among paramyxoviruses, ranging from 1 to 4, and their primary structures are poorly conserved. However, they are, in common, basic and relatively short (with some 200 amino acid residues) (reviewed in refs. 2, 11).

The V protein, on the other hand, is produced by a transcriptional frameshift ("co-transcriptional RNA editing"). The P mRNA is a faithful copy of the *P* gene, accounting for about three-fourths of the total transcripts and giving rise to the P protein, the smaller subunit of RNA polymerase complex. The remaining transcripts (one-fourth) are V mRNA, which carry a single guanine residue (+1G) (at nt 1052) inserted by the polymerase stuttering at a specific editing site (UUUUUCCC) located midway down the *P* gene; this insertion causes the frameshift (Fig. 3). The V protein therefore consists of a

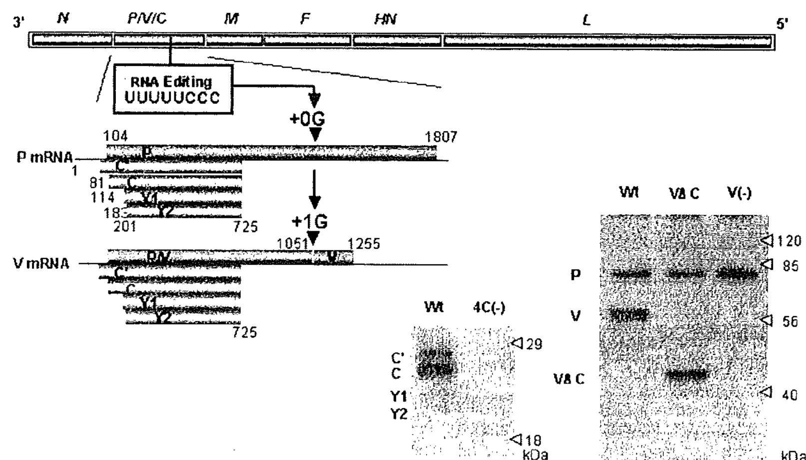

Figure 3. Expression of C and V proteins from SeV *P* gene and recovery of knock-out viruses: V(-) with the entire V protein deleted, VΔC with the V-unique region deleted, and 4C(-) with all four C proteins (C', C, Y1, and Y2) deleted. Deletion of the respective proteins was verified by Western blotting of cells infected with the recovered knock-out viruses. Wt: cells infected with wild-type SeV.

P/V common region (the N-terminal approximately three-quarters before the frameshift) and the V unique region (the C-terminal approximately one-quarter after the frameshift) (Fig. 3). The V unique region is highly conserved in the amino acid sequence among paramyxoviruses with a perfect preservation of seven cysteine residues that form a zinc finger-like motif and indeed, it binds two atoms of Zn^{2+} (reviewed in refs 2, 11).

The roles of accessory genes: Not mere accessories but rather the central players in *in vivo* pathogenesis

Without affecting the P ORF, mutations were introduced into the editing site or a stop codon was introduced just downstream of the editing site. Mutants with the total V protein deleted [V(-)] and only the C-terminal V-unique region deleted (VΔC) were respectively recovered without any difficulty (12, 13) (Fig. 3). These mutants replicated in cells as efficiently as the parental SeV, establishing that the *V* gene (protein) is nonessential. SeV causes fatal pneumonia for mice. When inoculated into mice, both V(-) and VΔC proliferated in the lung as efficiently as the parental wild-type (wt) SeV until day 1 post infection. However, they were then rapidly cleared, produced no lung lesion, and killed none of the mice, in contrast to the wt, which maintained a high viral load up to 8–10 days and killed all mice with severe hemorrhagic pneumonia (12, 13). A similar result was obtained with several mutants with the zinc finger-like motif disrupted and no capacity to bind zinc (14). Thus, the V protein that is fully dispensable for the viral life cycle was found to encode a luxury function required for pathogenesis, and the V unique region, particularly its zinc binding capacity, appeared to be crucial for this function.

