Nonviral Gene Transfer of Human Hepatocyte Growth Factor Improves Streptozotocin-Induced Diabetic Neuropathy in Rats

Naoki Kato,[1] Koichi Nemoto,[1] Kuniaki Nakanishi,[2] Ryuichi Morishita,[3] Yasufumi Kaneda,[3] Maki Uenoyama,[4] Tomosumi Ikeda,[4] and Kyosuke Fujikawa[1]

Peripheral neuropathy is common and ultimately accounts for significant morbidity in diabetes. Recently, several neurotrophic factors have been used to prevent progression of diabetic neuropathy. In this study, we gave repeated intramuscular injections of the human hepatocyte growth factor (HGF) gene percutaneously, using liposomes containing the hemagglutinating virus of Japan (HVJ), to examine therapeutic efficacy of nonviral gene transfer of HGF for experimental diabetic sensorimotor neuropathy in rats. Experimental diabetes induced by intraperitoneal injection of streptozotocin resulted in a marked tactile allodynia (but not in a thermal hyperalgesia), in a reduction of both the conduction velocity and the amplitude, and in a decreased laser Doppler flux of the nerve and the muscle at 6 weeks after the induction. All these changes were significantly reversed by repeated gene transfer of HGF. Furthermore, we analyzed the density of endoneurial capillaries and morphometrical changes of the nerve. The density of endoneurial capillaries, disclosing marked reduction in diabetic rats, was also reversed significantly by repeated gene transfer of HGF; however, no considerable differences were observed morphometrically in either myelinated or unmyelinated axons. These results suggest that nonviral HVJ liposome-mediated gene transfer of human HGF has potential for the safe effective treatment of diabetic sensorimotor neuropathy. *Diabetes* 54:846–854, 2005

Recently, several studies have demonstrated that systemic administration of various neurotrophins such as recombinant human nerve growth factor (1–3), neurotrophin-3 (1), and IGF (4,5) can be used effectively to reverse these symptoms of diabetic neuropathy using animal models. These observations raised hopes that neurotrophins might be useful for the clinical treatment of diabetic neuropathy. However, a human trial of recombinant human nerve growth factor to treat diabetic neuropathy failed to show a significant difference in the outcome (6). A short serum half-life of the recombinant protein or a blood-nerve barrier (analogous to a blood-brain barrier in the central nervous system) could be the explanation for this poor outcome to the therapeutic use of such factors. The presence of these obstacles has highlighted the need to develop innovative therapeutic strategies for more efficient delivery into the nervous system.

Gene transfer represents a novel means to express indentified transgenes in targeted locations in the nervous system. It appears to have advantages over the administration of single- or multiple-bolus doses of a recombinant protein because gene transfer can achieve an optimally high local concentration within the nervous system. Several studies have demonstrated that gene transfer of nerve growth factor, neurotrophin-3, or vascular endothelial growth factor could be potentially used as a treatment for diabetic neuropathy (7–9), and most of these studies were performed using viral vectors because of the high transfection efficiency.

In this study, we gave repeated intramuscular injections of nonviral hemagglutinating virus of Japan (HVJ) liposomes, which contain the coding sequence of hepatocyte growth factor (HGF), to streptozotocin (STZ)-induced diabetic rats. We have recently shown that this nonviral vector delivers DNA successfully into the nervous system from the injected muscle and has some advantages over conventional viral gene transfer (10). HGF was originally identified from plasma and serum as a molecule that could stimulate DNA synthesis in rat and human hepatocytes (11). Although it was at first considered that HGF could exert biological effects only on specific target cells, it has since been demonstrated that HGF also functions as a powerful neurotrophic factor (12,13) and as a powerful angiogenetic factor (14,15). These observations raised

From the [1]Department of Orthopaedic Surgery, National Defense Medical College, Tokorozawa, Saitama, Japan; the [2]Department of Pathology, National Defense Medical College, Tokorozawa, Saitama, Japan; the [3]Division of Gene Therapy Science, Osaka University Medical School, Suita, Osaka, Japan; and the [4]Division of Environmental Medicine, National Defense Medical College Research Institute, Tokorozawa, Saitama, Japan.

Address correspondence and reprint requests to Naoki Kato, MD, Department of Orthopaedic Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama, Japan 359-8513. E-mail: grd1505@gr.ndmc.ac.jp.

Received for publication 11 August 2004 and accepted in revised form 6 December 2004.

CMAP, compound motor action potential; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; HVJ, hemagglutinating virus of Japan; MNCV, motor nerve conduction velocity; STZ, streptozotocin.

hopes that the HGF protein might be useful for the clinical treatment of diabetic neuropathy.

The purpose of this study was to test the hypothesis that delivery and continuous expression of HGF within the nervous system by nonviral HVJ liposome–mediated gene transfer could improve experimental diabetic sensorimotor neuropathy.

RESEARCH DESIGN AND METHODS

A total of 146 male Wistar rats, ~4 weeks old, were assigned to this study. This experimental study was carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and the protocol was approved by the Committee on the Ethics of Animal Experiments in the National Defense Medical College.

Diabetes was induced by a single intraperitoneal injection of 70 mg/kg STZ (in 10 mmol/l citrate/0.9% saline buffer, pH 4.5) under sodium pentobarbital anesthesia (30–50 mg/kg i.p.). Plasma glucose level was measured 1 week later using a blood glucose test meter (Sanwa Kagaku Kenkyusyo, Nagaya, Japan), and all animals with <400 mg/dl were excluded from these studies. Body weight– and age-matched rats were used as nondiabetic control animals. Three weeks after the induction of diabetes, HVJ liposomes (100 μl), containing either 100 μg human HGF plasmid DNA or 100 μg pcDNA 3.1 (−) (as a control vector), were injected percutaneously into the proximal one-third of the tibialis cranialis of the right hindlimb via a 27-gauge needle. This injection was repeated at the same site at 3, 4, and 5 weeks after the induction of diabetes.

A total of 54 rats were used for electrophysiological studies and laser Doppler flux studies. These were divided into three equal groups as follows: diabetic + HGF gene transfer group (DM+HGF; $n = 18$), diabetic + control vector group (DM+Control; $n = 18$), and nondiabetic group ($n = 18$). Fifty rats from two groups (DM+HGF, DM+Control; $n = 25$ in each) were used to assess the expressions of human HGF protein, endogenous rat HGF protein, and human HGF mRNA. Specimens were taken from the injected muscle, the ipsilateral sciatic nerve, the ipsilateral dorsal root ganglia (DRG), and the spinal cord. Twenty-four rats from two groups (DM+HGF, DM+Control; $n = 12$ in each) were used for analysis of the distribution of the human HGF protein and mRNA expression. The remaining 18 were divided into three groups (DM+HGF, DM+Control, nondiabetic; $n = 6$ in each) and used for behavioral studies.

Construction of plasmid DNA. To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted between the *Eco*RI and *Not*I sites of the pUC-Sr α expression vector plasmid. In this plasmid, transcription of HGF cDNA was under the control of the Sr α promoter. As a control vector, we used a pcDNA 3.1 (−) plasmid DNA vector.

Preparation of HVJ liposomes complex vector. The HVJ liposome complex vector was prepared as described previously (16–18). Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in tetrahydrofuran. The lipid mixture was deposited on the sides of a flask by rotary evaporator. HMG (high mobility group)-1 was mixed with plasmid DNA in balanced salt solution, and the mixture was then added to the dried lipid. The liposome–DNA–HMG-1 complex suspension was mixed by a sequence of vortex, sonication. The purified HVJ (Z strain) was inactivated by ultraviolet irradiation immediately before use. The liposome suspension containing lipid was mixed with inactivated HVJ in balanced salt solution. Free HVJ was removed from the HVJ liposomes by sucrose-gradient centrifugation. The top layer containing the DNA-HVJ liposome complex was collected and used immediately.

