All animal protocols were approved by the Animal Ethics Committee of Asahikawa Medical University and by the Osaka University Committee on Animal Research.

Measurement of Human and Rat HGF

Tissue samples were harvested from the operative site of the rat tail at the each time point. Human HGF expression was measured by real-time reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized with the Thermo Script RT-PCR System (Invitrogen, Carlsbad, Calif). Relative gene copy numbers of HGF and glyceraldehyde phosphate dehydrogenase were quantified by real-time RT-PCR with TaqMan Gene Expression Assays (HS.00300159, Applied Biosystems, Foster City, Calif). The absolute number of gene copies was normalized with glyceraldehyde phosphate dehydrogenase and standardized by a sample standard curve.

Rat endogenous immunoreactive HGF concentration also was measured by an enzyme immunoassay using anti-rat HGF antibody. In brief, 96-well plates (Corning, NY) were coated with rabbit anti-rat HGF IgG at 4°C for 15 hours. After blocking with 3% bovine serum albumin in PBS, the conditioned medium was added to each well, and the preparation was incubated for 2 hours at 25°C. The wells were washed 3 times with PBS containing 0.025% Tween 20 (PBS-Tween); biotinylated rabbit anti-rat HGF IgG was added; and the preparation was incubated for 2 hours at 25°C. After washing with PBS-Tween, the wells were incubated with horseradish peroxidase–conjugated streptavidin-biotin complex in PBS-Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/mL *o*-phenylenediamine, 100 mmol/L sodium phosphate, 50 mmol/L citric acid, and 0.015% H_2O_2. The enzyme reaction was terminated by adding 1 mol/L H_2SO_4, and absorbance was measured at 490 nm.

Statistical Analysis

Statistical analysis was performed with StatView 5.0 software (SAS Institute, Inc, Cary, NC). All results are expressed as mean±SEM. Data were compared using ANOVA, followed by the Dunnett test for pairwise comparisons against control and by the Tukey test for multiple comparisons. Rat tail thickness data also were analyzed by repeated-measures ANOVA. To evaluate the effect of each treatment further, we used the mixed-effect model from day 1 to 35.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Development of LEC Culture System

cLECs were isolated from canine thoracic ducts (Figure 1A)[19] and grown in culture until confluence on day 14 (Figure 1B). Immunofluorescent staining of these cells was positive for the endothelial cell markers von Willebrand factor and PECAM-1 (Figure 1C and 1D) and for the lymphatic-specific markers VEGFR-3, LYVE-1, podoplanin, and Prox1 (Figure 1E through 1H). Because all cells were positive for these markers, the purities of cLEC cultures were considered to be >99%.

HGF Promotes LEC Proliferation and Migration

To investigate whether HGF promotes cLEC proliferation and migration, the cell was treated with several concentrations of human recombinant HGF. Treating cLECs with human recombinant HGF resulted in a dose-dependent increase in cellular proliferation (no treatment, 0.227±0.014 cells; 2 ng/mL, 0.303±0.023 cells; 10 ng/mL, 0.368±0.033 cells; 50 ng/mL, 0.367±0.031 cells; absorbance optical density (OD), 490 nm; $P<0.05$; Figure 2A) and migration (no treatment, 4.333±0.882 cells; 10 ng/mL, 228.000±5.033 cells; 50 ng/mL, 277.333±15.624 cells; $P<0.05$; Figure 2B). Furthermore, cells cotransfected with the c-*fos*–luciferase reporter gene and plasmid encoding human HGF showed increased c-*fos* promoter activity compared with cells transfected with GFP plasmid (as control) or with VEGF165 plasmid ($P<0.05$; Figure 2C).

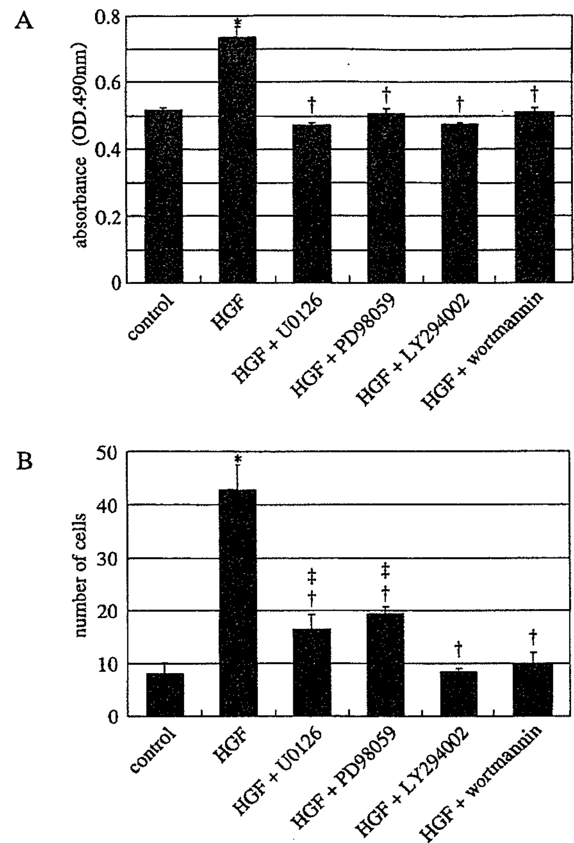

Figure 4. Inhibition of ERK and Akt in LECs. A, B, Effect of the MEK inhibitors U0126 (50 μmol/L) or PD98059 (30 μmol/L) and PI3K inhibitors LY294002 (50 μmol/L) or wortmannin (100 nmol/L) in the proliferative or migratory pathway of HGF on cLECs as demonstrated by the MTS and migration assay. n=8. *$P<0.0001$ vs control; †$P<0.0001$ vs HGF; ‡$P<0.05$ vs control.

Expression of c-Met was demonstrated by immunofluorescent staining (Figure 3A), suggesting that cLECs are responsive to HGF. Furthermore, ERK or Akt was phosphorylated from 5 to 15 minutes after the addition of HGF in cLECs, whereas total ERK or Akt protein levels were not altered by treatment with recombinant HGF (Figure 3B).

To investigate the role of ERK and Akt in the proliferative or migratory pathway of HGF, cLECs were pretreated with an MEK inhibitor (U0126 or PD98059) and a PI3K inhibitor (LY294002 or wortmannin). Treating cLECs with both inhibitors completely attenuated cellular proliferation ($P<0.0001$; Figure 4A). In cellular migration, both inhibitors significantly attenuated HGF-induced migration, whereas the effect of the PI3K inhibitors was more potent ($P<0.0001$; Figure 4B).

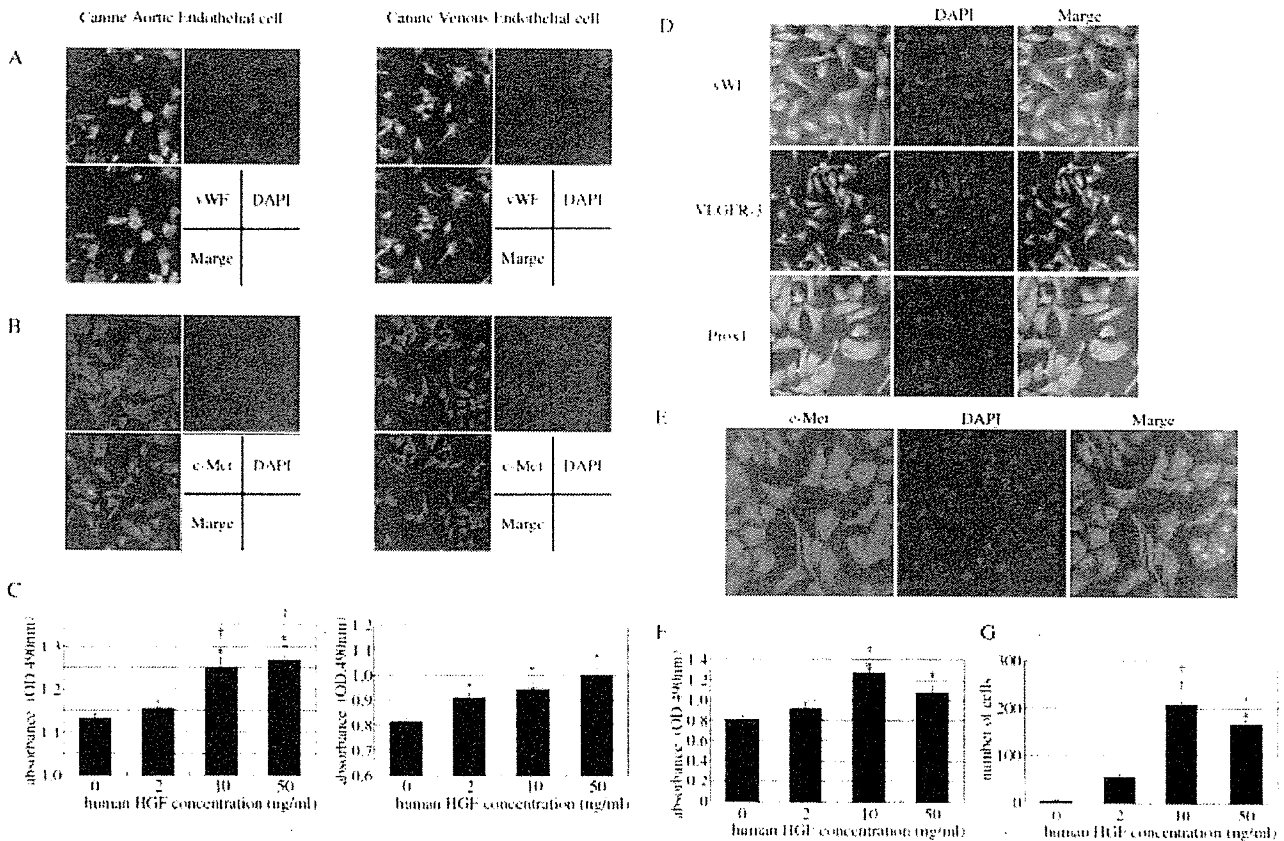

Figure 5. Effect of HGF on cAECs, cVECs, and human LECs. A, B, Representative pictures of immunofluorescent stains for the endothelial cell marker von Willebrand factor and the HGF receptor c-Met in cAECs (left) and cVECs (right) (×200 magnification). C, Effect of recombinant HGF on cAECs (left) and cVECs (right) as demonstrated by the MTS and migration assay. n=6. *$P<0.05$ vs HGF 0 ng/mL; †$P<0.05$ vs HGF 2 ng/mL. D, E, Representative pictures of immunofluorescent stains for von Willebrand factor (D, top), VEGFR-3 (D, middle), Prox1 (D, bottom), and c-Met (E) in human LECs (×200 magnification). F, G, Effect of recombinant HGF on human LECs as demonstrated by the MTS and migration assay. n=6. *$P<0.05$ vs HGF 0 ng/mL; †$P<0.05$ vs HGF 2 ng/mL.

