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Sendai Virus Vector-Mediated Transgene Expression in the Cochlea in vivo

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Key Words

Sendai virus vector · Gene transfer · Scala tympani · Scala media · Cochlea · Guinea pig

Abstract

We injected a recombinant Sendai virus (SeV) vector into the guinea pig cochlea using two different approaches – the scala media and scala tympani – and investigated which cell types took up the vector. The hearing threshold shift and distribution of transfected cells in animals using the scala media approach were different compared to those using the scala tympani approach. SeV can transfect very different types of cells, including stria vascularis, spiral ganglion neurons, and sensory epithelia of the organ of Corti, and fibrocytes of the scala tympani. Because SeV vectors can potentially deliver stimuli to the cochlea to induce hair cell regeneration, it may be a powerful tool for repairing the organ of Corti.

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Introduction

Gene transfer into inner ear organs is an attractive new approach for treating hearing disorders. This technology can also be useful for treating sensorineural hearing loss, such as inherited deafness [Kanzaki et al., 2002a]. Various viral vectors are capable of carrying out gene transfer. Adenoviruses (AVs) are the most commonly used vectors in experimental studies involving hearing disorders [Kanzaki et al., 2002b, c]. In guinea pigs, adeno-associated virus vectors [Di Pasquale et al., 2005; Lalwani et al., 1996] and herpes simplex virus vectors [Geschwind et al., 1996] have been successfully used for gene transfer into the inner ear. In addition, a new generation of AV vectors has been shown to successfully transfect a few sensory epithelial cells in the guinea pig cochlea [Luebke et al., 2001].

Vectors can be injected into the cochlea through two approaches – the scala media and scala tympani. Of the two, the scala tympani approach minimizes cochlear damage, and thus has been preferred for many gene transfer studies [Kanzaki et al., 2002c; Kawamoto et al., 2004; Yagi et al., 1999]. The scala tympani approach is particularly useful when targeting spiral ganglion neurons, because vectors are taken up selectively by scala tympani fibrocytes, which in turn act as carriers for the delivery to the spiral ganglion neurons. AV-glial cell line-derived neurotrophic factor injections into the scala tympani spare hair cells [Yagi et al., 1999] and spiral ganglion cells [Kanzaki et al., 2002c; Yagi et al., 2000], while effectively delivering glial cell line-derived neurotrophic factor to spiral ganglion neurons. On the other hand, the scala media approach is useful when targeting hair cells for regeneration [Kawamoto et al., 2003]. AV vector injections into the scala media result in reporter transgene expression in supporting cells [Ishimoto et al., 2002], indicating that this vector successfully reaches the sensory epithelial cells.

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Injection via the scala media with AV containing *Atoh1*, a mouse homolog of the *Drosophila* gene *atonal*, caused hair cell regeneration by inducing transdifferentiation of supporting cells [Izumikawa et al., 2005]. The drawback with scala media injections is that such injections may produce an excessive volume of the vector in endolymph or may result in mechanical contact.

Although AVs are effective in transferring genes in clinical situations [Verma and Weitzman, 2005], one problem is that the host response to gene therapy vector exposure involves both the innate and adaptive immune systems. The initial innate immune response also plays a significant role in acute toxicity owing to AV vector exposure [Nazir and Metcalf, 2005]. The cytopathic and immunogenic nature of AV, therefore, precludes its use as transgene vectors for treating hearing disorders in humans. Thus, to make this new technology feasible in humans, it is necessary to develop novel viral vectors that are efficiently transported within the middle and inner ear, but do not produce significant cytopathic and immunogenic responses.

One promising viral vector is Sendai virus (SeV), a member of the Paramyxoviridae family. SeV is an enveloped virus that has nonsegmented, negative-sense genomic RNA [Sakai et al., 1999]. Its replication and gene expression is driven by viral RNA polymerase strictly through a cytoplasmic mechanism [Nakanishi et al., 1998]. SeV vectors have been shown to deliver transgenes to respiratory [Inoue et al., 2004; Yonemitsu et al., 2000], vascular [Masaki et al., 2001], and muscle systems [Shiotani et al., 2001] and neurons [Shirakura et al., 2004]. Now SeV is tested in clinical trials for arteriosclerosis of the lower limbs in Japan.

There are three advantages to using SeV vectors for human gene therapy. First, SeV vectors are completely free of genotoxicity [Bitzer et al., 2003; Griesenbach et al., 2005]. SeV vectors replicate and transcribe transgenes only in the cytoplasm, importantly avoiding interaction with host chromosomes. Second, SeV vectors have remarkably high transfection efficiency in many tissues and cell types. Gene transfer to the respiratory airway via SeV vectors, for example, is at least 10-fold greater than that produced via AV vectors, and is 4- to 5-log-fold greater than that produced via cationic liposomes [Yonemitsu et al., 1996]. Finally, there is no evidence that SeV is pathogenic in humans. The SeV vector was designed using a rodent respiratory virus and has long been used for preparing hybridomas. In the retinal sensory system, an SeV vector produced high expression levels in retinal pigment epithelium after a brief vector-cell contact time, while AV did not [Ikeda et al., 2002]. Taken together, these data suggest that SeV might be a useful vector for delivering transgenes to other sensory systems, including the auditory system.

These properties make SeV vectors a prime candidate for the use in gene transfer therapy for treating hearing disorders. The aim of this study, therefore, was to evaluate the transfection efficiency of an SeV vector in the cochlea using two delivery pathways, the scala media and scala tympani.

Materials and Methods

Animals

We used 17 albino guinea pigs (250–350 g). All animal experiments were performed in accordance with the guidelines of the Keio University Committee for the Use and Care of Animals. The Keio University is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Experimental Groups

We assigned animals to one of two groups: group 1 animals received either SeV (n = 6) or sterile artificial endolymph (NaCl 1 mM, KCl 126 mM, $KHCO_3$ 25 mM, $MgCl_2$ 0.025 mM, $CaCl_2$ 0.025 mM and K_2HPO_4 1.4 mM; n = 3) [Ishimoto et al., 2002] injections into the cochlea via the scala media, and group 2 animals received either SeV (n = 5) or perilymph (sterile normal Ringer's solution; n = 3) [Kanzaki et al., 2002b] injections into the cochlea via the scala tympani. SeV transfection efficacy was assessed histochemically. Auditory brain stem responses (ABRs) were measured before SeV injection and 3 days after injection, prior to sacrifice, to assess shifts in hearing threshold.

SeV Vectors

The full-length SeV genome contains the following: 3′-end leader followed by six viral genes – nucleocapsid, phospho, matrix, fusion (F), hemagglutinin-neuraminidase, and large proteins – and a small 5′-end trailer sequence (fig. 1). Since the F protein conveys viral infectivity, we used F gene-deleted SeV vectors (SeV/ΔF) in our experiments. SeV/ΔF do not produce infectious progeny [Li et al., 2000]. We constructed SeV/ΔF carrying green fluorescence protein (GFP-SeV/ΔF) as previously described [Li et al., 2000]. Briefly, GFP cDNA was amplified with a pair of *Not*I-tagged primers that contained the following SeV-specific transcriptional regulatory signal sequences: 5′-ATTGGCCGCCGTACGGCCATGGTGAGCAAGGGCGAGGAG-3′ and 5′-ATTGGCCGCCGTACGATGAACTTTCACCCTAAGTTTTTCTTACTTCGGAGCTTTACTTGTACAGCTCGTCCATGCCG-3′. A GFP-SeV/ΔF cDNA (pGFP-SeV/ΔF) was constructed by introducing the amplified fragment into the *Not*I site of the parental pSeV18+/ΔF. pGFP-SeV/ΔF was transfected into LLC-MK2 cells infected with vaccinia virus vTF7–3, which expresses T7 polymerase [Fuerst et al., 1986]. The T7-driven recombinant GFP-SeV/ΔF RNA genome was encapsulated by nucleocapsid, phospho, and large proteins, which were derived from their re-

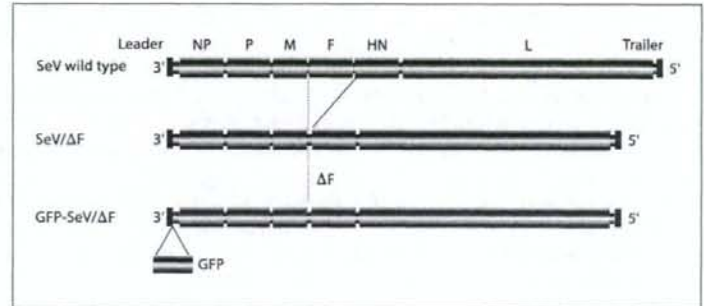

Fig. 1. The structures of the wild type of SeV, SeV/ΔF, and GFP-SeV/ΔF are shown. GFP-SeV/ΔF was used in this experiment. NP = Nucleocapsid; P = phospho; M = matrix; F = fusion; HN = hemagglutinin-neuraminidase; L = large proteins.

spective cotransfected plasmids. The recovered SeV vector was propagated using F protein-expressing packaging cell lines [Li et al., 2000]. Virus titers were determined, and infectivity was expressed as cell infectious units (CIU). The SeV vector was stored at –80°C until use.

