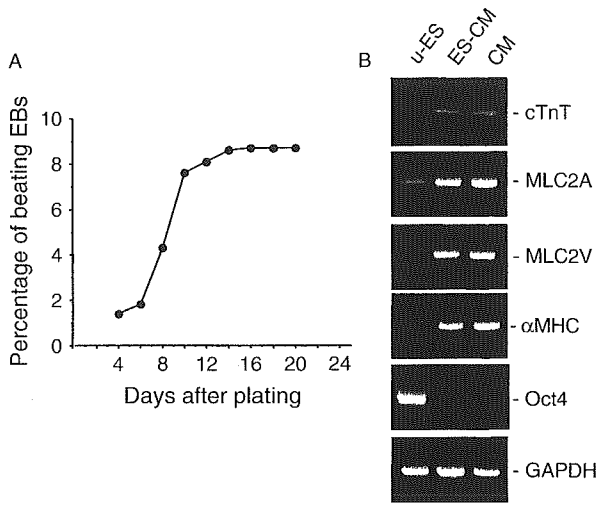

Figure 1. Differentiation of cardiomyocytes from cynomolgus ES cells. (A) Cumulative ratio of EBs derived from cynomolgus ES cells containing spontaneously contracting areas during differentiation. (B) Expression of specific cardiac markers. RNA samples from undifferentiated ES cells (u-ES), contracting area of EBs (ES-CM), and cynomolgus monkey cardiac tissues (CM) analyzed by RT-PCR for the expression of cardiac-specific markers: cTnT, MLC-2A, MLC-2V and αMHC. Oct-4 is undifferentiated ES cell marker. GAPDH served as internal standard

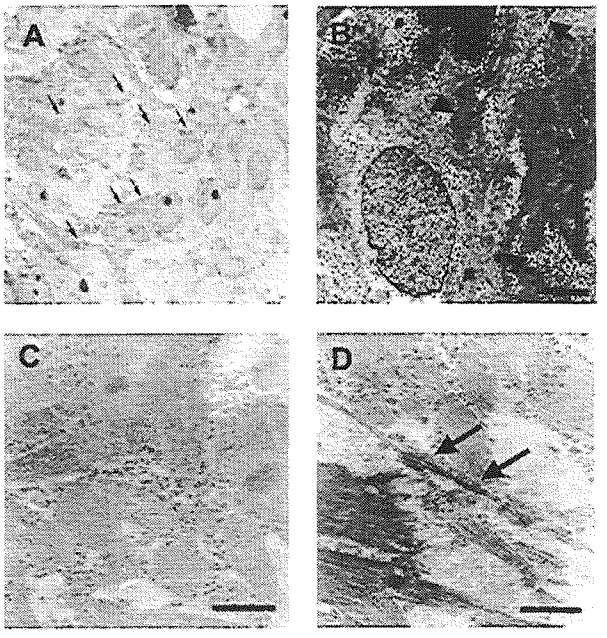

Figure 2. Morphological analysis of contracting EBs 14 days after plating. (A) High power light microscopy image stained with toluidine blue. Myofibers were observed (arrows). Magnification x1000. (B) Low power transmission micrograph revealed sarcomeric organization (arrowheads) and desmosomes (arrows). Scale bar: 2 μm. (C, D) High power transmission micrograph. Sarcomeric organization (C) and desmosomes (D, arrows) are evident. Scale bar: 500 nm

or CV-11 974 (Figure 3). We also confirmed the expression of cTnI and cardiac myosin in the contracting EBs by immunohistochemical staining (Figures 4B and 4E). Taken together, these results indicate that cynomolgus ES cells differentiate into cardiomyocytes *in vitro*.

Transgene expression with a SIV-based lentiviral vector

We used SIV vectors encoding the EGFP gene under the control of the CMV promoter to examine gene transduction in cardiomyocytes derived from ES cells. We detected EGFP expression in cultures by 5 days and this was maintained for at least 28 days (data not shown). Most staining of the transduced cells overlapped with cTnI or cardiac myosin (Figures 4C and 4F). The ratio (%) of EGFP-positive cells among cTnI-positive cells (4276 cells in eight samples) reached 97.1 ± 1.8% at 14 days after transduction (Figure 5). Cardiac differentiation and contractile function were not significantly altered in infected cultures.

Transplantation of cardiomyocytes derived from cynomolgus ES cells

We further investigated whether cardiomyocytes derived from cynomolgus monkey ES cells can survive in the rat myocardial infarction model myocardium. Transplanted cells transduced with the EGFP-SIV vector were identified in myocardial tissue section by green fluorescence, while cells stained with the cardiac-specific marker cTnI were identified by red fluorescence. The myocardial tissue co-expressed cTnI and EGFP 14 days after cell transplantation (Figure 6). In addition, the cTnI- and EGFP-expressed cells were normally stained with DAPI. These results confirmed that cardiomyocytes derived from transplanted ES cells survived in the injured myocardium.

Discussion

The present study demonstrates that cynomolgus monkey ES cells can differentiate into cardiomyocytes *in vitro*. Cardiomyocytic nature was confirmed by (1) the expression of cardiomyocyte-specific molecular markers such as cTnT, MLC-2A, MLC-2V, and αMHC, (2) the ultrastructural features of sarcomeric organization and desmosomes, and (3) intracellular calcium transience. Our cynomolgus ES cells formed contracting areas 3 days after EBs were plated. This point is in between the 1 day for murine ES cells and the 5–8 days for human ES cells after plating [2], reflecting the fetal developmental periods of these three species.

Transplantation of viable cardiomyocytes has emerged as a potential new therapy with which to treat the injured myocardium. Various types of cells including fetal and neonatal cardiomyocytes [18–20], skeletal myoblasts [21–23], and bone marrow cells [24,25] have been used as donor cells. However, the source of these cells might be limited and insufficient for clinical purposes. In

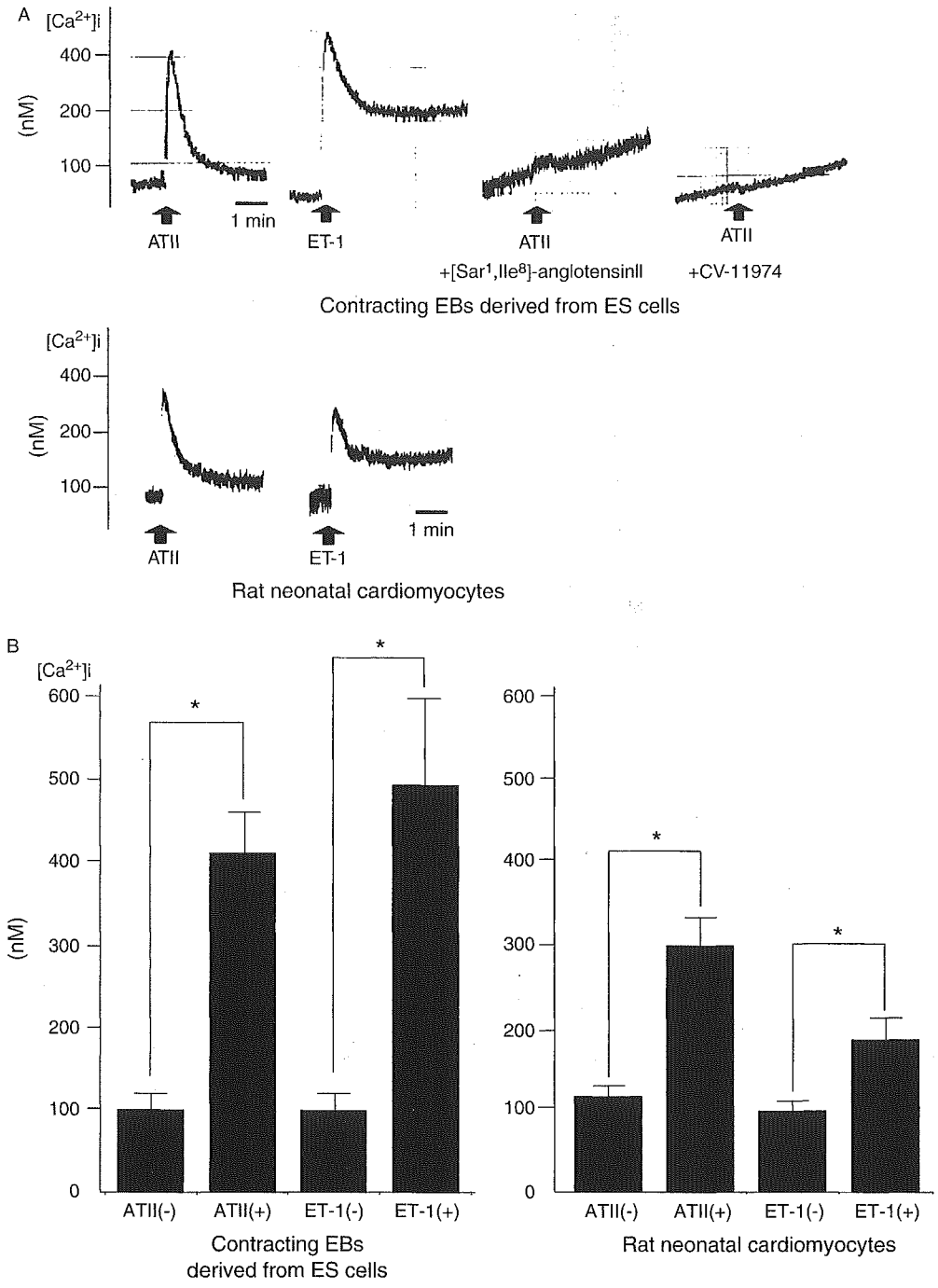

Figure 3. Functional analysis. Intracellular Ca^{2+} transience ($[Ca^{2+}]i$) of contracting EBs and rat neonatal cardiomyocytes determined by fura-2 fluorescence. (A) Typical Ca^{2+} transience appeared in response to 100 nM angiotensin II (ATII) and endothelin-1 (ET-1) in both cell types. The angiotensin-II-stimulated Ca^{2+} transience was inhibited by treatment with [Sar^1,Ile^8]-angiotensin II (non-selective antagonist) or CV-11974 (angiotensin II type 1 receptor selective antagonist) in contracting EBs. (B) Bar graph shows mean ± SD (n = 40). $^*p < 0.05$

addition, significant proportions of transplanted cells die after transplantation [26]. Since ES cells have a potent proliferative capacity, cardiomyocytes derived from ES cells are good candidates for cell transplantation therapy [4,27–29]. Cardiomyocytes derived from murine ES cells survive after intracardiac implantation [30]. We have shown here that cardiomyocytes derived from primate ES cells can also survive in the myocardium of myocardial infarction rats.

