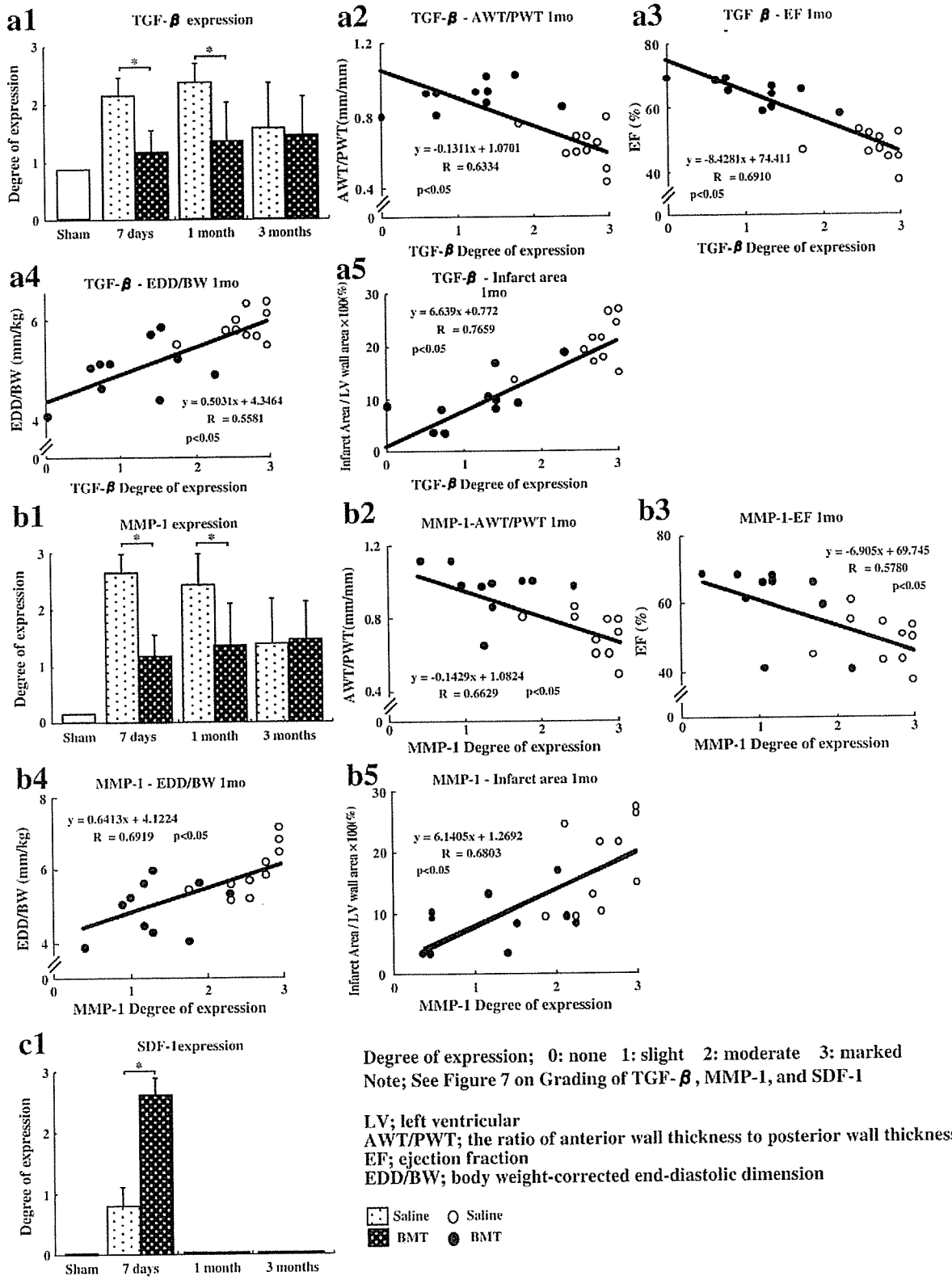

Fig. 8. BM-MNC treatment significantly down-regulated TGF-β and MMP-1 expression 7 days and 1 month post-MI, and up-regulated SDF-1 expression 7 days post-MI (a1, b1 and c1). Levels of TGF-β and MMP-1 were negatively correlated with AWT/PWT ratios and EFs and positively correlated with EDD/BW ratios and infarct size 1 month post-MI (a2–5 and b2–5).

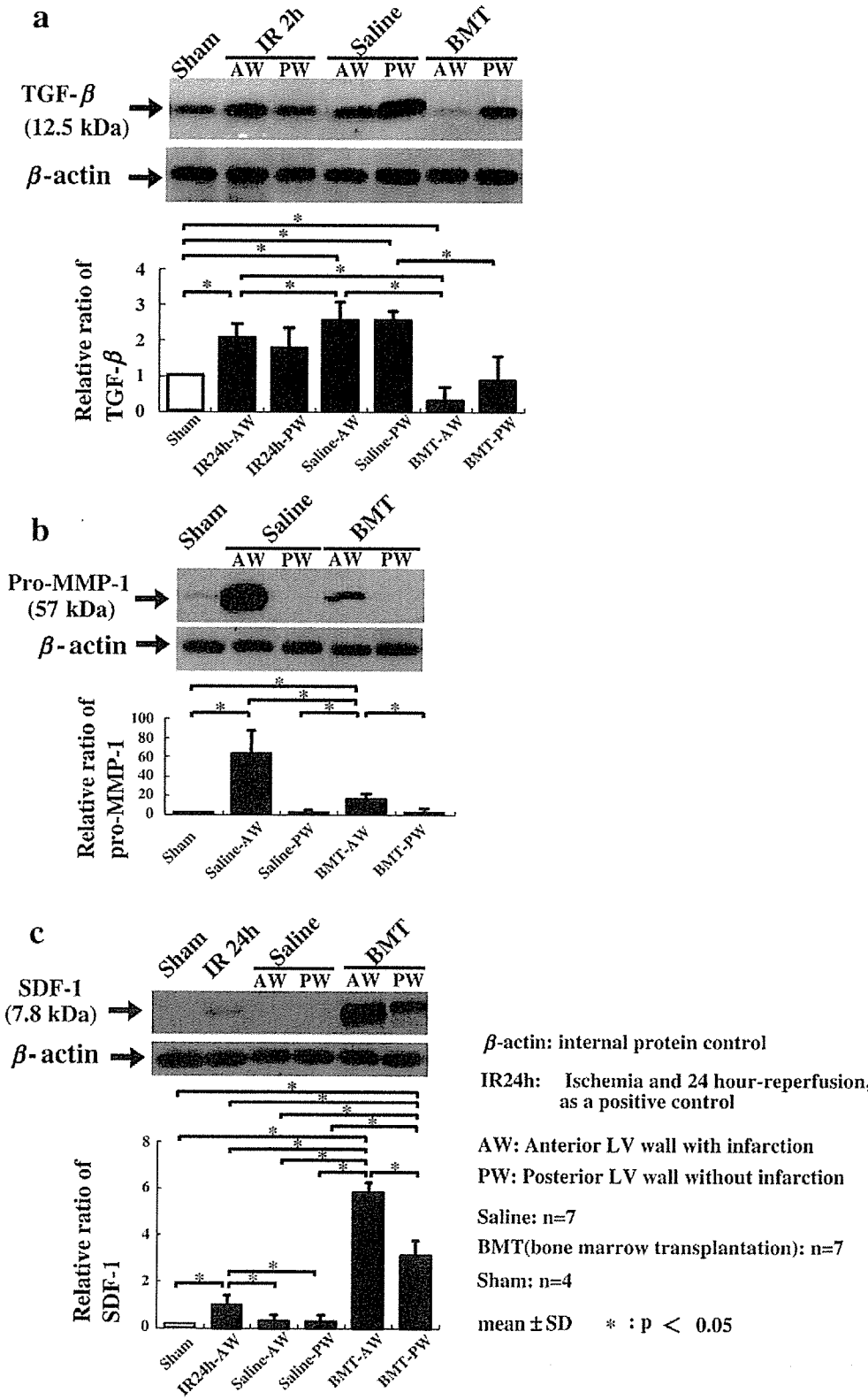

Fig. 9. Western blot analysis 7 days after infarction. Note the up-regulation of TGF-β in both the AW and PW is up-regulated in the Saline group, but down-regulated in the BM-MNC group. Note also that the expression of Pro-MMP-1 in the AW is up-regulated in the Saline, but down-regulated in the BM-MNC group. Finally, note the marked up-regulation of SDF-1 expression in both AW and PW of the BM-MNC group.

4.5. Clinical implications

The clinical implications of the present findings are related to the fact that (1) our model of ischemia–reperfusion corresponds more closely to standard strategies for the treatment of human acute MI than earlier models utilizing permanent occlusion; and that (2) intravenous injection of autologous BM-MNCs is less invasive than direct or intracoronary injection and removes some ethical and technical barriers, such as donor–recipient mismatch and demand–supply imbalance.

In the present study, injected bone marrow cells were aspirated before infarction. However, Dimmeler's group reported that bone marrow mononuclear cells derived from patients with ischemic heart disease showed impaired colony-forming capacity and migratory response to SDF-1 and VEGF. As bone marrow cells after the onset of infarction are available in the clinical setting, our findings based on the bone marrow cells obtained before infarction have limitations.

5. Conclusion

Intravenous transplantation of BM-MNCs leads to the development of BM-MNC-derived myocyte-like cells and regulates the expression of repair-related cytokines that facilitate repair in the post-MI heart.

Acknowledgements

We thank Rumi Maruyama, Akiko Tsujimoto, Hatue Oshika, Kazuko Goto and the staff of Kyoto Women's University (Yumiko Oi, Mayuko Goto, Chitose Suzuki and Yasuyo Hosokawa) for their technical assistance. This study was supported in part by Research Grants of Frontier Medicine (15209027, 16590721) from the Ministry of Education, Science, and Culture of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2005.11.001.