Surgery

Guinea pigs were anesthetized with xylazine (10 mg/kg; i.m.) and ketamine HCl (40 mg/kg; i.m.). Prior to vector or control injections, 0.5 ml of 1% lidocaine HCl was injected subcutaneously around the ear as local anesthesia. GFP-SeV/ΔF (titer: 5×10^7 CIU/5 μl) was injected into the cochlea as described below.

In group 1 animals, the ventral side of the left bulla was opened, and a hole was drilled in the lateral wall of the third turn (fig. 2) as previously described [Ishimoto et al., 2002]. In group 2 animals, the left side of the middle ear was exposed via a postauricular approach. Under the guidance of an operating microscope, a small fenestra was made with a sharp probe in the otic capsule at the base of the cochlea [Prieskorn and Miller, 2000; Stover et al., 1999]. To inject SeV, we used a 100-μl Hamilton syringe with an attached vinyl cannula and fine polyamide tip. In group 1 animals, 5 μl of either GFP-SeV/ΔF or endolymph were injected into the scala media, and in group 2 animals, 5 μl of either GFP-SeV/ΔF or perilymph were injected into the scala tympani. Ten minutes after injection, the cannula was removed and the fenestra was covered with a small piece of fascia that adhered to the otic capsule. The bulla defect was sealed with carboxylate cement (Durelon® ESPE America, Norristown, Pa., USA). Dexon® adsorbable suture was used to close the subdermal opening, and nylon suture was used to close the skin opening.

ABR Measurement

A needle electrode was subdermally inserted at the vertex, along the dorsal midline of the scalp between the external auditory canals. The reference electrode was placed below the pinna of the left ear, and the ground electrode was inserted below the contralateral ear. The auditory stimulus consisted of a 1-ms tone burst with a rise-fall time of 0.1 ms. Waveforms from 256 stimuli were delivered at a frequency of 9 Hz. ABR waveforms were recorded for 12.8 ms, sampled at 40000 Hz, bandpass-filtered (50–5000 Hz), and averaged using PowerLab system software (PowerLab2/20, AD Instruments, Castle Hill, Australia). ABR waveforms were recorded in 5-dB SPL intervals from a maximum amplitude until no waveform could be observed. ABRs were recorded at 4, 12, and 16 kHz. The ABRs were measured with the animals under xylazine and ketamine anesthesia (i.m.). The researcher who measured ABRs was blinded to the animal's group assignment.

Statistical Analysis

Unpaired t tests were performed to test for significant differences in ABR thresholds at each frequency between the two groups. SPSS version 13.0 (Chicago, Ill., USA) was used.

Epifluorescent Stereoscopy

Animals were sacrificed under deep anesthesia 3 days after being injected with either vector or control solutions. To perfuse the inner and middle ears locally, we decapitated the animals and removed the temporal bone. The entire cochlea or lateral wall was visualized using a stereoscopic zoom microscope (SMZ1500) configured with epifluorescent optics (Nikon, Tokyo, Japan).

Immunohistochemistry

The cochlea was fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and immersed in sucrose overnight for cryoprotection. After embedding in OCT compound [Whitlon et al., 2001], 10-μm-thick frozen sections were cut and collected. After permeabilization with 0.3% Triton X-100, the organ of Corti was stained for F-actin using a 1:100 solution of rhodamine phalloidin (Molecular Probes, Carlsbad, Calif., USA) for 30 min. The diluent for all solutions was PBS. Whole-mount preparations were observed using an Eclipse 80*i*® digital microscope configured with epifluorescent optics (Nikon).

Results

Transfection of the Cochlea after Injection into the Scala Media

GFP-positive cells were present in the cochlea and middle ear mucosa, including the otic capsule, of the ears receiving vector injections into the scala media

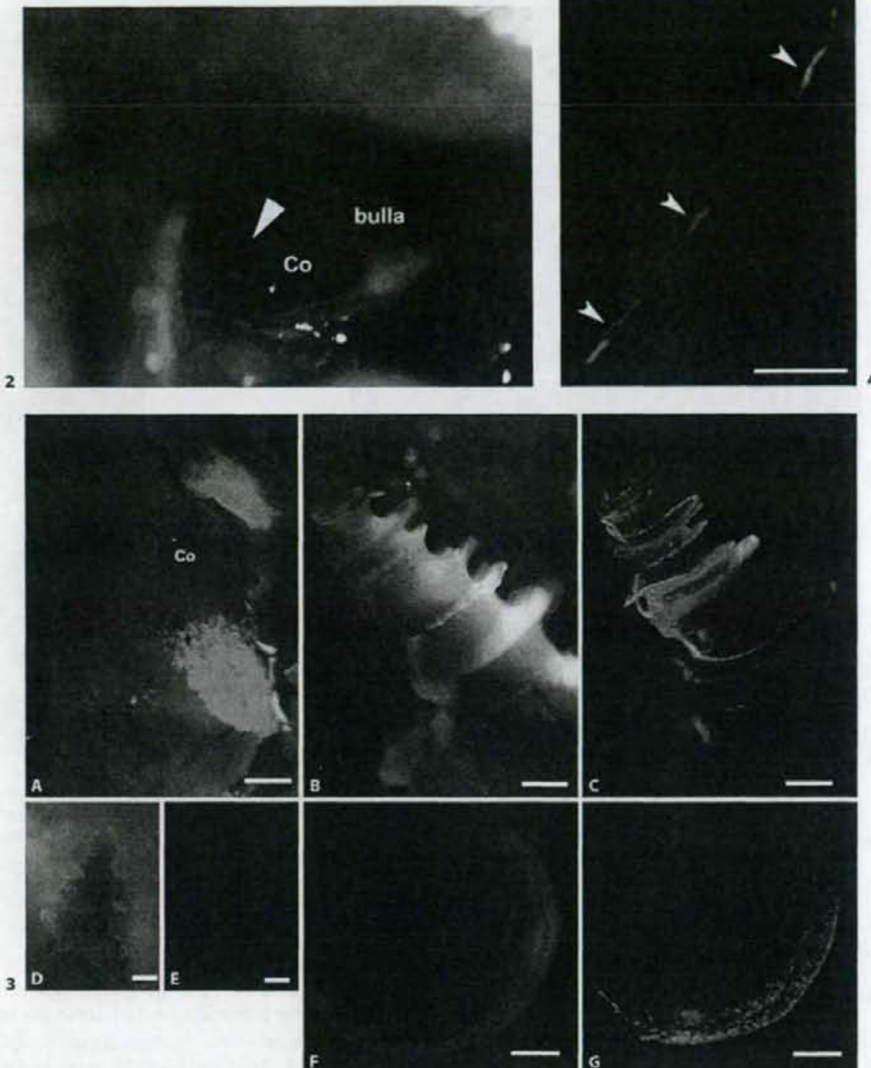

Fig. 2. Photomicrograph showing the surgical approach through the scala media. A ventrally located incision was made on the left ear, and then the window of the middle ear bulla was opened. A hole was made in the wall of the cochlear bone (arrowhead). Co = Cochlea; bulla = middle ear bulla.

Fig. 3. Photomicrographs of whole-mounted guinea pig cochlea showing the distribution of GFP-fusion protein after injection of GFP-SeV/ΔF into the scala media. **A** Fluorescent image showing the boney wall of the cochlea. **B**, **C** Light (**B**) and fluorescent (**C**) images of the cochlea of a SeV-inoculated ear. **D**, **E** Light (**D**) and fluorescent (**E**) images of the cochlea in an uninoculated, control ear. **F**, **G** Light (**F**) and fluorescent (**G**) images of the lateral wall of the inoculated ear. Scale bars: 1000 μm (**A–E**); 600 μm (**F**, **G**).

