the patients participating in exercise training. In addition, in those studies, exercise training was started relatively late (3–8 weeks) after the onset of infarction, which is not recent trend of early discharge and early return to work after AMI?[4] Thus, factors predicting subsequent LV remodeling in patients participating in exercise CR, starting early (approximately 2 weeks) after the onset of the AMI remain unknown. Recently, we demonstrated that patients with a low LVEF do not have aggravated LV remodeling after moderate intensity exercise training starting 2 weeks after the onset of AMI?[5] However, neurohumoral or exercise variables were not analyzed in that study.

*Present Study*

Compared with previous studies, the present study is unique because it comprehensively analyzed the predictive factors of LV remodeling using clinical, angiographic, neurohumoral, and exercise variables at baseline in postinfarction patients participating in exercise CR. As far as we know, no previous study has performed such a comprehensive analysis to identify determinants of postinfarction LV remodeling.

In addition, the present study is unique because our exercise rehabilitation program starts relatively early (approximately 2 weeks) after the onset of AMI. Although this timing may not be very early when compared with the recent trend of early discharge (3–5 days) after AMI,[4] it is much earlier than the timing (3–8 weeks after the onset) in the previous EAMI and the ELVD studies.

*Plasma BNP Concentration and Ventricular Remodeling*

The present findings that anterior infarct location and baseline plasma BNP concentration are major influencing factors of LV remodeling are in accordance with previous reports[12–14] We have shown in our previous studies that an initial elevation (>100 pg/ml on the 7th day after onset) and a subsequent sustained elevation (percentage decrease <25% from 30th to 90th day) of plasma BNP are reliable predictors of progressive LV remodeling after acute MI.[13,14] The present result indicates that our previous findings also hold true in patients participating in exercise CR after AMI. In addition, the present results suggest that patients with both anterior infarct location and an elevated BNP concentration (>500 pg/ml) at baseline are at high risk of subsequent LV remodeling.

Hama et al reported that the expression of the rat ventricular BNP gene after MI mainly occurred at the border of the infarcted region, where mechanical wall stress may be maximal?[26,27] Cerisano et al reported that LV dilatation occurring early after infarction is predictable from the Doppler-derived mitral deceleration time indicating an elevated LV filling pressure?[28] These findings suggest that a high plasma BNP concentration may reflect elevated LV wall stress, which is likely to accelerate LV remodeling and may explain why a high plasma BNP concentration is predictive of LV remodeling. Although the determination coefficient ($r^2$=0.09) of plasma BNP concentration for LV remodeling was not remarkably high in the present study, the results suggest that plasma BNP concentration is one of many determinants of the complex process of LV remodeling.

*Exercise Intensity and Ventricular Remodeling*

The present study found that exercise variables representing intensity, frequency, and total amount of exercise in CR do not affect LV remodeling, even when the exercise training is started relatively early (approximately 2 weeks) after the onset of AMI. The reasons for this result may be two-fold. First, the impact of the plasma BNP concentration is so powerful that the influence of exercise intensity or frequency on ventricular remodeling is masked. In other words, a transient increase in LV wall stress by exercise training may have no or only a small impact on the development of LV remodeling compared with the impact of baseline wall stress determined by infarct size. This possibility is supported by the result of the EAMI study showing that LV remodeling developed in patients with low LVEF regardless of exercise training?[9]

A second potential explanation is that the prescribed exercise intensity (40–60% of heart rate reserve) and frequency were at appropriate levels for not aggravating LV remodeling. In fact, a slightly lower exercise intensity (40–50% of heart rate reserve) was prescribed for patients with low LVEF (<40%) in the present study. This suggests that exercise intensity or frequency is not associated with LV remodeling, as long as they are within an appropriate range in the CR program. If more vigorous and excessive exercise had been imposed, LV remodeling might have occurred, a possibility that is supported by previous experimental results showing that vigorous swimming exercise in rats after a large MI aggravated LV remodeling?[29,30] whereas a moderate level of exercise either did not adversely affect[31] or even favorably attenuated LV remodeling in the same rat model?[32]

*Clinical Implications*

On the basis of the present results, plasma BNP concentration and infarct location are useful tools for predicting the likelihood of LV remodeling before beginning an exercise CR program after AMI. The present results also suggest that a moderate exercise intensity (50–60% heart rate reserve) for patients with LVEF >40% and a slightly lower intensity (40–50% heart rate reserve) for those with LVEF <40% may be safe and appropriate for average patients participating in exercise CR after AMI. Because patients with an anterior infarction and BNP concentration >150 pg/ml at approximately 2 weeks after the onset (especially, >500 pg/ml) are at high risk for subsequent LV remodeling and these patients often overlap with those with LVEF <40%, a relatively low level of exercise intensity (40–50% of heart rate reserve) is recommended for these patients. A low to moderate level (50% of peak $\dot{V}O_2$) of exercise has been reported to increase exercise capacity while minimizing ventricular wall stress in patients with LV dysfunction?[33] To determine whether a higher level of exercise intensity (50–70% heart rate reserve) aggravates LV remodeling, further studies are needed.

*Study Limitations*

The present study was a prospective observational study, and did not have a sedentary control group. Although this might have affected the association between exercise intensity and LV remodeling, the wide variation of exercise intensity and frequency does allow us to analyze correlations between exercise variables and delta-LVDd.

A one-dimensional measure, such as delta-LVDd, has a certain limitation in the assessment of 3-dimensional LV remodeling. We intentionally avoided LV end-systolic dimension (LVDs) as an index of LV remodeling, because LVDs suffers from a greater error than LVDd when local

asynergy of LV wall motion exists?[25]

The presence of myocardial ischemia may have affected both delta-LVDd and peak $\dot{V}O_2$; however, we excluded patients presenting with myocardial ischemia in the initial exercise test.

## Conclusion

In patients with AMI participating in exercise CR, baseline plasma BNP concentration and anterior infarct location, but not exercise intensity or frequency, are predictive factors of the development of LV remodeling. Patients with both anterior infarction and a BNP concentration >150 pg/ml at approximately 2 weeks after onset are at high risk for subsequent LV remodeling.

*Acknowledgment*

*This study was supported in part by the Research Grants for Cardiovascular Diseases (11C-7, 13C-3) from the Ministry of Health, Labor and Welfare, Japan.*

## References

1. Wenger NK, Froelicher ES, Smith K, Ades PA, Berra K, Blumenthal JA, et al. Cardiac rehabilitation: Clinical practice guideline No.17. Rockville, MD: US Department of Health and Human Services, Public Health Service, Agency for Health Care Policy and Research and the National Heart, Lung, and Blood Institute; AHCPR publication no. 96–0672, October 1995.
2. Fletcher GF, Balady GJ, Amsterdam EA, Chaitman B, Eckel R, Fleg J, et al. AHA scientific statement: Exercise standards for testing and training: A statement for healthcare professionals from the American Heart Association. *Circulation* 2001; **104:** 1694–1740.
3. Specchia G, De Servi S, Scire A, Assandri J, Berzuini C, Angoli L, et al. Interaction between exercise training and ejection fraction in predicting prognosis after a first myocardial infarction. *Circulation* 1996; **94:** 978–982.
4. Oldridge N, Furlong W, Feeny D, Torrance G, Guyatt G, Grove J, et al. Economic evaluation of cardiac rehabilitation soon after cardiac rehabilitation. *Am J Cardiol* 1993; **72:** 154–161.
5. Arvan S. Exercise performance of the high risk acute myocardial infarction patient after cardiac rehabilitation. *Am J Cardiol* 1988; **62:** 197–201.
6. Sullivan MJ, Higginbotham MB, Cobb FR. Exercise training in patients with severe left ventricular dysfunction: Hemodynamic and metabolic effects. *Circulation* 1988; **78:** 506–515.
7. Tavazzi L, Ignone G. Short-term haemodynamic evolution and late follow-up of post infarct patients with left ventricular dysfunction undergoing a physical training programme. *Eur Heart J* 1991; **12:** 657–665.
8. Jugdutt BI, Michorowski BL, Kappagoda CT. Exercise training after anterior Q wave myocardial infarction: Importance of regional left ventricular function and topography. *J Am Coll Cardiol* 1988; **12:** 362–372.
9. Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, et al. Long-term physical training and left ventricular remodeling after anterior myocardial infarction: Results of the Exercise in Anterior Myocardial Infarction (EAMI) trial. EAMI Study Group. *J Am Coll Cardiol* 1993; **22:** 1821–1829.
10. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L, for the ELVD Study Group. Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction. *Circulation* 1997; **96:** 1790–1797.
11. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction: Experimental observations and clinical implications. *Circulation* 1990; **81:** 1161–1172.
12. Gaudron P, Eiles C, Kugler I, Ertl G. Progressive left ventricular dysfunction and remodeling after myocardial infarction: Potential mechanisms and early predictors. *Circulation* 1993; **87:** 755–763.
13. Nagaya N, Nishikimi T, Goto Y, Miyao Y, Kobayashi Y, Morii I, et al. Plasma brain natriuretic peptide is a biochemical markaer for the prediction of progressive ventricular remodeling after acute myocardial infarction. *Am Heart J* 1998; **135:** 21–28.
14. Nagaya N, Goto Y, Nishikimi T, Uematsu M, Miyao Y, Kobayashi Y, et al. Sustained elevation of plasma brain natriuretic peptide levels associated with progressive ventricular remodeling after acute myocardial infarction. *Clin Sci* 1999; **96:** 129–136.
15. Sonnenblick EH, Anversa P. Models and remodeling: Mechanisms and clinical implications. *Cardiologia* 1999; **44:** 609–619.
16. Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiol Rev* 1999; **79:** 215–262.
17. Bolognese L, Cerisano G. Early predictors of left ventricular remodeling after acute myocardial infarction. *Am Heart J* 1999; **138:** S79–S83.
18. Uchida I, Takaki H, Kobayashi Y, Okano Y, Satoh T, Matsubara T, et al. $O_2$ extraction during exercise determines training effect after cardiac rehabilitation in myocardial infarction. *Circ J* 2002; **66:** 891–896.
19. Sakuragi S, Takagi S, Suzuki S, Sakamaki F, Takaki H, Aihara N, et al. Patients with large myocardial infarction gain a greater improvement in exercise capacity after exercise training than those with small to medium infarction. *Clin Cardiol* 2003; **26:** 280–286.
20. Goto Y, Sumida H, Ueshima K, Adachi H, Nohara R, Itoh H. Safety and implementation of exercise testing and training after coronary stenting in patients with acute myocardial infarction. *Circ J* 2002; **66:** 930–936.
21. Karvonen M, Kentala K, Mustala O. The effects of training on heart rate: A longitudinal study. *Annales Medicinae Expenrimentalis et Biologial Fennial* 1957; **35:** 307–315.
22. Borg G. Perceived exertion as an indicator of somatic stress. *Scand J Rehabil Med* 1970; **2:** 92–98.
23. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, et al. Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: Application of magnetic resonance imaging. *Circulation* 1997; **95:** 2060–2067.
24. Newby LK, Eisenstein EL, Califf RM, Thompson TD, Nelson CL, Peterson ED, et al. Cost effectiveness of early discharge after uncomplicated acute myocardial infarction. *N Engl J Med* 2000; **342:** 749–755.
25. Otsuka Y, Takaki H, Okano Y, Satoh T, Aihara N, Matsumoto T, et al. Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunction early after acute myocardial infarction. *Int J Cardiol* 2003; **87:** 237–244.
26. Hama N, Itoh H, Shirakami G, Nakagawa O, Suga S, Ogawa Y, et al. Rapid ventricular induction of brain natriuretic peptide gene expression in experimental acute myocardial infarction. *Circulation* 1995; **92:** 1558–1564.
27. Bogen DK, Rabinowitz SA, Needleman A, McMahon TA, Abelmann WH. An analysis of the mechanical disadvantage of myocardial infarction in the canine left ventricle. *Circ Res* 1980; **47:** 728–741.
28. Cerisano G, Bolognese L, Carrabba N, Buonamici P, Santoro GM, Antoniucci D, et al. Doppler-derived mitral deceleration time: An early strong predictor of left ventricular remodeling after reperfused anterior acute myocardial infarction. *Circulation* 1999; **99:** 230–236.
29. Oh BH, Ono S, Gilpin E, Ross J Jr. Altered left ventricular remodeling with beta-adrenergic blockade and exercise after coronary reperfusion in rats. *Circulation* 1993; **87:** 608–616.
30. Gaudron P, Hu K, Schambergen R, Budin M, Walter B, Ertl G. Effect of endurance training early or late after coronary artery occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction. *Circulation* 1994; **89:** 402–412.
31. Alhaddad IA, Hakim I, Siddiqi F, Lagenback E, Mallavarapu C, Nethala V, et al. Early exercise after experimental myocardial infarction: Effect on left ventricular remodeling. *Coron Artery Dis* 1998; **9:** 319–327.
32. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, et al. Favorable left ventricular remodeling following large myocardial infarction by exercise training: Effect on ventricular morphology and gene expression. *J Clin Invest* 1995; **96:** 858–866.
33. Demopoulos L, Bijou R, Fergus I, Jones M, Strom J, LeJemtel TH. Exercise training in patients with severe congestive heart failure: Enhancing peak aerobic capacity while minimizing the increase in ventricular wall stress. *J Am Coll Cardiol* 1997; **29:** 597–603.

