厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）

分担研究報告書

体外培養の増幅血管内皮前駆細胞移植による虚血性疾患治療に関する基礎・臨床研究

分担研究者　木原　康樹　神戸市立中央市民病院　部長

研究要旨

増幅血管内皮前駆細胞の心筋虚血組織への移植治療技術の開発を行った。同治療の将来の臨床適用を見据えて、大動物を用いた心筋虚血モデル・経カテーテル的な移植技術の確立に取り組んだ。

A. 研究目的

増幅血管内皮前駆細胞（Endothelial progenitor cell: EPC）の心筋・下肢虚血組織への移植治療技術を開発する。

B. 研究方法

大動物実験による細胞移植治療開発

新たに建設された大動物実験施設（神戸医療機器開発センター）において、先端医療センター血管再生研究グループおよびアイビーテック社と共同してブタ心筋虚血モデルの確立に取り組んだ。また、経カテーテル的な心筋内移植法も技術的に確立させた。

（倫理面への配慮）

上記の動物実験は、先端医療センターの動物実験審査委員会から実施の承認を得た後に開始した。

C. 研究結果

大動物実験成果

ブタ左回旋枝へのアメロイドコンストリクター装着による心筋虚血モデルの確立に取り組んだ。手術手技は十分に確立でき、術中死亡率は10%未満、術後４週間までの慢性期死亡率も20%程度と低く、満足すべき成績であった。さらに、４週後の冠動脈造影では、ほぼ全例で左回旋枝の完全閉塞が観察され、NOGA マッピングでも左室側壁の虚血が確認された。さらに、同マッピングガイド下に生食、血管新生因子遺伝子等の経カテーテル的な注入を試み、技術的な習熟を得た。

D. 考察および結論

今後は上記の大動物心筋虚血モデルを用いた細胞移植治療技術の開発を通して、臨床試験計画の完成に貢献していきたい。

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F. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

特記事項なし

2. 実用新案登録

特記事項なし

3. その他

特記事項なし

厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）

分担研究報告書

細胞培養センター（ＣＰＣ）を用いた血管前駆細胞（ＥＰＣ）体外増幅に関する研究

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研究要旨

ＧＭＰ基準に適合するＥＰＣの体外増殖培養技術確立と製造・品質及び衛生に関わる基準書・標準書・手順書・記録書等の管理文書類作成。

A. 研究目的

治療用の細胞を安全に患者様に、投与するためには、ＧＭＰ基準に従った細胞製剤の製造・品質管理が要求されている。今回の分担研究ではＣＰＣを用いた治療用の細胞の製造と品質管理に関する管理文書類を作成し、今後の細胞製剤製造手順の手引き書となることを目指す。

B. 研究方法

実際にＣＰＣ内でＥＰＣ細胞を培養し、感染症などの安全性・細胞増殖率・生存率等を検定する。それに応じ、出荷可能かどうかの品質管理と出荷判定基準を策定する。また使用するＣＰＣの衛生管理基準を参照しながら達成可能な清浄度及び清掃頻度・方法も策定する。

C. 研究結果

ＥＰＣの細胞培養を通じて下記のＣＰＣ製造・品質・衛生管理文書類を策定した。

記

製品標準書

管理基準書

製造管理基準書・品質管理基準書・衛生管理基準書

手順書

清浄度管理手順書 清掃・消毒記録 清掃・消毒区分

清掃・消毒作業手順書 清掃場所一覧

付着菌測定手順書 付着菌測定ポイント 付着菌測定頻度　ポイント 付着菌作業室別測定結果

落下菌測定手順書 落下菌測定ポイント

落下菌測定頻度　ポイント 落下菌作業室別測定結果

浮遊菌測定手順書 浮遊菌測定ポイント 浮遊菌測定頻度　ポイント 浮遊菌作業室別測定結果

浮遊微粒子測定手順書 浮遊微粒子測定ポイント 浮遊微粒子測定頻度　ポイント 浮遊微粒子作業室別測定結果

D. 考察と結論

このような文書体系を用いることにより(good tissue practice (GTP)、GMP規格を保証する体制の構築に役立った。今後は、技術員の教育と指導体制が課題となる。