The unique and rapid clearance of V(-) and VΔC viruses was no longer seen in interferon regulatory factor (IRF)-3 knockout (KO) mice, and both of the viruses were highly pathogenic for these KO mice (15). However, interferon (IFN)-β, one of the well-known IRF-3 inducible gene products, and IFN-α/β, whose synthesis is subsequently amplified via IRF-7 activation, and IFN-α/β signaling to induce an antiviral state, did not appear to be responsible for the rapid clearance of V(-) and VΔC viruses; both were cleared in IFN-α/β-receptor KO mice and STAT1 (signal transducer and activator of transcription 1) KO mice as efficiently as in the wild-type mice (15). These results predict the presence of a novel innate immunity mechanism induced by IRF-3 activation.

Through a difficult process, a viable clone, 4C(-) SeV, which expressed none of the four C proteins (C', C, Y1, or Y2), was recovered by disrupting the respective ORFs, indicating that SeV C proteins also fall into the category of nonessential accessory proteins (16) (Fig. 3). The difficulty of recovery was

the result of a reduction of 4C(-) virus infectivity by about 100-fold, and the success of the virus rescue was attributable to the high efficiency of our SeV rescue system, as noted above. The characterization of 4C(-) virus and a series of other C mutants with one or two C proteins deleted demonstrated the extreme versatility of the C proteins. They block IFN-α/β signaling (17) by inhibiting STAT2 phosphorylation (18). Actually all other paramyxoviruses also have been shown to encode anti-IFN functions (inhibition of IFN-α/β synthesis or IFN- α/β signaling, or both) (reviewed in ref. 11). However, the significance of such IFN antagonism, revealed in cell culture, for *in vivo* viral proliferation and pathogenesis was verified so far only for SeV (using another C mutant and STAT1 KO mice) (18a) and remains to be verified for other paramyxoviruses. The C proteins, nonstructural proteins that are not incorporated into virus particles but are abundantly expressed in cells, contribute greatly to viral morphogenesis by budding from the plasma membrane (19), possibly by escorting ERCRT (endosome-associated complexes required for transport) to the site of budding (20). ERCRT is thought to be required for pinching off cellular membrane at virus budding. In addition, the results supported the concept that the C proteins optimize the intracellular levels of SeV genomic RNA and mRNA for smooth progression of the viral lifecycle (16, 19).

SeV reverse genetics thus enabled investigation of accessory genes when no other means of doing so were available. The research clarified that, in the realm of *in vivo* pathogenesis, accessory genes are not mere accessories or minor players, but rather central players (reviewed in ref. 11). Mice appear to resist SeV by exerting at least two facets of innate immunity. One is the IFN system and the other is the IRF-3 dependent, as-yet-unidentified mechanism. To counteract these two antiviral mechanisms and persist in nature, SeV appears to have acquired the capacity to express both the C and the V proteins. The accessory genes thus should be highly relevant to virus biology in natural settings or to virus ecology.

5. Development of the SeV vector

Setting the stage—"Send in a sendai vector"

In parallel with the above studies to answer fundamental issues in paramyxo-virology by reverse genetics, we studied the possibility of inserting a foreign gene into SeV and expressing it under the control of SeV-specific transcriptional regulation. As mentioned above, in the SeV genome, the six basal genes are placed in tandem, in the order 3'-*N-P/V/C-M-F-HN-L*-5'. There is only a single promoter at the 3' end for RNA polymerase entry. By recognizing the start (S) (10 nt) and the end (E) (termination/polyadenylation) (11 nt) signals, the RNA polymerase gives rise to each mRNA sequentially