To examine the specificity of cLECs, we also examined the effect of HGF on aortic and venous endothelial cellular growth obtained from dog. Immunofluorescent staining of these cells was positive for the endothelial cell marker von Willebrand factor (Figure 5A) and the HGF receptor c-Met (Figure 5B). Treatment with human recombinant HGF resulted in dose-dependent increases in cellular growth (cAECs: no treatment, 1.132±0.011; 2 ng/mL, 1.156±0.016; 10 ng/mL, 1.249±0.024; 50 ng/mL, 1.268±0.033; cVECs: no treatment, 0.814±0.008; 2 ng/mL, 0.910±0.010; 10 ng/mL, 0.945±0.032; 50 ng/mL, 1.001±0.032; absorbance OD, 490 nm; $P<0.05$; Figure 5C), which were consistent with the previous results of human aortic endothelial cells.[26] Moreover, we also used human LECs to confirm the effect of HGF. As shown in Figure 5D and 5E, immunofluorescent analysis demonstrated that human LECs were positive for endothelial cell marker (von Willebrand factor), lymphatic-specific markers (VEGFR-3 and Prox1), and the HGF receptor c-Met. Treating human LECs with recombinant HGF resulted in an increase in cellular proliferation by MTS assay (no treatment, 0.810±0.035 cells; 2 ng/mL, 0.919±0.056 cells; 10 ng/mL, 1.278±0.035 cells; 50 ng/mL, 1.081±0.052 cells; absorbance OD, 490 nm; $P<0.05$; Figure 5F) and migration by modified Boyden chamber method (no treatment, 5.800±0.663 cells; 2 ng/mL, 55.200±6.127 cells; 10 ng/mL, 208.400±36.405 cells; 50 ng/mL, 166.600±7.991 cells; $P<0.05$; Figure 5G) in a dose-dependent manner. These results support the idea that HGF would induce LEC growth and migration.

Local Human HGF Gene Transfer Improves Lymphedema in a Rat Tail Model

Given the in vitro data, we hypothesized that overexpression of HGF would stimulate the growth of the lymphatic vascular system and alter the lymphedema. To investigate the effect of HGF gene transfer on the lymphedema, an operative model of rat tail lymphedema was created, and tail thickness was measured over the subsequent 35 days. Human HGF expression was detected by RT-PCR only in the human HGF-injected group, and maximal expression was detected 4 days after the operation. Of note, endogenous rat HGF concentrations were significantly upregulated in the human HGF-injected group at 4, 10, and 17 days after the operation ($P<0.001$; Figure 6A), which was consistent with a previous report.[27]

Tail thickness began to increase 1 day after the operation and was stable at 7 days in all animal groups, except for

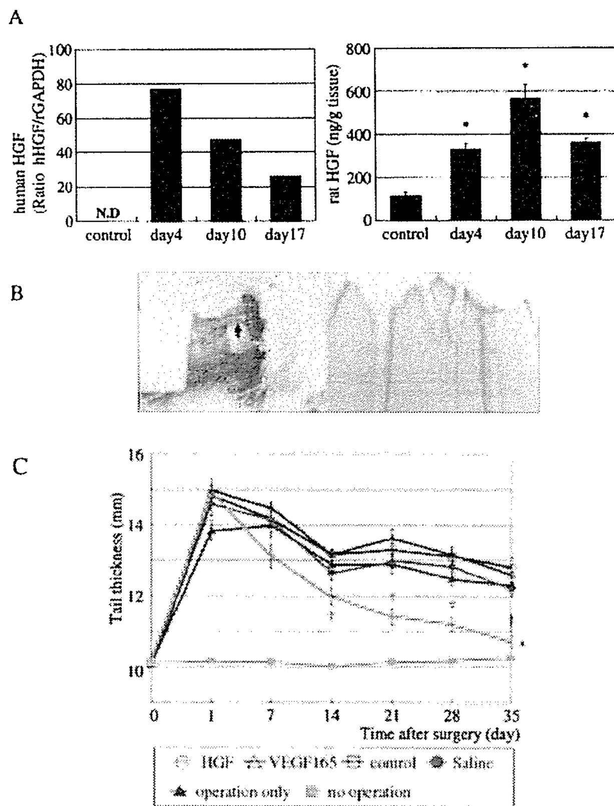

Figure 6. Gene therapy with HGF plasmid in an operative model of rat tail lymphedema. A, Expression of human HGF (left; RT-PCR) and rat HGF (right; enzyme immunoassay) on days 4, 10, and 17 after operation. Control indicates no injection of human HGF plasmid. n=7. *$P<0.001$ vs control. B, An operative model of rat tail lymphedema was created. For visualization of lymphatic vessels (arrow), 0.2 mL of 0.5% patent blue was injected into the tip of the tail (left). Tail thickness began to increase 1 day after the operation (right). C, Measurement of tail thickness in each group at baseline and at 1, 7, 14, 21, 28, and 35 days after operation. Each plasmid or saline was injected on days 1, 7, and 14 after operation. HGF indicates human HGF plasmid 200 μg/0.1 mL; VEGF165, human VEGF165 plasmid injection 200 μg/0.1 mL; control, indicates GFP plasmid injection 200 μg/0.1 mL); saline, saline injection 0.1 mL; and operation only, no injection. n=10. *$P<0.0001$ vs control analyzed by repeated-measures ANOVA; †$P<0.0001$ vs control analyzed by ANOVA followed by the Dunnett test.

those that did not undergo operation (Figure 6B). However, on days 14, 21, 28 and 35, tail thickness was decreased from the thickness noted on day 7 only in the HGF group compared with the control group ($P<0.0001$; Figure 6C). To analyze the total amount of HGF-induced effect on tail thickness during the recovery process of tail thickness, we performed repeated-measures ANOVA, including group, days, and interaction between group and days as factors. There are significant differences in group, days, and interactions between group and days ($P<0.0001$). To evaluate the effect of each treatment further, we repeatedly compared the curve in a mixed-effect model from day 1 to 35. Indeed, the curve of the HGF group was significantly different from that of the control group ($P<0.0001$; Figure 6C).

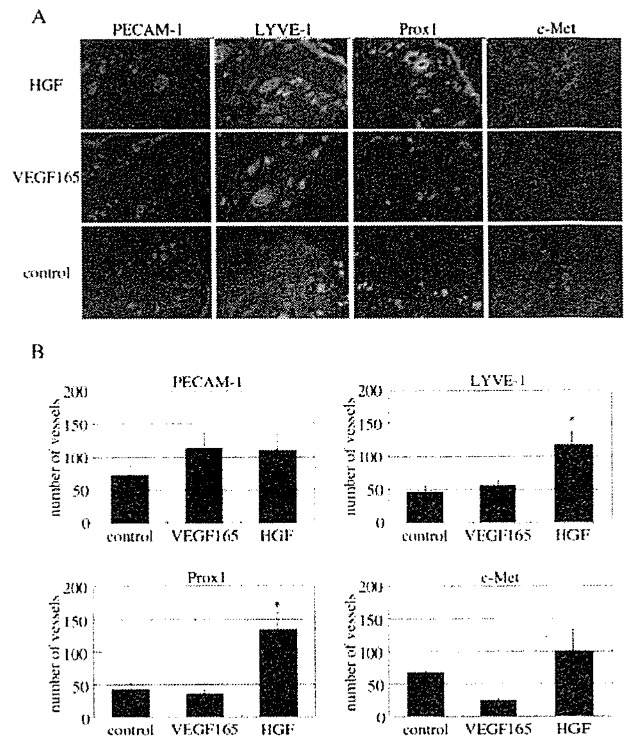

Figure 7. Immunofluorescent staining of endothelial cells in the rat tail. A, Representative pictures of immunofluorescent stains for the endothelial cell marker PECAM-1 and the LEC markers LYVE-1, Prox1, and c-Met in HGF, VEGF165, and control groups (×100 magnification). B, Quantitative analysis of stained vessels in each group. n=5. *$P<0.01$ vs control.

In the injection area of the tail, the expression of the endothelial cell marker PECAM-1 was increased in both the HGF- and VEGF165-injected groups (Figure 7A). However, expression of the LEC markers LYVE-1 and Prox1 was increased only in the HGF-injected group ($P<0.01$; Figure 7B).

Discussion

Chronic lymphedema is a disabling condition characterized by thickening of the skin resulting from fibrofatty deposition in underlying tissues and disfiguring swelling of affected limbs. Most cases of secondary lymphedema in humans are due to disruption or depletion of lymphatic vessels.[3–5,28] The present study demonstrated that human HGF gene transfer promoted local lymphangiogenesis and attenuated the phenotypic changes associated with secondary lymphedema.

The present study used an adult LEC system for various experimental protocols. Although most previous studies that investigated lymphangiogenesis have used fetal or neonatal LECs,[12,18] expression levels of lymphatic markers such as Prox1 or LYVE-1 in those studies have been variable. Because LECs differentiate and bud from embryonic veins during early embryogenesis, these lymphatic markers also are expressed in early venous endothelial cells, and LECs can transdifferentiate into venous endothelial cells.[29] In the adult LEC system used in the present study, LECs were purified with LYVE-1–conjugated magnetic beads, and lymphatic markers were stably expressed in this system. Moreover, the

HGF receptor c-Met also was expressed in the adult LEC, and ERK and Akt phosphorylation increased with HGF exposure, suggesting that these adult LECs were HGF responsive, similar to the manner in which aortic endothelial cells respond to HGF.[26] Because the inhibitors of MEK and PI3K significantly attenuated the cellular viability and migration, we speculate that ERK and Akt could be important in lymphangiogenesis and arterial angiogenesis. Interestingly, the effect of the PI3K inhibitors was more potent in cellular migration, which was consistent with that in aortic endothelial cells. These data demonstrate that HGF promotes arterial and lymphatic angiogenesis after surgical disruption of these vessels.

In terms of therapeutic angiogenesis using human HGF plasmid, the safety and efficacy of HGF plasmid DNA in patients with critical limb ischemia have been investigated in a ongoing prospective, open-label, clinical trial.[30] Preliminary data suggest that HGF treatment results in a reduction in pain scale, an increase in the ankle pressure index, and a decrease in lower extremity ulcer size. Furthermore, there were no signs of systemic or local inflammatory reactions and no development of tumors or progression of diabetic retinopathy in this population. Of note, no edema was observed in this trial, in contrast to the transient lower-extremity edema reported with clinical gene therapy using the VEGF-A gene.[31] In terms of lymphedema therapy, VEGF-C also activates VEGFR-2,[32,33] which may induce angiogenesis and local edema through increases in vascular permeability. In contrast, HGF treatment has not been associated with edema in human trials or animal experiments.[34] Moreover, a recent study reported that HGF promotes lymphatic vessel formation by mechanisms that are independent of the VEGF-C pathway.[18] These data suggest that HGF is a promising therapeutic agent for the treatment or prevention of lymphedema.

Many cancer patients develop lymphedema after the dissection of lymph nodes that is part of the staging and treatment process of their disease. However, clinical trials of HGF therapy have excluded patients with preexisting cancer because of theoretical concerns about tumor progression in the context of an angiogenic stimulus.[35] We previously reported that local overexpression of HGF in tumor-bearing mice did not stimulate distant tumor growth despite local elevations in HGF concentrations.[36] Further investigation to characterize the risks of HGF therapy in patients with preexisting cancer would be of benefit.