References

[1] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–5.
[2] Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004;95:9–20.
[3] Kudo M, Wang Y, Wani MA, Xu M, Ayub A. Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. J Mol Cell Cardiol 2003;35:1113–9.
[4] Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428:664–8.
[5] Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428:668–73.
[6] Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10:494–501.
[7] Minatoguchi S, Takemura G, Chen X, Wang N, Uno Y, Koda M, et al. Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation 2004;109:2572–80.
[8] Ragnarson B, Bengtsson L, Haegerstrand A. Labeling with fluorescent carbocyanine dyes of cultured endothelial and smooth muscle cells by growth in dye-containing medium. Histochemistry 1992;97:329–33.
[9] Honig MG, Hume RI. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. J Cell Biol 1986;103:171–87.
[10] Maruyama R, Takemura G, Aoyama T, Hayakawa K, Koda M, Kawase Y, et al. Dynamic process of apoptosis in adult rat cardiomyocytes analyzed using 48-hour videomicroscopy and electron microscopy-Beating and rate are associated with the apoptotic process. Am J Pathol 2001;159:683–91.
[11] Chacko KJ. Observation on the ultrastructure of developing myocardium of rat embryos. J Morphol 1976;150:681–710.
[12] Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, et al. Fusion of bone marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003;425:9968–73.
[13] Pochampally RR, Neville BT, Schwarz EJ, Li MM, Prockop DJ. Rat adult stem cells (marrow stromal cells) engraft and differentiate in chick embryos without evidence of cell fusion. Proc Natl Acad Sci U S A 2004;101:9282–5.
[14] Kajstura J, Rota M, Wang B, Cascapera S, Hosoda T, Bearzi C, et al. Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion. Circ Res 2005;96:127–37.
[15] Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, et al. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 2003;108:863–8.
[16] Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 on ex vivo expanded endothelial progenitor cell recruitment for ischemia neovasculalization. Circulation 2003;107:1322–8.
[17] Abbott JD, Huang Y, Liu Dingang, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004;110:3300–5.
[18] Wojakowski W, Tendera M, Michalowska A, Majka M, Kucia M, Maslankiewicz K, et al. Mobilization of CD34/CXCR4$^+$, CD34/CD117$^+$, c-Met$^+$ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction. Circulation 2004;110:3213–20.
[19] Yelbuz TM, Waldo KL, Zhang X, Waldo K, Zhang X, Zdanowicz M, et al. Myocardial volume and organization are changed by failure of addition of secondary heart field myocardium to the cardiac outflow tract. Dev Dyn 2003;228:152–60.
[20] Deten A, Hoelzl A, Leicht M, Barth W, Zimmer H. Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation. J Mol Cell Cardiol 2001;33:1191–207.
[21] Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K, et al. Transforming growth factor-β function blocking prevents myocardial

fibrosis and diastolic dysfunction in pressure-overloaded rats. Circulation 2002;106:130–5.
[22] Danielsen CC, Wiggers H, Andersen HR. Increased amounts of collagenase and gelatinase in porcine myocardium following ischemia and reperfusion. J Mol Cell Cardiol 1998;30:1431–42.
[23] Spinale FG. Matrix metalloproteinase: regulation and dysregulation in the failing heart. Circ Res 2002;90:520–30.
[24] Kim HE, Dalal SS, Young E, Legato MJ, Weisfeldt ML, D'Armiento J. Disruption of the myocardial extracellular matrix leads to cardiac dysfunction. J Clin Invest 2000;106:857–66.
[25] Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:376–468.
[26] Kawamoto A, Gwon H, Iwaguro H, Iwaguro H, Yamaguchi J, Uchida S, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103:634–7.

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Hepatocyte Growth Factor Gene Therapy Slows Down the Progression of Diabetic Nephropathy in *db/db* Mice

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Key Words
Diabetes · Apoptosis · Nephropathy · Growth factors · Gene therapy

Abstract
Background: Effect of hepatocyte growth factor (HGF) has scarcely been determined on diabetic nephropathy. ***Methods:*** Adenovirus encoding human HGF gene or LacZ gene (as the control) was injected into the hindlimb muscles of the C57BL/KsJ-*db/db (db/db)* mice at the age of 12 weeks, a model of genetic diabetes. Diabetic nephropathy was then evaluated at the age of 24 weeks. ***Results:*** The urine volume and albumin excretion progressively decreased in the control, whereas they remained unchanged in the HGF-treated group during the 12-week follow-up. The HGF gene therapy did not affect glucose metabolism. However, it resulted in a better renal function as evaluated by creatinine clearance (Ccr) than the control; Ccr was progressively worsened in controls (0.14 ± 0.02 liters/day) whereas unchanged in the HGF gene-treated group (0.38 ± 0.09 liters/day, $p < 0.05$). Kidneys of the HGF gene-treated mice showed glomeruli with greater area and cell population, smaller glomerular sclerotic index, and less fibrosis in both glomeruli and renal tubules, where apoptotic rate of glomerular endothelial cells and that of tubular epithelial cells were significantly decreased. TGF-β1 expression was significantly decreased in kidneys of the HGF gene-treated group. Finally, the HGF treatment significantly improved the long-term survival of *db/db* mice. ***Conclusions:*** The HGF gene delivery thus appeared to slow down the aggravation of diabetic nephropathy in *db/db* mice by attenuating progression from the hyperfiltration phase into the sclerotic phase through antiapoptotic and antifibrotic actions. The present findings suggest that the HGF gene delivery can be a novel therapeutic approach against diabetic nephropathy.

Introduction

Diabetic nephropathy is one of the most problematic renal diseases because of the exponentially increasing number of patients entering chronic dialysis programs with renal failure resulting from diabetes, and the high mortality rates of these patients receiving dialysis [1]. Diabetic nephropathy, in addition to many other glomerular diseases, finally progresses into a hypocellular, sclerotic phase. One of the most conspicuous pathologic changes in glomeruli of this disorder is mesangial expansion ac-

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companied by mesangial matrix deposition that consists of collagen and glycoproteins [2, 3]; such excessive deposits are, at least in part, associated with overexpression of transforming growth factor-β (TGF-β) [4, 5]. In addition, apoptosis was documented in tubular epithelial cells, which may contribute to atrophy of tubular epithelium and tubulointerstitial fibrosis in diabetic nephropathy [6, 7]. Moreover, we recently reported an increased apoptosis of renal glomerular cells of the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of non-insulin-dependent diabetes mellitus [8]. Thus, both space occupation by mesangial matrix and cell loss via apoptosis could importantly account for glomerular sclerosis with decreased capillaries in diabetic nephropathy.

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes [9, 10], shows potent angiogenic and mitogenic activities in various cells, preferentially in most epithelial and endothelial cells [11, 12]. In addition to this, antiapoptotic and antifibrotic activities of HGF have recently been paid attention; its antiapoptotic effect was accompanied by activation of the phosphatidylinositol 3-kinase/Akt pathway [13, 14], while its antifibrotic effect was reported to work through the antagonistic action on TGF-β [15]. The therapeutic efficacy of HGF was actually confirmed on renal fibrosis caused by chronic glomerulonephritis or by urinary obstruction [16–18]. Recently, Mizuno et al. [19] found beneficial effects of a long-term treatment with recombinant HGF upon streptozotocin-induced diabetic nephropathy in mice. Moreover, Cruzado et al. [20] demonstrated that HGF gene therapy not only prevents but also reverts advanced diabetic nephropathy in the same animal model. Except for these studies, however, effects of HGF on diabetic nephropathy have scarcely been determined and not confirmed in the other animal models. In the present study, we examined the effects of HGF gene therapy with adenovirus encoding the human HGF gene on progression of diabetic nephropathy, glomerular sclerosis in particular. We used the C57BL/KsJ-*db/db (db/db)* mouse, a rodent model of genetic diabetes, which exhibits renal pathology and dysfunction resembling those observed in human diabetes [21–23].

Materials and Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Female 8-week-old diabetic C57BL/KsJ-*db/db (db/db)* mice were purchased from Clea Japan (Tokyo, Japan). Mice were designated *db/db* by the vendor on the basis of the appearance of obesity, which is usually detectable at about 5 weeks of age. As the non-diabetic control mice, C57BL/KsJ-*db/+m (db/+m)* mice with the same sex and age were used (Clea Japan). Animals were weighed on receipt and weekly thereafter, and were provided food and water ad libitum.

Recombinant Adenoviral Vectors

Adenoviral vector plasmid pAd-HGF, which comprises cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter and human HGF cDNA (Ad.CAG-HGF) was constructed by the in vitro ligation method (from Dr. Mark A. Kay, Stanford University School of Medicine) as described previously [24]. Control Ad-LacZ was prepared as described previously [25].

Ad.CAG-HGF (1×10^9 plaque-forming units [pfu]/mouse) was injected into the hindlimb muscles of the 12-week-old *db/db* mice (n = 8) and *db/+m* mice (n = 6). As the control, an adenovirus encoding the LacZ gene (Ad.CAG-LacZ) was similarly injected to the other 8 *db/db* and 6 *db/+m* mice. Mice were followed-up for 12 weeks. They were killed by cervical dislocation and examined at the age of 24 weeks.

Urine and Blood Examination

Twenty-four-hour urine collections were periodically obtained in each animal after placement in a metabolic cage by washing the collection apparatus with 10 ml of distilled water with a spray bottle. Approximately 50–100 μl blood was obtained from the tail veins.

Glucose, albumin, creatinine, blood urea nitrogen (BUN), and HbA1c in blood and/or urine were measured by the standardized methods.

Measurement of Human HGF Level in the Plasma

The plasma concentration of human HGF was measured using an ELISA kit (Bethyl Laboratories, Montgomery, Tex., USA).

Histological Examination

The kidney was fixed in 10% phosphate-buffered formalin solution, and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin, PAS, and Sirius red F3BA (0.1% solution in saturated aqueous picric acid) (Aldrich Chemicals, Milwaukee, Wisc., USA) [26]. The glomerular size was measured as the whole capillary tuft area using a computed image analyzer (LUZEX F, Nireco Co., Kyoto, Japan) for 50 glomeruli on each slide under light microscopy. The cell number in each glomerulus (n = 50) was calculated using preparations stained with hematoxylin and eosin. Glomerular sclerotic index was calculated as the percent of the PAS-positive area in the glomerular area in preparations stained with PAS [27].

Immunohistochemistry

On the 4-μm-thick sections, proliferating cell nuclear antigen (PCNA) and Flk-1 were immunohistochemically stained using ABC Elite kits (Vector, Burlingame, Calif., USA). The primary antibodies used were anti-PCNA antibody (clone PC10, Dako Japan, Kyoto, Japan) at a dilution of 1:100 and anti-Flk-1 antibody (clone A-3, Santa Cruz, Calif., USA) at 1:100. Pretreatment by microwave irradiation in 10 m*M* citrate buffer (pH 6.0) at 400 W for 5 min twice was performed to retrieve the antigenicities. Diaminobenzidine tetrahydrochloride (DAB) or VIP substrate (Vector) was the chromogen. Mouse intestine was used as the positive control tissue

sections. Unanimity on the positive immunohistochemical stainings was acquired for all sections between the two observers who were unaware of which group the sections belonged.

In situ Nick End Labeling (TUNEL)

TUNEL was performed in deparaffinized 4-μm-thick sections with an ApopTag kit (Intergene, Purchase, N.Y., USA) according to the supplier's instructions. DAB was the chromogen. Sections were then counterstained with hematoxylin.

Double Immunohistochemistry

Sections were stained first with anti-PCNA antibody or TUNEL as described above. Then the sections were stained with the second primary antibody against Flk-1 and visualized with VIP substrate.

Electron Microscopy

Tissue samples of the kidney were cut into 1-mm cubes and fixed for 4 h at 4°C in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer. They were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanols, and embedded in Epon. Thin sections (80 nm) were cut with a diamond knife, collected on 300-mesh copper or nickel grids, and double stained with uranyl acetate and lead citrate before examination using an electron microscope (H-700, Hitachi, Tokyo, Japan). Ten photographs were taken in each glomerulus with a magnification of ×3,000 and printed. Five points of glomerular basement membrane picked up in each photograph and the thickness was measured. 100–150 measurements were averaged on 2–3 glomeruli per specimen.

