levels of GUSB expression in multiple visceral organs; however, no obvious GUSB expression was detected in the brain.[30] Since the results in C3H mice described above suggested that vector infection and subsequent GUSB expression were achievable in the mouse brain by neonatal administration, we injected AxCAhGUS into newborn B6/MPSVII mice, and therapeutic efficacy in CNS lesions was evaluated. Approximately 200% of normal GUSB activity was found in the brain 30 days after the viral injection, and 20–30% of normal GUSB activity was still detected at 140 days (Figure 4A). Histochemical study showed that disseminated GUSB-positive cells were distributed in the cortex of the mouse brain (Figure 4Ba). On the other hand, no GUSB-positive cells were detected in the brain of B6/MPSVII mice treated at 30 days after birth (Figure 4Bc). Histopathological study demonstrated that no vacuolated cells were detected even 140 days after birth (Figures 4Bd and Be).

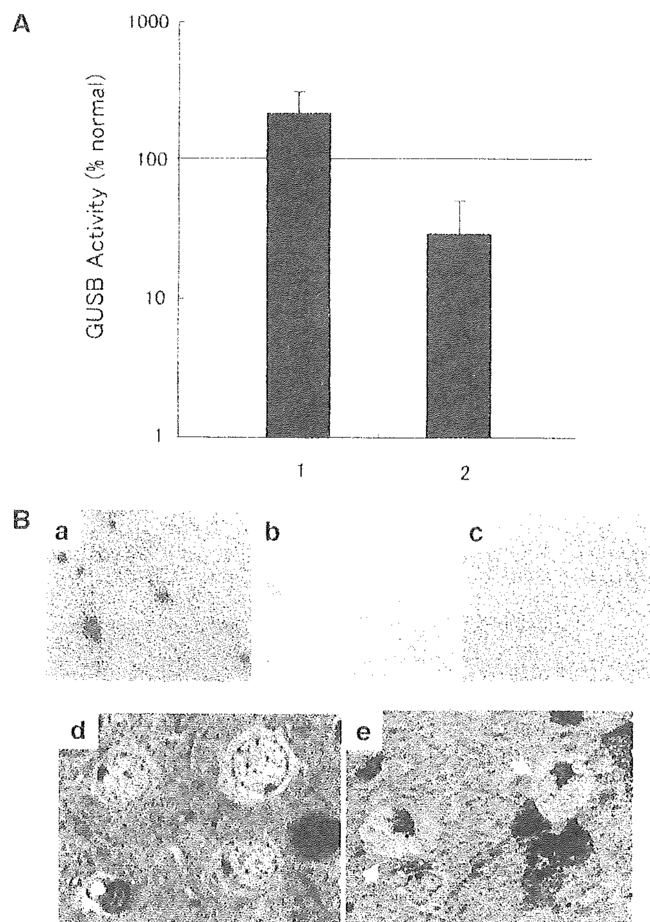

Figure 4 *Therapeutic efficacy in the brains of B6/MPSVII mice infected with AxCAhGUS at neonatal period. We injected 100 μl of viral solution containing 1 × 10*[illegible] *pfu of AxCAhGUS into newborn B6/MPSVII mice within 24 h of delivery, and evaluated the therapeutic effect in the CNS. (A) GUSB activity in the brain of B6/MPSVII mice treated with AxCAhGUS during the neonatal period is presented. Approximately 200% and 30% of the normal level of GUSB activity was detected at 30 days (lane 1) and 140 days (lane 2) after the treatment, respectively. Each bar represents the average and standard deviation from three treated animals. Results are expressed as a percentage of GUSB activity found in the brains from four age-matched B6 (+/+) mice. (B) Histochemical study of mouse brain was performed 140 days after the neonatal treatment. Disseminated strong GUSB-positive cells are observed in the treated mice (a), while faint and diffused GUSB staining is seen in the brain of an age-matched B6 (+/+) mice (b), and no GUSB staining is seen in the brain of the B6/MPSBVII mice treated 30 days after birth (c). (C) Histopathological analysis shows no lysosomal storage in the brain cortex in the mice treated during the neonatal period (d), while characteristic vacuolation (arrows) is observed in age-matched (140 days old) untreated B6/MPSBVII mice (e).*

Therapeutic efficacy of neonatal gene therapy against ocular complications

Retinal degeneration and corneal clouding are frequently observed as ocular complications in MPSVII or other mucopolysaccharidoses.[31,32] We studied the therapeutic efficacy in the retina and cornea of B6/MPSVII mice treated by systemic vector administration in the neonatal period. In the retina of the mice treated in adulthood, no or few GUSB-positive cells were identified among the retinal pigment epithelial cells where lysosomal storage is remarkable in the affected mice (Figures 5Aa and Ac). In contrast, these cells were clearly GUSB-positive in the mice treated in the neonatal period, and subsequent improvement of lysosomal storage was observed (Figures 5Ab and Ad). In the cornea, histochemical study indicated that disseminated GUSB-positive cells were present in the stroma, and subsequent amelioration of lysosomal storage was observed (Figures 5Bb and Bd) in the mice treated during the neonatal period. However, no obvious pathological correction was observed in the cornea of the mice treated in adulthood (Figures 5Ba and Bc). These results show that effective therapeutic results for ocular complications by systemic administration can be obtained only when the injection is carried out in the neonatal period.

Improvement of skeletal deformities by neonatal treatment

Skeletal deformity is another frequent and serious manifestation in patients suffering from all types of mucopolysaccharidoses. A flattened face is a characteristic phenotype of skeletal deformity in B6/MPSVII. All the mice that received neonatal treatment were of normal facial appearance even at 140 days after the treatment, and radiographic analysis showed morphological normalization of facial and cranial bones in the mice treated in the neonatal period (Figure 6). On the other hand, no obvious improvement in skeletal deformities was observed on adenovirus treatment in adult mice (data not shown), suggesting that neonatal or infantile gene therapy is essential for preventing skeletal deformities in MPSVII.

Feasibility of gene transduction by multiple vector administrations

The feasibility of gene transduction by multiple vector administrations was studied by quantification of serum GUSB activity in the MPSVII mice treated in the neonatal period. About 5–10 times higher than normal GUSB activity was observed 35 days after the treatment (Figure 7). Since no increase in the titers of anti-adenoviral neutralizing antibody was observed in the same serum samples (Table 1), we attempted a secondary viral administration at 35 days after birth. A rapid and marked increase of serum GUSB activity was identified 7 days after the secondary injection, and this high level of the activity was maintained for more than 100 days after birth (Figure 7). However, the mice generated significant

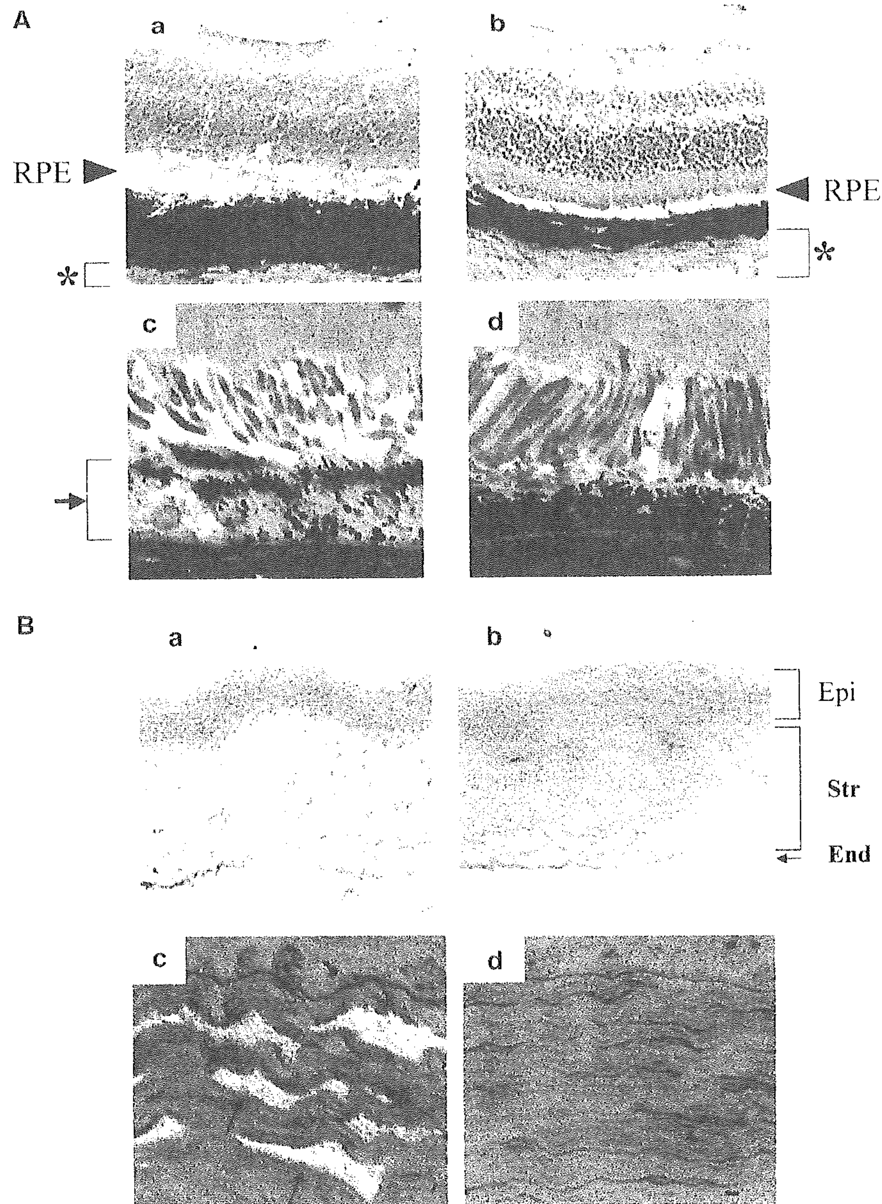

Figure 5 Therapeutic efficacy for ocular manifestations in B6/MPSVII mice infected with AxCAhGUS in the neonatal period. Newborn and adult B6 MPSVII mice were infected with AxCAhGUS, their eyes were enucleated 140 or 7 days after the treatment, respectively, and therapeutic efficacy in the retina (A) and cornea (B) was evaluated. (A) In the mice that received neonatal treatment (Ab), GUSB-positive cells are identified among retinal pigment epithelial cells (RPE) as well as in tissues outside of retina (asterisk), while in the mice treated in adulthood (Aa), GUSB-positive cells are only located in tissues outside of retina. Histopathological study shows amelioration of lysosomal storage in the mice treated during the neonatal period (Ad), but no pathological correction in the mice treated in adulthood (Ac). An arrow indicates vacuolated RPE cells due to lysosomal storage. (B) In corneal tissues, GUSB-positive cells are distributed in the corneal stroma (Str) only in the mice treated in the neonatal period (Bb), and no GUSB-positive cells are detected in adulthood (Ba). Elimination of lysosomal storage is observed in the mice that received neonatal treatment (Bd), but no correction is seen in the mice that treated in adulthood (Bc). Arrows indicate characteristic vacuolization due to lysosomal storage. Epi: epithelium; Str: stroma; End: endothelium.

levels of anti-adenoviral neutralizing antibody (32–128 titers) 2 months after the secondary injection (Table 1). Moreover, no increase of serum GUSB activity was observed 7 days after tertiary vector administration (Figure 7).