In conclusion, the present study demonstrated that genetic transfer of HGF plasmid DNA–attenuated lymphedema via the promotion of lymphangiogenesis. Further studies to determine the clinical utility of this approach would be of benefit to patients with lymphedema.

Acknowledgments

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Disclosures

None.

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CLINICAL PERSPECTIVE

Lymphedema is a disorder of the lymphatic vascular system characterized by impaired lymphatic return and swelling of the extremities. Treatment for this disabling condition remains limited and largely ineffective. The goal of the present study was to investigate the therapeutic efficacy of hepatocyte growth factor (HGF) in animal models of lymphedema. Immunofluorescent analysis demonstrated that canine primary lymphatic endothelial cells (cLECs) were positive for lymphatic-specific markers (vascular endothelial growth factor receptor-3, LYVE-1, podoplanin, and Prox1) and the HGF receptor (c-Met). Treating cLECs with human recombinant HGF resulted in a dose-dependent increase in cell growth and migration and increased activity of extracellular signal-regulated kinase and Akt. In human LECs, c-Met also was expressed, and treatment with HGF increased cell growth and migration in a dose-dependent manner. Transfection of human HGF plasmid DNA in cLECs also increased the *c-fos* promoter activity. Furthermore, weekly HGF gene transfer in a rat tail lymphedema model by disruption of lymphatic vessels resulted in a decrease in lymphedema thickness. Although expression of the endothelial cell marker PECAM-1 was increased in both HGF- and vascular endothelial growth factor 165–injected groups, expression of LEC markers (LYVE-1 and Prox1) was increased only in the HGF-injected group. These data demonstrate that expression of HGF via plasmid transfer improves lymphedema via promotion of lymphangiogenesis. Further study to determine the clinical utility of this approach would be beneficial to patients with lymphedema.

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Brief Report

Development of High-Throughput Functional Screening of Therapeutic Genes, Using a Hemagglutinating Virus of Japan Envelope Vector

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ABSTRACT

Isolation of effective therapeutic genes is critical for the advancement of gene therapy for various diseases, including vascular diseases and cancers. The goal of the present study was to screen a human cDNA library, using a hemagglutinating virus of Japan envelope (HVJ-E) vector, to isolate candidate genes with potent therapeutic potential. The advantages of a high-throughput functional screening system based on the HVJ-E vector include (1) rapid preparation of the vector containing the DNA library, (2) effective fusion-mediated transfer of the plasmids to various cells with minimal toxicity, and (3) easy cloning of candidate genes by transformation of *Escherichia coli*. These advantages resulted in a lower probability of damage to isolated clones and in minimization of the time needed to screen for candidate genes. Screening of a human heart library for candidate genes to regulate endothelial cell growth identified three growth-stimulating genes, as evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay and c-*fos* promoter activity, the products of which were more potent than vascular endothelial growth factor. Similarly, two growth-inhibiting genes were identified, the effects of which were similar to angiostatin. Overall, this novel system will help advance our understanding of cell biology and promote the utility of human gene therapy.

INTRODUCTION

COMPLETION OF SEQUENCING of the human genome has spurred tremendous efforts in the field of human genome functional analysis. Further, the promising field of gene therapy requires an efficient and accurate system of screening for and evaluating therapeutic genes, using high-throughput screening. Various expression cloning strategies have been established and used to clone cDNAs on the basis of biologic function. For example, NIH3T3 cell-based oncogene screening and the COS cell-based cloning method have been used for functional gene screening (Gluzman, 1981), and current models employ virus-based vectors, such as retrovirus and tobacco mosaic virus (Escobar *et al.*, 2003; Kitamura *et al.*, 2003). In a prior report, we suggested that the hemagglutinating virus of Japan envelope (HVJ-E) vector may possess some distinct advantages for use in high-throughput screening (Kaneda *et al.*, 2002). For example, approximately 20–30 copies of plasmid DNA can be incorporated into one HVJ-E particle when 200 μg of 7-kb plasmid is mixed with 3×10^{10} particles of inactivated HVJ. Further, use of the HVJ-E vector allows delivery of two different plasmids to the same cell, as confirmed by LacZ and green fluorescent protein (GFP) expression (our unpublished data). Other benefits include rapid

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preparation of the HVJ-E vector containing the DNA library (approximately 30 min) and easy cloning of candidate genes by transformation of *Escherichia coli* (12–16 hr). Therefore, the goal of the present study was to use the HVJ-E vector to screen a human cDNA library to isolate potent pro- and antiangiogenic therapeutic factors.

MATERIALS AND METHODS

Cell cultures

Human aortic endothelial cells (Clonetics HAECs) and human aortic smooth muscle cells (Clonetics HASMCs) (passage 3) were purchased from Cambrex Bio Science Walkersville (Walkersville, MD) and were maintained as previously described (Nakagami *et al.*, 2001). Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO_2 with medium changes every 2 days.

HVJ-E gene transfection and MTS assay

A SuperScript human heart cDNA library (Invitrogen, Carlsbad, CA) was amplified once with a Plasmid Maxi kit (Qiagen, Valencia, CA), and infused into HVJ-E vectors as described previously (Kaneda *et al.*, 2002). For the screening of inducible genes for endothelial cell (EC) growth, HAECs (10^3 cells per well) were seeded on 96-well collagen I-coated plates the day before transfection. Fifty hemagglutinating units (HAU) of HVJ-E/pDNA was added to medium containing protamine sulfate (150 μg/ml; Nacalai Tesque, Kyoto, Japan), and the 96-well plate was centrifuged at 2380 × *g* at 35°C for 1 hr. HVJ-E-containing medium was aspirated, and cells were maintained in 200 μl of endothelial basal medium 2 (EBM2) containing 1% fetal bovine serum (FBS) (Cambrex Bio Science Walkersville) for 7 days. Cell viability was measured on the basis of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay, using CellTiter 96 AQ_{ueous} One Solution reagent (Promega, Madison, WI). Several candidate genes contained within plasmids were separately transfected into HAECs or HASMCs that were seeded in 96-well plates.

c-fos *promoter assay*

Endothelial cells were seeded in 12-well plates for cotransfection with candidate plasmids, the c-*fos*-luciferase reporter gene and the *Renilla* luciferase reporter gene (phRG-TK; Promega), using Lipofectamine 2000 (Invitrogen) as previously described (Nakagami *et al.*, 2001). Twenty-four hours after transfection, luciferase activity was measured with the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instructions.

Plasmid DNA

pCMV-luciferase-GL2 was constructed by cloning the luciferase gene from the pGL2-promoter vector (Promega) into pcDNA3 (Invitrogen). Human hepatocyte growth factor (HGF) and human vascular endothelial growth factor (VEGF) plasmids were used as previously described (Hiraoka *et al.*, 2003), and pBLAST40 human angiostatin version 11 (InvivoGen, San Diego, CA) was used in this experiment.

Statistical analysis

All values are expressed as means ± SEM. Analysis of variance with subsequent Fisher's protected least significant difference (PLSD) test was employed to determine the significance of differences in multiple comparisons.

RESULTS AND DISCUSSION

The transfection efficiency of HVJ-E was assessed by luciferase activity (pLuc, 10 μg per 500 HAU of HVJ-E). Consistent with previous reports (Nishimura *et al*, 2001; Kaneda *et al.*, 2002), the addition of protamine sulfate significantly increased the transfection efficiency in a dose-dependent manner, and transfection efficiency increased with incubation time until 60 min with centrifugation, without affecting cell integrity. The results showed the best condition for centrifugation transfection of HVJ-E to HAECs (Fig. 1A). The transfection efficiency in HAECs was 2–10 times higher than that with other gene transfer reagents such as Lipofectamine 2000 (Invitrogen) (Kaneda *et al.*, 2002).

On the basis of these conditions, a human heart cDNA library (approximately 1 × 10^7 independent clones) was amplified, and approximately 4 × 10^6 clones were randomly infused into the HVJ-E vector, which was then transferred to HAECs that were seeded in 96-well plates, as shown in Fig. 1B. Seven days after transfection, the MTS assay revealed variable HAEC proliferation among individual wells (Fig. 2A). To characterize the candidate genes responsible for inducing endothelial cell proliferation, DNA was extracted from the cell population showing the highest degree of proliferation and then transformed directly into *E. coli*. After screening, 1588 independent clones were isolated and sent for sequencing to eliminate empty and repetitive plasmids. Seven hundred and eighty-one clones were selected and further examined for clones that exhibited multiple hits (i.e., ≥2; 291 clones). Each of the 291 clones was transfected into endothelial cells and evaluated by the MTS assay, using the effect of VEGF as a relative comparison for endothelial cell growth properties. Six genes were superior to the VEGF gene (Ferrara and Henzel, 1989) in terms of stimulating endothelial cell proliferation. These six candidate genes were further evaluated for proliferative activity by screening with a c-*fos* promoter assay, which also correlates with cellular growth (Nakagami *et al.*, 2001). This assay identified three genes that were superior to VEGF in terms of c-*fos* promoter activity (Fig. 2B).

The effect of gene 3743 was further examined by MTS assay relative to the effect of the hepatocyte growth factor (HGF) gene, which is an another potent endothelial cell growth factor (Morishita *et al.*, 2004). HAEC viability, but not vascular smooth muscle cell viability, was significantly greater with transfection of gene 3743 than with transfection of the HGF gene or in comparison with controls (Fig. 2C and D). Sequencing revealed that gene 3743 was a part of the human squamous cell carcinoma antigen recognized by T cells (Nakao *et al.*, 2000) (XM_004310.4, bp 2364–3958) and that gene 31008