Western Blotting for TGF-β1

Fifty micrograms of protein from fresh kidney homogenates in sample buffer (0.4 mol/l Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.02% bromophenol blue, and 2.5% 2-mercaptoethanol) was loaded per lane on 12 or 16% polyacrylamide gels and electrophoresed. Proteins were transferred, and the membranes were incubated with primary antibody using a 1:1,000 dilution of anti-TGF-β1 antibody (Promega, Madison, Wisc., USA). Alpha-tubulin was the loading control. The membranes were incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Amersham, UK). Immunoblots were developed by enhanced chemiluminescence system (ECL; Amersham Biosciences, Piscataway, N.J., USA) and exposed to imaging film (X-X-OMAT; Kodak, Rochester, N.Y., USA). The signals were quantified by densitometry.

Survival Study

For the survival study, female 12-week-old *db/db* mice were injected intramuscularly with Ad.CAG-HGF (1×10^9 pfu/mouse) (n = 15) or LacZ gene (1×10^9 pfu/mouse) (n = 15) and followed for 25 weeks.

Statistical Analysis

Values were expressed as the means ± SEM. Statistical comparisons were performed by Student's t test. Survival data were analyzed by the Kaplan-Meier method. $p < 0.05$ was considered significant.

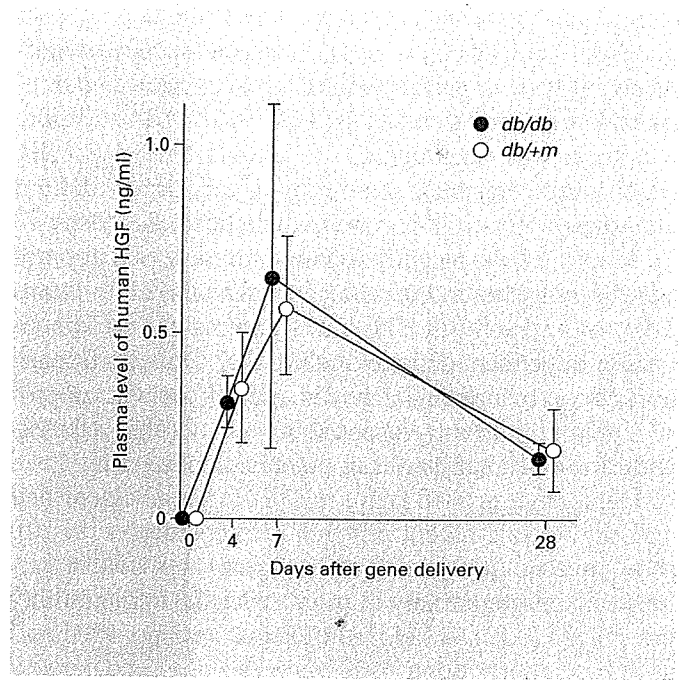

Fig. 1. Time course of human HGF levels in plasma of *db/db* and *db/+m* mice after HGF gene delivery. n = 3–6 at each time point in each group. In plasma of mice treated with LacZ gene, no HGF was detectable.

Results

Plasma Levels of Human HGF

As shown in figure 1, plasma human HGF was detectable after the HGF gene delivery in *db/db* and *db/+m* mice, whereas no human HGF was detected in the LacZ-treated mice. The plasma levels of human HGF in the *db/+m* mice after the HGF gene delivery were similar to those in the *db/db* mice (fig. 1). Effective bioactivity of human HGF on mice was confirmed in a previous study [28].

Renal Function

The blood glucose and HbA1c levels in *db/+m* and *db/db* mice were not significantly different between the LacZ- and HGF-treated groups at the age of 24 weeks (table 1), indicating no significant effect of the HGF gene delivery on glucose metabolism in these mice.

Figure 2 shows data of urine and blood chemistry on renal function in the LacZ- and HGF-treated *db/+m* and *db/db* mice before (at the age of 12 weeks) and at 12 weeks after the treatment (at the age of 24 weeks). As shown here, the *db/db* mice display far greater urine volume,

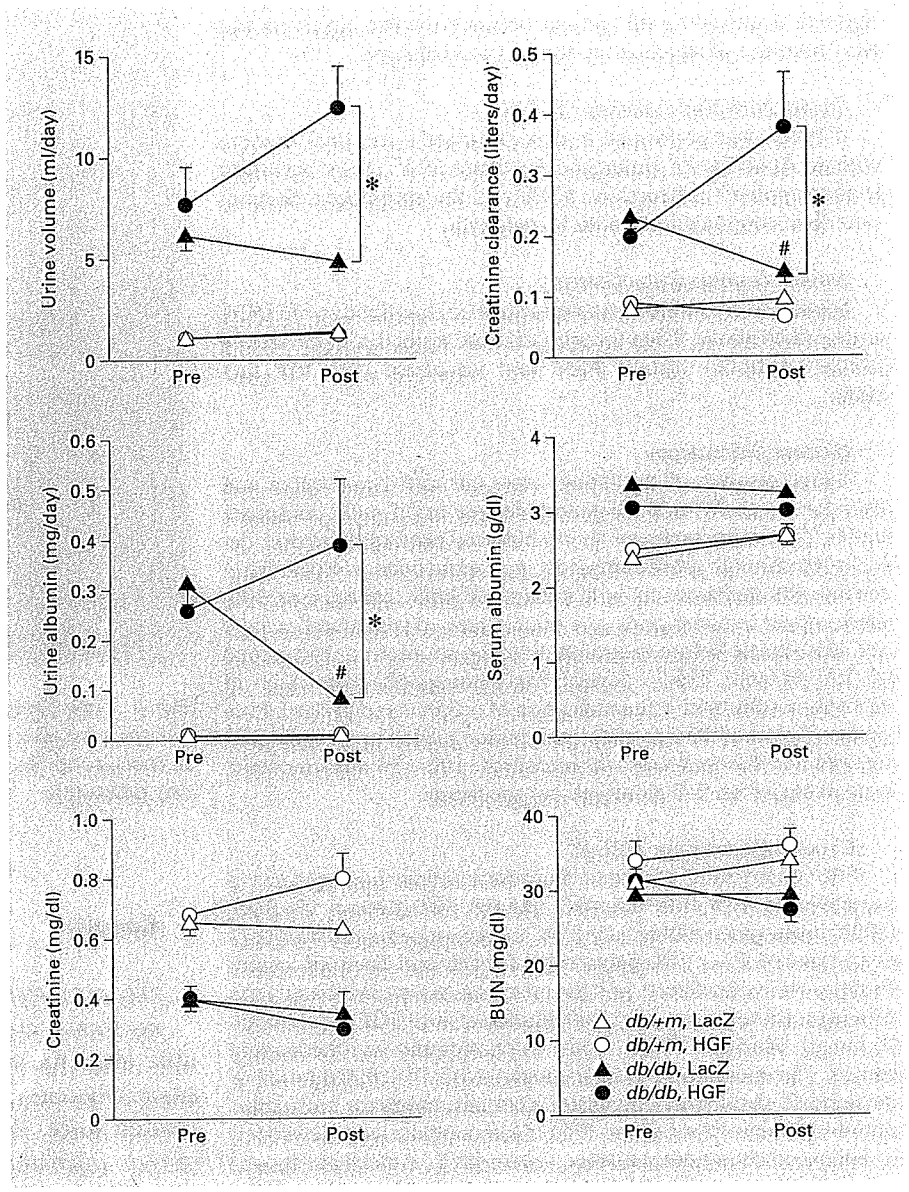

Fig. 2. Parameters of renal function in *db/db* and *db/+m* mice before and after LacZ- or HGF-gene therapy. # $p < 0.05$ compared with the pretreatment value. * $p < 0.05$ compared with the corresponding value of the LacZ group.

Table 1. Blood glucose and HbA1c values at the post-treatment with LacZ or HGF gene in *db/+m* and *db/db* mice

	db/+m		*db/db*	
	LacZ	HGF	LacZ	HGF
n	6	6	8	8
Blood glucose, mg/dl	201±11	211±8	836±33	801±26
HbA1c, %	2.8±0.11	2.9±0.06	13.7±0.2	12.9±0.3

greater urine albumin amount and concentration, smaller concentrations of serum creatinine and BUN, and greater creatinine clearance (Ccr), compared with the *db/+m* mice.

Although the HGF gene delivery did not affect any functional parameters of the *db/+m* mice, the renal function was significantly affected by the treatment in the *db/db* mice. Urine volume per day was greater in the HGF-treated *db/db* mice than in the LacZ-treated ones. Although urine albumin concentration was similar between the groups at the post-treatment, urine albumin amount was greater in the HGF-treated group; this is

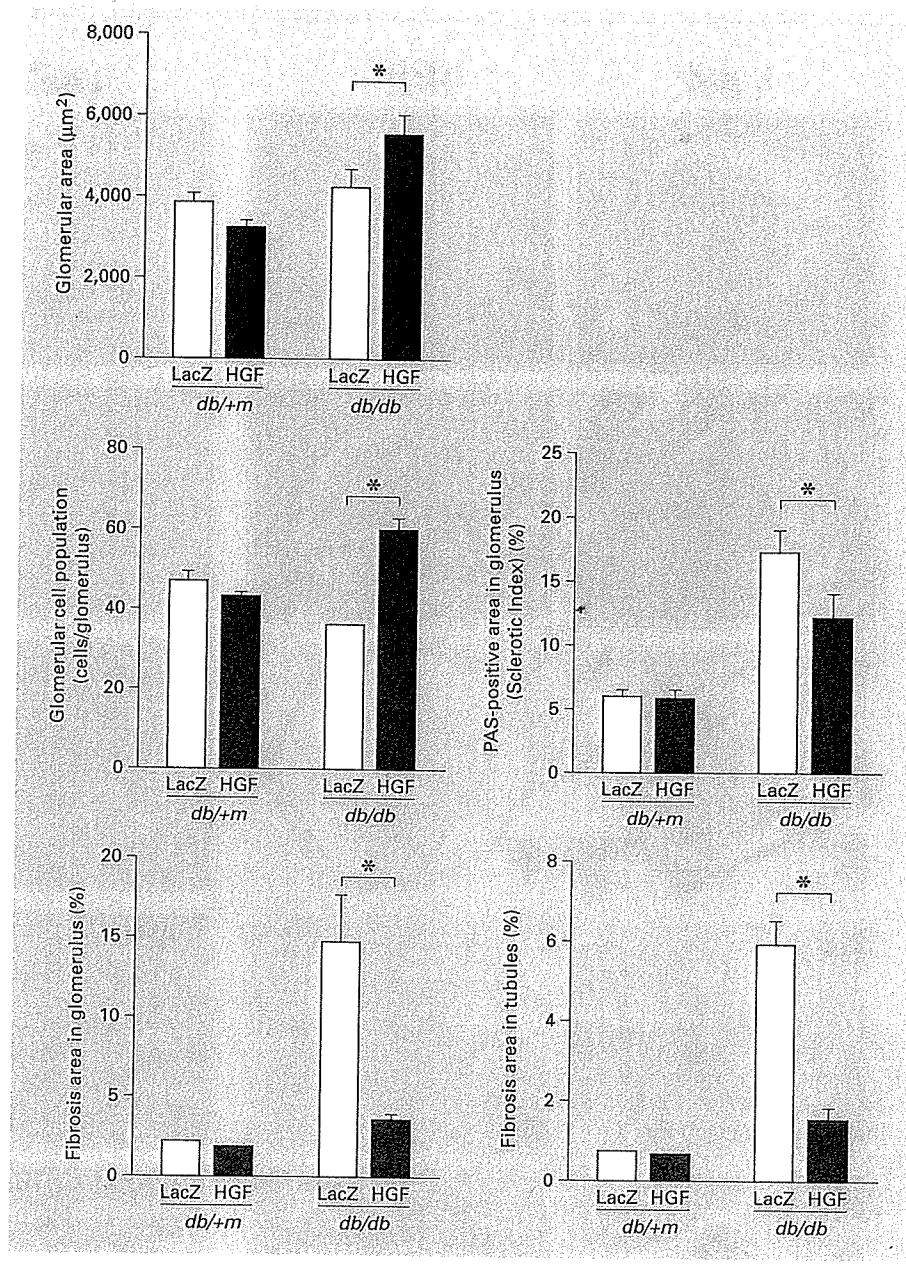

Fig. 3. Parameters of renal pathology in *db/db* and *db/+m* mice after LacZ or HGF gene treatment. * $p < 0.05$.

probably attributed in part to the greater amount of urine volume in that group. Serum albumin concentration was similar between the groups. Neither serum creatinine nor BUN level was significantly different between the groups at the age of 24 weeks, but the Ccr was significantly greater in the HGF-treated group; this value was worsened in the LacZ-treated group at the age of 24 weeks than at the age of 12 weeks, whereas in the HGF-treated group the value showed no significant change during the interval. These findings suggested that the glomerular hyperfiltration phase progressed into the sclerotic phase of nephropathy during the treated period for 12 weeks, in the control (LacZ-treated) *db/db* mice, while the progression was decelerated in the HGF-treated *db/db* mice.