# Adrenomedullin Gene Transfer Induces Therapeutic Angiogenesis in a Rabbit Model of Chronic Hind Limb Ischemia

## Benefits of a Novel Nonviral Vector, Gelatin

Noriyuki Tokunaga, MD; Noritoshi Nagaya, MD; Mikiyasu Shirai, MD; Etsuro Tanaka, MD; Hatsue Ishibashi-Ueda, MD; Mariko Harada-Shiba, MD; Munetake Kanda, MD; Takefumi Ito, MD; Wataru Shimizu, MD; Yasuhiko Tabata, PhD; Masaaki Uematsu, MD; Kazuhiro Nishigami, MD; Shunji Sano, MD; Kenji Kangawa, PhD; Hidezo Mori, MD

***Background***—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety of cardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigated whether AM gene transfer induces therapeutic angiogenesis in chronic hind limb ischemia.

***Methods and Results***—Ischemia was induced in the hind limb of 21 Japanese White rabbits. Positively charged biodegradable gelatin was used to produce ionically linked DNA-gelatin complexes that could delay DNA degradation. Human AM DNA (naked AM group), AM DNA-gelatin complex (AM-gelatin group), or gelatin alone (control group) was injected into the ischemic thigh muscles. Four weeks after gene transfer, significant improvements in collateral formation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with the control group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limb perfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highest content of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles was minimal in the AM-gelatin group.

***Conclusions***—AM gene transfer induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases. **(*Circulation*. 2004;109:526-531.)**

**Key Words:** peripheral vascular disease ■ angiogenesis ■ gene therapy ■ ischemia

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[1] AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells.[2–4] AM not only induces vasorelaxation but also regulates growth and death of these vascular cells.[5–10] These findings suggest that AM plays an important role in maintaining vascular homeostasis in an autocrine and/or paracrine manner.

A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis.[11–13] More recently, AM has been shown to activate the PI3K/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[7,14] These results raise the possibility that AM plays a role in modulating vasculogenesis and angiogenesis. However, whether AM induces therapeutic angiogenesis remains unknown.

Received May 20, 2003; revision received September 25, 2003; accepted September 26, 2003

From the Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan (N.T., M.S., M.K., H.M.); the Department of Cardiovascular Surgery, Okayama University Medical School, Okayama, Japan (N.T., S.S.); the Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan (N.N., T.I.); the Department of Internal Medicine, National Cardiovascular Center, Osaka, Japan (N.N., W.S., K.N.); the Department of Physiology, the Research Center for Genetic Engineering and Cell Transplantation, Tokai University School of Medicine, Isehara, Japan (E.T.); the Department of Pathology, National Cardiovascular Center, Osaka, Japan (H.I.-U.); the Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan (M.H.-S., K.K.); the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan (Y.T.); and the Cardiovascular Division, Kansai Rosai Hospital, Hyogo, Japan (M.U.).

Correspondence to Noritoshi Nagaya, MD, Department of Regenerative Medicine and Tissue Engineering or Hidezo Mori, MD, Department of Cardiac Physiology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail nagayann@hsp.nccve.go.jp or hidemori@ri.nccve.go.jp



*Circulation* is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000109700.81266.32

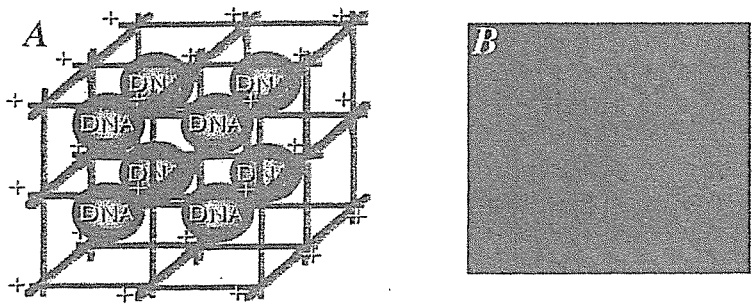



Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.[15,16] Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation.[15] Similarly, ionically linked DNA-gelatin complexes can delay gene degradation.[16] These findings raise the possibility that gelatin may serve as a nonviral vector for gene therapy.

Thus, the purposes of this study were (1) to investigate whether AM gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia and (2) to examine whether the use of biodegradable gelatin as a vector augments AM expression and thereby enhances the therapeutic effects of AM gene transfer.

## Methods

### Animal Model

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Twenty-one male Japanese White rabbits (body weight, 2.9±0.1 kg; Japan Animal Co. Osaka, Japan) were used for physiological and morphological assessment. In addition, 30 rabbits were used for radioimmunoassay, immunohistochemical examination, and Western blot analysis. After anesthetization with pentobarbital sodium (30 to 35 mg/kg), a longitudinal incision was made in the left thigh, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Hind limb ischemia was induced by ligation of the distal left external iliac artery and complete resection of the left femoral artery, as described previously.[17]

### Construction of Plasmid DNA

To construct the expression vector for human AM, the *EcoRI/XhoI* fragment of the full-length human AM cDNA was ligated into the EcoRI/XhoI fragment of the pcDNA1.1-CMV expression plasmid (Invitrogen). To verify that the pcDNA1.1-CMV vector encoding AM cDNA produces a biologically active AM protein, the expression vector was transfected into 293 cells, and AM activity in the transfected cells was measured by high-performance liquid chromatography and radioimmunoassay. The pcDNA1.1-CMV vector encoding β-galactosidase (LacZ) cDNA was used as a control DNA.

### Preparation of AM DNA-Gelatin Complex

Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30 μm, water content of 95%, and an isoelectric point (pI) of 9 after swelling in water.[15,16] Gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure (Figure 1A). Dried gelatin (4 mg, pI 9) was added to human AM DNA solution (500 μg/100 μL in phosphate-buffered saline, pH 7.4). After mixture of DNA and gelatin, DNA-gelatin complexes were incubated at 37°C for 2 hours.

To visualize incorporation of DNA into gelatin, AM plasmid DNA was labeled with rhodamine B isothiocyanate (RITC), as reported previously.[16] In brief, the coupling reaction of RITC to plasmid DNA was carried out by mixing the two substances in 0.2 mol/L sodium carbonate-buffered solution (pH 9.7), followed by gel filtration with a PD 10 column (Amersham-Pharmacia). RITC-labeled AM DNA was incorporated into positively charged gelatin (Figure 1B).

### Study Protocol

Ten days after the induction of hind limb ischemia (day 10), AM DNA (naked AM group, n=7), AM DNA-gelatin complex (AM-gelatin group, n=7), or gelatin alone (control group, n=7) was administered intramuscularly into 3 different sites in the ischemic adductor muscle and 2 different sites in the semimembranous muscle. In addition, Lac Z DNA-gelatin complex served as a control DNA (Lac Z-gelatin group, n=5). The amount of plasmid was 500 μg (1 mL) and that of gelatin was 4 mg. Morphological and angiographic analyses and measurements of calf blood pressure and laser Doppler flow were performed 4 weeks after gene transfer (day 38). After completion of these measurements, the adductor, semimembranous, and gastrocnemius muscles were weighed in each hind limb.[18] The muscle weight ratio was calculated for each muscle as follows: muscle weight ratio=muscle weight in ischemic hind limb/muscle weight in nonischemic hind limb. Specimens of the adductor muscle of the ischemic hind limb were obtained for histological examination.

### Measurement of Calf Blood Pressure

Calf blood pressure was measured on days 10 and 38 in both hind limbs with a Doppler flowmeter (Hayashi Denki Co. Ltd) and a 25-mm-wide cuff. The pulse of the posterior tibial artery was identified with the use of a Doppler probe, and the systolic blood pressure in both hind limbs was determined by standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic hind limb to that of the normal hind limb.[17]

### Laser Doppler Blood Perfusion Analysis

Blood flow of the ischemic hind limb was measured with the use of a laser Doppler blood perfusion image system (moorLDI, Moor Instruments) on day 38.

### Angiographic Analysis

Development of collateral arteries was evaluated by angiography on days 0 and 38. A 4F catheter was placed in the left internal iliac artery through the common carotid artery, and 3 mL contrast medium (Iopamiron 300, SCHERING) was injected with an automated angiography injector at a rate of 2.5 mL/s. Quantitative angiographic analysis of collateral vessel development in the ischemic hind limb was performed with the use of a 5-mm$^2$ grid overlay, as described previously.[17] The angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the ischemic medial thigh. The angiographic score was determined by 2 blinded observers.

### Morphological and Histological Examination

The degree of lower hind limb necrosis and thigh muscle necrosis was macroscopically evaluated on graded morphological scales (grade 1 to 3) for peripheral tissue damage and muscle necrosis area of the adductor, semimembranous, and medial large muscles. Capillary density of the ischemic hind limb was evaluated by alkaline phosphatase staining, as reported previously.[17] A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted under a ×40 objective. Capillary density was expressed as the mean number of capillaries per square millimeter. The number of myofibers in each field was also examined and the capillary/muscle fiber ratio calculated.

### Radioimmunoassay for Human AM

Human AM production was examined 1, 2, and 4 weeks after gene transfer in the naked AM group, AM-gelatin group, and control group (n=5 each). The muscles were harvested for radioimmunoassay and immunohistochemical examination. Immunoreactive human AM level in rabbit muscles was determined by immunoradiometric assay with the use of a specific kit (Shionogi Co, Ltd).[19] Tissue content of vascular endothelial growth factor (VEGF) was examined by ELISA kit (R&D systems).

### Immunohistochemistry for Human AM, Ki67 Antigen, and Phosphorylated Akt

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded 4-$\mu$m sections of ischemic thigh muscles 7 days after gene transfer. To elucidate AM expression after gene therapy, immunohistochemistry for human AM was performed with the use of a monoclonal antibody recognizing AM-(12–25) (1:100), as reported previously.[20] To evaluate the proliferative potential of AM, tissue sections were stained for Ki67, a marker for cell proliferation, with the use of monoclonal anti-Ki67 antibody (1:100) (DAKO). AM has recently been shown to promote proliferation of vascular endothelial cells at least in part through the PI3k/Akt pathway.[21] Thus, immunohistochemistry for phosphorylated Akt was performed with mouse monoclonal anti-phosphorylated Akt antibody (1:100) (Cell Signaling Technology).

### Western Blot Analysis

To identify Akt phosphorylation in ischemic muscles after AM gene transfer, Western blotting was performed with the use of a commercially available kit (PhosphoPlus Akt [Ser473] Antibody Kit, Cell Signaling Technology). Ischemic muscles in the 3 groups were obtained 7 days after AM gene transfer. These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the supernatant was used for Western blot analysis. The 50 $\mu$g of protein was transferred into sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane through the use of a wet blotting system. After blocking for 60 minutes, the membranes were incubated with primary antibodies (1:500) at 4°C overnight. The membranes were then incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling Technology), at a final dilution of 1:2000. Signals were detected through the use of LumiGLO chemiluminescence reagents (Cell Signaling Technology).

### Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated by 1-way ANOVA followed by Fisher's analysis, Scheffe's $F$ analysis, or Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

## Results

### Physiological and Morphological Assessment

Complete resection of the left femoral artery resulted in a similar decrease in calf blood pressure ratio among the 3 groups before the initiation of therapy (day 10) (Figure 2A). However, the calf blood pressure ratio on day 38 was highest in the AM-gelatin groups, followed by the naked AM group and subsequently the control group. The laser Doppler flow in hind limb was highest in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2B). The calf blood pressure ratio and laser Doppler flow 4 weeks after gene transfer did not significantly differ between the control group and Lac Z-gelatin group. Lower hind limb necrosis was minimal in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2C). Thigh muscle necrosis was also minimal in the AM-gelatin group. Similarly, the muscle weight ratio (ischemic/normal) on day 38 was highest in the AM-gelatin group (Table). Neither mean arterial pressure nor heart rate significantly differed among the 3 groups.

