Gene Transfer of Human Hepatocyte Growth Factor into Rat Skin Wounds Mediated by Liposomes Coated with the Sendai Virus (Hemagglutinating Virus of Japan)

Kuniaki Nakanishi,* Maki Uenoyama,*
Naruya Tomita,[†] Ryuichi Morishita,[‡]
Yasufumi Kaneda,[‡] Toshio Ogihara,[†]
Kunio Matsumoto,[§] Toshikazu Nakamura,[§]
Akie Maruta,[¶] Shigeo Matsuyama,[¶]
Toshiaki Kawai,^{||} Takashi Aurues,*
Takuya Hayashi,* and Tomosumi Ikeda*

From the Division of Environmental Medicine* and the
Departments of Laboratory Medicine[¶] and Pathology,^{||} National
Defense Medical College, Tokorozawa; and the Department of
Geriatric Medicine,[†] the Division of Gene Therapy Science,[‡] and
Division of Biochemistry, the Department of Oncology,[§]
Biomedical Research Center, Osaka University Medical School,
Suita, Japan

Hepatocyte growth factor (HGF) regulates cell growth, cell motility, and morphogenesis in various types of cells, including epithelial and endothelial cells, indicating that it probably promotes epithelial repair and neovascularization during wound healing. To better understand the effects of HGF on wound healing, we performed human HGF-gene transfer into skin wounds in rats. The rat HGF mRNA levels, and human and rat HGF protein concentrations in the wounds in HGF gene-transfer rats were significantly elevated at 3 days, 3 to 14 days, and 3 and 14 days after gene transfer, respectively. An expression of human HGF mRNA and protein was revealed in squamous cells in the epidermis, in endothelial cells and smooth muscle cells in blood vessels, and in fibroblasts in granulation tissues at 3, 7, and 14 days after gene transfer in HGF gene-transfer rats. The wound lesion area in HGF gene-transfer rats was significantly less than that in control rats from 3 to 7 days after gene transfer. The re-epithelialization rate, microvessel counts in granulation tissues, proliferating cell nuclear antigen index of fibroblasts in granulation tissues, and the proliferating cell nuclear antigen index in the epidermis of HGF gene-transfer rats were significantly increased at 3 and 7 days after gene transfer. Semiquantitative reverse transcriptase-polymerase chain reaction revealed that the expression levels of transforming growth factor- β 1 and Col α 2(I)

mRNAs in the wounds of HGF gene-transfer rats were significantly decreased at 7 and 14 days, respectively. The hydroxyproline concentration in the wound was significantly less in HGF gene-transfer rats than in control rats at 3 days after gene transfer. These results suggest that HGF gene transfer into a skin wound may aid re-epithelialization and neovascularization in the early phase of wound healing, and that HGF may play a role in modulating cutaneous wound healing. (Am J Pathol 2002, 161:1761–1772)

Wound healing, which consists of a series of coordinated events involving inflammation, angiogenesis, matrix synthesis, and collagen deposition, leads to re-epithelialization, neovascularization, and the formation of granulation tissues.^{1–3} The healing processes are regulated by a number of mitogens and chemotactic factors, including such growth factors and cytokines as fibroblast growth factor, transforming growth factor (TGF)- α , TGF- β , epidermal growth factor, platelet-derived growth factor, and vascular endothelial growth factor. However, few studies have dealt with the effects of hepatocyte growth factor (HGF) on wound healing.⁴

HGF, which is the same as scatter factor, is a plasminogen-related and mesenchyme-derived pleiotropic growth factor that regulates cell growth and cell motility in various types of cells.^{5–7} Furthermore, it is an important factor regulating morphogenic processes during embryogenesis and during organogenesis in the regeneration of several organs. For example, it is a strong mitogen for hepatocytes and other epithelial cells, including keratinocytes.⁸ It can stimulate angiogenesis, trigger the dissociation of cells, and initiate epithelial cell movement.^{9,10,11} Therefore, HGF may well promote epithelial repair and neovascularization during wound healing.