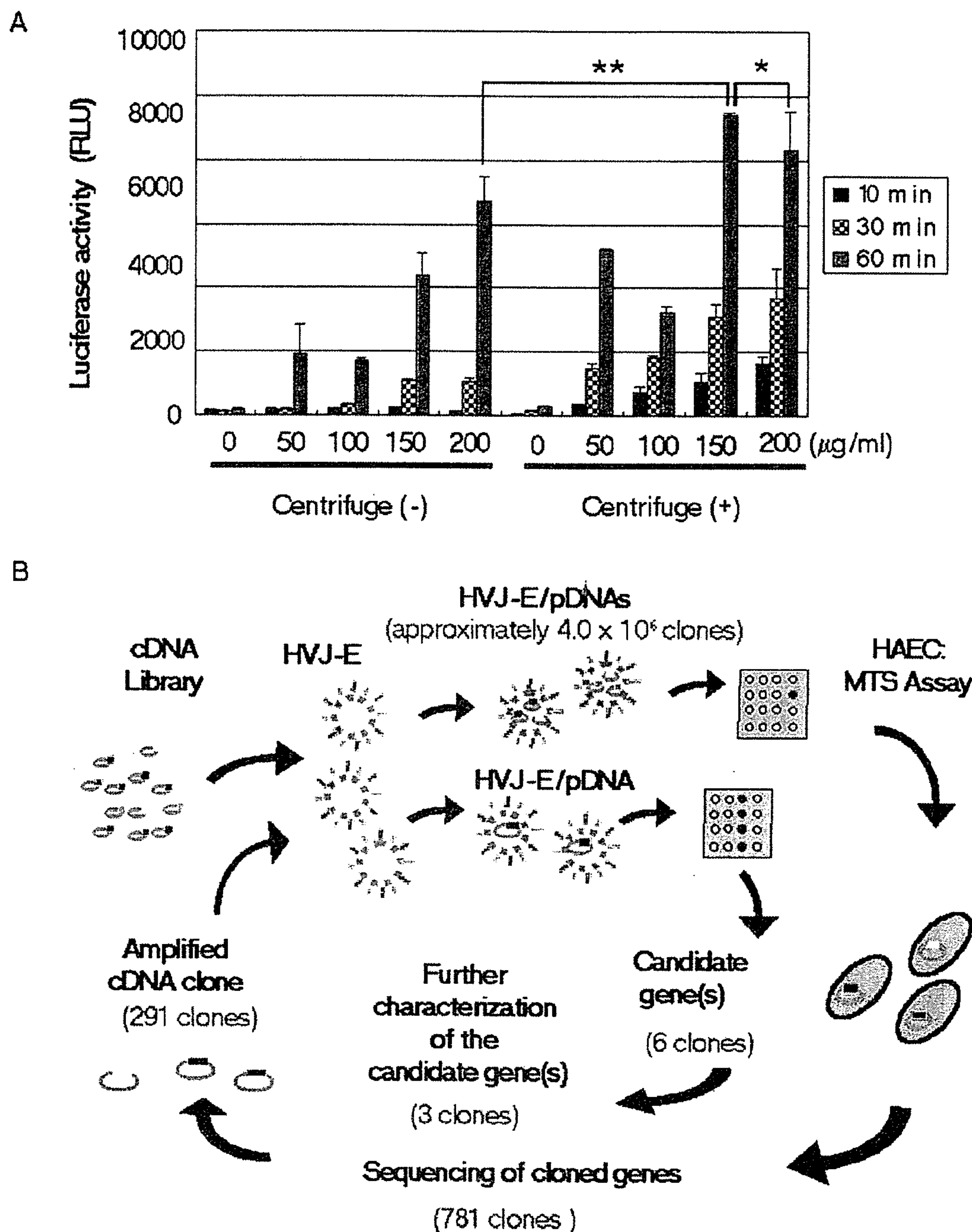

FIG. 1. (A) Transfection efficiency by luciferase activity using the HVJ-E vector under various conditions ($n = 8$). Fifty hemagglutinating units of HVJ-E/GL2 was added to medium containing protamine sulfate (0–200 μg/ml) for 5–60 min, with or without centrifugation at 2380 × *g*. $*p < 0.05$; $**p < 0.0001$. (B) Schematic drawing of a high-throughput functional screening system using HVJ-E vector. After screening, 1588 independent clones were isolated and sent for sequencing to eliminate empty and repetitive plasmids. Seven hundred and eighty-one clones were selected and further examined for clones that exhibited multiple hits (291 clones). Each of the 291 clones was transfected into endothelial cells and evaluated by MTS assay and the *c-fos* promoter assay. Three of these genes were more potent than VEGF in this assay.

was a part of the human caldesmon (NM_004342, bp 1379–1818). By contrast, gene 31306 had no homology with a human gene in a BLAST search.

Screening of the cDNA library for genes that inhibited endothelial cell growth was conducted with culture medium containing recombinant VEGF (10 ng/ml) and 1% FBS. HAECs were seeded (5×10^3 cells per well), and the MTS assay was performed on day 1. Forty-one colonies were collected and sequenced. Assessment of the effect of each clone on HAEC viability revealed that three different genes were similar to angiostatin in inhibiting endothelial cell proliferation (Fig. 3A). Further, the c-*fos* promoter assay demonstrated that two of

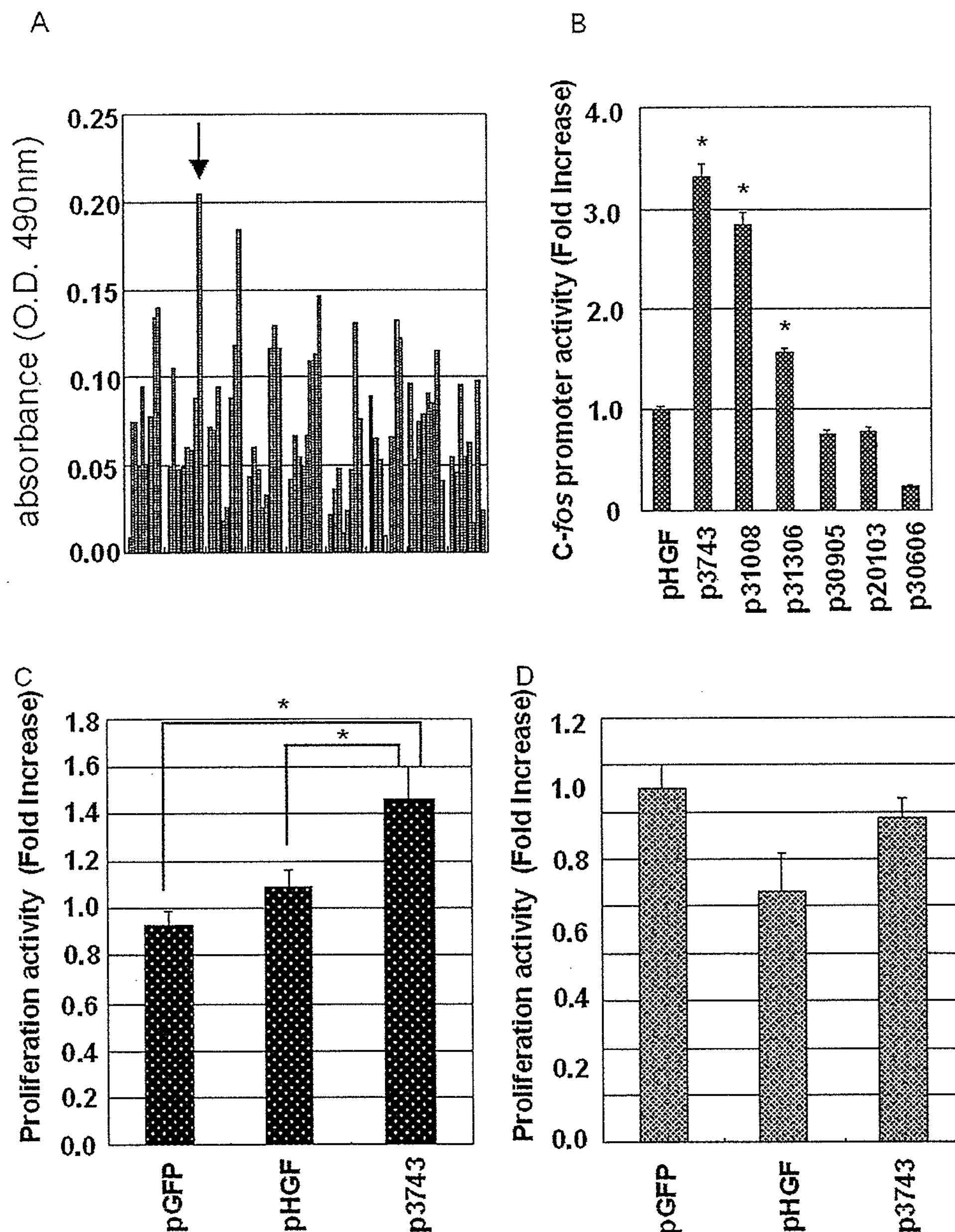

FIG. 2. (**A**) Representative example of MTS assay on first screening. The arrow indicates the well in which p3743 was identified. (**B**) c-*fos* promoter assay in HAECs ($n = 3$). Results are expressed as fold increase relative to the effect of VEGF. *$p < 0.05$, versus VEGF. (**C** and **D**) MTS assay in HAECs and HASMCs ($n = 8$). GFP, negative control; HGF, human HGF-overexpressing cells; p3743, 37-4-3-overexpressing cells. Results are expressed as fold increase relative to the effect of empty HVJ-E (HVJ-E without cDNA) for MTS assay. *$p < 0.05$.

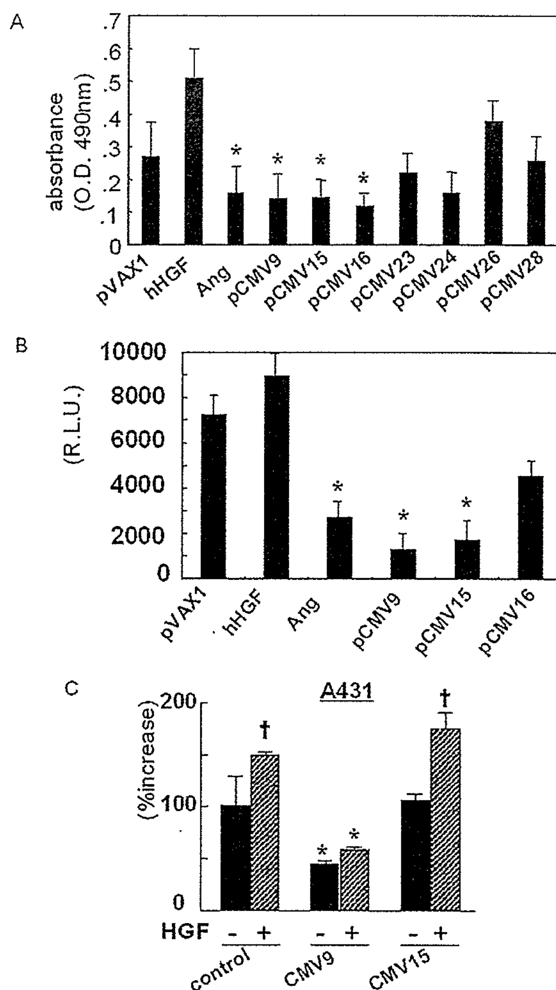

FIG. 3. (A) Representative examples of MTS assay and (B) c-*fos* promoter assay in HAECs ($n = 8$). PVAX, overexpressed empty vector (pVAX); HGF, overexpressed HGF; Ang, overexpressed angiostatin. The remainder of the data are labeled with individual candidate gene entry numbers. Results are expressed as fold increase relative to the effect of control. $^*p < 0.05$, versus pVAX. (C) c-*fos* promoter assay in A431 cells ($n = 8$). Control, overexpressed empty vector (pcDNA3); HGF, overexpressed HGF; pCMV9, overexpressed pCMV9 plasmid; pCMV15, overexpressed pCMV15 plasmid. Results are expressed as fold increase relative to the effect of control. $^*p < 0.05$, versus control; $^\dagger p < 0.05$ versus HGF(−).

these genes were similar to angiostatin in terms of growth-inhibitory activity (Fig. 3B). Sequencing of these genes showed that pCMV9 was also known as CHMP1B or CHMP1.5 (NM_020412) and that pCMV15 is a part of human myospryn (NM_153610, bp 11923–12282). Overexpressed pCMV9, but not pCMV15, significantly inhibited HGF-induced c-*fos* promoter activity in HGF receptor-expressing A431 cells (Matsuki *et al.*, 2004) (Fig. 3C).