Analysis of HGF protein expression. The concentration of each HGF protein was measured by enzyme-linked immunosorbent assays (ELISAs) using either anti-human HGF monoclonal antibody or anti-rat HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The human HGF ELISA system specifically detected human HGF, not endogenous rat HGF, and the rat HGF ELISA system specifically detected endogenous rat HGF, not human HGF (19). In addition, the colorimetric assay of protein concentration was performed using a DC Protein Assay System (Bio-Rad, Hercules, CA) and Benchmark Microplate Reader (Bio-Rad). The value measured by ELISA, as adjusted by the concentration of protein, was used as an expression of HGF protein. The measurement of the DRG was performed using all specimens collected at each defined time point due to the small volume of the specimen obtained from a given individual.

RT-PCR. The expression of human HGF mRNA was measured by RT-PCR. The total RNA was isolated using acid guanidinium isothiocyanate-phenol-chloroform extraction and ethanol precipitation. RT-PCR was performed using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied Biosystems, Alameda, CA) with a primer specific for human HGF. The primer was synthesized using an automated DNA synthesizer. Sequence information and the thermocycling condition was as follows: sense primer, 5′-CGACAGTGTT TCCCTTCTCG-3′; anti-sense primer, 5′-ATTGAGAACCTGTTTGCGTTTCT-3′; annealing temperature, 64°C; cycles, 40. PCR products (102 bp) were separated by electrophoresis in a 3% agarose gel and stained with ethidium bromide.

Immunohistochemistry. Specimens were embedded in an optimal cutting temperature compound and frozen at −80°C. Sagittal sections, 20 μm thick, cut serially on a cryostat were incubated overnight at 4°C with mouse monoclonal antibody against human HGF (1:20; Institute of Immunology). The sections were then incubated for 1 h in goat anti-mouse IgG biotinylated secondary antibody (Chemicon, Temecula, CA) diluted 1:200 and were immersed in fluorescein streptavidin (5 μg/ml; Vector Laboratories, Burlingame, CA).

In situ hybridization. The full-length human HGF cDNA, which was inserted between the *Eco*RI and *Not*I sites of the pUC-Sr α expression vector plasmid, was digested by the restriction enzymes of *Eco*RI, and the resulting fragment of HGF cDNA (848 bp) was then ligated between the *Eco*RI cloning sites of pGEM-7Zf (+) (Promega, Madison, WI). The antisense probe and the corresponding sense probe were labeled with biotin using SP6 and T7 polymerase, respectively, by means of an RNA labeling kit (Boehringer Mannheim, Postfach, Germany). Hybridization was performed in 50% (vol/vol) deionized formaide, 5× Denhardt's solution, 5% (vol/vol) dextran sulfate, 2× standard saline citrate, 0.3 mg/ml salmon sperm DNA, 5 mmol/l EDTA, and 0.01 μg/ml biotin-labeled probes. After performing a final stringency wash, hybridization was detected immunologically.

Behavioral studies. Tactile allodynia was determined by qualifying the withdrawal threshold of the hindpaw in response to a Semmes-Weinstein Monofilament Aesthesiometer (Kom Kare, Cincinnati, OH). A series of calibrated monofilaments were applied perpendicularly to the plantar surface of the right hindpaw with sufficient force to bend the filament for 6 s. Brisk withdrawal or paw flinching was considered a positive response. The mechanical stimulus producing a 50% likelihood of withdrawal response was calculated by the method described in detail previously (20). Each testing was repeated several times, and the mean value was used.

Thermal hyperalgesia was measured using a focused heat source directed onto the plantar surface of the right hindpaw. The thermal testing apparatus (MK-350B; Muromachi Kikai, Tokyo, Japan) was used to give the stimulus heat of 50 ± 0.1°C continuously. The withdrawal latency was recorded several times, and the mean value was calculated. A cutoff of 30 s was used to prevent potential tissue damage.

Laser Doppler flux studies. The right sciatic nerve was exposed in the mid-thigh region, and the injection site of the muscle was minimally exposed under anesthesia. A laser Doppler flowmeter (ALF 21R; Advance, Tokyo, Japan) was used for this study. Consecutive arbitrary flow units were recorded for 3 min, and the median of these values was used to represent whole-nerve (epineurial and endoneurial) blood flow and local blood flow of the muscle. The value measured by the laser Doppler flowmeter, as adjusted by the blood flow at week 0, was used as a relative unit of the blood flow in each tissue at each defined time point.

Electrophysiological studies. Immediately after the laser Doppler flux studies, the nerve was stimulated at two points using a hook-up electrode. The proximal stimulation point was the level of the sciatic notch, and the distal stimulation point was 15 mm distal to the proximal point. A surface pick-up electrode, specially designed for this study, was attached to the skin over the injected muscle. Motor nerve conduction velocity (MNCV) and compound motor action potential (CMAP) were measured with the aid of Neuropack instrumentation (Nihon Koden, Tokyo, Japan), and the mean values were calculated.

Histological studies. At 6 weeks after induction of diabetes, specimens were taken from the sciatic nerve at mid-thigh level. They were postfixed with 2% osmium tetroxide and then embedded in Epon resin (Quetol 512; Nisshin EM, Tokyo, Japan). Semi-thin (1 μm in thickness) transverse sections and ultrathin (70 nm) transverse sections were cut for examination under light microscopy and electron microscopy, respectively. Light micrographs were used to analyze the myelinated axons and the density of the endoneurial capillaries. Electron micrographs were used to analyze the unmyelinated axons. The computer system Toshiba Tospix (Toshiba, Tokyo, Japan) was used for the analysis.

Statistical analysis. The Student's t test or ANOVA for parametric values and Kruskal Wallis for nonparametric values were used. Statistical significance was set at $P < 0.05$.