To enhance the effects of cell transplantation therapy, gene modification of the donor cells might be useful for treating cardiac diseases. Angiogenic agents such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) might be candidates for gene transfer as they attenuate myocardial ischemia in patients with ischemic heart disease when administrated either into myocardium as a naked plasmid [31] or into coronary artery as a recombinant protein [32]. We used

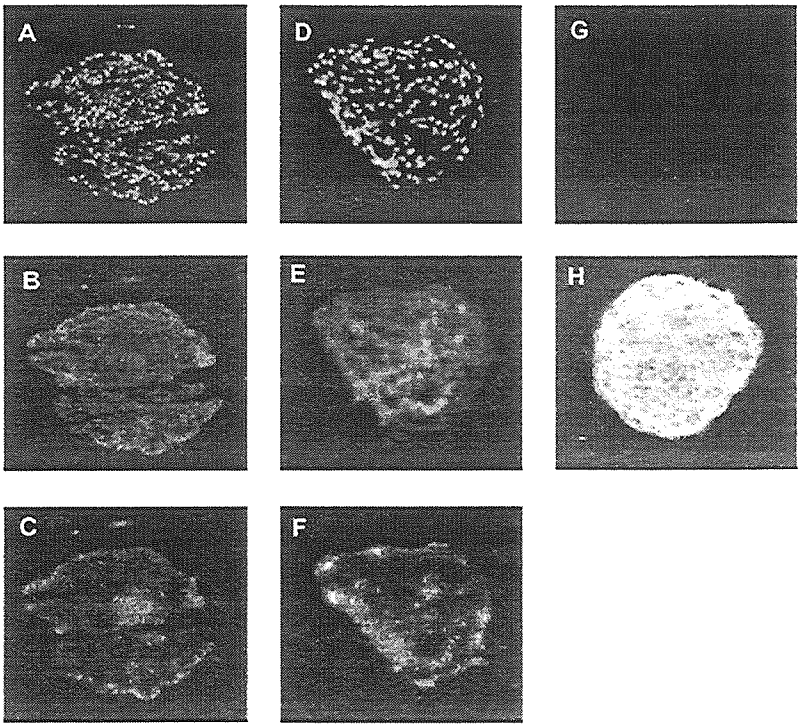

Figure 4. Immunohistochemical staining and EGFP expression. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated and stained with DAPI (A, D), cTnI (B) and cardiac myosin (E), then EGFP expression (C, F) was identified by fluorescent microscopy. Control staining in which mouse non-specific IgG was used as a primary antibody (G) and phase-contrast photograph (H). Magnification ×100

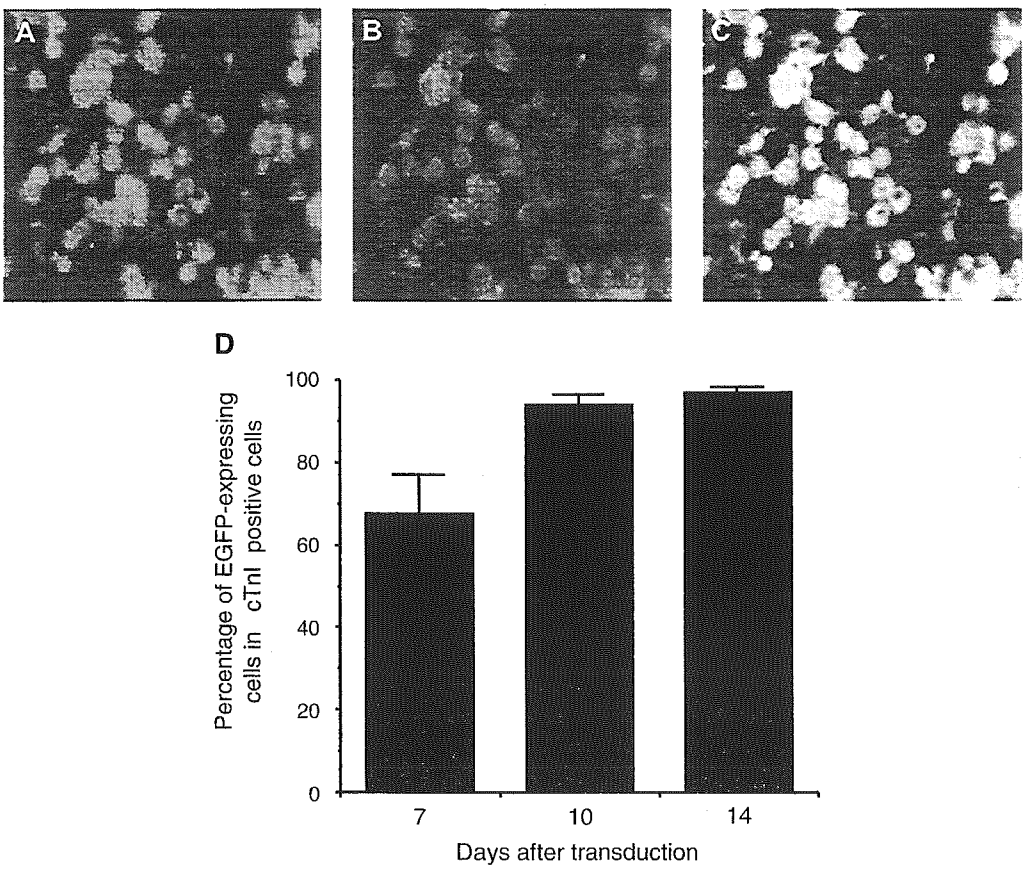

Figure 5. Transgene expression using SIV-based lentivirus vector. Cardiomyocytes derived from cynomolgus ES cells were transduced with EGFP-SIV vector. Contracting areas were isolated, trypsinized and stained with cTnI (A), then EGFP expression (B) was identified by fluorescent microscopy. (C) B merged with A. Magnification ×200. (D) Ratio (%) of cells expressing EGFP in cTnI-positive cells. Values are means ± SD of eight independent experiments

Figure 6. Expression of cTnI and EGFP in myocardium transplanted with cardiomyocytes derived from ES cells. Cardiomyocytes derived from cynomolgus ES cells transduced with the EGFP-SIV vector were implanted into injured myocardium. (A) DAPI staining, (B) cTnI staining, and (C) EGFP expression. Bar: 50 μm

a lentiviral vector based on SIV derived from the African green monkey (SIVagm) [17]. Lentiviral vectors based on either human immunodeficiency virus type 1 (HIV-1) or SIVagm are the only gene delivery vehicles that can efficiently transduce primate ES cells [14,33]. SIVagm-based vectors could offer safety advantages over those based on HIV-1 in human gene therapy. SIVagm is non-pathogenic in its natural host and in experimentally inoculated macaque monkeys, whereas HIV-1 causes severe pathogenicity in humans. In addition to the low homology of sequences between HIV-1 and SIVagm, most viral sequences were removed from our SIVagm vectors. Thus, this vector is unlikely to generate replication-competent virus by recombination between the two types of viruses in humans.

We efficiently and stably expressed EGFP in cardiomyocytes derived from cynomolgus ES cells using a lentiviral vector system based on SIV. Furthermore, we demonstrated that the implanted EGFP-positive cardiomyocytes derived from ES cells survived in the injured rat myocardium. Cell transplantation together with lentivirus-mediated gene modification offers considerable potential as a new therapeutic approach to treating cardiac diseases.

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We thank Naomi Takino, Chieko Muramatsu, and Yuki Onuma for excellent technical assistance. This study was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Science, Sports and Culture, the Japanese Government; by a Grant-in-Aid for the Development of Innovative Technology, the Kanae Foundation for Life and Socio-Medical Science; and by funds from The Cell Science Research Foundation.

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TRANSPLANTATION

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A NOVEL GENE THERAPY TO THE GRAFT ORGAN BY A RAPID INJECTION OF NAKED DNA I: LONG-LASTING GENE EXPRESSION IN A RAT MODEL OF LIMB TRANSPLANTATION

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Background. **It is important to develop a nontoxic gene transfer method for immunosuppressed patients. In this study, the authors applied a nonviral gene transfer method using rapid injection of naked DNA into the graft limb in rats.**

Methods. **Naked DNA (β-galactosidase, luciferase, or green fluorescent protein expressing plasmid) was used to test an intravascular gene transfer approach in various conditions on the Lewis rat limb. Then, in a rat limb transplantation model, these marker genes were administered preoperatively (day −2) or perioperatively (day 0) to the graft limb by the authors' "venous protocol." The expression level of luciferase was observed over a long period using a noninvasive living image acquisition IVIS system.**

Results. **Effective intravascular delivery of gene to the rat limb was achieved by a rapid bolus injection of naked DNA through the femoral caudal epigastric vein. Using this procedure, the limb graft with the marker gene perioperatively in place was safely transplanted. After limb transplantation, sustained marker gene expression was observed for more than 2 months.**

Conclusions. **This is the first report showing that the method of rapid injection of naked DNA into the limb can be applied to gene modification for organ transplantation.**

Recently, genetic modification of an allograft before transplantation has attracted interest because "ex vivo" gene transfer strategies show considerable potential in organ transplantation (*1–3*). Organ-specific gene transfer might also be beneficial in controlling local immune responses (*4*). However, clinical application to recombinant viruses for viral gene transfer is limited because of the risk of systemic viral illnesses in transplant recipients (*5*). Nonviral gene transfer to the graft therefore has great potential as a therapeutic approach for the compromised patient with chronic immunosuppression (*3*, *4*).

In the field of composite tissue transplantation, such as hand and limb transplantation, local gene therapy offers a promising strategy for genetic modification of allografts (*4*). Large quantities of donor skeletal muscle are available as a target for local gene transduction (*6*), and it is well known that muscle is a suitable tissue for nonviral gene transfer (*7–9*). In previous work, we demonstrated that sustained expression of the gene introduced on the muscle by means of a particle-mediated gene gun was detectable for more than 1 month (*10*). Recent studies have also found that intra-arterially delivered naked DNA was expressed in skeletal muscle and can be applied in genetic diseases such as Duchenne muscular dystrophy and hemophilia (*6*, *11*, *12*). Clinically, intravascular delivery of genes without multiple intraparenchymal injections into the target tissue is considered to be attractive.

In the present work, we tested the efficacy of rapid intravascular injection of naked DNA into the rat limb, comparing the arterial route to the venous route for gene delivery. We then applied a novel "venous protocol" to the graft limb and successfully achieved long-term marker gene expression in a limb transplantation model.

MATERIALS AND METHODS

Rats

Male Lewis (LEW) rats (major histocompatibility complex haplotype; RT1[l]), 8 to 12 weeks old, were purchased from Charles River Japan, Inc. (Yokohama, Japan) and used as donors and recipients. The animals were maintained under standard conditions and were fed rodent food and water in the Experimental Animal Center of Jichi Medical School. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Plasmids

pCAGGS-LacZ was constructed by inserting the *LacZ* cDNA into the *Eco*RI site of the *pCAGGS* (*13*) as described previously (*14*). The *photinus pyralis* luciferase expression plasmid, *pGL3*, was obtained from Promega (Madison, WI), and its gene was driven under an SV40 promoter. The expressing construct for an enhanced type of mutant in the green fluorescent protein (*GFP*) gene, *phGFP-105-C1*, driven under a cytomegalovirus (CMV) promoter, was kindly donated by Dr. T. Osumi (Himeji Institute of Technology, Hyogo, Japan). Large-scale preparation of plasmid DNA was performed by the alkaline lysis method, and closed circular plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Intravascular Rapid Injection of Naked DNA: Comparison of Arterial Route with Venous Route

To test the adequacy of injection routes for the graft limb, we compared the efficacy of introduced gene expression using arterial and venous routes for gene delivery. In all procedures, the rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). A medial skin incision (approximately 4 cm) was made in the thighs of the rat. For intravenous injection (venous group), a mi-

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crovessel clip was placed on the femoral vein at the proximal side of the bifurcation to the profunda femoris vein to block outflow of the blood to the leg. One hundred twenty-five micrograms of *pCAGGS-LacZ* or *phGFP-105-C1* in two volumes of 5 or 10 mL of normal saline was injected into the femoral caudal epigastric vein within 5 sec using 20-gauge catheters. To extend the duration of injection time to 45 sec while maintaining the venous pressure in the femoral vein, a microvessel clip was placed on the femoral artery during the injection. For intra-arterial injection (arterial group), the femoral artery was ligated and an equal volume of plasmid DNA was injected into the distal side of the femoral artery in the same manner. To observe gene expression without killing the experimental rats, 5 mL of 125-μg *pGL3* was injected in accordance with the venous protocol.