Use of this system allowed isolation of candidate genes within 1 month, because the HVJ-E vector does not require preparation of a viral library or packaging cell constructions. In addition, use of this vector allows easy cloning of candidate genes by transformation of *E. coli* (12–16 hr). By contrast, retrovirus-mediated expression is relatively more complicated and PCR is necessary to determine which cDNA is responsible for the phenotype. Thus, the advantages of HVJ-E may result in a lower probability of damage to isolated clones and in minimization of the time needed to screen for candidate genes.

The isolated genes in the current study may be of utility for therapeutic angiogenesis and cancer gene therapy. Further characterization of these genes to elucidate the biological functions and potency for clinical applications would be of benefit. Interestingly, one of the identified growth-suppressive genes is also known as CHMP1B or CHMP1.5, a human homolog of yeast SNF7p (*s*ucrose *n*on*f*ermenting). The CHMP family of proteins participates in intracellular membrane traffic events (Howard *et al.*, 2001), of which the endocytic pathway is an important component (Katzmann *et al.*, 2002). Further, one study reported that CHMP1B binds to spastin, a protein that is abnormal in the most common form of pure hereditary spastic paraplegia (Reid *et al.*, 2005). Further, the investigators reported that defects in intracellular transport and membrane trafficking may be common mechanisms for the neurodegeneration seen in hereditary spastic paraplegia (Reid *et al.*, 2005).

In summary, the present study demonstrated the successful use of a novel functional gene screening based on HVJ-E to isolate several candidate genes that modulate endothelial cell growth. We believe that HVJ-E-mediated gene transfer and expression cloning will continue to be an important tool for the advancement of our knowledge of the genome and cell biology.

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ORIGINAL ARTICLE

Acceleration of wound healing by combined gene transfer of hepatocyte growth factor and prostacyclin synthase with Shima Jet

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Although skin diseases are one of the target diseases for gene therapy, there has been no practical gene transfer method. First, we examined gene transfer efficiency of the spring-powered jet injector, Shima Jet, which was originally developed as a non-needle jet injector of insulin. Local gene expression was about 100 times higher when the luciferase plasmid was transferred by the Shima Jet than by a needle. Gene transfer of β-galactosidase revealed gene expression in the epidermis. Based on these results, we then examined the potential of gene therapy using the Shima Jet for wound healing. An increase of cellular proliferation of the epidermis and the number of microvessels in the granulation tissue was observed after hepatocyte growth factor (HGF) gene transfer. An increase in blood flow around the wound was observed after prostacyclin synthase (PGIS) gene transfer. Moreover, promotion on wound healing was observed in HGF gene transferred group, and further promotion was observed in combined gene transferred group as assessed by measuring wound area. These results indicate that co-transfer of HGF and PGIS genes by the Shima Jet could be an effective strategy to wound healing.
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Keywords: *Shima Jet; wound healing; hepatocyte growth factor (HGF); prostacyclin synthase (PS)*

Introduction

Gene therapy for skin diseases is evolving rapidly and several methods for gene transfer into the skin have been reported. Several viral vector methods such as recombinant adeno-associated virus vectors, lentiviral vectors and hemagglutinating virus of Japan-liposome method have been reported.[1–3] Viral vectors are often effective from the point of view of gene transfer, but concerns about safety, scalability of production and cost effectiveness need to be overcome before their clinical use can be established. It is true that these methods have advantages; however, thus far, the advantages do not seem to outweigh the disadvantages. On the other hand, non-viral vector methods such as gene gun,[4] electroporation,[5–7] liposome[8] and naked gene transfer[9–11] have been also reported. Non-viral methods are safer and cost effective compared to viral methods. Electroporation has been reported to improve transfer efficiency and improve wound healing. Jet injection of a solution containing naked plasmid has also been reported to have high efficiency of gene transfer to the skin in rats.[11] In this study, we examined whether the spring-powered jet injector, Shima Jet, which was originally developed as a non-needle jet injector of insulin, is effective for gene transfer into the skin aiming a therapeutic approach to skin diseases in humans. Because the Shima Jet has been already used in humans, the potential of gene therapy to skin diseases using the Shima Jet seems to be high.

Several basic diseases such as diabetes, arteriosclerosis obliterans, thromboangiitis obliterans, varicosity, vasculitis and decubitis ulcer cause a defect in the epidermis and dermis, and result in incurable ulcers, the main reason for which is a decrease in peripheral circulation. Wound healing is a complex programmed sequence of cellular and molecular processes, including inflammation, cell migration, angiogenesis, provisional matrix synthesis, collagen deposition and re-epithelialization.[12] In spite of proper wound treatment, some acute ulcers evolve into chronic non-healing wounds that show a dermal defect, ischemia and tissue necrosis. The healing processes are regulated by a number of growth factors such as basic fibroblast growth factor, transforming growth factor-*β*, epidermal growth factor, keratinocyte growth factor, platelet-derived growth factor, vascular endothelial growth factor and hepatocyte growth factor (HGF).[13–15] Interestingly, several growth factors that accelerate wound healing have been identified.[12] Hepatocyte growth factor was first found to be a potent

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mitogen for mature hepatocytes after purification and cloning.[16,17] In terms of wound healing, HGF enhances the migration, cell growth and DNA synthesis of keratinocytes and result in wound closure.[18,19] Hepatocyte growth factor also stimulates vascular endothelial cell migration, proliferation and organization into capillary-like tubes, leading to the promotion of angiogenesis.[20,21] On the other hand, prostacyclin (PGI_2) is a metabolite of arachidonic acid, which inhibits platelet aggregation and smooth muscle cell proliferation and induces vasodilatation. Prostacyclin is widely used orally to treat human patients with peripheral arterial disease or ischemic wounds. Prostacyclin synthase (PGIS) catalyzes the conversion of prostaglandin H_2 to PGI_2. In 1994, the amino-acid sequence of human PGIS was cloned.[22] Overexpression of the PGIS gene inhibits growth of vascular smooth muscle cells (VSMC), and transfer of PGIS gene ameliorates pulmonary hypertension and prevents neointimal formation after carotid balloon injury in rats.[23–25] Co-transfer of HGF and PGIS genes in a murine ischemic limb model has been reported to increase the concentration of HGF protein and resulted in a significant increase in blood flow and capillary density compared with transfer of HGF gene alone.[26,27] Based on these results, we examined the effects of co-transfer of HGF and PGIS genes by the Shima Jet on wound healing in this study.

Results

Histological analysis of β-galactosidase expression

Initially, we examined the potential of the Shima Jet to transfer genes into the skin using reporter genes, because there has been no report showing the successful gene transfer achieved by the Shima Jet into the skin. Microscopic expression of β-galactosidase could be observed in the epidermal layer of the rat skin at 48 h after transfer by the Shima Jet (Figure 1a and b). However, in control sections, in which the control plasmid was injected, no expression of β-galactosidase could be observed (Figure 1c and d).

Comparison of gene transfer efficiency into rat skin

So far, many researchers have reported the usefulness of needle injection for gene transfer into the skin. To evaluate the transfer efficiency, we compared luciferase activities in the rat skin at 24 h after transfer by the Shima Jet and by a needle (27-gauge). In the rat skin using the Shima Jet, the local expression of luciferase was about 100 times higher than that by a needle, as shown in Figure 2a. Moreover, the effect of gene transfer by the Shima Jet was dose-dependent, as shown in Figure 2a. We also measured gene expression in the skin in sequence after gene transfer by the Shima Jet. Luciferase activity could be observed until 7 days after gene transfer, as shown in Figure 2b. Then, we examined the gene transfer efficiency by measuring human HGF protein level in the skin. Higher expression of human HGF protein was observed in the group transferred by the Shima Jet, as shown in Figure 3. On the other hand, no expression of human HGF was observed in the group transferred by a needle. Moreover, in the control group transferred with the control plasmid, no human HGF could be detected.

Immunohistochemical analysis of hepatocyte growth factor and prostacyclin synthase

In the first experiment, we confirmed that gene expression of β-galactosidase was observed in the epidermis of the rat skin following transfer by the Shima Jet. Then, we examined whether HGF and PGIS gene expressions are produced in the same manner with the Shima Jet or not. As expected, strong expressions of human HGF and human PGIS were observed in the epidermis of the rat skin around injection sites transferred by the Shima Jet, using antibodies against human HGF and human PGIS,

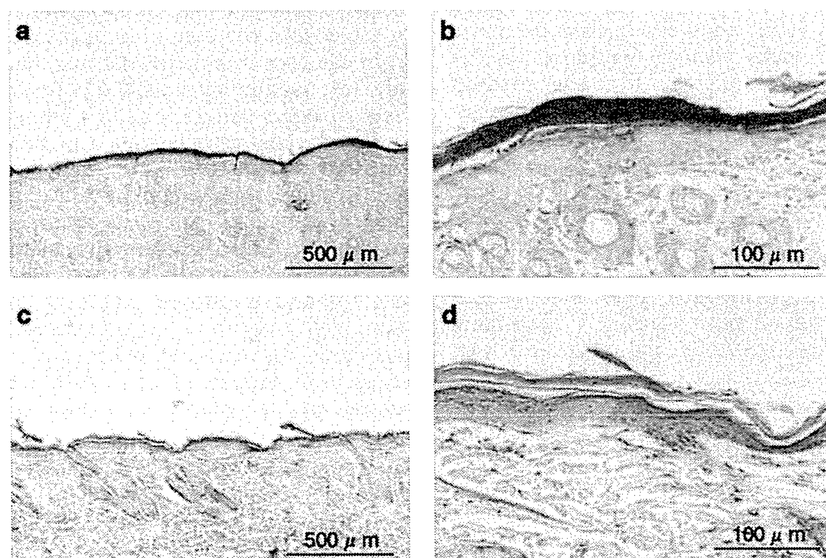


Figure 1 Representative photomicrographs of rat skin. Intense expression of β-galactosidase was observed in the epidermis after gene transfer of β-galactosidase plasmid (a and b), which was not observed in the skin of the control rat transferred with the control plasmid (c and d). Magnification: (a and c) ×40, (b and d) ×200.