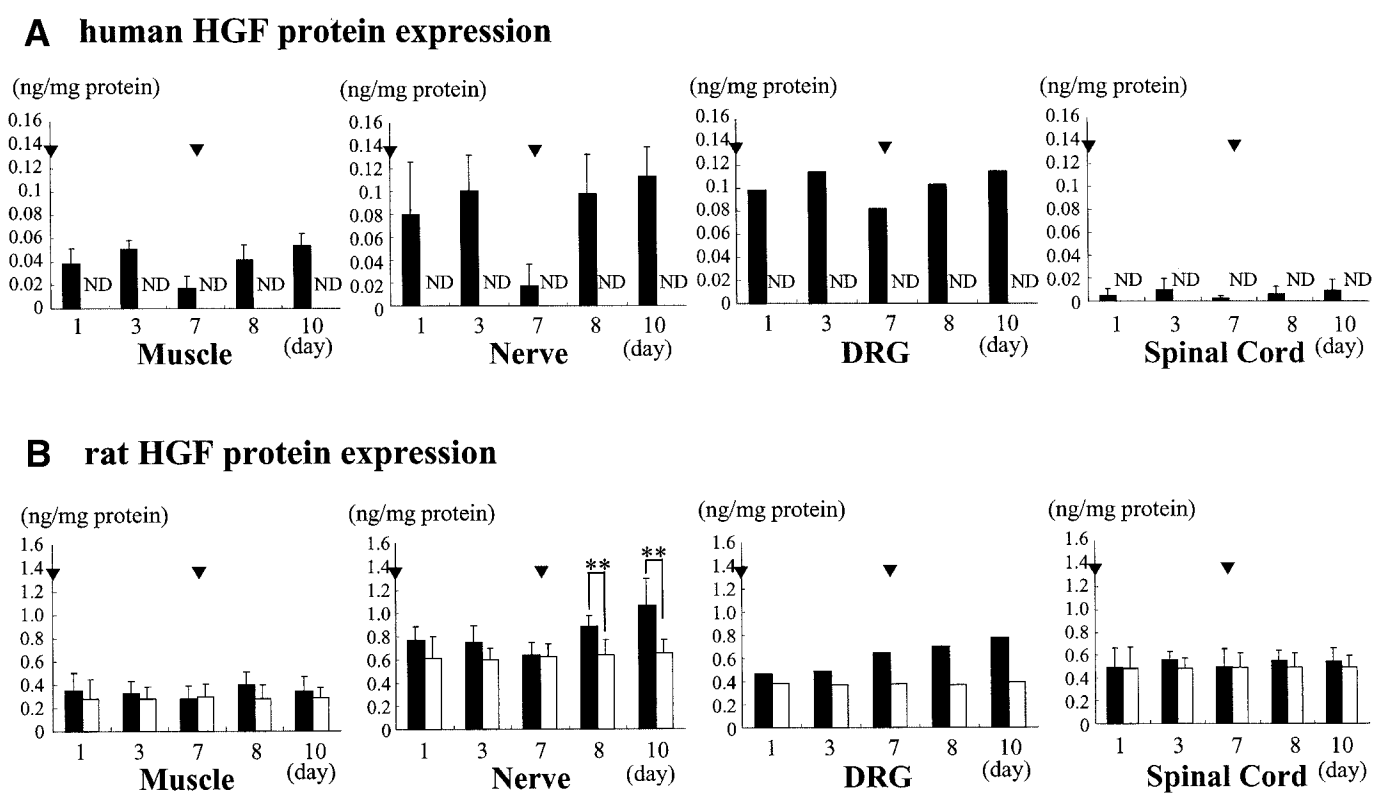

FIG. 1. Human and rat HGF protein expression (ELISA). The DM+HGF group (■) showed expression of the human HGF protein at each defined time point; however, the expression in the spinal cord was considerably lowered throughout the examination period. On the other hand, no expression could be detected in the DM+Control group (*A*). Both the DM+HGF group (■) and the DM+Control group (□) showed high levels of endogenous rat HGF protein in each tissue. The expression of the DM+HGF group was constantly higher than that of the DM+Control group, and, especially, the sciatic nerve of the DM+HGF group showed significantly higher expression than that of the DM+Control group at both 8 and 10 days after transfection (*B*). ND, no detection; ▼, gene transfection; **$P < 0.01$.

RESULTS

Analysis of HGF protein expression. In the DM+HGF group, the expression level peaked on day 3 and dropped off on day 7 after the first transfection. Subsequently, the expression showed a considerable resurgence after the second transfection, which was performed 7 days after the first transfection. No deterioration of the expression could be observed after the second transfection. On the other hand, no such expression could be detected at any defined time point in the DM+Control group (Fig. 1*A*).

Figure 1B shows the expression of endogenous rat HGF protein. Both the DM+HGF group and the DM+Control group showed high levels of rat HGF protein in all preparations at each defined time point. The expression in each tissue of the DM+HGF group was constantly higher than that of the DM+Control group, and, especially, the nerve of the DM+HGF group showed significantly higher expression than that of the DM+Control group at both 8 and 10 days after transfection ($P < 0.01$). In DRG, the DM+HGF group showed a consistent increase in the expression, and the expression was approximately twice as high as that of the DM+Control group on day 10 after the first transfection (day 3 after the second transfection).

RT-PCR. By RT-PCR, human HGF mRNA was specifically detected in the human liver RNA extracted from human liver tissues but not detected in the rat RNA extracted from rat liver. Therefore, our RT-PCR using specific primers against human HGF mRNA did not cross-react with rat HGF mRNA (Fig. 2*A*). The muscle and the nerve from the DM+HGF group showed strong expression on day 1 after the transfection and maintained its expression throughout the experimental period. On the other hand, DRG and the spinal cord from the DM+HGF group demonstrated moderate expression on day 1 but no expression on days 3 and 7 after the first transfection. Subsequently, the expression in DRG showed considerable resurgence on day 10 after the first transfection (day 3 after the second transfection) (Fig. 2*B*). No expression of human HGF mRNA could be detected in any tissue from the DM+Control group.

Immunohistochemistry. In the DM+HGF group, the sensory neurons in the ipsilateral DRG showed immunoreactivity and the ipsilateral nerve showed a granular coloration along the axon, and in the injected muscle, there were many immunopositive muscle fibers. On the other hand, in the DM+Control group, no immunopositive findings were observed in any tissue stained immunohistochemically with the same primary antibody (Fig. 3*A*).

In situ hybridization. In the DM+HGF group, some muscle fibers in the injected muscle showed strong expression of the human HGF mRNA, and the ipsilateral nerve exhibited localization of the expression along the axon. Strong expression of the human HGF mRNA was also observed in many sensory neurons in the ipsilateral DRG. On the other hand, all tissues harvested from the DM+Control group showed very weak levels of background grains when they were treated with the sense probe (Fig. 3*B*).

Behavioral studies. The thermal response latency and the tactile response threshold were measured every week after the induction of diabetes. Time-course change of the