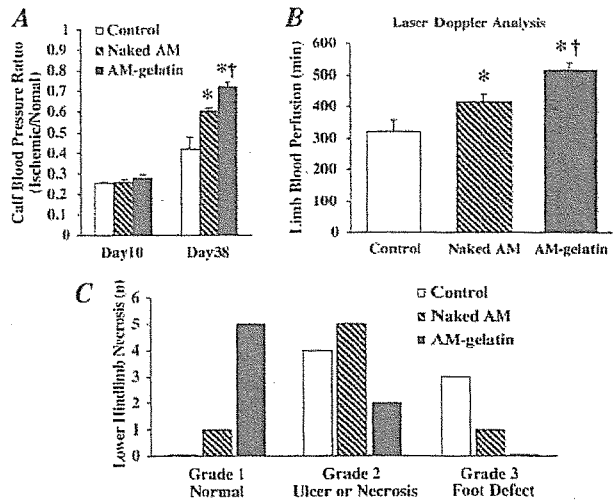



**Figure 2.** A, Calf blood pressure ratio (ischemic/normal hind limb) before (on day 10) and after (on day 38) gene transfer. B, Measurement of laser Doppler flow on day 38. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. C, Number of cases of each grade of lower hind limb necrosis on day 38. Lower hind limb necrosis was minimal in the AM-gelatin group. Number of necrosis or foot defect is statistically significant among the 3 groups ($P<0.05$ by Kruskal-Wallis test).

### Angiographic Analysis

Angiograms 4 weeks after gene transfer (day 38) showed the development of collateral arteries in the naked AM and

**Physiological Characteristics**

| | Control | Naked AM | AM-Gelatin |
|---|---|---|---|
| No. of rabbits | 7 | 7 | 7 |
| Body weight, kg | 2.46±0.06 | 2.65±0.10 | 3.16±0.09 |
| MAP, mm Hg | 112±3 | 114±3 | 116±2 |
| HR, beats/min | 269±12 | 253±5 | 262±7 |
| Muscle weight ratio | 0.71±0.03 | 0.84±0.02* | 0.95±0.02*† |

MAP indicates mean arterial pressure; HR, heart rate; and muscle weight ratio, ratio of muscle weight in ischemic hind limb to that in nonischemic hind limb. Data are mean SEM.

*$P<0.01$ vs control group; †$P<0.05$ vs naked AM group.

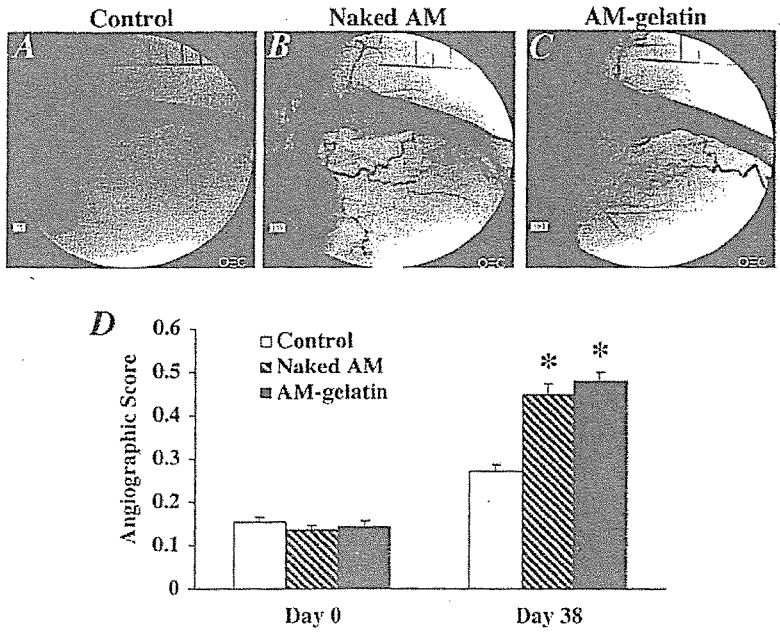



**Figure 3.** Representative angiograms of control group (A), naked AM group (B), and AM-gelatin group (C) on day 38. Collateral arteries were well developed in the naked AM and AM-gelatin groups. D, Angiographic score on days 0 and 38 in each group. Angiographic score on day 38 was significantly higher in the naked AM and AM-gelatin groups than in the control group. Data are mean±SEM. *$P<0.001$ versus control group.

AM-gelatin groups compared with that in the control group (Figure 3, A through C). Quantitative analysis of collateral vessels demonstrated that the angiographic score in both the naked AM and AM-gelatin groups was significantly higher than that in the control group (Figure 3D). Angiographic score did not significantly differ between the control group and Lac Z-gelatin group.

To examine the development of collateral vessels in an earlier stage, other rabbits (n=4 each) were examined 2 weeks after gene transfer (day 24). Angiograms showed significant collateral development in the naked AM and AM-gelatin groups compared with that in the control group.

## Histological Examination

Alkaline phosphatase staining of ischemic hind limb muscle showed marked augmentation of neovascularization in both the naked AM and AM-gelatin groups compared with the control group (Figure 4, A through C). Quantitative analysis demonstrated that capillary density of the ischemic adductor muscle was highest in the AM-gelatin group (Figure 4D). Analysis of the capillary/muscle fiber ratio yielded similar results. Seven days after gene transfer, intense immunostaining for Ki67 was observed in vascular endothelial cells of the naked AM and the AM-gelatin groups (Figure 4, E through G).

## AM Expression and Akt Phosphorylation After Gene Transfer

Seven days after gene transfer, modest immunostaining for human AM was observed in the naked AM group, whereas AM immunoreactivity was intense surrounding the gelatin in the AM-gelatin group (Figure 5, A through C). Tissue content of human AM was significantly increased both in the naked AM and the AM-gelatin groups 7 days after gene transfer (Figure 5D). The AM level in the AM-gelatin group was significantly higher that in the naked AM group. Two weeks after gene transfer, AM overexpression was observed only in the AM-gelatin group. The expression of endogenous VEGF and its receptors (Flt-1 and Flk-1) did not differ among the 3 groups (data not shown). Western blot analysis revealed that phosphorylated Akt in ischemic muscles was increased in both the naked AM and AM-gelatin groups 7 days after gene transfer (Figure 5E). Intense immunostaining for phosphory-

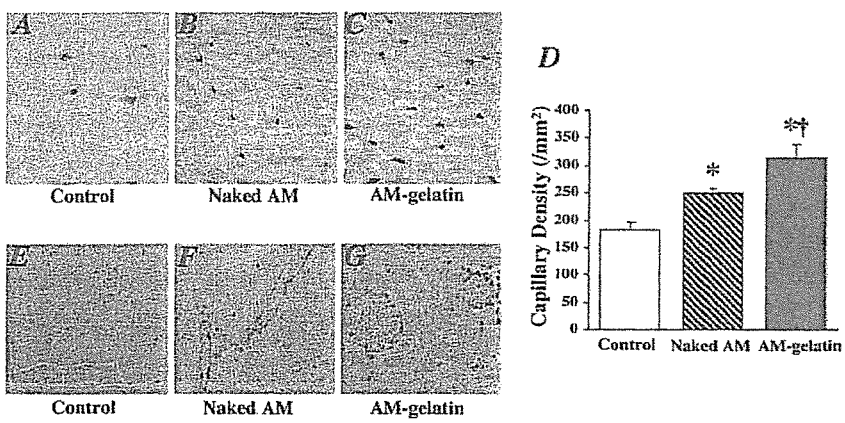



**Figure 4.** A through C, Representative examples of alkaline phosphatase staining in ischemic hind limb muscles. Magnification ×200. D, Quantitative analysis of capillary density in ischemic hind limb muscles. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. E through G, Immunohistochemical analysis of Ki67 antigen, a marker for cell proliferation. Magnification ×400.

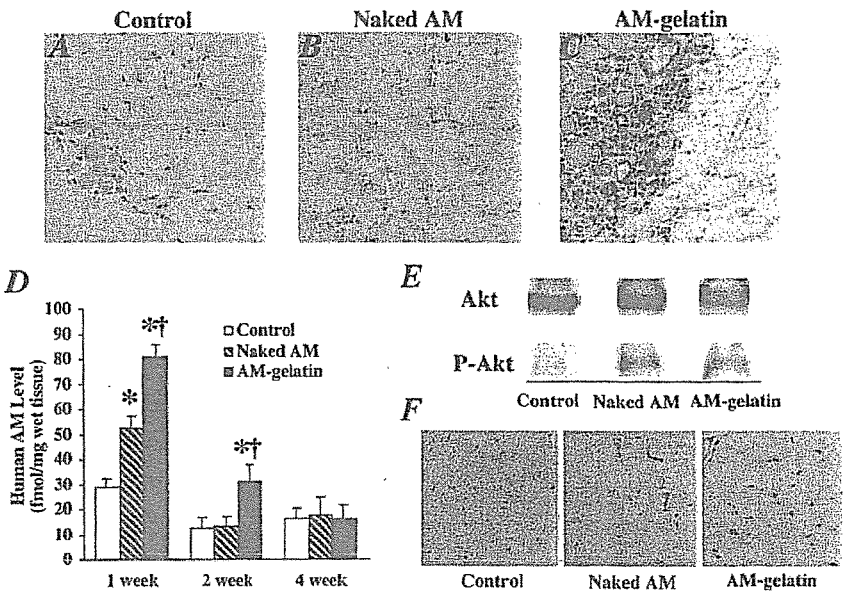



**Figure 5.** A through C, Immunohistochemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in ischemic muscles after gene transfer. Data are mean±SEM. *$P<0.01$ vs control group; †$P<0.01$ vs naked AM group. E, Western blot analysis for Akt phosphorylation in muscles. F, Immunohistochemical staining for phosphorylated Akt 7 days after gene transfer. Phosphorylated Akt was distributed at least in endothelial cells. Magnification ×400.

lated Akt was observed at least in endothelial cells of the Naked AM and the AM-gelatin groups (Figure 5F).

## Discussion

We demonstrated that (1) AM gene transfer induced hemodynamic and angiographic improvements in association with an increase in capillary density in a rabbit model of chronic hind limb ischemia. We also demonstrated that (2) administration of AM DNA-gelatin complexes markedly augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer.

AM has a variety of effects on the vasculature that include vasodilation,[1,5–7] inhibition of endothelial cell apoptosis,[8,9] and regulation of smooth muscle cell proliferation.[10] However, whether AM has angiogenic potential has remained unknown. In the present study, intramuscular administration of naked AM DNA augmented AM production in skeletal muscles, as indicated by increased tissue content and significant immunostaining of AM. As a result, AM gene transfer increased hind limb perfusion and ameliorated lower hind limb and thigh muscle necrosis in a rabbit model of hind limb ischemia. AM gene transfer may protect the ischemic hind limb partly by improving the blood flow in the ischemic hind limb because AM is originally identified as a potent vasodilating peptide.[1] Nevertheless, angiographic collateral development and high capillary density were observed in ischemic muscles after AM gene transfer. Ki67, a marker for cell proliferation, was detected in endothelial cells of microvessels after AM gene transfer. These results suggest that AM overproduction resulting from gene transfer may induce angiogenesis in a rabbit model of hind limb ischemia. Recent studies using AM gene knockout mice have shown that AM is essential for development of the vasculature during embryogenesis.[11–13] These studies support our results that AM may be an angiogenic factor. VEGF is known to induce angiogenesis and to regulate endothelial cell survival through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[22] Thus, the PI3K/Akt pathway is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[14] A recent study has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells at least in part through the PI3K/Akt pathway.[21] The present study demonstrated that phosphorylated Akt is increased at least in endothelial cells after AM gene transfer. AM gene transfer did not influence endogenous VEGF and its receptors. Taken together, it is interesting to speculate that AM may directly induce angiogenesis through the PI3K/Akt pathway.

In the present study, we used positively charged biodegradable gelatin as a nonviral vector. We have shown that basic fibroblast growth factor (bFGF) is ionically linked with gelatin, which enhances the angiogenic effects of bFGF by delaying protein degradation.[15] Thus, biodegradable gelatin has been used as a carrier of protein. However, little information is available regarding the therapeutic potential of gelatin as a nonviral vector for gene transfer. In the present study, we demonstrated that RITC-labeled AM DNA was incorporated into positively charged gelatin. In addition, intramuscular administration of AM DNA-gelatin complexes strongly enhanced AM production compared with that of naked AM DNA. These results suggest that biodegradable gelatin may serve as a vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hind limb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, laser Doppler flow, and muscle weight ratio and a decrease in necrosis of lower hind limb and thigh muscles. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. The angiogenic effects of AM-gelatin complexes were comparable to those of bFGF-gelatin complexes (data not shown). AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complex is readily phagocytosed by mac-

rophages, monocytes, endothelial progenitor cells, and so on, resulting in gene expression within these phagocytes.[23,24] These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer.