E. 研究発表

1. 論文発表

なし

2. 学会発表

なし

2. 実用新案登録

なし

3. その他

なし

F. 知的財産権の出願・登録状況

1. 特許取得

なし

厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）

分担研究報告書

体外培養の増幅血管内皮前駆細胞移植による虚血性疾患治療に関する基礎・臨床研究

分担研究者　福島　雅典　臨床研究情報センター研究部　事業統括

研究要旨

虚血性疾患を対象とした増幅血管内皮前駆細胞移植治療の臨床適用を目指して、臨床試験プロトコルの作成に着手した。同時に、プロトコル完成のために必要な前臨床研究項目を指摘し、今後早急に成果を挙げることを指示した。

A. 研究目的

増幅血管内皮前駆細胞（Endothelial progenitor cell: EPC）移植治療の臨床適用を目指して、同治療の臨床試験プロトコルを完成させる。

B. 研究方法

増幅EPCを臨床適用するためには、同治療の安全性を担保し、有効性を支持しうる基礎研究・前臨床研究・先行臨床研究成果が必須である。これまでの研究成果を先端医療センターの研究グループと検討した。

（倫理面への配慮）

臨床試験プロトコルは、完成後に先端医療センターの再生医療審査委員会、さらに厚生労働省での審査を請求する予定である。

C. 研究結果

基礎・前臨床研究として、移植細胞の至適用量を明らかにする必要があり、動物実験の追加を指示した。さらに現在施行されている第I/II相下肢血管再生治療臨床試験でのEPC移植治療（細胞の採取・分離・移植時から移植後１年まで）の安全性、有効性データを整理し、培養増幅EPC移植治療の方法の至適化、従来の方法に比しての優位性の検証を深めた。

D. 考察および結論

今後は上記の問題点を解決することで、倫理的でかつ再現性が高く、科学的評価に耐える臨床試験計画の完成に貢献していきたい。

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F. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

特記事項なし

2. 実用新案登録

特記事項なし

3. その他

特記事項なし

Ⅲ　研究成果の刊行に関する一覧表

Ⅲ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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浅原孝之，川本篤彦	血管内皮前駆細胞による血管再生・修復療法	心臓	38(2)	200-204	2006

Ⅳ　研究成果の刊行物・別刷

Synchrotron Radiation Coronary Microangiography for Morphometric and Physiological Evaluation of Myocardial Neovascularization Induced by Endothelial Progenitor Cell Transplantation

Hiroto Iwasaki, Kazuhito Fukushima, Atsuhiko Kawamoto, Keiji Umetani, Akira Oyamada, Saeko Hayashi, Tomoyuki Matsumoto, Masakazu Ishikawa, Toshihiko Shibata, Hiromi Nishimura, Hidekazu Hirai, Yutaka Mifune, Miki Horii, Kazuro Sugimura, Shigefumi Suehiro, Takayuki Asahara

Background—Therapeutic effect of stem cell transplantation (SCTx) for myocardial neovascularization has been evaluated by histological capillary density in small animals. However, it has been technically difficult to obtain imaging evidence of collateral formation by conventional angiography.

Methods and Results—Peripheral blood CD34+ and CD34− cells were isolated from patients with critical limb ischemia. PBS, CD34− cells, or CD34+ cells were intramyocardially transplanted after ligating LAD of nude rats. Coronary angiography of ex vivo beating hearts 5 and 28 days after the treatment was performed using the third generation synchrotron radiation microangiography (SRM), which has potential to visualize vessels as small as 20 μm in diameter. The SRM was performed pre and post sodium nitroprusside (SNP) to examine vascular physiology at each time point. Diameter of most collateral vessels was 20 to 120 μm, apparently invisible size in conventional angiography. Rentrop scores at day 28 pre and post SNP were significantly greater in CD34+ cell group than other groups ($P<0.01$). To quantify the extent of collateral formation, angiographic microvessel density (AMVD) in the occluded LAD area was analyzed. AMVD on day 28 post SNP, not pre SNP, was significantly augmented in CD34+ cell group than other groups ($P<0.05$). AMVD post SNP closely correlated with histological capillary density ($R=0.82$, $P<0.0001$).

