を目指し，異なるインフルエンザウイルス株の結合挙動を上記の糖アレイシステムを用いて検討した。すなわち，化学酵素法で合成，または市販の 6 種のシアル酸含有糖鎖とコントロールの 2 種類の糖鎖を固定化したアレイタイプのシュガーチップを調製し，実験に用いた。全部で 8 種類の糖鎖であるので，チップ上にはそれぞれの糖鎖は複数（4 ～ 8 点）スポットした。インフルエンザウイルスは，MDCK 細胞または鶏卵で培養し，ショ糖密度勾配法で精製後，鶏赤血球を用いたヘマグルチニン（HA）価で濃度を決定し，HA 価を合わせて実験に供した。図 4 に 1 例を示した。SPR イメージングで観測された輝度（a）を定量し，それぞれの糖鎖に対する絶対結合値

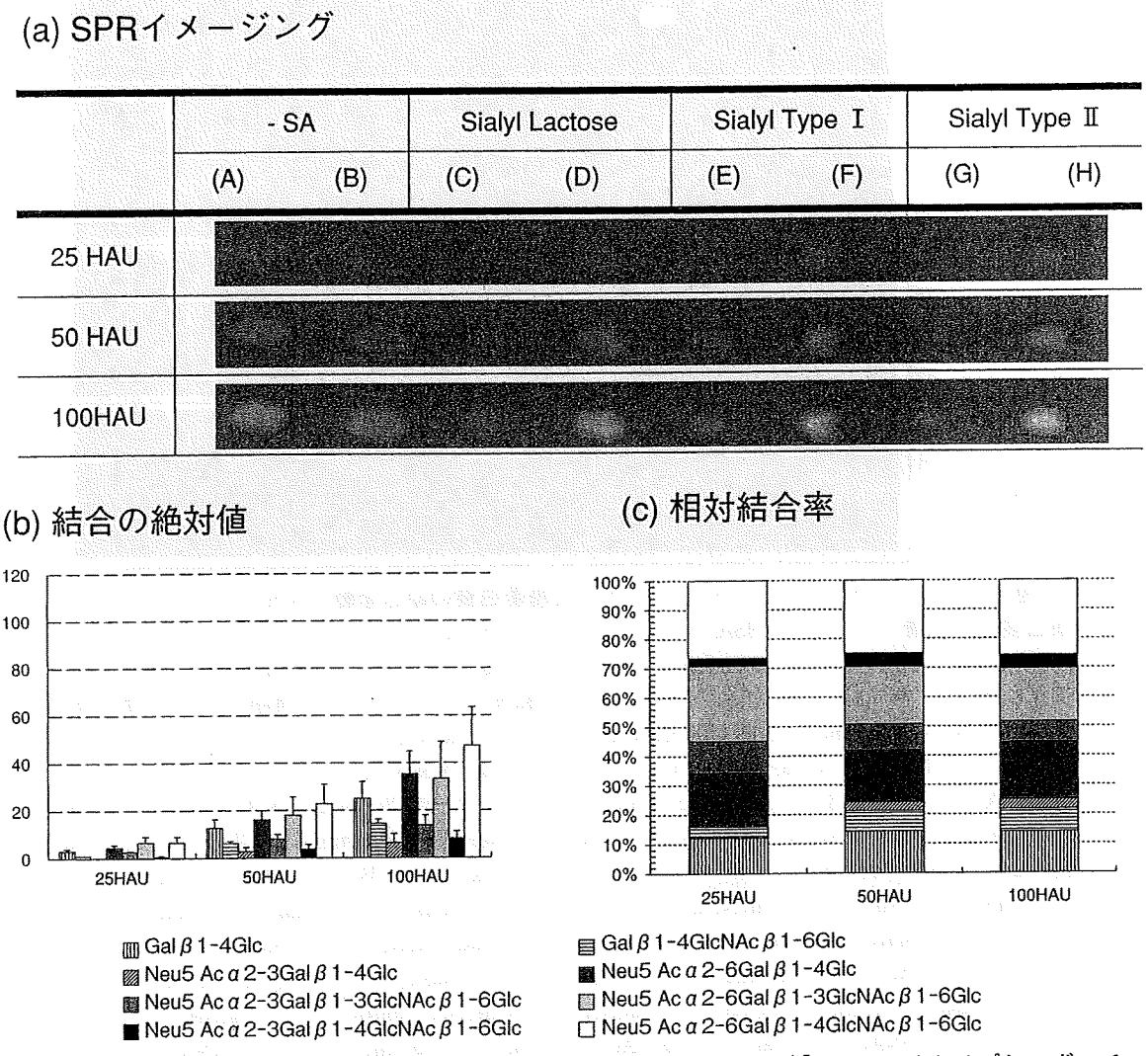

図 4　A 型インフルエンザウイルス福岡株［A/Fukuoka/C 29/85（H 3 N 2）］のアレイタイプシュガーチップへの結合挙動

(a) SPR イメージング；(b) 結合の絶対値；(c) 相対結合率。チップ上のアドレス（括弧内）にスポットした糖鎖リガンド複合体は以下の通り。Galβ1-4 Glc-mono(A)；Galβ1-4 GlcNAcβ1-6 Glc-mono(B)；Neu 5 Acα2-3 Galβ1-4 Glc-mono(C)；Neu 5 Acα2-6 Galβ1-4 Glc-mono(D)；Neu 5 Acα2-3 Galβ1-3 GlcNAcβ1-6 Glc-mono(E)；Neu 5 Acα2-6 Galβ1-3 GlcNAcβ1-6 Glc-mono(F)；Neu 5 Acα2-3 Galβ1-4 GlcNAcβ1-6 Glc-mono(G)；Neu 5 Acα2-6 Galβ1-4 GlcNAcβ1-6 Glc-mono(H)。

を算出した (b)。当然のことながら，HA 価が大きいほど，結合値は大きくなっている。ここで，(b) の絶対結合値を相対結合率に直すと，HA 価によらずそれぞれの糖鎖への相対結合率はほぼ同じであることが分かった (c)。そこで，ウイルスの種類を増やし，同じ系でウイルスの 8 種の糖鎖への相対結合率を測定することとした。現在までに，約 20 種類のインフルエンザウイルス株を対象とした測定を終了し，データベースの作成とそれに基づくウイルス株の識別アルゴリズム作成を開始している。

5　おわりに

アレイタイプの糖チップ（シュガーチップ）は，本稿で述べたインフルエンザウイルス株の同定など，今までの抗体を用いた方法では識別できなかった対象物の新しい検査・診断法に応用できる可能性がある。我々はインフルエンザウイルス以外のウイルスや，各種細菌の識別，さらには細胞表層の変化をアレイタイプのシュガーチップで観測する系を構築している。現在の実験上の問題点は，比較的大量のサンプル量（約 300μl）が必要であること，測定時のハンドリングが悪いこと，さらに測定方法が SPR イメージングであるため，定量化する際のダイナミックレンジが小さいことである。特に，後者は解離定数を求める時等に正確さを欠くときがあるので，我々はアレイタイプのシュガーチップは主としてスクリーニングに用いて，定性的情報を得るために使用し，正確な解離定数等は前述した 2 チャンネルの SPR 機器を使用して求めている。また，前者 2 つの問題をも一気に解決するために，我々は局所プラズモン共鳴法を原理とした光ファイバー型 SPR を別途開発しており，近い将来はそれに変えていきたいと考えている。

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Basic Science and Experimental Studies

In Vivo Hepatocyte Growth Factor Gene Transfer Reduces Myocardial Ischemia-Reperfusion Injury Through Its Multiple Actions

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ABSTRACT

Background: Hepatocyte growth factor (HGF) is reported to protect the heart against ischemia-reperfusion injury. However, whether in vivo adenovirus-mediated HGF gene transfer before ischemia is protective against ischemia-reperfusion and its precise mechanisms are still unknown.

Methods and Results: By using a rabbit model of ischemia-reperfusion injury, we demonstrate that HGF gene transfer is cardioprotective through its multiple beneficial actions, such as angiogenesis, Bcl-2 overexpression, and decreasing hydroxyl radicals, deoxyuride-5′-triphosphate biotin nick end labeling (TUNEL)-positive myocytes, and fibrotic area. After HGF gene transfer, the rabbits underwent 30 minutes of coronary occlusion and 30 minutes, 4 hours, 48 hours, and 14 days of reperfusion. The infarct size at 48 hours of reperfusion was significantly reduced in the HGF group (13.4% ± 2.3%) compared with that in the LacZ group (36.5% ± 2.0%) and saline group (40.3% ± 3.2%). At 14 days of reperfusion, HGF gene transfer improved left ventricular ejection fraction and fractional shortening, reduced the fibrotic area, and increased the capillary density in the risk area. At 4 hours of reperfusion, Bcl-2 protein was overexpressed and the incidence of TUNEL-positive myocytes was significantly decreased in the risk area in the HGF group compared with the LacZ and saline groups. The myocardial interstitial 2,5-dihydroxybenzoic acid level, an indicator of hydroxyl radical, increased during 30 minutes of ischemia and 30 minutes of reperfusion in the LacZ and saline groups, and was significantly inhibited in the HGF group.