Discussion

We previously reported the therapeutic efficacy of adenovirus-mediated gene therapy in adult MPSVII mice.[20] In the report, we demonstrated that high levels of GUSB activity in multiple extrahepatic organs such as spleen, kidney, lung, and heart were the result of *in vivo* cross-correction of GUSB delivered from the liver, where gene transduction had taken place. A rapid and complete amelioration of lysosomal storage was also observed in liver and spleen in the treated animals. These experiments, however, also disclosed the limitations of this treatment strategy. First, no significant GUSB activity was identified in the brain, which is one of the most important affected regions in MPSVII as well as other

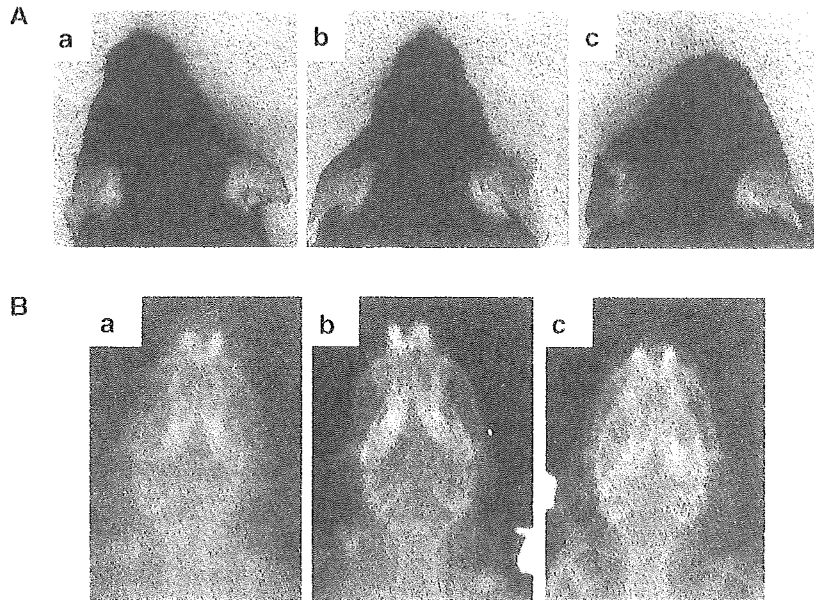

Figure 6 Therapeutic efficacy for skeletal deformity in B6/MPSVII. A flattened face is a characteristic symptom of skeletal deformity in B6/MPSVII. The faces of the mice (140 days old) that received neonatal adenoviral injection (Ab) are indistinguishable from those of the B6 (+/+) littermates (Aa), while an untreated MPSVII littermate show characteristic deformity (Ac). Radiographic analysis also indicates a remarkable improvement of facial and cranial bone deformities in the treated animal (Ba: normal littermate. Bb: a treated animal. Bc: untreated B6/MPSVII littermate).

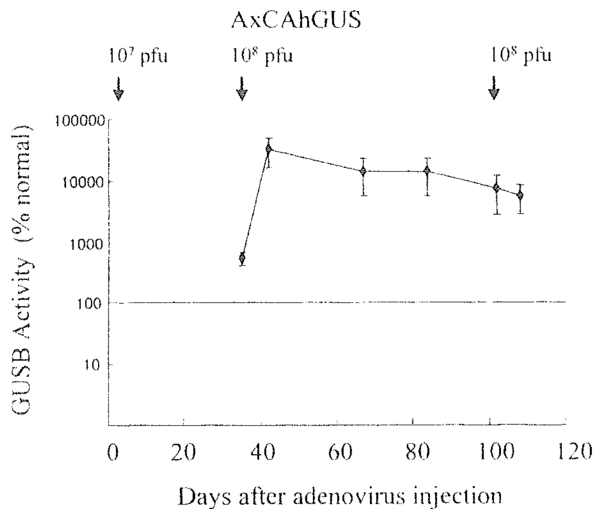

Figure 7 Time-dependent change of serum GUSB activity in the mice treated with AxCAhGUS. To investigate the feasibility of gene transduction by multiple vector injections, we performed periodic serum sampling from the mice infected with AxCAhGUS during the neonatal period. A 10-fold higher than normal GUSB activity was still identified in the mice 5 weeks after the treatment. Secondary vector administration was carried out just after the serum sampling. A week later, serum GUSB activities were measured again. A marked increase in serum GUSB activity was observed, and this high level of activity was maintained for more than 60 days. However, no obvious increase in GUSB activity was observed when a tertiary administration was performed 120 days after the first administration. Each point represents the mean and standard deviation from four treated animals. Results are expressed as a percentage of the GUSB activity in four age-matched B6 (+/+) mice. Arrows indicate the day when the vector injection was done. For the first administration, 100 µl of the vector solution containing 1×10^7 pfu of AxCAhGUS was injected into neonatal B6/MPSVII mice via superficial temporal veins. For secondary and tertiary administration, 1 ml of vector solution containing 1×10^8 pfu of AxCAhGUS was injected into B6/MPSVII mice via tail veins.

lysosomal storage disorders. Second, no pathological correction was observed in retina and cornea. Third, no improvement of skeletal deformities was found. Finally, the therapeutic effect did not persist for long.

Mental retardation is a frequent manifestation in several types of mucopolysaccharidoses as well as other lysosomal storage disorders.[33] We and other groups achieved successful pathological correction in the CNS by transplantation of GUSB-producing cells,[11,18] or by generation of GUSB-producing cells in the brain with direct vector administrations.[16] Although these therapeutic strategies may be effective, less invasive approach is required for widespread clinical applications. Sands *et al* showed that for BMT or ERT, it is necessary to start the treatments in the neonatal period to prevent degenerations of the CNS.[5,7,9] Daly *et al* have recently shown that neonatal gene transfer is also effective for treating the CNS in MPSVII using adeno-associated virus vectors.[27] They showed that the GUSB activity in the brain after AAV-mediated gene transduction was less than 10% of the activity in normal littermates. Although 1–5% of normal activity may be sufficient for the treatment in this particular disease model, a comparatively inefficient gene transduction may cause the problem in the treatment of human MPSVII or other lysosomal storage diseases. In the present study, we demonstrated that 20–30% of normal GUSB activity was detected in the mouse brain even 140 days after the adenovirus treatment. These observations indicate that an adenoviral gene transfer seems to be more efficient than the treatment with AAV vectors, although physiological and behavioral studies will have to be performed to prove this.

The blood–brain barrier is the main reason why both vector infection and GUSB transport were prevented in adult mouse brain.[34] The blood–retinal barrier is also a

hurdle in the delivery of drug to the cells in the retina from the systemic circulation.[35] In this paper, we have reported that no GUSB-positive cells were detected among retinal pigment epithelial cells, and lysosomal storage was not eliminated in the adult mice treated by adenovirus vectors. This finding suggests that the blood–retinal barrier prevented both vector infection and uptake of GUSB into the retinal pigment epithelial cells. On the other hand, long-term GUSB expression and pathological correction were observed in the mice treated in the neonatal period. Although it is unclear whether the blood–retinal barrier is immature in neonatal mice just like the blood–brain barrier, immaturity of the barriers may have enabled the vector infection to occur in retinal cells.[34]

Here, we also showed that lysosomal storage in corneal stroma was completely eliminated in the mice treated during the neonatal period, while no obvious efficacy was seen in the mice treated in adulthood. This finding is unexpected but favorable in terms of treatment of corneal clouding, which is the most frequent ocular manifestation in several types of mucopolysaccharidoses.[33]

We also showed improvement of skeletal deformities following neonatal treatment. In the mice treated after maturation, no obvious improvement of facial dysmorphology was achieved. However, the facial appearance of the mice treated in the neonatal period was completely normal. Radiographic analysis showed that their facial and cranial bones were also morphologically normal. These results demonstrate that a single vector injection during the neonatal period is sufficient for complete normalization of the facial and cranial bones in MPSVII.

One of the most important findings in this study is the remarkable difference in vector targeting organs after intravenous injection between neonatal and adult treatment. As we showed previously, most of the vectors were accumulated in the liver when we injected virus in adult mice.[20] On the other hand, the vectors were distributed in all organs examined when injection was carried out in the neonatal period. Fechner *et al* reported that anatomic vector barriers, in particular the endothelium structure, were the major factors in determining the vector-targeting organs, and the expression of viral receptors did not correlate with adenoviral vector targeting.[36] This suggests that immaturity of the blood–brain barrier and blood–retinal barrier is a major factor for determining vector-targeting organs, although the difference of the site of injection between newborn MPSVII and adult MPSVII might also partially account for the difference of the vector-targeted organs.

Since an exogenously transduced gene delivered by adenoviral vectors is episomal, dilution of the transgene should take place with the growth of the vector-infected organs or tissues. This indicates the necessity for multiple vector administrations to obtain long-term therapeutic efficacy especially for neonatal treatment. In this report, we demonstrated the feasibility of secondary gene transduction in mice treated initially during the neonatal period. However, anti-adenovirus antibody was generated after secondary treatment, and tertiary injection did not induce efficient gene transduction. These observations suggest that neonatal mice can avoid the deleterious immune response induced by infection with adenovirus vectors. Reducing the immune response that accompanies adenoviral vector infection is essential not only to prolong the transgene expression but also to carry out adenovirus-mediated gene therapy safely.