Gene Introduction to the Graft Limb and Its Transplantation

Before limb transplantation, a reporter gene (*pGL3* or *phGFP-105-C1*) was injected rapidly into the rat limb through the caudal epigastric vein. Reporter genes were introduced 2 days before limb transplantation (day −2 group) or just before limb transplantation (day 0 group). In the day 0 group, grafts were donated 10 to 15 min after DNA injection.

After gene transduction, limb transplantation was performed using the limb replantation model according to the procedure set out in our previous report (*15*). In summary, the rats were anesthetized with an intraperitoneal injection of pentobarbital, and both donor and recipient hind limbs were amputated at the midfemur. The donor limb with genetic modification was fixed orthotopically with an intramedullary pin constructed using 18-gauge injection needles. The femoral vessels and sciatic nerve were anastomosed with 10-0 nylon using a microsurgical technique. Muscle and skin were sutured with 4-0 nylon. After the surgery, the rats were maintained in a pathogen-free room under constant environmental conditions. Neither antibiotics nor immunosuppressants were used postoperatively in any of the experimental rats.

Assessment of Grafted Limb Survival

The skin of the donor limbs was observed daily and compared with that of the recipients' normal contralateral limb. Graft failure was defined as the presence of skin erythema for 24 hr, as described previously (*15*).

Detection of Marker Gene Expression

In the nontransplant model, the gene expressions after rapid intravascular injection of naked DNA into the rat limb were evaluated by the β-galactosidase (*LacZ*) reporter gene expression system. For histologic examination, rats were killed 2 days after gene transfer. Then, the anterior, posterior, and medial upper leg muscles and the posterior and anterior lower leg muscles were collected. After fixing with 0.2% glutaraldehyde, the samples were stained with β-galactosidase staining solution and expression of *LacZ* was observed as described previously (*14*, *16*).

The expression level was also evaluated without killing the animals using a marker gene of luciferase. In the nontransplant model, the expression was examined 2, 14, and 21 days after gene transfer; in the transplant model, it was examined 6, 12, and 30 days after limb transplantation. A cooled charge-coupled device camera, which is a noninvasive and sensitive optical imaging system, was used for detecting bioluminescence emitted from D-luciferin reacting with firefly luciferase in living animals. In vivo bioluminescence imaging was conducted on an IVIS system (Xenogen Corp., Alameda, CA) using Living Image acquisition and analysis software (Xenogen). n-Luciferin (potassium salt; Xenogen) was dissolved and diluted to 15 mg/mL in phosphate-buffered saline, filter-sterilized, and stored at −30°C. The rats were anesthetized with isoflurane and subsequently underwent intraperitoneal injection with luciferin (150 mg/kg body weight). Images were acquired 15 min after luciferin administration. A photographic image of the animal was taken in the chamber under dim illumination, followed by acquisition and overlay of the pseudocolor image representing the spatial distribution of photon counts produced by active luciferase within the animal. An integration time of 1 min with a binning of 100 pixels was used for luminescent image acquisition. The signal intensity was quantified as the sum of all detected photon counts within the region of interest.

Expression of the introduced *GFP* gene was visualized macroscopically under 489-nm wavelength excitation light as specified in previous reports (*10*, *17*). In the nontransplant model, rats were killed 70 days after gene transfer to evaluate the technical procedures. In the transplantation model, *GFP* expression in donor muscle fibers was analyzed by histologic examination, as described previously (*10*). In summary, frozen sections were obtained from the muscle of the grafted lower limbs on days 30 and 70 after transplantation, and *GFP* expression was observed immediately by fluorescent microscopy under 489-nm wavelength excitation light. After photographs of the *GFP*-expressing tissues were taken, the sections were stained with hematoxylin-eosin according to the standard procedure. The photographs of *GFP*-expressing sections and hematoxylin-eosin sections were merged using Photoshop software (Adobe Systems, Tokyo, Japan).

RESULTS

Assessment of the Differing Routes for Intravascular Delivery of Naked pCAGGS-LacZ *and* pGL3

The efficacy of intravascular transgene injection according to the β-galactosidase (*LacZ*) reporter gene detection system was compared between arterial and venous routes for gene delivery. As technical parameters, the duration of injection, injection volume, and injection route were assessed. Intravascular delivery of the transgene was evaluated by the degree of limb swelling at the time of injection and the detectability of *LacZ*-expressing cells 2 days after injection (Table 1). Almost all of the *LacZ*-positive cells were myofibers. Under the optimal injection conditions, up to 30% of myofibers expressed the *LacZ* in many areas of the muscles (Fig. 1).

We determined that effective intravascular delivery of the transgene was achieved by rapid injection of 5 mL of 125-μg naked DNA solution within 5 sec through the femoral artery or femoral caudal epigastric vein without occlusion of the arterial vessels leading into the hind limb. Our data also showed that high intravascular pressure and a solution volume of more than 5 mL were not necessary for efficient gene transduction. In accordance with this protocol, injecting into the femoral caudal epigastric vein, we confirmed massive luciferase gene expression in living rats using the in vivo bioluminescence imaging system, IVIS (data not shown).

TABLE 1. Influence of technical factor of rapid intravascular injection of 125-μg naked plasmid DNA (*pCAGGS-LacZ*) on rat limb muscles[a]

	Arterial group (femoral artery)	Venous group (femoral epigastric vein)	
Injection volume (mL)	5	5	10
Duration of injection time (sec)	30–42	2–5	3–8
LacZ expression	+~2+	2+	2+
Edema	Moderate	Moderate	Severe

[a] Rats were killed 2 days after gene transduction. The above technical factors were assessed by the degree of graft swelling and detectability of *LacZ* expression (n=3 in each group).

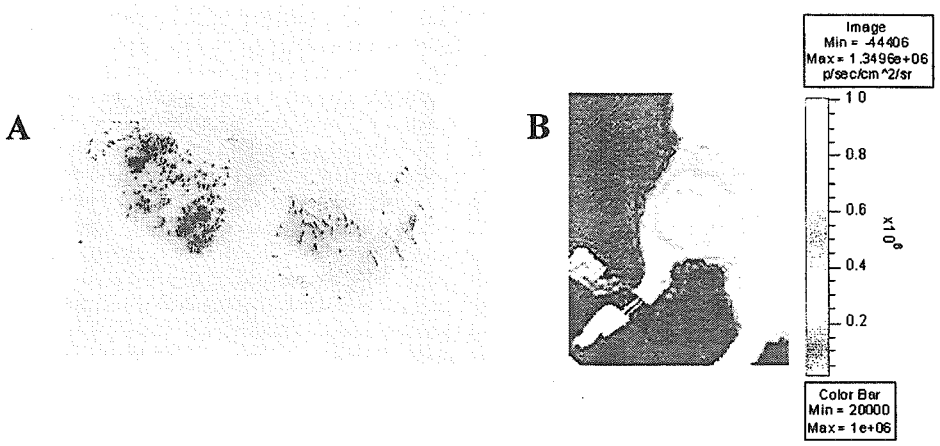

FIGURE 1. One of the representatives of well-detected *LacZ* activity in rat lower limb muscles stained with β-galactosidase staining solution. Samples were obtained on the second day after intravenous injection of 125-μg naked *pCAGGS-LacZ* through the caudal epigastric vein.

Long-Lasting Gene Expression in Rat Limbs after Intravenous Injection of Naked pHGFP-105-C1

We tested long-lasting gene expression on rat limbs using the *GFP* detection system. Five milliliters of 125-μg naked DNA (*phGFP-105-C1*) solution was administered intravenously through the femoral caudal epigastric vein within 5 sec. As shown in Figure 2, significant long-lasting expression of *GFP* in lower limb muscles was observed 30 days after gene transduction.

Successful Transplantation of the Grafted Limb with Introduced Marker Gene by the Preoperative and Perioperative Rapid Intravenous Injection Method

Using the venous protocol, we introduced reporter genes into the grafted limb before transplantation to study whether this strategy is suitable for limb transplantation. Five milliliters of solution of naked reporter gene (*pGL3* or *phGFP-105-C1*) was injected rapidly into rat limb through the femoral caudal epigastric vein preoperatively (day −2 group) or perioperatively (day 0 group). As shown in Table 2, all grafts survived for more than 30 days after limb grafting with gene expression. Although the grafts of the day 0 group were significantly swollen compared with those of day −2 group at the time of transplantation (15.7±0.1 and 14.0±0.4 g, respectively; $P<0.05$ by t test), there was no evidence of postoperative complications such as intravascular embolism, infection, and graft-versus-host disease in any experimental animal during the follow-up period in either group.

We then investigated whether the introduced reporter gene (*pGL3* or *phGFP-105-C1*) could be expressed in the donor limb after transplantation. Sustained *GFP*-expressing myofibers were detected macroscopically in the grafted limb at least 70 days after surgery in both administration time groups (data not shown). On the basis of histologic analysis, one of the representatives of obvious *GFP* expression in the muscles is shown in Figure 3. Moreover, strong luciferase activity was detected in the living transplanted limb at least 30 days after surgery by our bioluminescence-imaging system in both the day −2 group (data not shown) and the day 0 group (Fig. 4).

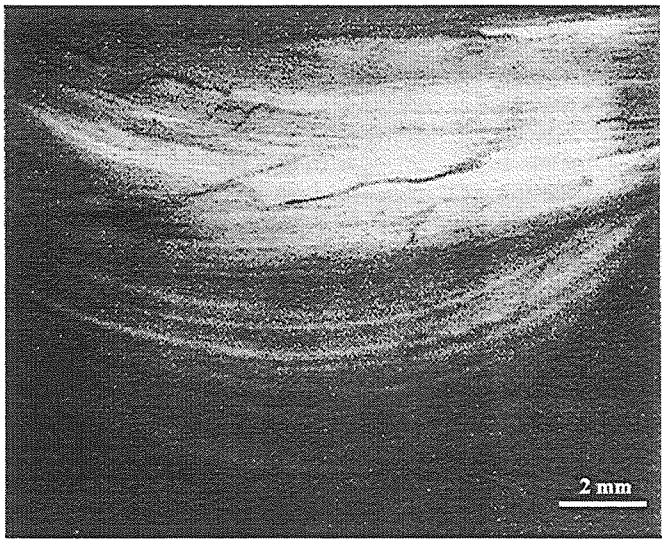

FIGURE 2. Representative expression of *GFP* in rat lower limb muscles resulting from intravenous injection of 125-μg *phGFP-105-C1* through the caudal epigastric vein. Samples were obtained 70 days after gene transduction and long-lasting expression of *GFP* was observed.