Conclusion: HGF gene therapy may be a novel therapeutic strategy against unstable angina pectoris or severe angina pectoris, which may progress to acute myocardial infarction. (*J Cardiac Fail 2007;13:874–883*)

Key Words: Angiogenesis, apoptosis, cardiac function, free radical, hepatocyte growth factor, infarct size.

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes,[1,2] was reported to have multiple actions such as mitogenic, angiogenic, antiapoptotic, and antifibrotic activities in various cells, preferentially in most epithelial and endothelial cells.[3,4] Recent studies, however, reported that human recombinant HGF is cardioprotective: HGF protected cardiomyocytes from acute ischemic death during acute myocardial infarction[5,6] and enhanced the survival of cardiomyocytes subjected to oxidative stress.[7,8] Taniyama et al.[9] recently reported the beneficial effects of HGF on cardiac function in an animal model of cardiomyopathy through its angiogenic and antifibrotic actions. Furthermore, we reported that postinfarction treatment with an adenoviral vector expressing HGF relieves chronic left ventricular remodeling and dysfunction in mice.[10] However, whether in vivo HGF gene transfer before ischemia reduces myocardial infarct size and improves left ventricular

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dysfunction and its precise mechanisms are still unknown. The hypothesis in the present study is that in vivo HGF gene transfer may reduce the myocardial infarct size and improve cardiac function via the antioxidant, angiogenic, antiapoptotic, and antifibrotic actions of HGF.

Methods

In this study, all rabbits received humane care in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Animal Selection

Japanese white male rabbits, each weighing approximately 2.0 to 2.5 kg, were used. None of the rabbits had any clinically evident infections.

Surgical Preparation

Rabbits were anesthetized with an intravenous injection of sodium pentobarbital (30–40 mg/kg), and additional doses were given when required throughout the experiment. They were orally intubated and ventilated with room air supplemented with a low flow of oxygen by mechanical ventilation (tidal volume 25–35 mL, respiratory rate 20–30 breaths/min) (Shimano, model SN-480-5, Tokyo, Japan). Serial blood gas analysis was performed, and ventilatory conditions were adjusted to keep the arterial blood gas within the physiologic range. Surgery was performed under sterile conditions. The carotid artery and jugular veins were cannulated to monitor the peripheral arterial pressure and to administer drugs. Next, the rabbits were systemically heparinized (500 U/kg). A thoracotomy was performed in the left third intercostal space, and the heart was exposed after excising the pericardium. A 4-0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle segment of the anterolateral surface of the left ventricle (LV). A small vinyl tube was passed into both ends of the silk suture, and the coronary branch was occluded by pulling the snare, which was fixed by clamping the tube with a mosquito hemostat. Myocardial ischemia was confirmed by ST-segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snare. All rabbits were allowed 20 minutes after completion of the surgical preparation to reach a steady state before starting the protocol.

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 using the in vitro ligation method (a gift from Mark A. Kay, MD, Stanford University School of Medicine) as described previously.[11] Control Ad-LacZ was prepared as described previously.[12]

Measurement of Human HGF Level in the Plasma and Cardiac Tissues

The plasma and cardiac tissue levels of human HGF were measured using an enzyme-linked immunosorbent assay kit (Institute of Immunology, Tokyo, Japan). The detection threshold was 5 pg/mL.

Expression of HGF in the Heart Detected by Immunoblotting

After 48 hours of myocardial infarction, rabbit hearts were divided into ischemic and nonischemic areas, and homogenized in lysis buffer on ice. Proteins (100 μg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Membranes were incubated with antibodies, antihuman HGF (R&D Systems Inc., Minneapolis, Minnesota), and β-actin (Sigma Chemical Co., St. Louis, Missouri), and subsequently with horseradish peroxidase-conjugated anti-goat immunoglobulin-G or anti-mouse immunoglobulin-G antibody (Dako Cytomation Inc., Carpinteria, California). Immunoreactive bands were detected using the chemiluminescent substrate (Amersham Biosciences, Piscataway, New Jersey) according to the manufacturer's protocol.

Protocol 1

After a thoracotomy was performed in the left third intercostal space and the heart was exposed after excising the pericardium, Ad.CAG-HGF (1×10^9 pfu/rabbit) was injected into the myocardium (HGF group). In the LacZ group, adenovirus encoding the LacZ gene was similarly injected into the myocardium. In the saline control group, saline was similarly injected into the myocardium. Three days after viral infection or saline injection, the rabbits underwent 30 minutes of coronary occlusion and 4 hours, 48 hours, or 14 days of reperfusion. In the sham group, rabbits received only a thoracotomy without induction of infarction. Hemodynamic parameters (systolic blood pressure, diastolic blood pressure, and heart rate) were monitored throughout the experiment. After the experiment, the chest was closed and the rabbits were allowed to recover from anesthesia for 48 hours or 14 days survival. The incidence of deoxyuride-5′-triphosphate biotin nick end labeling (TUNEL)-positive myocytes and the expression of Bcl-2 protein were detected after 4 hours of reperfusion. The myocardial infarct size was measured at 48 hours of reperfusion. The left ventricular function and the incidence of microvessels with positive CD31 were assessed after 14 days of reperfusion.

Physiologic Studies. Echocardiograms were recorded with an echocardiographic system (Aloka, Tokyo, Japan) equipped with a 7.5-MHz imaging transducer 14 days after myocardial infarction. After cardiac echocardiography, a micromanometer-tipped catheter (SPR 407, Millar Instruments, Houston, Texas) was inserted into the LV for recording ±dP/dt.

Infarct Size Measurement. Forty-eight hours after reperfusion, the rabbits were heparinized (500 μ/kg) and killed with an overdose of pentobarbital. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded, and Evans blue dye (4%, Sigma Chemical Co.) was injected into the aorta at 80 mm Hg. The area at risk was defined as the area without blue staining. The LV was sectioned into seven slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of triphenyl tetrazolium chloride at 37°C to visualize the infarct area, and photographed. The risk and infarct

areas were traced on each LV slice and multiplied by the slice's weight, then expressed as a fraction of the risk area or LV for each heart.

Histologic Analysis. The rabbits were killed at 4 hours, 48 hours, and 14 days after infarction. The hearts were removed and cut into seven transverse slices, and each slice was fixed with 10% buffered formalin and embedded in paraffin; 4-μm–thick sections were stained with hematoxylin-eosin and Masson's trichrome. Quantitative assessment of the fibrotic area as a percentage of the LV was performed using a multipurpose color image processor, LUZEX F (Nireco, Kyoto, Japan). The fibrotic area was measured within the infarct region.

Immunohistochemical Analysis. By using an indirect immunoperoxidase method, immunohistochemical staining was performed with monoclonal mouse antihuman CD31, endothelial cell antibody (Dako), and monoclonal mouse antihuman Bcl-2 (Dako), which cross-react with rabbit tissues. Morphometric analyses were performed by two persons blinded to the treatment. Hematoxylin-eosin staining was also performed in each slice, and localization was made on the basis of hematoxylin-eosin staining. The capillary density and Bcl-2–positive cardiomyocytes were counted in the ischemic area of the ventricle. There were 20 high-power fields (400×) for one slide. The capillary density was shown as the number of capillaries per high-power field, and the Bcl-2–positive cardiomyocytes were shown as a percentage of the total counts.

TUNEL. TUNEL assay was performed in deparaffinized 4-μm–thick sections with an ApopTag kit (Intergene, Purchase, New York). We counted 10,000 myocytes per heart. In the present study, we focused on the TUNEL-positive myocytes because we previously reported that TUNEL-positive myocytes are frequently observed at 4 hours of reperfusion after 30 minutes of ischemia in rabbits.[13]

Protocol 2

Measurement of Myocardial Interstitial Hydroxyl Radicals. Fourteen rabbits were used to investigate the effect of HGF gene transfer on the amount of myocardial interstitial hydroxyl radicals during ischemia-reperfusion. A microdialysis probe (PNF 1700; Asahi Medical, Tokyo, Japan; 20 mm in length, 0.31 mm OD, 0.2 mm ID; transverse type, 50,000 MW cutoff) for dialysate sampling was implanted in the risk region of the myocardium, which was served by the anterolateral coronary artery along the axis of the ventricular fibers and reached from the epicardial outer layer to the endocardial inner layer of the myocardium. Probe placement was confirmed at autopsy. The microdialysis probe was perfused with 1 mmol/L of salicylic acid dissolved in Ringer's solution at a rate of 10 μL/min. After a 60-minute rest following the completion of instrumentation, the dialysate was sampled during 30 minutes of pre-ischemia, 30 minutes of ischemia, and 30 minutes of reperfusion with intervals of 10 minutes in the saline (n = 7), LacZ (n = 7), and HGF (n = 7) groups. Dialysate samples were frozen at −83°C until further analysis. The measurement of the hydroxyl radical is based on the reaction between salicylic acid and hydroxyl radical; 1 mmol/L salicylic acid can trap approximately 10% of the theoretically generated hydroxyl radical, producing 2,3-dihydroxybenzoic acid (DHBA), 2,5-DHBA, and catechol as the derivatives in proportions of 49%, 40%, and 11%, respectively.[14] In the present study, we used 2,5-DHBA as an indicator of hydroxyl radical production because of its high specificity for hydroxyl radical.[15] The column used in the present study was an MCM C18 column (6 × 250; 5-120A; MC Medical Inc., Tokyo, Japan). The 2,5-DHBA, an indicator of hydroxyl radicals, was measured using high-performance liquid chromatography coupled with electrochemical detection, as described previously[14,15] with slight modifications.