Renal Pathology

Figure 3 shows quantitative data on renal pathology of the 24-week-old *db/+m* and *db/db* mice at the post-treatment with LacZ or HGF gene. The data were not different between treatments in the *db/+m* mice, where-

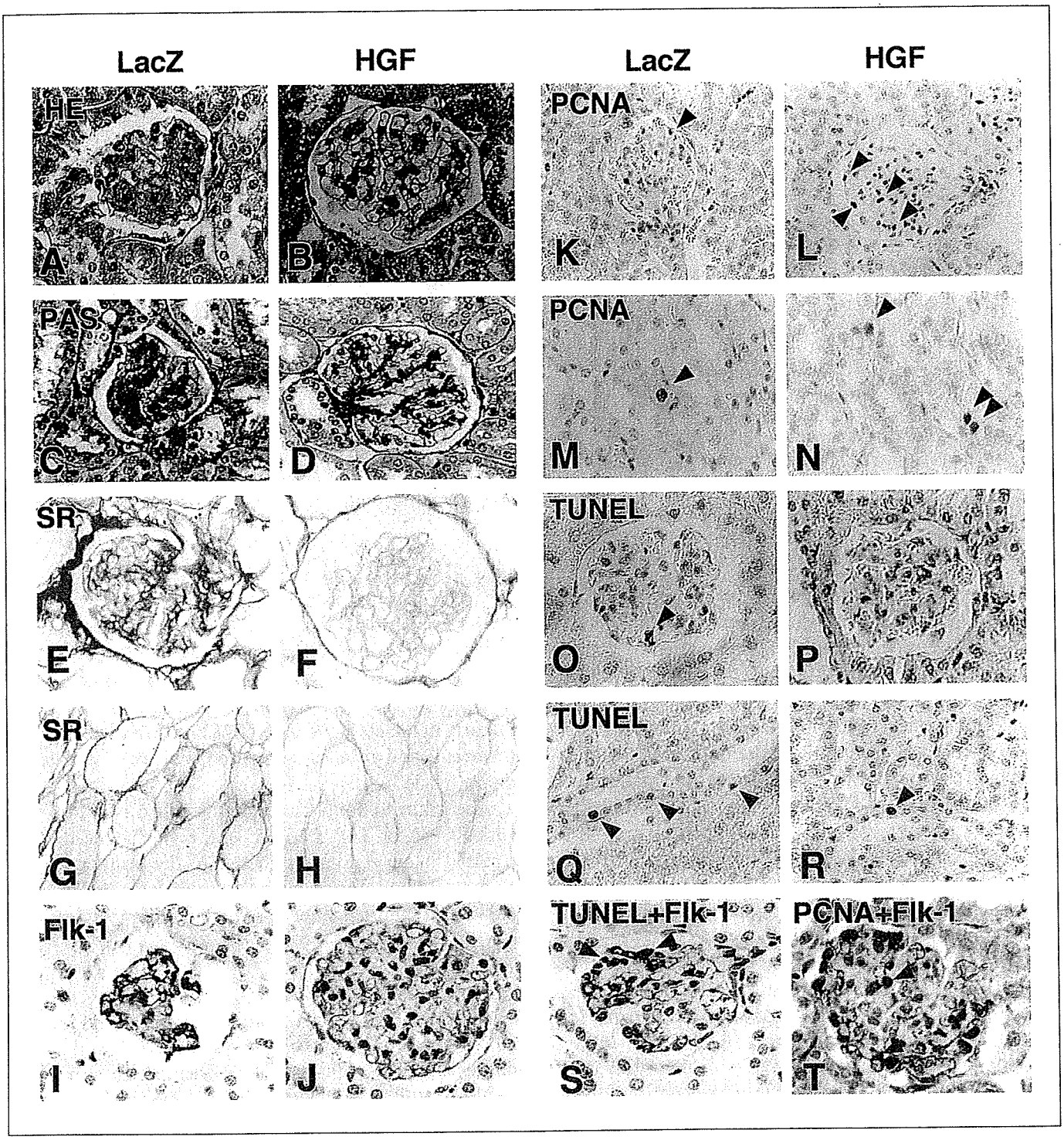

Fig. 4. Light microphotographs of renal glomeruli or tubules of *db/db* mice treated with LacZ (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, **Q**, **S**) or with HGF (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, **R**, **T**). **A**, **B** Hematoxylin-eosin stain. **C**, **D** PAS stain. **E**–**H** Syrius red stain. **I**, **J** Immunostain for Flk-1. **K**–**N** Immunostain for PCNA. **O**–**R** TUNEL stain. **S** TUNEL combined with Flk-1 immunostain. **T** Double immunostain for PCNA and Flk-1. Arrows indicate immunopositive cells. Original magnifications: ×400 in **S** and **T**; ×200 in the other panels.

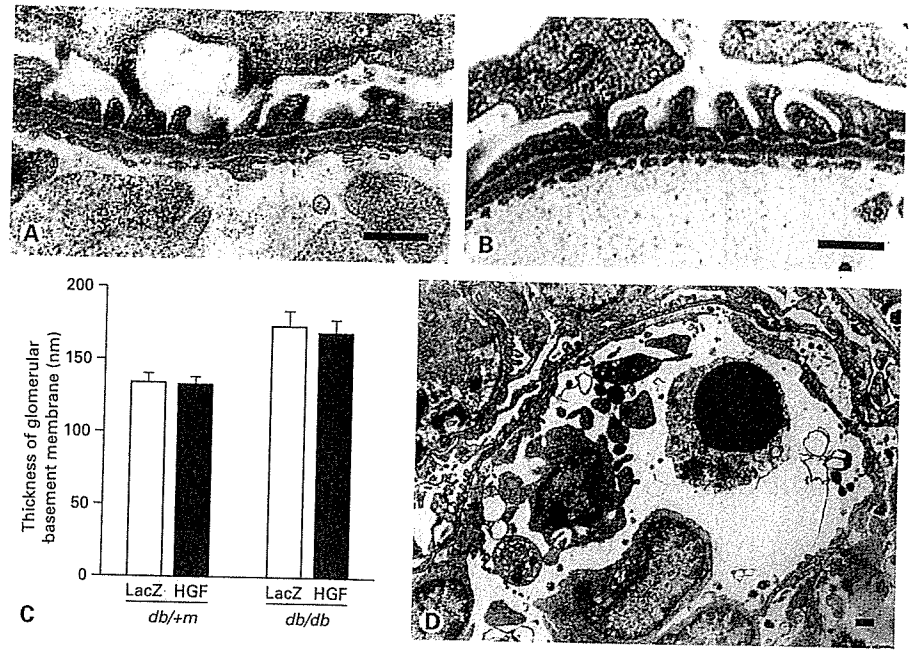

Fig. 5. Electron microphotographs of glomerular lesions observed in 24-week-old *db/db* mice. Glomerular basement membrane in the LacZ-treated (**A**) and HGF-treated *db/db* mouse (**B**). **C** Comparison of thickness of glomerular basement membrane between the LacZ- and HGF-treated mice. **D** Apoptosis of a glomerular capillary endothelial cell observed in the LacZ-treated *db/db* mouse. Bars = 1 μm.

as in the *db/db* mice, the gene therapy significantly affected the renal pathological parameters. The HGF-treated *db/db* mice showed significantly greater glomerular area and glomerular cell number than the LacZ-treated *db/db* mice (fig. 3, 4). Hypocellular, sclerotic lesions compatible with diabetic glomerulosclerosis were observed in glomeruli of the both groups under a light microscope, but its extent appeared smaller in the HGF-treated group. The glomerular sclerotic index calculated as the percent area of PAS-positive area in the glomerulus was reciprocally smaller in the HGF-treated mice. In addition, the percent area of fibrosis assessed by Sirius red stain was smaller in both glomerular and tubular areas.

Ultrastructural morphometry revealed that the thickness of the glomerular basement membrane was similar between the LacZ- (173 ± 4 nm) and HGF-treated (168 ± 4 nm) *db/db* mice, which were greater than those of *db/+m* mice (fig. 4).

Proliferation and Apoptosis

To seek the mechanisms for the increased glomerular cell population by the HGF treatment in the *db/db* mice, we next investigated proliferating activity and apoptosis of kidneys of the *db/db* mice. PCNA-positive cells were observed in glomeruli and tubules of both LacZ- and HGF-treated mice (fig. 4). The incidence of the PCNA-positive cells in glomeruli and that in tubules of the HGF-treated mice were similar between the groups (fig. 5). TUNEL positivity was noted in glomerular and tubular cells in both groups although in low incidences (fig. 4), and electron microscopy demonstrated apoptotic cells (fig. 6). The HGF treatment resulted in a significant reduction in the incidence of TUNEL-positive cells in both glomeruli and tubules, compared with the LacZ treatment (fig. 5).

According to double immunohistochemistry for Flk-1 with PCNA or TUNEL (fig. 4), the HGF gene therapy resulted in no significant change in the proliferation of glomerular endothelial cells and tubular epithelial cells, although a significant decrease in their apoptosis was noted (fig. 5).

Expression of TGF-β1

Consistent with the previous report [29], expression of TGF-β1 in the kidney was greater in the *db/db* mice than in the *db/+m* mice (data not shown). It was found that expression of TGF-β1 in the kidney was significantly reduced in the HGF-treated *db/db* mice, compared with the LacZ-treated ones (fig. 7).

