Therefore, the filter thickness should be increased as much as possible to decrease bremsstrahlung x rays of lower than K-absorption edge of iodine. Subsequently, using the barium sulfate filter, because the peak photon energy was nearly equal to the K-edge, aluminum filtering should be employed. In addition, cerium oxide filter is also useful in order to increase the peak energy and to decrease the low-photon-energy bremsstrahlung x rays.

In the present research, we employed a low-dose-rate x-ray generator in order to measure the x-ray spectra using a semiconductor detector. However, conventional medical x-ray generators with high dose rates can be employed to increase the tube current and to decrease the exposure time at a constant tube voltage.

With recent advances in angiography using MRI, if the density of gadolinium-based contrast media increases, enhanced K-edge angiography utilizing monochromatic x-ray generators, which produce K$\alpha$ rays of ytterbium, tantalum, and tungsten, will be fairly useful technique to decrease the absorbed dose during angiography.

**Acknowledgment**

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# Compact x-ray generator utilizing cerium-target tube for angiography

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## ABSTRACT

The cerium-target x-ray tube is useful in order to perform cone beam K-edge angiography because K-series characteristic x rays from the cerium target are absorbed effectively by iodine-based contrast mediums. The x-ray generator consists of a main controller and a unit with a high-voltage circuit and a fixed anode x-ray tube. The tube is a glass-enclosed diode with a cerium target and a 0.5 mm-thick beryllium window. The maximum tube voltage and current were 65 kV and 0.4 mA, respectively, and the focal-spot sizes were 1.3×0.9 mm. Cerium K-series characteristic x rays were left using a 3.0 mm-thick aluminum filter, and the x-ray intensity was 0.59 μC/kg at 1.0 m from the source with a tube voltage of 60 kV, a current of 0.40 mA, and an exposure time of 1.0 s. Angiography was performed with a computed radiography system using iodine-based microspheres 15 μm in diameter. In angiography of non-living animals, we observed fine blood vessels of approximately 100 μm with high contrasts.

**Key words:** x-ray tube, cerium target, quasi-monochromatic x rays, characteristic x rays, K-edge angiography

## 1. INTRODUCTION

Monochromatic parallel x-ray beams are the basis of radiography using synchrotrons in conjunction with single crystals, and these beams have been employed to perform enhanced K-edge angiography[1-3] and x-ray phase imaging.[4-6] In angiography, the beams with photon energies of approximately 35 keV are absorbed effectively by iodine-based contrast mediums. However, it is difficult to obtain sufficient machine times for various research projects, including medical applications.

In order to perform high speed medical radiography, although several different flash x-ray generator[7-13] utilizing

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cold-cathode tubes have been developed, plasma flash x-ray generators[14-18] are useful to produce quasi-monochromatic x rays without using a K-edge filter. Therefore, we have performed a demonstration of cone-beam K-edge angiography[19] utilizing a cerium plasma generator, since K-series characteristic x rays from the cerium target are absorbed effectively by iodine.

Recently, we have developed a steady state x-ray generator utilizing a cerium-target tube,[20] and have demonstrated enhanced K-edge angiography utilizing a barium sulfate filter. In the spectrum measurement, although Kα lines were produced, bremsstrahlung x rays with photon energies of lower than the barium K-edge (37.4 keV) were also observed. Therefore, optimum filtering for K-edge angiography should be selected to increase image contrast of fine blood vessels. In the present research, we employed a compact x-ray generator with a cerium target tube, and used it to perform a preliminary study on cone beam K-edge angiography achieved with cerium characteristic x rays utilizing an aluminum filter.

## 2. GENERATOR

Figure 1 shows the block diagram of the x-ray generator, which consists of a main controller and an x-ray tube unit with a Cockcroft-Walton circuit and a cerium-target tube. The tube voltage, the current, and the exposure time can be controlled by the controller. The main circuit for producing x rays is illustrated in Fig. 2, and employed the Cockcroft-Walton circuit in order to decrease the dimensions of the tube unit. In the x-ray tube, the negative high voltage is applied to the cathode electrode, and the anode (target) is connected to the tube unit case (ground potential) to cool the anode and the target effectively. The filament heating current is supplied by an AC power supply in the controller in conjunction with an insulation transformer. In this experiment, the tube voltage applied was from 45 to 65 kV, and the tube current was regulated to within 0.40 mA (maximum current) by the filament temperature. The exposure time is controlled in order to obtain optimum x-ray intensity. Quasi-monochromatic x rays are produced using a 3.0 mm-thick aluminum filter for absorbing soft bremsstrahlung rays.

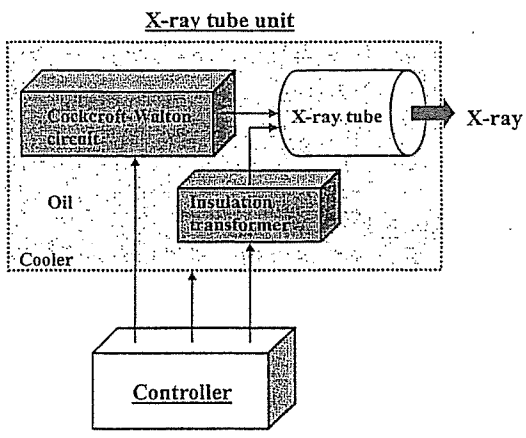


Fig. 1. Block diagram of compact x-ray generator with cerium-target radiation tube, which is used specially for K-edge angiography using iodine-based contrast mediums.