Currently, a highly efficient and safe gene delivery system is needed for gene therapy in humans. The present study demonstrated that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhanced the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases. However, the initial success of gelatin-mediated AM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

### Study Limitation

First, histological capillary density, calf blood pressure ratio, and laser Doppler flow were significantly higher in the AM-gelatin group than in the naked AM group. However, the angiographic score did not significantly differ between the two. This discrepancy raises the possibility that conventional angiography may have insufficient resolution to fully visualize the angiogenic microvessels. Second, human AM level was slightly elevated in the control group. This implies that the anti-human AM antibody used in this radioimmunoassay had some cross-reactivity with endogenous rabbit AM. Nevertheless, human AM level in the muscles was highest in the AM-gelatin group within 2 weeks after gene transfer. These results suggest that AM DNA-gelatin complexes induces potent and long-lasting AM production.

### Conclusions

Intramuscular administration of AM DNA induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases.

## Acknowledgments

This work was supported by a grant from the Japan Cardiovascular Research Foundation, HLSRG-RAMT-nano-001 and -RHGTEFB-genome-005, RGCD13C-1 from MHLW, grants from NEDO, a Grant-in-Aid for Scientific research from MECSST (13470154 and13877114), and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

## References

1. Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun*. 1993;192:553–560.
2. Sugo S, Minamino N, Kangawa K, et al. Endothelial cells actively synthesize and secrete adrenomedullin. *Biochem Biophys Res Commun*. 1994;201:1160–1166.
3. Sugo S, Minamino N, Shoji H, et al. Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem Biophys Res Commun*. 1994;203:719–726.
4. Kato J, Kitamura K, Kangawa K, et al. Receptors for adrenomedullin in human vascular endothelial cells. *Eur J Pharmacol*. 1995;289:383–385.
5. Shimekake Y, Nagata K, Ohta S, et al. Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and $Ca^{2+}$ mobilization, in bovine aortic endothelial cells. *J Biol Chem*. 1995;270:4412–4417.
6. Nagaya N, Satoh T, Nishikimi T, et al. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation*. 2000;101:498–503.
7. Nishimatsu H, Suzuki E, Nagata D, et al. Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. *Circ Res*. 2001;89:63–70.
8. Kato H, Shichiri M, Marumo F, et al. Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology*. 1997;138:2615–2620.
9. Sata M, Kakoki M, Nagata D, et al. Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. *Hypertension*. 2000;36:83–88.
10. Kano H, Kohno M, Yasunari K, et al. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J Hypertens*. 1996;14:209–213.
11. Shindo T, Kurihara Y, Nishimatsu H, et al. Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation*. 2001;104:1964–1971.
12. Caron KM, Smithies O. Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *Proc Natl Acad Sci U S A*. 2001;98:615–619.
13. Imai Y, Shindo T, Maemura K, et al. Evidence for the physiological and pathological roles of adrenomedullin from genetic engineering in mice. *Ann N Y Acad Sci*. 2001;947:26–34.
14. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res*. 2002;90:1243–1250.
15. Tabata Y, Hijikata S, Muniruzzaman M, et al. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. *J Biomater Sci Polym Ed*. 1999;10:79–94.
16. Fukunaka Y, Iwanaga K, Morimoto K, et al. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J Control Release*. 2002;80:333–343.
17. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest*. 1994;93:662–670.
18. Van Belle E, Witzenbichler B, Chen D, et al. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor. *Circulation*. 1998;97:381–390.
19. Ohta H, Tsuji T, Asai S, et al. A simple immunoradiometric assay for measuring the entire molecules of adrenomedullin in human plasma. *Clin Chim Acta*. 1999;287:B131–B143.
20. Nagaya N, Nishikimi T, Yoshihara F, et al. Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. *Am J Physiol Regul Integr Comp Physiol*. 2000;278:R1019–R1026.
21. Miyashita K, Itoh H, Sawada N, et al. Adrenomedullin promotes proliferation and migration of cultured endothelial cells. *Hypertens Res*. 2003;26:S93–S98.
22. Jiang BH, Zheng JZ, Aoki M, et al. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A*. 2000;97:1749–1753.
23. Tabata Y, Ikada Y. Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. *J Pharm Pharmacol*. 1987;39:698–704.
24. Nagaya N, Kangawa K, Kanda M, et al. Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation*. 2003;108:889–895.

Pediatr Cardiol 25:568–569, 2004
DOI: 10.1007/s00246-004-9013-4

Pediatric Cardiology



*Images in Pediatric Cardiology*

# Early Sympathetic Reinnervation Demonstrated by Iodine-123 Metaiodobenzylguanidine Imaging in a Child After Cardiac Transplantation

K. Watanabe,[1] K. Fukuchi,[2] S. Echigo[1]

[1]Department of Pediatrics, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan

[2]Department of Radiology, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan

A 2-year-old boy underwent orthotopic cardiac transplantation because of dilated cardiomyopathy. The procedure was performed at the Loma Linda University Medical Center (Loma Linda, CA, USA) when he was 17 months old. The patient returned to Japan 6 months after transplantation, and immunosuppressive therapy with cyclosporine and azathioprine was continued at our hospital. He is in good clinical condition.

We performed iodine-123 metaiodobenzylguanidine ($^{123}$I-MIBG) scintigraphy to assess cardiac sympathetic reinnervation 6 and 12 months after the cardiac transplantation. Before receiving 37 MBq of $^{123}$I-MIBG, the patient took no medication known to inhibit MIBG uptake, and thyroid uptake was blocked by potassium iodide. At the 6-month examination, partial reinnervation was observed in the basal anterior region in the left ventricle, which was confirmed by anterior planar imaging and single-photon emission computed tomography imaging (Fig. 1). At the second examination, 12 months after cardiac transplantation, the distribution of $^{123}$I-MIBG uptake included the septal, apical, and lateral walls, but the inferior wall was still not visualized (Fig. 2).

Previous studies showed that cardiac uptake of $^{123}$I-MIBG was not demonstrable in adults less than 1 year posttransplantation [1–4]. However, in our

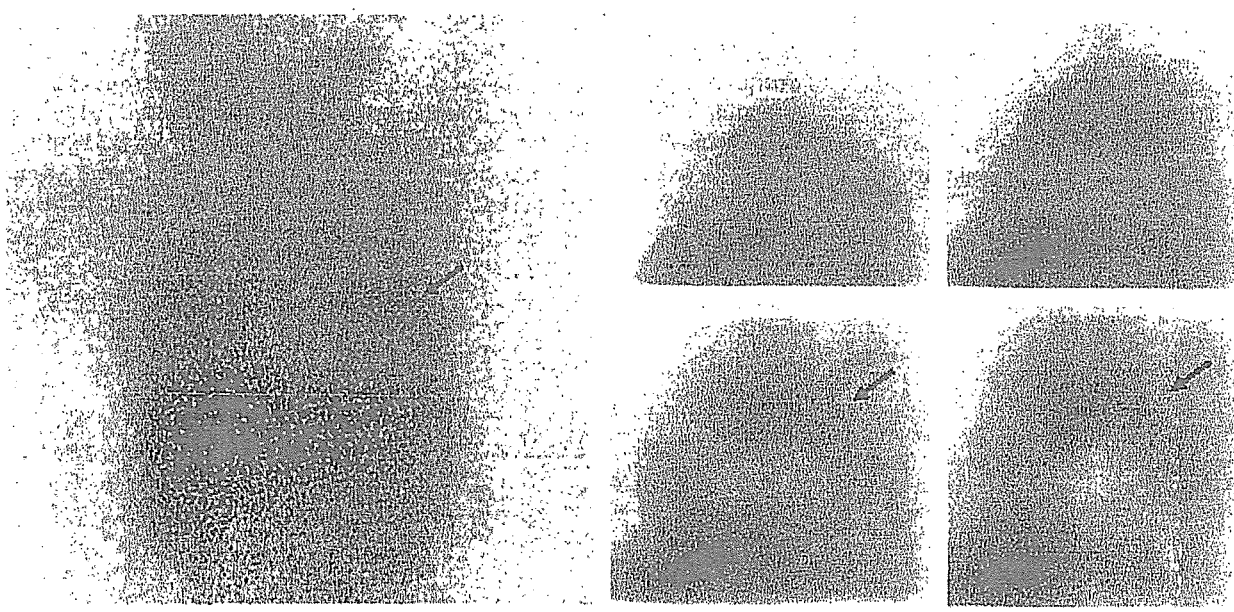


Fig. 1. Anterior planar and short-axis single-photon emission computed tomography images with iodine-123 metaiodobenzylguanidine (*MIBG*) 4 hours after injection. Regional MIBG uptake is seen in the basal anterior left ventricle (*arrows*) 6 months after the cardiac transplantation.

*Correspondence to:* K.Watanabe, *email:* kwatanab@hsp.ncvc.go.jp

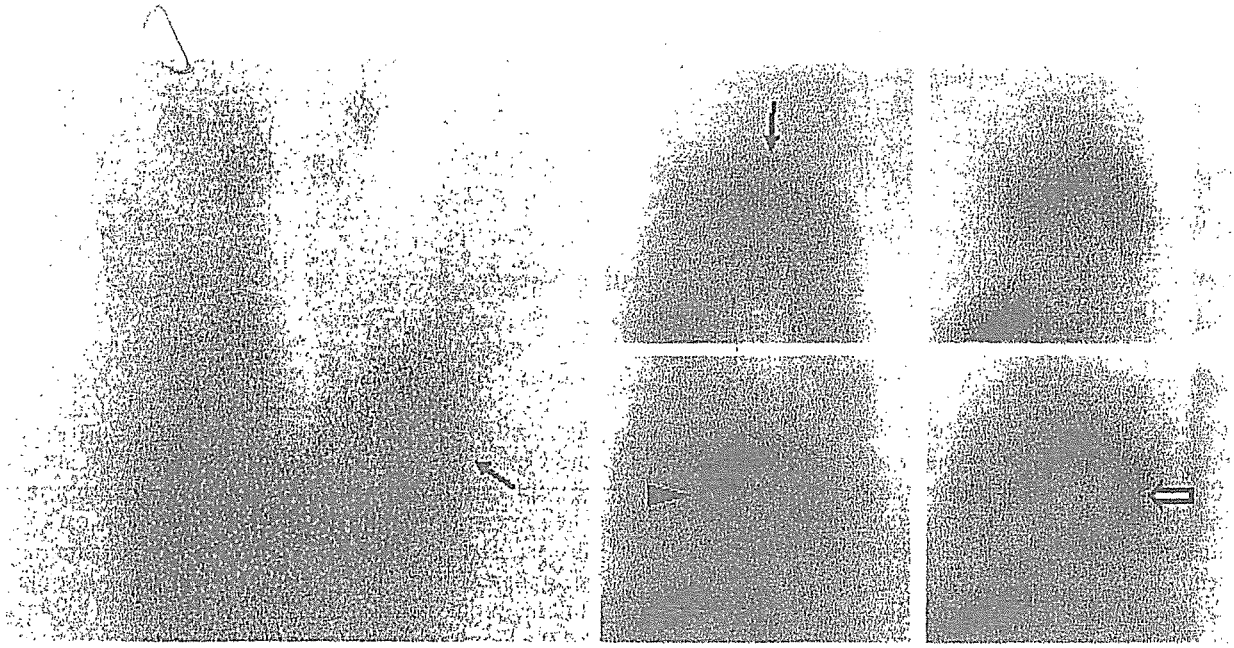


Fig. 2. A second iodine-123 metaiodobenzylguanidine study was performed 12 months after the cardiac transplantation, which showed further growth toward the apical (*arrow*), septal (*arrow head*), and lateral (*open arrow*) regions.

patient, cardiac $^{123}$I-MIBG uptake was clearly evident 6 months after cardiac transplantation, and at 12 months uptake showed obvious expansion. The present finding demonstrates, for the first time, that early (within 6 months after transplantation) sympathetic reinnervation is possible, at least in children.