toward the 5' end (3, reviewed in ref. 2). The E of the preceding gene and the S of the next gene are connected by a three-nucleotide intergenic (I) sequence. Thus, each gene junction consists of an E–I–S sequence (Fig. 4) that is highly conserved throughout the genome. We first inserted an 18-nt sequence containing a unique *Not*I restriction site between the S signal and the ORF of the N protein and recovered a virus without impairing virus infectivity (21). Then, we inserted into this unique site in a cassette-like fashion various foreign genes followed by a synthetic E–I–S sequence (Fig. 4), which had been PCR-amplified with a pair of *Not*I-tagged primers, recovered recombinant viruses, and expressed the inserted genes at surprisingly high levels in cultured cells (21, 22). The inserted genes were stably maintained during serial passages in the cells (21), although the RNA polymerase has no editing or repair capabilities and genes up to 4.5 kb could be accommodated in the 15.3-kb SeV genome (23).

Furthermore, we engaged in joint research with scientists at the Imperial College School of Medicine, London, UK, who were exploring modes of gene therapy for cystic fibrosis, a highly lethal disease frequently found in Caucasians. Our results demonstrated a surprisingly high performance of the SeV vector in reporter-gene delivery into and expression in the airway epithelial cells of various animal origins as well as human origin *in vitro*, *ex vivo*, or *in vivo* (24). In fact, the expression levels were several thousand-fold higher than those obtained by conventionally employed adenovirus vectors or cationic liposomes. These results gained wide attention; an article (p. 11 in News and Views of *Nature Genetics*, September 2000) stated "Send in a sendai vector. The results are encouraging. Perhaps this should not come as a surprise, as SeV shares the same receptor (sialic acid receptor) as the influenza virus, which seems to know a thing or two about penetrating the respiratory tract".

These preliminary results and the academic recognition encouraged us to develop the SeV vector for medical use. The research has been implemented mainly at DNAVEC Research Inc (DNAVEC Corp., at present), which was inaugurated as a government–private sector joint project in 1995, funded by the Organization for Drug ADR Relief, R&D Promotion and Product Review (then) under the Ministry of Health and Welfare (then) and seven major pharmaceutical manufacturing companies in Japan.

SeV vector as a novel class, non-genotoxic cytoplasmic RNA vector

The viral vectors developed for gene therapy and/or vaccination antigen delivery have often been derived from adenoviruses, adeno-associated viruses, and retroviruses. Incorporation of the vector itself and/or therapeutic gene(s) into chromosomes is a prerequisite for retrovirus gene expression. The genomes of

adenovirus and adeno-associated virus, while existing as episomes in the nucleus, are known to integrate into chromosomes at a certain frequency. Vectors based on these viruses therefore may cause injury to host genes or genotoxicity.

This genotoxicity was substantiated by the occurrence of infantile leukemia observed in France during the course of treatment of severe combined immunodeficiency using a retrovirus vector (reviewed in ref. 25). In contrast, the entire lifecycle of SeV is completed in the cytoplasm and has no nuclear phase. Thus, SeV vector theoretically exhibits no genotoxicity.

Because retroviruses (with the exception of lentiviruses) cannot infect non-dividing cells, conventional retrovirus vectors are basically usable only for dividing cells; they are not applicable to non-dividing cells such as central nerve cells and myocytes. SeV can infect and replicate in both dividing and non-dividing cells. In addition, its host range is remarkably broad, including cells of blood origin that adenovirus appears hardly to infect. In addition, no homologous recombination has been observed for SeV, theoretically eliminating fears of occurrence of unforeseen recombinant viruses. This feature is highly relevant to the construction of replication-incompetent vector constructs in complementing helper cells (see below); the seed stock should be free of contaminating replication competent virus generated by recombination events (reviewed in refs. 5, 8).

These features, many of which are shared by mononegaviruses, highlight the feasibility of developing the SeV vector as a novel class, non-genotoxic cytoplasmic RNA vector for wide medical use with safety.