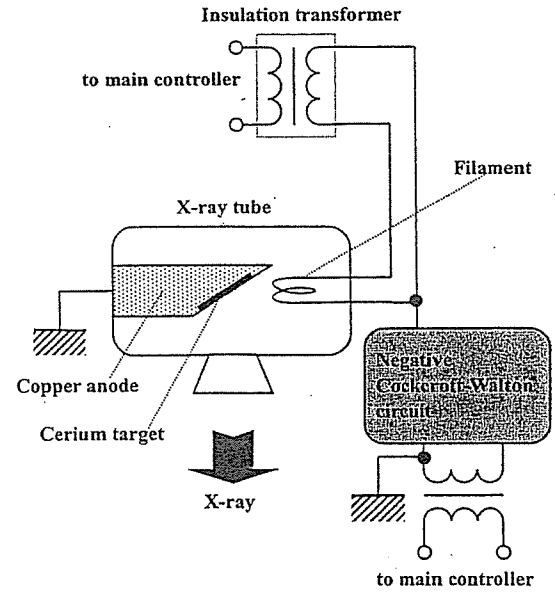


Fig. 2. Main circuit of x-ray generator.

## 3. CHARACTERISTICS

### 3.1 X-ray intensity

X-ray intensity was measured by a Victoreen 660 ionization chamber at 1.0 m from the x-ray source using the filter with

an exposure time of 1.0 s (Fig. 3). At a constant tube current of 0.40 mA, the x-ray intensity increased when the tube voltage was increased. In this measurement, the intensity with a tube voltage of 60 kV and a current of 0.40 mA was 0.59 μC/kg at 1.0 m from the source with errors of less than 0.2%.

### 3.2 Focal spot

In order to measure images of the x-ray source after the aluminum filtration, we employed a pinhole camera with a hole diameter of 50 μm (magnification ratio of 1:2) in conjunction with a Computed Radiography (CR) system[21] with a sampling pitch of 87.5 μm. When the tube voltage was increased, spot dimensions increased slightly and had values of 1.3×0.9 mm (Fig. 4).

### 3.3 X-ray spectra

In order to measure x-ray spectra, we employed a cadmium tellurium detector (CDTE2020X, Hamamatsu Photonics Inc.) (Fig. 5). Compared with a germanium detector, this detector has a lower energy resolution of 1.7 keV. When the tube voltage was increased, the characteristic x-ray intensities of Kα and Kβ lines increased, and both the maximum photon energy and the intensities of bremsstrahlung x rays increased.

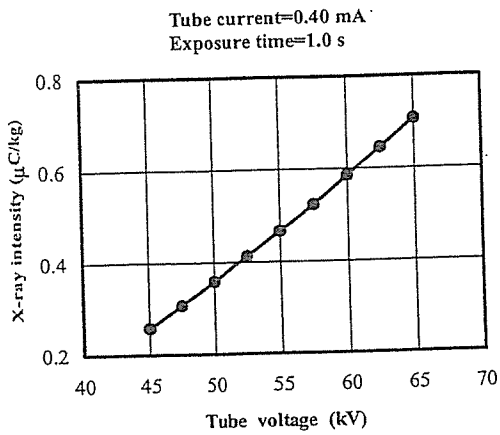


Fig. 3. X-ray intensity measured at 1.0 m from x-ray source according to changes in tube voltage.

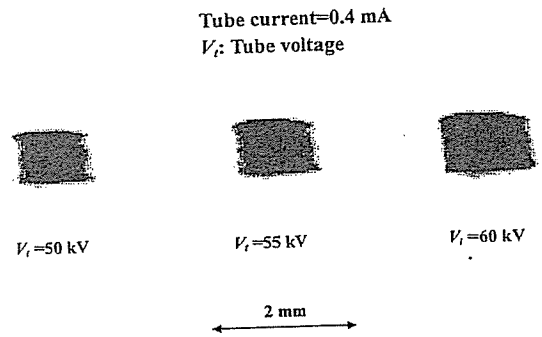


Fig. 4. Effective focal spots with changes in tube voltage.

## 4. ANGIOGRAPHY

Figure 6 shows the mass attenuation coefficients of iodine at the selected energies; the coefficient curve is discontinuous at the iodine K-edge. The average photon energy of the cerium Kα and Kβ lines are shown just above the iodine K-edge. Cerium is a rare earth element and has a high reactivity; however, the average photon energies of Kα and Kβ lines are 34.6 and 39.2 keV, respectively, and iodine contrast mediums with a K-absorption edge of 33.155 keV absorb the lines easily. Therefore, blood vessels were observed with high contrasts.

The angiography was performed by the CR system (Konica Regius 150) using the filter, and the distance (between the x-ray source and the imaging plate) was 1.5 m. Firstly, rough measurements of spatial resolution were made using wires. Figure 7 shows radiograms of tungsten wires coiled around a rod made of polymethyl methacrylate. Although the image contrast decreased somewhat with decreases in the wire diameter, due to blurring of the image caused by the sampling pitch of 87.5 μm, a 50 μm-diameter wire could be observed.