In general, gene therapy can be used as an approach to the treatment of a variety of clinical disorders.^{12,13} Of

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Address reprint requests to K. Nakanishi M.D., Division of Environmental Medicine, National Defense Medical College Research Institute, Tokorozawa 359-8513, Japan. E-mail: nknsknak@res.ndmc.ac.jp.

major importance in successful gene therapy is the selection of the appropriate vector for gene transfer. Viruses, adenoviruses in particular, have been the preferred vectors for gene transfer. However, viral infection-associated toxicity, immunological compromise, and possible mutagenic or carcinogenic effects make their use potentially dangerous. An alternative method, HVJ (hemagglutinating virus of Japan)-liposome-mediated gene transfer, which has been reported to be an efficient *in vivo* gene-transfer method, uses liposomes in combination with a viral envelope, and is associated with little toxicity.^{14,15} This method has been used successfully for *in vivo* gene transfer into various tissues, including liver, kidney, vascular wall, heart, and brain.^{10,14,16–18}

The purpose of the present study was to determine the effects of human HGF gene transfer into skin wounds in rats. We investigated 1) whether after gene transfer a distribution and deposition of human HGF mRNA and protein would be revealed within a full-thickness wound; 2) whether the genetically translated protein would be biologically active; and 3) whether the translated protein would have a biological influence in a pathological state, for example, on mitogenic activity involving several cell-types within a full-thickness wound, and on the rates of re-epithelization, neovascularization, and the deposition of extracellular matrix within the granulation tissues. In addition, we investigated whether these changes in the wound tissues would be associated with a secretion of TGF- β 1. We measured the wound lesion area, and human and rat HGF protein concentrations in wound tissues after HGF gene transfer, as well as the expressions of the mRNAs for TGF- β 1 and several other components, such as collagen type I [Col α 2(I)], collagen type III [Col α 1(III)], desmin, and vascular smooth muscle α -actin (α -SMA), which would be expected to be involved in wound healing. For this, we used the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), and we examined the morphological changes in the wounds by *in situ* hybridization and immunohistochemical methods.

Materials and Methods

One hundred male Wistar rats, ~11 weeks old and weighing 310 to 370 g, were assigned to one of two experiments, and then housed two per cage in a temperature-controlled room with a 12-hour light-dark cycle. All rats were given commercial chow and tap water *ad libitum*. This experimental study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the National Defense Medical College.

Sixty of these rats were divided into two groups (a HGF gene-transfer group and a control vector group) for later biochemical and histological examination of a round wound in the skin. As preparation for the making of this wound, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.5 ml/kg), then the hair on their back was clipped and the skin cleaned. A round

full-thickness wound measuring 14 mm in diameter was then made on the back of each animal using a 14-mm round scalpel. Three days later, the same rats (under pentobarbital anesthesia) received a subcutaneous injection of either HVJ liposomes (300 μ l) containing 100 μ g of a HGF cDNA or a control vector. This was delivered to the edge of the round wound using a 27-G needle. At 3, 7, or 14 days after the injection, they were decapitated under pentobarbital anesthesia.

The remaining 40 rats were subjected to measurement of round wound lesion area after gene (or control vector) transfer. In these rats, the round wound lesion area was measured from transparent tracings of the edge of the wound taken at 0, 3, 4, 5, 6, 7, 10, and 14 days after gene transfer. Measurements were made with the aid of an image analyzer (TOSPIX-U, AS3260C), and an image-analysis software package (both from Toshiba, Tokyo, Japan). The lesion areas are expressed as a percentage of the initial area (on day 0 after gene transfer). The day on which the full-thickness round wound was seen to be completely closed by epidermis was taken as the day of complete healing.

Construction of Plasmids

To produce a 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.⁵

Preparation of HVJ Liposomes

Transfection with HVJ-coated liposomes was performed essentially as previously described.^{10,14–18} Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed at a weight ratio of 1:4.8:2 in tetrahydrofuran. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of the solvent in a rotary evaporator. The high mobility group-1 (50 μ l) purified from calf thymus was mixed with plasmid DNA (200 μ g) in a total volume of 200 μ l of balanced salt solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 10 mmol/L Tris-HCl, pH7.6) at 20°C for 1 hour, and then the mixture was added to the dried lipid. The liposome-DNA-high mobility group-1 complex suspension was mixed by a sequence of vortex, sonication for 3 seconds, and shaking for 30 minutes. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm²/second) for 3 minutes immediately before use. The liposome suspension (0.5 ml, containing 10 mg lipid) was mixed with HVJ (30,000 hemagglutinating units) in a total volume of 3 ml of balanced salt solution. The mixture was incubated for 10 minutes at 4°C, and then for 30 minutes with gentle shaking at 37°C. Free HVJ was removed from the HVJ liposomes by sucrose-gradient centrifugation. The top layer of the sucrose gradient containing the HVJ-liposome-DNA complex was collected and used immediately.

