

Biodegradable Gelatin Hydrogel Potentiates the Angiogenic Effect of Fibroblast Growth Factor 4 Plasmid in Rabbit Hindlimb Ischemia

Hirofumi Kasahara, MD,* Etsuro Tanaka, MD, PhD,†‡§ Naoto Fukuyama, MD, PhD,†§ Eriko Sato, MD,* Hiromi Sakamoto, PhD,|| Yasuhiko Tabata, PhD,¶|| Kiyoshi Ando, MD, PhD,‡§ Harukazu Iseki, MD, PhD,‡ Yoshio Shinozaki, BS,† Koji Kimura, MD,* Eriko Kuwabara, MD,* Shirosaku Koide, MD, PhD,* Hiroe Nakazawa, MD, PhD,† Hidezo Mori, MD, PhD#
Isehara, Tokyo, Kyoto, and Suita, Japan

OBJECTIVES	We investigated the potentiation of gene therapy using fibroblast growth factor 4 (FGF4)-gene by combining plasmid deoxyribonucleic acid (DNA) with biodegradable gelatin hydrogel (GHG).
BACKGROUND	Virus vectors transfer genes efficiently but are biohazardous, whereas naked DNA is safer but less efficient. Deoxyribonucleic acid charges negatively; GHG has a positively charged structure and is biodegradable and implantable; FGF4 has an angiogenic ability.
METHODS	The GHG-DNA complex was injected into the hindlimb muscle (63 mice and 55 rabbits). Gene degradation was evaluated by using ¹²⁵ I-labeled GHG-DNA complex in mice. Transfection efficiency was evaluated with reverse-transcription nested polymerase chain reaction and X-Gal histostaining. The therapeutic effects of GHG-FGF4-gene complex (GHG-FGF4) were evaluated in rabbits with hindlimb ischemia.
RESULTS	Gelatin hydrogel maintained plasmid in its structure, extending gene degradation temporally until 28 days after intramuscular delivery, and improving transfection efficiency. Four weeks after gene transfer, hindlimb muscle necrosis was ameliorated more markedly in the GHG-FGF4 group than in the naked FGF4-gene and GHG-beta-galactosidase (control) groups ($p < 0.05$, Kruskal-Wallis test). Synchrotron radiation microangiography (spatial resolution, 20 μ m) and flow determination with microspheres confirmed significant vascular responsiveness to adenosine administration in the GHG-FGF4 group, but not in the naked FGF4-gene and the control.
CONCLUSIONS	The GHG-FGF4 complex promoted angiogenesis and blood flow regulation of the newly developed vessels possibly by extending gene degradation and improving transfection efficiency without the biohazard associated with viral vectors. (J Am Coll Cardiol 2003;41:1056-62) © 2003 by the American College of Cardiology Foundation

Angiogenic gene therapy using growth factors is widely studied to treat ischemic heart disease and severe limb ischemia (1,2). Of the two major methods of gene transfer, the use of virus vectors is efficient but biohazardous (3,4), while naked deoxyribonucleic acid (DNA) is safer, but less efficient (5). A highly efficient and safe drug delivery system without using a virus vector is needed for gene therapy in humans. We developed a new hydrogel consisting of amino acids, being biodegradable and, therefore, implantable, from gelatin (6). Hydrogel has been used to improve transfection efficiency in a hydrogel-coated balloon catheter (7). How-

ever, this hydrogel was not implantable because it consisted of carbohydrate and was not biodegradable. The purpose of the present study is to assess whether biodegradable gelatin hydrogel (GHG) improves the efficacy of gene therapy with the fibroblast growth factor 4 (FGF4)/hst1 gene; FGF4 is a growth factor discovered in human gastric cancer (8) and has a secretion signal domain (9). Its angiogenic ability has been confirmed both in vitro and in vivo (10).

METHODS

Experimental animals. All animal experiments were performed in accordance under the Guidelines of Tokai University School of Medicine on Animal Use, which conform to the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, DHEW publication No. (NIH) 86-23, revised 1985, Offices of Science and Health Reports, DRR/NIH, Bethesda, Maryland. Fifty-five Japanese white rabbits weighting 2.45 to 2.85 kg (Nihon Nosan Co., Tokyo, Japan) of both genders were used. The animals were anesthetized by intravenous injection of sodium pentobarbital (40 mg/kg), and hindlimb ischemia was created by the method of Takeshita et al. (11). Sixty-three

From the Departments of *Cardiovascular Surgery, †Physiology, ‡Internal Medicine, and §Research Center for Genetic Engineering and Cell Transplantation, Tokai University School of Medicine, Isehara, Japan; ||Genetics Division, National Cancer Center Research Institute, Tokyo, Japan; ¶Research Center for Biomedical Engineering, Kyoto University, Kyoto, Japan; #Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan. Supported by Grants-in-Aid for Scientific Research (13470154, 13470381, 13877114, 14657460, 14657461) from the MECSST; New Energy and Industrial Technology Development Organization; The Science Frontier Program of MESSC; The Research Grants for Cardiovascular Disease (H13C-1) and for Cancer Research (9-3, 10Shi-1) from the MHLW; HLSRG-H14nano001&genom005; the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

Manuscript received December 30, 2001; revised manuscript received July 2, 2002, accepted November 5, 2002.