Survival Study

Finally, we examined the effect of HGF gene therapy on the survival of *db/db* mice. Thirteen of 15 *db/db* mice (87%) treated with HGF gene survived over the subsequent 25 weeks, whereas only 7 of 15 *db/db* mice (47%)

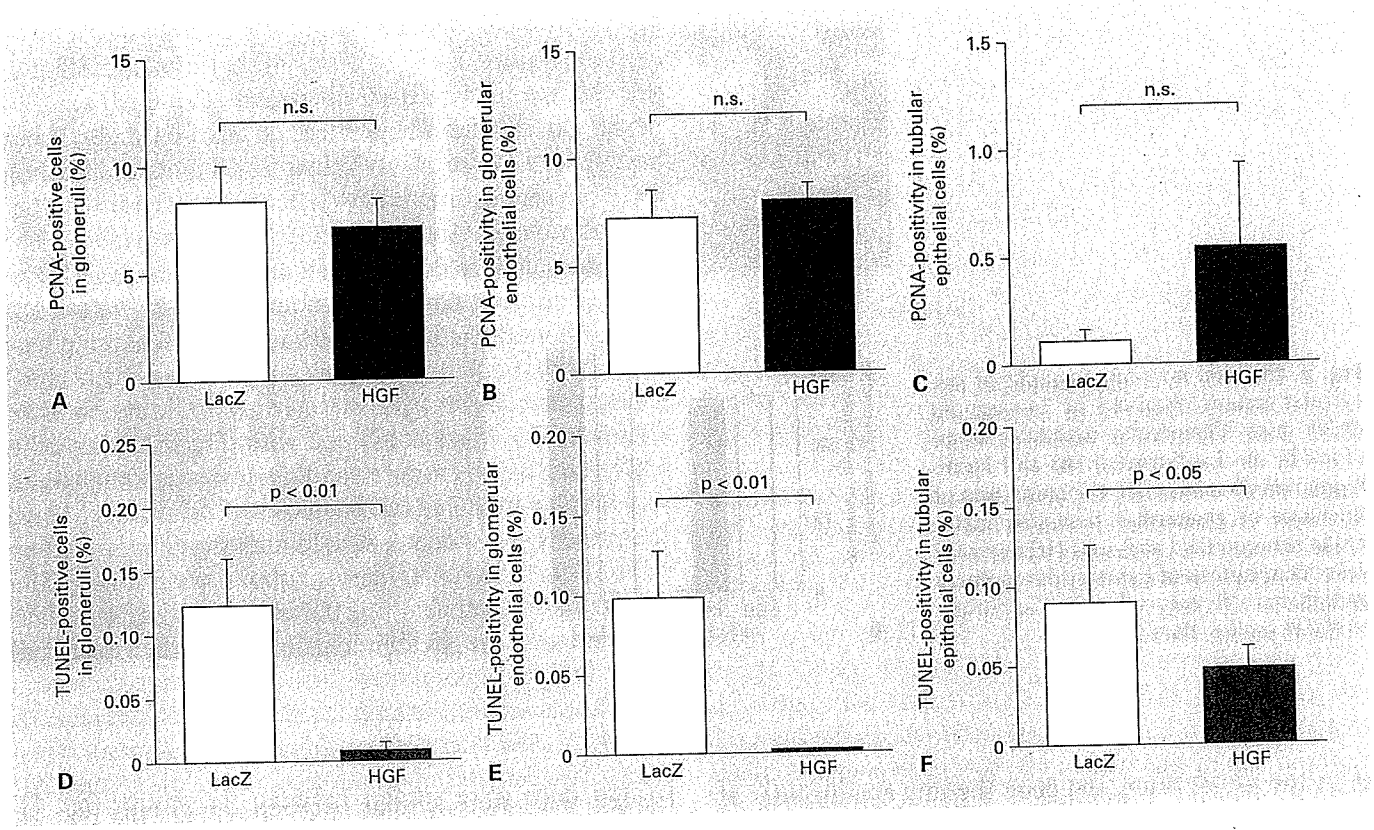

Fig. 6. Quantitative morphometry of PCNA- (**A–C**) or TUNEL-positive (**D–F**) cells in renal glomeruli or tubules of *db/db* mice treated with LacZ or HGF gene. Percent PCNA-positive cells in renal glomeruli (**A**) and in tubules (**C**). Percent TUNEL-positive cells in glomeruli (**D**) and in tubules (**F**). Percent PCNA-positive (**B**) or percent TUNEL-positive (E) glomerular endothelial cells assessed in double immunohistochemical preparations.

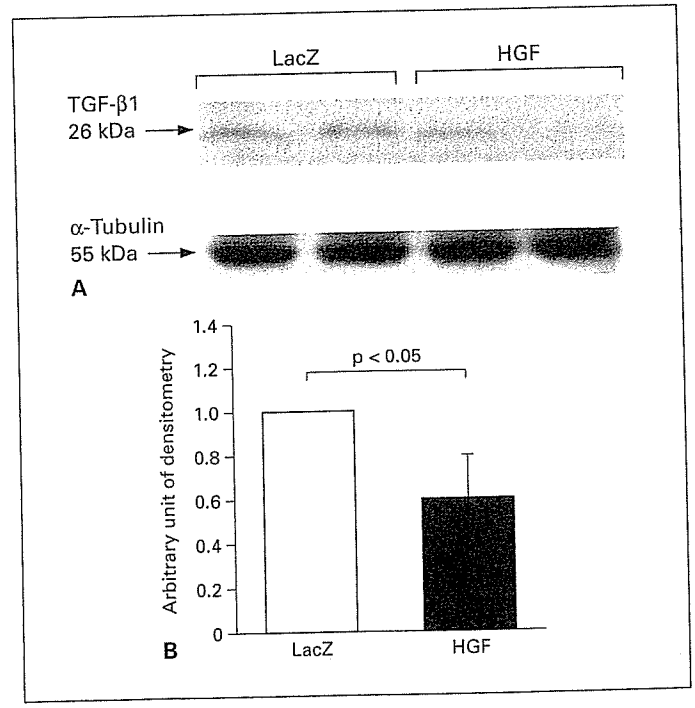

Fig. 7. Western blot for TGF-β1 in kidneys (**A**), and its densitometric analysis (**B**). Y-axis indicates arbitrary unit of densities.

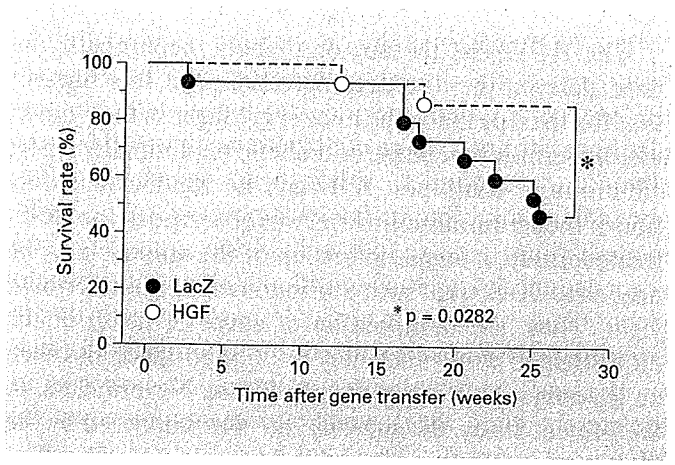

Fig. 8. Survival curve for the HGF- and LacZ-treated *db/db* mice. The mice were treated with HGF gene (n = 15) or LacZ gene (n = 15) at the age of 12 weeks and followed for 25 weeks. There was a significant difference (p = 0.0282) in the survival rate between the groups.

treated with the LacZ gene survived within the interval. Comparison of survival curves by the Kaplan and Meier method showed a significant difference between the HGF- and LacZ-treated groups (log-rank 4.814, p = 0.0282) (fig. 8).

Discussion

In general, diabetic nephropathy progresses from the glomerular hyperfiltration stage into the glomerular sclerotic stage with reduced glomerular filtration [30–32]. The diabetic *db/db* mice displayed far greater urine volume and Ccr than the nondiabetic *db/+m* mice, suggesting that the *db/db* mice at the beginning of the experiment were already suffering from the glomerular hyperfiltration stage of diabetic nephropathy. The *db/db* mice treated with the LacZ gene in the present study showed this progression into the glomerular sclerotic stage during the observation period, i.e. progressive reduction in urine volume and Ccr. However, the present study revealed that the HGF gene therapy markedly prevented this progression. Pathologically, the HGF gene therapy resulted in a significant prevention of formation of glomerular sclerotic lesions and of glomerular and tubular fibrosis that was marked in the LacZ-treated *db/db* mice. The glomerular cell population of the HGF-treated mice was also significantly greater than that of the LacZ-treated mice. Overall, it is apparent that the HGF gene therapy slowed down the progressive deterioration in functional and pathological aspects of diabetic nephropathy in the *db/db* mice. More importantly, this therapy improved survival of these mice. It was apparent that the beneficial effect of HGF on diabetic nephropathy was irrelevant to glucose metabolism since levels of blood glucose and HbA1c were similar between the HGF-untreated and -treated *db/db* mice. The present study also confirmed that such effects of the HGF gene therapy were not observed in nondiabetic *db/+m* mice.

The glomerular cell population, endothelial cell population in particular, was significantly greater in the HGF gene-treated *db/db* mice. According to immunohistochemistry for Flk-1 combined with TUNEL assay, the HGF gene therapy suppressed glomerular endothelial cell and tubular epithelial cell apoptosis in *db/db* mice. Thus, the present study demonstrated an anti-apoptotic effect of HGF on those cells. In particular, preservation of the glomerular endothelial cell population by such mechanism might have greatly contributed to the functional maintenance of glomerular filtration, because the endothelial cells secure the area for glomerular filtration. Although the thickness of the glomerular basement membrane was also an important regulator of glomerular filtration, the ultrastructural morphometry of the present study did not confirm this contribution.

Previous studies reported an anti-fibrotic effect of HGF in the renal tubulointerstitium of animals with chronic renal diseases including experimentally induced diabetic nephropathy [17–20]. Consistent with these findings, the present HGF gene therapy resulted in suppression of fibrosis not only in the glomerulus but also in the tubulointerstitium of the *db/db* mice. Moreover, similar to the capillary endothelial cells, tubular epithelial cells showed decreased apoptosis. Thus, HGF exerted beneficial effects by affecting both glomerular and tubulointerstitial lesions of diabetic nephropathy. The HGF gene therapy resulted in a smaller glomerular sclerotic index and a more reduced fibrotic area in glomeruli of diabetic nephropathy, compared with the LacZ gene treatment. Thus, the HGF gene therapy alleviated the progression to glomerular obsolescence in diabetic nephropathy through a reduction in the mesangial matrix and fibrosis and also through maintenance of the glomerular cell component, endothelial cells in particular. An opposite effect of HGF against TGF-β was reported previously [15, 33, 34]. In the present study, the Western blot analysis actually demonstrated greatly attenuated expression of TGF-β1 in the HGF-treated kidneys of *db/db* mice. Since TGF-β, in addition to platelet-derived growth factor (PDGF), accelerates transformation of mesangial cells into myofibroblasts

that potentially produce collagen fibers [35, 36], such an anti-TGF-β effect of HGF may be important for attenuation of glomerulosclerosis in diabetic nephropathy. Alternatively, as Mizuno et al. [17] previously suggested, an endothelial protective effect of HGF may play an important role for the prevention of glomerulosclerosis.