Table 1. Primer Sequences, TaqMan Probe Sequences, and Thermocycle Conditions Used for RT-PCR

mRNA	Sense primer (5'–3')	Anti-sense primer (5'–3')	TaqMan probe (5'–3')	Annealing temperature (°C)	Cycles	Sizes of PCR products (bp)
Human HGF	CGACAGTGTTCCTCTCG	ATTGAGAACCTGTTTGCCTTCT		64	40	102
Rat HGF	CCAGCTAGAAACAAA GACTTGAAAGA	GAAATGTTTAAGAT CTGTTTGCCTT	AATCCATGATGTCCATGAG AGAGGCG	62	40	101
GAPDH	CTTCACCACCATGGA GAAGGC	GGCATGGACTGTGG TCATGAG	CCTGGCCAAGGTCATCCAT GACAACTTT	60	40	238
TGF- β 1	TGAGTGGCTGTCTTT TGACGTC	GCAGTTCTTCTCTG TGGAGCTG	CAGTGGCTGAACCAAGGAG ACGGAAT	64	50	301
Col α 2(I)	GGCTGCTCCAAAAAG ACAAATG	CCAGAGGTGCAATG TCAAGGAA	ATACAAAACGAATAAGCCA TCTCGCCTGCC	60	40	97
Col α 1(III)	GTGAAAGAGGATCTG AGGGCTC	GAGTTCAGGGTGGC AGAATTT	TGCTGCCATTGCTGGAGTT GGA	64	50	302
α -sm-actin	CGATAGAACACGGCA TCATCAAT	GCATAGCCCTCATA GATAGGCA	AACTGGGACGACATGGAAA AGATCTGG	60	50	301
Desmin	AGCGCAGAATTGAGT CACTCAA	TGTCGGTATTCCAT CATCTCCT	CTCAGGGACATCCGTGCTC AGTATGAGA	60	50	301

HGF, hepatocyte growth factor; GAPDH, rat glyceraldehyde-3-phosphate dehydrogenase; TGF- β 1, rat transforming growth factor- β 1; Col α 2(I), rat α -2 type I collagen, segment 2; Col α 1(III), rat collagen type III α -1; desmin, rat desmin; α -sm-actin, rat vascular smooth muscle α -actin.

Blood Sampling and Autopsy

Before autopsy, rats were decapitated under anesthesia and blood samples were taken for the determination of HGF. The blood samples were placed in chilled tubes containing ethylenediaminetetraacetic acid (EDTA) (2 mg/ml), and then centrifuged. The plasma was frozen immediately and stored at -80°C until assay.

At autopsy, the skin was removed. Tissues from 10 rats in each group were weighed, then cut in half. One half was frozen in liquid nitrogen, and stored at -80°C until use. The wound tissues so obtained were subjected to RNA and peptide extractions. The other half was fixed in periodate-lysine-paraformaldehyde solution for *in situ* hybridization and immunohistochemistry.

Histomorphometric Analysis

After hematoxylin and eosin staining of specimens obtained from maximal cross-sections of the wound, the epithelial gap (distance between the two epithelial edges) was measured to assess re-epithelialization, whereas granulation-tissue area was measured as an index of wound contraction. The epithelial gap was measured using a calibrated lens micrometer. The percentage of re-epithelialization (taking the original 14-mm wound diameter as 100%) was calculated on 3, 7, and 14 days after gene transfer. The area of granulation tissue was calculated 3, 7, and 14 days after gene transfer with the aid of the image analyzer and image-analysis software package mentioned above.

Total RNA Extraction and Semiquantitative RT-PCR

The expression of various mRNAs in skin tissues obtained from 10 rats in each group was examined by semiquantitative RT-PCR.¹⁹ In the skin tissues of each rat, we did not separate the epidermis from the granula-