Abbreviations and Acronyms

ANOVA	= analysis of variance
cDNA	= complementary deoxyribonucleic acid
DNA	= deoxyribonucleic acid
FGF4	= fibroblast growth factor 4
GHG	= gelatin hydrogel
lacZ	= beta-galactosidase
NIH	= National Institute of Health
PBS	= phosphate-buffered saline
pI	= isoelectric point
RNA	= ribonucleic acid
RT-nested PCR	= reverse transcription-nested polymerase chain reaction

mice (male ddY mice, six to seven weeks old, Shizuoka Animal Center, Shizuoka, Japan) were also used.

Preparation of GHG-DNA complex. DNA encoding FGF4, beta-galactosidase (lacZ) with the cytomegalovirus enhancer-chicken β -actin hybrid promoter comprising a cytomegalovirus enhancer, and chicken beta-actin promoter were constructed (12); GHG was prepared from bovine bone (6). The GHG used in this study was characterized by a spheroid shape with a diameter of approximately 200 μ m, water content of 95%, and an isoelectric point (pI) of 11 after swelling in water, without special statement.

The efficiency of incorporation of DNA into positively and negatively charged GHG was evaluated. Dried GHG (4 mg, pI 11 or 5) was added to lacZ DNA solution (500 μ g/100 μ l in phosphate-buffered saline [PBS], pH 7.4), mixed with a vortex mixer for 5 s, and allowed to stand at 37°C; the solution immediately settled. The absorbance (260 nm) of the supernatant was measured. In a sham control experiment, GHG was added to pure PBS solution. Positively charged GHG (pI 11) was immediately impregnated with naked DNA, and was stable at pH 7.4 for at least 120 h, whereas the negatively charged one (pI 5) was not.

Experimental protocols. PROTOCOL 1: DNA DEGRADATION AND THE IMPROVEMENT OF TRANSFECTION EFFICIENCY BY GHG. To examine the temporal extension of gene degradation by GHG, the decay sequence of 125 I-labeled DNA impregnated into unlabeled GHG, 125 I-labeled GHG, and 125 I-labeled DNA solution was compared (63 mice). Plasmid DNA and GHG were radioiodinated with 125 I, with TiCl_3 and Bolton and Hunter reagent (Amersham Pharmacia Biotech Ltd., Buckinghamshire, United Kingdom) (13), respectively. To impregnate GHG with DNA, dried GHG (2 mg) was added to 100 μ l of naked lacZ solution (50 μ g/100 μ l in PBS), mixed for 5 s, and allowed to stand at 37°C for 2 h. Each complex was injected into the hindlimb muscle. On days 1, 3, 5, 7, 14, 21, or 28, the muscle was collected, and radioactivity was measured with a gamma counter (ARC-301B, Aloka Co., Ltd., Tokyo, Japan) in three mice each.

The following experiment was performed in 16 rabbits to assess spatial potentiation of gene expression by GHG. Intramuscular gene transfer was performed 10 days after

modeling hindlimb ischemia. The DNA solution (FGF4-gene or lacZ 500 μ g/100 μ l PBS) mixed with GHG (4 mg; GHG-FGF4 complex, n = 2; GHG-lacZ complex, n = 2) and the original FGF4-gene solution (naked FGF4-gene, n = 2) were diluted with 0.4 ml saline and slowly injected through a 23-gauge needle at a single point in the adductor muscle marked with a 4-0 nylon suture. Tissue samples from the transfected left adductor muscle (the injection site and the adjacent region 10 mm apart from the injection site), the right adductor muscle, stomach, liver, spleen, testes, kidneys, heart, lungs, and brain were retrieved and immediately frozen in liquid nitrogen on day 17; FGF4-gene expression was evaluated by reverse transcription-nested polymerase chain reaction (RT-nested PCR). In the remaining 10 rabbits, gene expression was evaluated with lacZ gene; GHG-lacZ complex (n = 5) and naked lacZ solution (n = 5) were injected at a single point in the adductor muscle in the same way as the GHG-FGF4 injection on day 10. On day 17, a muscle sample at the injection site was dissected out, and expression of lacZ was determined by X-Gal histostaining (14).