Mizuno et al. [19] recently reported beneficial effect of injection of recombinant human HGF on streptozotocin-induced diabetic nephropathy in mice. Although our findings were mostly consistent with theirs, there are some discrepancies in some aspects. They found that HGF treatment reduced glomerular area and suppressed glomerular hyperfiltration, which were opposite to our findings. It may be most likely that the discrepancy was caused by the difference of animal models with different stage and progression of diabetic nephropathy; our model might be at the later stage than that of Mizuno et al. as it was already at the hyperfiltration stage at the beginning of the experiments. Laping et al. [37] reported that diabetic nephropathy in *db/db* mice was aggravated by an HGF supplement. However, the dose of HGF used in the study seemed to be too low to attain and sustain physiological HGF level as Mizuno et al. [19] pointed out. In our study, the plasma HGF levels were considered sufficiently high because the level was greater at any time point measured than that produced by the plasmid DNA injection in another study [38], which effectively prevented progression of liver cirrhosis in an animal model. The way in which the very low dose of HGF has different effects awaits results from additional studies.

The HGF gene therapy on diabetic nephropathy indeed delayed the disease progression, but this suggests that the therapy made the mice stay longer in the glomerular hyperfiltration phase of diabetic nephropathy where albuminuria continues. Although no significant reduction of the serum albumin level was observed in the HGF-treated group, a longer follow-up of the animals may be needed to check what such continuous albuminuria would yield. Thus, the examination of cause of death of the HGF-treated *db/db* mice may be of importance and such an investigation is warranted in future. Nevertheless, in the present study, the survival rate was improved by the HGF gene treatment. There has been no clinically effective treatment for the pathological condition of patients with diabetic nephropathy. Even a strict control of hyperglycemia does not necessarily guarantee deceleration of disease progression [32], and many patients have to enter chronic dialysis programs with renal failure. Effective HGF supply would delay the start of the dialysis program, and it is suggested that it would be one of the promising candidates for preventing, or at least slowing down the progression of clinical and pathophysiological manifestations of diabetic nephropathy.

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References

1 Ritz E, Orth SR: Nephropathy in patients with type 2 diabetes mellitus. N Engl J Med 1999; 341:1127–1133.
2 Woodrow D, Moss J, Shore I, Spiro RG: Diabetic glomerulosclerosis: Immunogold ultrastructural studies on the glomerular distribution of type IV collagen and heparan sulphate proteoglycan. J Pathol 1992;167:49–58.
3 Mohan PS, Carter WG, Spiro RG: Occurrence of type VI collagen in extracellular matrix of renal glomeruli and its increase in diabetes. Diabetes 1990;39:31–37.
4 Border WA, Noble NA: Transforming growth factor-β in tissue fibrosis. N Engl J Med 1994; 331:1286–1292.
5 Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN: Increased glomerular and tubular expression of transforming growth factor-β1, its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. Am J Pathol 2001;158: 1653–1663.
6 Ishii N, Ogawa Z, Suzuki K, Numakami K, Saruta T, Itoh H: Glucose loading induces DNA fragmentation in rat proximal tubular cells. Metabolism 1996;45:1348–1353.
7 Ortiz A, Ziyadeh FN, Neilson EG: Expression of apoptosis-regulatory genes in renal proximal tubular epithelial cells exposed to high ambient glucose and in diabetic kidneys. J Invest Med 1997;45:50–56.
8 Murata I, Takemura G, Asano K, Sano H, Fujisawa K, Kagawa T, Baba K, Maruyama R, Minatoguchi S, Fujiwara T, Fujiwara H: Apoptotic cell loss following cell proliferation in renal glomeruli of Otsuka Long-Evans Tokushima fatty rats, a model of human type 2 diabetes. Am J Nephrol 2002;22:587–595.
9 Nakamura T, Nawa K, Ichihara A: Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 1984; 122:1450–1459.
10 Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S: Molecular cloning and expression of human hepatocyte growth factor. Nature 1989; 342:440–443.
11 Birchmeier C, Gherardi E: Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 1998;8:404–410.
12 Kosai K, Matsumoto K, Funakoshi H, Nakamura T: Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice. Hepatology 1999;30:151–159.
13 Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, Testa JR: Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci USA 2001;98:247–252.

14 Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Matsumoto K, Nakamura T, Kaneda Y, Horiuchi M, Ogihara T: Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. Hypertension 2001;37:581–586.
15 Mizuno S, Matsumoto K, Kurosawa T, Mizuno-Horikawa Y, Nakamura T: Reciprocal balance of hepatocyte growth factor and transforming growth factor-beta 1 in renal fibrosis in mice. Kidney Int 2000;57:937–948.
16 Matsumoto K, Nakamura T: Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. Kidney Int 2001;59:2023–2038.
17 Mizuno S, Kurosawa T, Matsumoto K, Mizuno-Horikawa Y, Okamoto M, Nakamura T: Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. J Clin Invest 1998;101:1827–1834.
18 Mizuno S, Matsumoto K, Nakamura T: Hepatocyte growth factor suppresses interstitial fibrosis in a mouse model of obstructive nephropathy. Kidney Int 2001;59:1304–1314.
19 Mizuno S, Nakamura T: Suppressions of chronic glomerular injuries and TGF-beta 1 production by HGF in attenuation of murine diabetic nephropathy. Am J Physiol 2004;286: F134–F143.
20 Cruzado JM, Lloberas N, Torras J, Riera M, Fillat C, Herrero-Fresneda I, Aran JM, Alperovich G, Vidal A, Grinyo JM: Regression of advanced diabetic nephropathy by hepatocyte growth factor gene therapy in rats. Diabetes 2004;53:1119–1127.
21 Hummel KP, Dickie MM, Coleman DL: Diabetes, a new mutation in the mouse. Science 1996;153:1127–1128.
22 Like AA, Lavine RL, Poffenbarger PL, Chick WL: Studies in the diabetic mutant mice. Diabetologia 1974;10:607–610.
23 Cohen MP, Clements RS, Hud E, Cohen JA, Ziyadeh FN: Evolution of renal function abnormalities in the db/db mouse that parallels the development of human diabetic nephropathy. Exp Nephrol 1996;4:166–171.
24 Mizuguchi H, Kay AM: A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum Gen Ther 1999; 10 2013–2017.
25 Chen SH, Chen XH, Wang Y, Kosai K, Finegold MJ, Rich SS, Woo SL: Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci USA 1995;92: 2577–2581.
26 Junqueira LC, Bignolas G, Brentani RR: Red sirius staining plus polarizing microscopy: a specific method for collagen detection in tissue sections. Histochem J 1979;79:445–447.
27 Matsumae T, Jimi S, Uesugi N, Takebayashi S, Naito S: Clinical and morphometrical interrelationships in patients with overt nephropathy induced by non-insulin-dependent diabetes mellitus: a light- and electron-microscopy study. Nephron 1999;81:41–48.
28 Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, Goto K, Takahashi T, Hayakawa K, Koda M, Kawase Y, Maruyama R, Okada H, Minatoguchi S, Mizuguchi H, Fujiwara T, Fujiwara H: Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. Circulation 2003;107:2499–2506.
29 Ziyadeh FN, Hoffman BB, Han D-C, Iglesias-de la Cruz MC, Hong S-w, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-β antibody in db/db diabetic mice. Proc Natl Acad Sci USA 2000;97:8015–8020.
30 Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. J Clin Invest 1984;74:1143–1155.
31 Fabre J, Balant LP, Dayer PG, Fox HM, Vernet AT: The kidney in maturity onset diabetes mellitus: a clinical study of 510 patients. Kidney Int 1982;21:730–738.
32 Viberti G, Wiseman MJ, Pinto JR, Messent J: Diabetic Nephropathy; in Kahn CR, Weir GC (eds): Joslin's Diabetes mellitus, ed 13. Malvern, Lea & Febiger, 1997, pp 691–737.
33 Yasuda H, Imai E, Shiota A, Fujise N, Morinaga T, Higashio K: Antifibrogenic effect of a deletion variant of hepatocyte growth factor on liver fibrosis in rats. Hepatology 1996;24:636–642.
34 Taniyama Y, Morishita R, Nakagami H, Moriguchi A, Sakonjo H, Shokei-Kim, Matsumoto K, Nakamura T, Higaki J, Ogihara T: Potential contribution of a novel antifibrotic factor, hepatocyte growth factor, to prevention of myocardial fibrosis by angiotensin II blockade in cardiomyopathic hamsters. Circulation 2000; 102:246–252.
35 Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van GY, Tarpley JE, Yee JS: Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. Am J Pathol 1996:148:1169–1180.
36 el Nahas AM, Muchaneta-Kubara EC, Zhang G, Adam A, Goumenos D: Phenotypic modulation of renal cells during experimental and clinical renal scarring. Kidney Int 1996;54: S23–S27.
37 Laping NJ, Olson BA, Ho T, Ziyadeh FN, Albrightson CR: Hepatocyte growth factor: a regulator of extracellular matrix genes in mouse mesangial cells. Biochem Pharmacol 2000;59: 847–853.
38 Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R, Matsumoto K, Nakamura T, Takahashi H, Okamoto E, Fujimoto J: Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 1999;5:226–230.

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Local overexpression of HB-EGF exacerbates remodeling following myocardial infarction by activating noncardiomyocytes

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Insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) are cardiogenic and cardiohypertrophic growth factors. Although the therapeutic effects of IGF and HGF have been well demonstrated in injured hearts, it is uncertain whether natural upregulation of HB-EGF after myocardial infarction (MI) plays a beneficial or pathological role in the process of remodeling. To answer this question, we conducted adenoviral HB-EGF gene transduction in *in vitro* and *in vivo* injured heart models, allowing us to highlight and explore the HB-EGF-induced phenotypes. Overexpressed HB-EGF had no cytoprotective or additive death-inducible effect on Fas-induced apoptosis or oxidative stress injury in primary cultured mouse cardiomyocytes, although it significantly induced hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts. Locally overexpressed HB-EGF in the MI border area in rabbit hearts did not improve cardiac function or exhibit an angiogenic effect, and instead exacerbated remodeling at the subacute and chronic stages post-MI. Namely, it elevated the levels of apoptosis, fibrosis, and the accumulation of myofibroblasts and macrophages in the MI area, in addition to inducing left ventricular hypertrophy. Thus, upregulated HB-EGF plays a pathophysiological role in injured hearts in contrast to the therapeutic roles of IGF and HGF. These results imply that regulation of HB-EGF may be a therapeutic target for treating cardiac hypertrophy and fibrosis.
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Keywords: apoptosis; gene transfer; growth factor; myocardial infarction; remodeling

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF-family of growth factors, is synthesized as a type I transmembrane protein (proHB-EGF).[1] Membrane-bound proHB-EGF is cleaved at its juxtamembrane domain by a specific metalloproteinase, resulting in shedding of soluble HB-EGF.[2] Whereas soluble HB-EGF is a potent mitogen for a number of cell types, including vascular smooth muscle cells, fibroblasts, keratinocytes, and hepatocytes,[3–5] the activity of proHB-EGF may be mitogenic or growth inhibitory depending on cell type.[6]

HB-EGF has been implicated in a number of physiological and pathological processes. HB-EGF may play a role in the development of atherosclerosis resulting from smooth muscle cell hyperplasia,[4,7,8] pulmonary hypertension, and oncogenic transformation.[9,10] In contrast, HB-EGF is

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therapeutic for the skin,[11,12] kidney,[13,14] liver, and small intestine.[3,5,15,16] HB-EGF is markedly upregulated during the acute phase of injury and plays an essential role in epithelial cell repair, proliferation and regeneration in these organs.[5,11,13,15,17] Further direct evidence of therapeutic benefit was provided by studies of administration of recombinant HB-EGF in animal ischemic disease models.[16] Thus, HB-EGF plays a number of physiological roles, and its effects are diverse and even opposing in nature depending on the tissues examined.