Fig. 4. Photomicrograph of histological section of guinea pig middle ear after injection of GFP-SeV/ΔF. GFP signal was observed in the middle ear mucosa (arrowheads). Scale bars: 10 μm.

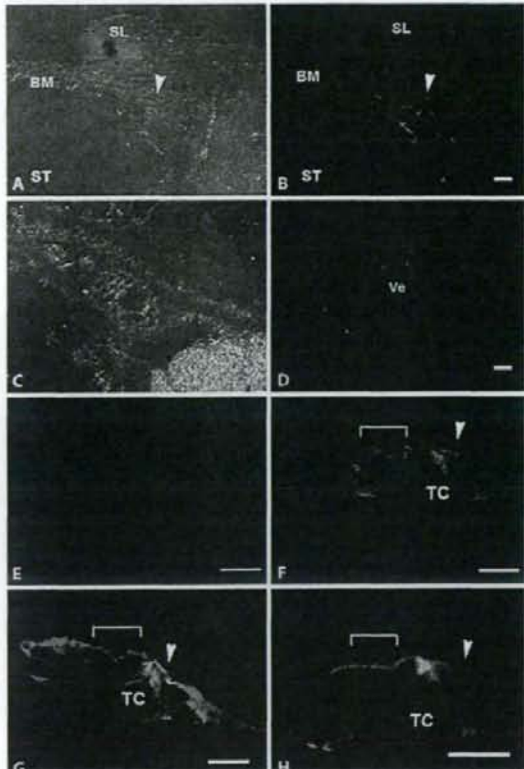

Fig. 5. Photomicrographs of histological sections of guinea pig scala media after injection of GFP-SeV/ΔF. **A**, **B** Spiral ganglion neuron (arrowhead); DIC image (**A**) and fluorescent image (**B**). **C**, **D** Cochlear blood vessel; DIC image (**C**) and fluorescent image (**D**). **E** Stria vascularis. **F–H** Organ of Corti with the arrowhead pointing to the inner hair cell and the bracket suggesting the outer hair cell region; the organ of Corti from an inoculated ear (**F**, **G**) and an uninoculated control ear (**H**). Red = F-actin stained with rhodamine phalloidin; green = GFP-SeV/ΔF-transfected cells; BM = basement membrane; SL = spiral limb; ST = scala tympani; TC = tunnel of Corti; Ve = cochlear blood vessel. Scale bars: 100 μm (**A–D**), 50 μm (**E**), 10 μm (**F–H**).

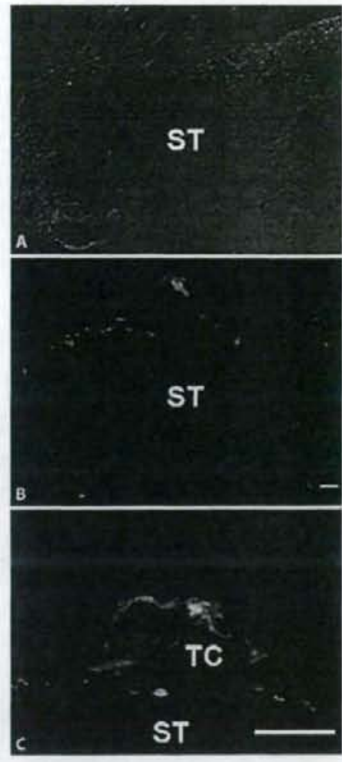

Fig. 6. The cochlea from an ear inoculated with GFP-SeV/ΔF via a scala tympani approach. **A–C** The organ of Corti and fibrocytes of the scala tympani; DIC image (**A**) and fluorescent image (**B**), and higher magnification (**C**); numerous labeled fibrocytes in the scala tympani. ST = Scala tympani; TC = tunnel of Corti. Scale bars: 100 μm (**A**, **B**), 10 μm (**C**).

(fig. 3A–C, fig. 4). This suggests that the injection leaked out of the cochlea and onto the middle ear mucosa. There were no GFP-positive cells in the contralateral ear (fig. 3D, E). GFP was also found in the lateral wall (fig. 3F, G). In one of the inoculated cochlea, GFP localized to several types of cells in several spiral ganglion neurons, in the outer layers of cochlear blood vessels and in the stria vascularis (fig. 5A–E). Transfected cells were found in the outer hair cell layer but not in the inner hair cell layer. In several animals, supporting cells (pillar, Hensen, Deiters, and Claudius cells) and inner and outer sulcus cells were also transfected (fig. 5F, G). We observed no GFP-positive cells in the uninoculated cochlea (fig. 5H).

Transfection of the Cochlea after Injection into the Scala Tympani

GFP-positive signals were found in many fibrocytes in the scala tympani of the inoculated ears (fig. 6A–C) but not in the contralateral or uninoculated ears (control)

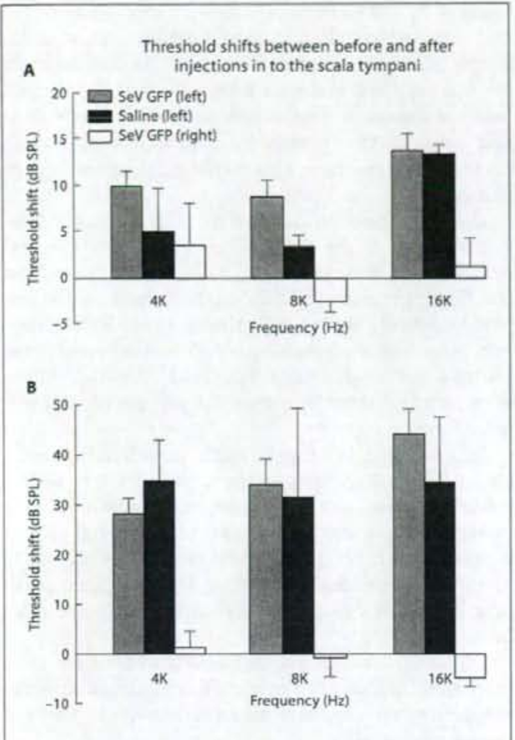

Fig. 7. ABR threshold shifts observed before and after injections via scala media (**A**) and scala tympani (**B**) approaches.

(data not shown). No GFP-positive cells were found in the scala media and vestibuli.

ABR Threshold Shift before and after Vector Injections

Hair cells disappeared in the animals that received SeV injections into the scala media. No obvious damage was seen, however, in the cochlea or middle ear of animals that received injections into the scala tympani. This is consistent with our ABR results, showing that pre- and postinjection threshold shifts were lower in animals operated through the scala tympani approach than in animals operated through the scala media approach. There were no statistically significant differences in hearing threshold shift among the animals that received SeV vector, endolymph, or perilymph injections, indicating that the SeV vector did not affect hearing function.

Table 1. Distribution of transgene expressions after SeV injection via scala media and tympani

Via scala media approach (n = 6)	Animals
Distribution of expression	
All (four) turns	3
Two turns	2
One turn	1
Site of expression	
Scala media	
Stria vascularis	2
Spiral ganglion neurons	1
Vessel	1
Reissner's membrane	1
Organ of Corti	
Inner hair cell	1
Outer hair cell	2
Pillar cells	2
Deiters cells	2
Hensen cells	6
Claudius cells	6
Inner sulcus cells	2
Scala tympani	
Fibrocytes	1
Via scala tympani approach (n = 5)	**Animals**
Distributions of expression	
All (four) turns	1
Two turns	2
One turn	2
Site of expression	
Scala tympani	
Fibrocytes	5

In group 1 (scala media approach), GFP-positive expressions were found at all turns (n = 3 ears), two turns (n = 2), and one turn (n = 1). GFP-positive transfection was found at a wide variety of cells at the scala media and tympani including the organ of Corti. In group 2 (scala tympani approach), GFP-positive expressions were found at all turns (n = 1), two turns (n = 2), and one turn (n = 1). GFP-positive transfection was found at numerous fibrocytes of the scala tympani but not scala media.