DISCUSSION

Recent advances in immunosuppressive drugs and surgical techniques have led to attempts at human hand and limb transplantation (*18–20*). However, the allergenic composite tissues, including skin, muscle, bone, cartilage, and bone marrow, have unique immunologic characters with different antigenicities, so that composite limb allotransplantation demands heavy immunosuppression (*21*, *22*). To overcome these problems, we have attempted a local nonviral gene therapy to the grafted limb using a particle-mediated gene gun with the aim of reducing the dose of immunosuppressant drugs (*4*).

TABLE 2. Graft survival in rat limb transplantation after rapid intravenous injection of naked DNA

Gene transfer[a]	No.	Survival (days)	Success rate
Day −2	4	>30[b], >30, >30, >70[b]	100% (4/4)
Day 0	7	>30[b], >30, >30, >30, >70[b], >70, >70	100% (7/7)

[a] Naked DNA was injected into donor limb preoperatively (day −2 group) or perioperatively (day 0 group).

[b] Rats were killed to examine gene expression.

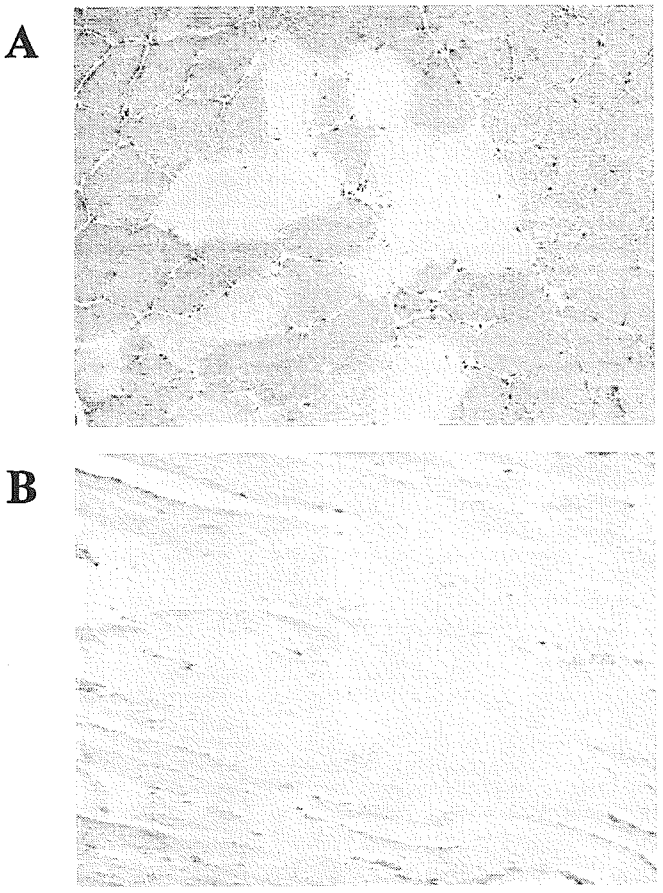

FIGURE 3. Representative histologic analysis of *GFP* expression in the muscle cross-section (A) or longitudinal section (B) from the grafted lower limb 30 days after transplantation (day −2 group). Two days before transplantation, 5 mL of 125-μg naked *phGFP-105-C1* solution was administered intravenously within 5 sec through the caudal epigastric vein.

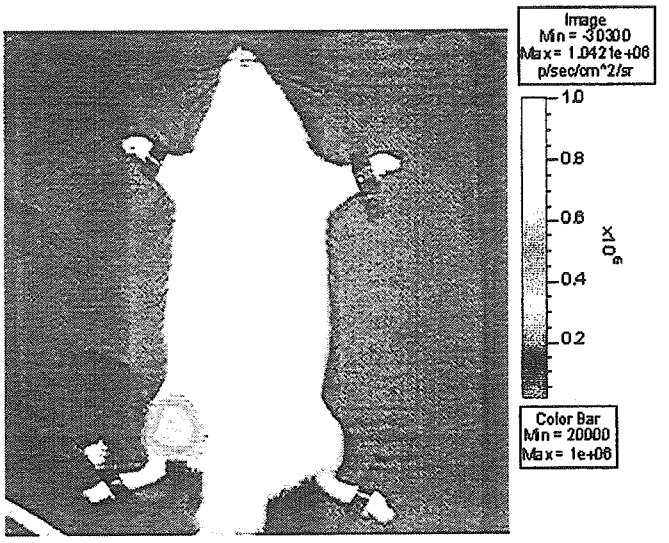

FIGURE 4. Representative location and magnitude of luciferase expression in living rat limb 30 days after limb transplantation (day 0 group). The average photon count (p/sec/cm^2/sr/min) in the graft limb was 2.0×10^7. Just before transplantation, 5 mL of 125-μg naked *pGL3* solution was administered intravenously within 5 sec through the caudal epigastric vein.

Recently, intra-arterial delivery of naked DNA into the muscles has been reported experimentally, and its clinical use has been proposed (*6*, *11*, *12*). Although the mechanism of intravascular delivery of naked DNA is still unclear, a rapidly elevated intravascular hydrostatic pressure is essential for successful gene transfer (*6*, *11*, *12*). Because the muscle endothelium has a large number of small pores, plasmid DNA is capable of crossing microvascular walls by extravasations under conditions of high intravascular pressure (*6*).

In the present study, we first confirmed that the intravenous delivery of naked DNA into the limb had high efficiency, matching that of intra-arterial delivery (Table 1). Using our protocol, we were able to observe sustained reporter gene expression in the muscle for more than 30 days using our *GFP* expression detection system. This is consistent with our previous finding that marker gene expression in the muscle is mediated by the nonviral gene transfer method; the gene gun, modified with bupivacaine, was detected persistently for more than 1 month (*10*). Both results clearly indicate the potential of muscle as a target tissue for somatic gene therapy.

Second, we applied our venous protocol in rat limb transplantation using the femoral epigastric vein as a route for gene transfer. Naked DNA was administered rapidly intravascularly through the femoral caudal vein, both preoperatively (day −2) and perioperatively (day 0). In both the day −2 group and the day 0 group, the grafted limbs were completely accepted without any postoperative complications. The introduced reporter gene was expressed successfully in the grafted limb, and sustained expression was observed in both groups. The venous protocol also has many advantages over the arterial protocol. First, the femoral artery and vein can be left intact for anastomosis, which is essential in microsurgical techniques for rat limb transplantation. Second, soon after gene transduction through the femoral caudal epigastric vein, the microvessel clip was removed from the femoral vein and the limb was able to be reperfused immediately. This reperfusion process might be central to effective gene transfer to the donor limb, because we found with the gene gun that ex vivo transfection was not effective (*23*). Effective gene transfection might therefore be achieved only in living tissue where the blood supply is sufficient. Although the evidence of gene expression in the day 0 group suggests that this method could be applied in clinical limb transplantation from cadaveric heart-beating donors, we must confirm this protocol in a large animal as a preclinical study.

Although nonviral gene transfer is a promising strategy in the field of transplantation, targeted gene delivery has been limited by the efficiency of in vivo DNA transfer (*4*). Several experimental approaches have been established to enhance the expression levels of transgenes (*3*, *12*, *24*, *25*). It has also been reported that cell-permeable molecules can be used as carrier sequences for introducing biologically active hydrophilic molecules such as DNA or peptide moieties into cells in vitro and in vivo (*26*). Recently, these cell-permeable peptides have been used with viral gene transfer, improving gene expression at reduced titers of virus in vivo (*27*). These pep-

tides could therefore be used together with the intravascular nonviral gene transfer method to improve the efficiency of the transgene and reduce the amount of DNA administered and thus reduce the graft swelling after bolus injection of DNA solution.

CONCLUSION

We have achieved successful gene transfer into the limb muscle by an intravenous injection of plasmid DNA. In rat limb transplantation, our results clearly indicate the feasibility of this strategy for gene modification to the graft limb before transplantation. There seemed to be no obvious adverse effects in the rat limb transplant model. However, a large-volume infusion caused capillary vascular damage, and it might be serious in other graft organs, such as liver and kidney. Further studies using functional genes in a large animal should determine the clinical relevance of this study.

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DNA IMMUNIZATION OF THE GRAFTED LIVER BY PARTICLE-MEDIATED GENE GUN[1]

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Background. **Direct DNA vaccination of liver allografts before transplantation may provide an effective strategy for inducing protective immunity to infection and malignancy.**

Methods. **In this study, the authors examined the feasibility of gene gun-mediated vaccination of liver grafts. Using plasmids expressing luciferase and green fluorescent proteins, their expression was tested in a graft liver.**

Results. **Protein expression was observed in the graft liver and significantly enhanced in hepatectomized rats. A short course of tacrolimus (FK506) also evoked the expression of these proteins. Effects of primary immunization to the liver on the humoral response were then tested using an expression plasmid encoding hepatitis B virus surface (HBs) antigen and were compared to that of skin immunization alone. The results showed that local immunization to the liver strongly induced antibody formation. Furthermore, the combination of an immunized partial liver graft with tacrolimus significantly enhanced antibody production against HBs antigen.**

Conclusions. **A DNA vaccine to the liver may be one strategy for preventing infectious disease associated with liver transplantation under tacrolimus treatment.**

Many studies have demonstrated successful genetic modification of liver allografts before transplantation (*1*, *2*). Almost all of these have used recombinant viral vectors as the foreign gene vehicle, perfusing the grafts in situ or ex vivo during the transplantation. However, although viral vectors such as recombinant adenoviruses and adenoassociated viruses can provide a high level of gene delivery, their clinical application is limited because of the risk of systemic viral illness in the transplant recipient (*3*). The gene gun offers an alternative nonviral approach to gene delivery. Originally described by Williams et al. (*4*), the gene gun can directly achieve plasmid DNA expression in cells in situ. Particle-mediated gene transfer also has the advantages of being fast, simple, and versatile. Previously, we have demonstrated successful gene transfer into a rat liver by using a gene gun (*5*). Now, the focus of our research is to effectively introduce protective immunity against infectious (*6*) or malignant (*7*, *8*) diseases by use of direct DNA vaccination using a gene gun. Recent preclinical results in immunization against malaria, which has a hepatocyte target, have strongly supported our hypothesis that the liver is a suitable target for gene immunization (*9*).

In this study, we first tested the feasibility of applying gene gun-mediated gene transfer to a rat liver using a plasmid expressing luciferase and green fluorescent proteins with or without tacrolimus treatment. Plasmid-encoded hepatitis B virus surface (HBs) antigen was then used to demonstrate the humoral immune response to DNA immunization. Finally, an immunized partial graft was heterotransplanted and followed up to observe antibody formation.