## References


1. Dae MW, De Marco T, Botvinick EH, et al. (1992) Scintigraphic assessment of MIBG uptake in globally denervated human and canine hearts—implications for clinical studies. *J Nucl Med 33*:1444–1450
2. De Marco T, Dae M, Yuen-Green MSF, et al. (1995) Iodine-123 metaiodobenzylguanidine scintigraphic assessment of the transplanted human heart: evidence for late reinnervation. *J Am Coll Cardiol 25*:927–931
3. Estorch M, Campreciòs M, Flotats A, et al. (1999) Sympathetic reinnervation of cardiac allografts evaluated by $^{123}$I-MIBG imaging. *J Nucl Med 40*:911–916
4. Toba M, Ishida Y, Fukuchi K, et al. (1998) Sympathetic reinnervation demonstrated on serial iodine-123-metaiodobenzylguanidine SPECT images after cardiac transplantation. *J Nucl Med 39*:1862–1864

*Circ J* 2004; **68:** 304–307

# Exercise-Induced Hepatocyte Growth Factor Production in Patients After Acute Myocardial Infarction

## —— Its Relationship to Exercise Capacity and Brain Natriuretic Peptide Levels ——

Satoshi Yasuda, MD; Yoichi Goto, MD; Hiroshi Takaki, MD; Yasuhide Asaumi, MD; Takeshi Baba, MD; Shunichi Miyazaki, MD; Hirohi Nonogi, MD

**Background** The hepatocyte growth factor (HGF) is a multifunctional cytokine with cardioprotective properties and potent myogenic activity for vascular endothelium. In patients after acute myocardial infarction, exercise training has the beneficial effects on cardiovascular adaptations. We hypothesized that exercise induces HGF production in those patients. If this hypothesis is correct, HGF production may be associated with clinical parameters of cardiovascular function.

**Methods and Results** In 20 patients after acute myocardial infarction, HGF levels in the pulmonary artery ($HGF_{PA}$) and aorta ($HGF_{Ao}$) were determined at rest and during supine submaximal exercise, with cardiac output (CO) measured by catheterization. Exercise-induced HGF production was calculated by using the following equation: $[(HGF_{PA}-HGF_{Ao})\times CO$ during exercise$]-[(HGF_{PA}-HGF_{Ao})\times CO$ at rest]. On a separate day, peak oxygen uptake ($\dot{V}O_2$) was determined during a symptom-limited upright cardiopulmonary exercise test. Exercise increased HGF production (from 1.6±3.0 to 9.0±6.3 $\mu$g/ml, $p<0.001$). Exercise-induced HGF production was inversely related to peak $\dot{V}O_2$ ($r=-0.664$, $p<0.01$) and positively related to levels of brain natriuretic peptide (BNP), a biochemical marker for post-infarction ventricular remodeling ($r=0.686$, $p<0.01$).

**Conclusions** Exercise significantly increases HGF production. This phenomenon may play an important role in post-infarction patients, particularly with reduced exercise tolerance and elevated BNP levels. (*Circ J* 2004; **68:** 304–307)

**Key Words:** Exercise; Growth substances; Myocardial infarction; Rehabilitation

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocyes, has mitogenic, motogenic, morphogenic, and anti-apoptotic activities in a variety of cells through its receptor, c-Met[1,2] HGF is a unique growth factor to act protectively against endothelial dysfunction[3–5] myocardial ischemia/infarction and remodeling[6–8] Thus, the HGF system (HGF and its receptor c-Met) is attracting increasing attention in the field of cardiovascular pathophysiology[9]

In patients with acute myocardial infarction (AMI), exercise training has the beneficial effects on cardiovascular systems[10] Cardiac effects include attenuation of post-infarction ventricular remodeling,[11,12] for which brain natriuretic peptide (BNP) is a useful biochemical marker[13] Vascular effects include an increase in the density of skeletal-muscle capillaries[14] and improvement in endothelial-dependent vasodilation,[15,16] which are important determinants for exercise tolerance and symptoms.

In the present study, we hypothesized that exercise induces HGF production, mediating the beneficial effects of exercise training in patients with AMI. If so, HGF production may be associated with clinical parameters of cardiovascular function.

*(Received August 4, 2003; revised manuscript received December 24, 2003; accepted January 8, 2004)*

*Division of Cardiology, Department of Medicine, National Cardiovascular Center, Osaka, Japan*

Mailing address: Satoshi Yasuda, MD, PhD, Division of Cardiology, Department of Medicine, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita 565-8565, Japan. E-mail: syasuda@hsp.ncvc.go.jp

## Methods

*Study Patients*

The study group included 20 male patients (aged 61± 12 years [mean±SD]) after AMI. The infarction site was anterior in 13 patients (65%), and inferior/lateral in 7 patients (35%). All patients underwent reperfusion therapy (percutaneous transluminal coronary angioplasty in 17 patients and intravenous administration of tissue-type plasminogen activator in 3 patients) on admission. The peak level of serum creatine kinase was 2,995±2,043 [mean± SD] U/L. The severity of heart failure ranged from New York Heart Association functional class I to II. The baseline patient characteristics are summarized in Table 1. No patients had liver (elevated levels of aminotransferases), kidney (elevated levels of creatinine or urea), or lung dysfunction (restrictive or obstructive pattern in spirometry). No patients had prior myocardial infarction. Medications remained unchanged during the entire study.

The study was approved by the institutional review committee. The protocol was fully explained, and all patients gave their written informed consent to participate in the study.

Table 1 Baseline Characteristics of Patients

| | |
|---|---|
| *NYHA, n (%)* | |
| *I* | *12 (60)* |
| *II* | *8 (40)* |
| *LVEF (%)* | *43±9* |
| *LVEDVI (ml/m²)* | *70±11* |
| *LVEDP (mmHg)* | *13±6* |
| *Coronary risk factors, n (%)* | |
| *Diabetes mellitus* | *11 (55)* |
| *Hyperlipidemia* | *9 (45)* |
| *Hypertension* | *7 (35)* |
| *Medications, n (%)* | |
| *ACE-inhibitor* | *11 (55)* |
| *$Ca^{2+}$-antagonist* | *12 (60)* |
| *Nitrates* | *9 (45)* |
| *Aspirin* | *20 (100)* |
| *Diuretics* | *8 (40)* |
| *Digoxin* | *7 (35)* |
| *β-blockers* | *4 (20)* |

*NYHA, New York Heart Association classification; LVEF, left ventricular ejection fraction; LVEDVI, left ventricular end-diastolic volume index; LVEDP, left ventricular end-diastolic pressure; ACE, angiotensin-converting enzyme; $Ca^{2+}$, calcium.*

Table 2 Changes in Hemodynamics and HGF Levels in Response to Supine Exercise

| | *Baseline* | *Peak exercise* | *p value* |
|---|---|---|---|
| *HR (beats/min)* | *68±11* | *117±17* | *<0.001* |
| *Aosyst (mmHg)* | *126±17* | *169±20* | *<0.001* |
| *PAsyst (mmHg)* | *34±7* | *58±14* | *<0.001* |
| *PAdiast (mmHg)* | *11±4* | *19±5* | *<0.001* |
| *CO (L/min)* | *6.8±1.6* | *14.7±3.9* | *<0.001* |
| *$HGF_{PA}$ (ng/ml)* | *7.72±3.50* | *7.82±3.53* | *NS* |
| *$HGF_{Ao}$ (ng/ml)* | *7.45±3.32* | *7.14±3.14* | *NS* |
| *ΔHGF(PA–Ao) (ng/ml)* | *0.27±0.44* | *0.68±0.58* | *<0.01* |
| *CO×ΔHGF (μg/min)* | *1.6±3.0* | *9.0±6.3* | *<0.001* |
| *$S\dot{V}O_2$ (%)* | *67±4* | *34±10* | *<0.001* |

*HGF, hepatocyte growth factor; HR, heart rate; Ao, aorta; PA, pulmonary artery; syst, systolic pressure; diast, diastolic pressure; CO, cardiac output (by the thermodilutional method); ΔHGF (PA–Ao), the difference in HGF levels between pulmonary artery and aorta; $S\dot{V}O_2$, mixed venous $O_2$ saturation.*
*p values were assessed with the paired student t-test.*

*Cardiac Catheterization and Supine Exercise Test*

From the right brachial artery through a 6F sheath, chronic phase coronary angiography and left ventriculography were performed according to the conventional Judkins technique, 28±7 [mean±SD] days after the onset of myocardial infarction[17] Heparin was initially administered at a dose of 5,000 IU into the distal brachial artery. For angiographic evaluation of left ventricular volumes, ventricular silhouettes in 30° right anterior oblique projections were digitized with an ANCHOR ventriculography analysis system (Siemens-Elema, Solna, Sweden). By the area-length method, the left ventricular end-systolic and end-diastolic volume indices and ejection fraction were calculated. Left ventricular pressure was measured with a 2F high-fidelity micromanometer catheter (model SPC-320; Miller Instruments, Houston, TX, USA) advanced into the left ventricle via the lumen of a 6F pig tail catheter.

A 7.5 F Swan-Ganz thermodilution catheter (Opticath®; Abbott Laboratories, North Chicago, IL, USA) was inserted through the left subclavian vein, to measure cardiac output (CO) and pulmonary artery (PA) pressure.

After sampling blood and measuring hemodynamic parameters at baseline, the supine bicycle exercise test was performed by using a Siemens Ergometry System 930B, and the mixed venous $O_2$ saturation ($S\dot{V}O_2$), and pressure of the PA and aorta (Ao) were monitored. We also monitored arterial blood $O_2$ saturation continuously using a Biox III pulse oximeter (Omeda, Louisville, KY, USA). The workload was increased at 3-min intervals in 30-W increments followed by a 0-W bicycling period for 1 min. The exercise was finished at 30 W in 2 patients, 60 W in 8 patients and 90 W in 10 patients. This final workload was the submaximal level for each patient, because the peak heart rate was approximately 80% of the maximal heart rate achieved at the symptom-limited cardiopulmonary exercise test, as described below. Before and immediately after the supine exercise test, blood samples were taken from the PA and the Ao. The samples were centrifuged at 4°C and stored at −80°C until assayed.

*Cardiopulmonary Exercise Test*

On a separate day (3±1 [mean±SD] days before the cardiac catheterization), patients underwent the symptom-limited cardiopulmonary exercise test (CPX), with determination of peak oxygen uptake ($\dot{V}O_2$), workload and heart rate. The exercise test was performed on a calibrated, electronically braked bicycle in an upright position (Examiner, Lode B.V., Groningen, Netherlands). Ramp protocols began at a workload of 0 W for 1 min and increased in 15-W increments at 1-min intervals. Expired gas analysis was performed by using a respiromonitor AE-280 (Minato Products, Tokyo, Japan). The $\dot{V}O_2$ was measured on a breath-by-breath basis, and was averaged over contiguous 30-s intervals, except at peak exercise, when 18-s averaging was used.

*Hepatocyte Growth Factor Measurements*

Hepatocyte growth factor levels in the pulmonary artery ($HGF_{PA}$) and aorta ($HGF_{Ao}$) were determined with specific enzyme-linked immunosorbent assay kits (Otsuka Assay Laboratories, Tokushima, Japan). Microtiter plates coated with an anti-HGF murine monoclonal antibody were incubated with standard HGF or serum samples, and an anti-HGF rabbit polyclonal antibody was added. After adding first the anti-rabbit goat immunoglobulin G-peroxidase conjugate and then o-phenylene diamine, the absorbance was read at 492 nm using a plate reader[18] The sensitivity of the HGF kit was 0.1 ng/ml. This assay system detects only bioactive, heterodimeric (mature) forms of HGF in the blood samples[18,19] Previous studies have demonstrated that there is a strong (r=0.986) positive correlation between HGF levels measured by this assay system and those measured by bioassay (determined by stimulating DNA synthesis of rat hepatocytes in primary cultures)[18,19]

The BNP levels were determined with a specific immunoradiometirc assay kit (Shionogi Co, Osaka, Japan), as previously reported[17] The sensitivity of this BNP kit is 2 pg/ml. Brain natriuretic peptide has been considered as a biochemical marker of ventricular remodeling after myocardial infarction.

*Data Analysis*

Exercise-induced HGF production (μg/min) was calculated by using the following equation:

$[(HGF_{PA}-HGF_{Ao})\times CO$ at peak exercise$]-[(HGF_{PA}-HGF_{Ao})\times CO$ at rest].