Discussion

Distribution of SeV-Transfected Cells

Our findings indicate that SeV vectors (5.0×10^7 CIU/5 μl) can efficiently transfer transgenes to a wide variety of cell types in the organ of Corti, including stria vascularis cells, spiral ganglion neurons (through scala media injections) and fibrocytes (through scala tympani injections) (table 1). The extent of SeV transfection is comparable to that obtained with AV vectors. When AV

vectors are injected into guinea pig cochlea at 1.0×10^{11} virus particles/ml (approximately 5.0×10^8 virus particles/5 μl) via a scala media approach, AV vectors reach supporting cells such as interdental cells, inner sulcus cells, and Hensen cells [Ishimoto et al., 2002].

SeV may reach the sensory epithelial cells, spiral ganglion neurons, and stria vascularis by traversing the endolymphatic space and the lateral wall of the scala media. The route by which SeV vectors transfect spiral ganglion neurons is unknown. The vectors may penetrate the habenulae perforatae, in which the spiral ganglion nerve runs through a hole in the tympanic lip of the osseous spiral lamina. When injected via the scala tympani approach, SeV cannot penetrate the basement membrane and thus does not reach the organ of Corti as well as does AV.

SeV Does Not Affect Hearing Function

We find no significant differences in the hearing threshold of animals that received SeV, endolymph, or perilymph injections, indicating that SeV does not affect hearing function and that SeV may have lower levels of (cochlear) toxicity than do other viruses [Bitzer et al., 2003; Griesenbach et al., 2005]. We did find, however, that hearing function was differentially affected by the two routes of injection through the scala media and scala tympani. ABRs of animals operated via the scala tympani approach were lower than those of animals operated via the scala media approach. This disparity may be due to mechanical damage to the scala media. When AV is injected into the scala tympani, damage to auditory hair cells is limited [Han et al., 2004], whereas when it is injected into the scala media, numerous outer hair cell are damaged [Ishimoto et al., 2002]. This is consistent with our findings that injections into the scala media are more traumatic than injections into the scala tympani because injection via the scala media made pressure of endolymph followed by the rupture of Reissner's membrane which may mix cochlear fluid including perilymph and endolymph. Mixing fluids change Na^+ and K^+ ion in the inner ear and may not maintain homeostasis. The injection hole was also made in spiral ligament and stria vascularis and the direct flow pressed the organ of Corti through the tectorial membrane. This may be sufficient to damage hair cells.

Our data demonstrated that no GFP-positive expression was seen in the contralateral ear. Previous reports demonstrated that injection of 5 μl of AV vector did not transfect into the contralateral ear both via the scala tympani and media approach [Ishimoto et al., 2002]. However, Stover reported that 25 μl of AV was delivered to the contralateral ear [Ishimoto et al., 2002]. The distribution of transfection may depend on the amount of viral volume.

In conclusion, SeV may be useful for delivering a variety of therapeutic genes to the organ of Corti. Recent studies suggested that supporting cells can be induced to undergo mitosis and differentiate into hair cell phenotypes [Raphael, 1992]. Thus, SeV vectors can be used to promote hair cell regeneration by delivering gene products that induce differentiation of supporting cells to hair cells.

Our data demonstrate that SeV may be a powerful therapeutic tool for sensorineural hearing loss diseases to regenerate hair cells and may prevent spiral ganglion neuron degeneration.

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センダイウイルスベクターを利用したワクチン技術の開発

特集　臨床応用が迫る遺伝子治療の動向と国産技術の開発

井　上　　誠*

Development of vaccine technology using Sendai virus vector

The cytoplasmic RNA vector would be promising for use in gene vaccines to large population of patients because of its important genotoxicity-free nature. The candidate intranasal gene vaccines have been developed using Sendai virus(SeV) vector for both infectious and chronic diseases.

センダイウイルス(Sendai virus)ベクターは，"細胞質型 RNA ベクター"という新規概念の遺伝子デリバリーシステム(ベクター)である．このベクターが有する遺伝毒性がないという性質は，不特定多数のヒトあるいは動物(家畜)を想定したワクチン療法の場合，今後，必須条件となる．センダイウイルスベクターを利用して，新興・再興感染症を含む一群の感染症および非感染症疾患の治療に対しての有効性・安全性試験を実施しており，エイズワクチンなどはすでに開発段階にある．当該技術利用による，真に有効で安全な遺伝子ワクチン製剤の提供が望まれている．

*Makoto Inoue**

key words : Sendai virus, gene vaccine, nasal immunization, AIDS vaccine, genotoxicity-free

20世紀の後半に各種ワクチンの開発と普及が成功し，感染症，特に伝染病の制圧に成功したかに捉えられていたが，現在も依然として感染症による犠牲者が多く存在している．また，近年では新興・再興感染症とよばれる感染症が増加し，HIV，SARS あるいは鳥インフルエンザなどが人類の脅威となっている．動物においても同じであり，動物を介したヒトへの感染症の広がりだけでなく，動物種内特異的な感染症も多く知られている．

これらの一連の感染症に対しては，理論的にはワクチン療法が最も有効であり，個々のウイルスに対して開発が行われているが，充分に有効なものは開発されていないのが現状である．また，非感染症の神経系疾患をはじめとする各種慢性疾患に対しても，免疫系賦活化によるワクチン療法の開発は多く試みられており，医療的および社会的ニーズがきわめて高い分野である．

センダイウイルス(Sendai virus)ベクターは，"細胞質型 RNA ベクター"という新規概念の遺伝子デリバリーシステム(ベクター)であり，染色体と相互作用しない，すなわち遺伝毒性のないベクターである(図1)．ヒトあるいは動物(家畜)への先端医療としての遺伝子ワクチンは多く検討・報告されているが，DNA 型ワクチンが大半である．遺伝毒性がないという性質は，不特定多数のヒトあるいは動物(家畜)を想定したワクチン療法の場合，今後，必須条件となると考えられ，当該センダイウイルスベクターを利用して遺伝子治療および遺伝子ワクチンシステムを構築することはきわめて重要である．

また，センダイウイルスベクターは，もともとマウスの呼吸器病ウイルスをベースとしているため，鼻粘膜を含む呼吸器系上皮細胞への遺伝子導入効率がきわめて高いことがわかっている(図2)．より投与が簡便で，また，全身免疫と粘膜免疫の両者を誘導できる"点鼻ワクチン"としての利用が可能である．センダイウイルスベクターをプラットフォーム技術として，細胞質遺伝子治療(cytoplasmic gene therapy)および細胞質遺伝子ワクチン(cytoplasmic gene vaccine)の開発を推進し，これらの新興・再興感染症を含む一群の感染症および神経変性疾患などの非感染症疾患の治療に対して，真に有効で安全な遺伝子ワクチン製剤を提供することを目的として

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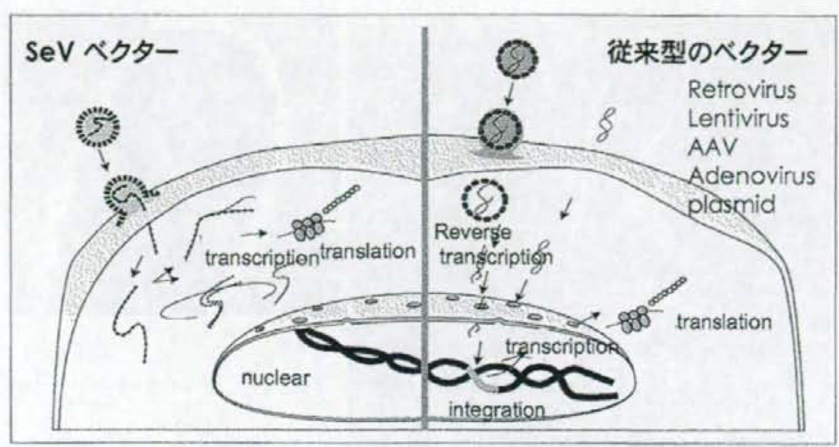

図1　細胞質型 RNA ベクターと遺伝子組込み型ベクターの遺伝子発現様式
センダイウイルスベクターのような細胞質型 RNA ベクターは，細胞質内で複製と転写翻訳を行い，核内へは移行しない．また，DNA への変換もない．一方，遺伝子組込み型ベクターは，DNA として核内に移行し，染色体にインテグレートされ，遺伝子は転写・翻訳される．