Statistical Analysis

Data are expressed as the group mean ± standard error of the mean. To compare the group means of hydroxyl radical levels, area at risk, infarct size, and hemodynamic parameters, one-way analysis of variance (ANOVA) was performed. If the ANOVA result was significant, a modified unpaired t test was performed to assess which group was significantly different. The effects of treatments on hemodynamics were analyzed with one-way repeated-measures ANOVA. A post-ANOVA adjustment was made by the Bonferroni method. Differences with a P value less than .05 were considered statistically significant.

Results

Mortality and Animal Exclusion

Initially, 92 rabbits were enrolled in protocol 1. Among these rabbits, eight died of ventricular fibrillation during ischemia-reperfusion (Table 1). Of the remaining rabbits, eight died after the first day of the experiment. Thus, the experiments were completed in the remaining 76 rabbits, and the data from these animals were used for the analysis.

X-gal Staining

Figure 1 shows X-gal staining of the LV infected with Ad-LacZ. Hematoxylin-eosin staining showed that myocytes were stained blue, revealing the adenovirus-infected myocytes.

Plasma and Cardiac Tissue Levels of Human HGF

The cardiac tissue level of human HGF level reached 263 ng/g tissue 3 days after the viral transfection in the HGF-treated rabbits but not in the LacZ-treated rabbits. No human HGF was detected in the plasma, both in the LacZ-treated and HGF-treated rabbits.

Table 1. Survival Rate

Group	No.	VF	Premature Death	Survivors (%)
Sham	5	0	0	5/5 (100)
Saline 4 h	6	1	0	5/6 (83)
LacZ 4 h	7	1	0	6/7 (85)
HGF 4 h	6	0	0	6/6 (100)
Sham	5	0	0	5/5 (100)
Saline 48 h	10	1	1	8/10 (80)
LacZ 48 h	10	1	1	8/10 (80)
HGF 48 h	8	0	1	7/8 (87)
Sham	5	0	0	5/5 (100)
Saline 14 d	10	1	2	7/10 (70)
LacZ 14 d	10	2	1	7/10 (70)
HGF 14 d	10	1	2	7/10 (70)

VF, ventricular fibrillation; HGF, hepatocyte growth factor.

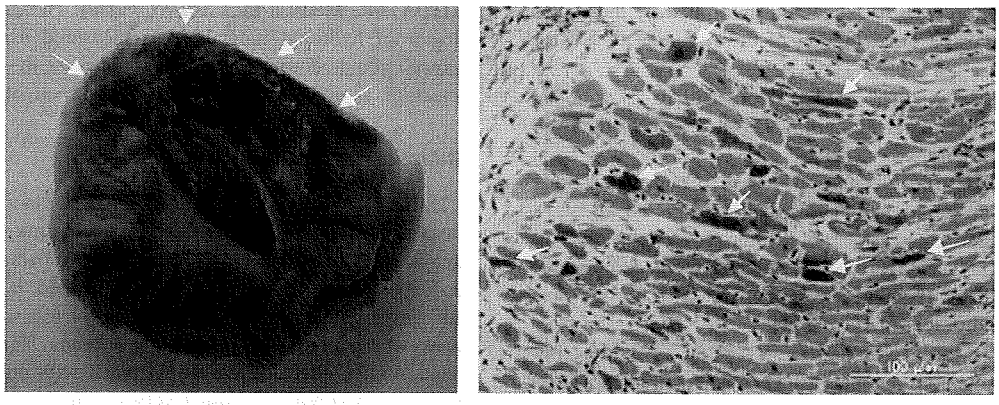

Fig. 1. X-gal staining of LV infected with Ad-LacZ. Macroscopic image (*left*). Cardiac tissues were stained blue, suggesting the infection of adenovirus. Myocytes were stained blue, suggesting the infection of adenovirus (*right*). Infection of adenovirus (*arrows*). Cardiac tissue was obtained 48 hours after myocardial infarction.

Immunoblot Analysis

As shown in Figure 2, the expression of human HGF protein was confirmed in the ischemic area in the HGF group but not in the LacZ group.

Physiologic Studies

According to echocardiography and cardiac catheterization 14 days post-myocardial infarction, the saline-treated and LacZ-treated rabbits showed decreased cardiac function, decreased LV ejection fraction percentage, LV fractional shortening percentage, and ±dP/dt (Fig. 3). HGF gene therapy significantly improved each of these conditions, indicating the improvements of post-infarct cardiac function. There was no difference in systolic and diastolic blood pressures and heart rate among the saline, LacZ, and HGF groups before, during, and after 30 minutes of ischemia.

Pathologic Studies

Infarct Size. There was no significant difference in the area at risk as a percentage of the LV between the LacZ and HGF groups. The infarct size as a percentage of the area at risk was significantly reduced in the HGF group (13.4% ± 2.3%) compared with the LacZ group (36.5% ± 2.0%) and saline group (40.3% ± 3.2%) 48 hours after infarction (Fig. 4).

TUNEL-Positive Myocytes. HGF gene therapy resulted in a significant reduction of the incidence of TUNEL-positive myocytes (2.6% ± 1.0%, $P < .05$) compared with the saline group (10.8% ± 1.9%) and LacZ group (11.1% ± 2.2%) (Fig. 5).

Immunohistochemical Staining. The capillary density in the infarcted area assessed by immunostaining of CD31 was significantly higher in the HGF group than in the saline and LacZ groups 14 days after infarction (Fig. 6). The expression of Bcl-2 was significantly increased in the risk area after 4 hours of reperfusion in the HGF group compared with the saline and LacZ groups (Fig. 7).

Fibrotic Area. Fibrotic area as a percentage of the LV was significantly reduced in the HGF group compared with the saline and LacZ groups (Fig. 8).

Effect on Myocardial Interstitial 2,5-DHBA Levels. As shown in Figure 9, the myocardial interstitial levels of 2,5-DHBA, an indicator of the hydroxyl radical, were significantly increased at 10 and 20 minutes after the start of coronary occlusion and peaked at 10 minutes after the start of reperfusion compared with the preischemic period in the saline and LacZ groups. However, HGF gene therapy significantly attenuated the increase of the myocardial interstitial 2,5-DHBA levels during ischemia and reperfusion periods.

Discussion

The present study revealed that in vivo adenovirus-mediated HGF gene transfer before ischemia reduced the

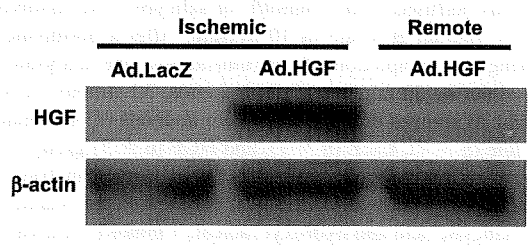

Fig. 2. Expression of HGF in the heart detected by immunoblotting. The expression of human HGF protein was observed in the ischemic area of the LV in the HGF group but not in the LacZ group. The expression of human HGF was not observed in the remote area of the LV in the HGF group. Cardiac tissue was obtained 48 hours after myocardial infarction. HGF, hepatocyte growth factor.