Conclusions—The SRM, capable of visualizing microvessels, may be useful for morphometric and physiological evaluation of coronary collateral formation by SCTx. The novel imaging system may be an essential tool in future preclinical/translational research of stem cell biology. (***Arterioscler Thromb Vasc Biol.* 2007;27:000-000.**)

Key Words: synchrotron radiation microangiography ■ image ■ CD34+ cells ■ neovascularization ■ myocardial infarction

Stem/progenitor cell transplantation (SCTx) investigated since the early 1990s is a novel approach for vascular regeneration therapy in ischemic diseases.[1–3] One of the examples of the SCTx is transplantation of adult peripheral blood CD34+ cells that are endothelial progenitor cell (EPC)-enriched population. Transplantation of CD34+ cells prevents left ventricular (LV) dilatation and wall thinning, inhibits myocardial fibrosis and apoptosis, and preserves LV function through augmentation of myocardial neovascularization and blood flow.[4–9] Evidence of increased vascularity by therapeutic neovascularization such as CD34+ cell transplantation has been obtained by histological assessment of capillary density and physiological evaluation of tissue perfusion has been by microsphere methods in small sized animals (mice and rats) with acute MI.[10] However, the histological examination has limitation for precise assessment of vascular physiology in response to environmental stress. Though microsphere assessment was performed to evaluate physiological blood flow, it was pointed out to lack significant reproducibility in small animal models. Several research groups have utilized other approaches such as corrosion casts[11,12] and angiography[13,14] to visualize collateral vessels.

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Corrosion casts allow the visualization of small arteries less than 100 μm in diameter,[11] although it is impossible to examine in live animals or ex vivo beating hearts, and the complete distension of the vessels depends on several factors such as elastic properties of the vessel wall, viscosity of the infused material, and pressure of infusion. Conventional angiography, which has been widely performed in clinical practice, can be also undergone in live animals repeatedly. However, previous reports indicated that conventional angiography systems, which could not visualize arteries less than 200 μm in diameter,[15,16] have insufficient resolution to visualize full extent of collateral formation. The resolutional limitation may lead to underestimation of angiogenic potential of the SCTx, because improvement of collateral-dependent flow typically results from the proliferation of vessels less than 180 μm in diameter.[17–19] The indispensable angiographic assessment in small animals has never been established.

Synchrotron radiation (SR) has been investigated as a novel approach for animal studies because intravenous coronary angiography, a relatively less invasive technique compared with selective coronary angiography, was begun at the end of 1970's. Research groups have improved imaging systems in SR facilities for future clinical application.[20] Aside from the intravenous coronary angiography, Mori et al[15,16] recently developed a new angiography system called SR microangiography (SRM), which was an intraarterial microangiography system. In this system, monochromatic SR is used as an x-ray source, which energy was adjusted to 33.2 keV just above the iodine K-edge energy to produce the highest contrast image of the iodine contrast material, and a high-fidelity video system is also used as a detector, which has the potential to visualize small vessels (diameter <50 to 100 μm). Thereafter, many researchers have used the SRM to visualize penetrating transmural coronary arteries in the canine hearts,[21] collateral microvessels following therapeutic angiogenesis in rat model of hind limb ischemia,[22] vasodilatation of arterial circle of cerebrum and its branches of the dogs,[23] and tumor-derived angiogenic vessels of the rabbits[24] at the Photon Factory in Tsukuba, Japan. However, the previous SRM system was unable to visualize coronary arteries, their branches, and collateral vessels in beating hearts of small animals because of the still inappropriate image quality. Currently, new SRM system with spatial resolution in the μm range has been developed in the SPring-8 (Japan Synchrotron Radiation Research Institute) in Sayo, Japan. Recently, Kidoguchi et al[25] have applied the new SRM system to visualize branches of rat middle cerebral arteries and successfully depict the vessels as small as 30 μm in diameter at 9.5 μm of detector pixel size. In this study, we used the new generation SRM to visualize rat coronary vessels as small as 20 μm in diameter at 4.5 μm of pixel size and evaluated coronary vascular function in response to vasodilator under fast beating condition. Here, we report usefulness of the third generation SRM to visualize collateral vessels and quantify the effect of therapeutic neovascularization by bone marrow (BM)-derived CD34+ cell transplantation in rats with MI.