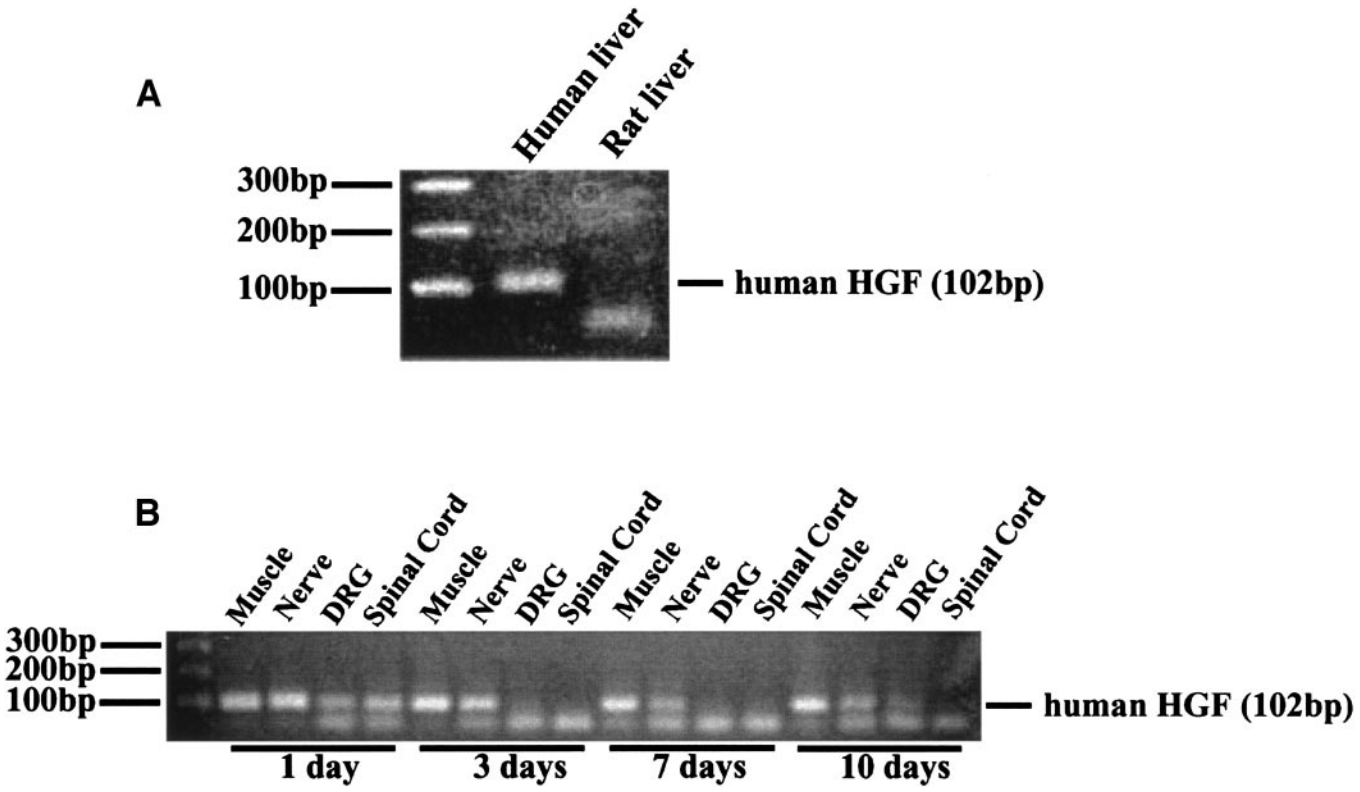

FIG. 2. RT-PCR. Human HGF mRNA was specifically detected in the human liver RNA extracted from human liver tissues, but not detected in the rat RNA extracted from rat liver (*A*). The muscle and the nerve from the DM+HGF group showed strong expression on day 1 after the transfection and maintained its expression throughout the experimental period. On the other hand, DRG and the spinal cord from the DM+HGF group demonstrated moderate expression on day 1 but no expression on days 3 and 7 after the first transfection. Subsequently, the expression in DRG showed considerable resurgence on day 10 after the first transfection (day 3 after the second transfection) (*B*).

thermal response latency showed no statistical differences between the three groups at any defined time points (Fig. 4*A*). On the other hand, diabetic rats (the DM+HGF and DM+Control group) displayed a considerable reduction in tactile response threshold at 1 week after the induction of diabetes. This became more pronounced with time, reaching a 57% reduction in paw withdrawal threshold at 3 weeks after the induction. As shown in Fig. 4*B*, both the DM+HGF group and the DM+Control group demonstrated significantly lower tactile nociceptive threshold than the nondiabetic group throughout the examination period ($P < 0.01$). However, this impaired tactile nociception was significantly improved in the DM+HGF group compared with the DM+Control group and reached significant difference at 5 and 6 weeks after the induction ($P < 0.05$ and $P < 0.01$, respectively) (Table 1).

Electrophysiological studies. At 3 weeks after the induction of diabetes, the mean MNCV of diabetic rats was 31.0 ± 3.8 m/s, against 41.9 ± 4.1 m/s for the nondiabetic rats ($P < 0.01$). After repeated gene transfer, the DM+HGF group demonstrated significant restoration of the mean MNCV, whereas the DM+Control group showed a steady decrease. There was a significant difference between the DM+HGF group and the DM+Control group at 6 weeks after the induction (35.1 ± 3.4 and 28.2 ± 3.1 m/s, respectively). The mean CMAP of diabetic rats also demonstrated a significant decrease compared with that of the nondiabetic rats at 3 weeks after the induction ($P < 0.01$). After repeated gene transfer, the DM+HGF group showed a considerable resurgence, whereas the DM+Control group decreased steadily. There was a significant difference between the DM+HGF and DM+Control group at 6 weeks after the induction (25.7 ± 3.9 and 17.1 ± 2.9 mV, respectively) (Fig. 5, Table 1).

The laser Doppler flux studies. Sciatic nerve of diabetic rats in both the DM+HGF group and the DM+Control group failed to show an increase in whole-nerve laser Doppler flux compared with the nondiabetic group and reached significant difference at 3 weeks after the induction of diabetes ($P < 0.01$). Subsequently, the whole-nerve laser Doppler flux in the DM+HGF group considerably increased and reached significance compared with the DM+Control group at 6 weeks after the induction ($P < 0.01$). There was no significant difference between the DM+HGF group and the nondiabetic group. Local muscle laser Doppler flux followed approximately the same pattern as that observed for the sciatic nerve, with the exception that there was still a significant difference between the DM+HGF group and the nondiabetic group at 6 weeks after the induction ($P < 0.01$) (Fig. 6, Table 1).

Histological studies of the nerve. The semi-thin transverse sections of sciatic nerve from the three groups are shown in Fig. 7*A*. Size/frequency distributions of myelinated axons showed two peaks at around 2.00 and 4.00 μm. There was a trend toward a decrease in the DM+Control group compared with the nondiabetic group, and this trend was slightly reversed by HGF gene transfer. Representative electron microscopic findings of the sciatic nerve from the three groups are shown in Fig. 7*B*. Size/frequency distribution of unmyelinated axons showed a trend toward an increase in both the DM+Control group and the DM+