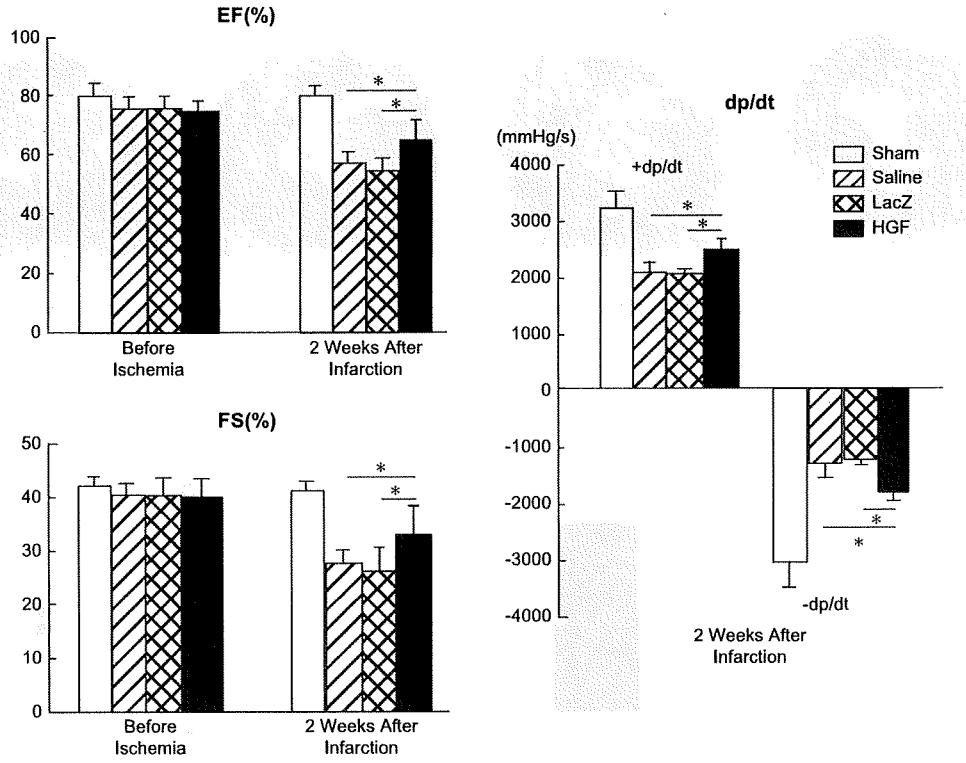

Fig. 3. Hemodynamic parameters assessed by echocardiography and cardiac catheterization 14 days after infarction. Note the improvement of ejection fraction, fractional shortening, and ± dp/dt in the HGF group. EF, ejection fraction; FS, fractional shortening; HGF, hepatocyte growth factor. $*P < .05$.

myocardial infarct size and improved left ventricular function via multiple beneficial actions, such as antioxidant, antiapoptotic, and antifibrotic actions, and enhancement of Bcl-2 expression and angiogenesis.

Expression of Human HGF

With the use of enzyme-linked immunosorbent assay and Western blot analysis, we detected human HGF in the cardiac tissue of HGF-treated rabbits but not in the LacZ-treated rabbits. We could not detect human HGF in the plasma or cardiac tissue in the LacZ group. However, Nakamura et al.[5] reported increased plasma levels of HGF after myocardial ischemia-reperfusion. This discrepancy may be explained by the fact that we used adenoviral vector plasmid, which produces human HGF, and antihuman HGF antibody (R&D Systems, Inc., Minneapolis, Minnesota) to assess whether human HGF was produced in the plasma and cardiac tissue of the rabbit, whereas Nakamura et al. used anti-rat HGF antibody to detect the plasma level of HGF in a rat model of myocardial infarction.

In the present study, we did not examine how long the expression of "exogenous" HGF continued in the myocardium of the rabbits. However, we previously reported that adenoviral vector-mediated transfer of HGF increased plasma levels of human HGF persistently for 4 weeks in mice.[16]

Mechanisms of Beneficial Effects of HGF on Infarct Size and Left Ventricular Function

The mechanisms responsible for the beneficial effects of HGF on the infarct size and left ventricular function seemed to be complicated, probably reflecting multifunctions of HGF. It has been reported that recombinant human HGF administered immediately after reperfusion after 20 minutes of ischemia reduced the infarct size and improved cardiac function through antiapoptotic effects in rats.[5] This is consistent with the result of the present study, although Nakamura et al.[5] used recombinant human HGF and we used adenovirus-mediated HGF gene transfer. In vitro studies showed that HGF has an antioxidant effect.[7,8] Gene transfer of human cDNA HGF has been reported to improve cardiac function in isolated rat hearts.[6] Moreover, it has been reported that HGF induces angiogenic and antifibrotic effects in a model of cardiomyopathy and heart failure.[9] The results of the present study demonstrated that gene transfer of HGF before ischemia 1) reduced the production of hydroxyl radical during ischemia and reperfusion, 2) reduced the incidence of TUNEL-positive myocytes, 3) enhanced the expression of Bcl-2 protein, 4) increased angiogenesis, 5) reduced the fibrotic area, 6) reduced the infarct size, and 7) improved LV dysfunction.

As mentioned above, the present study demonstrated that in vivo gene transfer of HGF before ischemia showed

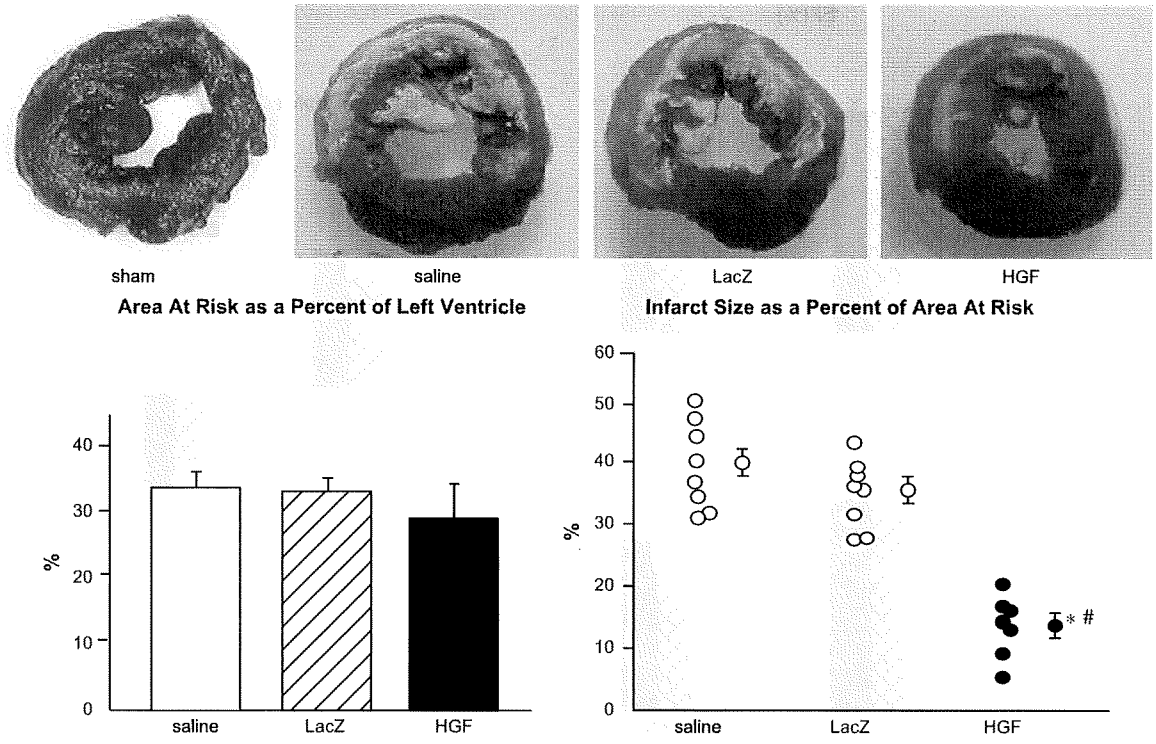

Fig. 4. Infarct size as a percentage of the LV. Typical photographs of the LV stained with triphenyl tetrazolium chloride and Evans blue dye (*top*). Note that the infarct area is smaller in the HGF group than in the LacZ group. Area at risk as a percentage of LV (*left*). Infarct size as a percentage of area at risk (*right*). Note that the infarct size was significantly reduced in the HGF group than in the LacZ group. Cardiac tissue was obtained 48 hours after myocardial infarction. HGF, hepatocyte growth factor. * $P < .05$ versus control. #$P < .05$ versus LacZ group.