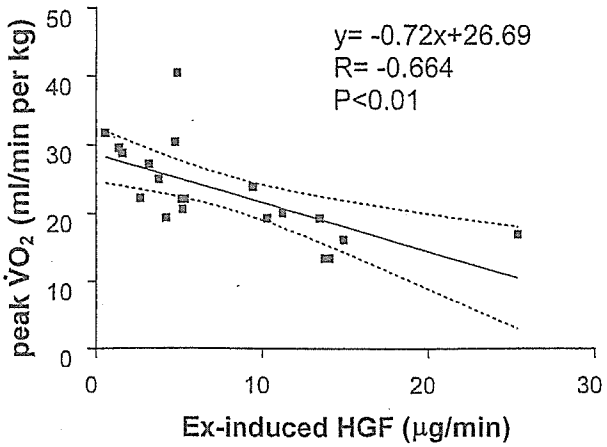



Fig 1. Correlation of the exercise (Ex)-induced hepatocyte growth factor (HGF) production with peak oxygen uptake (peak $\dot{V}O_2$) in 20 patients after acute myocardial infarction (AMI).

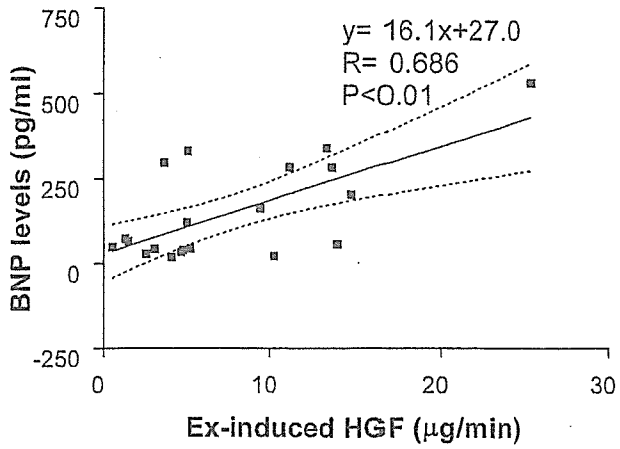



Fig 2. Correlation of the exercise Ex-induced HGF production with brain natriuretic peptide (BNP) levels.

The $\chi^2$ test was used for comparison of categorized variables. The Student's t-test or Mann-Whitney U-test rank test was used for comparisons of mean values to determine significance of difference between the 2 groups. Linear regression curves and correlations were calculated according to the least squares method. All data are presented as mean ± SD. Differences were considered significant at $p<0.05$.

## Results

*Changes in Hemodynamics and HGF in Response to Supine Exercise*

Table 2 shows the changes in hemodynamics and HGF levels, at baseline (=before exercise) and at peak exercise during the catheterization. At baseline, there were no significant differences in HGF levels between PA and Ao. The supine exercise (74±18 W in intensity, 10±2 min in duration) significantly increased heart rate, Ao pressure, PA pressure, and CO, whereas it decreased $S\dot{V}O_2$. Although the absolute HGF levels in PA and Ao appear unchanged, the difference in HGF levels between PA and Ao (ΔHGF) significantly increased by approximately 3-fold after the exercise. When assessed based on the fold change compared with the baseline level, exercise increased the $HGF_{PA}$ to 1.02±0.11-fold ($p<0.05$), but did not change $HGF_{Ao}$ (0.96±0.09-fold). Finally, in the patients of the present study, exercise-induced HGF production ([ΔHGF×CO at peak exercise]−[ΔHGF×CO at baseline]) was calculated to be 7.4±6.3 μg/min, on average.

*Correlations With HGF Production*

Peak $\dot{V}O_2$, workload and heart rate determined during the symptom-limited upright cardiopulmonary exercise text (CPX) performed on a separate day were 23±7 ml/min per kg, 130±37 W, and 140±24 beats/min, respectively.

As shown in Fig 1, exercise-induced HGF production correlated inversely with peak $\dot{V}O_2$ ($r=-0.664$, $p<0.01$). Eight patients with peak $\dot{V}O_2<20$ ml/min per kg had greater exercise-induced HGF production (13.4±5.9 vs 3.9±2.4 μg/min, $p<0.05$) and higher prevalence of angiographically significant stenosis in major coronary arteries (>60%) (50 vs 8%, $p<0.05$) in comparison with the remaining 12 patients with $\dot{V}O_2\geq20$ ml/min per kg.

Also, as shown in Fig 2, exercise-induced HGF production correlated positively with BNP levels at baseline ($r=0.686$, $p<0.01$). However, there were no significant relations with cardiac function at rest (left ventricular end-diastolic volume index and ejection fraction), percentage increase in heart rate, Ao pressure and PA pressure in response to the submaximal supine exercise (data not shown).

## Discussion

The major finding of the present study is that HGF production is induced during exercise in accordance with the severity of exercise intolerance and the increase in BNP levels.

In patients after AMI, exercise training is now emerging as an important component of the therapy[10,20] It attenuates post-infarction ventricular remodeling, which is associated with heart failure and increased mortality[21] and is accompanied by an elevated level of BNP[13] Regular exercise training also increases the density of skeletal muscle capillaries[14] and induces repetitive increases in vascular blood flow and shear stress[22] thereby improving endothelium-dependent vasodilation[15,16] Both central (cardiac) and peripheral (skeletal muscle and vascular) effects of exercise training may consequently improve exercise tolerance and symptoms[10] From the data obtained in the present study, a causal relationship cannot be clearly determined. However, the several effects of exercise training are potentially mediated through HGF in patients after AMI.

As shown in Table 2, exercise increases the concentration gradients of HGF levels between PA and Ao, indicating exercise-induced HGF production. The vessel wall may be a potential source of circulatory HGF[23] Fig 1 shows that exercise-induced HGF production is associated with reduced peak $\dot{V}O_2$. In particular, patients with peak $\dot{V}O_2<20$ ml/min per kg were sensitive towards the HGF response to exercise. These patients with reduced exercise capacity had a higher prevalence of coronary artery stenosis. Myocardial ischemia appears to be one of the determinants for exercise capacity and is known to induce upregulation of non-cardiac HGF systems[24] HGF promotes angiogenesis as a potent growth factor of endothelial cells[25] and promotes the functional recovery of nitric-oxide-mediated vasodila-

tion?[26] thus improving myocardial blood flow. Also, as shown in Fig 2, exercise-induced HGF production is associated with increased levels of BNP. The HGF may be systemically released during exercise in response to left ventricular dysfunction and may exert wound healing and cardioprotective actions against myocardial ischemia/infarction?[27,28] In a mouse myocardial infarction model, HGF gene therapy attenuated left ventricular remodeling and dysfunction?[28]

The recent clinical studies also suggest a possibility that HGF may contribute to improving myocardial ischemia and dysfunction. In the CAPTURE (c7E3 Anti-Platelet Therapy in Unstable REfractory angina) trial studying the patients with acute coronary syndromes, elevated HGF levels are associated with reduced incidence of death and myocardial infarction?[29] In another study in patients with coronary artery disease, elevated coronary sinus HGF levels were associated with collateral formation and with left ventricular dysfunction?[30] Thus, interventions that enhance HGF levels could be beneficial in the management of those patients. Exercise is a potential approach. However, further studies are required to determine whether short-term benefits of exercise could translate into long-term effects?[20]

In conclusion, the present study provides a novel aspect of exercise training as cytokine-mobilization. HGF may have a therapeutic implication in patients after AMI.

*Acknowledgment*

*This study was supported, in part, by grants from the Uehara Memorial Foundation, the Osaka Heart Club, Japan Cardiovascular Research Foundation (Dr Yasuda), and the Ministry of Health and Welfare, Japan (Dr Goto).*

## References

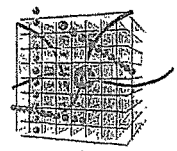
1. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of hepatocyte growth factor. *Nature* (*Lond.*) 1989; **342:** 440–443.
2. Boros P, Miller CM. Hepatocyte growth factor: A multifunctional cytokine. *Lancet* 1995; **345:** 293–295.
3. Nakamura Y, Morishita R, Nakamura S, Aoki M, Moriguchi A, Matsumoto K, et al. A vascular modulator, hepatocyte growth factor, is associated with systolic pressure. *Hypertension* 1996; **28:** 409–413.
4. Nakano N, Moriguchi A, Morishia R, Kida I, Tomita N, Matsumoto K, et al. Role of angiotensin II in the regulation of a novel vascular modulator, hepatocyte growth factor (HGF), in experimental hypertensive rats. *Hypertension* 1997; **30:** 1448–1454.
5. Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Kim S, et al. Anti-apoptotic action of hepatocyte growth factor through mitogen-activated protein kinase on human aortic endothelial cells. *J Hypertens* 2000; **18:** 1411–1420.
6. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest* 2000; **106:** 1511–1519.
7. Taniyama Y, Morishita R, Nakagami H, Moriguchi A, Sakonjo H, Kim S, et al. 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.
8. Ueda H, Nakamura T, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. *Cardiovasc Res* 2001; **51:** 41–50.
9. Morishita R. Recent progress in gene therapy for cardiovascular disease. *Circ J* 2002; **66:** 1077–1086.
10. Ades PA. Cardiac rehabilitation and secondary prevention of coronary heart disease. *N Engl J Med* 2001; **345:** 892–902.
11. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, et al. Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: Application of magnetic resonance imaging. *Circulation* 1997; **95:** 2060–2067.
12. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: Results of the Exercise in Left Ventricular Dysfunction (ELVD) trial. *Circulation* 1997; **96:** 1790–1797.
13. Nagaya N, Nishikimi T, Goto Y, Miyao Y, Kobayashi Y, Morii I, et al. Plasma brain natriuretic peptide is a biochemical marker for the prediction of progressive ventricular remodeling after acute myocardial infarction. *Am Heart J* 1998; **135:** 21–28.
14. Richardson RS, Wagner H, Mudaliar SR, Saucedo E, Henry R, Wagner PD. Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *Am J Physiol* 2000; **279:** H772–H778.
15. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res* 1994; **74:** 349–353.
16. Gielen S, Schuler G, Hambrecht R. Exercise training in coronary artery disease and coronary vasomotion. *Circulation* 2001; **103:** E1–E6.
17. Yasuda S, Goto Y, Sumida H, Noguchi T, Baba T, Miyazaki S, et al. Angiotensin-converting enzyme inhibition restores hepatocyte growth factor production in patients with congestive heart failure. *Hypertension* 1999; **33:** 1374–1378.
18. Tsubouchi H, Niitani Y, Hiroko S, Nakayama H, Gohda E, Arakaki N, et al. Levels of the human hepatocyte growth factor in serum of patients with various diseases determined by an enzyme-linked immunosorbent assay. *Hepatology* 1991; **13:** 1–5.
19. Arakaki N, Kawakami S, Nakamura O, Ohnishi T, Miyazaki H, Ishi T, et al. Evidence of the presence of an inactive precursor of human hepatocyte growth factor in plasma and sera of patients with liver diseases. *Hepatology* 1995; **22:** 1728–1784.
20. Pina IL, Apstein CS, Balady GJ, Belardinelli R, Chaitman BR, Duscha BD, et al. American Heart Association Committee on exercise, rehabilitation, and prevention. Exercise and heart failure: A statement from the American Heart Association Committee on exercise, rehabilitation, and prevention. *Circulation* 2003; **107:** 1210–1225.
21. White HD, Norris RM, Brown MA, Brandt PW, Whitlock RM, Wild CJ. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. *Circulation* 1987; **76:** 44–51.
22. Niebauer J, Cooke JP. Cardiovascular effects of exercise: Role of endothelial shear stress. *J Am Coll Cardiol* 1996; **28:** 1652–1660.
23. Wajih N, Walter J, Sane DC. Vascular origin of a soluble truncated form of the hepatocyte growth factor receptor (c-met). *Circ Res* 2002; **90:** 46–52.
24. Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Enhanced expression of hepatocyte growth factor/c-Met by myocardial ischemia and reperfusion in a rat model. *Circulation* 1997; **95:** 2552–2558.
25. Belle EV, Witzenbichler B, Chen D, Silver M, Chang L, Schwall R, et al. Potential angiogenetic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor. The case for paracrine amplification of angiogenesis. *Circulation* 1998; **97:** 381–390.
26. Hayashi K, Nakamura S, Morishita R, Moriguchi A, Aoki M, Matsumoto K, et al. In vivo transfer of human hepatocyte growth factor gene accelerates re-endothelialization and inhibits neointimal formation after balloon injury in rat model. *Gene Therapy* 2000; **7:** 1664–1671.
27. Shimada Y, Yoshiyama M, Jissho S, Kamimori K, Nakamura Y, Iida H, et al. Hepatocyte growth factor production may be related to the inflammatory response in patients with acute myocardial infarction. *Circ J* 2002; **66:** 253–256.
28. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, et al. 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. Heeschen C, Dimmeler S, Hamm CW, Boersma E, Zeiher AM, Simoons ML. CAPTURE (c7E3 Anti-Platelet Therapy in Unstable REfractory angina) Investigators. Prognostic significance of angiogenic growth factor serum levels in patients with acute coronary syndromes. *Circulation* 2003; **107:** 524–530.
30. Lenihan DJ, Osman A, Sriram V, Aitsebaomo J, Patterson C. Evidence for association of coronary sinus levels of hepatocyte growth factor and collateralization in human coronary disease. *Am J Physiol* 2003; **284:** H1507–H1512.