Here we showed that a single adenoviral infection in the neonatal period is sufficient to prevent the progression of multiple tissue lesions in the CNS, ocular tissues and skeletal system in MPSVII mice. Since humans are rather more developed at birth than mice in terms of the blood–brain barrier, we may have to consider fetal gene therapy (*in utero* gene therapy) to achieve the similar therpeutic efficacy in human MPSVII. The molecular genetics of lysosomal storage diseases has been intensively investigated, and prenatal diagnosis of lysosomal storage diseases is now available. Although several safety issues have to be resolved before clinical applications, neonatal or fetal treatment with an adenoviral vector combined with prenatal diagnosis is a promising strategy for MPSVII and other lysosomal storage disorders.

Table 1 Serum anti-adenovirus titers of the mice treated with AxCAhGUS

	Titers of serum anti-adenovirus
1 month after neonatal treatment	<2 (*n*=7)
2 months after secondary treatment	32, 32, 64, 128
1 month after the treatment in adult mice	128, 256, 256

Materials and methods

Adenoviruses

The two adenoviruses, AxCAhGUS and AxCALacZ, used in this study were E1/E3-deleted recombinant adenoviruses expressing human β-glucuronidase (GUSB) and *E. coli* β-galactosidase (lacZ), respectively.[19,20] Both viruses were generated based on the COS-TPC method described previously.[37] Briefly, a cosmid, pAxCAhGUS, which contained an expression cassette of human GUSB under the control of the CAG promoter,[38] was constructed by subcloning cDNA for human GUSB at a unique *Swa*I site of pAxCAwt. Then 293 cells were co-transfected with the cosmid pAxCAhGUS and an adenoviral DNA–terminal protein complex, which had already been digested at several sites with *Eco*T22I. A recombinant adenovirus was generated through homologous recombination in the transfected cells. Another adenoviral vector AxCALacZ was supplied by Dr I Saito.[39]

Animals

Syngeneic B6/MPSVII mice were obtained from a pedigree colony of B6.C-H-2bml/ByBir-gusmps/+ mice maintained at the National Children's Medical Research Center.[2,3] Normal C3H mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). All mice were maintained in accordance with the guidelines of the animal committee of the facility.

Neonatal injections

Viral solution (100 μl) containing 1×10^7 pfu of AxCAhGUS was injected into newborn B6/MPSVII mice (*n*=8)

or normal C3H mice (n=30) via the superficial temporal veins within 24 h of delivery.[27,28] For comparison, adult B6 (+/+) mice (n=12) were injected with 1 ml of viral solution containing 1×10^8 pfu of AxCAhGUS via tail veins.

Detection of viral DNA in mouse organs

The total DNA of tissue samples was extracted using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Viral DNA in each tissue was detected using PCR to amplify the partial cDNA for human GUSB based on a method described previously.[20] Briefly, the forward and reverse primers were synthesized from the sequence of exons 6 and 7. Expected products are 240-bp fragments from human cDNA encoded in the viral genome of AxCAh-GUS. Since these primer sequences were identical to the corresponding regions of the murine GUSB gene with two mismatches, 454-bp DNA fragments were amplified when PCR was carried out using mouse genomic DNA as template.

Quantitative and histochemical analysis of GUSB activity

GUSB activity in tissue was quantified using a fluorometric assay described previously.[40] Briefly, tissues were homogenized in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 1 mM dithiothreitol. When necessary, the samples were incubated for 2h at 65°C to inactivate endogenous GUSB in normal C3H mice. GUSB activity was measured using 4-methylumbelliferyl β-D-glucuronide (Sigma, St. Louis, MO, USA) as substrate. Histochemical detection of GUSB activity was performed on 6-μm-thick frozen sections using naphtol AS-BI β-D-glucuronide (Sigma) as substrate. Tissues were then counterstained with methyl green (Muto Chemistry, Tokyo, Japan).

Histopathological analysis of lysosomal storage

Tissues were isolated from the mice, and immediately immersed in cold 2% glutaraldehyde in 0.1 M. cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol solutions, and embedded in Spurr's Medium (Polyscience, Warrington, PA, USA). Three micrometers thick frozen section were stained with toluidine blue for evaluation of lysosomal storage. Histological sections were evaluated morphologically by light microscopy.[18]

Anti-adenoviral neutralizing antibodies

Anti-adenovirus neutralizing antibodies in serum were measured as described previously with minor modifications.[41] Briefly, the serum samples were heat-inactivated at 55°C for 30 min and diluted in the medium in two steps. Each serum dilution (0.1 ml) was mixed with 5×10^5 pfu of AxCALacZ (10 μl), incubated at 37°C for 90 min, and applied to nearly confluent 293 cells in a 96-well plate for 10 h. The supernatant, containing serum and viruses, was then replaced with normal medium for 18h. Cells were fixed and stained with X-gal. In the absence of neutralizing antibody, all of the cells stained blue. Titers of the neutralizing antibody for each serum sample were reported as the highest dilution at which less than 25% of the cells stained blue.

Acknowledgements

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Prolonged Survival of Rat Liver Allograft With Adenoviral Gene Transfection of Human Immunodeficiency Virus Type 1 *nef*

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HIV-1 *nef* is believed to allow immune evasion by modifying cell surface molecules because of certain mechanisms such as downregulation of the major histocompatibility complex (MHC) class I molecule complex as well as upregulation of FasL. In the present study, we successfully generated a recombinant adenovirus vector containing HIV-1 *nef*. We detected the expression of *nef* in liver infected with AxCANef by immune staining and Western blotting, and confirmed its expression as persistent for more than 4 weeks. Furthermore, the surface expression of MHC class I was downregulated in AxCANef-infected hepatic cells. In addition, we also observed *nef*-induced FasL upregulation of gene-transfected hepatic cells. Using a DA-to-Lewis orthotopic liver transplantation model, we transfected AxCANef to a liver graft to determine whether *nef* expression could have an effect on recipient survival. AxCANef significantly prolonged recipient survival time (14.5 days) compared with the uninfected group (11 days) ($P < .001$) and the AxCALacZ-infected group (11 days) ($P < .001$). Histologic analysis showed reduction in the number of accumulated inflammatory cells and an increase in apoptotic cells in grafts expressing *nef*. In conclusion, we showed that the *nef* gene could prolong survival of rat liver allografts, and this result suggested the potential clinical use of its transfection. (*Liver Transpl 2003;9:805-813.*)

Transplantation medicine is entering a new age and is slowly undergoing an important paradigm shift away from the traditional chemical immunosuppression regimens that dominate the clinic today and toward modalities with tissue and cell specificity. This shift promises to be more evident in the years to come, when gene therapy will be less of an issue for debate and more of a challenge to clinicians, transplantation immunologists, and biologists.[1] Gene therapy strategies to prolong graft survival involve gene transfer and expression of immunomodulatory or graft-protecting molecules. An allograft could be genetically engineered to express tolerogenic molecules, or to produce immunosuppressive cytokines that might be an impaired alloimmune response of recipient and intragraft apoptosis. The local production of immunosuppressive molecules has the potential to reduce their systemic side effects and to increase their bioavailability and hence, their therapeutic efficiency. Therefore, it could be a promising approach for attenuating graft rejection and facilitating engraftment.[2]

In our previous study,[3,4] the systemic administration of adenovirus vector via a tail vein resulted almost completely in intrahepatic expression of the exogenous gene. This lack of expression in the extrahepatic organs is one of the features in gene therapy using the adenoviral vector. Therefore, it is feasible to modulate liver grafts with some immunoregulatory genes. Furthermore, we have indicated that adenovirus-mediated gene transfer is very effective in rodents and leads to efficient gene expression.[3,4]

The virus including the adenovirus vector was a foreign body for the recipient. Therefore, the immune system was shown to play an essential role in viral clearance because of its antigenicity to the host's antigen-presenting cells. The activation of the host's immune system diminishes the stable transgene-expression level produced by the adenovirus vector in a time-dependent manner.[5,6] Generally, to achieve growth, survival, and transmission in the host, viruses splendidly evade the host's immune system using immune modulators. More than 50 different virus genes have been identified as immune modulators.[7] For instance, viral interleukin-10 (vIL-10), encoding the Epstein-Barr virus (EBV) genome, is highly homologous with human and murine IL-10. It has immunosuppressive properties

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equal to or greater than IL-10, but seems to lack the immunostimulatory properties of human IL-10.[8] vIL-10 was thus one of the key factors of EBV that evaded the immune system in the host. Using adenovirus vector expressing vIL-10, Zuo et al[9] showed prolongation of rat cardiac allograft survival. Furthermore, Efrat et al[10] also showed prolonged survival of pancreatic islet allografts mediated by adenovirus immunoregulatory transgene, early region 3 (E3). In the previous study, we also reported prolonged survival of rat liver allografts after expression of cytokine response modifier A (CrmA), a cowpox virus encoding a serpin-like protease-inhibitor gene using adenovirus vector.[11]

The human and simian immune deficiency viruses (HIV and SIV) encode three signatory retroviral genes (*gag*, *pol*, and *env*) and seven regulatory genes (*tat*, *rev*, *tev*, *vup*, *vpr*, *vif*, and *nef*) that may be associated with the pathogenesis of the virus infection.[12] The *nef* of HIV-1, HIV-2, and SIV seems to be a substantial virulence factor and is critical for the development of AIDS.[13] Although its function in vivo still remains unclear, in vitro studies have well established that it has great implication in cell function. *Nef* is responsible for compromising CD4[14] and MHC class I[15] cell-surface expression, upregulation of FasL[16-19] on the surface of HIV-infected cells, and serious interruptions in different signal transduction pathways.[20-23] These mechanisms might serve as a means to induce a local sanctuary in a host's immune surveillance system.[24] Therefore, the *nef* might be associated with the great evasion of the response of cytotoxic T lymphocytes (CTL) that are a potent antiviral defense mechanism of the host's immune system. Previously, we have reported prolonged survival of rat recipients with a gene transfer of FasL, CrmA, and CTLA4Ig using adenovirus vector.[25-27] However, we have yet to find a satisfactory gene for clinical use of the adenovirus vector. Therefore, we applied the HIV strategy using the *nef* for allotransplantation, to explore *nef*'s own potential for clinical usage. Our results showed that the *nef* effectively prolonged the survival of liver allografts and inhibited infiltration of leucocytes. This indicates a potential therapeutic use of *nef* for protection from cellular damage caused by allorejection.