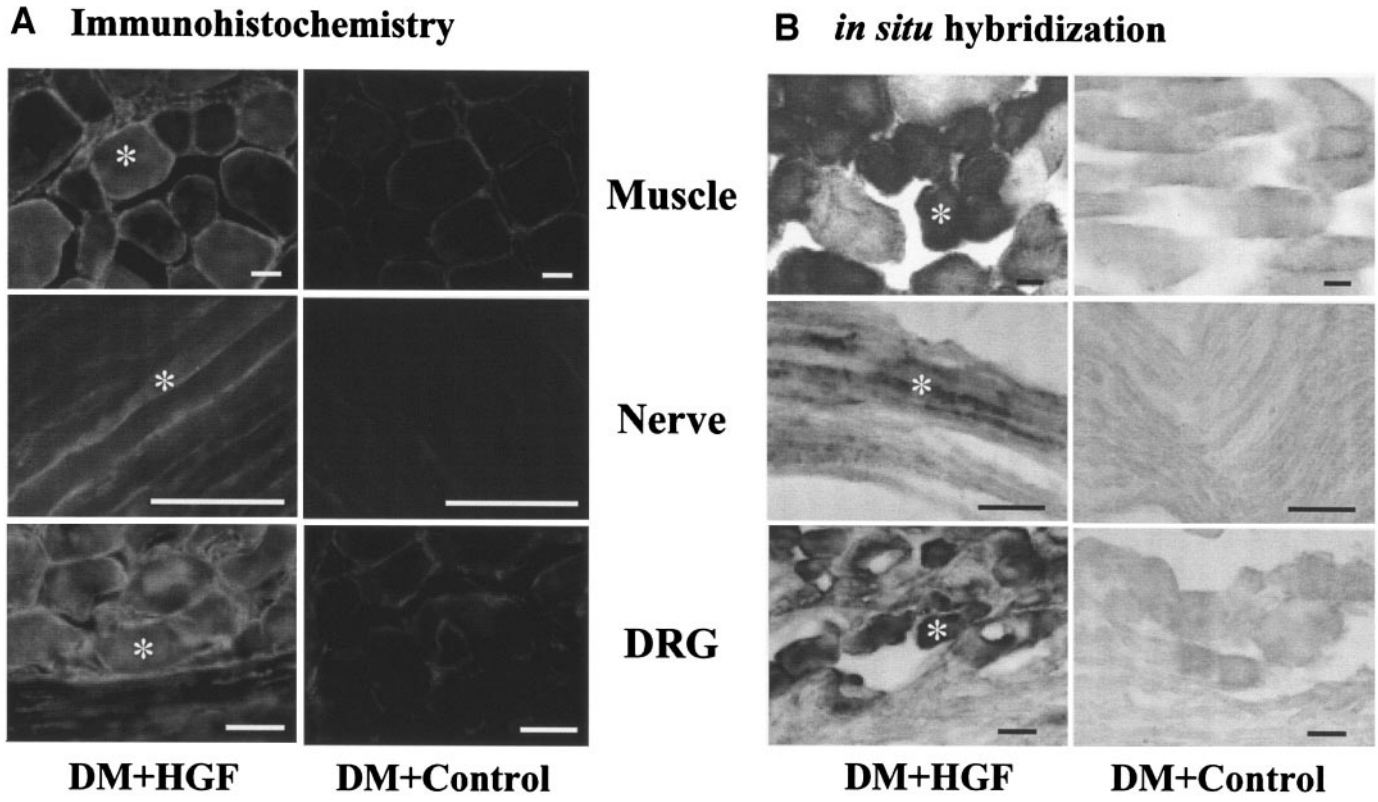

FIG. 3. Immunohistochemistry (fluorescein isothiocyanate) and in situ hybridization. *A*: In the DM+HGF group, the injected muscle, the ipsilateral sciatic nerve, and the ipsilateral sensory neurons in the ipsilateral lumbar DRG showed immunoreactivity. On the other hand, in the DM+Control group, no immunopositive findings were observed in any tissue stained immunohistochemically with the same primary antibody. *B*: In the DM+HGF group, some muscle fibers in the injected muscle showed strong expression of the human HGF mRNA, and the ipsilateral nerve exhibited localization of the expression along the axon. Strong expression of the human HGF mRNA was also observed in many sensory neurons in the ipsilateral DRG. On the other hand, all tissues harvested from the DM+Control group showed very weak levels of background grains when they were treated with the sense probe. Scale bar: 50 μm.

HGF group, in comparison with that in the nondiabetic group.

The mean diameter of myelinated axons in the nondiabetic group was 3.73 ± 0.27 μm, the DM+Control group was 3.58 ± 0.32 μm, and the DM+HGF group was 3.68 ± 0.35 μm. There were no significant differences in the mean diameter of the axons between the three groups. The mean diameter of unmyelinated axons in the nondiabetic group was 0.65 ± 0.03 μm, the DM+Control group was 0.66 ± 0.04 μm, and the DM+HGF group was 0.66 ± 0.06 μm. There were no significant differences in the mean diameter of the axons among the three groups. The density of the endoneurial capillaries in the nondiabetic group was 38.7 ± 3.0 number/mm^2, the DM+Control group was

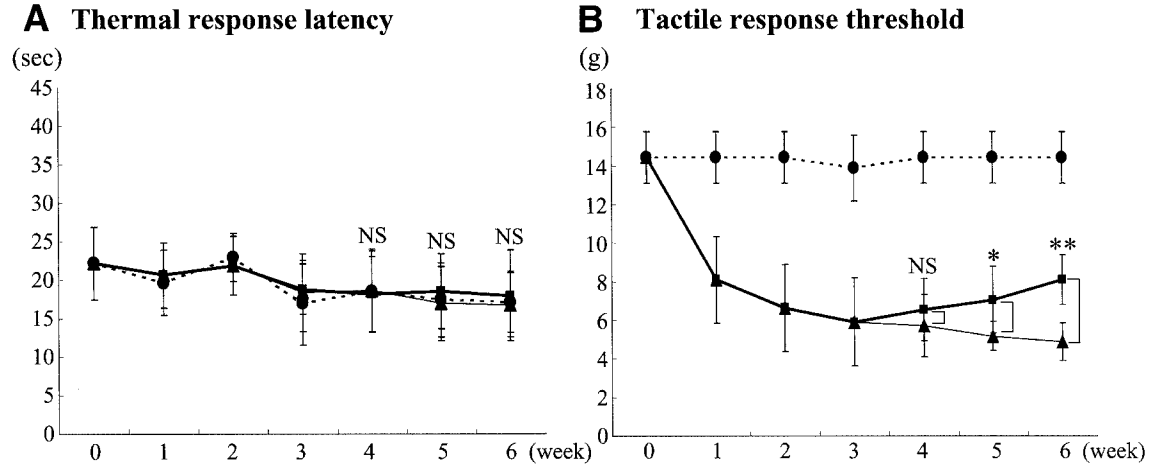

FIG. 4. Behavioral tests for the thermal response latency and the tactile response threshold. Time-course change of the thermal response latency (*A*) and the tactile response threshold (*B*) of the nondiabetic group (●), the DM+HGF group (■), and the DM+Control group (▲) is shown. The thermal response latency showed no statistical differences between the three groups. On the other hand, both the DM+HGF group and the DM+Control group demonstrated significantly lower tactile response threshold than the nondiabetic group throughout the examination period ($P < 0.01$). This impaired tactile nociception was significantly improved in the DM+HGF group compared with the DM+Control group and reached significant difference at 5 and 6 weeks after the induction. *$P < 0.05$; **$P < 0.01$.

TABLE 1
Effects of streptozotocin-induced diabetes and influence of human HGF gene therapy

	Nondiabetic	DM+HGF	DM+Control	*P*
Body weight (g)	315.0 ± 17.9	158.7 ± 36.7	158.3 ± 16.0*	<0.01
Plasma glucose level (mg/dl)	103.2 ± 12.2	568.7 ± 40.1	597.5 ± 29.6*	<0.01
Thermal response latency (s)	17.2 ± 3.98	18.0 ± 5.9	16.7 ± 4.2	NS
Tactile response threshold (g)	14.5 ± 1.3	8.1 ± 1.3	4.9 ± 1.0†	<0.01
MNCV (m/s)	42.2 ± 3.3	35.1 ± 3.4	28.2 ± 3.1†	<0.01
CMAP (mV)	31.1 ± 2.5	25.7 ± 3.9	17.1 ± 2.9†	<0.01
Laser doppler flux (relative unit)				
Muscle	1.87 ± 0.14	1.56 ± 0.16	1.18 ± 0.18†	<0.01
Nerve	1.38 ± 0.12	1.25 ± 0.15	0.86 ± 0.17†	<0.01
Myelinated axon diameter (μm)	3.73 ± 0.27	3.68 ± 0.35	3.58 ± 0.32	NS
Unmyelinated axon diameter (μm)	0.65 ± 0.03	0.66 ± 0.06	0.66 ± 0.04	NS
Density of endoneurial capillaries (number/mm^2)	38.7 ± 3.0	39.3 ± 2.6	31.8 ± 3.7†	<0.01

Data are means ± SE at 6 weeks after induction of diabetes. *$P < 0.05$, nondiabetic vs. DM+HGF and DM+Control; †$P < 0.05$, DM+Control vs. DM+HGF.

31.8 ± 3.7 number/mm^2, and the DM+HGF group was 39.3 ± 2.6 number/mm^2. The DM+Control group disclosed a significant reduction in the density compared with the nondiabetic group and the DM+HGF group ($P < 0.01$). There was no significant difference between the DM+HGF group and the nondiabetic group (Table 1).