検討を行っている．

センダイウイルスベクターの開発

世界ではじめて実用化に成功したセンダイウイルスベクターは，遺伝毒性のない"細胞質型 RNA ベクター"として，細胞質遺伝子治療，RNA ワクチンなどの新規治療製剤を提供できると期待されている．センダイウイルスを遺伝子導入ベクターとして開発するに当たって(1995 年から開発開始)，筆者らはセンダイウイルスゲノムから遺伝子を欠失していく手法をとってきた(図 3)．遺伝子を欠失することで安全性を高めるとともに，治療対象疾患に則したベクターへ改良することに成功している．宿主細胞への侵入に関わる膜融合蛋白質(F 蛋白)遺伝子をゲノムから欠失させることにより，二次感染性のない，非伝播型ベクターへ改良することに成功した[1]．

このタイプのベクターを用いて，九州大学付属病院において臨床研究が計画され，厚生労働省・厚生科学審議会科学技術部会末梢性血管疾患遺伝子治療臨床研究作業委員会における議論を経て，2005 年 12 月 12 日，厚生労働省の厚生科学審議会・科学技術部会において，当該遺伝子治療の臨床試験計画が了承された．臨床研究においては，FGF-2(fibroblast growth factor 2：塩基性線維芽細胞増殖因子)遺伝子を搭載したベクターを利用して，慢性動脈硬化症などを原因とする重症の虚血下肢(重症虚血肢)に対する血管新生を目的とした遺伝子治療が実施されており，すでに数名の患者への投与が実施されている．ベクターの選択とともに，搭載遺伝子の選択にも工夫が凝らされた計画である．この臨床研究は国内開発ベクターによるはじめての細胞質遺伝子治療となるが，臨床研究実施に向けて GMP 対応ベクター生産も実施している．つまり，この新しいベクターの臨床レベルの基本技術は出来上がっている状況である．

また，ウイルス粒子のアセンブリーと出芽の中心的役割を担っているマトリクス蛋白(M 蛋白)遺伝子を欠失することで，感染細胞からの粒子放出を原理的に抑え，さらに，F 蛋白を浸潤転移性がん特異的に発現が亢進しているマトリクスプロテアーゼ(MMP-2,9 など)依存的に活性化するように改変することで，浸潤転移性がん特異的な細胞融合死を生じる新しいタイプの腫瘍崩壊性ベクターの構築にも成功した[2]．さらに，複数の遺伝子の欠失にも成功

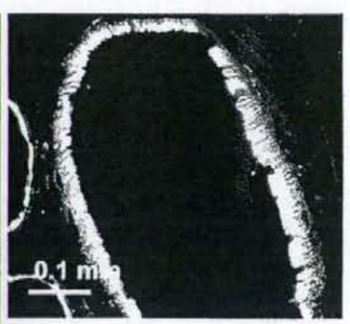

図2
センダイウイルスベクターによる
効率的な遺伝子デリバリー
GFP 遺伝子を組み込んだセンダイウイルスベクター(GFP-SeV/ΔF：1×10^7 CIU)のマウス鼻腔内投与による鼻腔上皮細胞への効率的な遺伝子導入

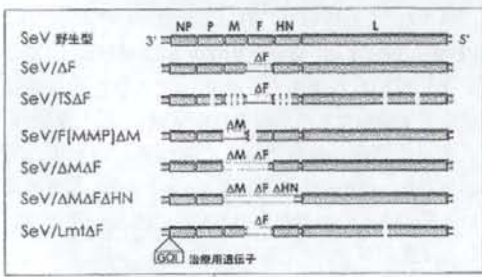

図3 各種遺伝子欠失型センダイウイルスベクター
目的によって各種遺伝子欠失型ベクターを使いわける．また，目的遺伝子(GOI)の期待する発現量により，遺伝子搭載位置を変更してベクターを構築する．

しており[3,4]，安全性の向上，免疫原性などの減弱を確認している．その他，変異導入による細胞傷害を伴わない発現系の確立，ミニゲノム系の利用などベクターの改良は確実に進歩しており，多くの使用用途に応じたベクターの提供が可能になりつつある．

このように，GMP 対応製造法をすでに確立し，臨床応用が実施されているとともに，ベクター改良による多種多様なベクターデザイン開発も順調に実施しており，ベクターのラインナップがなされている．さらに，当該ベクターの基本特許を含め，一連のベクター改良・応用法についても随時特許出願を実施しており，事業としてのベクター開発による具体的な社会貢献が可能な状況である．

エイズワクチンへの応用

2006 年 12 月の WHO の発表によると，世界の HIV の感染者は 3,950 万人であり，2006 年の 1 年間で 430 万人の新規感染者が増加し，アジアでも 860 万人の感染者がいるとされている．実際の数字はこれをはるかに超えるとの推定もある．日本でも患者・感染者を合わせた数は 1 万人を超えており，先進国で唯一患者数が増加している．

HIV の複製を抑制し，エイズの発症を遅延する有効な治療法として，多剤併用治療法などが開発されているが，高価であるうえにウイルスを体内から完全に排除するのは困難である．アフリカ・アジアでの患者数の増加，および真に有効な薬剤の不足から，安価で有効なワクチンを開発することが，治療的にも予防的にも急務となっている．現在，世界的規模での開発活動が行われている．

エイズウイルスは CD4 を受容体として認識する

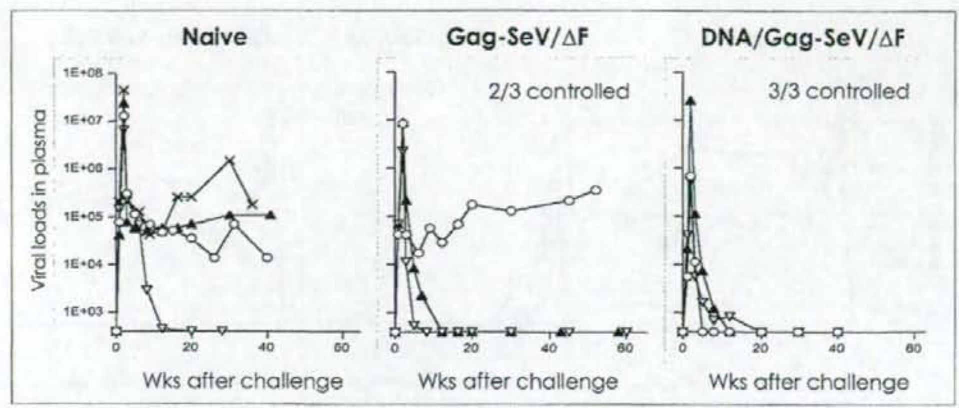

図4 急性発症モデル(SHIV89.6P)におけるワクチン効果

SIV(SHIV89.6P)チャレンジ後の血漿中のウイルス量を示す．センダイウイルスワクチン単独投与では(Gag-SeV/ΔF)3頭中2頭でSIVの増殖を抑制し，プラスミドプライムと組み合わせることで(DNA/ Gag-SeV/ΔF)，全頭(3頭中3頭)でよく抑制した．このワクチンは2年間以上にわたってSIV増殖を抑制した．(東京大学医科学研究所 俣野哲郎先生提供)

ため，CD4陽性T細胞を標的として感染している．エイズに感染してもCD4陽性T細胞は一定期間正常範囲にあるが(5〜10年)，閾値以上に減少すると免疫不全に陥りエイズを発症する．エイズワクチンとしての有効性(HIV複製を抑制)のためには，液性免疫(抗体)よりも感染細胞をターゲットにした細胞性免疫(細胞傷害性Tリンパ球：CTL)がより重要であると認識されている．

そこで，Gagを感染防御抗原とし，CTLを誘導するワクチン戦略に焦点をおき，DNAワクチンで初回免疫(プライム)し，Gag発現センダイウイルスベクターで追加免疫(ブースト)する方法を採用して評価した．エイズワクチンの評価のためには，霊長類(マカクサル，カニクイサル)とサル免疫不全ウイルス(SIV)を用いた評価系が有効に評価可能なモデルである．センダイウイルスベクターの点鼻投与により霊長類にも有効に遺伝子導入が可能であるという基礎的観察のあとに[5]，同モデルを用いて評価を行った．