tion tissues. The total RNA in the skin tissues (including the epidermis) was isolated using acid guanidinium isothiocyanate-phenol-chloroform extraction and ethanol precipitation.²⁰ RT-PCR was performed using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied Biosystems, Alameda, CA) with several primers. The following primers were synthesized using an automated DNA synthesizer: human HGF, rat HGF, TGF- β 1, Col α 2(I), Col α 1(III), desmin, α -SMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequence information for all of the PCR primers and TaqMan probes used is listed in Table 1 together with the thermocycling conditions.²¹ TaqMan probes were labeled at the 5' end with the reporter dye molecule, FAM (6-carboxyfluorescein), and at the 3' end with the quencher dye, TAMRA (6-carboxytetramethylrodamine). The reaction master mix was prepared according to the manufacturer's protocol to give final concentrations of $1\times$ reaction buffer, 300 $\mu\text{mol/L}$ dATP, 300 $\mu\text{mol/L}$ dCTP, 300 $\mu\text{mol/L}$ dGTP, 600 $\mu\text{mol/L}$ dUTP, 3 mmol/L $\text{Mg}(\text{OAc})_2$, 0.1 U/ μl rTth DNA polymerase, 0.01 U/ μl AmpErase UNG, 900 nmol/L primers, and 200 nmol/L TaqMan probe. To perform PCR, the RT reaction was incubated at 60°C for 30 minutes, followed by incubation at 95°C for 5 minutes to deactivate AmpErase UNG. PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems). During each cycle of the PCR, the $5' \rightarrow 3'$ exonuclease activity of rTth DNA polymerase cleaves the TaqMan probe, thereby increasing the fluorescence of the reporter dye at the appropriate wavelength. The increase in fluorescence was proportional to the concentration of template in the PCR (Figure 1A). The threshold cycle was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the SD of the baseline). In other words, signals were regarded as positive if the fluorescence intensity exceeded $10 \times$ the SD of the baseline fluorescence. The standard curve was obtained using the threshold cycle established separately for four wells for each applied RNA amount (Figure 1B).

Figure 1. RT-PCR for collagen type I [Colα2(I)] mRNAs. **A:** Amplification plot for Colα2(I) mRNA obtained in real time using a semiquantitative RT-PCR method. Total RNA (200 ng, 40 ng, 8 ng, and 1.6 ng) extracted from normal rat skin was subjected to RT-PCR. **B:** Standard curve for threshold cycle of RT-PCR. The threshold cycle (obtained in quadruplicate) was plotted for each of the four RNA amounts applied.

PCR products were separated by electrophoresis in a 3% agarose gel and stained with ethidium bromide.

In Situ Hybridization

For *in situ* hybridization of HGF, deparaffinized 4% paraformaldehyde-fixed sections obtained from 10 rats in each group were treated with 0.2 N HCl for 20 minutes, then incubated in 2× standard saline citrate for 10 minutes at 37°C, and finally incubated in 5 μg/ml proteinase K for 10 minutes at 37°C. Sections were subsequently postfixed in 4% paraformaldehyde for 5 minutes, then incubated for 10 minutes in 0.1 mol/L triethanolamine buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride to prevent nonspecific binding because of oxidation of the tissue. The full-length human HGF cDNA, which was inserted between the *EcoRI* and *NotI* sites of the pUC-SRα expression vector plasmid, was digested by the restriction enzymes of *EcoRI*, and the resulting fragment of HGF cDNA (848 bp) was then ligated between the *EcoRI* cloning sites of pGEM-7Zf(+) (Promega, Madison, WI). The anti-sense probe and the corresponding sense probe were labeled with digoxigenin using SP6 and T7 polymerase, respectively, by means of a RNA labeling kit (Boehringer Mannheim, Postfach, Germany). Hybridization was performed overnight at 42°C in 50% (v/v) deionized formamide, 5× Denhardt's solution, 5% (w/v) dextran sulfate, 2× standard saline citrate, 0.3 mg/ml salmon sperm DNA, 5 mmol/L EDTA, and 0.01 μg/ml digoxigenin-labeled probes. After performing a final stringency wash at 55°C for 20 minutes, hybridization was detected immunologically.

Immunohistochemistry

For immunohistochemistry, the indirect immunoperoxidase method was applied to deparaffinized sections obtained from 10 rats in each group. This method used a

mouse monoclonal antibody against HGF (1:20; Institute of Immunology, Tokyo, Japan), a polyclonal antibody against factor VIII (1:100; DAKO Inc., Glostrup, Denmark), mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA) (PC-10, 1:100; DAKO Inc.), and a horseradish peroxidase-labeled secondary antibody against rabbit immunoglobulins (diluted 1:250; Chemicon International Inc.). The mouse monoclonal antibody against human HGF specifically detected human HGF, not rat HGF. Autoclave pretreatment in 0.01 mol/L of citrate buffer, pH 6.0, was performed for 15 minutes at 120°C for immunohistochemistry against PC-10. For the negative control, the incubation step with the primary antibody was omitted.