Experimental Procedures

Cell Culture and Transient Transfections

RPMI1640, Dulbecco's Modified Eagle (DME) medium, and bovine serum were purchased from Sigma Ltd (St. Louis, MO) and Life Technologies, Inc (Rockville, MD). AH44 cells, a rat-hepatocyte cell line[28] (a kind gift from Dr. Fukumoto, Yamaguchi University), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and Kanamycin 75 mg/L. They were subjected to transient transfection with a Lipofectamine Pulus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

Plasmid and Adenovirus Vector

The CD95L-486 plasmid was kindly provided by Dr. Gary A. Koretzky. The plasmid of Cytomegalovirus (pCMV)/nef-containing *nef* sequences were kindly provided by Drs. Y. Koyanagi and N. Yamamoto, Tokyo Medical and Dental University.[29] A recombinant adenovirus vector, AxCANef, was constructed based on the cosmid cassettes-terminal protein complex (COS-TPC) method described previously.[30] AxCALacZ was kindly provided by Dr. I. Saito, University of Tokyo.[31] AxCANef and AxCALacZ were the parental virus genomes. We subsequently propagated the recombinant viruses with 293 cells and stored the prepared vector solution at −80°C.

Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was extracted from isolated by using Isogen (Nippon gene, Tokyo, Japan) as described previously.[32] The quality of the RNA was confirmed on formaldehyde-agarose gels. One microgram total RNA was used for first-strand cDNA synthesis in 20 μL of 100 mM Tris-HCl, 500 mM KCl, 5 mM $MgCl_2$, 1 mM dNTP, 1 U/μL RNase inhibitor, random 9 mers primer, and 0.25 U/μL avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan). The polymerase chain reaction (PCR) amplification was conducted in 100 μL of reaction mixture, containing 200 μM of each of the regular dNTPs, 10 pmol of each primer, and 2.5 U Taq DNA polymerase (TaKaRa). The primers used were Nef (319 bp), 5′-CAGCTGTTGCTG-GACT TACAGG-3′ and 5′-CACAGTTGATGGCTCAT-CATCG-3′; FasL (123bp), 5′-GACTT CACAGAGGAT-ACCC-3′ and 5′-T AAGTTGTTCTTCACAAACTCC-3′; and β-actin (461 bp) 5′-CATCGTGGGCCGCTCTA GGCA-3′ and 5′-CCGGCCAGCCAAGT CCAGACGC-3′. We used the TaKaRa Thermal Cycler 480 PCR system and the hot start technique to increase specificity. The thermal-cycling parameters were denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension for 90 seconds at 72°C. There were 40 cycles. PCR products (10 μL) were analyzed on 1.0% to 1.8% agarose gels. The prominent bands of the correct size were visualized with ethidium bromide staining.

Luciferase Assay

Luciferase activity was assayed in cell-culture lysates using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Luciferase activity, expressed in arbitrary light units, was determined in triplicate for each experimental condition using a luminometer (PerkinElmer Life Sciences Inc, Boston, MA).

Flow Cytometry

MHC class I expression was detected in the AH44 cells through incubation with mouse anti-Nef monoclonal antibody (Advanced Biotechnologies, Inc, Columbia, MD) in phosphate-buffered saline (PBS) for 60 minutes at room temperature (RT), followed by staining for 30 minutes at 4°C

with fluorescein isothiocyanate-conjugated goat anti-mouse Ig antibody (Pharmingen, San Diego, CA). The fluorescent cells were analyzed by a fluorescence-activated cell sorter (FACSort, Becton Dickinson, San Jose, CA) using a 488-nm argon-ion laser. Green fluorescence was monitored through a 530/20 nm band-pass filter.

Western Blotting

Fifty micrograms of cytosolic protein extracts were loaded onto each lane of a 12.5 % sodium dodecyl sulfate-polyacrylamide gel, separated, and then blotted to a nitrocellulose membrane (Bio-Rad, Hercules, CA). We used mouse anti-Nef monoclonal antibody diluted 1:1,000 in PBS to detect Nef protein. A secondary antibody, a horseradish peroxidase-coupled goat anti-mouse Ig antibody (Pharmingen), was incubated at a dilution of 1:1,000 for 2 hours at 4°C. The specific protein complexes were identified using an enhanced chemilaminescence (ECL) substrate chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Animals

Adult male Lewis ($RT1^l$) rats weighing 210 to 250 g were used as recipients, and DA ($RT1^a$) rats, same weights, were used as donors. The animals were maintained under standard conditions and fed rodent food and water, according to the laboratory animal care principles and the guide for the care and use of laboratory animals in our institution.

Liver Cell Isolation

We isolated the rat hepatocytes and nonparenchymal cells using a modifying method, as described previously.[33]

Immunocytochemistry

After cytospin centrifugation, the cells were fixed for 2 minutes with methanol/acetone (1:1) at RT. The cells were then incubated with mouse anti-Nef antibody, diluted 1:150 with PBS in a humidified chamber. The slides were washed with PBS, then incubated for 1 hour with a second antibody (anti-mouse IgG) consisting of alkaline phosphatase–conjugated goat antibody (Santa Cruz Biochemicals, Santa Cruz, CA) diluted at 1/100 in the working solution. Excess antibody binding was removed by washing the sides with PBS. The specific signal was visualized after incubation with Vector Red substrate (Vector Laboratories, Burlingame, CA). Counterstaining was performed with hematoxylin-eosin (HE).

Orthotopic Liver Transplantation

Transplantation surgery was performed on the rats under anesthesia with ether, according to Kamada and Calne's technique.[34] The DA rat liver was flushed with 8 mL of cold lactated Ringer's solution through the portal vein, then removed and preserved in the same solution. The graft was subsequently transplanted into the Lewis rat without reconstruction of the hepatic artery. The day of grafting was considered as day 0 and the day of recipient death as the final day of survival. The recipients were divided into the following three groups: group 1 (control, n = 8), syngeneic control consisting of Lewis-to-Lewis liver grafting; group 2 (allogeneic grafts, n = 12), allografts without any treatment; group 3 (AxCALacZ-transfected allografts, n = 12); and group 4 (AxCANef -transfected allografts, n = 12). There was no significant difference in the periods of graft preservation and portal clamp times among the groups. We injected the AxCA-Nef or AxCALacZ with 1 $\times$ 10^9 plaque forming units (pfu) immediately after orthotopic liver transplantation via the tail vein of recipients.

Specimens

Aside from the recipients for the survival study, three from each group were killed under ether anesthesia on day 5 after grafting, and the liver grafts were removed. The graft blocks up to 1 cm^3 were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Elkhart, IN) and snap-frozen in isopentane; 6-μm frozen sections were then cut in a cryostat for DNA fragmentation analysis and immunohistology.

Immunohistochemistry

The slides with sectioned samples were air-dried and incubated at 4°C overnight with the primary mouse monoclonal antibody specific for rat CD2 (Serotec, Oxford, UK). The slides were then incubated for 1 hour with a second antibody (anti-mouse IgG) consisting of alkaline phosphatase–conjugated goat antibody (Santa Cruz Biochemicals) diluted at 1/100 in the working solution. Color development was performed with an alkaline phosphatase substrate kit. The sections were counterstained with HE.

In Situ Assay for DNA Fragmentation

The detection of apoptotic cells was assayed using an ApopTag Plus In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD), as described previously (32).

Histologic Studies

Liver tissues fixed in 10% phosphate-buffered formalin were embedded in paraffin, and their 5-μm-thick sections were stained with HE for standard microscopy.

Statistical Analysis

The Student's *t*-tests were used to compare paired and unpaired analysis, and *P* values of $< .05$ were considered statistically significant. A statistical evaluation for graft survival was performed using the Kaplan-Meier test.

Results

Generation of a Recombinant Adenovirus Vector Containing HIV-1 *nef* Expression Cassette and In Vitro Expression of *nef* in AH44 Cells Infected With AxCANef

Recombinant adenoviral vectors expressing *nef* (AxCA-Nef) were generated based on the COS-TPC method described previously.[30] Briefly, a cosmid containing an

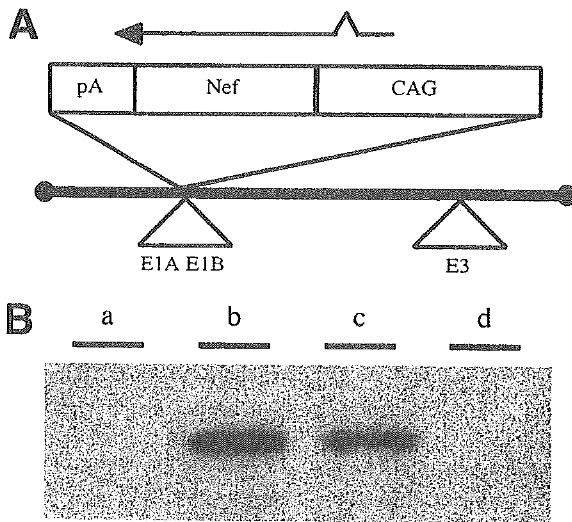


Figure 1. Recombinant adenoviral genome structure and in vitro expression of *nef* in AH44 cells.

(A) Schematic of recombinant adenoviruses containing a Nef expression cassette. The expression cassette is inserted in the adenoviral E1 region, from right to left in AxCANef.

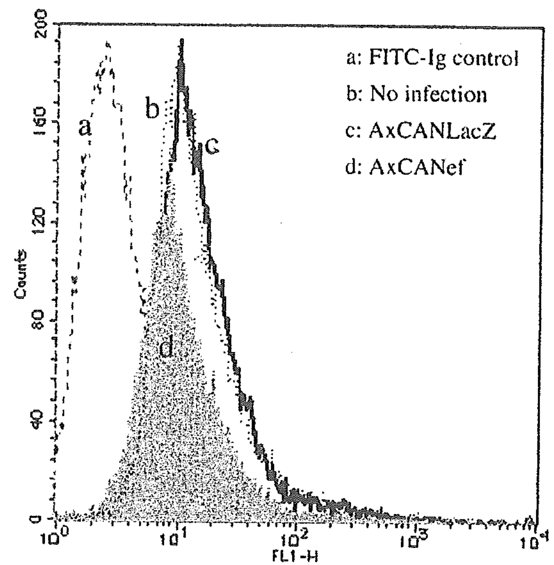
(B) Western blotting detection of Nef in AH44 cells infected with nef-expressing vector. Lane a, uninfected. Lane b, AxCANef (MOI. 1.0). Lane c, pCMV/nef. Lane d, AxCA-LacZ (MOI. 1.0).

expression cassette coding for the *nef* cDNA[29] under the control of the CAG promoter[35] was constructed. Subsequently, 293 cells were cotransfected with the cosmids and the adenovirus DNA-terminal protein complex, digested at several sites with *Eco*T22I.[31] A recombinant adenovirus, AxCANef, was generated through homologous recombination in the 293 cells. The genomic structure of a recombinant adenovirus vector, AxCANef, is shown in Figure 1A. After AH44 cells were infected with AxCANef, the expression of the *nef* protein was detected by Western blotting, using an anti-nef antibody (Fig. 1B).