はじめに，攻撃ウイルスとしてSHIV89.6Pを使用したが，センダイウイルスベクターによるきわめて高いワクチン効果が確認された(図4)[6,7]．SHIV89.6Pは，エンベロープといくつかのアクセサリー遺伝子がヒトHIV-1のものに置き換えたキメラSIV(Gagなど主要内部蛋白はSIV由来)であり，接種後2週程度でサル末梢血のCD4陽性細胞が激減するという特徴のため早期の評価が可能である．しかし，個体間感染様式の違い，およびサルの免疫系にコントロールされやすいという特徴から評価系としての問題点が指摘されている．この系においては，センダイウイルスベクターのほかに，アデノウイルスとワクシニアウイルスベクターを用いたプロトコールにおいても，この攻撃ウイルスに対する防御を誘導した結果が報告されている．

そこで自然感染経過に，より近いSIV株を攻撃ウイルスに使用した評価系として，SIVmac239株を用いて同様の実験を行った[8,9]．SIVmac239に対してもGag発現センダイウイルスベクターのプロトコールはきわめて有効であり，SIVに対する細胞性免疫の有意な亢進と，高いワクチン効果が確認された(図5)．一方で，アデノウイルスベクターやワクシニアウイルスベクタープロトコールは不成功に終わったことが報告されている[10,11]．その後，アデノウイルスベクターも，ある程度有効であることが報告された[12]．

以上のような事実から，センダイウイルスベクターはエイズワクチン戦略において，既存の遺伝子ワクチンのなかでは最も高い効果を示すワクチンの

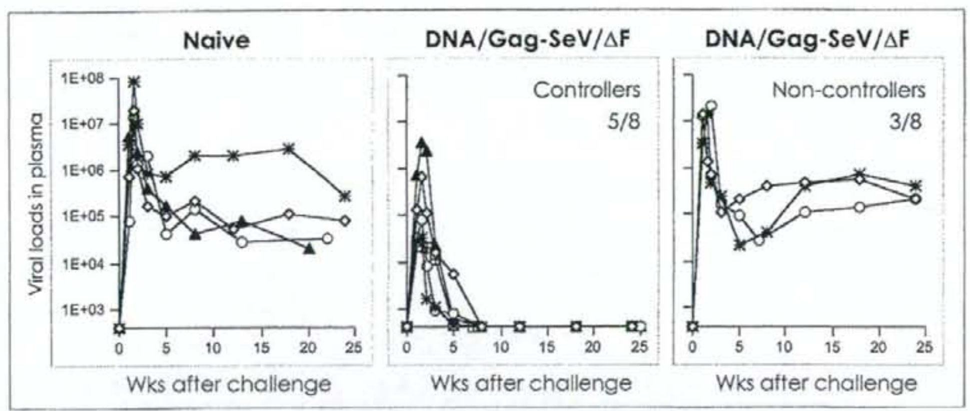

図5　自然経過モデル(SIVmac239)におけるワクチン効果

SIV(SIVmac239)チャレンジ後の血漿中のウイルス量を示す．処置した8頭中5頭でSIV増殖をよく抑制したが，3頭では増殖が観察された．これらの動物においてもSIVに対するCTLは維持されており，エピトープに差がある可能性があると予想し解析している．(東京大学医科学研究所 俣野哲朗先生提供)

一つであると捉えられている．

エイズワクチンの世界的規模での開発活動のなかで，(ペプチド・蛋白抗原以外に)遺伝子ワクチンとして多くのベクターが開発されている．特に，プラスミドベクターなどの非ウイルスベクターのみではなく，ウイルスベクターの応用例が多い．

たとえば，ワクチニアウイルス・アンカラ株ベクター(modified vaccinia Ankara (MVA)：NIAID-LVD and therion biologics)[13]，カナリア痘ウイルスベクター(canarypox：Sanofi Pasteur)[14]，鶏痘ウイルスベクター(fowlpox：Therion Biologics)[15]などのオルソポックスウイルス類，非複製型アデノウイルスベクター(adenovirus：Merck and NIH VRC)[16]，また，センダイウイルスと同じ(−)鎖RNAウイルスである，弱毒化水疱性口内炎ウイルスベクター(Vesicular stomatitis virus：AlphaVax)[17,18]，(＋)鎖RNAアルファウイルスであるベネズエラウマ脳炎ウイルスベクター(Venezuelan equine encephalitis virus (VEE)：GlobeImmune)[19]なども開発されている．アデノウイルスベクターやポックスウイルス類ベクターは，すでに臨床試験での検討が実施されている．また，追加免疫をするため，種々の組合せによるコンビネーションも数多く検討されている．

これらのベクターには複製型ベクターも多く含まれており，弱毒化ワクチン株を使用するなど安全性を確保したうえで，より有効性を高めるための工夫を行っているとともに，世界的なエイズ問題の深刻さ・緊急性を再認識することが出来る．センダイウイルスと類似のゲノム構造の一本鎖RNAベクターもすでに検討されているが，ベクターの改良・製造システム，点鼻ワクチンとしての粘膜上皮への遺伝子導入能など差別化が可能である．後述するように，センダイウイルスベクターの臨床応用も具体的に進行しており，早期の開発が望まれている．

神経変性疾患治療ワクチンへの応用

いわゆる高齢化社会への変遷に伴い，神経変性疾患の患者数は増加の一途を辿っており，たとえばアルツハイマー病患者数は日本で100万人，アメリカでは450万人，世界的には1,500万人以上いるとされ，今後20年間で2倍以上の患者数に達すると予想されている．治療薬は存在しているものの，病状が進行した段階の治療法，あるいは早期であっても進行抑制効果の高い治療法，さらには発症そのものを予防する方法などの開発が望まれており，新規の治療法に対する社会的ニーズはきわめて高い．

アルツハイマー病の病態仮説として，アミロイドβ(Aβ)の凝集および沈着による老人斑の形成が原因であるとする"アミロイドカスケード仮説"が有力である．この仮説をもとに，アルツハイマー病に対する新規治療法として，Aβを標的とするワクチン療法が注目されており，この有効性のためには液性免疫(抗Aβ抗体)の誘導が重要であると考えられている．実際に，凝集Aβペプチドをアジュバントとともに投与することで，モデルマウスにおける脳のアミロイド沈着が減少したことが報告された[20]．これらの結果をもとに，Elan社およびWyeth社によって，合成Aβペプチド(AN-1792)をアジュバント(QS21)とともに投与する臨床試験が行われ，被験者の血清中に抗Aβ抗体の上昇が確認され[21]，高次脳機能の改善も報告された[22]．

しかし，2001年にはじまった軽度〜中等度のアルツハイマー病患者を対象に行われたphase II試験において6%(298名中18名)に髄膜脳炎が発生し[23]（最終的には，372人の被験者のなかの5人との報告もある），治験は2002年1月に中止された．死亡した患者の脳組織の病理学的解析によると，新皮質での老人斑が消失と，それに伴うアストロサイトの増殖や変性軸索も消えている例がみられていた．髄膜脳炎を発症した原因については，アジュバントを必要とするワクチン療法だったため，一部の患者においてアジュバント誘導性の細胞性免疫により，AβまたはAPPに反応するTh1タイプのCD4陽性T細胞が脳内に浸潤することで，アレルギー性実験的脳脊髄炎様の髄膜脳炎を引き起こしたのではないかと推察されている．ワクチン療法そのものは有効であると認識されており，髄膜脳炎のない，より安全なワクチン技術の開発が望まれている．

その目的のために，Th2タイプのアジュバント(aluminun など)を使用する免疫法による臨床試験(Vaccine ACC-001，Wyeth)，あるいはAβに対する抗体を直接投与する受動免疫法[24]による臨床試験(bapineuzumab =AAB-001，Elan and Wyeth；RN-1219，Rinat/Pfizer：LY-2062430，Eli Lilly)などが実施されている．ただし，受動免疫においては，大量の抗体を長期間にわたって投与することによる抗イディオタイプ抗体の出現，および血管へのアミロイド沈着により出血傾向に陥ることが懸念されている．

一方で，遺伝子ワクチンの検討も進んでいる．プラスミド[25,26]，アデノウイルスベクター[27,28]アデノ随伴ウイルスベクター[29,30]などを利用した例が報告されている．特に，国立長寿医療センターのグループは，アデノ随伴ウイルスベクターを用いてTh2 T細胞が誘導されやすい腸管粘膜免疫系を利用した経口内服治療法を創出しており[30]，東京都神経科学総合研究所のグループはNovartis社と組んで，プラスミドの繰り返し投与による有効性を示している[26]．遺伝子ワクチンについても国内技術での進展がみられている例である．センダイウイルスベクターによるアルツハイマー病ワクチンの研究開発においては，変異型アミロイド前駆体蛋白質を発現するトランスジェニックマウスを用いた検討において，Aβペプチド遺伝子をはじめとした組換えセンダイウイルスベクターの経鼻投与による治療効果の検討が進んでいる[31]．