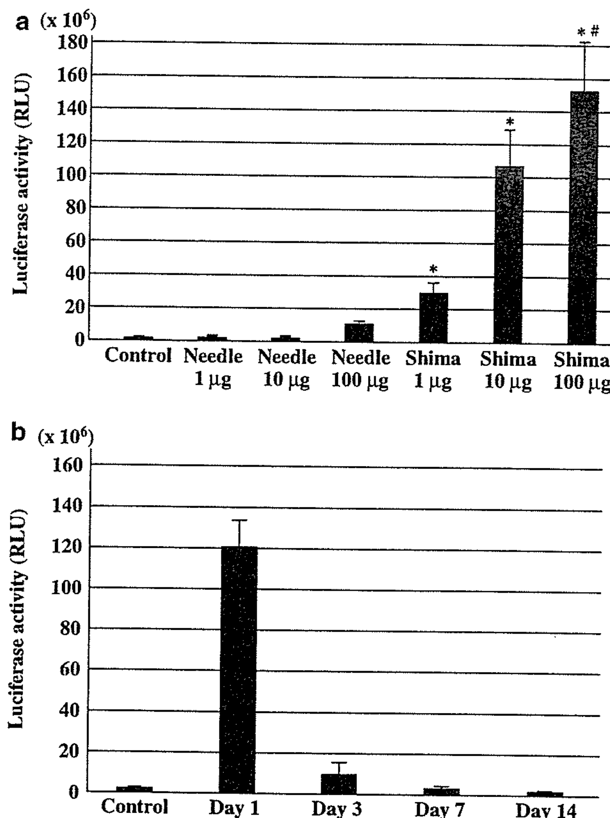


Figure 2 Comparison of gene transfer efficiency with needle and Shima Jet assessed by luciferase activity. (a) Comparison of luciferase activity achieved by gene transfer of luciferase plasmid by a needle and by the Shima Jet. Local expression of luciferase was about 100 times higher by the Shima Jet than by a needle. Control = samples from rats transferred with the control plasmid; needle = samples from rats transferred with luciferase plasmid by a needle; Shima = samples from rats transferred with luciferase plasmid by the Shima Jet. 1, 10 and 100 μg mean the amount of plasmid transferred. *$P<0.01$ vs needle 1, 10 and 100 μg; #$P<0.01$ vs Shima 1 and 10 μg. (b) Time course of luciferase activity after gene transfer of 100 μg/100 μl of luciferase plasmid by the Shima Jet. The significant expression of luciferase could be observed until day 7. Days 1, 3, 7 and 14 mean days after gene transfer. Control = samples from rats transferred with the control plasmid.

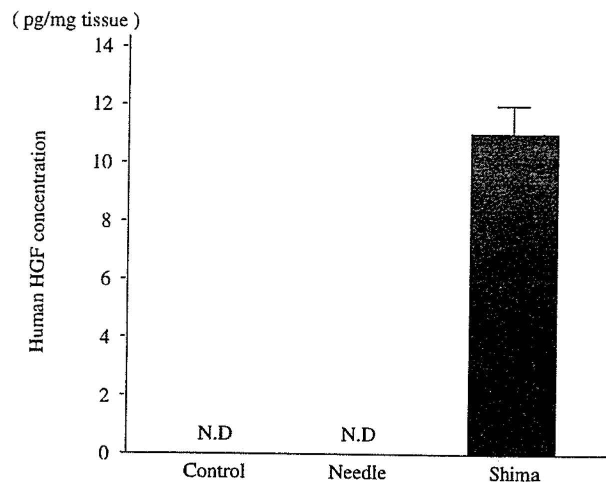


Figure 3 Comparison of gene transfer efficiency with needle and Shima Jet assessed by hepatocyte growth factor (HGF) activity. Human HGF concentration in the skin after transfer of 100 μg/100 μl of human HGF gene by a needle and by the Shima Jet is shown. Higher expression of human HGF protein was observed in the group transferred by the Shima Jet. On the other hand, no expression of human HGF was observed in the group transferred by a needle. Moreover, in the control group, no human HGF was detected. ND = not detected; needle = samples from rats transferred by a needle; Shima = samples from rats transferred by the Shima Jet.

respectively, at 2 days after gene transfer (Figure 4a and d). Weaker expression of HGF or PGIS was observed in the epidermis after gene transfer by a needle (Figure 4b and e). On the other hand, sections from control rats transferred with the control plasmid showed no positive staining for either human HGF or human PGIS (Figure 4c and f).

Comparison of blood flow by Laser Doppler imaging

The image of the Laser Doppler imaging (LDI) (Moor Instruments, Axminister, UK) analyzer showed that blood flow in the rat skin was not increased apparently on day 4 after transfer with human HGF gene, as shown in Figure 5a. Quantitative analysis also showed no tendency for an increase in blood flow (Figure 5b). This result does not seem to be consistent with our previous report;[26,27] however, this assay was performed on day 4 and it seemed too short to induce angiogenesis, indicating that our results of this study are reasonable. On the other hand, additional PGIS gene transfer to HGF gene transfer increased the blood flow even at 4 days (Figure 5a). A significant increase in the blood flow in PGIS plasmid transferred group compared to other groups was confirmed by quantification (Figure 5b).

Comparison of granulation tissue area

There was no significant difference in the granulation tissue area between each group at 3 days after gene transfer (Figure 6a). However, there was a tendency for decreasing in groups transferred with HGF gene and co-transferred with HGF and PGIS genes.

Comparison of neovascularization in granulation tissue and cell proliferation in epidermis

The capillary vessels (microvessels) within the granulation tissue were detected by immunohistochemical-staining for factor VIII. The number of vessels was significantly increased in HGF gene transferred group, and HGF and PGIS genes co-transferred group at 7 days after transfer compared with those in the control group (Figure 6b and c). The number of proliferating cell nuclear antigen (PCNA)-positive cells in the epidermis on the edge of the round wound was significantly increased in HGF gene transferred group at 3 days after gene transfer compared with those in the control group ($P<0.05$) (Figure 6d and e).

Wound lesion area

The model we used in this study showed natural recovery around 2 weeks after induction of wound, even when we used prednisolone (PZ) (Shionogi Ltd, Osaka, Japan) to induce the wound impairment. Until 7 days, control wounds did not show any decrease in area; so,

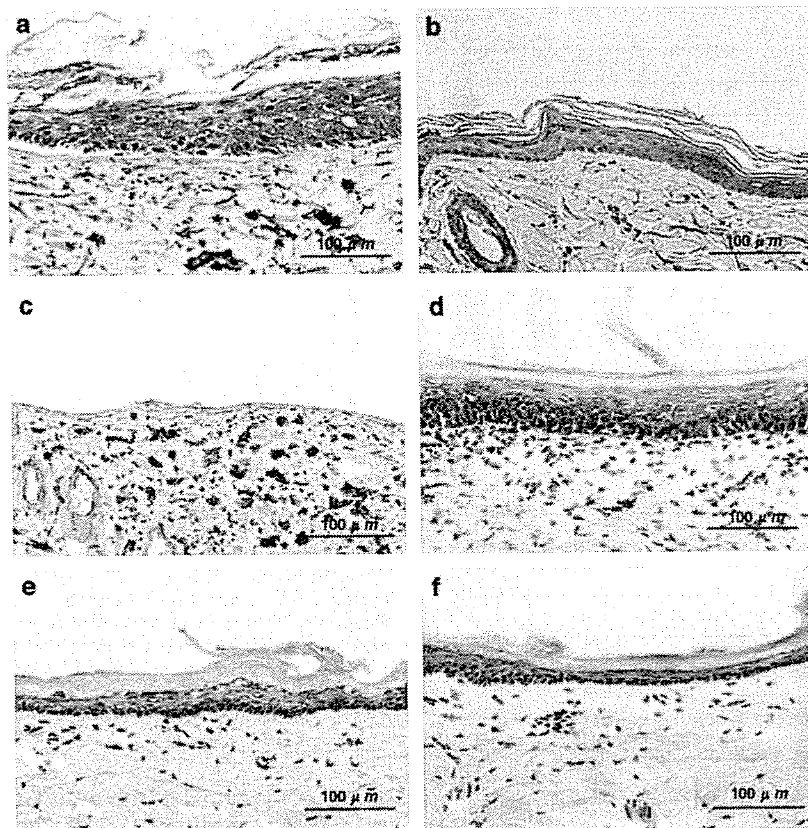


Figure 4 Immunohistochemical analysis of hepatocyte growth factor (HGF) and prostacyclin synthase (PGIS). Immunohistochemical demonstration of human HGF or human PGIS expression in the rat skin after gene transfer by the Shima Jet or by a needle is shown. Intense immunostaining for human HGF and human PGIS was observed in the epidermis after transfer of HGF and PGIS genes, respectively, by the Shima Jet (**a** and **d**) weaker staining was observed by a needle (**b** and **e**), and none was observed in the rat skin after transfer of the control plasmid by the Shima Jet (**c** and **f**). Magnification ×200.

the effect of gene transfer should be considered until 7 days. On day 0, wound lesion area was taken as 100% in each group. Hepatocyte growth factor gene transfer by the Shima Jet significantly decreased the wound lesion area as compared to the control group at day 6 after gene transfer (Figure 7a and b). Moreover, even on day 4, a tendency for a decrease but not significant in wound lesion area could be observed in HGF gene transferred group. On the other hand, co-transfer of HGF and PGIS genes showed a significant decrease in wound lesion area as compared to the control group at days 4 and 6 ($P<0.05$) (Figure 7a and b), indicating that on both days 4 and 6, additional wound closure effect could be observed by the additional gene transfer of PGIS. Prostacyclin synthase gene transfer alone did not improve the wound closure rate. We also compared the wound closure rate after HGF and PGIS gene transfer by the Shima Jet by a needle. We could not observe a significant wound closure after gene transfer by a needle (Figure 7c).

Discussion

Wound healing disorders following systemic glucocorticoid treatment are a commonly observed and experimentally proven phenomenon. Glucocorticoid-mediated effects are multifunctional and prevent the early inflammatory phase, which is essential for efficient wound repair, and also intervene in the regulation of pro-inflammatory cytokines, growth factors, matrix proteins and matrix proteases, which seem to have an impact on wound healing.[28] Glucocorticoid is also known to inhibit angiogenesis and fibroblast proliferation.[29] Topical application of recombinant growth factor proteins has already been used to treat healing wounds. However, this approach has disadvantages, such as the requirement for large amounts of purified recombinant protein, the short half-life of the growth factors owing to proteases at the wound site, the potential toxicities of repeated high doses of growth factors and difficulty with appropriate delivery of growth factors in the wound.

DNA delivery into the skin may be useful for the treatment of skin diseases requiring local or systemic distribution of a transgene product. However, the development of an effective, consistent and patient-friendly transfer system into the skin remains a challenge. The success of gene delivery is largely dependent on the development of methods to deliver genes to target cells with minimal toxic side effects selectively and efficiently. At present, the vast majority of efforts directed toward gene delivery *in vivo* have focused on viral vectors. Although often effective, viral vectors have significant drawbacks, including concerns about safety,