Regulation of Surface MHC Class I and FasL Molecules in Nef-Expressing Cells

In lymphoid cells, although surface expression of MHC class I was reduced in stable expressed *nef*, no reports indicated reduction of MHC class I expressed *nef* in hepatic cells. Furthermore, no study reported that *nef* downregulated MHC class I expression using HIV-1 $_{\text{JR-CSF}}$ strain. We analyzed surface expression of the MHC class I on AH44 hepatic cells using flow cytometry. The MHC class I molecules on the surface of the AxCANef-infected AH44 cells were reduced compared with control cells (Fig. 2A.)

To determine whether Nef $_{\text{JR-CSF}}$ expressed in AH44 cells upregulated FasL expression, we cotrans-



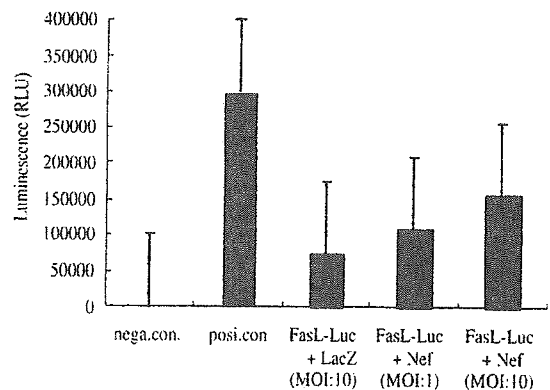


Figure 2. AdexNef infection regulated the MHC class I and FasL expression.

(A) Downregulation of the MHC class I molecular expression after AdexNef infection in AH44 cells. Surface expression of MHC class I was analyzed by cytofluorimetry. a: FITC-Ig (2nd antibody) control. b: Uninfected. c: AxCANef. d: AxCALacZ.

(B) Upregulation of FasL transcription by Nef. Untreated AH44 cells (nega. cont.; negative control: 1) Transient cotransfection of a FasL promoter/luciferase reporter with an AxCANef (MOI: 10) into Jurkat cells (posi. cont.; positive control), AxCALacZ (MOI: 10) FasL-Luc.+LacZ (MOI:10); negative control; 2) AxCA-Nef (MOI:1), FasL-Luc.+Nef (MOI:1), AxCANef (MOI: 10), and FasL-Luc.+Nef (MOI:10) into AH44 cells. The expression of the reporter gene was determined as described in Experimental Procedures. Bars indicate the mean ± SD of three experiments.

fected CD95L-486 plasmid, which contains a luciferase reporter gene, under the transcriptional control of the FasL promoter,[36] and AxCANef to AH44 cells. Using this method, we indirectly monitored FasL transcription by measuring luciferase activity after Nef expression. As shown in Figure 2B, the AH44 cells expressing *nef* increased the luciferase activity in an multiplicities of infection-dependent manner, indicating that FasL transcription may be upregulated by Nef expression.

In Vivo Expression of Nef in Rat Liver Cells Infected With AxCANef

When we injected a rat intravenously with AxCANef, the expression level of the Nef was detected in the gene-transfected tissues using Western blotting (Fig. 3A). The Nef was expressed for at least 28 days in rat liver. Either isolated hepatocytes or nonparenchymal cells from the AxCANef-infected rat were expressed Nef, although the level of the expression was different (Fig. 3B). Furthermore, administering AxCANef at 10^9 pfu in the rat positively stained approximately 60% of the hepatocytes and nonparenchymal cells (Fig. 3C).

Reverse Transcriptase–PCR Detection of the FasL Expression After Being Infected With AxCANef In Vivo

Next we discuss the function of the *nef* gene as it induced the FasL expression. The isolated hepatocytes and nonparenchymal cells from the AxCANef–infected rat were subjected to reverse transcriptase (RT)–PCR. As shown in Figure 4, top column, both hepatocyte and nonparenchymal cells isolated from the AXCANef-infected rat expressed *nef*, whereas control virus-infected cells were not expressed. Moreover, consistent with in vitro data, we found that FasL was upregulated after *nef* was expressed in hepatocytes and nonparenchymal cells (Fig. 4, middle column).

Recipient Survival Time After Liver Grafting

As shown in Table 1, all recipients with syngeneic grafts survived indefinitely. The median survival time in allogeneic grafts that were not infected by any adenoviral vector

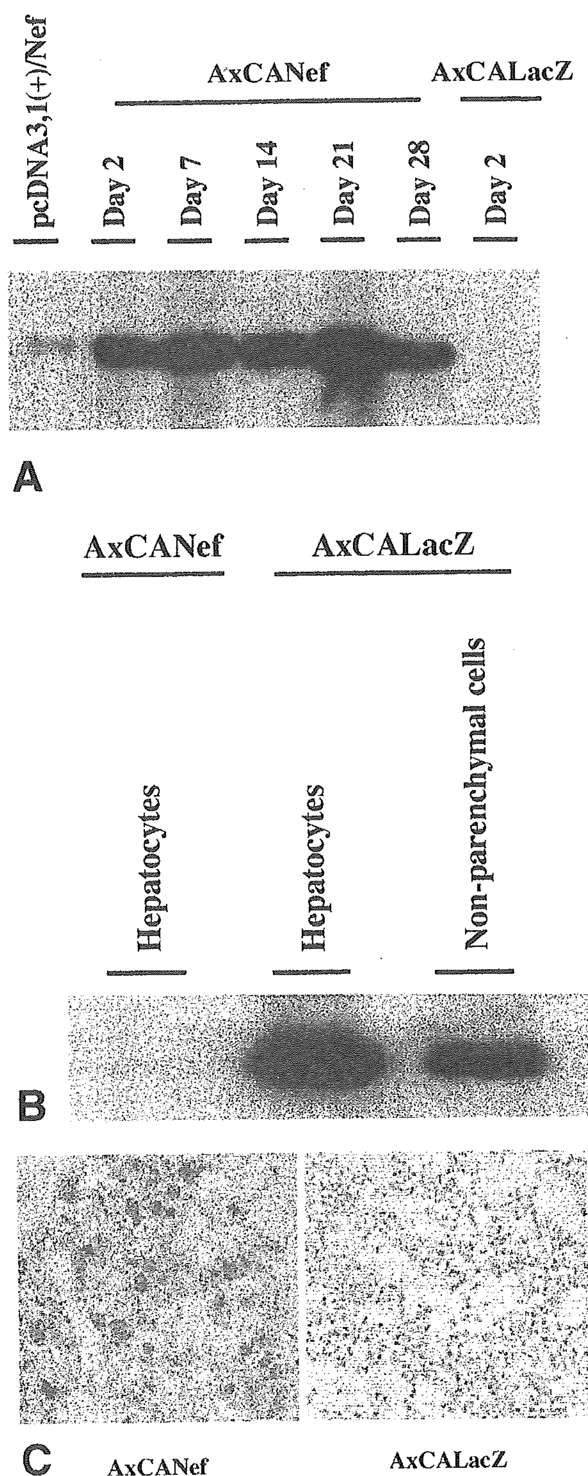


Figure 3. Detection of Nef in the liver tissues and isolated rat liver cells from AxCANef-infected rats.

(A) *Nef* expression on liver tissues was detected by immunoblotting in the days after infection with AxCANef. AH44cells transfected with pcDNA3.1(+)/*Nef* were used for positive control, and liver tissues infected with AxCALacZ were used for negative control. Hepatocytes and nonparenchymal cells were isolated from AxCANef-infected rats.

(B) *Nef* expression on either hepatocyte or nonparenchymal cells was detected by Western blotting.

(C) Immunocytochemical study of Nef. (C-a) Isolated rat hepatocytes expressing Nef, indicating that 50% to 60% of hepatocytes are positive after adenovirus infection. (C-b) Isolated rat nonparenchymal cells were also confirmed by an anti-Nef antibody. We injected 10^9 pfu of AxCANef or AxCALacZ via the rat tail vein.

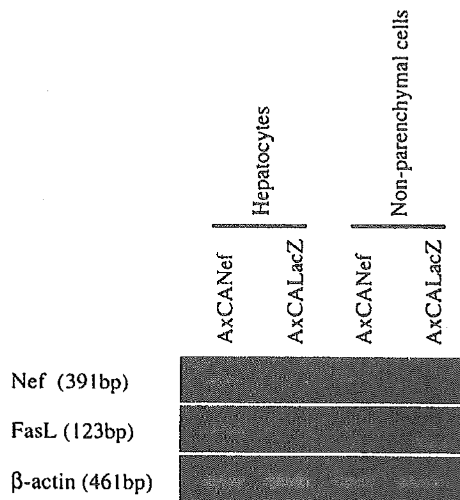


Figure 4. RT-PCR for Nef and FasL mRNAs in the isolated rat liver cells from AxCANef-infected rats. Representative data show the intensity of RT-PCR products obtained from hepatocyte and nonparenchymal cells in each AxCANef-infected or AxCANLacZ-infected rat. The AxCALacZ-infected cells showed no expression of mRNAs for Nef and FasL. Data are representative of three independent experiments.

was 11 days (range, 10 to 15 days), and in control grafts infected by AxCALacZ it was 11 days (range, 10 to 12 days). However, the survival times of recipients infected by AxCANef were significantly prolonged ($P < .001$) to 14.5 days in median (range, 12 to 30 days).

HE and Immunohistochemical Staining

The graft biopsy samples on day 5 after grafting were stained with HE and examined with light microscopy. We observed extensive infiltrated mononuclear cells and severe hepatocyte destruction in the control rats (Fig. 5A). Furthermore, we stained CD2 using mouse monoclonal antibody for the rat T cells. Immunohistochemical staining showed that CD2-positive cells infiltrated the graft (Fig. 5C). In AxCANef-injected recipients, however, hepatocyte destruction was reduced and infiltration of monocytes including CD2-positive cells was improved (Figs. 5B to 5D).