PROTOCOL 2: SALVAGE OF HINDLIMB ISCHEMIA WITH GHG-PLASMID COMPLEX ENCODING FGF4. The angiogenic effect of three sets of GHG-DNA complexes were compared in 39 rabbits with hindlimb ischemia: 1) GHG impregnated with lacZ plasmid (GHG-lacZ: control); 2) naked FGF4-plasmid (naked FGF4-gene); and 3) GHG impregnated with FGF4 plasmid (GHG-FGF4). The amount of plasmid was 500 μ g (1.0 ml) and that of GHG was 4 mg. On day 10 of ischemia, the gene complex was injected at five points 20 mm apart in the adductor muscle with a 23-gauge needle.

In 18 rabbits, on days 10 and 38 of ischemia, calf systolic blood pressure was measured by the Doppler flow signal from the posterior tibial artery (ES-100V2, Hayashi Denki Co., Kawasaki, Japan) with a 25-mm wide cuff. The calf blood pressure ratio of each rabbit was defined as the ratio of the systolic pressure of the ischemic limb to that of the normal limb. Regional blood flow was measured by the microsphere method (15) at baseline on days 0 and 38. On day 38, adenosine (100 μ g/kg/min) was administered 30 min after baseline flow measurement (vasodilatory condition). A 4F catheter was introduced into the ascending aorta via the common carotid artery for microsphere injection and adenosine administration. Microspheres (15- μ m diameter, 3×10^6) labeled with one of four sets of stable heavy elements (In, I, Ba, or Ce, Sekisui Plastic, Osaka, Japan) (15) were suspended in 0.05% sodium dodecyl sulfate at a concentration of 5×10^6 /ml and injected into the ascending aorta. After killing the animals, the adductor, semimembranous, and gastrocnemius muscles were dissected out and weighed. The X-ray fluorescence of the labeled microspheres was measured in 4 to 8 g of the dissected muscles to calculate the regional blood flow (15) and expressed as the ratio of flow in the ischemic limb to flow in the normal limb.

Table 1. Morphologic Evaluation of Gene Therapy

Muscle Necrosis (Area)	GHG-lacZ	Naked FGF4	GHG-FGF4*†
Grade 0 (0 cm ²)	0	0	0
Grade 1 (<1 cm ²)	0	0	50% (3/6)
Grade 2 (<3 cm ²)	17% (1/6)	33% (2/6)	50% (3/6)
Grade 3 (<5 cm ²)	0	67% (4/6)	0
Grade 4 (<10 cm ²)	50% (3/6)	0	0
Grade 5 (>10 cm ²)	33% (2/6)	0	0
Muscle weight ratio (%)	48 ± 67 (6)	62 ± 14 (6)	79 ± 11* (6)

Morphologic indexes in 18 gene-transferred rabbits on day 38. The ischemic limb was macroscopically evaluated by using graded morphological scales for muscle necrosis area (the adductor, semimembranous, medial large, and gastrocnemius muscles) (grade 0 to 5); GHG-FGF4 group had significantly less muscle necrosis compared with naked FGF4 and GHG-lacZ groups. Muscle weight ratio was significantly different between GHG-FGF4 and GHG-lacZ groups. $p < 0.05$ vs. *GHG-lacZ, †naked FGF4 (Kruskal-Wallis test, analysis of variance).

FGF4 = fibroblast growth factor 4; GHG = gelatin hydrogel; lacZ = β -galactosidase.

Sufficient mixing of microspheres injected into the aorta (not into the left atrium) was confirmed by a preliminary study in which two different sets of microspheres were simultaneously injected into the aorta. The liner regression analysis on the two different sets of flows yielded an almost identical regression line ($y = 1.011x - 0.003$, $r = 0.98$, $Sy \cdot x = 0.032$). The remaining muscle tissue was used for histological analysis. An investigator blinded to the treatment macroscopically evaluated the ischemic limb on graded morphological scales for area of muscle necrosis (the adductor, semimembranous, medial large, and gastrocnemius muscles; grade 0 to 5; Table 1).

Synchrotron radiation microangiography characterized by high-resolution and high-sensitivity (16) was performed in 21 rabbits as previously described (15,17,18). The system is capable of separating adjacent lead lines only 20 μ m apart on the resolution bar chart with 640 \times higher sensitivity than charge-coupled device camera system. This system allows detection and functional analysis of small vessels with a diameter of 200 to 500 μ m in situ (15,17,18). Contrast material containing 37% nonionic iodine (Iopamidol, Nihon Schering Co., Tokyo, Japan) was injected via a 4F catheter placed immediately above the aortic bifurcation under baseline condition and during adenosine administration (100 μ g/kg/min) (vasodilatory condition) via the same catheter. Vessel density in the midzone collateral was evaluated as an angiographic score (11,15,18).