その他の遺伝子ワクチン応用と実用化への取組み

新興・再興感染症とよばれる感染症には数多くのものがある．そのなかで鳥インフルエンザに対するワクチン療法について，センダイウイルスベクターを用いた共同研究開発をすでに開始している．動物種内の感染症についても同様に実施している．エイズワクチンは具体的に臨床開発の段階に入っている．世界で最初の遺伝子治療製剤(p53-adenovirus)を上市した中国Shenzhen SiBiono GeneTech社と技術導出に関する契約を締結し，中国での臨床試験の準備に入っている．さらに，世界のエイズワクチン開発を推進している国際エイズワクチン推進構想(International AIDS Vaccine Initiative：IAVI)と提携し，特にアジア・アフリカ地域他に有効なワクチンを供給するべく共同開発を実施している．神経変性疾患治療ワクチンなどその他疾患に関しても同様であり，特にアルツハイマー病ワクチンに関して，エーザイ社とワクチン療法の創薬研究に関する契約を締結し，共同開発を目指している．

センダイウイルスベクターの遺伝子ワクチンと

しての開発の過程で，このベクターの免疫学的特長が明らかになってきた．なかでも遺伝子発現量とともに特筆すべきは，その免疫賦活能の高さであり，その性質はがんの樹状細胞療法にも適用されている[32]．この特長は，モノクローナル抗体作製にも応用することができ，一般的な細胞内抗原やI型膜蛋白だけでなく，特に抗体取得が難しいとされているG蛋白質共役型受容体(G protein-coupled receptors：GPCR)などの複数回膜貫通型蛋白に対してきわめて有効に作用した．モノクローナル抗体作製の応用範囲は広く，抗体医薬開発への貢献度は非常に高いと捉えられている．

おわりに

以上のように，筆者らは，センダイウイルスベクターが有効なワクチン効果を有することを示し，具体的なワクチン製剤様態を提案している[33]．センダイウイルスを利用した遺伝子ワクチンは，① 遺伝毒性がない安全な遺伝子ワクチンを提供できる，② ベクターに搭載する遺伝子による免疫系の制御が可能である，③ 経鼻ワクチンとして効果的で簡便な治療製剤となりうる，という有効な性質を有している．また，一方で個々の疾患において要求される免疫系反応(および投与形態)が異なるために，それぞれの最適化も必要である．このとき，ベクター技術をもとにしたシステムとしての最適化が必要であるが，センダイウイルスベクターシステムは，個々の疾患に求められるベクター様態に確実に対応し，治療デバイスを含めて具体的に遺伝子ワクチン製剤を提供することが可能である．

また，筆者らの研究開発は，日本発のベクターシステムを用いて，国内機関のみではなく海外機関との共同研究・共同開発の推進に結びつくよい実例になっている．このような取組みによって，社会的要請度の高い感染症疾患および神経変性疾患を含む重症疾患の治療に対して，効果的なワクチン製剤を提供するとともに，研究，保健行政，国際協力など多くの貢献に繋がるものと期待したい．

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Improved Safety of Hematopoietic Transplantation with Monkey Embryonic Stem Cells in the Allogeneic Setting

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Improved Safety of Hematopoietic Transplantation with Monkey Embryonic Stem Cells in the Allogeneic Setting

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Key Words. Cynomolgus monkey • Hematopoiesis • Embryonic stem cell • In utero transplantation • Teratoma • Purging Tumor prevention

ABSTRACT

Cynomolgus monkey embryonic stem cell (cyESC)-derived in vivo hematopoiesis was examined in an allogeneic transplantation model. cyESCs were induced to differentiate into the putative hematopoietic precursors in vitro, and the cells were transplanted into the fetal cynomolgus liver at approximately the end of the first trimester ($n = 3$). Although cyESC-derived hematopoietic colony-forming cells were detected in the newborns (4.1%–4.7%), a teratoma developed in all newborns. The risk of tumor formation was high in this allogeneic transplantation model, given that tumors were hardly observed in immunodeficient mice or fetal sheep that had been xeno-transplanted with the same cyESC derivatives. It turned out that the cyESC-derived donor cells included a residual undifferentiated fraction positive for stage-specific embryonic antigen (SSEA)-4 (38.2% ± 10.3%) despite the rigorous differentiation culture. When an SSEA-4-negative fraction was transplanted ($n = 6$), the teratoma was no longer observed, whereas the cyESC-derived hematopoietic engraftment was unperturbed (2.3%–5.0%). SSEA-4 is therefore a clinically relevant pluripotency marker of primate embryonic stem cells (ESCs). Purging pluripotent cells with this surface marker would be a promising method of producing clinical progenitor cell preparations using human ESCs. STEM CELLS *2006;24:1450–1457*

INTRODUCTION

Human embryonic stem cells (hESCs) hold great potential in the treatment of a variety of diseases and injuries because embryonic stem cells (ESCs) have the ability to proliferate indefinitely in culture and to differentiate into any cell type [1, 2]. Because ESCs are able to form teratomas when transplanted into immunodeficient mice, safety concerns would be raised against the clinical application of hESCs [3, 4]. It will be necessary to test the safety of these cells in animal transplantation models before clinical application. Nonhuman primate transplantation models would be desirable for this purpose; however, there have been only a few reports on these models [5–7]. The successful engraftment of transplanted cells in primates will not be achieved unless the immune rejection of transplanted cells is circumvented (e.g., through immunosuppressive treatment) [6]. The early gestational fetus may be a good recipient with which to circumvent immune rejection because the immune system is premature [8]. In addition, in the animal fetus, transplanted cells would engraft without conditioning of recipients such as irradiation or immunosuppressive treatment [9–12]. We have previously established a system for allogeneic transplantation of cynomolgus ESCs (cyESCs) using preimmune fetal monkeys as recipients [5].

We have also reported a novel method for hematopoietic engraftment from cyESCs in sheep [13]. The method is a combination of three steps: (a) differentiation in vitro to generate the putative hematopoietic precursors [14]; (b) transplantation of the cells in utero [15]; and (c) development into hematopoietic cells in vivo using the hematopoietic microenvironment of the fetal liver [16]. In the present study,

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we have examined the safety as well as the efficacy of hematopoietic engraftment of cells derived from cyESCs in the allogeneic transplantation model.

Materials and Methods

Animals

Pregnant cynomolgus monkeys (16–22 years old) were obtained by mating and were reared at the Tsukuba Primate Research Center in accordance with Rules for Animals Care and Management set forth by the Research Center and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan. Experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. The animals were free of intestinal parasites and were seronegative for herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus.

Cell Preparation

A cyESC line (CMK6G) stably expressing green fluorescent protein (GFP) was established after transfection of the parental cyESC line (CMK6) with the enhanced GFP gene (Clontech, Palo Alto, CA, http://www.clontech.com) [17]. cyESCs were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, http://www.kyowa.co.jp)-treated mouse (ICR or BALB/c; Clea Japan, Tokyo, http://www.clea-japan.com) embryonic fibroblasts as previously described [18]. The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% fetal calf serum (FCS; Invitrogen) [19].

cyESCs were induced to differentiate into the putative hematopoietic precursors as previously described [13]. Briefly, undifferentiated cyESCs were transferred onto mitomycin C-treated confluent OP9 cells and cultured for 6 days in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8% FCS, 8% horse serum (Invitrogen), 5×10^{-6} M hydrocortisone (Sigma, St. Louis, http://www.sigmaaldrich.com), and multiple cytokines, including 20 ng/ml recombinant human (rh) bone morphogenetic protein-4 (R&D Systems, Minneapolis, http://www.rndsystems.com), 20 ng/ml rh stem cell factor (Biosource, Camarillo, CA, http://www.biosource.com), 20 ng/ml rh vascular endothelial growth factor (VEGF; R&D Systems), 20 ng/ml rh Flt-3 ligand (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), 20 ng/ml rh interleukin-3 (PeproTech), 10 ng/ml rh interleukin-6 (PeproTech), 20 ng/ml rh granulocyte colony-stimulating factor (PeproTech), and 2 IU/ml rh erythropoietin (Roche, Basel, Switzerland, http://www.roche.com). The cells were resuspended in 0.1% human serum albumin (Sigma)/Hanks' balanced saline solution (Sigma) for transplantation.