In Situ Visualization of Apoptosis in the Grafted Liver

The TUNEL analysis was performed in the grafts on day 5 after transplantation. As shown in Figures 5E to 5H, the ratio of apoptotic cells was higher in the AxCA-Nef-injected grafts than in the control allografts.

Discussion

Targeted gene expression using adenovirus vectors has been achieved in the liver at a high level,[4,37] suggesting that it can be a useful means of in vivo gene transfection in liver transplantation. However, a major disadvantage of adenovirus vectors has been the low-level production of viral late proteins and the subsequent host immune responses against these protein antigens, resulting in the limited persistence of transgene expression.[38,39] For that reason, adenovirus-mediated gene therapies with some immunoregulatory genes were able to achieve a prolonged gene expression period.[39,40] Recently, we and other investigators indicated that adenovirus-mediated gene therapy with CTLA4Ig resulted in indefinite survival in the recipient of a rat liver allograft.[25,40] Its mechanisms may involve blockage of costimulatory signals between B7 on antigen-presenting cells and CD28 on activated CTL, inducing the anergy. Furthermore, we found prolonged survival of the FasL and/or CrmA transfected rat liver allografts using adenoviral vector.[11,26]

Purgus et al[41] reported an interesting case involving a renal transplant patient with a long-term nonprogressive HIV-1 infection and who was asymptomatic despite sustained immunosuppression. It has suggested that it is a possibility to be able to use HIV-1 genes for immunosuppressive therapy. Our purpose in this study is to use the HIV strategies of immune evasion for transplantation; in other words, to apply this genetically engineered CTL immune escape (induced by HIV *nef*) in adenovirus vector to a rat liver allograft.

Table 1. Prolongation of the Survival of the HIV-1 Nef-Expressed Liver Allograft in the Rat

Group	Graft Survival (d)	Median (d)
G-1 (syngeneic)	>100 × 8	>100
G-2 (control allograft)	10 × 3, 11 × 4, 12 × 3, 13, 14	11
G-3 (LacZ-treated allograft*)	10, 11 × 6, 12 × 5	11
G-4 (Nef-treated allograft*)	12 × 2, 14 × 4, 15 × 2, 16 × 3, 25, 30	14.5†

*Reciepients were administered 1 × 10^9 pfu AxCALacZ or AxCANef via tail vein.
†The recipient's survival in Nef-expressed allografts was significantly prolonged compared with LacZ-treated allografts ($P < .001$).

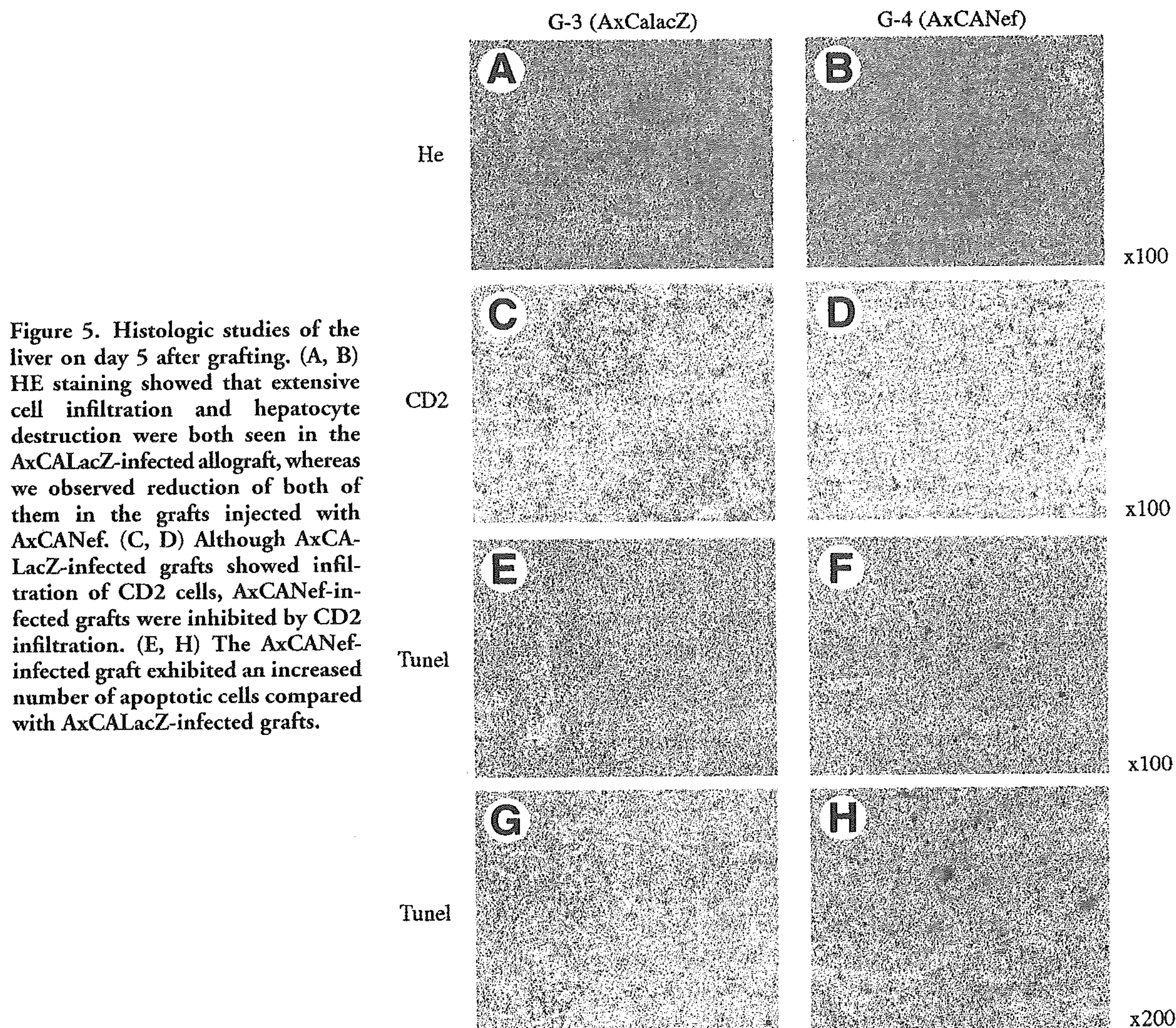


Figure 5. Histologic studies of the liver on day 5 after grafting. (A, B) HE staining showed that extensive cell infiltration and hepatocyte destruction were both seen in the AxCALacZ-infected allograft, whereas we observed reduction of both of them in the grafts injected with AxCANef. (C, D) Although AxCALacZ-infected grafts showed infiltration of CD2 cells, AxCANef-infected grafts were inhibited by CD2 infiltration. (E, H) The AxCANef-infected graft exhibited an increased number of apoptotic cells compared with AxCALacZ-infected grafts.

Our in vitro studies showed that *nef* downregulates MHC class I in hepatic cells. Accordingly, a part of the immunosuppressive effect was thought to be a downregulation of MHC class I in graft liver cells. Downregulation of MHC class I was one of the important factors in HIV-1–mediated immune escape.[42] Although we did not try Nef-mediated downregulation of MHC class I in antigen-presenting cells in the liver, De et al[43] showed that Nef does not downregulate MHC class I in macrophage. Furthermore, MHC class I was not downregulated in dendritic cells.[44] However, Andrieu et al[45] showed that Nef downregulates MHC class I surface expression on human dendritic cells, impairing presentation to HIV-specific CD8+ cells. These two reports contradict each other. Therefore, the role of *nef* that downregulates MHC class I in dendritic cells needs more clarification.

Downregulation of MHC class I was a passive protection strategy of *nef*. In the contrast, FasL expression was thought to be positive protection.[17-19] In our in vitro and in vivo studies, FasL expression was shown in either hepatocytes or nonparenchymas. Because Nef expression in rat liver can be detected for at least 28 days, we speculated that the upregulation of the FasL mRNA would be maintained for the same time, although we did not confirm it. Previously, we and other investigators reported that exogenous FasL expression on the allograft effectively prolonged survival of rat liver[26,27] and renal[46] grafts, which correlated with an increased apoptosis of infiltrated cells and reduction of arteritis. In our rat liver allograft model, that FasL expression in the allograft with plasmid vector[27] and adenoviral vector[26] prolonged survival longer than the control vector-treated allograft. Furthermore, we showed that a FasL-expressing graft induced apoptosis in infiltrated cells. In that study, double-staining with the graft sections for T lymphocyte and the TUNEL assay showed that the apoptotic cells was clas-

sified as T lymphocyte. In the present study, more apoptosis was observed in the AxCANef-injected allografts than in the control graft. Although further clarification is needed to identify the cell type as being apoptosis, the previous reports[26,27] and histologic and RT-PCR data suggested that the infiltration cell apoptosis was induced by FasL expression in the graft, and it might be a mechanism of prolonging the allograft survival.

In this study, we did not investigate whether MHC downregulation or Fas-L upregulation is the means by which AxCANef transfection prolongs allograft survival. We now try to construct two types of Nef which were not downregulated MHC class I and one that was not upregulated FasL expression, in an attempt to find the causes of allograft survival by AxCANef.

Using immunomodulatory molecules, prolonged survival of allograft may be achieved in human beings. However, adenovirus vector was contained by antigenicity for the host and removed by the immune system from host. Therefore, improvement of adenovirus vector was needed for clinical application.

The transplant setting can offer a unique opportunity for ex vivo whole-organ transduction before as well as after donor procurement. We attempted to transfect adenovirus vector using ex vivo a clamp technique by trapping vector perfusate within the liver graft during the cold preservation period. The ratio of gene-transfected cells was comparable with transferred cells via a donor tail vein 3 days before transplantation (unpublished data). Therefore, there is some possibility of pretreatment of the donor liver by adenovirus vector.

Although in this study we did not try ex vivo perfusion in hepatectomized liver, we attempted to transfect adenovirus in the partial hepatectomized (70%) liver via tail vein. The ratio of gene transfected cells was equal to or greater than whole liver because of the tissue mass (unpublished data). These results indicated that hepatectomized liver was also able to transfect exogenous genes by an adenovirus vector satisfactorily.