Methods

Isolation of CD34+ Cells From Patients With Critical Limb Ischemia

Peripheral blood total mononuclear cells (tMNCs) were obtained from 3 male patients 71, 63, and 60 years of age with atherosclerotic peripheral artery disease by apheresis after 5-day subcutaneous administration of G-colony stimulating factor (CSF) (10 μg/kg/d). CD34+ cells or CD34− cells were isolated from the tMNCs by a magnetic cell sorting system, CliniMACS (Miltenyi Biotec).[26] The CD34+ cell fraction had a purity of >99%, as determined by fluorescence-activated cell sorting (FACS) analysis using a monoclonal antibody specific for human CD34 (Becton Dickinson). CD34+ cells in this study were $CD31^{bright}$, $AC133^{bright}$, and $CD45^{dim}$ but negative for KDR and VE-cadherin. In contrast, CD34− cells were positive for CD45 and CD31, but negative for AC133, KDR, and VE-cadherin. The FACS results suggest that freshly-isolated CD34+ cells are immature population responsible for hematopoietic stem cells, endothelial progenitor cells, and hemangioblasts, whereas the CD34− cells are not considered to be either immature or mature endothelial lineage cells (supplemental Figure I, available online at http://atvb.ahajournals.org).

These patients received intramuscular transplantation of 10^5 CD34+ cells/kg according to the protocol of a phase I/II dose-escalation clinical trial. Remaining CD34+ or CD34− cells were used for following experiments. Informed consent regarding the cell therapy and experimental use of the remaining cells was obtained from each patient before the case registration. The clinical study protocol was approved by the Institutional Ethics Committees of Kobe Institute of Biomedical Research and Innovation and Kobe City General Hospital.

Animals

Female athymic nude rats (F344/N Jcl rnu/rnu; CLEA Japan, Tokyo, Japan) aged 7 to 8 weeks and weighing 145–160 g were used in this study. The Institutional Animal Care and Use Committees of RIKEN Center for Developmental Biology approved all animal procedures including human cell transplantation. All of our experiments on imaging of the rat hearts with MI also conformed to the SPring-8 Guide for Care and Use of Laboratory Animals in SRM examination.

Induction of Myocardial Infarction and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 mg/kg and 10 mg/kg, respectively, IP). MI was induced by ligating left anterior descending coronary artery (LAD) as described previously.[7–9] Twenty minutes after MI, rats received intramyocardial transplantation of 1×10^5 CD34− cells or 1×10^5 CD34+ cells resuspended with 100 μL of PBS or the same volume of PBS without cells (n=9 in each group). To evaluate incorporation and development of the transplanted cells in MI tissue, CD34+ cells or CD34− cells labeled with fluorescent carbocyamine 1, 1′-dioctadecyl1–1 to 3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes, Carlsbad, CA) were intramyocardially transferred into athmic nude rats (n=3) after MI.[9]

Imaging System

SRM experiments were performed at the 2nd optical hatch of the BL28B2 beamline in the SPring-8. Monochromatic synchrotron radiation with an energy level of 33.2 keV was obtained from the beamline. An X-ray imaging system needs to have high shutter speed to make sharp and blur-free images of fast-moving hearts, and for this purpose we developed a shutter system using a rotating disk with radial slots rotating around an axis parallel to the X-ray beam. The shortest shutter open time was 0.1 ms. X-rays transmitted through the object are detected by the X-ray direct-conversion type detector incorporating the X-ray SATICON pick-up tube. For high-resolution, real-time imaging (7.0 μm or 4.5 μm pixel size, 30 frames/second), the monochromatized x-ray obtained from the third generation SR source and the new rotating disk shatter were used.