MATERIALS AND METHODS

Animals and Plasmids

Inbred Lewis rats (MHC haplotype; $RT1^l$) were derived from animals bred at Jichi Medical School from animals originally obtained from Charles River Ltd. (Yokohama, Japan). Female rats (150–250 g) were used to study the effects of hepatectomy or tacrolimus treatment on anti-HBs antibody production. Female (150–200 g) and male (280–370 g) rats were used as donors and recipients, respectively, in the auxiliary liver transplantation (ALT) experiment. The rats were maintained under standard conditions at the Animal Center of Jichi Medical School and had free access to standard chow and tap water.

The *Renilla reniformis* luciferase expression plasmid *pRL-thymidine kinase* (*TK*) was obtained from Promega (Madison, WI), and its expression was driven by a herpes simplex virus (HSV) TK promoter. The expression construct for an enhanced type of mutant in the green fluorescent protein (*GFP*) gene, *phGFP-105-C1*, driven under a cytomegalovirus (CMV) promoter, was kindly donated by Dr. T. Osumi, Himeji Institute of Technology (Hyogo, Japan) (*10*). For the HBs antigen expression plasmid, DNA covering the entire S gene sequence of a hepatitis B virus (HBV) clone (pNDR260: genotype C/subtype adr) (*11*) was inserted into the pCMV expression vector carrying the CMV promoter, generating *pCMV-HBs*. The mouse interleukin (IL)-12 expression plasmid, *pCAGGS-IL-12*, was kindly provided by Dr. S. Wolf, Genetic Institute (Cambridge, MA), and contained both IL-12 p35 and p40 cDNA driven by a chicken β-actin gene promoter (*7*). A large-scale plasmid DNA preparation was performed by an alkaline lysis method, and closed circular plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

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Particle-Mediated Gene Transfer and Plasmid DNA

The Helios gene gun system (Bio-Rad Laboratories, Tokyo, Japan) was used according to the manufacturer's instructions. Plasmid DNA was precipitated onto 1.6-μm gold particles as previously described (*5–7*). The plasmid DNA-coated gold particle suspension was introduced into a Tefzel tube (Bio-Rad) placed on a flow of nitrogen (0.4 mL/min) for 15 min. The tube was then cut into 1.2-cm-long cartridges and stored in a container with silica gel at −20°C. One milligram of gold particles contained 2.0 μg of plasmid DNA, resulting in the delivery of 1.0 μg of DNA per shot.

Luciferase Assay and GFP *Expression*

The *phGFP-105-C1* and *pRL-TK* expression plasmids were mixed to a 1:9 ratio and then precipitated onto gold particles as described above. The expression area of the introduced *GFP* gene was easily visualized by excitation at 489 nm (Leica MZ FLIII; Leica Microsystems, Tokyo, Japan). An area 6 mm in diameter was punched out at the indicated time after transfection. The luciferase activity was measured using a luciferase reporter assay system (Promega). Each assay was independently repeated several times, and the average value was indicated. The activity depended on the incubation time and protein concentration used for the assay.

ALT

ALT was performed according to our cuff method as previously shown (*12*). Briefly, the common bile duct of the donor liver was divided and the liver side cannulated with a 22-gauge, 5-mm-long polyethylene tube. The gastroduodenal, left gastric, and splenic arteries were all divided and then ligated. Finally, the superior mesenteric artery, right renal vein, and portal vein were ligated and cut, and the infrahepatic cava was mobilized from surrounding tissue. The liver was perfused with 10 mL of cold University of Wisconsin solution before removal. Then, the graft was prepared in cold University of Wisconsin solution. The suprahepatic inferior vena cava (IVC) and the proximal end of the aorta were ligated with 6-0 silk sutures and the infrahepatic IVC was cuffed with a 14-gauge, 2-mm-long polyethylene tube. A heterotopic liver transplant was performed in the left kidney bed of the recipient. The left kidney was mobilized together with the ureter that was divided, and the distal end was stented with a 20-gauge polyethylene tube. The renal artery and vein were separated, and the artery was divided and cuffed with a 20-gauge, polyethylene tube 1 mm in diameter. After clamping the renal vein, an anterior venotomy was made, which was anastomosed to the cuff of the donor IVC. Arterialization was achieved by inserting the cuffed renal artery of the recipient into the distal end of the donor aorta. Finally, the bile duct tube of the graft was inserted into the jejunum of the recipient.

Transfection using the gene gun into the donor livers was performed according to the following protocols: (1) ex vivo bombardment after retrieval; (2) in vivo bombardment immediately after the donor laparotomy (40–60 min before donation); and (3) in vivo bombardment into the remaining 70% of the liver after a 30% hepatectomy. Then, all livers were heterotopically transplanted as detailed above.

In the nonresected liver group, the liver was exposed through a midline abdominal incision. The wound was closed with a continuous suture. *GFP* and luciferase expression was assessed on days 1, 3, 7, 14, 21, and 28 (n=4 for each day). In the resected liver group, a transverse abdominal incision was performed and a 30% hepatectomy (left lobectomy) was completed, followed by bombardment of the remaining liver using the gene gun.

For immunosuppressant administration, after the first bombardment the rat was given tacrolimus (FK506; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) or saline for a control. Tacrolimus was administered intramuscularly at a dosage of 0.64 mg/kg (*13*) for 14 days after gene gun bombardment into the liver.

Gene Immunization

For immunization of HBs antigen to the nontransplanted liver, rats were subjected to 30% hepatectomy with or without tacrolimus. For the initial vaccination, IL-12 cDNA was mixed with HBs cDNA at a 1:1 ratio, and they were delivered into the normal liver, the 30% hepatectomized liver, and the freshly shaved abdominal wall (the skin group). Each group (n=5) was boosted with HBs cDNA alone by the gene gun in the rat abdominal skin after the first bombardment at 1-week intervals for 4 weeks. Rats were bled from the tail vein at prebombardment and at 2, 4, and 6 weeks postvaccination. The blood was spun at 4,000 rpm for 10 min. The serum was collected using a serum separator for enzyme-linked immunosorbent assay (ELISA) analysis to assay the serum titer of anti-HBs antibodies.

To assess the degree of immunization achieved in the transplanted liver, rats underwent heterotopic transplantation with the syngeneic liver graft that had been bombarded in vivo just before grafting and the 30% host hepatectomy. Immunization with HBs cDNA was then performed to the abdominal skin at 1-week intervals up to and including the fourth week. The rat serum was collected at weeks 2, 4, and 6 after ALT, and the anti-HBs antibody titer was assayed by ELISA as described below.

Measurement of Anti-HBs Antibodies

Antibodies to HBs antigen were measured by IMx AUSAB (Abbott Laboratories, Chicago, IL), which is an automated microparticle enzyme immunoassay. The anti-HBs antigen level was determined by comparison with standard reference preparations and was expressed in milli-international units per milliliter.

Statistical Analysis

Data are presented as the mean±SD. Statistical differences between the two groups were evaluated using the unpaired Student *t* test. The calculations were performed with StatView software (Abacus Concepts, Berkeley, CA). Values of $P<0.05$ were considered significant.

RESULTS

Particle-Mediated Gene Transfer into the Rat Liver

To evaluate whether particle-mediated gene transfer is effective in treating the liver in the rat, we transfected the liver tissue in normal rats with *GFP* and luciferase reporter genes driven by a CMV and an HSV-TK promoter, respectively. *GFP* expression was also easily detected by 489-nm excitation at 1 day postbombardment by the Helios gene gun. The expression decreased and reached a minimum at day 7 (Fig. 1). *GFP* expression was clearly observed at days 1 and 3, but few positive cells were seen at day 7 after transfection of the *GFP* gene. *GFP*-positive cells were detected on the liver surface, and the bombarded point was severely damaged and inflammatory cells infiltrated around the damaged hepatocytes (Fig. 2). A section of the expressing area 6 mm in diameter was also punched out for further measurement of luciferase activity. The relative fluorescent intensities in the liver showed a correlation between luciferase and *GFP* expression levels (Fig. 3). These results demonstrated that *GFP* expression induced by the gene gun in the liver could be useful for easy detection of protein production. Because the gene gun sometimes causes mechanical damage resulting from the microcarrier shot, especially with pressurized gas-accelerated particles, an optimum pressure should be determined for successful in vivo gene transfer to avoid tissue damage (*4, 5*). On the basis of the expression levels of the transfected *GFP* expression plasmid in the liver with various

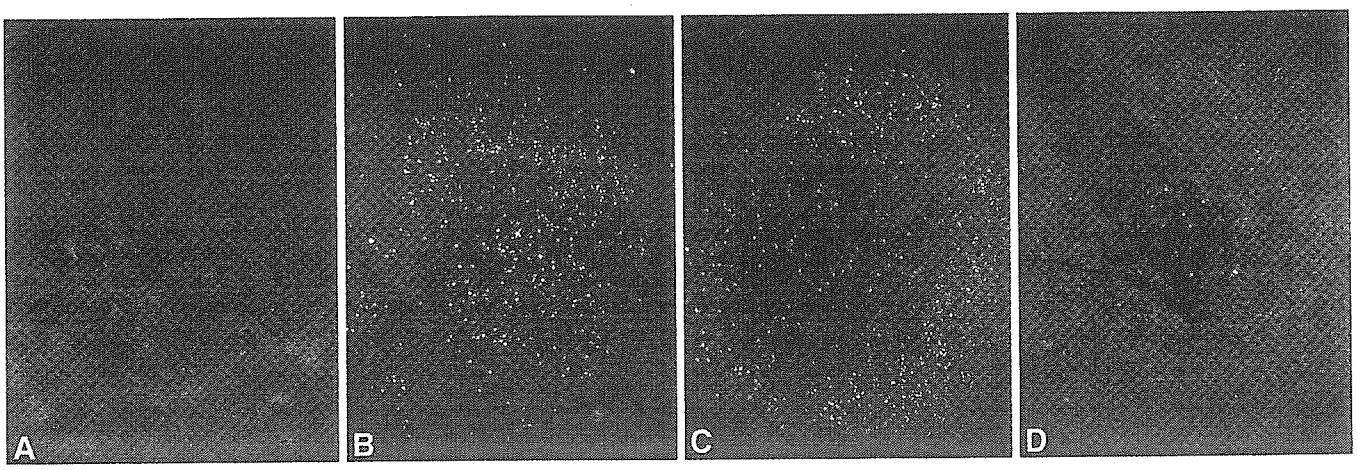

FIGURE 1. Time course expression of *GFP* expression in the rat liver resulting from use of the Helios gene gun. The *phGFP-105-C1* expression plasmid was transfected into the liver using a Helios gene gun at 250 psi. The expression of the introduced *GFP* gene was visualized by excitation at 489 nm. *GFP* expression in the liver at 0 day (A), at 1 day (B), at 3 days (C), and at 7 days (D) after gene gun transfection. Luciferase expression in vivo peaked at 1 day postbombardment by the Helios gene gun and decreased to baseline levels by 7 days.

gas pressures, an optimum pressure of 250 psi was selected as previously shown (*5*).