In summary, we successfully constructed a recombinant adenovirus expressing the HIV-1 *nef* gene and identified Nef expression in hepatic cells in vitro and in rat liver cells after they were infected with AxCANef in vivo. *Nef*-expressing cells were downregulated as the expression of the MHC class I molecularly, and FasL expression was upregulated transcriptionally. The rat liver allograft with Nef expression had an effectively prolonged survival because of its function in the downregulation of the MHC class I and upregulation of the FasL expression compared with the control graft. These results suggest that the *nef* gene may be a potent candidate for therapeutic use in clinical patients undergoing immune-mediated allograft rejection.

Acknowledgement

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■特集：家族性腫瘍の若年者診断の倫理的問題

小児の遺伝学的検査の特徴

小須賀基通*　奥山虎之*

小児の遺伝学的検査は，先天性遺伝性疾患に対する確定診断を目的に行われることが多い．しかし，疾患によっては小児の保因者診断，発症前診断を行うことも可能である．また，小児の遺伝学的検査の結果をもとにして発症していない家族やクライアントについての保因者診断，発症前診断，または次子の出生前診断などが可能となる．このような問題に関して，遺伝医療に従事する者は小児の遺伝学的検査においては小児の被検者の利益の保護に立った立場から，検査適応の是非，年齢や理解度に応じた本人に対する遺伝カウンセリングやインフォームド・コンセント，自由意思の確認や自己決定の尊重をするよう心がけねばならない．さらには親による代諾の是非，代諾の場合における結果の本人への告知の時期・方法，診断結果の本人以外の家系内への影響の考慮についても慎重に配慮しなければならない．

キーワード：確定診断，出生前診断，発症前診断，保因者診断，代諾

I.　はじめに

遺伝学的検査とは，遺伝性疾患の診断のためにDNA・RNA・染色体・蛋白・代謝産物などを解析，測定する検査である．近年，多くの遺伝性疾患の病態が解明され，責任遺伝子が同定されると同時に，遺伝学的検査の技術の開発が進み，遺伝学的診断は飛躍的な進歩を遂げている．それと同時に遺伝学的診断における遺伝カウンセリングの必要性，診断によって得られた個人の遺伝子情報や診断に用いた生体試料の取り扱い，およびその他の倫理的，法的，社会的問題について慎重に検討すべき問題も数多く生じてきた．これまでに日本人類遺伝学会からは「遺伝カウンセリング・出生前診断に関するガイドライン (1994)」[1]，「遺伝性疾患の遺伝子診断に関するガイドライン (1995)」[2]，「遺伝学的検査に関するガイドライン (2000)」[3] が提案され，家族性腫瘍研究会からは「家族性腫瘍における遺伝子診断の研究とこれを応用した診療に関するガイドライン (2000)」[4] が提案されてきた．平成15年8月には遺伝医学関連団体である10学会および研究会が，『遺伝学的検査に関するガイドライン』[5] を発表し，これを遵守して遺伝学的検査を行うよう提言している．一般にヒト生殖細胞系列における遺伝子変異もしくは染色体異常以外の，たとえば癌などの体細胞に限局し次世代に受け継がれることのない遺伝子変異や染色体異常の検査，細菌・ウイルスなどの病原体の検査などは遺伝学的検査に含まれない．

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II.　小児の遺伝学的検査

小児の遺伝学的検査は，一般には先天的に遺伝性疾患を発症した患者の確定診断のために行われることが多いが，その遺伝学的検査の情報をもとにして発症していない家族やクライアントについての保因者診断，発症前診断，または次子の出生前診断などが行われることもある．『遺伝学的検査に関するガイドライン』[5] では，遺伝医療に従事する者は，これらの小児の遺伝学的検査についても，小児の被検者の利益の保護に立った立場から，検査適応の是非，年齢や理解度に応じた本人に対する遺伝カウンセリングやインフォームド・コンセント，自由意思の確認や自己決定の尊重，親による代諾の是非，代諾の場合における遺伝学的情報の本人，あるいは本人以外への開示の時期・方法，診断結果の本人以外の家系内への影響に対する考慮について，慎重な配慮を心がけるよう提言している．

III.　出生前診断

遺伝学的検査の技術は胎児の遺伝性疾患の診断にも応用されるようになったが，出生前の遺伝子診断の適応となる疾患については厳密な決まりはない．日本産婦人科学会や日本人類遺伝学会はおのおのガイドライン[1,6] を発表し，これに準拠して出生前診断における遺伝学的検査を行うよう提言している．出生前に遺伝学的検査を行う場合，発端者の病名が明らかになっているだけでは充分ではなく，遺伝子変異部位が同定されているか，診断に利用できる遺伝子多型が明らかになっていることが必要である．胎児の染色体やDNAを採取するためには一般に羊水検査と絨毛検査が行われる．羊水検査は妊娠15～18週に行われ，羊水細胞を2～3週間培養してから染色体検査や遺伝子検査を行うため，最終的な結果が得られるまでに時間がかかるこ

とが多い．絨毛検査の適応は原則的に羊水検査と同じであるが，妊娠9～11週から採取でき，培養をしなくても十分なDNA量が得られるため，羊水検査より早期診断が可能である．以前は，手技的に困難であり絨毛採取後の流産率も若干高いことから，遺伝的にハイリスクな症例に行われることが多かったが，熟練した医師が行えば流産率も自然流産率と変わらなくなっている．また，最近では母体末梢血液から胎児由来細胞を採取することも可能となってきている．一般に出生前診断の対象疾患は，疾患の重篤度，治療方法の有効性，診断の精度，夫婦の環境などを考慮して決められるべきである．また遺伝学的検査前後の遺伝カウンセリングの実施，診断によって得られた個人の遺伝子情報や生体試料の取り扱いなどにも当然，十分な配慮が必要である．

本稿では小児期の遺伝学的検査および出生前診断において考慮すべき点について，いくつかの疾患を具体的にあげて解説する．

Duchenne型筋ジストロフィー

Duchenne型筋ジストロフィーは，ジストロフィン蛋白の異常により発症する進行性の筋疾患である．新生児・乳児期には目立った症状は認めないことが多く，2～4歳頃から走れない，転びやすい，階段の昇降困難など歩行に関する異常が認められるようになり，さらに進行すると10歳前後で歩行不能となり，20歳前後で人工呼吸器が必要となることが多い．本症の頻度は男児出生4000人に1人，人口10万人に3～5人といわれている．X連鎖劣性遺伝形式をとり，一般的に遺伝子変異を有していても発症しない保因者である母親から，子どもに遺伝子変異が伝わる．その場合，男児は50％が正常で，50％が罹患し，女児は50％が正常で，50％が保因者である．保因者である母親は一般に症状は認めず，血清CK値の軽度上昇を認めることがある．ジストロフィン遺伝子はX染色体短腕（Xp21.2）に存在しており，2300 kbのサイズで，79のエクソンからなる．本疾患の遺伝子異常は，ジストロフィン遺伝子の欠失が50～60％，重複が約10％で，残りの30～40％は点変異である．ジストロフィン遺伝子の欠失の遺伝子診断はmultiplex PCR，定量的サザンブロット法，SSCP法（single strand conformational polymorphism），直接シークエンス法，変異遺伝子異常により合成された異常蛋白を解析するPTT（protein truncation test）などの方法が行われる．これらの方法により約90％の症例の遺伝子異常が検出可能である．本症の約1/3は突然変異による発症であるが，約2/3の症例は母親からのジストロフィン遺伝子変異を受け継いだと考えられる．したがって児が本症と診断された場合，母親が保因者である可能性を考慮しなければならない．母親が保因者であった場合，発症者の同胞女児が保因者である可能性，母親の家系内における保因者の存在の可能性，次子の発症の可能性といった影響が生じてくる．小児のDuchenne型筋ジストロフィーの遺伝学的検査を行う際は，遺伝カウンセリングによりこれらの点をよく留意すべきである．

筋強直性ジストロフィー

筋強直性ジストロフィーは常染色体優性遺伝形式をとり，19番染色体長腕（19q13）に存在するMT-PK（myotonin protein kinase）遺伝子の3'側非翻訳領域にあるCTGの3塩基の繰り返し配列の増加が原因である．正常人ではCTGの繰り返し配列は5～30回程度だが，患者では50～2000回にも達する．このリピート数と臨床症状は相関し，また親から子，子から孫へと世代を経るにしたがってこのリピート数が増加して症状が重くなる表現促進（anticipation）が認められる[7]．成人における臨床症状は，収縮した筋肉が弛緩しにくくなるミオトニア現象や筋力低下・筋萎縮による特異顔貌，眼瞼下垂，嚥下障害などがみられ，筋症状に加えて白内障，心病変，中枢神経病変を伴うことがある．筋強直性ジストロフィーの母親から生まれた子どもに新生時期から重度の筋強直低下と中枢神経症状をみることがあり，先天性筋強直性ジストロフィーと呼ばれる．この場合，CTGのリピート数は，親より子どものほうが圧倒的に増加している．先天性筋強直性ジストロフィーの患児が出生するのは，母親が筋強直性ジストロフィーの罹患者であることがほとんどで，父親が罹患者の場合は先天性筋強直性ジストロフィーの患者が生まれることは極めて稀であることが知られている．したがって，臨床所見から先天性筋強直性ジストロフィーを疑い，児の遺伝子検査を行う場合は，病識や臨床症状のない親の診断にもつながるので慎重にすべきである．まず両親，特に母親についてもよく観察し，罹患者あるいは保因者である可能性についても検討し，必ずこれらの点に考慮した十分な遺伝カウンセリングを行ってから児の遺伝子診断に臨むべきである．その場合，親の症状は軽いので白内障や心伝達異常等の精査などのメリットがあることも強調すべきである．また本症に対する出生前遺伝子診断を検討する際には，先天性筋強直性ジストロフィーの患児は出生児期の呼吸・哺乳障害を克服すれば生命予後・機能的予後はDuchenne型筋ジストロフィーや福山型筋ジストロフィーに比べるとはるかによいことなども考慮しなければならない．

軟骨無形成症

軟骨無形成症は，常染色体優性遺伝形式をとり4番染色体長腕（4p16.3）に存在するFGFR3（fibroblast growth factor receptor 3, 線維芽細胞増殖因子受容体3型）遺伝子の変異が原因である．臨床症状は，軟骨内骨化の異常による四肢短縮の小人症と長管骨・短管骨の短縮により引き起こされる神経学的，歯科的，耳鼻科的合併症であるが，一般に生命予後，知的予後は良好である．軟骨無形成症の約80％は健常両親からの新生突然変異であるが，両親のいずれかが本症の場合は浸透率がほぼ100％であるため，50％の確率で児に発症する．患者の約97％においてはFGFR3遺伝子における遺伝子変異が同一であるため[8,9]，遺伝子診断は比較的容易である．小児期には臨床像，全身の骨レントゲン所見および遺伝子診断で診断が可能である．出生前診断の場合においても，胎児期の超音波所見と遺伝子診断とを合わせると正確な確定診断が可能である．しかし，本