2

# 病院設置型微小血管造影装置の開発と臨床応用の可能性

Masaaki Chiku ◎ 知久正明*
Kazuhiro Nishigami ◎ 西上和宏*
Eizo Mori ◎ 盛　英三†
Toshiaki Kawai ◎ 河合敏昭§
Ryo Mochizuki ◎ 望月　亮#

*国立循環器病センター心臓血管内科／†同 心臓生理部
§浜松ホトニクス㈱／#NHKエンジニアリングサービス



**Summary**

骨髄単核球細胞移植や末梢血幹細胞移植による血管再生療法が末梢動脈閉塞症に対し臨床応用され，疼痛や皮膚潰瘍など臨床症状の改善が報告されている．しかし，血管造影などの臨床検査では十分に評価できていない現状である．これは，現在使用されている解像度の低い血管撮影装置では，約100$\mu$m前後の微小な新生血管の描出が困難なためである．一方，シンクロトロン放射光は100$\mu$m以下の微小血管を評価することが可能であるが，臨床導入にはその莫大なコストが問題になる．そこで，血管再生療法後の新たな診断方法として，病院に設置可能な微小血管造影装置を開発した．

**Key words**

◎血管再生療法　◎病院設置型微小血管造影装置
◎新生血管　◎シンクロトロン
◎単色X線

## 循環器領域における微小血管造影

狭心症や心筋梗塞などの虚血性心疾患や閉塞性動脈硬化症に対する治療選択は，①薬物治療，②カテーテル治療，③外科的バイパス術が主な治療法である．しかし，血行再建が困難な重症例や，糖尿病を合併した微小血管病変を伴う症例が高頻度にみられるようになった．このような症例は既存の治療法では限界があり，新しい治療戦略として血管再生治療が期待されている．Asaharaらは成人末梢血中の単核球分画に，血管内皮細胞に分化し得る内皮前駆細胞の存在を報告した[1]．単核球は主に骨髄に存在するため，骨髄単核球細胞移植をすることにより，血管新生や側副血行路が発達し，動物実験の虚血モデルで下肢血流量増加作用や心機能が改善することが確認された[2)3)]．これらの基礎および臨床結果に基づき，カテーテル治療や外科的バイパス術による血行再建が困難な重症末梢性動脈疾患に骨髄単核球細胞移植が臨床導入され，その有効性が報告された[4]．当施設でも4例のバージャー病患者に骨髄細胞移植を施行し，全例とも安静時疼痛が改善し，皮膚潰瘍を呈する3症例も完全に回復した(図1)．しかし，骨髄細胞移植治療の評価に関しては，血管造影を

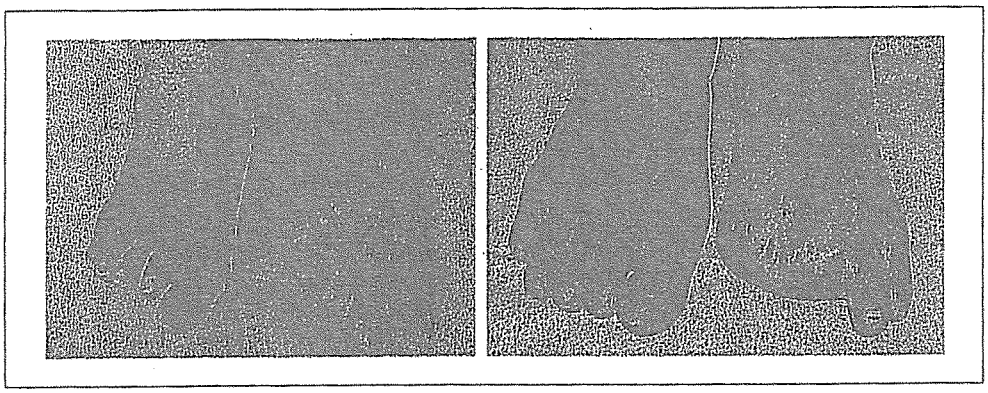


図1．バージャー病の下肢皮膚潰瘍
移植治療前は左第1趾から第3趾まで広範な皮膚潰瘍を認めたが(左)，移植治療3ヵ月後には完全に上皮化された(右)．

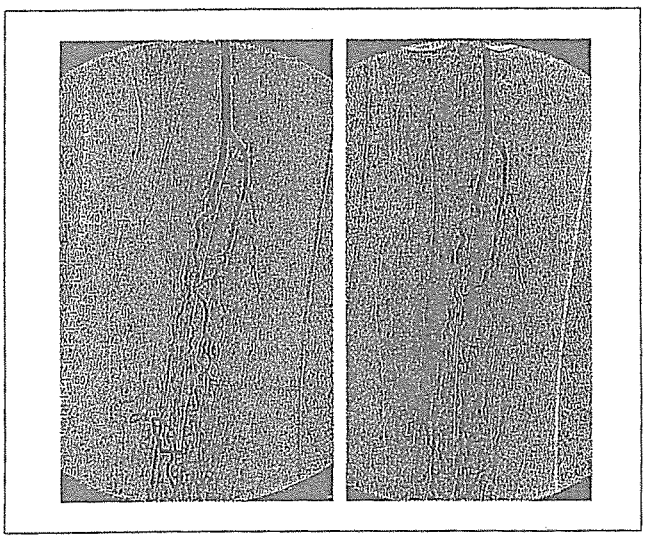


図2．下肢血管造影
移植治療前の血管造影と比較し(左)，移植治療1ヵ月後に血管周辺にもやもや像は認めるが，新生血管の評価は困難である(右)．

含めた臨床検査では臨床症状の改善を十分には反映しなかった(図2)．一般に，治療効果の判定として血管造影法(DSA)が施行される．しかし，既存の血管造影装置の解像度は約200～300 $\mu$mであり，再生される新生血管は約100 $\mu$m程度の微小血管であり，その評価は困難である．症例によっては血管数の増加がみられることがあるが，側副血行路の発達(arteriogenesis)と考えられており，再生した血管そのものが造影されているわけではない．そこで，再生された新生血管が臨床症状の改善に関与していることを証明するには，再生血管を描出することが重要である．これら，再生された微小血管を血管造影検査で評価するためには，微量の造影剤を検出できる装置が必要となる．その要素としてはX線が高輝度で，平行化，単色化の性質を持ち，検出系を高感度，高解像度化することが重要である．

現在のところ，これらの要素をすべて取り揃えているのが放射光施設内の微小血管造影装置であり，微小血管の検出を可能としている[5]．放射光の詳細については他稿に委ねるが，簡単に列記する．放射光とは広域のスペクトルを持つ白色光であり，太陽光のように限りなく平行に近い性質がある．その白色光に対しシリコン結晶を用い，ヨード吸収端の直上に設定することにより単色化が可能となる．現在のところ，微小血管の描出を可能にしているのは放射光を用いた微小血管造影法だけである．しかし，放射光施設は多額のコストと広大な敷地を必要とし，臨床導入するには時間的・空間的にも問題がある．そこで微小血管造影法が臨床応用できるように，新エネルギー・産業技術開発機構(NEDO)の支援により，病院設置型の微小血管造影装置を浜松ホトニクス・NHKエンジニアリング

の協力を得て，共同開発した．

## 微小血管造影法に必要な要素

### 1．高輝度

輝度は単位面積あたりのX線のフォトン量であり，イメージングプレートに像を映し出す重要な要素である．X線は被写体を通過する時に散乱・吸収されるため，検出器に到達する前にそのフォトン量は顕著に減衰する．こうした理由で既存の血管造影装置は，X線のフォトン量を維持するために白色光で撮影している．放射光のX線は，既存のX線装置より約$10^8$倍も輝度が高く，被写体によりX線の減衰を受けても，十分なフォトン量を維持することが可能である．X線を単色化する行程で，著しくフォトン量を減少させたとしても，既存の白色X線と同等のフォトン量を検出器に確保することができる．この要素は，微小血管造影には必要不可欠である．

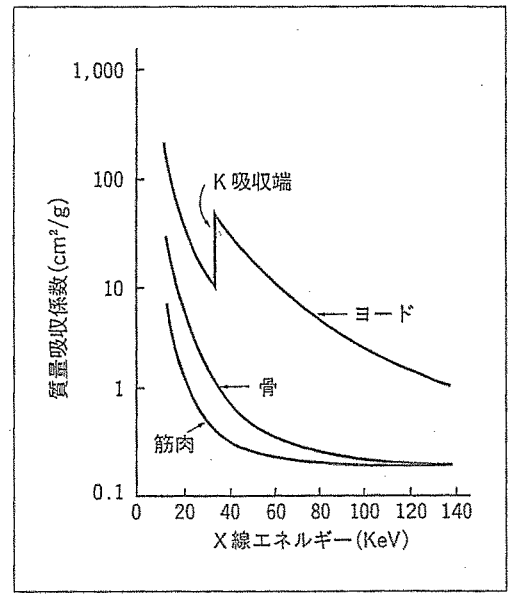



図3．X線エネルギーと質量吸収係数の関係
33.3keV直上でヨードK吸収端が上昇し，組織との質量吸収係数を最大にする．

### 2．単色化

ヨードは33.3 keVのエネルギーレベルでK吸収端を持つ．これは質量吸収係数が不連続に上昇し，X線のエネルギーをヨードのK吸収端の直上のエネルギーに変換すると，ヨードと周囲組織との質量吸収係数の差が最大となる．組織とヨードとのコントラストが最良となるため，微量のヨードを検出できやすくする効果がある(図3)．放射光をシリコン結晶によるBragg反射を応用し，その角度により必要な単色エネルギーを得ることができる(図4)．

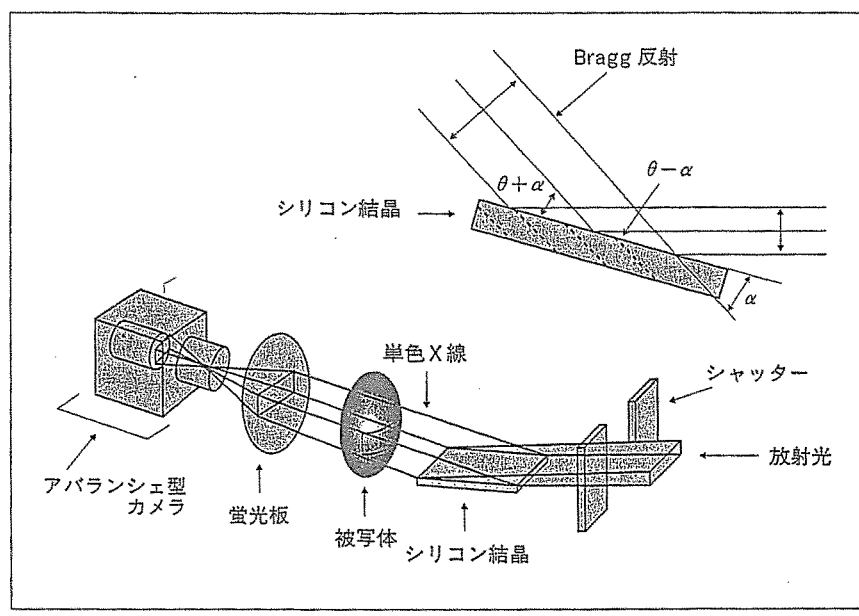



図4．放射光施設の微小血管造影システム
放射光をシリコンを用いBragg反射させ，その角度を調節し，単色X線を得る．その像は高感度，高解像度のアバランシェ型カメラで撮影される．

## 3．平行化

平行線により映し出された像は，理論的にいえば等大の大きさになる．非平行光線は被写体から像との距離が離れるほど像は拡大し，辺縁がぼやけてしまう(図5)．放射光のX線は限りなく平行な性質であり，これも微小血管造影に理想的である．一方，既存のX線は平行線ではなく，微小血管レベルの評価に影響するため，微小血管造影には不向きである．浜松ホトニクスが開発した微細な孔を多数有するキャピラリープレートを用いると，X線を平行化することが可能である．X線源と被写体の距離を近づけた場合，平行化すると像の拡大を防げるが，平行化しないと像が拡大してしまうことをコンピュータシミュレーションで証明した(図6)．