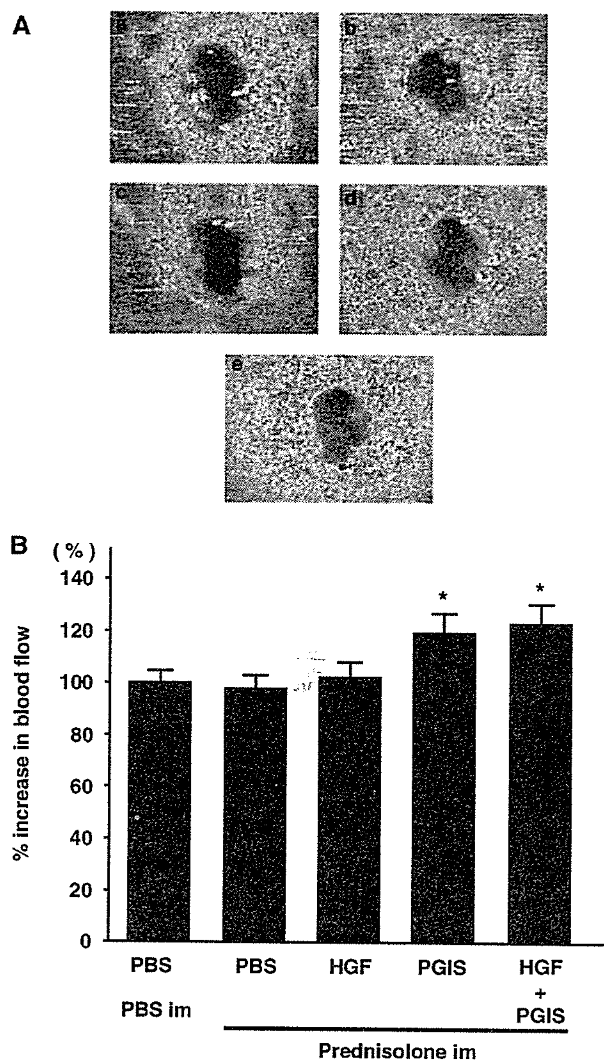


Figure 5 Effect of intradermal transfer of hepatocyte growth factor (HGF) or prostacyclin synthase (PGIS) gene for blood flow. Effects of intradermal transfer of human HGF or human PGIS gene for blood flow around the wound were evaluated. (A) Typical images of blood flow analyzed by Laser Doppler imaging at 4 days after transfer by the Shima Jet. An increase in blood flow was observed around the wound in rats transferred with human PGIS gene (d and e). (a) Rats treated with phosphate-buffered saline (PBS) injection after induction of wound by PBS (group 1: PBS/PBS); (b) rats treated with PBS injection after induction of wound by prednisolone (PZ) (group 2: PZ/PBS); (c) rats transferred with human HGF gene after induction of wound by PZ (group 3: PZ/HGF), (d) rats transferred with human PGIS gene after induction of wound by PZ (group 4: PZ/PGIS); (e) rats co-transferred with both human HGF and PGIS genes after induction of wound by PZ (group 5: PZ/HGF+PGIS). (B) A significant increase in blood flow was observed in PGIS gene transferred groups 4 and 5 (PGIS and HGF+PGIS) as compared to other groups by quantification.

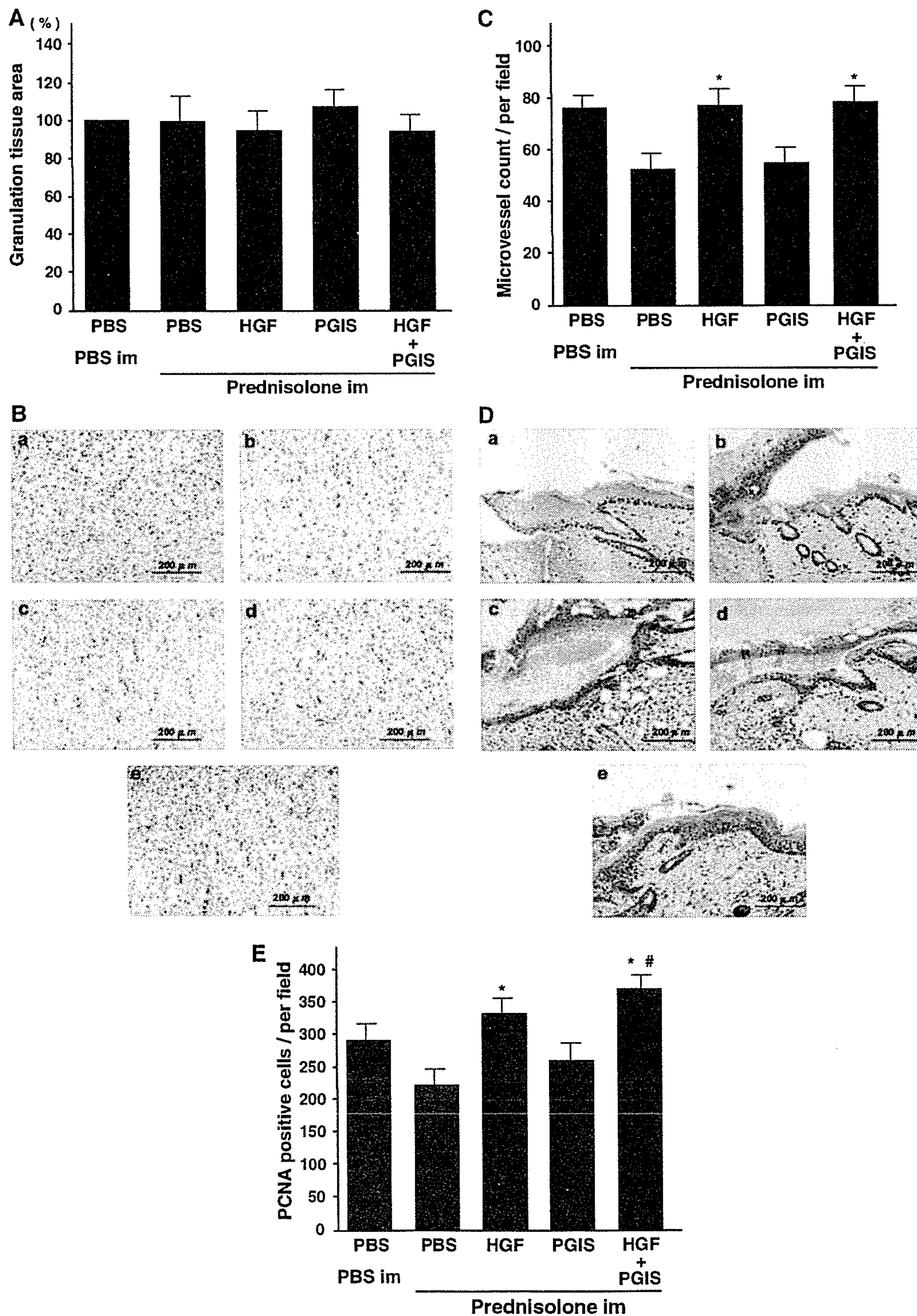
scalability of production and cost effectiveness. Non-viral approaches are generally safer and less expensive, but also less effective, and some gene delivery methods need specialized equipment or. training for wound treatment. To be able to take full advantage of targeting skin diseases for gene therapy, it is important to establish an efficient and reproducible delivery system. Jet injection as a tool for gene delivery of plasmid DNA is a strong candidate to meet these criteria.[10] From this point of view, we evaluated the efficiency of intradermal injection of naked plasmid into the skin by a spring-powered jet injector, Shima Jet, which was originally developed as a non-needle jet injector of insulin for diabetic patients. Previously, naked DNA has been shown to be delivered and efficiently expressed following direct injection into the skin, without the need for a DNA carrier. In our study, gene expression in the skin was mainly observed in the epidermis. The mechanisms controlling the topical delivery of naked plasmid DNA are poorly understood and need to be further investigated. The advantage of using plasmid DNA alone in gene delivery is that it may avoid potential host responses elicited by viral DNA carriers and that DNA can be repeatedly injected into the desired area to compensate for the loss of gene expression over time. Furthermore, gene transfer efficiency by a Jet injection is higher compared to a needle injection, as reported before in a porcine model.[30]

Gene transfer into the skin by the Shima Jet uses naked plasmid without any vectors, is a local therapy, is easy to handle, has high efficiency of transfer and causes little pain. Based on these advantages, gene transfer into the skin by the Shima Jet might have high potential for clinical application. Increased expression of HGF and its receptor, c-met, was observed in response to wounding.[15] Hepatocyte growth factor has a significant effect on vascularization and granulation tissue formation during wound healing *in vivo*.[31] Moreover, HGF stimulates wound healing, with less susceptibility to cutaneous scarring.[32] In this study, re-epithelialization and microvasculation of the wound were promoted after HGF gene transfer. We measured the expression of human HGF protein in the skin around the wound. Although not measured in this study, the level of rat endogenous HGF may be upregulated after human HGF gene transfer.[3,33]

In the present study, we showed the effectiveness of co-transfer of HGF and PGIS genes by the Shima Jet for wound healing. The process of cutaneous wound healing is characterized by four overlapping repair phases involving hemostasis, inflammation, proliferation and remodeling. After injury, new tissue formation starts with re-epithelialization and is followed by granulation tissue formation. The latter process includes macrophage accumulation, fibroblast growth, matrix deposition and angiogenesis. In our study, by PGIS gene transfer, the blood flow in the skin increased. Prostacyclin has been reported to have a cytoprotective action on endothelial cells and to inhibit VSMC growth. Prostacyclin is an unstable metabolite of arachidonic acid that is produced by endothelial cells and non-enzymatically hydrated. In this study, improvement in wound closure was not detected by PGIS gene transfer alone, which may result from the short half-life of PGI_2. Previous studies showed that PGI_2 administration in humans induced cutaneous flushing, and intravenous PGI_2 infusion raised skin blood flow as measured by Laser Doppler flowmetry.[34] Thus, PGI_2 is thought to relax the smooth muscle of both large and small arteries, and PGI_2 analogues are widely used for the treatment of peripheral arterial disease. In another study, increased local HGF production from vascular cells by a PGI_2 analogue was suggested

to improve endothelial dysfunction induced by high glucose, in addition to the direct vasodilator effects on VSMC.[35] Beraprost sodium improves walking distance in patients with peripheral arterial occlusive disease.[36] On the other hand, recently, a beneficial effect of beraprost sodium on rat with anti-glomerular basement membrane serum-induced nephritis was reported.[37] Beraprost sodium has multiple effects directed to improve microcirculation, such as anti-platelet, vasolidator and anti-inflammatory effects.[38]

Taken together, the present results suggest that blood flow would be increased by vasodilator effect of



human PGIS gene transfer, re-epithelialization would be promoted through the regeneration of epithelial cells by human HGF gene transfer, and finally, wound healing would be accelerated by the effects of human HGF and PGIS gene co-transfer in this model. These days, the numbers of patients suffering from incurable ulcers caused by diabetes mellitus or ischemic arterial diseases are increasing. Co-transfer of human HGF and PGIS genes using the Shima Jet as demonstrated in this study would be an ideal and effective strategy for wound healing.

Materials and methods

Construction of plasmids

To produce expression vectors, human HGF cDNA (2.2 kb)[21,22] or human PGIS cDNA (1.5 kb)[22,23] was inserted in the PVAX expression vector, which utilizes the cytomegalovirus (CMV) promoter/enhancer. The vector used as control was the CMV vector plasmid, which does not contain HGF or PGIS cDNA. The β-galactosidase gene expression plasmid (pCMV-LacZ) driven by CMV promoter was purchased from a commercial source (Promega Corporation, Madison, WI, USA). The control plasmid for pCMV-LacZ was also purchased from Promega Corporation.

In vivo *gene transfer by Shima Jet*

This experimental study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (NIH). The protocol was approved by the committee on the Ethics of Animal Experiments in Osaka University. Twelve-week-old female Wister rats were used in this study. All rats were anesthetized intramuscularly with a mixture of xylazine (7 mg/kg) and ketamine (35 mg/kg). The rats' backs were clipped. Naked pCMV-LacZ (100 μg/100 μl/body) or the control plasmid (100 μg/100 μl/body) was transferred into the skin by the Shima Jet. Skin sections were obtained 48 h post-transfer as described previously.[3] Sections were fixed in 1.0% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min, washed three times in PBS and then stained for β-galactosidase activity according to the method described in previous reports.[39] Sections were counterstained with hematoxylin and eosin (HE).