Angiograms of rabbit hearts are shown in Fig. 8. These two images were obtained using iodine and cerium microspheres of 15 μm in diameter at a tube voltage of 60 kV. In the case where the cerium spheres were employed, the coronary arteries were barely visible. Figure 9 shows an angiogram of a larger dog heart using the cerium target at a tube voltage of 60 kV using iodine spheres. For comparison, we show 3-dimensional image of the coronary arteries constructed from x-ray CT images by Pascal (Digital Culture Tech. Corp.) with a tungsten x-ray tube (Fig. 10). Using this imaging technique, fine blood vessel were not observed at all.

## 5. DISCUSSION

In summary, we employed an x-ray generator with a cerium-target tube and succeeded in producing cerium characteristic x rays, which can be absorbed easily by iodine-based contrast mediums. The characteristic x-ray intensities increased with increases in the tube voltage, and low-photon-energy bremsstrahlung rays were absorbed effectively by the filter. Although the cerium x-ray generator used in this research produces both the characteristic and the bremsstrahlung x rays, bremsstrahlung intensity can be decreased effectively by considering the angle dependence without using the filter, since bremsstrahlung rays are not emitted in the opposite direction to that of electron acceleration. Subsequently, the generator produced maximum number of characteristic photons was approximately 35M photons/cm$^2$· s at 1.0 m from the source, and the photon count rate can be increased easily by improving the target.

As compared with 3-dimensional blood images constructed from x-ray CT images by Pascal, fine blood vessels were visible. Because the sampling pitch of the CR system is 87.5 μm, we obtained spatial resolutions of approximately 100 μm. In order to observe fine blood vessels of less than 100 μm, the spatial resolution of the CR system should be improved as much as possible.

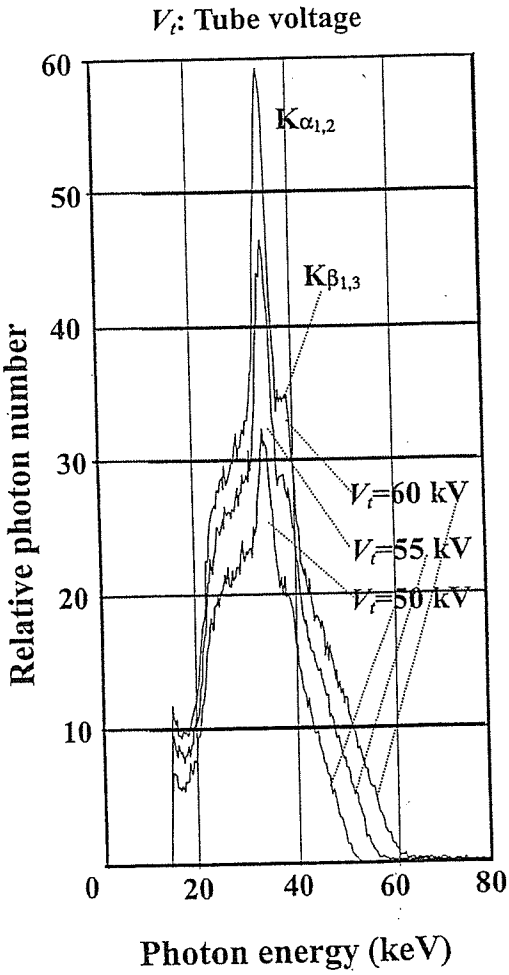


Fig. 5. X-ray spectra measured using cadmium tellurium detector with changes in tube voltage.

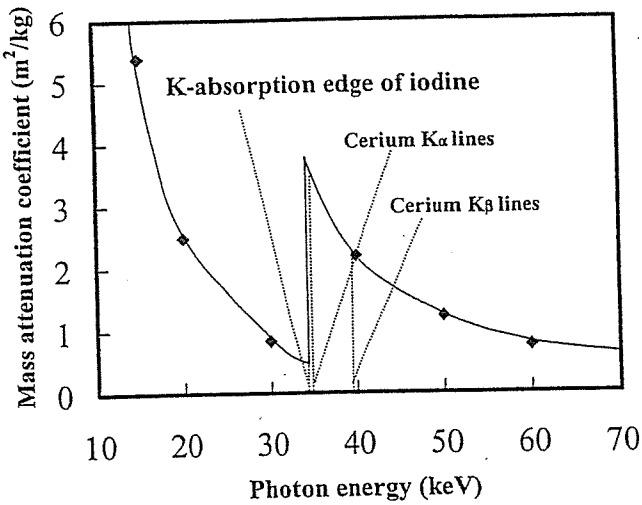


Fig. 6. Mass attenuation coefficients of iodine, and average photon energies of cerium Kα and Kβ lines.

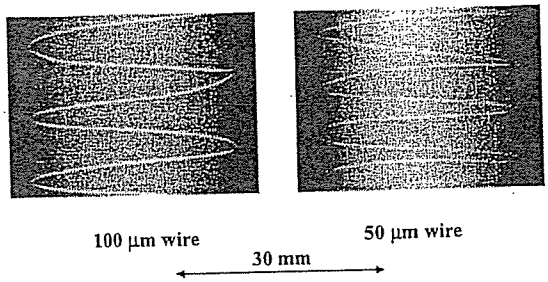


Fig. 7. Radiograms of tungsten wires in PMMA rod with tube voltage of 60 kV.