Construction of non-transmissible versions

SeV is thought to have little or no implication in human diseases. Nevertheless, SeV vectors with full-length genomes may be inappropriate for pharmaceutical use because they can be propagated and transmitted to persons other than the targeted patient. The use of SeV vectors for therapeutic purposes in various clinical settings would therefore necessitate construction of non-propagating, non-transmissible versions through deletion of one or more viral genes other than the *N, P,* and *L* genes essential to maintain transgene expression.

We established an F-expressing packaging cell line with a Cre/*lox P*-inducible expression system and succeeded in recovering SeV with the *F* gene deleted (SeV/ΔF) and with the envelope F proteins trans-supplied (10) (Fig. 4). We examined efficiency of transfer into and expression in cells in culture of the green fluorescence protein (GFP)-encoding gene inserted into the SeV/ΔF vector. The transgene expression was remarkably high in efficiency in a wide variety of cells including primary hepatocytes, primary lung epithelial cells, primary smooth muscle cells, CD34-positive blood stem cells, primary cerebral cortex neurons, primary dorsal root ganglion neurons, and embryonic stem (ES)

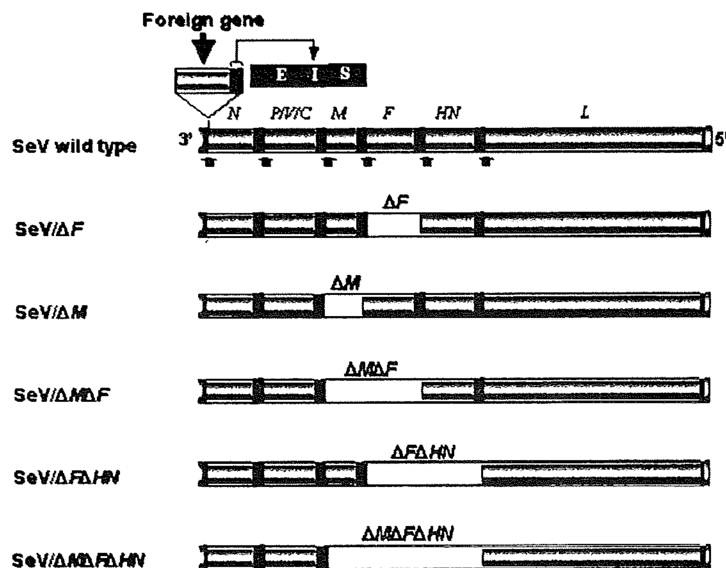

Figure 4. Wild-type and various gene-deleted (Δ) SeV vectors. For details see the text. Foreign genes can be inserted at various locations (indicated by arrows) depending on the intended purpose.

cells of human or animal origin (10). Moreover, *in vivo* gene transfer and expression were also remarkable as exemplified by strong GFP expression in the ependymal cells and pyramidal neurons, when the vector was stereotaxically introduced into the intraventricular and hippocampal regions, respectively, of rats (10) (Fig. 5), as well as into the respiratory tract epithelial cells, myocytes, vascular endothelial cells, nasal mucosa epithelial cells, and subretinal neurons of rats or mice. These results demonstrate that one of the essential SeV genes can be deleted without sacrificing the transgene expression efficiency and the wide host spectrum. Also to be noted is that the titers of ΔF vectors generated in cell cultures were as high as those of the wild-type vectors, reaching 10^8 infectious units (IU)/ml in the supernatant (10).

Our SeV technology was able to generate other single-gene deletion vectors, ΔM and ΔHN, double gene-deletion vectors, $\Delta M\Delta F$ and $\Delta F\Delta HN$, and a triple gene-deletion vector, $\Delta M\Delta F\Delta HN$ (26, 27, 28) (Fig. 4).