Plasmid. Complementary deoxyribonucleic acid (cDNA) of human hst1/FGF4 (19), or bacterial β -galactosidase was inserted into the expression vector pRC/CMV (Invitrogen Corp., Carlsbad, California) and designated as pRC/CMV-HST1-10 (human stomach tumor) and pRC/CMV-lacZ, respectively. Preparation and purification of the plasmid from cultures of pRC/CMV-HST1-10-, or pRC/CMV-lacZ-transformed *Escherichia coli* were performed by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

RT-nested PCR. Ribonucleic acid (RNA) was extracted from tissues with ISOGEN (Nippon Gene, Tokyo, Japan).

The extracted RNA was treated with DNase twice to eliminate DNA contamination. In each set of experiments, 0.5 μ g of total RNA was denatured at 70°C for 5 min, and reverse transcription was carried out at 37°C for 60 min; RT-nested PCR was carried out in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer, Wellesley, Massachusetts) with primers designed to selectively amplify the FGF4 cDNA. The external primers EcoHST1f3 (forward primer: GGA ATT CAC TGA CCG CCT GAC CGA CGC ACG GCC CTC G) and SalHST1r2 (reverse primer: GCG TCG ACC CCG AGG CTG AGG CAA GGG TCC TCT) were used for the first round (generating a 704-base pair [bp] fragment). The second round of the amplification (nested PCR) was performed with two internal primers HST1LGC1 (forward primer: AGC TCT CGC CCG TGG AGC GG) and HST1AA-r (reverse primer: CTC TGG AGG GTC ACA GCC TG) (generating a 282-bp fragment). The PCR reactions were performed as follows. The thermal cycle conditions for the first round were 30 cycles (95°C for 1 min, 59°C for 1 min, 72°C for 1 min), and for the second round were 25 cycles (94°C for 1 min, 72°C for 2 min), followed by incubation at 72°C for 10 min, respectively. Amplification products were detected after electrophoresis on 3.5% agarose gels by staining with ethidium bromide. A primer set of beta-actin (generating a 506-bp fragment) was used as a positive control for RT-PCR analysis.

Statistical analysis. Data are presented as mean values \pm SD. Differences were assessed by using the paired t test, Kruskal-Wallis test, or analysis of variance (ANOVA) for factorial or repeated measures with the Scheffé F test when applicable. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Protocol 1: DNA degradation and the improvement of transfection efficiency by GHG. The radioactivity of radiolabeled DNA impregnated into GHG in the limb muscle remained above the detection limit for four weeks (solid line in Fig. 1). The radiolabeled DNA impregnated into GHG had decay sequences almost identical to those of the radiolabeled GHG (dotted line in Fig. 1). By contrast, the radioactivity of naked DNA (dashed line in Fig. 1) decreased to <10% of the baseline within a day.

Gelatin hydrogel improved transfection efficiency in vivo; RT nested-PCR analyses revealed FGF4 expression at all injection sites in the left adductor muscle in the FGF4-gene-treated animals ($n = 4$), as shown in lanes 1 (naked FGF4-gene) and 4 (GHG-FGF4) in Figure 2; FGF4 expression was also detected in the adjacent region 10 mm apart from the injection site in the left adductor muscle in the GHG-FGF4-treated animals (lane 5), but not in the naked FGF4-gene-treated animals (lane 2). No expression was detected in any animal at remote sites, such as the right adductor muscle (lanes 3 and 6), stomach, liver, spleen,

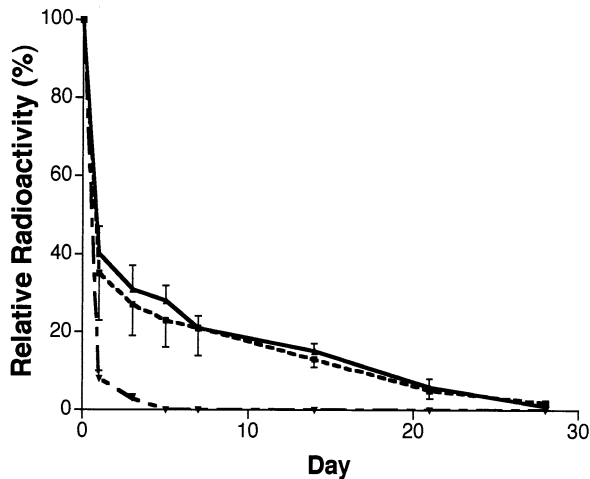


Figure 1. Decay sequences of radiolabeled deoxyribonucleic acid (DNA), gelatin hydrogel (GHG), and DNA combined with GHG in the hindlimb muscles of mice. Unlabeled GHG impregnated with ¹²⁵I-labeled DNA (solid line), ¹²⁵I-labeled GHG (dashed line), and ¹²⁵I-labeled DNA solution (dotted line) were injected into the hindlimb muscles.

testes, kidneys, heart, lungs, or brain (data not shown); lacZ-treated animals showed no FGF4 expression at any sites (lanes 7 and 8). Beta-actin expression was detected in all samples (lower panel), but neither beta-actin nor FGF4 expression was detected in any control samples that were not treated with reverse transcriptase; lacZ expression of naked DNA (500 μg) was localized to the injection site (Fig. 3A), whereas GHG-DNA complex (DNA amount 500 μg) showed a spatially expanded expression on day 17 (Fig. 3B). The degree of gene expression in myocytes was also augmented by GHG.