Then, luciferase expression was compared between normal and 30% hepatectomized livers. An approximately 30% partial hepatectomy was performed as a complete left lobectomy after a transverse abdominal incision, followed by gene gun bombardment into the remaining liver. As shown in Figure 4, relative luciferase activity was sevenfold higher in the group with the 30% partial hepatectomy than with the control treatment at day 1 postbombardment. The expression peaked at day 2 and decreased nearly to baseline levels at day 7 postbombardment. Luciferase expression in the 30% partial hepatectomized rats was enhanced by tacrolimus administration (0.64 mg/kg), which in a previous report suppressed the acute rejection of liver allografts in rats (*13*). The luciferase activity in the group receiving tacrolimus treatment was superior to that of the group receiving the 30% partial hepatectomy alone. These results indicated that the liver subjected to a partial hepatectomy combined with tacrolimus has a great potency in enhancing foreign gene expression.

Efficient Gene Expression by Gene Gun in the Grafted Livers

Next, the possibility of applying the gene gun-mediated transfection method to rat liver transplantation using the *GFP* expression plasmid was examined. Transfection into donor livers was performed using each of the following procedures; briefly: (1) ex vivo bombardment after retrieval, (2) in vivo bombardment immediately after the donor laparotomy (40–60 min before donation), and (3) in vivo bombardment against the remaining 70% liver after a 30% partial hepatectomy. All livers were then transplanted heterotopically as described (see *Materials and Methods*). Fluorescent microscopic analysis under a 480-nm excitation light showed that *GFP* was significantly expressed in the grafts of in vivo bombardment (procedure 2), and its expression was also augmented in the grafts of the reduced-size livers (procedure 3) (Fig. 5). However, the *GFP* expression level was extremely

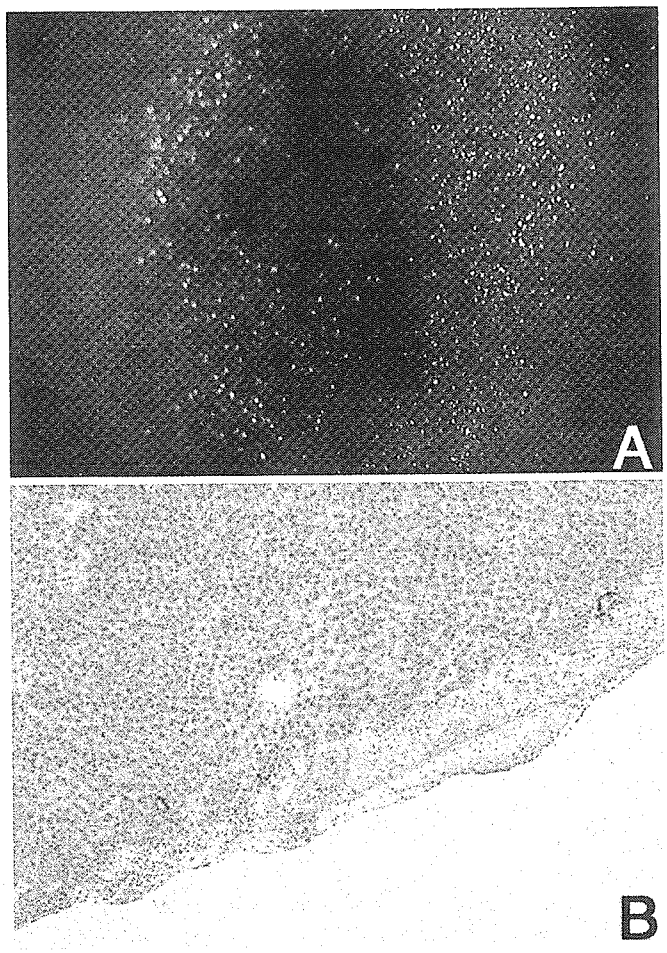

FIGURE 2. Coexistence of *GFP*-positive cells and damaged liver cells after gene gun bombardment. (A) *GFP* expression on the bombarded liver surface. *GFP*-positive cells were abundantly seen around the bombarded point, but few were present at the focal point. (B) A lengthwise section. Several *GFP*-positive cells were at a 2-mm depth. The liver surface was damaged.

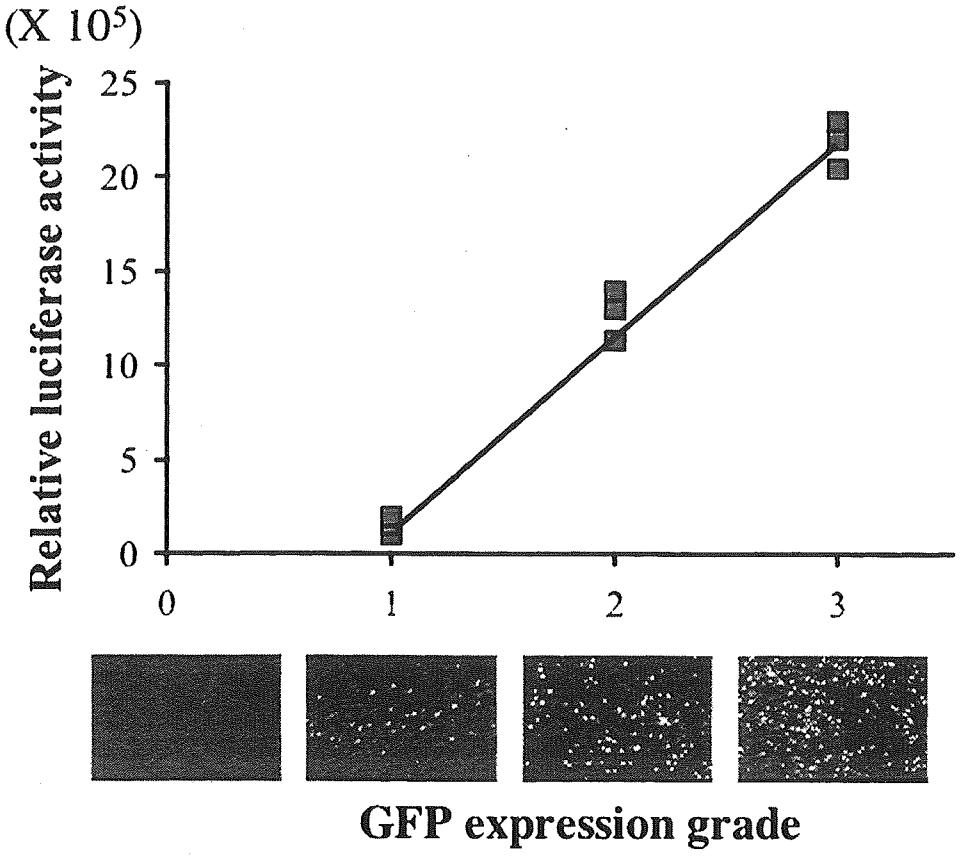

FIGURE 3. Correlated expression between *GFP* and luciferase genes in the rat liver. The *phGFP-105-C1* and *pRL-TK* expression plasmids were mixed at a 1:9 ratio and then were transfected into the rat liver using the gene gun at 250 psi. The expression area of the introduced *GFP* gene was visualized by excitation at 489 nm, and an area 6 mm in diameter was punched out at the indicated period after transfection. The luciferase activity was then measured as described (see *Materials and Methods*). Relative luciferase activity at 1 day postbombardment by the gene gun correlated with the *GFP* expression grade ($y=1\times10^6x-7\times10^6$, $r^2=0.99$). The *GFP* expression grade was determined as the indicated lower panel (grades 1–3). The activity depended on the incubation time and protein concentration used for the assay.

low in the livers of the ex vivo bombardment model (procedure 1). These results suggested that effective gene transfer into the liver graft requires in vivo bombardment before the graft donation, and that reduced-size liver grafting may be highly effective in genetic modification.

DNA Vaccination with HBs Antigen cDNA to the Rat Liver under the Use of Tacrolimus

Many recent studies have demonstrated successful genetic modification of liver allografts before transplantation (*1*, *2*), and immunization with DNA vaccines has a great potential to introduce humoral and cellular immune responses that protect against infectious diseases (*6*, *9*). To test the possibility of DNA vaccination, we used a plasmid expressing HBs antigen to introduce antibodies serologically. The effect of tacrolimus on antibody production was evaluated from a clinical viewpoint because, after liver grafting, patients must be treated with calcineurin inhibitors. Serum levels of antibody production were measured by ELISA in normal and 30% partial hepatectomy rats with or without tacrolimus. An immunostimulatory genetic adjuvant, IL-12 cDNA, was combined with HBs antigen cDNA for the first immunization, after which the rats were boosted with HBs antigen cDNA by percutaneous gene gun bombardment at 1-week intervals for 4 weeks. As shown in Table 1, at week 10 postimmunization, the level of anti-HBs antibodies was significantly increased in the group that had the combined 30% hepatectomy and tacrolimus treatment (359.6 mIU/mL). However, the results from the percutaneous vaccination and the normal liver immunization with or without tacrolimus did not achieve the serum level in the group with the 30% partial hepatectomy with tacrolimus.

Augmentation of Antibody Production by Immunized Liver Grafting Coupled with Tacrolimus

Finally, we examined the serum level of anti-HBs antibodies when the HBs cDNA was immunized into the pregrafted liver and sequentially followed by ALT. Rats were heterotopically given a syngeneic liver graft, with in vivo bombardment just before grafting, and a 30% partial hepatectomy was performed. The rats were immunized with the HBs cDNA at the abdominal skin at 1-week intervals for 4 weeks after liver grafting. The serum was assayed by ELISA at weeks 2, 4, and 6 after ALT. Each case is shown separately in Table 2. The serum level of the anti-HBs antibodies was highest in the group with the 30% host hepatectomized and the reduced-size liver grafting coupled with tacrolimus treatment (>635 mIU/mL) compared with all other groups. These results dem-

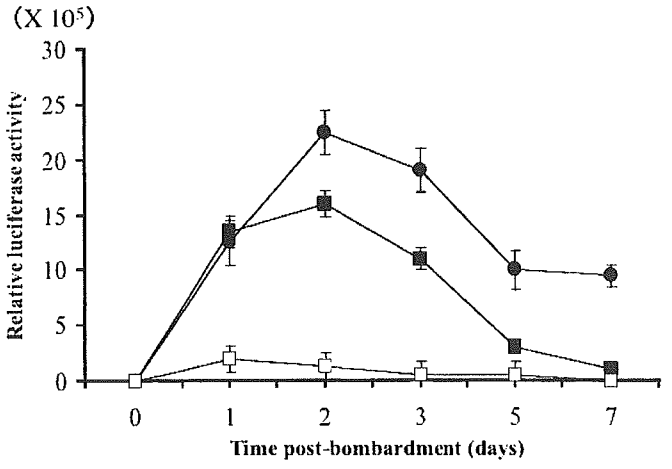

FIGURE 4. Time course expression of luciferase in the hepatectomized rat with or without tacrolimus (FK506) administration. The *phGFP-105-C1* and *pRL-TK* expression plasmids were mixed to a 1:9 ratio and then transfected into the normal liver and into the 30% hepatectomized liver with or without tacrolimus administration (0.64 mg/kg) using the gene gun at 250 psi. The expression area of the introduced *GFP* gene was visualized under an excitation of 489 nm, and an area 6 mm in diameter was punched out at the indicated period after transfection. Then, luciferase activity was measured. (*open squares*) Transfection into the normal liver; (*filled squares*) transfection into a 30% hepatectomized liver; (*filled circles*) transfection into a 30% hepatectomized liver plus tacrolimus administration (0.64 mg/kg). Luciferase activity was sevenfold higher in the group with the 30% hepatectomy than with the control treatment at 1 day postbombardment. Luciferase expression in the 30% hepatectomized liver group was enhanced by the administration of 0.64 mg/kg of tacrolimus ($P<0.005$, on days 2, 3, 5, and 7). Each assay was independently repeated three times and the average value is indicated. The activity depended on the incubation time and protein concentration used for the assay. Error bar, SD (n=3).

onstrated that DNA vaccination by the cDNA-bombarded liver can potentially introduce humoral immune responses and that antibody production was augmented by a combination of the host hepatectomy and tacrolimus.