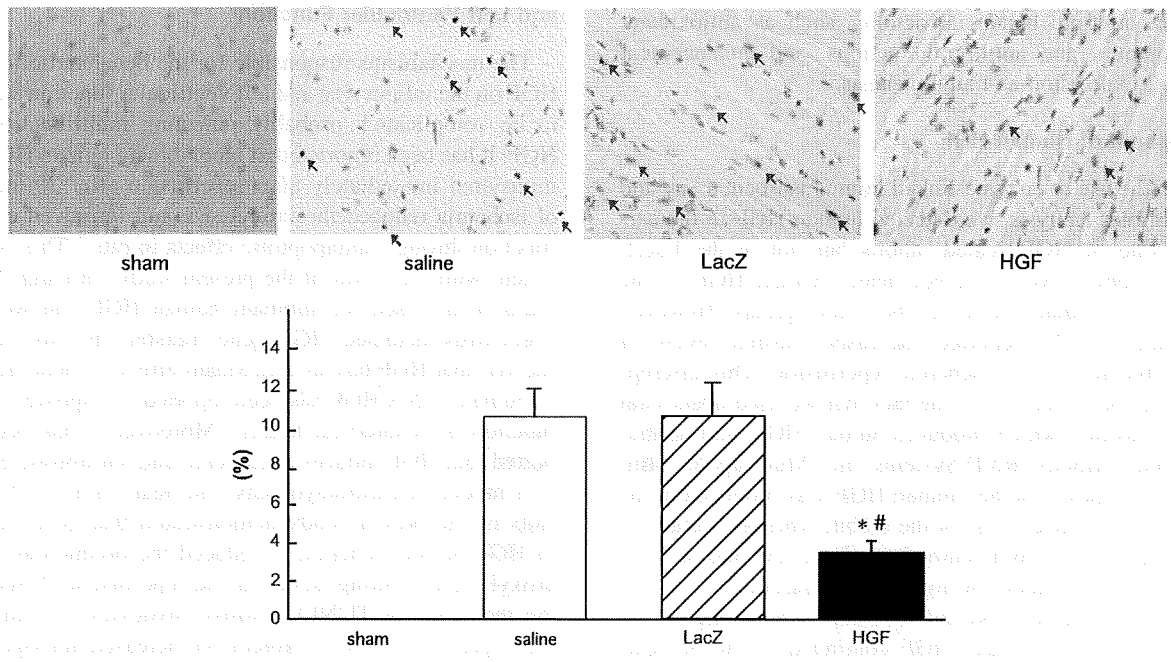

Fig. 5. TUNEL-positive myocytes in the infarcted myocardium. Photomicrograph (light microscopic TUNEL analysis) of myocardium from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 4 hours of reperfusion (×400) (*top*). Brown TUNEL-positive nuclei are seen in the infarcted area. Incidence of TUNEL-positive myocytes in the LacZ and HGF groups (*bottom*). *$P < .05$ versus LacZ group. #$P < .05$ saline group. HGF, hepatocyte growth factor.

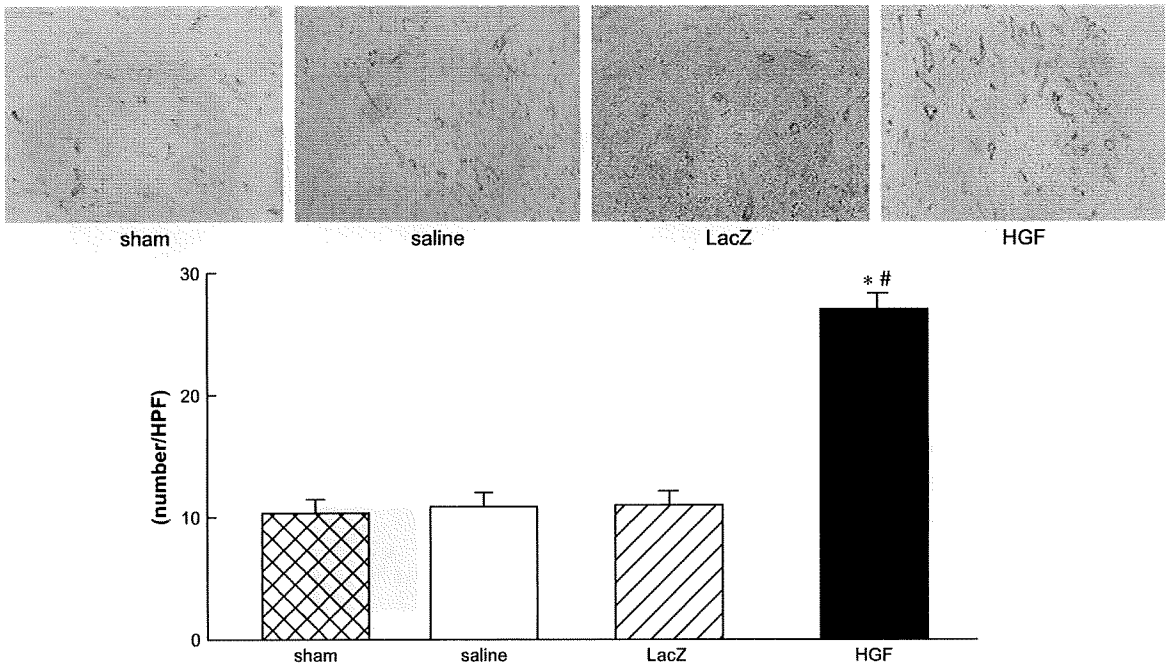

Fig. 6. Capillary density assessed by immunostaining of CD31. Photomicrograph of myocardium immunostained with CD31 from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Brown stained microvessels are seen in the infarcted area. Incidence of CD31-positive microvessels in the LacZ and HGF groups (*bottom*). *$P < .05$ versus LacZ group. #$P < .05$ saline group. HPF, high-power field; HGF, hepatocyte growth factor.

multiple beneficial actions, and all of these effects might contribute to the reduction in the infarct size and the improvement of left ventricular dysfunction after infarction. Among the multiple effects of HGF, the most remarkable finding in the present study was that HGF gene therapy strikingly decreased hydroxyl radical during ischemia-reperfusion. Reactive oxygen species, such as superoxide and hydroxyl radicals, have been suggested to be

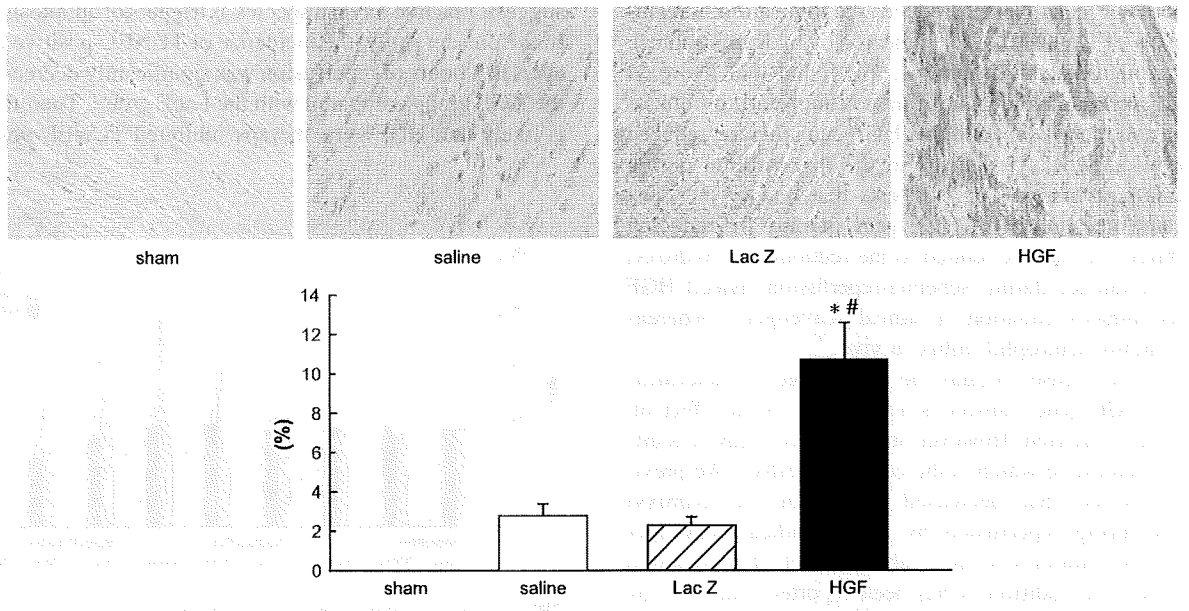

Fig. 7. Expression of Bcl-2 protein in the infarcted myocardium. Photomicrograph of myocardium immunostained with Bcl-2 from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Brown stained myocytes are seen in the infarcted area. Incidence of Bcl-2–positive myocytes in the LacZ and HGF groups (*bottom*). Bcl-2 protein was predominantly expressed at the border of infarcted and intact areas. *$P < .05$ versus LacZ group. #$P < .05$ saline group. HGF, hepatocyte growth factor.