For the analysis of PC-10, and on the basis of the immunoreaction in at least 1000 tumor cells, the percentage of nuclei with a positive immunoreaction was determined (PCNA index). Microvessel counts in the wound (as detected by immunohistochemistry for factor VIII) were assessed by light microscopy in the areas containing the highest numbers of vessels. The number of vessels was determined in one ×200 field (×20 objective and ×10 ocular; 0.0925 mm² per field).

Extraction of Tissues and Determination of HGF Proteins and Hydroxyproline in the Wound

Tissue samples obtained from 10 rats in each group were homogenized for 1 minute in 4 vol of 20 mmol/L Tris-HCl buffer, pH 7.5, containing 0.1% 2 mol/L NaCl, 0.1% Tween-80, 1 mmol/L phenylmethyl sulfonyl fluoride, and 1 mmol/L EDTA by means of a polytron homogenizer (24,000 rpm; Kinematica AG, Lucerne, Switzerland). The homogenate was centrifuged at 15,000 × g for 30 minutes at 4°C, and the supernatant and pellet were stored at −80°C until enzyme-linked immunosorbent assay (ELISA) for HGF protein, and amino acid analysis for hydroxyproline, respectively. The concentration of human HGF protein was measured by ELISA using anti-human-HGF monoclonal antibody, and the concentration of rat HGF was measured, also by ELISA, using anti-rat-HGF monoclonal antibody (Institute of Immunology). The human HGF ELISA system specifically detected human HGF, not rat HGF. The plasma concentration of HGF protein was measured in 50 μl of rat plasma using the ELISA described above. For the hydroxyproline assay, the pellet was hydrolyzed in 6 N HCl for 16 hours at 110°C. The hydroxyproline content was determined using an amino acid analyzer (model 835; Hitachi Ltd., Tokyo, Japan). The protein content was determined using a Lowry assay (DC protein assay; Bio-Rad Laboratories, Hercules, CA).

Statistic Analysis

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

Figure 2. RT-PCR for human and rat HGFs. **A:** Detection of PCR products. Rat skin (HGF): rat RNA extracted from the wound tissues at 3 days after HGF gene transfer in HGF gene-transfer rat. Rat skin (HGFCV): rat RNA extracted from the wound tissues at 3 days after HGF control vector (HGFCV) gene transfer in HGFCV gene-transfer rat. Human HGF PCR: RT-PCR with human primer pair. Rat HGF PCR: RT-PCR with rat primer pair. **B:** Results of semiquantitative RT-PCR for rat HGF in wound tissues in HGF gene-transfer rats and control rats. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group).

Results

Expression Levels of Human and Rat HGF mRNAs and Concentration of Human and Rat HGF Proteins in Wound Tissues and/or Plasma

By RT-PCR, human HGF mRNA was specifically detected in the human RNA extracted from human liver tissues and

in the rat RNA extracted from the wound tissues at 3, 7, and 14 days after HGF gene transfer in HGF gene-transfer rats. However, it was not detected in the rat RNA extracted from the wounds on any days in control rats. Rat HGF mRNA was specifically detected in the rat RNA extracted from wound tissues in HGF gene-transfer and control rats, but not at all in human RNA (Figure 2A). When the rat HGF mRNA level in the wound tissues of

Figure 3. Human and rat HGF concentrations in the wound after gene transfer. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group).

Figure 4. Localization of human HGF mRNA (**a–e**) in the wound in HGF gene-transfer rats and control rats. **a** and **b**: HGF gene-transfer rats at 3 days after gene transfer. **c**: Control rats at 3 days after gene transfer. **d**: HGF gene-transfer rats at 7 days after gene transfer. **e**: HGF gene-transfer rats at 14 days after gene transfer. Scale bars, 200 μ m.

HGF gene-transfer rats was compared with that in control rats (Figure 2B), the former was significantly higher at 3 days after HGF gene transfer and tended to be higher at 14 days.