Protocol 2: salvage of hindlimb ischemia with GHG-plasmid complex encoding FGF4. Functional evaluation of ischemic hindlimbs showed amelioration of the ischemia

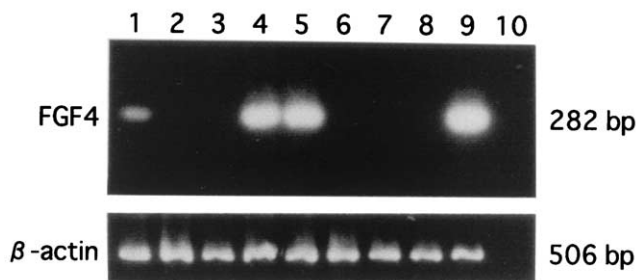


Figure 2. Representative transgene expression demonstrated by reverse-transcription nested polymerase chain reaction (RT-nested PCR). The left adductor muscle of the rabbits was injected with naked fibroblast growth factor 4 (FGF4) gene (lanes 1 to 3), gelatin hydrogel (GHG)-FGF4 (lanes 4 to 6), or GHG-lacZ (lanes 7 and 8). Each sample was obtained from the injection site (lanes 1, 4, and 7) and the adjacent region 10 mm apart from the injection site (lanes 2, 5 and 8) in the left adductor muscle, and from the contralateral adductor muscle (lanes 3 and 6). The RT-nested PCR products from ribonucleic acid of each sample were analyzed on agarose gel; FGF4 expressed Cc1/16 cells as a positive control (lane 9) and no deoxyribonucleic acid (DNA) template as a negative control (lane 10). A housekeeping beta-actin gene was amplified as a complementary DNA loading control.

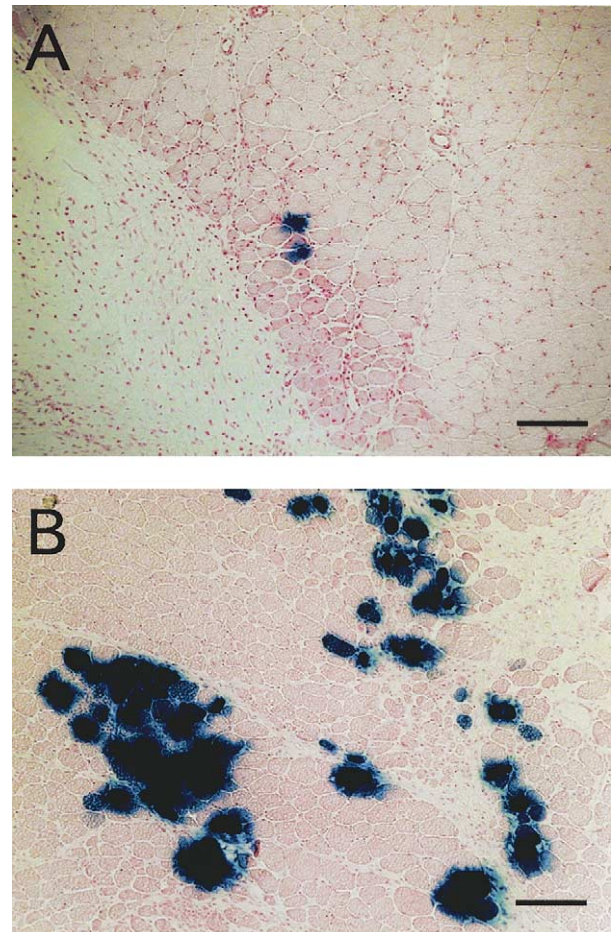


Figure 3. Representative gene expression of lacZ in the ischemic adductor muscle in rabbits on day 17. Naked deoxyribonucleic acid (DNA) (A) or gelatin hydrogel-DNA complex (B) was injected into the adductor muscle 10 days after the ischemic insult. X-Gal stain; original magnification ×20; bar = 200 μm.

by the FGF4-gene and potentiation of the amelioration when GHG was used as a delivery device. The augmentation of regional blood flow with GHG was more evident under vasodilatory conditions than at the baseline.