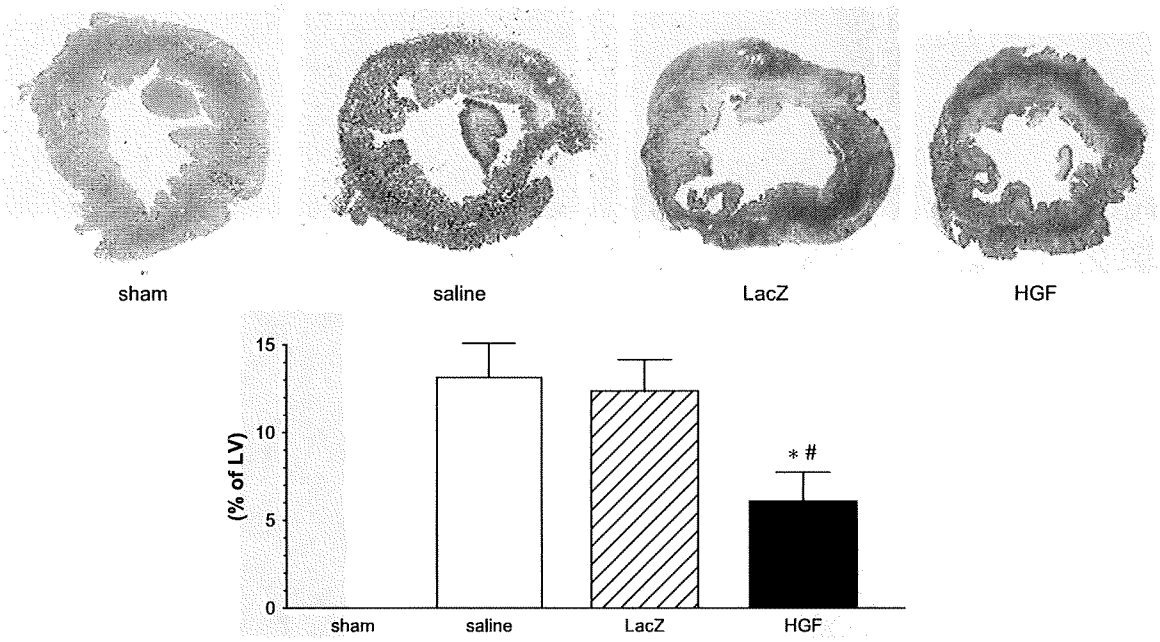

Fig. 8. Fibrosis area determined by Masson's trichrome staining. Photomicrograph of myocardium with Masson's trichrome staining from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Blue stained areas are seen in the infarcted area. The fibrosis area as a percentage of LV in the LacZ and HGF groups (*bottom*). $*P < .05$ versus LacZ group. $\#P < .05$ saline group. LV, left ventricle; HGF, hepatocyte growth factor.

predominant mediators of ischemia-reperfusion injury.[17] Among the reactive oxygen species, the hydroxyl radical is highly reactive and plays a critical role in post-ischemic myocardial damage during reperfusion.[18] We investigated whether HGF gene therapy reduces the myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radical, using a microdialysis technique. We found that hydroxyl radicals were generated in the interstitium during both ischemia and reperfusion and that HGF gene therapy reduced the level of hydroxyl radicals in the myocardium during ischemia-reperfusion. This suggests that one of the most important mechanisms of HGF gene therapy for reducing the infarct size may be related to the reduction of hydroxyl radical production during ischemia-reperfusion. Indeed, HGF directly induces glutation, a radical scavenger,[19] whereas HGF inhibits neutrophil influx in vivo.[20]

In a strict sense, it may be impossible to determine whether HGF gene transfer is a cause of, or an effect of, the infarct limitation. However, in an in vivo study it is difficult to determine which is the cause and effect. We previously reported that decreased production of hydroxyl radicals during reperfusion by a free radical scavenger reduced the infarct size in a rabbit model of myocardial infarction.[21] In addition, it has been reported that HGF by itself has an anti-oxyradical effect.[7,8] Therefore, it may be possible that HGF gene therapy, which produced human HGF in the ischemic area, attenuated the level of hydroxyl radicals during ischemia-reperfusion and led to the infarct limitation.

It has been reported that the reactive oxygen species released from myocytes after ischemia-reperfusion may trigger both necrosis and apoptosis.[22] Free radical scavengers have been reported to inhibit the appearance of apoptosis, which suggests reactive oxygen species as triggers of apoptosis.[23,24] In the present study, the incidence of TUNEL-positive myocytes at 4 hours of reperfusion was significantly decreased in the HGF group compared with the LacZ group. Therefore, it is likely that HGF gene therapy inhibited TUNEL-positive

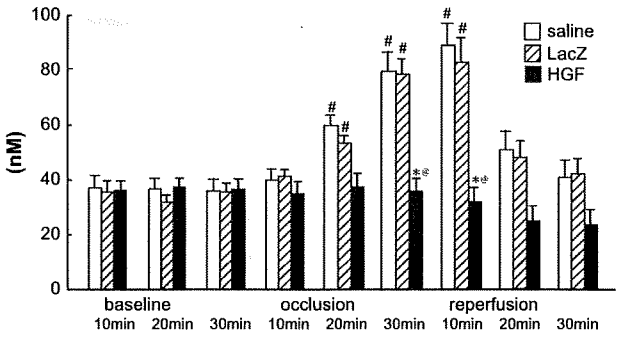

Fig. 9. Effect of HGF gene transfer on myocardial interstitial 2,5-DHBA levels, an indicator of hydroxyl radicals before, during 30 minutes of ischemia, and 30 minutes of reperfusion at an interval of 10 minutes. $*P < .05$ versus LacZ group. $\#P < .05$ versus baseline value. $@P < .05$ versus saline group. HGF, hepatocyte growth factor.

myocytes by reducing the burst of oxygen free radicals such as hydroxyl radicals.

Apoptosis is governed by a member of the regulating genes mediated by apoptotic signals. Bcl-2 is one of the members of the Bcl-2 family, such as Bcl-2, Bcl-XL, and Bcl-w, which act as inhibitors of apoptosis. Cytochrome C released from the mitochondria has been reported to bind to Apaf-1 to activate caspase 9, which can cleave and activate caspase 3, leading to the formation of various death substrates that produce apoptosis.[25] Bcl-2 can interfere with cytochrome C release and suppress the actions of Apaf-1 and provide protection from apoptosis.[26] It has been reported that ischemia followed by reperfusion induced a time-dependent reduction in the expression of Bcl-2 protein.[27] In the present study, HGF gene therapy significantly enhanced the expression of Bcl-2 protein in the ischemic area. Therefore, the antiapoptotic effect of HGF gene therapy may be attributed to the up-regulation of Bcl-2 expression. HGF has been shown to activate GATA4,[28] and GATA-4 can regulate Bcl-2 expression;[29] thus, GATA-4 may be involved in the overexpression of Bcl-2.

It has been reported that HGF treatment enhances angiogenesis as assessed by vessel density[10,30] after myocardial infarction. In the present study, we also found that the incidence of CD31-positive microvessels in the infarcted area was significantly higher in the HGF group than in the LacZ group, suggesting that HGF gene transfer increased the angiogenesis in the infarcted area. This might have contributed to the reduction in the infarct size and the improvement of cardiac function via an increase of myocardial regional blood flow. HGF has also been reported to have an antifibrotic effect in several organs, such as the liver, kidney, lung, and heart.[10,30–34] We also demonstrated that the fibrosis area determined by Masson's trichrome staining 14 days after myocardial infarction was significantly smaller in the HGF group than in the LacZ group, suggesting that HGF gene transfer has an antifibrotic effect on post-infarction myocardium. However, reduced fibrosis and preserved function may be an effect of reduced infarct size. The precise cellular mechanism by which HGF reduces the infarct size still remains to be investigated. However, one of the likeliest candidates for the infarct size-reducing effect by HGF may be an effect of decreasing oxyradicals, such as hydroxyl radicals, during ischemia-reperfusion because free radical scavengers that scavenge hydroxyl radicals have been reported to reduce the infarct size.[21,35]

Study Limitations

Cytokines other than HGF were not examined in the present study. However, several cytokines are known to influence cardiac function and may play important roles, particularly in pathologic situations such as acute myocardial infarction.[36,37] It is possible that HGF modulates the ischemia-reperfusion injury and post-infarct process through interaction with other cytokines. In the present study, we did not perform an experiment on survival. Whether the protective effects of HGF gene therapy translate into survival benefits still remains to be elucidated.

Clinical Implications

The present findings demonstrate a novel therapeutic strategy against unstable angina pectoris or severe angina pectoris, which may progress into acute myocardial infarction despite conventional therapy.

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最新学際情報

増殖制御型アデノウイルスによる遺伝子治療

癌はいまだわが国を含む多くの先進諸国の最多死因であり，わが国だけでも毎年約32万人が癌で死亡している。つまり近年に治療成績の向上がみられる早期癌とは対照的に，多発性遠隔転移の進行癌にはいまだ効果的な治療法は確立されておらず，そのため遺伝子治療のような革新的治療法の開発が切望されている。

遺伝子治療の臨床成績は，まず90年代にレトロウイルスベクターによる*ex vivo*遺伝子治療(切除癌に*in vitro*の培養下でサイトカインなどの治療遺伝子を導入し，放射線で増殖不能化したのちに体内に戻す)が行われた。しかしこれは不十分な治療効果に加え，多大な労力に伴う治療の不確実性と高額の経費の問題もあり，一般医薬化には至っていない。

90年代後半より，*in vivo*遺伝子導入(直接癌部にベクター注入)を可能とする非増殖型アデノウイルス(ADV)ベクターが，癌遺伝子治療の中心ベクターとなり，臨床試験数も増加してきた[1]。現在まで世界で約1,300の遺伝子治療の臨床プロトコールが発表されているが，その2/3は癌であり，使用されているベクターはADVが最多である。このような臨床試験(研究)での結論は，「遺伝子治療は癌には安全な一般医薬となりうるが，治療効果は当初期待されたレベルには達していない」ということである。この原因の1つは，「非」増殖型のベクターでは*in vivo*で体内の全癌細胞にもれなく治療遺伝子を導入することは「物理的に」不可能であるため，癌の再発が起こりえるという問題である。