DISCUSSION

We have now confirmed that delivery and continuous expression of human HGF within the nervous system significantly improved the experimental diabetic sensorimotor neuropathy induced by STZ. In this study, diabetic rats showed tactile allodynia, which was evident within 1 week after the induction of diabetes and lasted for at least 6 weeks (the end of the study) but failed to show any significant alterations in thermal response latency as reported previously (21). It is considered that tactile nociception is predominantly mediated by myelinated afferents, whereas thermal nociception is mainly transmitted through unmyelinated c-fiber afferents (22). It would therefore be possible that myelinated afferents mediating tactile nociception are more vulnerable than c-fiber afferents mediating thermal nociception in these diabetic rats. In addition, we observed a significant reduction of MNCV and an overall reduction of whole-nerve blood flow at 3 weeks after the induction of diabetes. This result corroborates previous reports demonstrating that diabetic neuropathy has been related to microangiopathy and endoneurial ischemia (23,24) and also supports other reports showing that reduced blood flow or ischemia directly affects nerve conduction velocity (25–27).

Furthermore, we have conducted detailed morphometric studies for both myelinated and unmyelinated axons to determine the structural changes that may underlie these functional abnormalities. In this study, there were no significant differences in the mean diameter of either myelinated or unmyelinated axons between the three groups. This discrepancy between functional abnormalities and morphometrical abnormalities has been frequently observed in diabetic rats (28,29). On the other hand, the size/frequency distributions of both myelinated and unmyelinated axons showed unique morphometrical changes. Malone et al. (30) reported that morphometrical abnormalities were confirmed only when diabetes had been induced in immature rats and therefore suggested that hyperglycemia would impair growth and maturation of nerves rather than degeneration of mature nerves. Considering that diabetes was induced in 4-week-old rats in this study, we suppose the impairment of growth and maturation of specific axons might be one explanation for these unique morphometrical changes.

It has been reported that HGF has a potential to exert multifunctional biological effects. In the nervous system, HGF plays an important role in the maturation and function of neurons (12) and also prevents neuronal death by its antiapoptotic effect (13). Thus, it should function as a powerful neurotrophic factor in this diabetic neuropathy model. However, it is likely that it also functioned as a

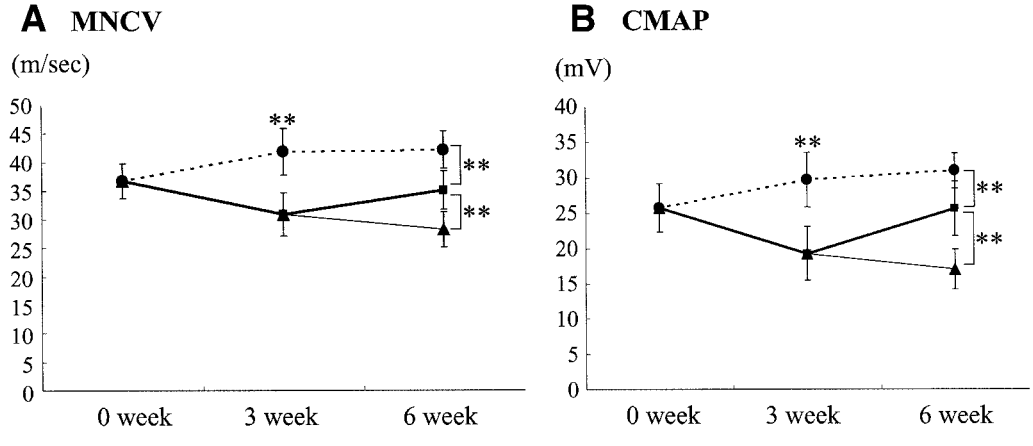

FIG. 5. Electrophysiological studies. MNCV (*A*) and CMAP (*B*) of the nondiabetic group (●), the DM+HGF group (■), and the DM+Control group (▲) are shown. Both MNCV and CMAP showed marked reduction in the DM+Control group; however, this reduction was prevented significantly in the DM+HGF group. **$P < 0.01$.

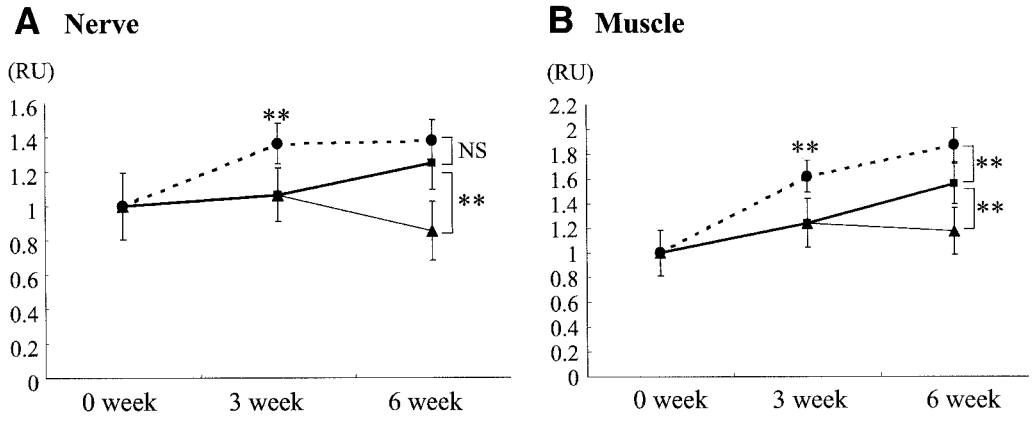

FIG. 6. The laser Doppler flux studies. The laser Doppler flowmetry of the sciatic nerve (*A*) and the injected muscle (*B*) of the nondiabetic group (●), the DM+HGF group (■), and the DM+Control group (▲) are shown. Both the sciatic nerve and the injected muscle showed marked reduction in the DM+Control group; however, this reduction was prevented significantly in the DM+HGF group. **$P < 0.01$.

powerful angiogenetic factor (14,15). Indeed, our laser Doppler flux studies showed that whole-nerve blood flow in diabetic rats was fully restored to the control level at 3 weeks after the first gene transfer, and the density of the endoneurial capillaries that disclosed a reduction in diabetic rats was significantly reversed by repeated HGF gene transfer. Considering that no remarkable expression of human HGF protein could be observed in the spinal cord, it seemed more plausible that the improvement of MNCV was achieved mainly by virtue of the angiogenetic effect, not the neurotrophic effect of HGF.

For repeated HGF gene therapy, we used a nonviral HVJ liposome–mediated gene transfer method that had been used successfully for in vivo gene transfer into various tissues (14,31–35). HVJ contains two distinct glycoproteins (hemagglutinating neuroaminidase and fusion protein) in its envelope that are involved in cell fusion. HVJ liposomes can fuse with plasma membrane, and DNA can be directly introduced into cell cytoplasm without lysosomal degradation. It is considered that HVJ liposomes do not replicate or recombine to form an infectious agent and may not evoke inflammatory or immune responses (33). Recently, gene transfer into the nervous system has been performed in experimental animals using viral vectors because of the high transfection efficiency (36–42). However, these methods are potentially hazardous because of viral infection–associated toxicity, immunological compromise (which makes repeated in vivo gene transfer inefficient), and most importantly, deleterious side effects (43). In this respect, we believe this nonviral gene transfer method could be considered suited to clinical applications.