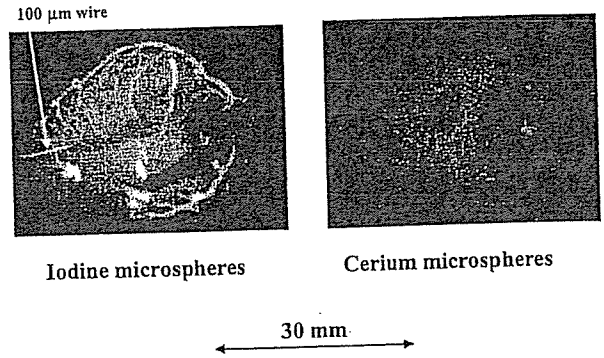


Fig. 8. Angiograms of extracted rabbit hearts using iodine and cerium microspheres with tube voltage of 60 kV.

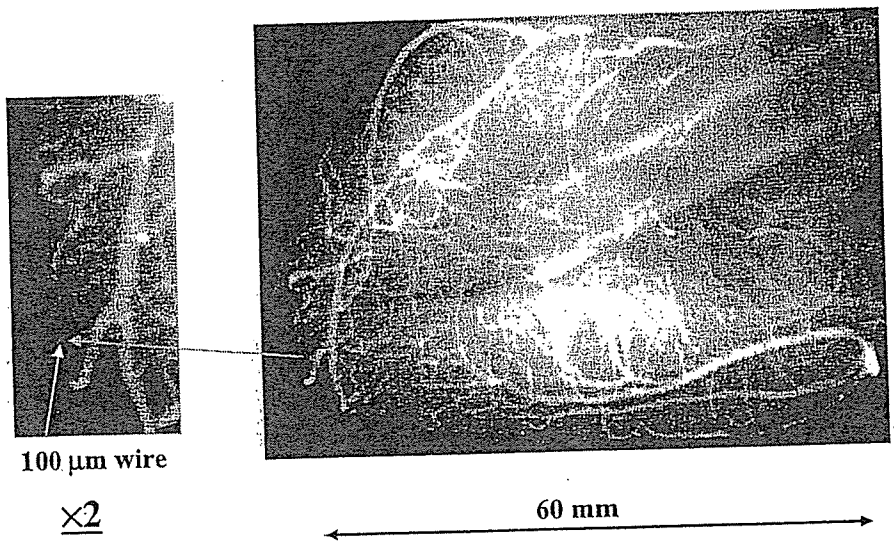


Fig. 9. Angiograms of extracted dog heart using iodine microspheres with tube voltage of 60 kV.

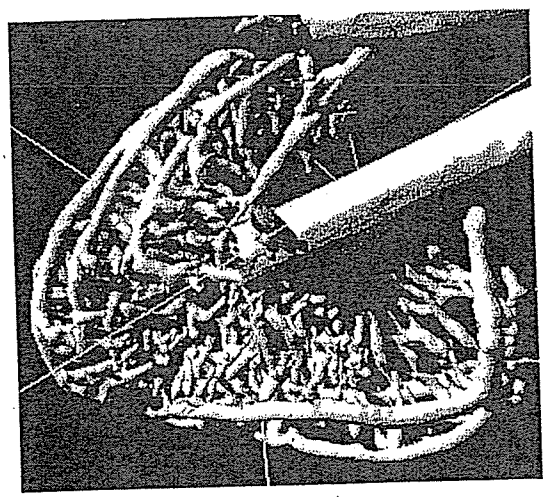

Fig. 10. 3-dimensional image of coronary arteries constructed from x-ray CT images by Pascal.

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# Cerium X-ray Spectra without Filtering and their Application to High-contrast Angiography

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**Abstract**

The cerium-target x-ray tube is useful in order to perform cone-beam K-edge angiography because K-series characteristic x rays from the cerium target are absorbed effectively by iodine-based contrast media. The x-ray generator consists of a main controller, an x-ray tube unit with a high-voltage circuit and an insulation transformer, and a personal computer. The tube is a glass-enclosed diode with a cerium target and a 0.5-mm-thick beryllium window. The maximum tube voltage and current were 65 kV and 0.4 mA, respectively, and the focal-spot sizes were 1.2×0.8 mm. Sharp cerium K-series characteristic x rays were observed without using a filter, and the x-ray intensity was 209 $\mu$Gy/s at 1.0 m from the source with a tube voltage of 60 kV and a current of 0.40 mA. Angiography was performed with a computed radiography system using iodine-based microspheres 15 $\mu$m in diameter. In angiography of non-living animals, we observed fine blood vessels of approximately 100 $\mu$m with high contrasts.