By ELISA, the human HGF protein level was found to be greatly increased at 3, 7, and 14 days after HGF gene transfer in the wound tissues of HGF gene-transfer rats (Figure 3A). The level of human HGF protein was highest at 3 days after gene transfer, and decreased from 3 to 14 days after gene transfer. However, no human HGF was detected at all in the wounds in control rats. No human HGF protein was detected in any of the plasma samples from HGF gene-transfer rats. In contrast, the rat HGF levels in the wound tissues of HGF gene-transfer rats were increased by comparison with those in control rats after gene transfer (Figure 3B). Statistical significance was reached between HGF gene-transfer rats and control rats at both 3 and 14 days after gene transfer ($P < 0.05$).

Expression of Human HGF mRNA and Protein in Wound Tissues

In the wound tissues of HGF gene-transfer rats, HGF mRNA was found to be present 3 days after gene transfer in squamous cells in the epidermis on the edge of the round wound, in the endothelial cells and smooth muscle

cells of blood vessels, and in fibroblasts in the granulation tissues (Figure 4). The extracellular components showed no detectable expression of this mRNA. In contrast, it was not detected at all in control rats. Likewise, human HGF protein was detected in the same cell types (squamous cells in the epidermis on the edge of the wound, in the endothelial cells and smooth muscle cells of blood vessels, and in fibroblasts in the granulation tissues) in HGF gene-transfer rats, but not in control rats (Figure 5). Thereafter, these expressions were sustained until 14 days after gene transfer (the end of the study).

Wound Lesion Size, Re-Epithelialization Rate, and Wound Contraction

The round wound lesion area (expressed as a percentage of the original round wound lesion area on day 0 after gene transfer) was significantly decreased in HGF gene-transfer rats from 3 to 7 days after gene transfer (compared ζ that in control rats) ($P < 0.05$; Figure 6). However, there was no difference in the number of days required for complete healing between HGF gene-transfer rats and control rats. The degree of re-epithelialization of the wound in HGF gene-transfer rats [expressed as a percentage of the original round wound diameter (14 mm)] was significantly increased at 3 and 7 days after gene transfer (compared to

Figure 5. Localization of human HGF protein (**a–e**) in the wound in HGF gene-transfer rats and control rats. **a** and **b**: HGF gene-transfer rats at 3 days after gene transfer. **c**: Control rats at 3 days after gene transfer. **d**: HGF gene-transfer rats at 7 days after gene transfer. **e**: HGF gene-transfer rats at 14 days after gene transfer. Scale bars, 200 μ m.

that in control rats) ($P < 0.05$; Figure 7). However, there was no difference in the granulation tissue area (used as an index of wound contraction) between HGF gene-transfer rats and control rats (Figure 8). In fact, the tendency was for the granulation tissue areas to be smaller in the former group than in the latter.

Cell Proliferation and Neovascularization in the Wound

The PCNA indexes in the epidermis on the edge of the round wound and in the fibroblasts of granulation tissues were both significantly increased in HGF gene-transfer rats at 3 and 7 days after gene transfer (compared with those in control rats) ($P < 0.05$; Figures 9 and 10). However, in the endothelium of blood vessels in granulation tissues the PCNA indexes did not change throughout the experiment (Figure 10B). The microvessel counts in granulation tissues, which contained a large number of vessels (as detected by immunohistochemistry for factor VIII), were significantly increased in HGF gene-transfer rats at 3 and 7 days after gene transfer ($P < 0.05$; Figure 11).

Expression of Skin Components in Wound

By semiquantitative RT-PCR, the expressions of TGF- β 1 and Col α 2(I) mRNAs in the wound tissues of HGF gene-

transfer rats were found to be significantly decreased (compared to those in control rats) at 7 and 14 days after gene transfer, respectively ($P < 0.05$; Figure 12).

Hydroxyproline Concentration in the Wound

The hydroxyproline concentration in the wound was found to be significantly lower in HGF gene-transfer rats than in control rats at 3 days after gene transfer ($P < 0.05$; Figure 13).

Figure 6. Wound lesion area after gene transfer as a percentage of original wound lesion area. *, $P < 0.05$ versus value for control rats ($n = 20$ for each group).

Figure 7. Degree of re-epithelialization of the wound after gene transfer (epithelial gap expressed as a percentage of original wound diameter). *, $P < 0.05$ versus value for control rats ($n = 10$ for each group).