この問題を克服するため，現在世界中で盛んに研究が進められているのが，癌特異的増殖型ADV(CRA)である。CRAは，ウイルス増殖が正常細胞では阻止され，癌細胞内では旺盛に起こるように遺伝子改変されたADVで，生体内で効率的かつ癌細胞特異的な遺伝子導入が可能となる[2]。またさらにCRA自身が，増幅したウイルス(蛋白)により癌細胞を特異的に殺す「溶解性ウイルス」医薬となる利点も併せもつ。その原理は，非増殖型ADVではウイルス増殖に必須のE1領域を欠損させて治療遺伝子に置換していたが，CRAではE1領域を改変(一部欠失変異化と，内因性プロモーターの置換の2戦略あり)することで，ウイルス増殖が癌のみで起こるようにするというものである[2]。CRAは基礎研究，臨床研究の両方でその有用性が示されている一方で，完全に理想的なCRAを開発するためには，2つの問題が残っていた。第1には，このような一因子で癌と正常の細胞を完全に識別するレベルの癌特異化は困難ということである。また最大の問題は，このような一因子制御のCRAでさえ，効率的・標準化作製技術が確立されていないため，研究開発がきわめて非効率ということであった。

著者らはこの問題を克服するため，従来の単一因子のCRAとは一線を画く「多数」の異なる癌特異化因子で，精密なウイルス増殖制御が可能なCRAであるm-CRAを，迅速・効率的に作製可能な「標準化」作製技術を初めて開発した[3]。その原理と作製法の詳細は拙著[2,3]に譲るが，これにより7因子以上の癌特異化因子を挿入する次世代のm-CRAが作製可能となった。

著者らはこの独自技術でさまざまな癌治療m-CRA医薬を作製，評価しているが，本稿ではサバイビン依存性m-CRAを紹介する[4]。サバイビンはIAP(inhibitor of apoptosis)ファミリーとして同定されたが，その後，骨軟部腫瘍を含むほとんどの種類の癌で高発現している一方，分化した正常細胞では発現が認められないことがわかり，現在は癌治療のターゲット分子としても注目されている。著者らはこのサバイビン遺伝子プロモーターでADVのE1を発現制御する

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Surv.m-CRAを開発した。

Surv.m-CRAは調べた全種類の癌細胞で，癌細胞特異的なウイルス増殖と細胞死を誘導し，さらに骨肉腫の動物モデルで高い治療効果を示した。さらに既報告のCRAのなかでは最良のテロメラーゼ(TERT)依存性m-CRA(Tert.m-CRA)と詳細な比較実験をしたところ，Surv.m-CRAはTert.m-CRAを，癌治療効果と癌特異性(安全性)の両面でしのぐという有望な成果が得られた[3),4)]。このように骨軟部腫瘍はもとより，ほぼ全種類の癌を効率よく安全に治療でき，既存のm-CRAより優れたSurv.m-CRAは，早期の臨床応用化が期待される。また，癌特異性(安全性)も癌治療効果もさらに増殖した高度m-CRA化の種々の改良型Surv.m-CRA，あるいは第二，第三弾の新規m-CRAの開発も進めている。

将来のわが国の国民福祉と経済の向上につながるのは，「基盤技術からわが国で開発し，基本特許の知財を確保した医薬」であるため，著者らのm-CRAの医薬化は重要であると思われる。遺伝子治療の臨床化の公的支援の体制が十分でないわが国では困難もあるが，著者ら自身でのm-CRA医薬開発と併せて，わが国全体のm-CRA研究開発の発展に寄与できる体制づくりや産業化も計画している。(堀川良治／小宮節郎 鹿児島大学大学院医歯学総合研究科運動機能修復学講座整形外科学，小戝健一郎　鹿児島大学大学院医歯学総合研究科細胞生物構造学講座)

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教授就任記念講演
先端医学開発の研究と医学教育

小　戝　健一郎

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教授就任記念講演

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Ⅰ．緒　　言

遺伝子治療と再生医学は21世紀の最先端医療の代表として，また脳の高次機能の解明は21世紀の生物学の最大の課題として，いずれも世界中で盛んな研究開発が行われている。本稿では，私の取り組んでいるこの三大分野の研究において，遺伝子治療は「癌遺伝子治療における独自ベクターと新規治療法の開発」，再生医学は「生体内再生医学とES細胞での再生医学」，脳は「Rett症候群の病態解明・治療法の開発と高次脳機能のエピジェネティック分子制御の解明」の研究について，その背景や方向性も踏まえて紹介したい。

Ⅱ．遺伝子治療

(1)　第一，第二世代の癌遺伝子治療

遺伝子治療は，(1982年に行われた無謀な臨床試験を除けば) 1990年に米国で行われた臨床試験が最初であり，まだ20年にも満たない新しい医療である。但しその後は現在まで世界で1000以上の臨床プロトコールが発表・実施され，一般医薬も近々発売されるといわれているように，臨床化のスピードは決して遅くない。その臨床プロトコールの４分の３は癌であるように，現時点の遺伝子治療の代表的な対象疾患は癌である。一方，既存の癌治療法の現状は，近年の診断，治療技術の進歩により，早期の癌に関しては治療成績の向上がみられている。しかし進行癌，特に末期の遠隔転移癌に対しては，既存治療法では限界があるのは明白であり，このため遺伝子治療のような革新的治療法の開発が切望されているのである。今回は，我々の種々の疾患に対する遺伝子治療研究のうち，癌に対する研究開発について紹介する。

まず癌遺伝子治療の歴史を概説すると，第一世代の癌遺伝子治療は，1980年代に基礎研究が進み，90年代に臨床研究が行われたレトロウイルスベクターによる*ex vivo*遺伝子治療である。これは切除した癌に，*in vitro*の培養下にレトロウイルスベクターでサイトカイン遺伝子などを導入し，放射線で増殖不能化した後に体内に戻し，抗腫瘍免疫を賦活化するという戦略である。しかし臨床試

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験で明らかな治療効果が見られなかった上に，一人の患者の治療に多大な労力と多額の経費がかかるということから，一般医療化されるには至らなかった。

私が米国留学した1990年初頭は，現在は癌遺伝子治療の主流のベクターとなったアデノウイルスベクター（ADV）が開発されたばかりであり，第二世代の癌遺伝子治療となる，*in vivo*遺伝子治療（癌結節に直接ベクターを注射して遺伝子導入する）の研究が限られた専門施設で始まっていた。我々の米国ベイラー医科大学の研究グループは，世界に先駆けADVを用いた「コンビネーション癌遺伝子治療法」を開発した[1,2]。これは強力な癌細胞死誘導効果を持つ自殺遺伝子の単純ヘルペスウイルス・チミジンキナーゼ（HSV-tk）遺伝子と，種々のサイトカイン遺伝子を導入・発現する2～3種のADVを同時に癌結節に注入し，その後にガンシクロビル（GCV）を投与するという戦略である。HSV-tk遺伝子/GCVは癌細胞優位な殺傷，強力なバイスタンダード効果（僅か数%の癌細胞に遺伝子導入できれば結節内の多くの癌細胞が細胞死に陥る）で腫瘍を減らすと同時に癌抗原を散らばらせ，そこでサイトカインで免疫細胞を誘導すれば，細胞性免疫を中心とする特異的な全身性抗腫瘍免疫が効率的に誘導できるという，理想的な免疫療法である。一部は米国の共同研究者が臨床試験を行っているが，私自身もその後は本邦で，自殺遺伝子の至適発現レベル[3]，サイトカインの至適発現レベル[4]，同所性モデルでの前臨床研究など[5,6]，臨床化における新たな重要事項を明らかにしてきた。

(2) 第三世代癌遺伝子治療の癌優位増殖型アデノウイルス（CRA）と，我々が開発した次世代の「多因子による癌特異的増殖制御型アデノウイルス」(m-CRA)

前述のように，第一世代，第二世代の癌遺伝子治療は米国を中心に数多く臨床試験（研究）がなされてきたわけであるが，その結論は，「遺伝子治療は癌には安全で一般医療の一つと成り得るが，但しその治療効果は当初期待されたような癌治療のブレークスルーというレベルではない」というものである。治療効果が劇的なものになっていない主因の一つは，*in vitro*でいくら遺伝子導入効率の高いベクターでも，「非」増殖型のベクターでは*in vivo*で体内の全癌細胞にもれなく遺伝子を導入することは「物理的」に不可能である（ベクター液が達しない癌細胞には当然，遺伝子は導入されない）ため，遺伝子「未」導入癌細胞からの再発が起こりえるからである。この問題を認識していない戦略も数多く臨床試験がなされ，その結果が社会的失望も招いてしまった感もあるが，ただ我々は当初よりこの問題を最大の克服課題と正確に捉え，前述のコンビネーション遺伝子治療のように，当初より「遺伝子未導入癌細胞も治療可能な戦略」を開発してきた。