**Keywords:** x-ray tube, cerium target, x-ray spectra, characteristic x rays, K-edge angiography, energy-selective radiography

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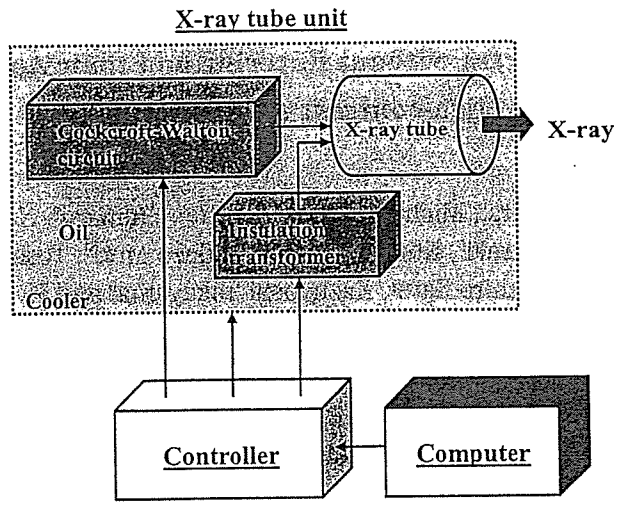


Fig. 1: Block diagram of compact x-ray generator with cerium-target radiation tube, which is used specially for K-edge angiography using iodine-based contrast media.

## 1. Introduction

The principle basis of quality assurance for enhanced K-edge angiography is the discontinuity of the absorption coefficient at the K-absorption edge of iodine-based contrast media, and angiography has been performed using monochromatic parallel x-ray beams with synchrotrons.[1-3] Subsequently, monochromatic x-ray computed tomography at two different energies has provided information on the electron density of human tissue.[4] In addition, a compact pulsed tunable monochromatic x-ray source has been designed, developed, and tested.[5] From the source, conical x-ray beams from 10 to 50 keV with pulse widths of 8 ps have been produced, and these beams are useful for biomedical imaging and protein crystallography.

In order to perform high-speed medical radiography, although several different flash x-ray generators[6-10] utilizing cold-cathode tubes have been developed, plasma flash x-ray generators[11-14] are useful to produce quasi-monochromatic x rays without using a K-edge filter. Therefore, we have performed a demonstration of cone-beam K-edge angiography[19] utilizing a cerium plasma generator, since K-series characteristic x rays from the cerium target are absorbed effectively by iodine.

Recently, we have developed a steady-state x-ray generator utilizing a cerium-target tube, and have demonstrated enhanced K-edge angiography utilizing a barium sulfate filter.[15] In this research, K$\alpha$ lines (34.6 keV) were left by absorbing K$\beta$ lines (39.2 keV), and bremsstrahlung x rays with photon energies of lower than the barium K-edge (37.4 keV) were also observed. However, because cerium K$\beta$ lines are also absorbed effectively by iodine, both K$\alpha$ and K$\beta$ lines should be selected to perform angiography. In measurements of x-ray spectra, although we usually employed a cadmium tellurium detector with a photon energy resolution of 1.7 keV, the resolution should be improved as much as possible to measure the characteristic x-ray intensity.

In the present research, we measured the x-ray spectra from a cerium-target tube using a germanium detector, and performed a preliminary study on cone-beam K-edge angiography achieved with cerium characteristic x rays without using a filter.

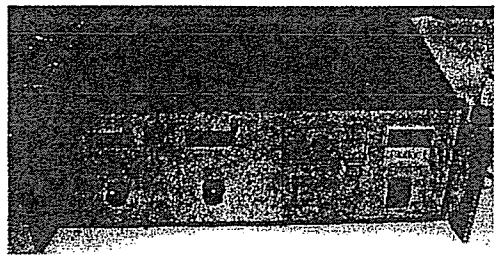

Fig. 2: Main controller.

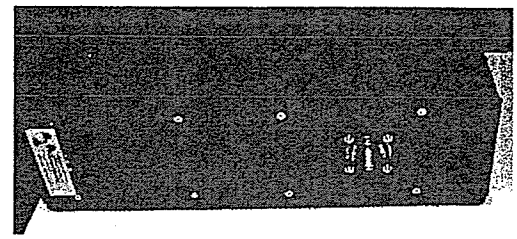

Fig. 3: X-ray tube unit.

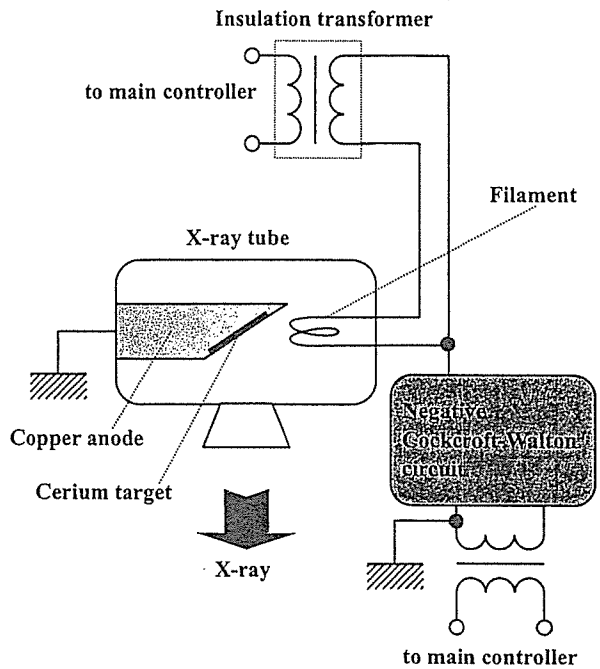


Fig. 4: Main circuit of x-ray generator.