DISCUSSION

Recent studies showed that DNA vaccines represent a novel means of expressing antigens in vivo for the generation of both humoral and cellular immune responses (*6*, *9*). They have also elicited protective immunity in a number of preclinical models of diseases (*9*). Because of the simplicity of altering constructs or of mixing different plasmids, DNA vaccines have been used to explore the effects of various vaccination conditions, such as the use of different forms of an antigen (secreted or cell membrane-associated), and the effects of co-expressed cytokines. In light of efficient DNA vaccination, particle-mediated gene transfer is superior to the intramuscular injection of expression plasmids (*8*). Although recent advances in gene therapy have improved a number of recombinant virus vectors, their recombinant viruses may remain restricted from the standpoint of clinical application because of the risk of systemic complications in the transplant recipient. Therefore, efficient methods and administration routes for DNA immunization using nonviral gene transfer are required for safe clinical application, and the gene gun would provide a great advantage to clinical gene immunization.

We showed particle-mediated gene transfer by the gene gun into the rat liver. *GFP* expression in the liver induced by this method immediately reached the maximum level, but gradually declined. This early decrease might depend on liver tissue specificity. Although the expression within the muscle by the gene gun was detectable for more than 1 month (*8*), the damaged liver resulting from gene gun bombardment might induce liver regeneration locally. In this experiment, we used the donor liver as a site of primary immunization for vaccination because the evidence from our experiments (*6*) and others (*9*) showed that the beneficial effect of immunization to the liver was observed in malaria, in which the liver is the primary target. Partial hepatectomy was also performed to enhance foreign gene expression transiently in the liver. This transient expression of a foreign protein in the damaged tissue might cause a strong immune response. Effective gene transfection by the gene gun might be achieved in the "living" tissue where the blood supply was sufficient. In contrast, the gene expression level of ex vivo transfection into the liver was low. Regenerating the liver by innervation and blood supply seems to lead to increased transcription of the plasmid DNA. Our data also supported the evidence that calcineurin inhibitors stimulate proliferation of the liver cells after hepatectomy (*14–16*). On the basis of the significant increase of the bromodeoxyuridine labeling index after a hepatectomy, tacrolimus administration significantly enhances hepatocyte proliferation (*17*). However, hepatocyte growth factor (HGF) and transforming growth factor-β1 do not appear to contribute to tacrolimus-induced liver regeneration because their mRNA expression was not affected in the liver (*18*). Recently, the deletion variant of HGF (dHGF), which exerts mitogenic and antifibrotic effects, has also been reported to augment the regeneration and functional recovery of partial liver grafts and to reduce hepatocyte injury in acute cellular rejection (*18*). It is likely that there is a possible pathway to mimic the hepatotropic effect of tacrolimus, though it remains to be elucidated how another immunosuppressant could have a hepatotropic effect after partial hepatectomy. In addition to these results, we also observed increased transgene expression in regenerating muscle after inflammation (*8*). Together, these findings suggest that cell proliferation coupled with tissue regeneration may be essential for efficient transcription of a foreign gene in vivo.

In this study, we tested the ability of gene gun vaccination to induce humoral immunity using HBs cDNA plasmid and demonstrated enhanced antibody production under tacrolimus treatment in the rat liver ALT model. Although hyperimmunoglobulin plus lamivudine has been shown to prevent HBV recurrence effectively in patients after liver transplantation (*19*), it is expensive and might cause a mutant HBV. Tacrolimus is a powerful immunosuppressant that strongly suppresses T-cell–mediated immune responses and possibly limits B-cell–mediated antibody production. In this study, we have focused on humoral immunity because Th1 immunity was significantly enhanced by immunization to liver (*6*) and muscle (*8*) using the gene gun. Although the repeated penetration through skin was required, better immunization was

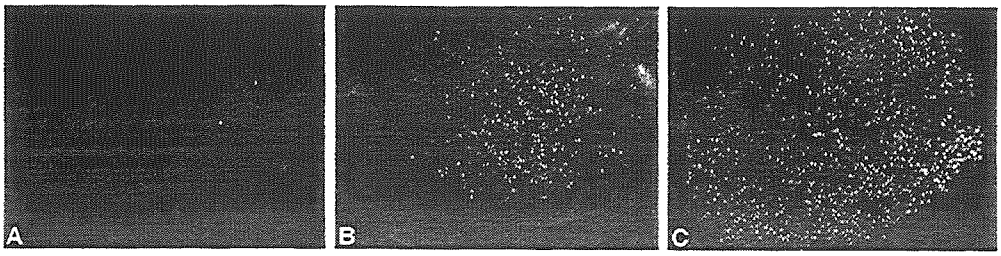

FIGURE 5. ***GFP*** **expression in the grafted liver after particle-mediated gene transfection. The possibility of applying the gene gun-mediated transfection method to rat liver transplantation was examined by using the *GFP* expression plasmid in various settings. Transfection into the donor livers was performed using each of the following procedures: (1) ex vivo bombardment after retrieval; (2) in vivo bombardment immediately after the donor laparotomy; and (3) in vivo bombardment against the remaining 70% liver after a 30% hepatectomy. The liver grafts were then transplanted heterotopically as described (see *Materials and Methods*), and *GFP* expression was monitored by fluorescent microscopy (489-nm excitation). *GFP* expression in procedure 1 (A), procedure 2 (B), and procedure 3 (C). *GFP* was significantly expressed in the grafts from the in vivo bombardment (procedure 2), and *GFP* expression was also augmented in those grafts subjected to a 30% hepatectomy (procedure 3). However, the level of *GFP* expression was low in the livers of the ex vivo bombardment model (procedure 1).**

TABLE 1. Titer of anti-HB antibodies in various immune routes

Group	No.	Immunized route	FK506[a]	Antibody titer (mIU/mL)			
				2 wk	4 wk	7 wk	10 wk
1	5	Skin	–	ND*	5.1±2.3	131.1±31.2	158.1±24.3
2	5	Skin	+	ND	22.1±7.3	164.2±17.1	104.7±37
3	5	Normal liver	–	ND	2.4±1.3	19.6±4.9	90.9±11.4
4	5	Normal liver hepatectomized	+	ND	1.9±0.8	28.9±3.3	155.2±17.3
5	5	Liver hepatectomized	–	ND	4.4±1.3	26.3±3.1	107.2±9.3[b]
6	5	Liver	+	ND	16.3±2.1	215.2±25.4	361.2±29.3[b]

[a] Administration for 2 wk.
[b] $P < 0.001$.
ND, Not detectable.

TABLE 2. Titer of anti-HB antibodies under FK506 treatment

Group	No.	Hepatectomy	FK506	Antibody titer (mIU/mL)		
				2 wk	4 wk[a]	7 wk[b]
1	1	+	+	ND	106.9	635.5
	2	+	+	ND	180.8	694.0
	3	+	+	ND	218.3	770.6
2	1	–	+	ND	88.0	443.1
	2	–	+	ND	90.4	586.4
	3	–	+	ND	89.3	486.3
3	1	+	–	ND	406.6	581.9
	2	+	–	ND	523.3	599.9
	3	+	–	ND	220.5	219.0

[a] No statistical difference among the three groups.
[b] Significant difference ($P < 0.05$) between group 1 and the others.
ND, Not detectable.

obtained through the first bombardment to the liver. A tendency toward hypogammaglobulinemia has been reported recently in heart and renal allograft recipients treated with tacrolimus (*20*, *21*) but not in patients who received liver grafts. It remains to be determined whether tacrolimus may affect antidonor antibody synthesis, although calcineurin inhibitors have been known to decrease transgene expression. Our findings suggest that liver allograft recipients have a potential to respond to infection during tacrolimus treatment for antibody production. The anti-HBs antibody titers in the ALT group were significantly higher than those in the non–liver-transplant group. Thus, the transplanted liver might act to immunize more strongly than a normal liver.

The efficacy in HBV carriers who develop fulminant hepatitis and undergo liver transplantation also remains unclear. However, local DNA immunization to the graft liver may be a promising strategy for preventing infectious diseases associated with liver transplantation under tacrolimus treatment.

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Depletion of Mitochondrial DNA in HIV-1-Infected Patients and Its Amelioration by Antiretroviral Therapy

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Mitochondrial DNA (mtDNA) of peripheral blood mononuclear cells (PBMCs) collected from Human immunodeficiency virus 1 (HIV-1)-infected patients and healthy controls were measured longitudinally using real-time polymerase chain reaction to evaluate the effects of antiretroviral agents on mtDNA synthesis in vivo and to assess the value of monitoring mtDNA in PBMCs to predict adverse events amongst these patients. MtDNA levels in PBMCs were significantly decreased in treatment-naive HIV-1-infected patients compared with healthy people. MtDNA levels were not only significantly correlated with $CD4^+$ T-cell count, but also inversely correlated with HIV-1 viral load. MtDNA levels in untreated patients and healthy controls were stable during the period of observation. On the other hand, amongst patients treated with regimens containing AZT/3TC or d4T/3TC, mtDNA increased during treatment and recovered to levels comparable to healthy controls. In contrast, mtDNA decreased immediately after the initiation of an AZT/ddC-containing regimen. We did not find a correlation between mtDNA levels and changes in clinical parameters. There was no significant difference in mtDNA levels between patients with and those without lipoatrophy. Furthermore, there was no obvious difference in mtDNA levels amongst those patients exhibiting signs and symptoms of peripheral neuropathy. In conclusion, the decrease in mtDNA levels in PBMCs amongst HIV-1-infected patients and its amelioration by antiretroviral therapy may suggest the influence of direct effects on mitochondria or mtDNA by HIV-1 infection. Further investigations are needed to elucidate the mechanisms contributing to decreased mtDNA and the value of mtDNA measurement in the care of HIV-1-infected individuals. ***J. Med. Virol. 70:497–505, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: $CD4^+$ T cell; viral load; nucleoside analogue reverse transcriptase inhibitors; apoptosis; peripheral neuropathy; lipoatrophy

INTRODUCTION

Since the introduction of highly active antiretroviral therapy (HAART), the morbidity and mortality of human immunodeficiency virus 1 (HIV-1) infection has been reduced dramatically. Attention has now focused on adverse events resulting from long term antiretroviral therapy. It is thought that most side effects of nucleoside analogue reverse transcriptase inhibitors (NRTIs) such as macrocytic anemia, peripheral neuropathy, pancreatitis, myopathy, hepatic steatosis and lactic acidosis, are attributable to mitochondrial damage [Kakuda, 2000; Moyle, 2000; White, 2001]. Moreover, the association between NRTIs, particularly d4T, and lipoatrophy has been suggested by several lines of observation [Brinkman et al., 1999; Saint-Marc and Touraine, 1999; Saint-Marc et al., 1999; Carter et al., 2001; Cossarizza et al., 2001; Lichtenstein et al., 2001; Martinez et al., 2001; Saves et al., 2002]. It is thought

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that NRTIs exhibit a high affinity to DNA polymerase γ, the only polymerase used in mitochondrial DNA (mtDNA) replication, and inhibit its synthesis, resulting in mitochondrial damage and thereby reducing cellular energy production.