Regional blood flow analysis and angiographic analysis further confirmed the background mechanism for amelioration of hindlimb ischemia by GHG-FGF4 (Table 2). On day 38, blood flow during adenosine administration (vasodilatory condition) in the GHG-FGF4 group ($105 \pm 13\%$ in terms of ischemic/normal flow ratio) was significantly higher than in either the naked FGF4-gene group ($68 \pm 18\%$, $p < 0.05$) or the GHG-lacZ group ($50 \pm 12\%$, $p < 0.05$, ANOVA). The differences between the naked FGF4-gene and GHG-lacZ groups were not significant (ANOVA). The adenosine-dependent flow-augmentation (responsiveness to vasodilatory stimulation; comparison between adenosine and baseline values on day 38) was noted only in the GHG-FGF4 group (from $79 \pm 16\%$ to $105 \pm 13\%$, $p < 0.05$, ANOVA), and not in the other two groups. A similar tendency was noted in flow under baseline conditions on day 38 in all three groups; however, the

Table 2. Functional Evaluation of Gene Therapy

	GHG-lacZ	Naked FGF4	GHG-FGF4
Blood flow ratio (%) (ischemic/normal)			
Day 0 (BL)	33 ± 8 (6)	36 ± 11 (6)	37 ± 10 (6)
Day 38 (BL)	47 ± 12‡ (6)	58 ± 16‡ (6)	79 ± 16*‡ (6)
Day 38 (Ad)	50 ± 12‡ (6)	68 ± 18‡ (6)	105 ± 13*‡§ (6)
Angiographic score			
Day 38 (BL)	0.37 ± 0.12 (7)	0.39 ± 0.13 (7)	0.42 ± 0.11 (7)
Day 38 (Ad)	0.36 ± 0.10 (7)	0.41 ± 0.13 (7)	0.56 ± 0.15*‡§ (7)
Blood pressure (%) (ischemic/normal)			
Day 10	31 ± 8 (6)	33 ± 5 (6)	26 ± 7 (6)
Day 38	56 ± 4 (6)	63 ± 6 (6)	70 ± 11* (6)

Angiographic score was calculated on the synchrotron radiation microangiogram. $p < 0.05$ vs. *GHG-lacZ, †naked FGF4, ‡day 0 (BL), and §day 38 (BL) (analysis of variance).

Ad = during adenosine administration; BL = under baseline condition; FGF4 = fibroblast growth factor 4; GHG = gelatin hydrogel; lacZ = β -galactosidase.

differences in baseline flow among the three groups were less marked than during adenosine administration.

Synchrotron radiation microangiography revealed microvessel responsiveness to the vasodilatory stimulation in the GHG-FGF4-treated rabbits (Figs. 4C and 4D), whereas vascular density was somewhat decreased by adenosine treatment in some of the GHG-lacZ-treated rabbits (Figs. 4A and 4B). Angiographic score analysis yielded quantitative evidence (Table 2). The angiographic score during adenosine administration (vasodilatory condition) was significantly higher in the GHG-FGF4 group (0.56 ± 0.15) than in either the naked FGF4-gene group (0.41 ± 0.13 , $p < 0.05$) or the GHG-lacZ group (0.36 ± 0.10 , $p < 0.05$, ANOVA). By contrast, under baseline conditions, the angiographic scores of the three groups were not significantly different.

On day 38, the GHG-FGF4 group had the highest calf-blood pressure ratio ($70 \pm 11\%$), and it was lower in the

naked FGF4-gene group ($63 \pm 6\%$), and even lower in the GHG-lacZ group ($56 \pm 4\%$, $p < 0.05$ vs. the GHG-lacZ group, ANOVA, Table 2). On day 10 (the time of gene transfer), the degrees of decrease in the three groups were not significantly different (26% to 33% in the mean).

Tissue damage was least in the GHG-FGF4-treated rabbits (Table 1). Limb muscle necrosis was $<3 \text{ cm}^2$ and grade 1 or 2 in all of the animals in the GHG-FGF4 group, and significantly less than in the other two groups ($p < 0.05$, Kruskal-Wallis test). A similar difference was noted in the degrees of toe necrosis (data not shown). The muscle weight ratio (ischemic/normal) on day 38 was highest in the GHG-FGF4 group ($79 \pm 11\%$), lower in the naked FGF4-gene group ($62 \pm 14\%$), and even lower in the GHG-lacZ group ($48 \pm 67\%$), and the difference between the GHG-FGF4 group and GHG-lacZ group was significant ($p < 0.05$, ANOVA). The muscle weight ratio values (a morphological index) positively correlated with vascular responsiveness to adenosine (adenosine/baseline blood flow ratio [%]; a functional index) ($r = 0.50$, $n = 18$, $Sy \cdot x = 0.15$, $Sy \cdot x/y = 0.23$, $p = 0.045$). Thus, the blood flow, microangiographic, and morphologic analyses demonstrated a greater ameliorative effect of the GHG-FGF4 complex compared with the naked FGF4-gene (Tables 1 and 2).

Minimal inflammatory infiltrates, such as neutrophil and lymphoplasmacytic cell infiltrates, were noted at the injection site, but histological analysis showed that the infiltrates were localized. There was no evidence of fibrous proliferation or tumor formation in the transfected muscles or in other organs (right adductor muscle, stomach, liver, spleen, testes, kidneys, heart, lungs, and brain) in any of the groups.