## 2. Generator

Figure 1 shows the block diagram of the x-ray generator, which consists of a main controller (Fig. 2), a cerium-target x-ray tube unit (Fig. 3) with a Cockcroft-Walton circuit and an insulation transformer, and a personal computer. The tube voltage, the current, and the exposure time can be controlled by both the controller and the computer. The main circuit for producing x rays is illustrated in Fig. 4, and employs the Cockcroft-Walton circuit in order to decrease the dimensions of the tube unit. In the x-ray tube, the negative high-voltage is applied to the cathode electrode, and the anode (target) is connected to the tube unit case (ground potential) to cool the anode and the target effectively. The filament heating current is supplied by an AC power supply in the controller in conjunction with an insulation transformer. In this experiment, the tube voltage applied was from 45 to 65 kV, and the tube current was regulated to within 0.40 mA (maximum current) by the filament temperature. The exposure time is controlled in order to obtain optimum x-ray intensity.

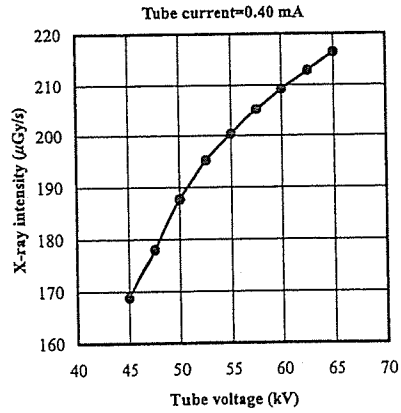


Fig. 5: X-ray intensity measured at 1.0 m from x-ray source according to changes in tube voltage.

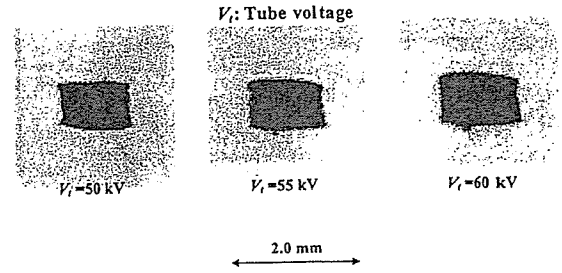


Fig. 6: Effective focal spots with changes in tube voltage.

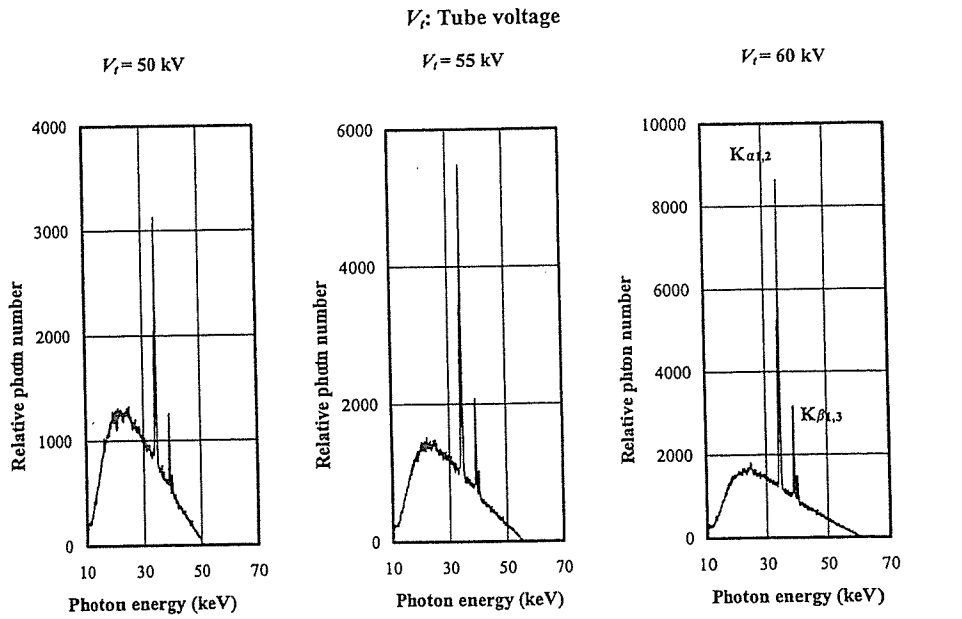


Fig. 7: X-ray spectra measured using germanium detector.

## 3. Characteristics

### 3.1 X-ray intensity

The x-ray intensity rate was measured by a Victoreen 660 ionization chamber at 1.0 m from the x-ray source (Fig. 5). At a constant tube current of 0.40 mA, the x-ray intensity increased when the tube voltage was increased. In this measurement, the intensity with a tube voltage of 60 kV and a current of 0.40 mA was 209 $\mu$Gy/s with errors of less than 0.2%.

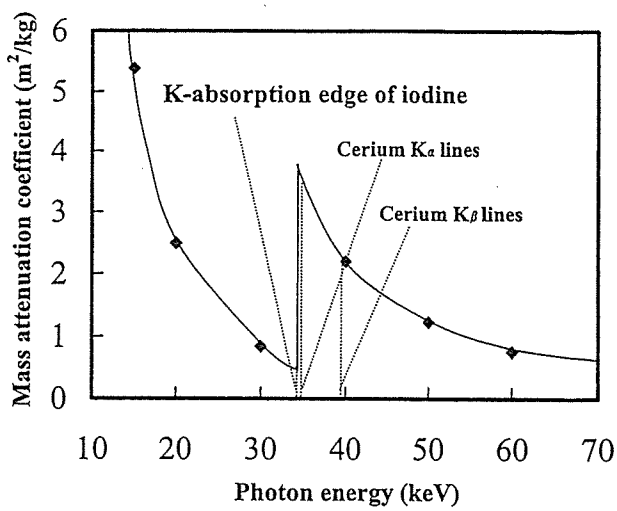


Fig. 8: Mass attenuation coefficients of iodine, and average photon energies of cerium K$\alpha$ and K$\beta$ lines.