Viral vector-derived transduction *in vivo* often results in a short gene-expression period ranging from several days to, at most, a few weeks, except for the chromosomally integrated vectors with no encoded virus proteins. Such short periods of expression are the result of the induction of the immune response

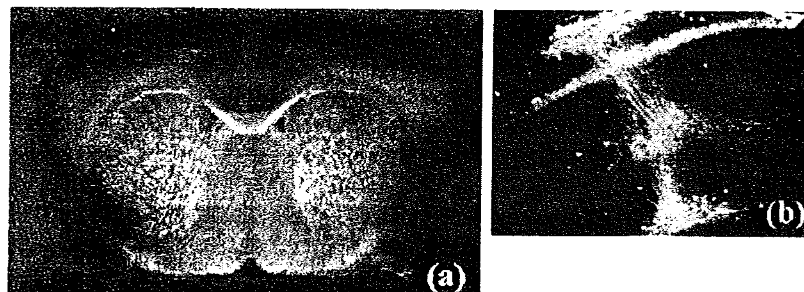

Figure 5. Efficient GFP gene expression using SeV/ΔF in rat ependymal cells (a) and pyramidal neurons (b). Adapted from Li et al. 2000 with permission.

against the administered virus vectors. Once established, the immune response invalidates the re-administration. SeV vector is also subject to this problem. Indeed, the vector was found to be rapidly cleared in safety tests in monkeys (see section 7), and its re-use was hampered for a period of several months. However, when re-administering it 8 months later, we observed remarkable levels of transgene expression in both monkeys and mice. The possibility of re-use as a vaccine vector was suggested by the rapid and robust expansion of cellular immune responses in monkeys to the transgene products as well as to SeV that was achieved by the second administration made about one year after the first administration (see section 6 and ref. 36).

Specific neutralizing antibodies to the virus vectors and cytotoxic T cells are primarily responsible for the vector elimination. The primary targets of neutralizing antibodies are principally the envelope proteins; research is currently under way on the extent of possible evasion from the immune responses by deletion of all three envelope genes: *F*, *HN*, and *M*. Because there is antigenic cross reactivity between SeV and human parainfluenza virus type 1 (hPIV1), certain human population who have been exposed to hPIV1 may be immune, albeit not completely, to SeV. The performance of SeV vectors should be evaluated in hPIV1 seropositive and seronegative targets.

6. Application of the SeV vector

Gene therapy

The SeV vector is attracting attention and interest from the academic and clinical sectors as well as from preventive medicine practitioners worldwide because of its unparalleled high performance in terms of gene transfer and expression level, its extremely broad target range, and the success of generating a series of gene-deleted versions. The quality-control methods for

large-scale production and purification of the vectors to ensure their safety and efficacy have been established so that industrial production based on good manufacturing practice will be possible.

The most advanced gene therapy targets angiogenesis for severely ischemic limbs (critical limb ischemia) arising from chronic arterial sclerosis or other similar diseases (29). It uses the SeV/ΔF vector carrying the fibroblast growth factor (FGF)-2 gene. Pre-clinical studies with mouse and rabbit ischemic limb models conducted at Kyushu University Graduate School of Medicine demonstrated notable therapeutic efficiency. The clinical trial planned at Kyushu University Hospital was approved by the Ministry of Health, Labor and Welfare of Japan and has just begun. This trial would be the first instance of a clinical application of Japanese-developed virus vectors.

Possible clinical applications of the same type of vectors to treatment of ischemic heart diseases are also under investigation. The treatment involves introduction of the vector into the infarct area to enhance angiogenesis, and its efficacy has been shown in a porcine model of chronic ischemic heart failure (unpublished data). The above-mentioned application to cystic fibrosis therapy is in progress as a joint investigation with the Cystic Fibrosis Gene Therapy Consortium, UK.

Cancer treatment

A group of researchers at Chiba University Graduate School of Medicine has been studying the feasibility of gene therapy for esophageal cancer, prostate cancer, and brain tumors (glioma). For instance, local intra-tumor administration of an SeV vector carrying the interleukin 2 gene, in combination with vaccination with irradiated autologous tumor cells, has yielded marked shrinkage or disappearance of tumors, presumably associated with systemic immune activation and induction of the local immune response (30). The SeV vector not only effectively introduced genes into dendritic cells but also appeared to induce powerful tumor antigen-presentation ability by potently activating the dendritic cells. A prominent curative effect in a multiple number of murine tumor models was observed with the application of dendritic cell therapies (31).