DISCUSSION

We demonstrated that GHG potentiated the angiogenic effect of the FGF4-gene (protocol 2) by prolonging DNA degradation and improving transfection efficiency (protocol 1). Thus, GHG might facilitate the gene therapy of intractable circulatory disorders with genes for angiogenic growth factors.

Gelatin hydrogel augmented the effect of the FGF4-gene

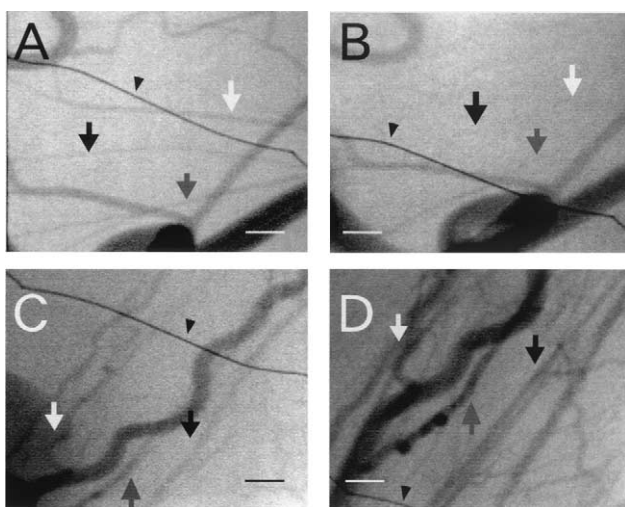


Figure 4. Representative synchrotron radiation microangiograms of the hindlimb ischemia in the rabbits. Synchrotron radiation microangiograms were taken under baseline conditions (A and C) and after repeated adenosine administration (B and D) on day 38. (A and B) Gelatin hydrogel (GHG)-lacZ-treated rabbit; (C and D) GHG-fibroblast growth factor 4-treated rabbit. **Arrows** indicate the same point in the vessels. **Arrowheads** reference copper wires with a diameter of $130 \mu\text{m}$; **bar** = 1 mm.

therapy by improving gene biodegradation and transfection efficiency. We demonstrated that GHG rapidly absorbed plasmid DNA and did not release it in vitro (see Methods section). In the radioiodine experiment, the radioactivity of naked DNA was reduced to less than 10% of the baseline within a day, whereas the radioactivity of DNA impregnated into GHG remained for four weeks (Fig. 1). In the experiment using the rabbit hindlimb ischemia model, the PCR analysis suggested that GHG expanded the gene transfer spatially (lanes 2 and 5 in Fig. 2). The maker gene experiment (protocol 1) confirmed that the use of GHG augmented both the number of transfected myocytes and the degree of gene expression in these cells, and also supported the spatially expanded gene expression (Fig. 3). The superiority of the therapeutic effects of the GHG-FGF4-gene complex on hindlimb ischemia compared with naked FGF4-gene treatment was confirmed in the rabbit experiments in protocol 2 (Fig. 4, Tables 1 and 2); GHG-FGF4-treated rabbits were characterized by less severe tissue damage in the ischemic limb (Table 2) and more marked vascular responsiveness to adenosine than either the naked FGF4-treated or GHG-lacZ-treated rabbits (Fig. 4, Table 2, Protocol 2 in the Results section). Under the baseline conditions, blood flow in normal muscle tissue is set at a relatively low level in preparation for an abrupt increase in flow demand (approximately 5× and 30× in the heart and in the skeletal muscle, respectively) during exercise, etc. (responsiveness to the vasodilatory stimulation). In other words, normal muscle tissue has a sufficient flow reserve (20,21), and, thus, the presence or absence of vascular responsiveness to adenosine administration can be used as an index of fundamental vascular function in angiogenic vascular segments. The demonstration of a positive correlation between flow responsiveness to adenosine and the muscle weight ratio further supports our hypothesis. Baseline flow may reflect the total number of angiogenic vessels, if it does not respond to vasodilatory stimulation. However, if the vasodilatory mechanism is present, baseline flow alone does not necessarily reflect the quality and/or quantity of angiogenic vascular segments. The amelioration of the ischemic tissue in the GHG-FGF4 group may be related to an adequate flow reserve (20,21) of so-called "well-tempered angiogenic vessels" (22).