症は生命予後，知的予後は良好であるため，出生前診断は倫理的に問題があるという指摘もある．

カルバミルリン酸合成酵素I欠損症

カルバミルリン酸合成酵素I欠損症は，尿素サイクルの酵素の一つであるCPSI（Carbamoylphosphate Synthetase I）の欠損によって引き起こされる高アンモニア血症の一つである．新生児期発症の場合，生後数日から哺乳力不良，嘔吐，高アンモニア血症，さらに意識障害，痙攣などの症状を起こす予後不良の疾患である．本疾患は常染色体劣性遺伝形式をとり，CPSI遺伝子は2番染色体長腕（2q35）に存在し[10]，いくつかの変異遺伝子がすでに報告されている[11,12]．

本センターにおける本症の出生前診断の症例を提示する．第1子は，出生直後の高アンモニア血症などから臨床的に新生児型CPSI欠損症と診断され，その後，早期に死亡している．患児のCPSI遺伝子を解析したところ，一方のアレルは1529delGのためフレームシフトを起こしており，もう一方のアレルはC2161Tの遺伝子変異のためアルギニンがストップコドンに変化しているナンセンス変異となっており，患者のCPSI遺伝子はこれらの複合ヘテロ接合体であることがわかった．さらに両親のCPSI遺伝子の解析を行ったところ，父親は一方のアレルに1529delGの遺伝子変異を持ち，母親の一方のアレルはC2161Tの遺伝子変異を持ったヘテロ接合体，すなわち健康保因者であることが判明した（ここでいう保因者は遺伝子変異を有しているが，将来にわたってそれに起因する疾患の発症のないものをいう）．その後，これらの遺伝学的情報を基に，次子の妊娠時に出生前診断を行った．絨毛細胞から得られたDNA検体の遺伝子検査から，胎児は父親と同じ1529delGのヘテロ接合体であり，罹患者ではなく健康保因者であることが判明し，その後，出生に至った（Fig.1, Fig.2）．本件においては，第1子の遺伝学的検査により，患児の確定診断がなされ，次に両親の保因者診断がなされた後に，さらにこれらの結果が出生前診断に行われたケースである．

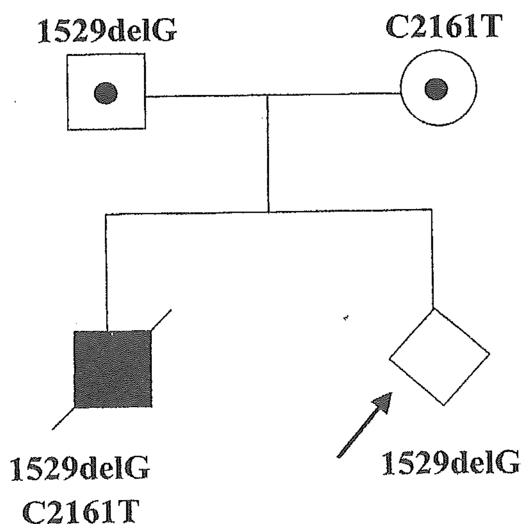


Fig.1. Patient pedigree of the family with CPS1 deficiency.
発端者である第1子は1529del GとC 216Tの複合ヘテロ接合体でCPSI欠損症を発症した．父は1529del Gのヘテロ接合体で，母はC 216Tのヘテロ接合体であった．今回，第2子の出生前遺伝子検査を行い，父と同様の1529del Gのヘテロ接合体であった．

21水酸化酵素欠損症（21-OHD）

21水酸化酵素欠損症（21-OHD）は，副腎における糖質コルチコイドおよび鉱質コルチコイド合成に必要な21水酸化酵素（P450 c21）の欠損による先天性副腎過形成症の一つである．21水酸化酵素が欠損した結果，コルチゾールおよびアルドステロン産生の障害と，アンドロゲンの過剰分泌が起こる．コルチゾールおよびアルドステロンの産生が障害されることにより，新生児期から低血糖，哺乳不良，血圧低下，電解質異常，循環障害，ショックなどが起こる．また，アンドロゲン過剰分泌によって出生時より女児外性器の男性化（陰核肥大，陰唇の癒合）が起こり，一方，男児においては成長促進，早期の男性化を起こす．本症は常染色体性劣性遺伝形式をとり，21水酸化酵素遺伝子（CYP21）は6番染色体短腕上に，その偽遺伝子（CYP21P）とともに存在している．遺伝子解析によりCYP21遺伝子の欠失の割合は欧米では15～30%，本邦では10～15%と報告されている．遺伝子の欠失が認められない症例では，CYP21遺伝子からCYP21P遺伝子への変換，点突然変異などが同定されており，本症の約90%は遺伝子検査で診断可能となっている[13]．本症は新生児マススクリーニングの対象疾患であり，ほとんどの症例が早期に発見され，治療が行われるようになった．しかし胎児期にアンドロゲンに過剰暴露されることにより，すでに女児の外性器の男性化や胎児脳の性分化障害が進行していることから，現在では出生前診断で本症と診断されれば積極的に胎児期の治療が行われるようになった．出生前診断と胎児治療の実際としては，すでに21-OHDと診断された児を持った母親が次児を妊娠した場合が対象となる．児の妊娠が確認された時点から母体にデキサメサゾンを投与する．絨毛採取によりDNAを抽出し，まずFISH法により性別を確認し，男性と診断した場合は母体へのデキサメサゾン投与を中止する．胎児が女性の場合，その後の遺伝子解析により本症と診断された場合は妊娠末期までデキサメサゾン投与を継続する．胎児治療による有効性は75%程度と報告されている．本症は比較的頻度が高く，出生前診断により有効な胎児治療が可能な数少ない疾患であるため，出生前診断に対する倫理的な問題は少ないと考えられる．

IV. おわりに

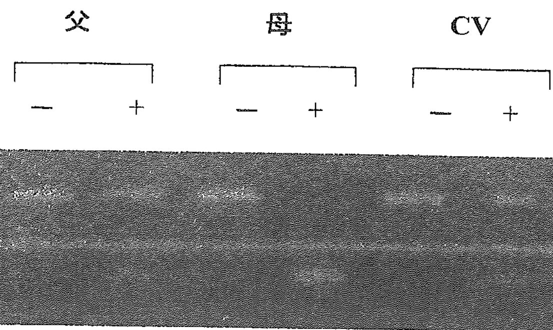
「遺伝学的検査に関するガイドライン」においては，遺伝性疾患の診断は高い倫理的配慮と遺伝カウンセリングの充実などの条件下に極めて慎重になされるべきであると提言されている．特に（1）検査を受ける人（被検者）の人権を尊重・保護する倫理的配慮，（2）診断的検査技術の精度の向上，診断自体の正確性と客観性，（3）インフォー

1529delG（父親由来の変異）の検出

Hae III **Digestion**

287bp （Mutant） →

146+141bp（Normal） →



C2161T（母親由来の変異）の検出

BsmAI **Digestion**

148 bp (Mutant) →

116 bp (Normal) →

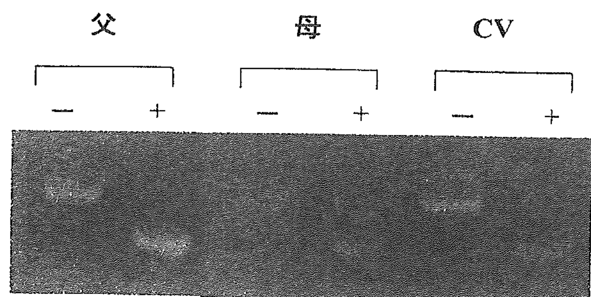


Fig.2. Detection of the 1529del G and C 216T mutations by PCR-RFLP.
胎児絨毛細胞（CV）を用いてPCR-RFLPを行った．1529delGの保因者（父親とCV）の場合，287bpのPCR産物は制限酵素HaeIIIで切断されず287bpのままである．点変異のない場合（母親）は287bpのバンドが146bpと141bpに切断される．C216Tの保因者（母親）の場合，148bpのPCR産物は，制限酵素*BsmAI*により116bpと31bp（検出されず）に切断されず148bpのままである．点変異のない場合（父親とCV）は148bpのバンドが116bpと31bpに切断される．したがってCVは1529delGのみの保因者である．

ムド・コンセントの実施，(4) 医療・倫理・検査技術に関する責任体制の確立，ならびに (5) 検査前後の遺伝カウンセリングの実施，などに留意して行われるべきであることが明記されている．小児の遺伝学的検査において特に重要となるのは，(1) (3) (5) についてである．またアメリカ人類遺伝学会は，1995年に「Points to Consider: Ethical, Legal, and Psychosocial Implications of Genetic Testing in Children and Adolescents」の声明を発表し，小児の遺伝学的診断に従事する者は子どもの利益と親・家族の利益の双方について慎重に検討しなければならない．さらに，子どもに最良の医療を提供するために医学的・心理社会的・生殖に関する諸問題を家族とともに考慮すべきである．また遺伝学的検査から得られた情報の影響力と遺伝学的検査の今後の利用拡大を踏まえると，遺伝学的診断に従事する者は小児に対する遺伝学的検査の是非における遺伝カウンセリングを行ううえでますます重要になる，と結論づけている．今後も，遺伝学的検査の必要性は高まっていくと考えられるが，これらの提言を遵守して，小児の遺伝学的検査の実施に望むべきである．

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The Characteristic of Genetic Testing for Children with Inherited Genetic Diseases

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Genetic testings for children with inherited genetic diseases could be done for the purpose of the diagnosis, carrier diagnosis and the presymptomatic diagnosis. Moreover, the carrier diagnosis, presymptomatic diagnosis for a family or a client whose symptoms have not appeared yet and prenatal diagnosis could be performed using the genetic information. Our medical staffs that treat genetic issuers have to try to carry out proper genetic counseling, informed consent for protect rights of children and fetus.

Key words : prenatal diagnosis, presymptomatic diagnosis, carrier diagnosis

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