Because the SeV M protein plays a key role in the assembly of viral components at the plasma membrane and virus particle formation and release by budding from the plasma membrane, virtually no virus particle was produced in cells infected with SeV/ΔM (26). Instead, the viral glycoproteins HN and F accumulate on the cell membrane. These cells effectively fuse with the adjacent cells, leading to the formation of large syncytia of several tens or more cells in the entire culture. At the same time, the RNPs transferred from cell to cell are further multiplied to incur cell injury, leading to lysis of the syncytia. The ability of such massive syncytia formation and cell lysis characteristic

of SeV/Δ*M* appeared to be well suited for the treatment of solid cancers that form a mass of malignant cells.

The SeV F glycoproteins, which exhibit fusion activity and confer infectivity to the virus, are synthesized as inactive precursors and converted to an active form at the cell surface through proteolytic processing by trypsin-like enzymes produced by host cells and secreted into extracellular space. This type of virus-activating protease is not ubiquitous in the host organism but highly specific to certain tissues. SeV thus targets only limited types of organs or tissues, such as the respiratory tract of rodents (reviewed in ref. 32). The above-mentioned cell fusion induced by the SeV/Δ*M* vector was observed in the presence of trypsin added to the culture medium. On the other hand, malignant metastatic tumor cells are known to often overproduce and secrete tumor-specific proteases such as matrix metalloproteinase (MMP). Accordingly, we generated a new type of SeV/Δ*M* expressing GFP [SeV/F(MMP)Δ*M*], in which the original tryptic cleavage site of the F glycoprotein was replaced with a sequence susceptible to cleavage by MMP (33) (Fig. 6a). As expected, this protease-activation SeV/Δ*M* mutant can no longer spread by cell–cell fusion or kill the cells in the presence of trypsin but can do so by the addition of MMP2 or MMP9 (Fig. 6b) and in cancer cells robustly expressing MMP (33); when injected into a mass of human fibrosarcoma HT1080 transplanted into nude mice, this mutant manifested marked tumor growth-suppressing activity (Fig. 6c) (33).

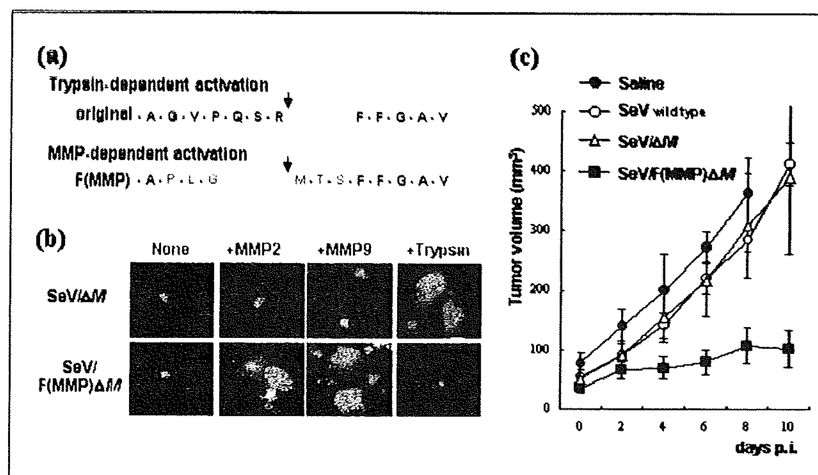

Figure 6. Generation of MMP-dependent SeV/Δ*M* expressing GFP and its anti-tumor effects. For details see the text. p.i., post infection. Adapted from Kinoh et al. 2004 with permission.