Discussion

Although there have been several studies of the transfer into wounds of the genes for such factors as insulin-like growth factor, platelet-derived growth factor, and epidermal growth factor,^{22–25} none of the previous reports have focused specifically on the quantitative and qualitative changes in a number of the factors involved in wound healing or on histopathological effects after HGF gene transfer. In our study, we observed increases in the amounts of human and rat HGF mRNAs and proteins in the wound tissues in HGF gene-transfer rats compared to control rats (using RT-PCR, *in situ* hybridization, ELISA, and immunohistochemistry), as well as more rapid re-epithelialization, intensive proliferation of several types of cells, and intensive neovascularization within the wound (using immunohistochemistry). In contrast, during the wound healing we saw evidence of down-regulation of TGF- β 1 mRNA and Col α 2(I) mRNA (using semiquantitative RT-PCR) and decreases in the amount of hydroxyproline within the wound. Taken together, these results suggest that HGF gene transfer into a skin wound may aid re-epithelialization and neovascularization as part of the early phase of the process of wound healing. It might also suppress scar formation (see below).

Figure 8. Area of granulation tissues after gene transfer ($n = 10$ for each group).

Figure 9. Expression of PCNA in the epidermis on the edge of the wound in rats after gene transfer. **A:** Percentage of PCNA-positive cells in the epidermis after gene transfer. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group). **B:** Expression of PCNA. PCNA was detected in the nuclei of squamous cells in the epidermis and fibroblasts in the subepidermis. PCNA-positive cells were counted in squamous cells in the suprabasal area (**a–c**: 3, 7, and 14 days, respectively, after gene transfer in HGF-gene transfer rats; **d–f**: days 3, 7, and 14, respectively, in control rats). Scale bars, 200 μ m (**a–f**).

In the present study, we demonstrated that human HGF production within the wound was sustained until 14 days after gene transfer (the end of the study), although the level declined from 0.90 pg/mg at peak (at 3 days after gene transfer) to 0.66 pg/mg at 14 days. Despite the low-level expression of the human HGF protein in the wound tissues in HGF gene-transfer rats, the level of rat HGF mRNA in the wound tissues at 3 days after HGF gene transfer in these rats was 1.5 times the level seen in control rats. Furthermore, the rat HGF concentration in the wound in HGF gene-transfer rats was higher than in the control rats by some 2 to 6 pg/mg throughout the study, with a significant difference being detected at 3 and 14 days after gene transfer. Therefore, 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. Yo and colleagues²⁶ revealed that when the HGF concentration in the conditioned medium (48 hours after transfection) from endothelial cells transfected with a human HGF vector was measured by ELISA using anti-human and anti-rat HGF antibodies, the immunoreactive levels of human and rat HGF were, respectively, ~0.29 ng/ml and 14 ng/ml higher in the conditioned medium from the human HGF vector group than in that

Figure 10. Expression of PCNA in granulation tissue in rats after gene transfer. **A:** Percentage of PCNA-positive fibroblasts in granulation tissue after gene transfer. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group). **B:** Percentage of PCNA-positive endothelial cells in blood vessels in granulation tissue after gene transfer ($n = 10$ for each group). **C:** Expression of PCNA. PCNA was detected in the nuclei of endothelial cells (arrowheads) and fibroblasts (arrows) in granulation tissue (**a–c:** 3, 7, and 14 days, respectively, after gene transfer in HGF gene-transfer rats; **d–f:** days 3, 7, and 14, respectively, in control rats). Scale bars: 200 μ m (**a–f**).

from the control vector group. They therefore suggested that HGF may itself regulate local HGF production by an autoloop-positive feedback mechanism, and thus operate in an autocrine-paracrine manner.^{26,27} Our data support this hypothesis.

Figure 11. Microvessel count in granulation tissue in rats after gene transfer detected by immunohistochemistry for factor VIII. **A:** Microvessel count in granulation tissue. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group). **B:** Immunohistochemistry for factor VIII. Factor VIII was detected in endothelial cells in granulation tissue (**a–c:** 3, 7, 14 days, respectively, after gene transfer in HGF gene-transfer rats; **d–f:** 3, 7, 14 days, respectively, in control rats). Scale bars: 200 μ m (**a–f**).

In our immunohistochemical study, using an antibody against human HGF protein that did not cross-react with rat HGF, a positive reaction was revealed in keratinocytes, fibroblasts, and the endothelial cells and smooth muscle cells of blood vessels within the wound. Likewise, human HGF mRNA was found in these cells after gene transfer. However, in skeletal muscle cells around the wound we detected neither human HGF protein nor HGF mRNA using immunohistochemistry and *in situ* hybridization. Although the explanation for this phenomenon is unclear, one possibility is that the entry of HVJ liposomes may be prevented by the muscle's fascia and/or sarcolemma.