It has been observed that HB-EGF-null mice develop severe heart failure associated with dilated ventricular chambers, diminished cardiac function, and grossly enlarged cardiac valves,[18,19] indicating that HB-EGF is an essential cardiogenic factor. HB-EGF is found in the adult heart under normal physiological conditions,[20] and the HB-EGF and/or EGF receptor (EGFR) families are further upregulated under pathological conditions such as cardiac hypertrophy[21] or myocardial infarction (MI).[22,23] Together with the recent finding that shedding of proHB-EGF results in cardiac hypertrophy,[24] it has recently been suggested that HB–EGF-induced cardiomyocyte hypertrophy plays a central role in hypertensive heart disease.[24,25] However, several previous studies demonstrated that overexpression of hepatocyte growth factor (HGF) and insulin-like growth factor (IGF), which are also cardiogenic growth factors, significantly induced cardiac hypertrophy but had potent therapeutic rather than pathologic effects in injured hearts, including those damaged by MI.[26–29] This led us to question whether HB-EGF might also possess therapeutic activity in the injured heart. Intriguingly, were HB-EGF to prove pathogenic, it could be the result of a secondary biological effect of this molecule separate from its promotion of hypertrophy. Thus, we endeavored to settle the questions raised by these conflicting reports in the most direct way possible, through targeted overexpression of HB-EGF in heart lesions.

One obvious approach by which to overexpress a target gene and explore the resulting effects would be to use transgenic mouse (TgM) technology, currently one of the most powerful approaches to elucidate directly the physiological and pathological roles of a gene of interest. In the present study, we opted instead to use an adenoviral gene transduction strategy, which allowed us to answer the same biological question while at the same time enabling a first assessment of the use of HB-EGF in gene therapy. Additionally, the use of an adenoviral vector allowed for greater spatial and temporal control of HB-EGF expression compared with the TgM approach, as persistent overexpression of the transgene (from the embryonic stage and before the onset of a disorder) may have produced data artefacts. Previous studies demonstrated that the expression of HB-EGF and EGFR family mRNAs was significantly increased around MI lesions.[22,23] In this context, adenoviral HB-EGF gene transduction around the MI area following onset of MI may serve to highlight the effect of HB-EGF on both cardiomyocytes and noncardiomyocytes following MI, offering a means to elucidate the role of this intriguing molecule in the development of heart disease.

Materials and methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors (Ads), Ad.HB-EGF and Ad.LacZ, which express HB-EGF or LacZ gene under the transcriptional control of a Rous sarcoma virus long-terminal repeat, were constructed as described previously.[27,30,31] All Ads were amplified in 293 cells, purified twice on CsCl gradients, and desalted.[27,30,31]

Injury Models in Primary Cultured Cardiomyocytes and Cardiac Fibroblasts

Cardiomyocytes and cardiac fibroblasts were isolated from 1-day-old neonatal Balb/c mice as previously reported.[32] The cardiomyoctes were incubated in Dulbecco's modified Eagle's medium (D-MEM, Sigma Chemical Co., St Louis, MO, USA) containing 5% fetal bovine serum (Sigma Chemical Co.) at 37°C for 24 h, and subsequently infected with Ads at various multiplicities of infection (MOI), followed by incubation in serum-free D-MEM for 48 h. In injury models of apoptosis and oxidative stress, cells were incubated with either 1 μg/ml agonistic anti-Fas antibody[33] (Jo2, Beckton-Dickinsion Biosciences, San Jose, CA, USA) with 0.05 μg/ml actinomycin D (Sigma Chemical Co.) for 24 h, or with 100 μM H_2O_2 (Wako Pure Chemical Industry, Osaka, Japan) for 1 h as previously described.[34,35] Cell viability was determined by WST-8 assay (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's protocol 24 h after the induction of cell death.

For proliferation assays, cardiac fibroblasts were incubated in D-MEM supplemented with 5% fetal bovine serum, and were used following three or four passages. The purity of these cultures was >95% cardiac fibroblasts as confirmed by vimentin-positive, desmin-negative and α-smooth muscle. actin-negative stainings as previously described.[36] WST-8 assay was performed at 24, 48 and 72 h after infection with Ads or addition of recombinant human HB-EGF (R&D Systems Inc., Minneapolis, MN, USA).

Immunocytochemistry and Analysis of Primary Cultured Cardiomyocytes

At 24 h following adenoviral infection at MOI 30, primary cultured cardiomyocytes were fixed in 4% paraformaldehyde, permeabilized with 0.05%

Triton-X and stained with primary goat anti-human HB-EGF antibody (R&D Systems Inc.), secondary donkey anti-goat IgG Alexa 488 antibody (Molecular Probes, Inc., Eugene, OR, USA), rhodamine phalloidin (Molecular Probes, Inc.) and Hoechst 33342 (Molecular Probes, Inc.). Digital images captured using a laser-confocal microscope system (LSM510, Carl Zeiss, Oberkochen, Germany) were employed for morphometric and quantitative analyses using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

Animal Studies

Male Japanese white rabbits weighing 2–2.5 kg underwent a 30-min occlusion of the left coronary artery, followed by reperfusion, in order to generate MI as previously described.[37] Ad.HB-EGF or control Ad.LacZ (1×10^{11} viral particles) (each group, $n=16$) was directly injected into the border area between the risk and the intact areas at the time of reperfusion. Echocardiograms were recorded just before and 2 or 4 weeks after generation of MI. The rabbits were killed either 2 or 4 weeks later (each, $n=8$) and the hearts were collected, weighed, and then processed to obtain histological sections. In the sham control group ($n=4$), the chests of the rabbits were opened and closed under anesthesia without occlusion of the coronary artery or adenoviral injection, and echocardiograms and histological analyses were performed 2 or 4 weeks later. All animal studies were performed in accordance with the guidelines of the National Institute of Health as dictated by the Animal Care Facility at the Gifu University School of Medicine.

Adenoviral Gene Transduction Efficiencies and X-Gal Staining

The efficiency of *in vitro* and *in vivo* adenoviral gene transduction was analyzed by Ad.LacZ infection and X-gal staining, as previously described.[27,30,31]

Pathological Examination in Animal Experiments

The estimation of the risk and MI areas has been described previously.[37] Briefly, the coronary branch in the excised heart was reoccluded and 4% Evans blue dye (Sigma Chemical Co.) was injected via the aorta to determine the risk area. The LV was sectioned into seven slices parallel to the atrioventricular ring. Each slice was incubated in 1% solution of triphenyl tetrazolium chloride (TTC) to visualize the infarct area.

For histological analysis, the heart was fixed in 10% formalin and embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin (H–E) or Masson's trichrome for regular or fibrotic estimation, respectively. The sizes of individual cardiomyocytes were measured using the LUZEX F system (Nireco, Kyoto, Japan). Apoptotic cells were detected under light microscopy by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay (ApopTag kit, Intergen Co., Purchase, NY, USA) in accordance with the manufacturer's protocol. The immunohistochemical staining for proliferating cells, α-smooth muscle actin (SMA-positive cells), rabbit macrophages and vascular endothelial cells were carried out with anti-Ki-67 (MIB-1, Dako), anti-SMA (1A4, Dako), anti-RAM11 (Dako) and anti-CD31 (JC/70A, Dako) antibody, respectively, as described previously.[37] For fluorescent immunohistochemistry and TUNEL assay, 6-μm frozen sections were fixed in 4% paraformaldehyde and stained using a fluorescein-FragEL DNA fragmentation detection kit (Oncogene Research Products, San Diego, CA, USA) together with individual antibodies, according to the manufacturer's instructions.

Statistical Analysis

Data are represented as means $\pm$ standard error of the mean. Statistical significance was determined using Student's *t*-test. One-way ANOVA was used in multiple comparisons. $P<0.05$ was considered to be statistically significant. All statistical analysis was performed with StatView software (SAS Institute Inc., Cary, NC, USA).

Results

Adenoviral Gene Transduction Efficiency *In Vitro*

The adenoviral constructs demonstrated high infectivity in primary cultured mouse cardiomyocytes; infection of cardiomyocytes with Ad.LacZ at MOIs of 10 and 30 resulted in approximately 80% and over 90% successful gene transduction, respectively, without morphological changes, cell damage or death (Figure 1). Cardiac fibroblasts also demonstrated high infectivity, although the accurate quantification was difficult.

Effects of Adenoviral HB-EGF Gene Transduction on Cardiomyocytes and Cardiac Fibroblasts *In Vitro*

To explore the direct effects of HB-EGF on cardiomyocytes, we examined cell viability following Ad.HB-EGF infection in two representative injury models of primary cultured cardiomyocytes, Fas-induced apoptosis[34,35] and H_2O_2 oxidative stress injury[34,35] (Figure 2a and b). Both types of stimulus at the predetermined doses efficiently induced cell death in approximately 80% of the cultured cells, and adenoviral HB-EGF gene transduction did not result in significant changes in viability in either of these models at any MOI (Figure 2a and b). However,