AIDS vaccine development

Preliminary studies

Excepting a few subunit vaccines, virtually all AIDS vaccine candidates now in the pipeline focus on eliciting cell-mediated immunity against HIV, especially cytotoxic T lymphocyte (CTL) responses, according to AIDS VACCINE BLUEPRINT 2006, issued by the International AIDS Vaccine Initiative, New York. Efforts also have to be made to develop candidates that elicit other potentially protective immune responses, in particular neutralizing antibodies. Simian immunodeficiency virus (SIV) infection of non-human primates (macaques) provides virtually the sole available model for research and development of AIDS vaccines.

Our efforts using SeV vectors expressing SIV proteins also focus on induction of virus-specific CTL responses. Initially, we used the V(-) backbone of full-length SeV because, compared with the standard (wild-type) SeV, the V(-) version could express several-fold higher amounts of foreign genes (22) and was also expected to be safer for monkeys and humans (see section 4). We found that cynomolgus monkeys immunized intranasally with V(-)SeV expressing just a single protein, Gag, of the SIVmac239 strain [V(-)SeV-Gag] tolerated well the challenge infection with the homologous virus (34).

V(-)SeV-Gag–infected cells were able to stimulate Gag-specific T cells to produce IFN-γ *in vitro*. Gag-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses were elicited after intranasal V(-)SeV-Gag vaccination in rhesus macaques. A single intranasal SeV-Gag inoculation into macaques could protect most of the monkeys from acute $CD4^+$ T-cell depletion after an intravenous challenge with the CXCR4-tropic strain SHIV89.6P and could control well the challenge virus replication in the macaques (35). These results indicated the prophylactic potential of this SeV-Gag vaccination by inducing effective CTL responses. The potential of the SeV vector for inducing virus-specific cellular immune responses as a therapeutic AIDS vaccine has also been proven in macaque AIDS models (36). In this study, we made two administrations of Gag or Tat expressing SeV into monkeys with an interval of about one year and observed rapid and remarkable expansion of SIV-specific and SeV-specific $CD4^+$ and $CD8^+$ T cells after the second administration.

DNA-prime/SeV-boost regimen

Various prime/boost regimens using DNA vaccines/viral vectors are thought to be a promising method for efficient CTL induction. The DNA prime/V(-)SeV-G boost regimen efficiently induced Gag-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in rhesus macaques, nicely controlled viral replication, and almost perfectly protected the macaques following an intravenous CXCR4-tropic SHIV89.6P challenge (35). Essentially the same results were obtained

when the Gag-expressing SeVΔF (with the intact *V* gene) was used instead of full-length V(-)SeV-Gag (37). Several other prime/boost regimens such as DNA-prime/modified vaccinia virus Ankara (MVA) vector-boost (38) and DNA-prime/adenovirus (AdV) vector-boost (39) also induced good CTL responses and succeeded in controlling the acute AIDS model of SHIV89.6P challenge infection. However, many of these included the Env immunogen, in addition to the internal Gag and Pol proteins, which may induce neutralizing antibodies. Env-independent consistent viral control has been found only in the DNA-prime/AdV-boost and DNA-prime/SeV-boost regimens (35, 39).

The strain SHIV89.6P, commonly used as a challenge virus in the above-described vaccine models, is a chimera SIV in which the envelope and some accessory genes are replaced with the HIV-1 counterparts, whereas the major internal proteins such as Gag and Pol are derived from SIV, and uses CXCR4 as the co-receptor. The reason for the use of this strain as a challenge virus was simply that experimental outcomes are obtained in a much shorter time frame because CD4-positive T cells in simian peripheral blood drastically decline about two weeks following injection. In the natural realm, however, HIV-1 and SIV use co-receptor CCR5 and develop AIDS following a long period (over years) of chronic infection. Moreover, SHIV89.6P tends to be readily controlled by the simian immune system for unknown reasons despite its highly acute and highly pathogenic nature. SHIV89.6P may be compared to "a sheep in wolf's clothing" (40). Thus, the use of SHIV89.6P as a challenge virus is not justified for efficacy tests in SIV models, and the use of CCR5-tropic, chronic SIV strains such as SIVmac239 has been strongly recommended, reflecting more closely the natural course of human AIDS (40).