In addition, there were three other notable findings in this study. First of all, stable transfection efficiency could be obtained in the nerve and DRG by repeated intramus-

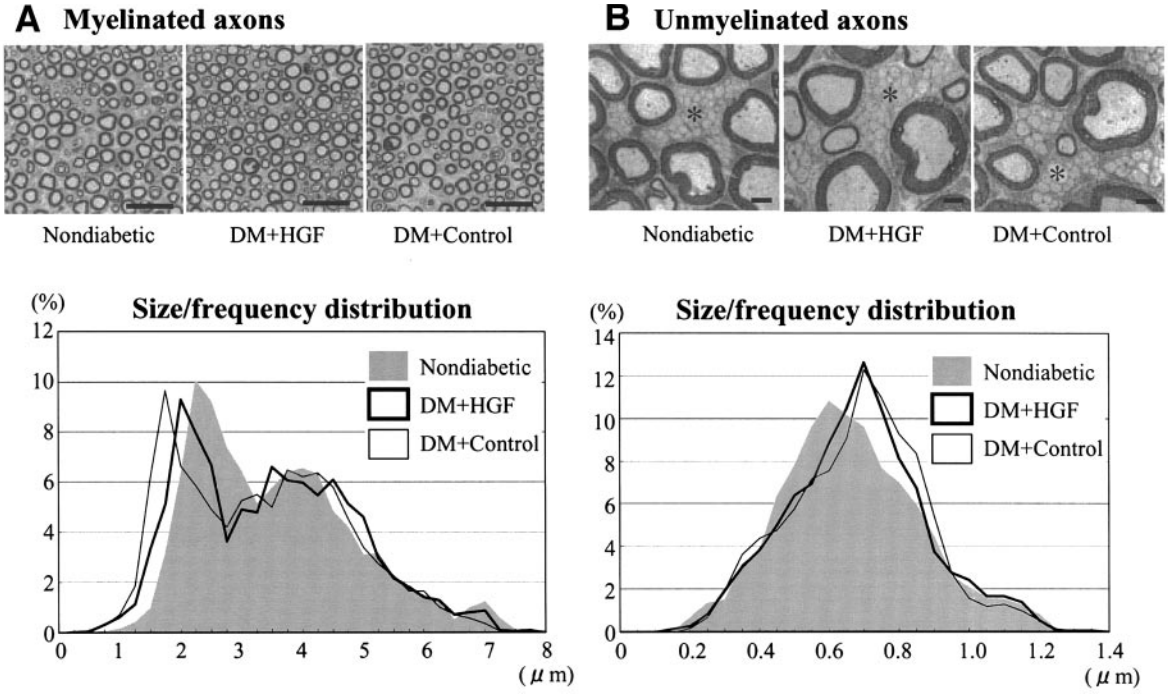

FIG. 7. Histological studies of the nerve. *A*: Myelinated axons. The semi-thin transverse sections of sciatic nerve obtained at 6 weeks after induction of the diabetes are shown. Large-diameter axons and small-diameter axons were found to coexist in each group. There was no infiltration of inflammatory mononuclear cells into the nerve in either group. The size/frequency distributions of myelinated axons showed two peaks at around 2.00 and 4.00 μm. There was a trend toward a decrease in the DM+Control group compared with the nondiabetic group, and this trend was slightly reversed by HGF gene transfer. *B*: Unmyelinated axons. Representative electron microscopic findings of the sciatic nerve obtained at 6 weeks after the induction of the diabetes (*unmyelinated axons) are shown. The size/frequency distribution of unmyelinated axons showed a trend toward an increase both in the DM+Control group and in the DM+HGF group, in comparison with that in the nondiabetic group.

cular injections. In this study, the expression of the human HGF mRNA was confirmed in the nerve and the DRG by RT-PCR, and its distribution was identified using the in situ hybridization technique. Therefore, it is evident that gene transfer into the nervous system did occur via retrograde axonal transport, and this transported exogenous HGF gene produced human HGF protein detected in each tissue. This result demonstrates the practical feasibility of gene therapy for the diabetic sensorimotor neuropathy via retrograde axonal transport.

Second, it is notable to see that the present human HGF gene transfer increased the production of endogenous rat HGF, especially in the nerve and DRG. This result seems to support the idea that transfection into the nervous system with the human HGF gene has the potential to boost the expression of endogenous rat HGF. It is possible that the human HGF gene may serve as a positive regulator of the production, secretion, and/or posttranslational modification of rat HGF. This phenomenon was also reported in previous experimental studies (19,34).

Third, the concentration of endogenous rat HGF in each tissue was at least 10-fold higher than that of human HGF in this study. This considerable difference in the concentration between human HGF and rat HGF was also observed in previous reports where endothelial cells had been transfected with a human HGF gene and the conditioned medium from these cells was measured by ELISA. They revealed that the immunoreactive levels of human and rat HGF were ~0.29 and 14 ng/ml higher, respectively, than those from the control group and therefore suggested that HGF might itself regulate local HGF production by an autoloop-positive feedback mechanism and thus operate in an autocrine-paracrine manner (44). Our data support this hypothesis.

On the basis of our results, we believe that repeated intramuscular injection of nonviral HGF-HVJ liposomes is an efficient way of preventing functional nerve degeneration in a model of STZ-induced diabetic sensorimotor neuropathy. We commend HGF gene transfer as a novel therapeutic paradigm for the treatment of diabetic sensorimotor neuropathy.

ACKNOWLEDGMENTS

This work was supported by grants from the Nakayama Foundation for Human Science and the Nakatomi Foundation.