さてこの問題を根本的に解決するものとして近年期待されているのが，癌特異的に増殖する変異ウイルスの開発であり，その中でも特に癌優位増殖型ADV（CRA; Conditionally replicating adenovirus）の研究が盛んである[7,8]。CRAは，ウイルス増殖が正常細胞では阻止され，癌細胞内では旺盛に起こるため，生体内で高効率かつ癌細胞特異的な遺伝子導入を可能とするものである。またさらにCRA自身が，癌細胞内で増幅されたウイルス蛋白により癌細胞を特異的に殺す「溶解性ウイルス療法」の医薬となる利点も併せ持つため，CRAは新世代の癌遺伝子治療として期待されている[7,8]。

その要点のみ述べると，「非」増殖型ADVベクターではウイルス増殖に必須のE1領域を治療遺伝子に置換する方法をとっているが，CRAはこのE1領域を改変することでウイルス増殖を制御し，癌と正常の細胞でのウイルス増殖に違いを持たせるというものである（図1）。E1領域の一部欠失変異化と，内因性プロモーターを癌特異的遺伝子プロモーターへ置換するという二つの戦略があるが，両者とも基礎研究，臨床試験で良好な結果が示されている。その一方，たかだか一（あるいは二）因子で癌特異化を試みる既存のCRAでは，癌と正常の細胞を「完全に」識別可能とするレベルの癌特異化は困難で，特に正常細胞でも僅かながらウイルスが増殖するという潜在的な問題が残されていた。また最大の問題は，CRAに関しては未だ効率的・標準化作製技術が確立されていないことであり，このためCRAの開発研究は一部の専門施設に限られ，その「手作り」状態は多大な時間と労働力を要するため，研究は極めて非効率ということであった。真のCRAを開発するために我々は，従来の単一因子で癌特異化を試みるCRAとは一線を画す「多数」の異なる癌特異化因子で精密なウイルス増殖の制御が可能なCRA（m-CRA）を，簡単・迅速・効率よく作製，改良可能な

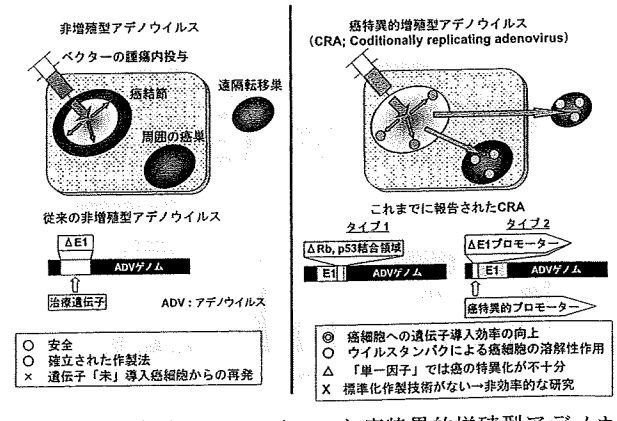

図1．非増殖型アデノウイルスと癌特異的増殖型アデノウイルス（CRA）の比較

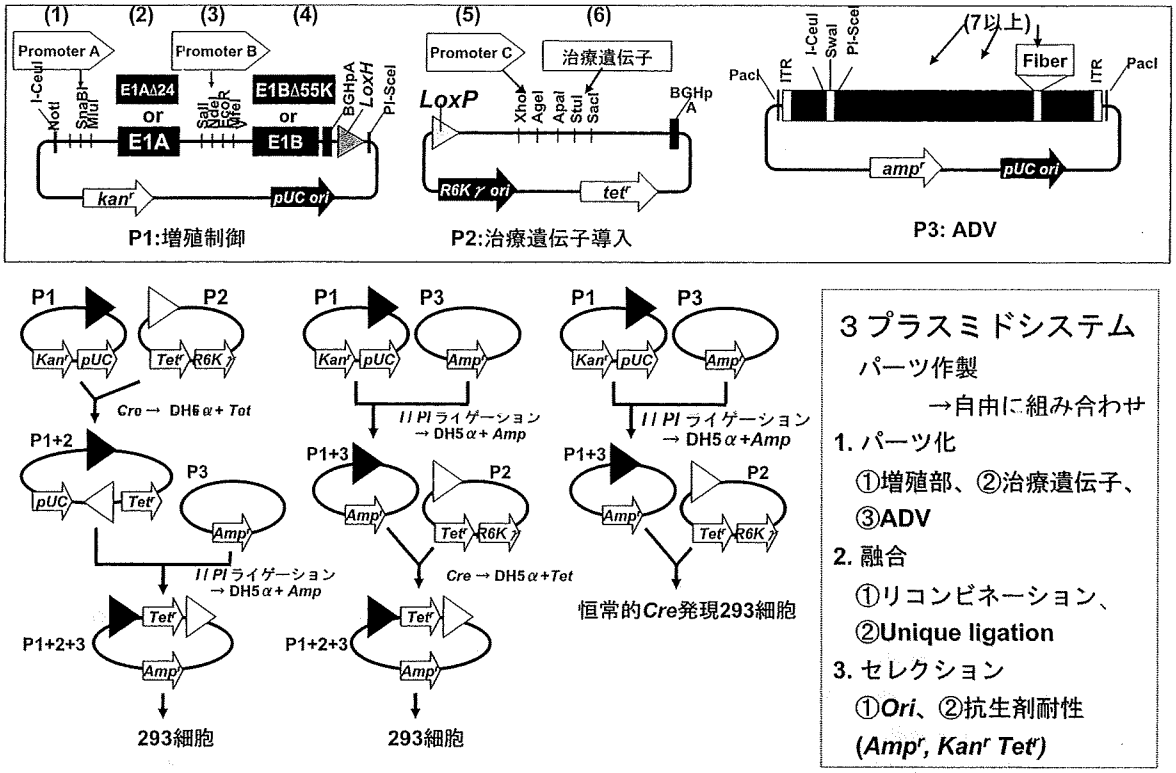

図２．我々が開発したm-CRA作製技術

「標準化」作製技術を独自開発した[7-9]。その基本的な発想は，「それぞれのパーツを独立して作製し，後で自由に組み合わせる」という「３プラスミドシステム」である（図２）。つまりウイルス増殖制御部（P1），治療（導入）遺伝子（P2），ADVゲノム（P3）の３要素を独立した３つのプラスミドに収載させて各パーツの個別の自由設計を可能とし，様々な遺伝子組換え技術を導入することで，簡単・確実にこの３プラスミドを融合させ一つのm-CRA（プラスミド）にできるようにした。これにより７因子以上の癌特異化因子の挿入／ADVの修飾が，各プラスミドの通常の遺伝子組換え作業で簡単に行うことが可能となった。

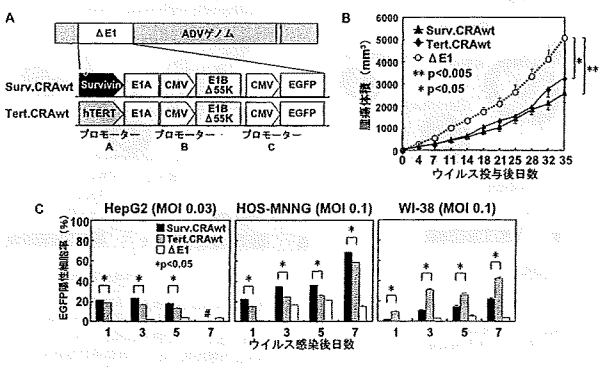

図３．サバイビン依存性m-CRAとテロメラーゼ依存性m-CRAの性能比較

我々はこのように基盤となるm-CRA作製技術から開発し，実際にこの技術で革新的な癌治療m-CRA医薬として様々なものを作製し，機能を評価しているが，本稿では第一弾となるサバイビン依存性m-CRAを紹介する（図３）[10]。サバイビンはIAP（Inhibitor of apoptosis）ファミリーとして同定されたが，その後，サバイビンはほとんどの種類の癌で高発現している一方，分化した正常細胞では発現がみとめられないことが分かった。さらにサバイビンの発現レベルと癌患者の予後が相関するということも分かり，現在はサバイビン自体が癌治療の新たなターゲット分子として注目されている。我々は，サバイビン遺伝子プロモーターでADV E1Aを発現制御するサバイビン依存性m-CRA（Surv.m-CRA）を開発し，実際にこのSurv.m-CRAは極めて高い癌治療効果と癌特異性の両面を兼ね備える画期的な新規CRAであることを明らかにした[10]。さらに我々は，これまでに報告されたCRAの中では最良のテロメラーゼ（TERT；telomerase reverse transcriptase）依存性m-CRA（Tert.m-CRA）も同様に作製し，詳細な比較実験まで行った（図３）。その結果，Surv.m-CRAはTert.m-CRAを，癌治療効果と癌特異性（即ちウイルス増殖と細胞死誘導効果が，癌細胞ではより旺盛である一方，逆に正常細胞ではより消退する）の「両面」で凌ぐということが明確となり，つまりSurv.m-CRAは現時点では最高性能を持つ新規CRAの一つという有望