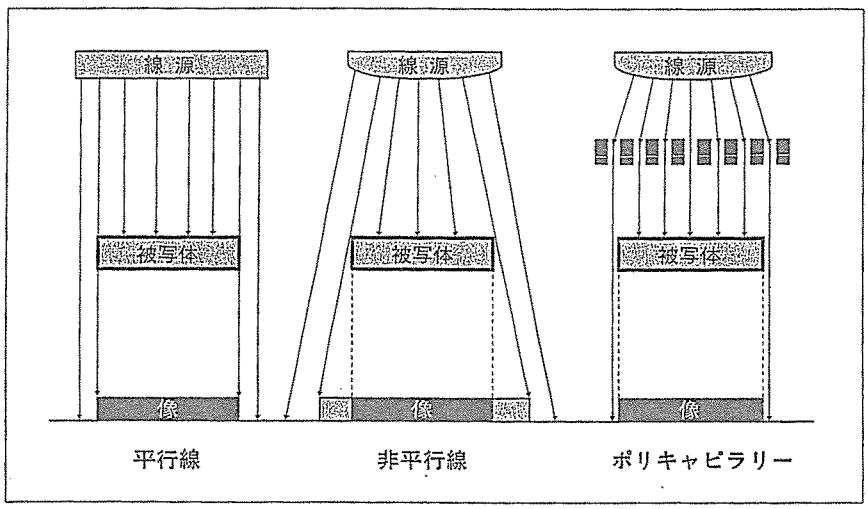



図5．平行化
平行線では被写体と像は同一の大きさであるが(左)，非平行線では像が拡大し，辺縁はぼやける(中)．ポリキャピラリーを用いて平行化させると，像は被写体の大きさに限りなく近づく(右)．

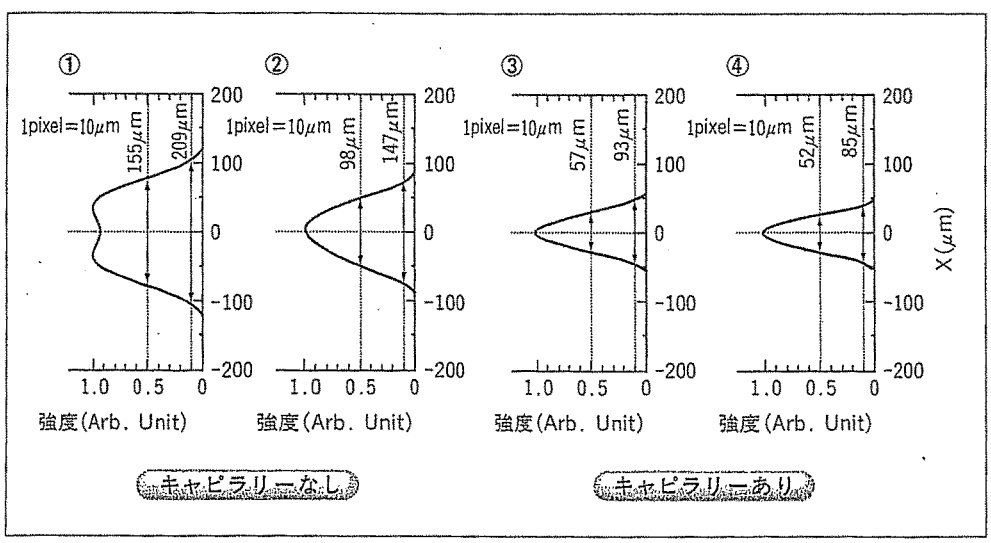



図6．ポリキャピラリーのコンピュータシミュレーション
①②は線源とスリットとの距離がそれぞれ50 cmと80 cmで，キャピラリーによる平行化がない状態である．この時に検出器に入るX線幅は，それぞれ155 μm，98 μmに拡大される．③④も同じく距離を50 cmと80 cmとし，キャピラリーで平行化した状態である．平行化した場合は，52～57 μm程度の誤差しかない．
1 pixel＝10 μm．

### 4．高解像度・高感度

検出器の解像度をチャート撮影から得ることができる．1 mm 幅に 4 本のライン(2 ラインペア)を識別できれば 1,000 μm÷4 で 250 μm の解像度，同じく 20 本のライン(10 ラインペア)識別できれば 50 μm の解像度となる．既存の血管造影装置では約 2 ラインペアであり，空間分解能は 250 μm といえる(図 7)．感度を高くすると微量の X 線を検出できるため，微小血管を造影するにはイメージングプレートとして高感度蛍光板の使用が好ましい．

## 病院設置型微小血管造影装置

新エネルギー・産業技術開発機構(NEDO)の支援により，病院設置型微小血管造影装置を開発した．X 線源は既存の大容量大出力を持つ CT 用の X 線管を用い，検出系は NHK エンジニアリングの技術により超高感度ハイビジョンカメラシステムを導入した．

### 1．X 線管球

普及型微小血管造影装置の CT 用の X 線管は，最大陽極熱容量が 5 MHU と世界最大級の大きさである．X 線高電圧装置も大出力化し，市販の装置では不可能な 70 kVp・800 mA で高輝度の X 線を得ることができる．連続 20 秒照射が可能であり，撮影は動画で観察できる．優れた冷却性能を持ち，480 秒の休止で繰り返し照射が可能である．

### 2．疑似単色化

疑似単色化はランタノイド系の金属を複合したフィルターで，ヨードの K 吸収端(33.3 keV)上にピークを持つ疑似単色 X 線を得ることができる．

### 3．平行化

焦点に起因するボケを低減する目的で，マルチファイバーからなるコーン型のコリメーターを開発した．チャンネルサイズと素子の厚みにより，出射角と透過効率が決定されるため，実用となる条件を求め試作した(図 8)．これにより，被写体が検出器から離れる場合に生じる解像度の低下を防ぐことができる．

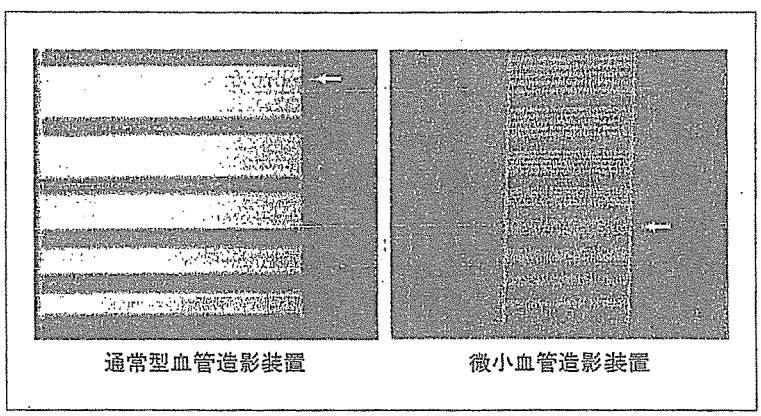



図 7．チャートによる解像度の評価
通常型血管造影装置(左)の解像度は 250 μm(1 mm/4=2 ラインペア)，普及型微小血管造影装置(右)の解像度は 50 μm(1 mm/20=10 ラインペア)を示す．

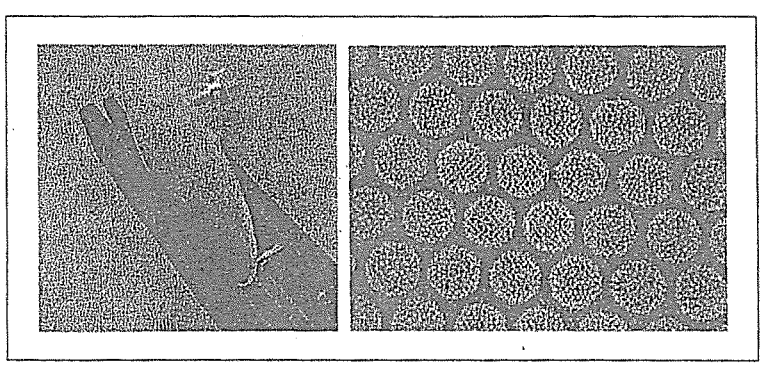


図 8．ポリキャピラリー
多数の孔を持ったポリキャピラリーが，X 線を平行化する．

### 4．超高感度ハイビジョンカメラの開発

検出系は高解像度・高感度蛍光板で作成した蛍光像を，超高感度・高精細撮像管であるアバランシェ型ハイビジョンモノクロ新 Super-HARP カメラで撮影する．これらの検出器系から高解像度(50 μm)が得られる．CCD を用いたハイビジョンカメラは，画素あたりの光子数が減少し感度が低下するため，高精細画像として微小血管を描出するには限界がある．アバランシェ型ハイビジョン用撮影管は高解像度で，高感度の撮影が可能である．非セレン膜で構成された光伝導電層は，高電圧操作下で電子なだれ現象を生じさせ，実効量子効率が数百倍の光電変換ができる．新 Super-HARP カメラは，空間分解能は 25 μm で，CCD カメラより 100 倍以上の感度があり，25 μm の非セレン膜の構造をとっている[6)-9)]．

### 5．トータルシステム

本装置は，既存の血管装置と同様にCアーム保持型を有しており，さまざまな角度からの撮影が可能である(図 9)．今までのハイビジョン映像は，高画質

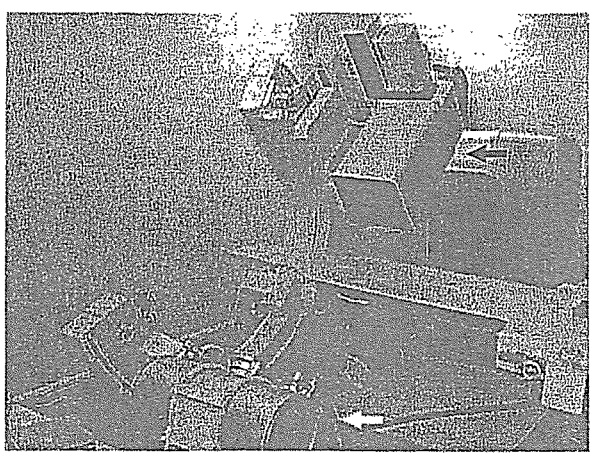


図 9．トータルシステム
筒型のX線管は，現在臨床で使用されているCT用の高出力線源である(白矢印)．Cアーム上端には蛍光板を有した検出器と HARP 管を有するカメラを搭載している(黒矢印)．

200 万画素の動画映像 1 秒間 30 フレームを長時間記録する装置が必要であり，放送局用の VTR しかなかった．これは，専門家しか扱えなく，装置から得られた画像の分析も複雑であった．そこで，デジタル高精細画像処理・表示装置を導入した．ハードディスクへの直接記録方式をとり，数十分の長時間記録が可能となり，小型で安価な装置となった．性能は，10 ビットデジタル画像データが記録できる大容量ハードディスク 144GB(1,000×1,000 画素×10 ビットを 30 フレーム/秒を 40 分記録可能)を搭載しており，映像データは直ちに再生・逆再生・スチル再生が可能であり，マウスのみで機械操作ができる．

### 6．直接X線量と散乱線量

安全性の検討として，直接 X 線量と散乱 X 線量を計測した．X 線発生装置から 1 m に検出器を設置し，管電圧 70 kV，管電流 500 mA で 20 秒照射した場合，0.547 Sv(62.7R)であった(図 10)．検査の撮影条件としては，最低でも一検査あたり 100 R 以下(3 R/sec)を目標としており，妥当な線量と考えられる．また，X 線発生装置から 1 m の距離にファントムを置き，50 cm 側方で散乱 X 線を検出した場合の散乱 X 線量は 0.0225 mSv(2.58 mR)であった(図 11)．放射線医療従事者の年間被曝量の限度は 50 mSv であり，術者に対する影響は少ないと考えられた．

### 7．微小血管画像

イヌ冠動脈ファントムを用い，既存の血管造影装置と比較した．ファントムは冠動脈にヨードを含むマイクロスフィアーを注入後に結紮し，作成した．微小血管造影装置では，イヌ冠動脈の中隔枝が末梢まで分岐するたびに血管径が細くなっていくのが観察できたが，既存の X 線装置では観察できなかった(図 12)．また，ウサギ下肢血管を結紮し，慢性期に形成された新生血管を微小血管造影装置で観察した．さらに，血管拡張剤であるアデノシンを動脈に投与したところ，微小血管が拡張する現象も確認できた(図 13)．現在のとこ