REFERENCES

1. Tomlinson DR, Fernyhough P, Diemel LT: Role of neurotrophins in diabetic neuropathy and treatment with nerve growth factors. *Diabetes* 46:S43–S49, 1997
2. Unger JW, Klitzsch T, Pera S, Reiter R: Nerve growth factor (NGF) and diabetic neuropathy in the rat: morphological investigations of the sural nerve, dorsal root ganglion, and spinal cord. *Exp Neurol* 153:23–34, 1998
3. Elias KA, Cronin MJ, Stewart TA, Carlsen RC: Peripheral neuropathy in transgenic diabetic mice: restoration of c-fiber function with human recombinant nerve growth factor. *Diabetes* 47:1637–1642, 1998
4. Ishii DN, Lupien SB: Insulin-like growth factors protect against diabetic neuropathy: effects on sensory nerve regeneration in rats. *J Neurosci Res* 40:138–144, 1995
5. Zhuang HX, Snyder CK, Pu SF, Ishii DN: Insulin-like growth factors reverse or arrest diabetic neuropathy: effects on hyperalgesia and impaired nerve regeneration in rats. *Exp Neurol* 140:198–205, 1996
6. Apfel SC, Schwartz S, Adornato BT, Freeman R, Biton V, Rendell M, Vinik A, Giuliani M, Stevens JC, Barbano R, Dyck PJ: Efficacy and safety of recombinant human nerve growth factor in patients with diabetic polyneuropathy: a randomized controlled trial. *JAMA* 284:2215–2221, 2000
7. Pradat PF, Kennel P, Naimi-Sadaoui S, Finiels F, Orsini C, Revah F, Delaere P, Mallet J: Continuous delivery of neurotrophin 3 by gene therapy has a neuroprotective effect in experimental models of diabetic and acrylamide neuropathies. *Human Gene Ther* 12:2237–2249, 2001
8. Schratzberger P, Walter DH, Rittig K, Bahlmann FH, Pola R, Curry C, Silver M, Krainin JG, Weinberg DH, Ropper AH, Isner JM: Reversal of experimental diabetic neuropathy by VEGF gene transfer. *J Clin Invest* 107: 1083–1092, 2001
9. Goss JR, Goins WF, Lacomis D, Mata M, Glorioso JC, Fink DJ: Herpes simplex-mediated gene transfer of nerve growth factor protects against peripheral neuropathy in streptozotocin-induced diabetes in the mouse. *Diabetes* 51:2227–2232, 2002
10. Kato N, Nakanishi K, Nemoto K, Morishita R, Kaneda Y, Uenoyama M, Ikeda T, Fujikawa K: Efficient gene transfer from innervated muscle into rat peripheral and central nervous systems using a nonviral HVJ (hemagglutinating virus of Japan)-liposome method. *J Neurochem* 85:810–815, 2003
11. Nakamura T, Nawa K, Ichikawa A, Kaise N, Nishimoto T: Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Lett* 224:311–316, 1987
12. Korhonen L, Sjoholm U, Takei N, Kern MA, Schirmacher P, Castren E, Lindholm D: Expression of c-Met in developing rat hippocampus: evidence for HGF as a neurotrophic factor for calbindin D-expressing neurons. *Eur J Neurosci* 12:3453–3461, 2000
13. Hayashi K, Morishita R, Nakagami H, Yoshimura S, Hara A, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y, Sakai N: Gene therapy for preventing neuronal death using hepatocyte growth factor: in vivo gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Ther* 8:1167–1173, 2001
14. Aoki M, Morishita R, Taniyama Y, Kida I, Moriguchi A, Matsumoto K, Nakamura T, Kaneda Y, Higaki J, Ogihara T: Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis, ets. *Gene Ther* 7:417–427, 2000
15. Koike H, Morishita R, Iguchi S, Aoki M, Matsumoto K, Nakamura T, Yokoyama C, Tanabe T, Ogihara T, Kaneda Y: Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene. *FASEB J* 17:779–781, 2003
16. Oku N, Nojima S, Inoue K: Studies on the interaction of HVJ (Sendai virus) with liposomal membranes: HVJ-induced permeability increase of liposomes containing glycophorin. *Virology* 116:419–427, 1982
17. Kaneda Y, Morishita R, Dzau VJ: Prevention of restenosis by gene therapy. *Ann N Y Acad Sci* 811:299–308, 1997
18. Tsuboniwa N, Morishita R, Hirano T, Fujimoto J, Furukawa S, Kikumori M, Okuyama A, Kaneda Y: Safety evaluation of hemagglutinating virus of Japan: artificial viral envelope liposomes in nonhuman primates. *Human Gene Ther* 12:469–487, 2001
19. Nakanishi K, Uenoyama M, Tomita N, Morishita R, Kaneda Y, Ogihara T, Matsumoto K, Nakamura T, Maruta A, Matsuyama S, Kawai T, Aurues T, Hayashi T, Ikeda T: Gene transfer of human hepatocyte growth factor into rat skin wounds mediated by liposomes coated with the Sendai virus (hemagglutinating virus of Japan). *Am J Pathol* 161:1761–1772, 2002
20. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63, 1994
21. Raz I, Hasdai D, Seltzer Z, Melmed RN: Effect of hyperglycemia on pain perception and on efficacy of morphine analgesia in rats. *Diabetes* 37:1253–1259, 1988
22. Ossipov MH, Bian D, Malan TP Jr, Lai J, Porreca F: Lack of involvement of capsaicin-sensitive primary afferents in nerve-ligation injury induced tactile allodynia in rats. *Pain* 79:127–133, 1999
23. Dyck PJ: Hypoxic neuropathy: does hypoxia play a role in diabetic neuropathy. *Neurology* 39:111–118, 1989
24. Stevens EJ, Carrington AL, Tomlinson DR: Nerve ischemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models. *Diabetologia* 37:43–48, 1994
25. Cameron NE, Cotter MA, Low PA: Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol* 261:E1–E8, 1991
26. Tesfaye S, Harris RM, Ward JD: Exercise-induced conduction velocity increment: a marker of impaired peripheral nerve blood flow in diabetic neuropathy. *Diabetologia* 35:155–159, 1992
27. Young MJ, Veves A, Smith JV, Walker MG, Boulton AJM: Restoring lower

limb blood flow improves conduction velocity in diabetic patients. *Diabetologia* 38:1051–1054, 1995
28. Yagihashi S: Pathology and pathogenetic mechanisms of diabetic neuropathy. *Diabetes Metab Rev* 11:193–225, 1995
29. Walker D, Carrington A, Cannan SA, Sawicki D, Sredy J, Boulton AJ, Malik RA: Structural abnormalities do not explain the early functional abnormalities in the peripheral nerves of the streptozotocin diabetic rat. *J Anatomy* 195:419–427, 1999
30. Malone JI, Lowitt S, Korthals JK, Salem A, Miranda C: The effect of hyperglycemia on nerve conduction and structure is age dependent. *Diabetes* 45:209–215, 1996
31. Morishita R, Gibbons GH, Ellisoon KE, Nakajima M, Zhang L, Kaneda Y, Ogihara T, Dzau VJ: Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. *Proc Natl Acad Sci U S A* 90:8474–8478, 1993
32. Yamada K, Moriguchi A, Morishita R, Aoki M, Nakamura Y, Mikami H, Oshima T, Ninomiya Y, Kaneda Y, Higaki J, Ogihara T: Efficient oligonucleotide delivery using the HVJ-liposome method in the central nervous system. *Am J Physiol* 271:R1212–R1220, 1996
33. Dzau VJ, Mann MJ, Morishita R, Kaneda Y: Fusigenic viral liposome for gene therapy in cardiovascular diseases. *Proc Natl Acad Sci U S A* 93:11421–11425, 1996
34. Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R, Matsumoto K, Nakamura T, Takahashi H, Okamoto E, Fujimoto J: Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 5:226–230, 1999
35. Hagihara Y, Saitoh Y, Kaneda Y, Kohmura E, Yoshimine T: Widespread gene transfection into the central nervous system of primates. *Gene Ther* 7:759–763, 2000
36. Finiels F, Ribotta MG, Barkats M, Samolyk ML, Robert JJ, Privat A, Revah F, Mallet J: Specific and efficient gene transfer strategy offers new potentialities for the treatment of motor neurone diseases. *Neuroreport* 6:2473–2478, 1995
37. Kuo H, Ingram DK, Crystal RG, Mastrangeli A: Retrograde transfer of replication deficient recombinant adenovirus vector in the central nervous system for tracing studies. *Brain Res* 705:31–38, 1995
38. Shy ME, Tani M, Shi YJ, Whyatt SA, Chbihi T, Scherer SS, Kamholz J: An adenoviral vector can transfer lacZ expression into Schwann cells in culture and in sciatic nerve. *Ann Neurol* 38:429–436, 1995
39. Sørensen J, Haase G, Krarup C, Gilgenkrantz H, Kahn A, Schmalbruch H: Gene transfer to Schwann cells after peripheral nerve injury: a delivery system for therapeutic agents. *Ann Neurol* 43:205–211, 1998
40. Haase G, Pettmann B, Vigne E, Castelnau-Ptakhine L, Schmalbruch H, Kahn A: Adenovirus-mediated transfer of the neurotrophin-3 gene into skeletal muscle of pmn mice: therapeutic effects and mechanisms of action. *J Neurol Sci* 160:S97–S105, 1998
41. Yamamura J, Kageyama S, Uwano T, Kurokawa M, Imakita M, Shiraki K: Long-term gene expression in the anterior horn motor neurons after intramuscular inoculation of a live herpes simplex virus vector. *Gene Therapy* 7:934–941, 2000
42. Glatzel M, Flechsig E, Navarro B, Klein MA, Paterna JC, Bueler H, Aguzzi A: Adenoviral and adeno-associated viral transfer of genes to the peripheral nervous system. *Proc Natl Acad Sci U S A* 97:442–447, 2000
43. Marshall E: Gene therapy death prompts review of adenovirus vector. *Science* 286:2244–2245, 1999
44. Yo Y, Morishita R, Yamamoto K, Tomita N, Kida I, Hayashi S, Moriguchi A, Kato S, Matsumoto K, Nakamura T, Higaki J, Ogihara T: Actions of hepatocyte growth factor as a local modulator in the kidney: potential role in pathogenesis of renal disease. *Kidney Int* 53:50–58, 1998