Differences in Characteristics of Synchrotron Radiation System Between the Photon Factory and the SPring-8

	Photon Factory	SPring-8
Input field of view, mm	50×50 or 20×20	7.0×7.0 or 4.5×4.5
Pixel size, μm	48×48 or 19×19	7.×7.0 or 4.5×4.5
Spatial resolution, μm	30	6
Minimum detectable vessel diameter, μm	50–100	20
Shortest shutter open time, msec	17	2

Sequential images were obtained with an input field of view of 7.0 mm × 7.0 mm or 4.5 mm × 4.5 mm. Image signals were converted into digital format and stored in a frame memory with a 1024×1024 pixels format and 10-bit resolution. Improved points in the new generation SR imaging system in the Spring-8 compared with the previous version in the Photon Factory in Tsukuba, Japan is shown in the Table.

Coronary Microangiography

Transplanted immunodeficient rats were anesthetized with pentobarbital and anticoagulant heparin intraperitoneally. After thoracotomy, the heart and aortic arch were rapidly excised and immersed in perfusion solution. The pericardium was quickly removed under immersion and aorta was prepared for cannulation. The heart was mounted on an aortic cannula, and then pulmonary artery was cut near its origin. Throughout the experiment, aortic retrograde perfusion at a constant flow rate (4.0 mL/min) with oxygenated perfusion solution drawn from a temperature-regulated reservoir (37°C) was started according to the Langendorf technique, as described in detail previously.[27] The perfusion solution was of the following composition (in mmol/L): NaCl, 118.5; $NaHCO_3$, 25.0; KCl, 3.2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.4; glucose, 11.0. The solution was filtered before use and gassed continuously with 90% O_2/10% CO_2 (pH 7.4 at 37°C). Perfusion fluid was directed into coronary arteries to perfuse the entire ventricular mass of the heart. Contractile function and regular heart rhythm returned within a few seconds, and maximum function was established in several minutes. After stabilization of heart rate and perfusion pressure in the ex vivo beating hearts under the Langendorf perfusion, SRM at baseline was performed in each animal. The microangiographic images were taken at base, mitral papillary muscle, and apical levels. Microangiography was performed with an automated injector (Nemoto Kyorindo) which was programmed to reproducibly deliver 0.4 mL/sec of nonionic contrast media containing 37% iodine (Iopamiron 370; Nihon Schering) for 4 sec. After the baseline angiography were taken, sodium nitroprusside (SNP) (Roche), an endothelium-nondependent vasodilator, was added to oxigenized Krebs-Henseleit solution while keeping the perfusate concentrations and the flow rate. The concentration of SNP used in this study was 1×10^{-6} mol/L, which corresponds to values validated as the most suitable concentration of SNP to assess the vasodilating effect in a previous study.[28] Microangiography was similarly performed to visualize dilated coronary vessels. Each imaging started 1 to 2 seconds before contrast media infusion, so that background pictures without contrast media could be taken for later computed analysis.

Tissue Harvest

After SRM, hearts were sliced in a broad-leaf fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap frozen in liquid nitrogen (LN_2), and stored at −80°C for immunohistochemistry. Rat hearts in OCT blocks were sectioned, and 5-μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 minutes and stained immediately. Total RNA was isolated by selective dissection of peri-infarct area in LV myocardium for reverse transcriptase-polymerase chain reaction (RT-PCR).