### 3.2 Focal spot

In order to measure images of the x-ray source without filtration, we employed a pinhole camera with a hole diameter of 50 $\mu$m (magnification ratio of 1:2) in conjunction with a Computed Radiography (CR) system[16] with a sampling pitch of 87.5 $\mu$m. When the tube voltage was increased, spot dimensions increased slightly and had values of 1.2 × 0.8 mm (Fig. 6).

### 3.3 X-ray spectra

In order to measure x-ray spectra, we employed a germanium detector (GLP-10180/07-P, Ortec Inc.) (Fig. 7). When the tube voltage was increased, the characteristic x-ray intensities of K$\alpha$ and K$\beta$ lines substantially increased, and both the maximum photon energy and the intensities of bremsstrahlung x rays increased.

## 4. Angiography

Figure 8 shows the mass attenuation coefficients of iodine at the selected energies; the coefficient curve is discontinuous at the iodine K-edge. The average photon energy of the cerium K$\alpha$ and K$\beta$ lines are shown just above the iodine K-edge. Cerium is a rare earth element and has a high reactivity; however, the average photon energies of K$\alpha$ and K$\beta$ lines are 34.6 and 39.2 keV, respectively, and iodine contrast mediums with a K-absorption edge of 33.155 keV absorb the lines easily. Therefore, blood vessels were observed with high contrasts.

The angiography was performed by the CR system (Konica Regius 150) without using a filter, and the distance (between the x-ray source and the imaging plate) was 1.5 m. Firstly, rough measurements of spatial resolution were made using wires. Figure 9 shows radiograms of tungsten wires in a rod made of polymethyl methacrylate with a tube voltage of 55 kV. Although the image contrast decreased somewhat with decreases in the wire diameter, due to blurring of the image caused by the sampling

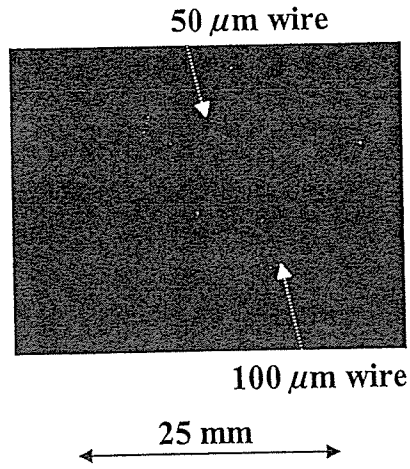


Fig. 9: Radiogram of tungsten wires in PMMA rod with tube voltage of 55 kV.

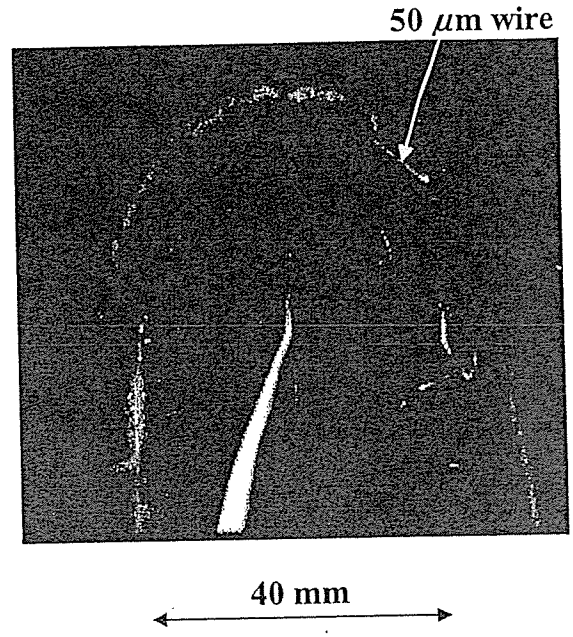


Fig. 10: Angiogram of rabbit ear with tube voltage of 50 kV.

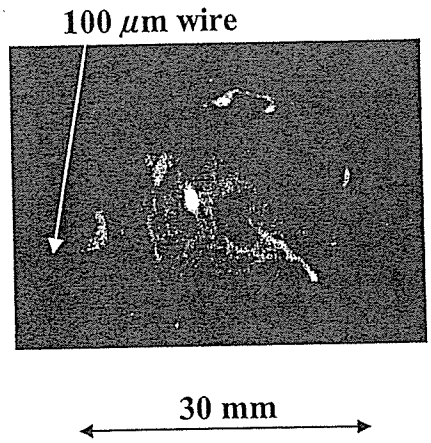


Fig. 11: Angiogram of extracted rabbit heart with tube voltage of 50 kV.

pitch of 87.5 μm, a 50 μm-diameter wire could be observed.