Whereas several preclinical trials of prime/boost vaccines have uncovered a difficulty in controlling SIV replication in macaques (41, 42, 43, 44), our DNA-prime/SeV-boost vaccine regimen was successful, inducing Env-independent, CTL-based control of SIV replication with undetectable setpoint plasma viremia (45). However, the success has been limited to several populations of target monkeys because of efficacy restriction to particular class I MHC haplotypes.

7. Safety issues

Before its use in humans, we conducted a series of studies to assess the toxicity of SeVΔF carrying the FGF-2 gene (see section 6) in nonhuman primates. The first was an acute toxicity study with a single administration at a high dose; control animals received phosphate buffered saline. The vector sample at a dose volume of 1 ml/kg was intramuscularly injected into four sites of the thigh and two sites of the calf muscle of cynomolgus monkeys at doses of 5×10^8 (n=3) and 5×10^9 (n=3) IU/kg. Each monkey in this and subsequent

studies weighed approximately 3 kg. No apparent clinical manifestation was seen. A slight inflammatory reaction at the site of injection was observed histopathologically. An increase in the level of local FGF-2 was remarkable at the injection site, but there was no systemic increase in FGF-2 levels. No vector virus genome was detected in blood and urine by RT-PCR.

The second study was bio-distribution (vector distribution in the body) after a single administration. A dose of 5×10^8 IU/0.3ml/kg was injected into one site of the femoral muscle of cynomolgus monkeys. Two monkeys each were sacrificed on days 2, 4, 7, and 14, respectively, for examining vector distribution in the body. On day 4, the vector virus genome was detected in the cardiac muscle and the inguinal lymph node of the injection side but not in the other organs and tissues. The virus genome was not found on the other days in any organs and tissues. Again, no adverse effect was found throughout the observation period up to 14 days post injection.

The third was a sub-acute toxicity and bio-distribution study using repeated intramuscular administration. The vector sample was injected every day up to 14 days into one site of the femoral muscle of rhesus monkeys at doses of 7×10^7 (n=6) and 7×10^8 IU /kg (n=6) in a volume of 0.3 ml/kg. The monkeys were euthanized and autopsy was done on the day following the last administration (n=4) and 28 days later (n=2). The vector virus was detected at the injection site of one of the animals on the day following the last injection in the high dose group but not in the others. No clinically abnormal sign was observed in any animal throughout the observation period including the first 14 days of vector administration and the subsequent recovery period of 28 days.

Safety and bio-distribution of V(-)SeV-Gag were also examined in macaque AIDS models (46). Following intranasal vaccination with 10^8 IU of V(-)SeV-Gag into six cynomolgus macaques, two of the animals were euthanized at days 4, 7, and 13, respectively, and processed for autopsy. Robust Gag expression was observed in the nasal mucosa, with much lower but significant expression in the local retropharyngeal and submandibular lymph nodes (LN). Expression peaked within a week and lasted at least until week 2 after the vaccination. The vector virus was recovered from nasal swabs at day 4 but not at all at the end of week 2. Gag expression was undetectable in the lung or in the thymus, spleen, or inguinal LN, indicating restricted virus spread. None of the SeV-immunized macaques displayed appreciable clinical manifestations. In addition, examination of naive macaques living in a cage together with acutely V(-) SeV-Gag–infected macaques suggested that transmission of this vector from the targeted macaques to naive ones was very inefficient (46).

Finally, the data available indicated that the wt SeV intranasally administered to adult humans as a live vaccine against hPIV1 gave rise to no severe adverse outcome (47). This trial was based on a rationale that

considered the antigenic cross reactivity between hPIV1 and SeV and because SeV has been believed to be only poorly, if at all, pathogenic for humans.

Taken together, the findings suggest that it is more than likely that SeV is not pathogenic for humans or for nonhuman primates.

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