Flow Cytometry

Primary antibodies (Abs) used in the present study were anti-human CD34 monoclonal Ab (mAb; BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), anti-human CD31 mAb (Pharmingen), anti-human CD45 mAb (Pharmingen), anti-human vascular endothelial (VE) cadherin mAb (Pharmingen), rabbit anti-human VEGF receptor (VEGFR)-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), and anti-stage-specific embryonic antigen (SSEA)-4 mAb (Chemicon, Temecula, CA, http://www.chemicon.com). All of them cross-reacted to cynomolgus counterparts as previously demonstrated [18, 20–22]. Secondary Abs were phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (Ig) Ab (DakoCytomation, Glostrup, Denmark, http://www.dako.com) and Alexa Fluor 647-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Cells stained with unlabeled primary Abs were incubated with fluorescence-labeled secondary Abs. Cells were incubated with either primary or secondary Ab for 20–60 minutes at 4°C. Regarding staining with the anti-VEGFR-2 Ab, the cells were incubated with biotin-conjugated goat anti-rabbit IgG Ab (Beckman Coulter, Miami, http://www.beckmancoulter.com), followed by PE-conjugated streptavidin (Beckman Coulter). Fluorescence-labeled cells were analyzed with a FACS Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Data analysis was performed using the CellQuest software (Becton, Dickinson and Company). Isotype-matched, irrelevant mAbs (DakoCytomation or Beckman Coulter) served as negative controls. Nonviable cells were excluded from analysis by propidium iodide (Sigma) costaining.

Cell Sorting

Cell sorting was performed to purge SSEA-4^{+} cells from among the cultured cyESCs in vitro. Cells were incubated with the anti-SSEA-4 mAb for 1 hour at 4°C and washed twice with Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were then incubated with the PE-conjugated anti-mouse Ig Ab for 1 hour at 4°C and washed twice again. GFP-positive and SSEA-4-negative cells were sorted using an Epics Elite cell sorter (Beckman Coulter). Data acquisition was performed using the Expo2 software (Beckman Coulter).

Transplantation and Delivery

Transplant procedures were previously described [5]. Briefly, animals were anesthetized via an intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, http://www.sankyo.co.jp) and received 0.5%–1.0% isoflurane by inhalation by means of an endotracheal tube. Cells (0.16–46×10^{6} cells per fetus; Table 1) were injected into the fetal liver through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester. The fetuses were delivered by cesarean section at 2–3 months after transplant (gestation 120–157 days, full term 165 days).

Colony Polymerase Chain Reaction

Cynomolgus clonogenic hematopoietic colonies were produced as previously described [20]. After cells were cultured in methylcellulose medium for 10–14 days, well-separated individual colonies were plucked into 50 μl of distilled water and digested with 20 μg/ml proteinase K (Takara, Shiga, Japan, http://www.takara-bio.com) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μl) was used for a nested polymerase chain reaction (PCR) to detect the *GFP* gene sequence. The outer primer set was 5′-AAGGACGACGGCAACTACAA-3′ and 5′-ACTGGGTGCTCAGGTAGTGG-3′, and the inner primer set was 5′-GCATCGACTTCAAGGAGGAC-3′ and 5′-GTTGTGGCGGATCTTGAAGT-3′. Amplification conditions for both the outer and inner PCR were 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The

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Table 1. ESC-derived hematopoiesis and tumor formation

Animals	Animal no.	Transplanted cells	Purging SSEA-4+ cells	Cell number per fetus (×10^6)	Donor-derived CFU in recipients[a] at birth (donor/total colony number)	Tumor formation	Observation period (months)
Monkeys	0031	Undifferentiated ESCs	−	3.90	n.d.	+	3
	2311		−	0.16	n.d., Dead	+	2
	0321		−	0.21	n.d., Dead	+	2
	0841	Day-6 ESC-derived cells	−	10	4.1% (2/49)	+	3
	1551		−	46	n.d., Dead	+	2.5
	0021		−	46	4.7% (4/85)	+	3
	0691	Day-6 ESC-derived cells	+	0.16	3.2% (2/62)	−	3
	0381		+	1.40	5.0% (4/80)	−	3
	0022		+	0.17	2.3% (2/86)	−	3
	0981		+	0.31	4.1% (3/73)	−	3
	0051		+	0.31	n.d., Dead[b]	−	3
	1552		+	0.75	4.4% (2/45)	−	4
Sheep[c]	57	Day-6 ESC-derived cells	−	50	1.1% (1/91)	−	18
	55		−	50	1.1% (1/91)	−	26
	141		−	78	1.1% (1/91)	−	26
	182		−	14	1.6% (1/63)	−	21

[a]Percentage of donor-derived CFU was calculated by dividing the number of CFU positive for the green fluorescent protein gene sequence by the number of CFU positive for the β-actin gene sequence. Donor-derived CFU were analyzed at delivery.
[b]Death due to ablation of placentae. Other deaths were presumably tumor-related.
[c]As published by Sasaki et al. [13].
Abbreviations: CFU, colony-forming units; ESC, embryonic stem cell; n.d., not done; SSEA, stage-specific embryonic antigen.

outer PCR products were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA, http://www.qiagen.com). Simultaneous PCR for the β-actin sequence was also performed to ensure DNA amplification of the sample in each colony. The primer set for β-actin was 5′-CATTGTCATGGACTCTGGCGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGGGC-3′. Amplification conditions for β-actin PCR were 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplified GFP (131 bp) and β-actin (234 bp) products were resolved on 2% agarose gel (Sigma) and visualized by ethidium bromide (Invitrogen) staining.

RNA PCR

Total RNA was extracted from cells of interest using the EZ1 RNA universal tissue kit (Qiagen). RNA was reverse-transcribed at 50°C for 30 minutes using the RNA LA PCR kit (Takara) with oligo dT primer. The resulting cDNA was then subjected to PCR. Regarding PCR for Oct-4, the primer set was 5′-GGACACCTGGCTTCGGATT-3′ and 5′-TTCGCTTTCTCTTTCGGGC-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 1.5 minutes. Regarding PCR for Scl, the primer set was 5′-GGGCGGAAAGCTGTTTGCGATT-3′ and 5′-TCGCTGAGAGGCCTGCAGTT-3′. The PCR conditions were 35 cycles of 95°C for 30 seconds, 63°C for 1 minute, and 72°C for 1 minute. A simultaneous PCR for β-actin was also conducted on each cDNA sample as an internal control as described above. Amplified Oct-4 (697 bp), Scl (201 bp), and β-actin (234 bp) products were resolved on 2% agarose gel and visualized by ethidium bromide staining.

Results

In Utero Transplantation and Delivery

cyESCs stably expressing GFP were used in this study [17]. In the setting of allogeneic transplantation, GFP was used as a genetic tag to track transplanted cell progeny. We employed the OP9 stromal cell coculture method instead of the embryoid body formation method to facilitate the hematopoietic differentiation [19, 23, 24] (Fig. 1A, 1B). According to the flow cytometric analysis, CD34, CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]), CD144 (VE-cadherin), and VEGFR-2 (Flk-1) were all upregulated on day 6 but decreased thereafter (Fig. 1C–1E, 1G). Among the markers examined, CD34 is a widely used surface marker of hematopoietic stem cells in both human and monkey subjects [25–27]. The others are key markers of hemangioblasts (which generate endothelial and hematopoietic lineages) in both mice and humans [14, 28]. Cells positive for both VEGFR-2 and VE-cadherin emerged on day 6 (Fig. 1H). CD45, however, was not detected until day 12 (Fig. 1F). Despite the hemangioblast marker expression on day 6, the hematopoietic *Scl* gene was upregulated at this time point as assessed by RNA PCR (Fig. 1I), implying that the hematopoietic commitment might have already occurred on day 6 [29, 30]. We therefore designated the day 6 cyESC-derived progenitor cells as putative hematopoietic precursors. The time course profiles presented here were similar to those of hESCs [14, 24]. The GFP expression was stable during the 6-day culture (Fig. 1A, 1B) and afterward (data not shown).

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