Angiographic Assessment of Collateral Vessel Formation

Collateral flow filling to the LAD territory pre and post SNP was graded angiographically in a blinded manner by use of the Rentrop scoring system.[7] To quantify development of collateral vessels, angiographic microvessel density (AMVD) in the occluded LAD area both pre and post SNP was measured by following computed analysis. Hearts were divided into 4 parts from ligation point to apex, then we measured vessel densities in each part. Region of interest was determined in LAD perfusing area but without visible major branches of the LAD. The images immediate before (background) and during contrast media infusion were captured by an image scanner. After the image capture, vessel density in each part was obtained by subtracting the background density from the angiographic density processed with the NIH image program (v. 1.62) as described previously.[29] Average value of the vessel densities in 4 portions was calculated as the AMVD for each imaging procedure. The ratio of AMVD post SNP to pre SNP (AMVD ratio) was also calculated. These data analyses were performed by 2 blinded observers.

Morphometric Evaluation of Capillary Density

Histochemical staining with isolectin B4 (Vector Laboratories) was performed, and capillaries were recognized as tubular structures positive for isolectin B4. Histological capillary density was evaluated by morphometric examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD.[7–9] All morphometric studies were performed by 2 examiners who were blinded to treatment.

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc). All values were expressed as mean±SE. Paired *t* tests were performed for comparison of data between day 5 and day 28, and between pre and post SNP infusion. The comparisons among 3 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Fisher protected least significant difference test. Correlation between histological and microangiographic vessel densities was analyzed by linear regression test. Differences of $P<0.05$ were considered statistically significant.

Results

Rentrop Score Pre and Post SNP Infusion

SRM was performed to evaluate collateral vessel development by elucidating Rentrop score, a semiquantitative grading of collateral flow filling into the occluded coronary artery,[10] 5 and 28 days after cell transplantation. SRM on day 5 demonstrated that the LAD was totally occluded at the ligation point and collateral flow filling into the distal LAD was not well visualized in all groups (Figure 1a). Angiographic Rentrop score at day 5 was not significantly different in each group (Figure 1c). In contrast, SRM on day 28 revealed better visualization of collateral vessels into the distal LAD area in CD34+ cell group compared with both CD34− cell and PBS groups. Collateral vessels were generated from left circumflex artery or proximal site of LAD. Diameter of the collateral vessels was generally 20 to 120 μm, which is apparently invisible size in conventional angiography (Figure 2a). Rentrop score at day 28 was significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 1.6±0.2; CD34−, 0.6±0.2; PBS, 0.4±0.2, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 2c).