Figures 10 and 11 show angiograms of a rabbit ear and heart, respectively. These images were obtained using iodine microspheres of 15 μm in diameter at a tube voltage of 50 kV. Fine blood vessels in the ear and the coronary arteries in the heart were visible. Figure 12 shows an angiogram of a larger dog heart at a tube voltage of 60 kV using iodine spheres. For comparison, we show 3-dimensional image of the coronary arteries constructed from x-ray CT images by Pascal (Digital Culture Tech. Corp.) with a tungsten x-ray tube (Fig. 13). Using this imaging technique, fine blood vessels were not observed at all.

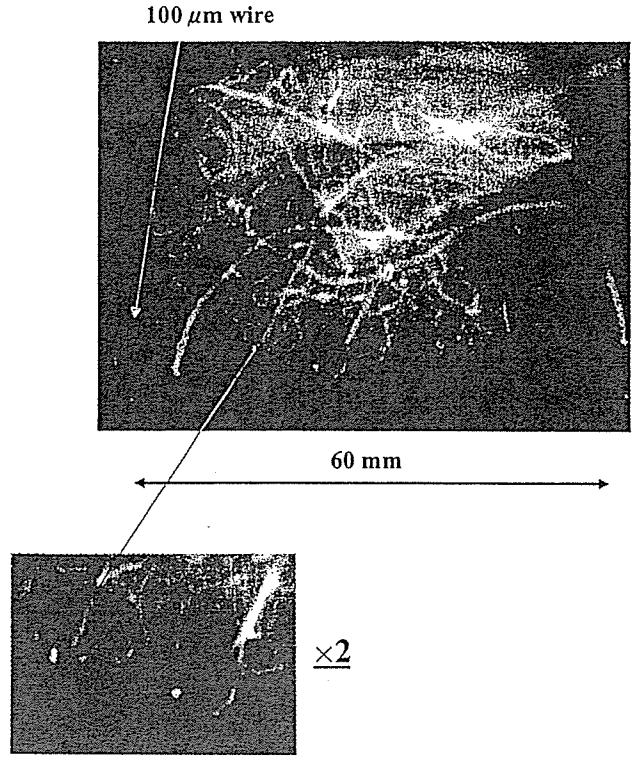


Fig. 12: Angiogram of extracted dog heart using iodine microspheres with tube voltage of 60 kV.

## 5. Discussion and results

In summary, we employed an x-ray generator with a cerium-target tube and succeeded in producing cerium characteristic x rays, which can be absorbed easily by iodine-based contrast media. Both the characteristic and the bremsstrahlung x-ray intensities increased with increases in the tube voltage, low-photon-energy bremsstrahlung x rays with energies of less than the iodine K edge should be absorbed by filtering to perform angiography. Without using the filter, bremsstrahlung intensity can be decreased effectively by considering the angle dependence, since bremsstrahlung rays are not emitted in the opposite direction to

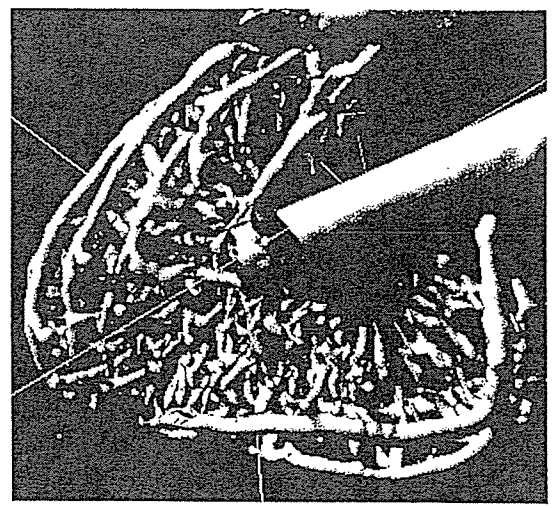

Fig. 13: 3-dimensional image of coronary arteries constructed from x-ray CT images by Pascal.

that of electron acceleration.

The x-ray intensity was limited because the thermal contact between the target and the anode was not good. However, the intensity can be increased by welding the target or using a cerium-alloy target. In addition, a rotation anode tube can be developed by sputtering of cerium.

As compared with 3-dimensional blood images constructed from x-ray CT images by Pascal, fine blood vessels were visible. Because the sampling pitch of the CR system is 87.5 $\mu$m, we obtained spatial resolutions of approximately 100 $\mu$m. In order to observe fine blood vessels of less than 100 $\mu$m, the spatial resolution of the CR system should be improved.

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

## Benefits of a Novel Nonviral Vector, Gelatin

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***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.

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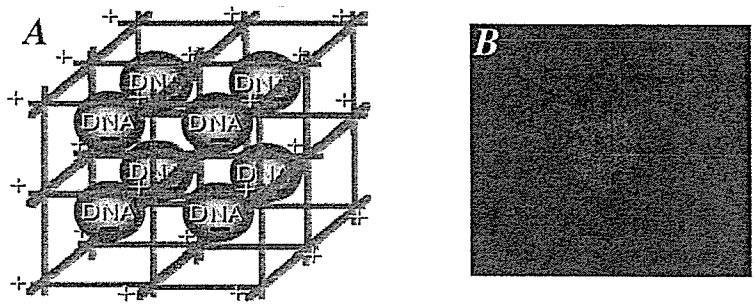


Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

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.