Consequently, GHG offers several advantages as a new gene delivery system: 1) it has a positively charged structure, so it holds negatively charged nucleic acids, proteins, and drugs within its structure; 2) GHG is biodegradable and implantable; the biodegradable nature is from the gelatin itself but not from the hydrogel state. The substance bound to the GHG is gradually released as the gelatin degrades in situ. The degradation period can be adjusted to two to four weeks by varying the water content. Thus, the prolonged release of the DNA held in GHG was presumably responsible for the augmentation of gene therapy. The use of hydrogel-coated balloon-angioplasty-catheter has been reported (7). However, this gel is much different from our

hydrogel. The present GHG consists of amino acids and is biodegradable and implantable, whereas their hydrogel consists of carbohydrate and is not biodegradable nor implantable. 3) The isoelectric point and the shape of the GHG can be modified. Negatively charged GHG holds positively charged substances such as basic-FGF protein (6,23), and disk-shaped GHG has been found to be effective for reconstruction of bone defects (23); 4) GHG is less biohazardous than adenovirus vectors. Gelatin is already used in the clinical field, and its safety is established. Thus, the use of GHG with naked DNA improves its transfection efficiency without causing serious cytotoxicity or biohazards, which are inconvenient side effects of virus vectors (24). Therefore, the nonvirus vector GHG is useful for various gene therapies including the treatment of cardiovascular disorders.

Acknowledgments

The authors wish to thank Chiharu Tada, Akiko Hori, Sachie Ueno, and Takayuki Hasegawa for their technical work.

Reprint requests and correspondence: Dr. Hidezo Mori, Department of Cardiac Physiology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita 565-8565, Japan. E-mail: hidemori@ri.ncvc.go.jp.

REFERENCES

1. Asahara T, Bauters C, Zheng LP, et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation* 1995;92:417-27.
2. Aoki M, Morishita R, Taniyama Y, et al. Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis. *Gene Ther* 2000;7:417-27.
3. Yang Y, Trinchieri G, Wilson JM. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nat Med* 1995;1:890-3.
4. Zabner J, Ramsey BW, Meeker DP, et al. Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. *J Clin Invest* 1996;97:1504-11.
5. Takeshita S, Isshiki T, Sato T. Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in rats. *Lab Invest* 1996;74:1061-5.
6. Tabata Y, Hijikata S, Muniruzzaman M, Ikada Y. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. *J Biomater Sci Polym Ed* 1999;10:79-94.
7. Isner JM, Pieczek A, Schainfeld R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patients with ischaemic limb. *Lancet* 1996;348:370-4.
8. Sakamoto H, Mori M, Taira M, et al. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc Natl Acad Sci USA* 1986;83:3997-4001.
9. Fuller PF, Peters G, Dickson C. Cell transformation by kFGF requires secretion but not glycosylation. *J Cell Biol* 1991;115:547-55.
10. Yoshida T, Ishimaru K, Sakamoto H, et al. Angiogenic activity of the recombinant hst-1 protein. *Cancer Lett* 1994;83:261-8.
11. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994;93:662-70.
12. Miyazaki J, Takaki S, Araki K, et al. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* 1989;79:269-77.

13. Bolton AE, Hunter WM. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. *Biochem J* 1973;133:529–39.
14. Ueno H, Li JJ, Tomita H, et al. Quantitative analysis of repeat adenovirus-mediated gene transfer into injured canine femoral arteries. *Arterioscler Thromb Vasc Biol* 1995;15:2246–53.
15. Tanaka E, Hattan N, Ando K, et al. Amelioration of microvascular myocardial ischemia by gene transfer of vascular endothelial growth factor in rabbits. *J Thorac Cardiovasc Surg* 2000;120:720–8.
16. Tanioka K, Yamazaki J, Shidara K, et al. Avalanche-mode amorphous selenium photoconductive target for camera tube. *Adv Electronics Electron Phys* 1988;74:379–87.
17. Mori H, Hyodo K, Tanaka E, et al. Small-vessel radiography in situ with monochromatic synchrotron radiation. *Radiology* 1996;201:173–7.
18. Takeshita S, Isshiki T, Ochiai M, et al. Endothelium-dependent relaxation of collateral microvessels after intramuscular gene transfer of vascular endothelial growth factor in a rat model of hindlimb ischemia. *Circulation* 1998;98:1261–3.
19. Taira M, Yoshida T, Miyagawa K, Sakamoto H, Terada M, Sugimura T. cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity. *Proc Natl Acad Sci USA* 1987;84:2980–4.
20. Austin RJ, Aldea GS, Coggins DL, Flynn AE, Hoffman JI. Profound spatial heterogeneity of coronary reserve: discordance between patterns of resting and maximal myocardial blood flow. *Circ Res* 1990;67:319–31.
21. Coggins DL, Flynn AE, Austin RJ, et al. Nonuniform loss of regional flow reserve during myocardial ischemia in dogs. *Circ Res* 1990;67:253–64.
22. Blau H, Banfi A. The well-tempered vessel. *Nat Med* 2001;7:532–4.
23. Yamada K, Tabata Y, Yamamoto K, et al. Potential efficacy of basic fibroblast growth factor incorporated in biodegradable hydrogels for skull bone regeneration. *J Neurosurg* 1997;86:871–5.
24. Marshall E. Gene therapy death prompts review of adenovirus vector. *Science* 1999;286:2244–5.