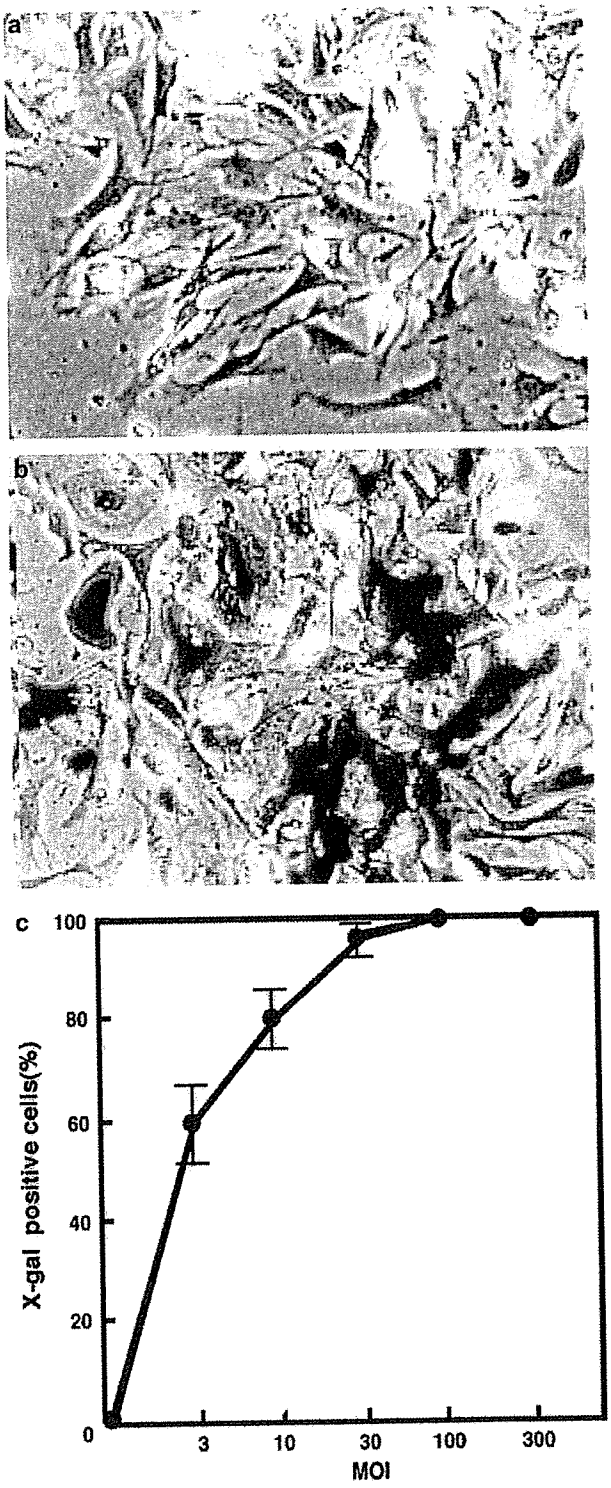

Figure 1 Efficiency of adenoviral gene transduction in primary cultured mouse cardiomyocytes. X-gal staining after Ad.LacZ infection at (a) MOI 0 (ie, no infection as a negative control) and (b) MOI 10. (c) Graph showing adenoviral gene transduction efficiency at various MOIs.

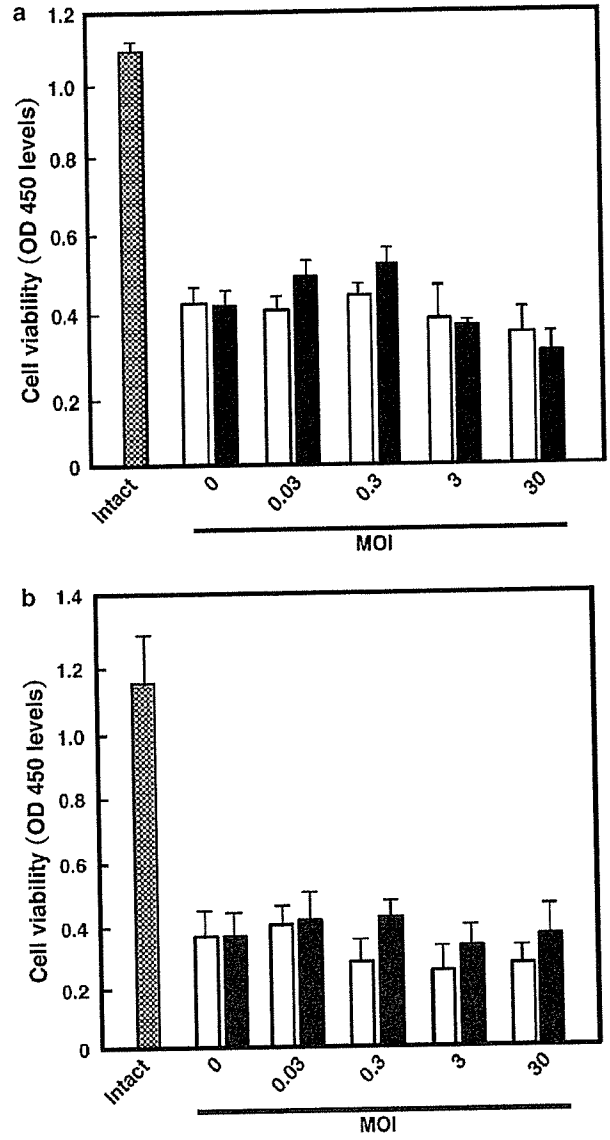

Figure 2 Cell viability after adenoviral HB-EGF gene transduction in two injury models of primary cultured cardiomyocytes. Primary cultured cardiomyocytes were infected with Ad.LacZ (white bar) or Ad.HB-EGF (black bar), and were then exposed to 1 μg/ml anti-Fas antibody and 0.05 μg/ml actinomycin D for 24 h (a) or 100 μM H_2O_2 for 1 h (b); cell viability was evaluated by WST-8 assay. 'Intact' indicates the control (untreated cells).

cardiomyocytes became significantly enlarged, and their F-actin-containing myofibrils were drastically condensed, enlarged and increased in number following adenoviral HB-EGF gene transduction (Figures 3 and 4).

Next, we explored whether HB-EGF exhibited an inhibitory or stimulatory effect on the growth of cardiac fibroblasts. Both the addition of recombinant HB-EGF and adenoviral HB-EGF gene transduction significantly accelerated the growth of cardiac fibroblasts. (Figure 5). Thus, HB-EGF gene

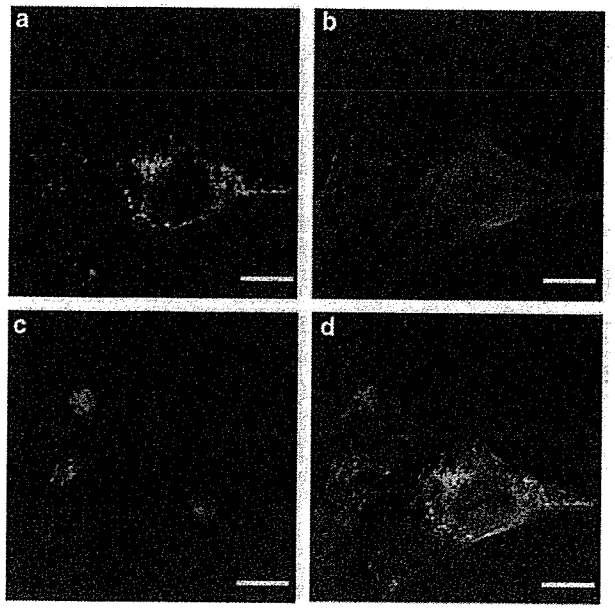

Figure 3 Microscopic images of HB-EGF gene-transduced cardiomyocytes. Confocal microscopic analysis demonstrated apparent hypertrophic changes in individual cardiomyocytes overexpressing HB-EGF after Ad.HB-EGF infection at MOI 30. (**a**) Staining with goat anti-human HB-EGF antibody and Alexa 488-labeled donkey anti-goat secondary antibody, (**b**) staining using rhodamine phalloidin-labeled F-actin, (**c**) staining using Hoechst 33342 (nuclei), and (**d**) merged image. Scale bar = 20 μm.

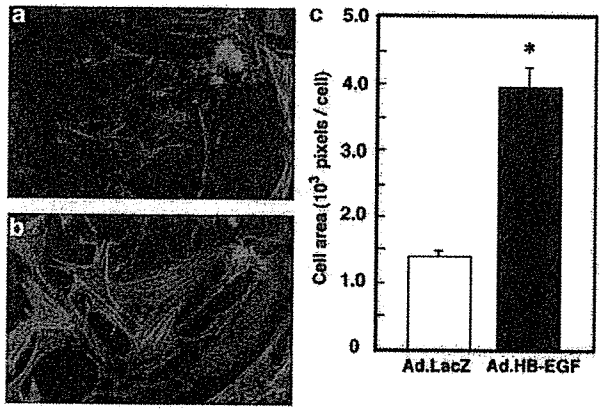

Figure 4 Morphometric and quantitative analysis of HB-EGF gene-transduced cardiomyocytes after Ad.LacZ (**a**) or Ad.HB-EGF (**b**) infection at MOI 30. Hypertrophy as shown by condensation of rhodamine phalloidin-labeled F-actin and enlargement of cell area. Original magnification, × 100. (**c**) Graphic depiction of cell area determined for 500 cardiomyocytes infected with Ad.HB-EGF or Ad.LacZ. *$P<0.001$.

transduction and overexpression conferred a direct hypertrophic effect on cardiomyocytes and a growth-stimulating effect on cardiac fibroblasts, but did not have additive death-inducible or cytoprotective effects on cardiomyocytes.

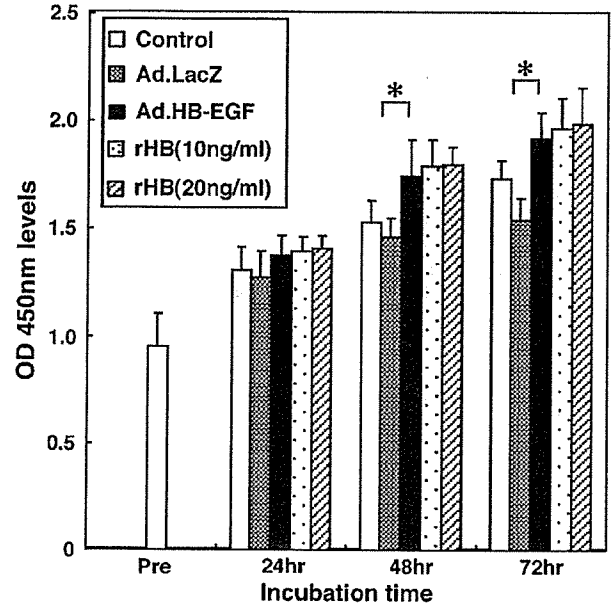

Figure 5 Proliferation of cardiac fibroblasts overexpressing HB-EGF. Cardiac fibroblasts (cell density; 1×10^5/ml) were infected with Ad.LacZ at MOI 30 (shaded bar) or Ad.HB-EGF at MOI 30 (black bar), or were exposed to 10 or 20 ng/ml rHB-EGF for 24, 48, and 72 h; cell proliferation was evaluated by WST-8 assay at OD 450 nm. *$P<0.05$.

Macroscopic Findings after Adenoviral HB-EGF Gene Transduction in the Rabbit MI Model

Recent studies have demonstrated that HB-EGF and EGFR family mRNAs were significantly upregulated around MI lesions,[22,23] and it is for this reason that we injected our adenovirus constructs specifically into this region. X-gal staining after Ad.LacZ injection into this area showed that we were able to drive gene transduction predominantly around the MI (Figure 6a). A number of previous studies have demonstrated retention of transgene expression for at least 3 weeks following *in vivo* adenoviral gene transduction into the heart.[38–40] We have previously described the pathological process in the rabbit MI model in detail, including granulation consisting of myofibroblasts, macrophages and neovasculation at 2 weeks (the subacute stage) and scar formation at 4 weeks (the chronic stage).[37] Together, these results suggested that the period of transgene expression resulting from adenoviral gene transduction would be sufficient for the purposes of this study. In this context, we injected Ad.HB-EGF into the MI injury border area, estimated the risk area and the MI area by TTC and Evans blue dye after 2 weeks (as shown in Figure 6b) and ultraechographically analyzed cardiac function 2 and 4 weeks later. There was no difference in risk area between the two groups (Figure 6c). On the other hand, the ratio of MI area to risk area was seemingly reduced to a small degree by Ad.HB-EGF at 2 weeks post-injection due to hypertrophic changes; however, this reduction was