Re-epithelialization of a round wound takes place by the migration of keratinocytes from the edges of the wound toward its center. *In vitro*, HGF enhances migration, cell growth, and DNA synthesis in keratinocytes cultured under physiological Ca^{2+} conditions.⁸ Furthermore, HGF has been found to promote epithelial wound resealing in T84 intestinal monolayers as a result of en-

Figure 12. RT-PCR for TGF-β1 (TGF-β1), collagen type I [Colα2(I)], vascular smooth muscle α-actin (α-sm-actin), desmin, and collagen type III [Colα1(III)] mRNAs in the wound in rats after human HGF gene transfer. **A:** Detection of PCR products. **B:** Results of semiquantitative RT-PCR. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group).

hanced cell turnover.²⁸ *In vivo*, administration of recombinant HGF has been shown to promote the regeneration of epithelial cells in rat kidneys injured by anti-tumor drugs.¹⁰ However, in gastric ulcers produced in rats by cryoinjury, subcutaneous administration of recombinant HGF had no effect on the ulcer-healing rate, although the human HGF concentration in the serum was increased,

Figure 13. Hydroxyproline concentration in the wound in rats after gene transfer. *, $P < 0.05$ versus value for control rats ($n = 10$ for each group).

and epithelial cell proliferation was increased in the ulcer margin on days 8 to 15 after the cryoinjury.⁴ On the basis of the macroscopic and histological evidence provided by the present study, HGF gene-transfer rats would seem to exhibit a more rapid decrease in round lesion area and a more rapid increase in re-epithelialization than control rats, as well as a hyperproliferation of the basal cell layers of the epidermis at the edge of a round wound. Therefore, we suggest that in this model, the increased HGF mRNA and protein expressions may enhance epithelial mitogenesis and migration into and across the wound by a paracrine and/or autocrine action.

HGF has been reported to be a potent angiogenic growth factor on the basis of its observed action as both a mitogen for endothelial cells and a potent inducer of tube formation *in vitro*.^{6,7} Furthermore, other investigators have found *in vivo* evidence that angiogenesis is induced by HGF both in rat hearts (with a noninfarcted or infarcted myocardium)¹⁷ and in rat corneas.⁹ In the present study, we also observed an increase in the number of blood vessels within granulation tissues in HGF gene-transfer rats. These findings support the idea that HGF has a potent angiogenic action.

A transient up-regulation of TGF-β expression is an important event in wound healing. TGF-β has been

shown to stimulate fibroblasts to produce a wide spectrum of matrix proteins, matrix protease inhibitors, and integrin receptors, thereby increasing matrix formation and modulating cell-cell interactions within the wound site.²⁹ In the present study, using semiquantitative RT-PCR we observed that TGF- β 1 mRNA expression was decreased in HGF gene-transfer rats at 7 days after gene transfer (by comparison with control rats). Furthermore, Col α 2(I) mRNA expression and hydroxyproline production were decreased at 3 days after gene transfer, respectively, although no difference in the granulation tissue area (an index of wound contraction) was seen between HGF gene-transfer rats and control rats. On this basis, we suggest that scar formation in HGF gene-transfer rats may be suppressed through a down-regulation of TGF- β 1 synthesis, although HGF gene transfer may induce fibroblast proliferation. In contrast, a dysregulated and sustained overexpression of TGF- β 1 would presumably contribute to an enhancement of tissue fibrosis because an increased expression of TGF- β 1 mRNA has been reported in the tissues of patients with cutaneous fibrosis (eg, hypertrophic scars and keloids).³⁰ Furthermore, addition of TGF- β -neutralizing antibodies to adult wounds not only reduced the cellularity in wound granulation tissues, but also improved the architecture of the neodermis.³¹ Our results suggest that although HGF gene transfer into a skin wound might induce re-epithelialization and neovascularization as part of the process of wound healing, it might not promote scar formation. If this is so, HGF gene transfer into a skin wound may prove to be a useful treatment for patients with cutaneous fibrosis.

In conclusion, manipulation of re-epithelialization and neovascularization by means of a gene transfer-induced overexpression of HGF shows promise as a new therapeutic option in the field of wound healing.

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