There are much in vitro data supporting this polymerase γ hypothesis [Martin et al., 1994; Medina et al., 1994; Benbrik et al., 1997], and a hierarchy in inhibitory properties against DNA polymerase γ and mtDNA synthesis, ddC > ddI > d4T > 3TC > AZT > ABC, has been previously proposed [Kakuda, 2000; White, 2001]. Concerning the quantification of mtDNA in vivo, some studies have demonstrated depletion of mtDNA in muscle biopsy specimens from patients with AZT-induced myopathy [Arnaudo et al., 1991; Casademont et al., 1996; Masanes et al., 1998]. Recently, Shikuma et al. demonstrated mtDNA depletion in subcutaneous adipose tissues of HIV-1-infected patients with lipoatrophy [Shikuma et al., 2001]. In contrast, Zaera et al. detected mtDNA deletion, but not depletion, in muscle biopsy specimens from patients with lipodystrophy [Zaera et al., 2001]. Recently, two studies quantified mtDNA in peripheral blood amongst HIV-1-infected patients with lipodystrophy or lipoatrophy, but failed to detect mtDNA depletion [Cossarizza et al., 2002; McComsey et al., 2002]. All of these were cross-sectional studies. Since mtDNA levels are variable amongst individuals, longitudinal studies are needed. Very recently, Cote et al. reported depletion and recovery of mtDNA content in buffy coats before and after cessation of treatment in 8 patients with symptomatic hyperlactatemia, respectively [Cote et al., 2002]. On the other hand, McComsey et al. found no mtDNA depletion in one patient with lactic acidosis [McComsey et al., 2002]. Both studies were quite small. Since different cell types should be expected to differ with respect to intracellular NRTI-metabolism and transportation, biopsy specimens of target organs are optimal specimens to explore the causal relationship between the mtDNA and adverse effects. However, longitudinal evaluation does not warrant invasive procedures; therefore, we have chosen PBMCs as a source for DNA. In this study, we set out to determine whether mtDNA monitoring can predict adverse events in patients on antiretroviral therapy. We also wished to explore the effects of various NRTIs on changes in levels of mtDNA.

MATERIALS AND METHODS

Patients and Healthy Controls

A total of 46 HIV-1-infected patients, who had been followed at the Research Hospital of the Institute of Medical Science, University of Tokyo between 1995 and 2001, and 29 HIV-1-sero-negative healthy volunteers were enrolled in this study. All participants were Japanese. Thirteen of 46 patients remained untreated for their HIV-infection during this period, while the remainder of the patients (n = 33) were treated with various regimens containing at least one NRTI. Only the patients with good compliance greater than 90% of doses reported taken were selected. The background characteristics of participants are listed in Table I. To increase the homogeny of our samples, 25 of 33 treated patients taking a combination of 2 NRTIs, namely AZT/3TC (n = 8), d4T/3TC (n = 12) or AZT/ddC (n = 5), were selected. Twenty-two of 25 subjects were treatment naive patients and the other three had not been exposed to any antiretroviral agents for at least 3 months before initiation of the regimen. Only one patient, who was treated with AZT/ddC/NFV, had a short treatment interruption less than 1 month during the study period.

DNA Extraction from PBMCs

A total of 306 DNA samples, 251 from 46 HIV-1-infected people and 55 from 29 healthy controls were extracted from PBMCs which had been separated from whole blood using Ficoll-Conray and stored in liquid nitrogen. DNA extraction was performed using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instruction. DNA samples were stored at −80°C until use.

Quantitative Real-Time PCR

For mtDNA quantification, the TaqMan PCR system (PE Applied Biosystems, Foster City, CA) was used. Two sets of primers and TaqMan probes [conjugated with the fluorphores FAM (6-carboxyfluorescein) at the 5′ end, and TAMRA (6-carboxytetramethylrhodamine) at the 3′ end] were designed to amplify the mtDNA (GenBank: NC001807). The sequences of primers and probes were as follows: for Mt1 (11149-11218) amplification, forward primer: (5′-TCC CCA CCT TGG CTA TCA TC-3′), reverse primer: (5′-GGG TGT AGA ATA GGA AGT ATG TGC C-3′) and TaqMan probe: (5′-ATG AGG CAA CCA GCC AGA ACG CCT-3′). For Mt2 (2638-2720), forward primer: (5′-TCC ACG AGG GTT CAG CTG TC-3′), reverse primer: (5′-TCG TCT TGC TGT GTT ATG CCC-3′) and TaqMan probe: (5′-CCA GTG AAA TTG ACC TGC CCG TGA A-3′). To normalize the input DNA amount, a segment of β-actin gene (exon3: 2141-2435) was amplified in a separate tube (forward primer: 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′, reverse primer: 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′ and TaqMan probe: 5′-ATG CCC TCC CCC ATG CCA TCC TGC GT-3′). Mt1 was amplified in all samples, but Mt2 was not amplified in some treated patients. The primers and probes for the mtDNA were designed using Primer Express software (v1.5a; PE Applied Biosystems). For β-actin, the primers and probe described in the TaqMan PCR Reagent kit protocol (PE Applied Biosystems) were used. Concentrations of primers and probe were optimized according to the manufacturer's instructions. Each reaction volume (50 μl) included 25 μl of TaqMan Universal PCR Master Mix[2X solution composed of AmpliTaq Gold DNA polymerase, AmpErase uracil-N-glycosylase(UNG), dATP, dCTP, dGTP, dUTP, passive reference (ROX), and optimized buffer components; PE Applied Biosystems], primers (500 nM for mtDNA or 300 nM for β-actin), TaqMan probe

TABLE I. Characteristics of Patients and Healthy Controls

	All treated patients	AZT/3TC	d4T/3TC	AZT/ddC	No treatment controls	Healthy controls
No. of patients	33	8	12	5	13	29
No. of samples	199	42	58	23	52	55
No. of sampling per a person[a]	6.03 ± 2.28	5.25 ± 1.98	4.83 ± 1.19	4.60 ± 2.07	4.00 ± 1.58	3.36 ± 1.43[b]
Duration of follow-up (days)[a]	986.7 ± 567.2	754.0 ± 364.7	654.7 ± 313.9	451.8 ± 135.5	1156.5 ± 401.8	494.6 ± 354.9[b]
Age[a]	38.4 ± 8.8	40.4 ± 9.5	40.33 ± 9.52	35.0 ± 8.1	29.2 ± 7.7	33.1 ± 8.0
Male:Female	30:3	8:0	9:3	5:0	11:2	25:4
Mode of transmission						
MSM[c]	18	3	7	3	7	
Heterosexual	14	5	4	2	3	
Hemophilia	1	0	1	0	3	
Baseline $CD4^+$ cell counts ($/mm^3$)[a]	200.8 ± 118.4[d]	201.9 ± 101.2[e]	210.2 ± 149.6[e]	277.2 ± 70.1[e]	535.5 ± 155.4[d]	
Baseline HIV RNA (copies/ml)[a]	86103 ± 98569[f]	39600 ± 67063[e]	101992 ± 77648[e]	24350 ± 15973[e]	3865 ± 3526[f]	
Coadministered PIs or NNsRTIs		Nothing: 3	NFV: 8	Nothing: 2		
		IDV: 2	RTV/SQV: 2	NFV: 3		
		SQV: 1	IDV: 2			
		EFV: 3	RTV/IDV: 1			
			EFV: 2			
			NVP: 1			

HIV, human immunodeficiency virus; IDV, indinavir; SQV, saquinavir; RTV, ritonavir; NFV, nelfinavir; NVP, nevirapine; EFV, efavirenz.
[a]Mean ± 1 SD.
[b]Mean of 11 healthy people in whom multiple sample were available.
[c]Men who had sex with men.
[d]There was a statistically significant difference in baseline $CD4^+$ T-cell count between treated patients and no treatment controls ($P = 0.000000003$, Student's t-test).
[e]There was no statistically significant difference among the three regimen groups (Kruskal-Wallis H-test).
[f]There was a statistically significant difference in baseline HIV-RNA copies between treated patients and no treatment controls ($P = 0.0009$, Mann Whitney U-test).

(200 nM), 1 μl of DNA and distilled water. UNG was added to prevent carry over of PCR products from previous sessions. Thermal cycler conditions were as follows: 50°C for 2 min to optimize AmpErase UNG enzyme activity, 95°C for 10 min to inactivate UNG, denature native DNA and activate AmpliTaq GOLD DNA polymerase, 40 cycles of 95°C for 15 seconds and 60°C for 1 min. PCR was carried out on the ABI PRISM 7700 sequence detection system (PE Applied Biosystems). Raw data was analyzed using Sequence Detection Systems software (version 1.7a; PE Applied Biosystems). For creating standard curves, a sample of DNA prepared from one PBMC stock of a single healthy person was used throughout the study. Standard curves were created for both mtDNA and β-actin in duplicate for each session using the plots of five different DNA concentrations (2.3, 4.6, 11.5, 23 and 115 ng/μl). All samples were tested in triplicate. Mean values and standard deviations were calculated. MtDNA amounts were expressed in relation to levels of the chromosomal β-actin gene, and the mtDNA/β-actin gene ratio of the sample used for creating standard curves was defined arbitrarily as 1.0 and was not included in the evaluation of healthy controls.

Definitions of Lipoatrophy and Peripheral Neuropathy

Patients self-reporting fat loss in the face or extremities and confirmed by physicians were regarded as lipoatrophic patients. Drug-induced peripheral neuropathy was diagnosed by a neurologist when symptoms appeared after starting therapy.

Statistical Analysis

Correlations between mtDNA levels (measured as Mt1) and various parameters, [$CD4^+$ T-cell count, $CD8^+$ T-cell count, HIV-RNA copies, white blood cell count (WBC), hemoglobin level (Hgb), red blood cell count (RBC), mean corpuslar volume of RBC (MCV), platelet count (Pt), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), blood sugar level (BS), total cholesterol (TC), triglyceride (TG), glutamyl transpeptidase (γ-GTP), and amylase (AMY)] were determined statistically using Spearman's ranks test. Unfortunately, blood samplings for lactate level were not coordinated with the PBMC stock used for DNA extraction. Therefore, we chose mtDNA measured within 3 months of lactate sampling and analyzed these values using Peason's correlation coefficients test. Changes of mtDNA levels during the period of observation were analyzed using the paired t-test. Differences in clinical parameters between various populations was analyzed using Student's t-test, the Mann-Whitney U-test or the Kruskal-Wallis H test. The choice of statistical models and data analysis was undertaken with the consultation of a statistician. All reported