Luciferase activity assay

Luciferase expression plasmid driven by the SV-40 promoter and the control plasmid for luciferase expression plasmid were obtained from a commercial source (Promega Corporation). Rats were killed 1, 3, 7 and 14 days after transfer of luciferase plasmid (50 μg/50 μl/body or 100 μg/100 μl/body). In this study, we compared the efficiency of gene transfer into the skin by the Shima Jet and by a needle. Moreover, we also compared gene expressions at each time point in the skin after gene transfer by the Shima Jet. Luciferase activity was measured with a luciferase assay system according to the manufacturer's protocol (Promega Corporation).

Measurement of hepatocyte growth factor in skin

Naked human HGF gene (100 μg/100 μl/body) or the control plasmid (100 μg/100 μl/body) was transferred into the skin of rats by the Shima Jet. Tissue samples around injection sites obtained from rats were washed with PBS and homogenized in extraction buffer (20 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl, 0.1% Tween 80, 1 mM ethylenediamine tetraacetic acid and 1 mM phenylmethyl sulfonyl fluoride) by means of a polytron homogenizer (Kinematica AG, Lucerne, Switzerland). The homogenate was centrifuged at 15 000 r.p.m., at 4°C for 30 min, and the resultant supernatant served as the tissue extract.[3] The concentration of HGF in the skin was measured using an enzyme-linked immunosorbent assay kit for human HGF protein (Institute for Immunology, Tokyo, Japan).

Impaired wound healing model

Seven-week-old male Wister rats weighing 200–250 g were used to make this model. We used 30 rats and divided them into five groups (six rats for each group). The impaired wound healing rat model was made as described previously.[40] Briefly, PZ suspended in PBS was intramuscularly (i.m.) injected twice (3 days and immediately before the wounding) at a dose of 30 mg/kg with a needle to rats of groups two, three, four and five, and PBS instead of PZ was injected into rats of group 1 as control. The rats' backs were clipped and depilated with Epilat (Kanebo, Tokyo, Japan). One full-thickness wound (1.6 cm in diameter) was made on the back of each rat. To measure the wound closure, the wound was traced on tracing paper and the traced area was calculated by NIH imaging software on days 0, 4, 6 and 12 (National Institutes of Health, Bethesda, MD, USA).

Figure 6 Histological analysis of wound healing. (**A**) Granulation tissue areas after gene transfer are shown. There was no significant difference between each group 3 days after transfer. (**B**) Representative photographs of immunohistochemistry for factor VIII in granulation tissue 7 days after transfer. The numbers of microvessels were apparently increased in groups 3 and 5 (c and e). (a) rats treated with phosphate-buffered saline (PBS) injection after induction of wound by PBS (group 1: PBS/PBS); (b) rats treated with PBS injection after induction of wound by PZ (group 2: PZ/PBS); (c) rats transferred with human HGF gene after induction of wound by PZ (group 3: PZ/HGF); (d) rats transferred with human prostacyclin synthase (PGIS) gene after induction of wound by PZ (group 4: PZ/PGIS); (e) rats co-transferred with both human HGF and PGIS genes after induction of wound by PZ (group 5: PZ/HGF+PGIS). (**C**) Microvessel counts in granulation tissue are shown. The results are consistent with those from immunohistochemistry. Microvessels in granulation tissue were evaluated by counting factor VIII-positive cells (endothelial cells) in immunohistochemistry. The real numbers of microvessels were significantly increased in groups 3 and 5 (HGF and HGF+PGIS). $^{*}P<0.05$ vs group 2 (PBS). (**D**) Expression of PCNA in the epidermis 3 days after gene transfer. Intense staining for PCNA was observed in groups 3 and 5 (HGF and HGF+PGIS). (a) rats treated with PBS injection after induction of wound by PBS (group 1: PBS/PBS); (b) rats treated with PBS injection after induction of wound by PZ (group 2: PZ/PBS); (c) rats transferred with human HGF gene after induction of wound by PZ (group 3: PZ/HGF); (d) rats transferred with human PGIS gene after induction of wound by PZ (group 4: PZ/PGIS); (e) rats co-transferred with both human HGF and PGIS genes after induction of wound by prednisolone (group 5: PZ/HGF+PGIS). (**E**) The number of PCNA-positive cells in the edge of epidermis per field. $^{*}P<0.05$ and $^{\#}P<0.01$ vs rats treated with PBS injection after induction of wound by prednisolone.

Local transfer of hepatocyte growth factor and prostacyclin synthase genes into rat skin

On day 0, the same day of making the wound, human HGF gene (100 μg, five points) and human PGIS gene (100 μg, five points) were transferred around the wound by the Shima Jet. Human HGF gene was transferred to rats of group 3 (PZ/HGF), PGIS gene was transferred to rats of group 4 (PZ/PGIS), both genes were transferred to rats of group 5 (PZ/HGF+PGIS) and PBS instead of functional genes was injected into rats of groups 1 (PBS/PBS) and 2 (PZ/PBS). As an additional experiment, the same amount of HGF gene and PGIS gene was transferred around the wound by a needle.

Measurement of blood flow by Laser Doppler imaging

Measurement of blood flow with LDI (Moor Instruments) was performed as described previously.[26,27] Because Laser Doppler flow velocity correlates with capillary density, we measured skin blood flow by LDI. Consecutive measurements were obtained over the same regions of interest by averaging four sites around the wound.

Immunohistochemical analysis of hepatocyte growth factor, prostacyclin synthase and proliferating cell nuclear antigen

For immunohistochemistry, skin samples were fixed with 4% paraformaldehyde, and deparaffinized and sectioned. To analyze the localization and expression of human HGF, factor VIII and PCNA tissue samples were incubated with a rabbit polyclonal antibody against human HGF (diluted 1:10; Institute of Immunology), human factor VIII (diluted 1:400; Sigma-Aldrich, St Louis, MO, USA), mouse monoclonal antibodies against PCNA (PC-10, 1:50; Dako Inc., Kyoto, Japan) and biotinylated secondary antibody against rabbit and mouse immunoglobulin (Vectastatin Elite ABC kit) (Vectastatin, Burlingame, CA, USA). The rabbit polyclonal antibody against human HGF specifically cross-reacts only with human HGF but not with rat HGF.[16–18] To analyze the localization and expression of human PGIS, skin samples were snap frozen in liquid nitrogen and sectioned. Skin sections were fixed in acetone and incubated with a mixture of rabbit polyclonal antibody against human PGIS (1:1000) and biotinylated goat polyclonal secondary antibody against rabbit immunoglobulin (1:300) (Dako Inc.). Then, sections were incubated with peroxidase-conjugated streptavidin (Dako Inc.).

Measurement of granulation tissue area

After HE staining of sections obtained from the wound, granulation tissue area was measured as an index of wound contraction. The area of granulation tissue was

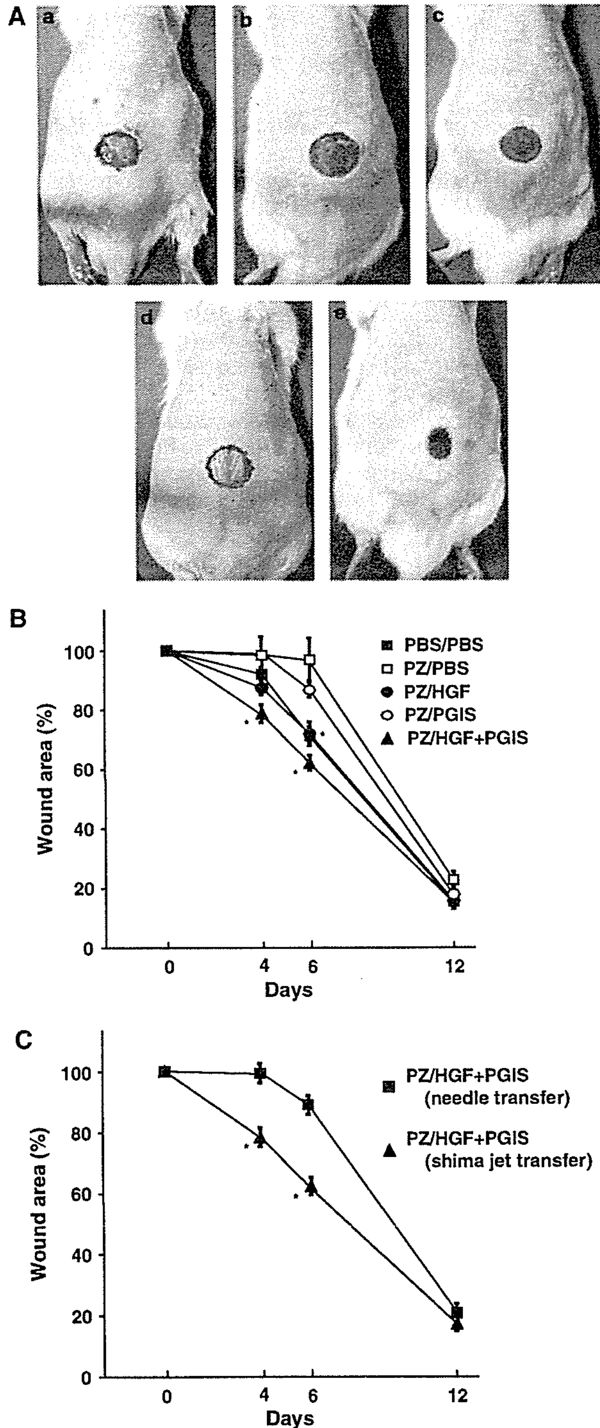


Figure 7 Time course of excisional wound area after gene transfer. (**A**) The representative photograph of wound at day 6 after gene transfer. (**a**) Rats treated with phosphate-buffered saline (PBS) injection after induction of wound by PBS (group 1: PBS/PBS); (**b**) rats treated with PBS injection after induction of wound by predonisolone (PZ) (group 2: PZ/PBS); (**c**) rats transferred with human HGF gene after induction of wound by PZ (group 3: PZ/HGF); (**d**) rats transferred with human prostacyclin synthase (PGIS) gene after induction of wound by PZ (group 4: PZ/PGIS); (**e**) rats co-transferred with both human HGF and PGIS genes after induction of wound by PZ (group 5: PZ/HGF+PGIS). (**B**) Excisional wound area after gene transfer by the Shima Jet. Values are shown as the percentage of the original wound lesion area on day 0. $^*P<0.01$ vs rats treated with PBS injection after induction of wound by PZ (group 2: PZ/PBS). (**C**) Comparison of excisional wound area after gene transfer by the Shima Jet and by a needle. Values are shown as the percentage of the original wound lesion area on day 0. $^*P<0.01$ vs rats co-transferred with HGF and PGIS genes by a needle after induction of wound by prednisolone.

calculated 3 days after gene transfer. The image was obtained by stereomicroscope (Leica MZ16) and analyzed by NIH imaging software (National Institutes of Health).

Statistical analysis

The results are expressed as the mean±s.e.m. Fisher's protected least-significant difference test was applied to the data when significant F ratios were obtained in analysis of variance. Differences are considered to be significant at $P<0.05$.

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