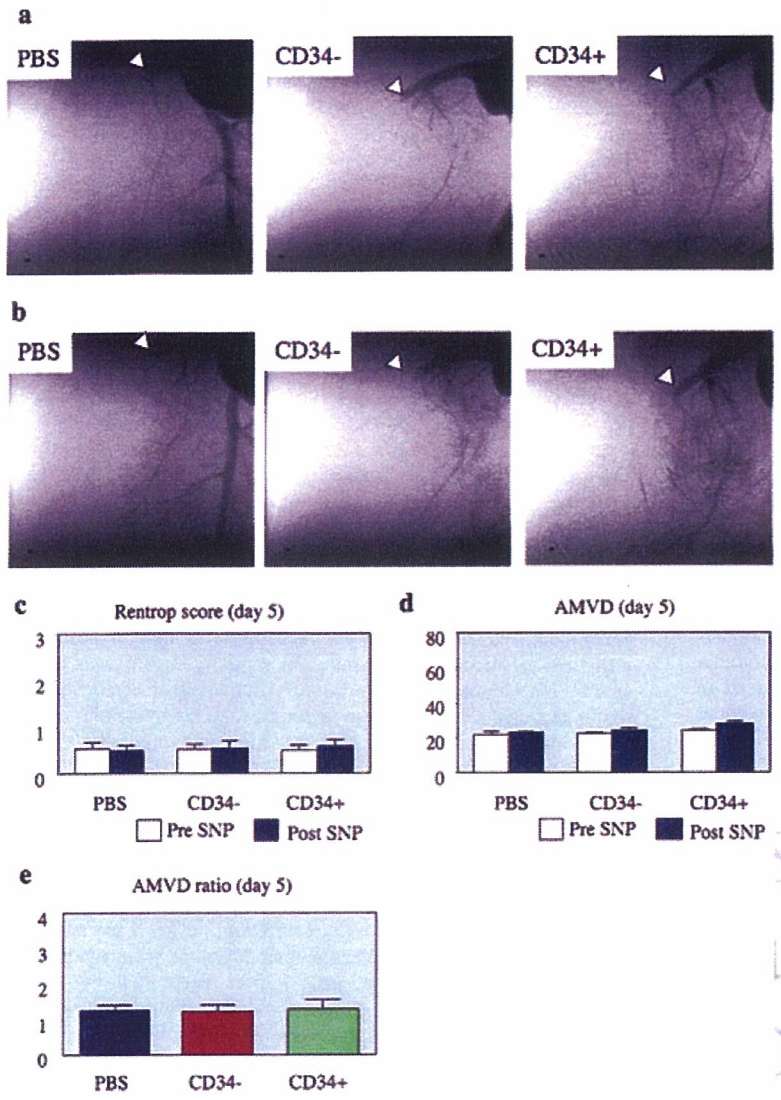

Figure 1. a, Representative images of synchrotron radiation microangiography (SRM) 5 days after PBS, CD34−, or CD34+ cell transplantation (pre sodium nitroprusside [SNP]; 7.0×7.0 mm). Collateral vessels were poorly visualized in all groups. Arrowhead shows ligation point (scale bar; 100 μm). b, Representative SRM images post SNP at day 5 (7.0×7.0 mm). Collateral vessels were poorly visualized in all groups (scale bar; 100 μm). c, Rentrop score of collateral development pre and post SNP in each group at day 5. d, Angiographic microvessel density (AMVD) pre and post SNP in each group at day 5. e, Ratio of AMVD post SNP to pre SNP (AMVD ratio) in each group at day 5.

SRM post SNP was similarly performed to evaluate the augmentation of new microvasculature 5 and 28 days after transplantation. SRM on day 5 revealed slightly better visualization of the new microvasculature post SNP compared with pre SNP in each group (Figure 1b). However, Rentrop score post SNP at day 5 was not significantly different in each group (Figure 1c). SRM on day 28 in CD34+ cell group, not in CD34− and PBS groups, revealed that new microvasculature in the occluded LAD area was better visualized post SNP than pre SNP (Figure 2a and 2b). Rentrop score at day 28 in CD34+ cell group was significantly greater post SNP than pre SNP (CD34+ post SNP, 1.8±0.1; CD34+ pre SNP, 1.6±0.2, $P<0.05$). However, in PBS or CD34− cell group, Rentrop score at day 28 post SNP was not significantly different from that pre SNP (Figure 2c).

SRM post SNP at day 28 revealed better visualization of collateral vessels into the distal LAD area in CD34+ cell group compared with both CD34− cell and PBS groups (Figure 2b). Rentrop score post SNP at day 28 was significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 1.8±0.1; CD34−, 0.7±0.2; PBS, 0.5±0.2, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 2c).

Thus, the new generation SRM system enabled visualization and evaluation of new microvasculature created by CD34+ cell transplantation in the fast beating rat hearts. These results suggest that CD34+ cell transplantation may enhance collateral blood flow in the ischemic myocardium and may also improve collateral vascular function in response to SNP infusion.

Angiographic Microvessel Density (AMVD) in SRM

To quantify the activity of collateral vascular formation in the occluded LAD area, we measured the AMVD in SRM by computed analysis. AMVD pre and post SNP on day 5 was not significantly different in CD34+ cell group from that in other groups (Figure 1d). The ratio of AMVD post SNP to pre SNP (AMVD ratio) on day 5 was similar in all groups (Figure 1e).

AMVD pre SNP on day 28 was not significantly different in CD34+ cell group from that in other groups (CD34+, 27.3±3.2; CD34−, 23.2±0.8, PBS, 21.6±2.7). However, in CD34+ cell group, not in other groups, AMVD on day 28 was significantly greater post SNP than

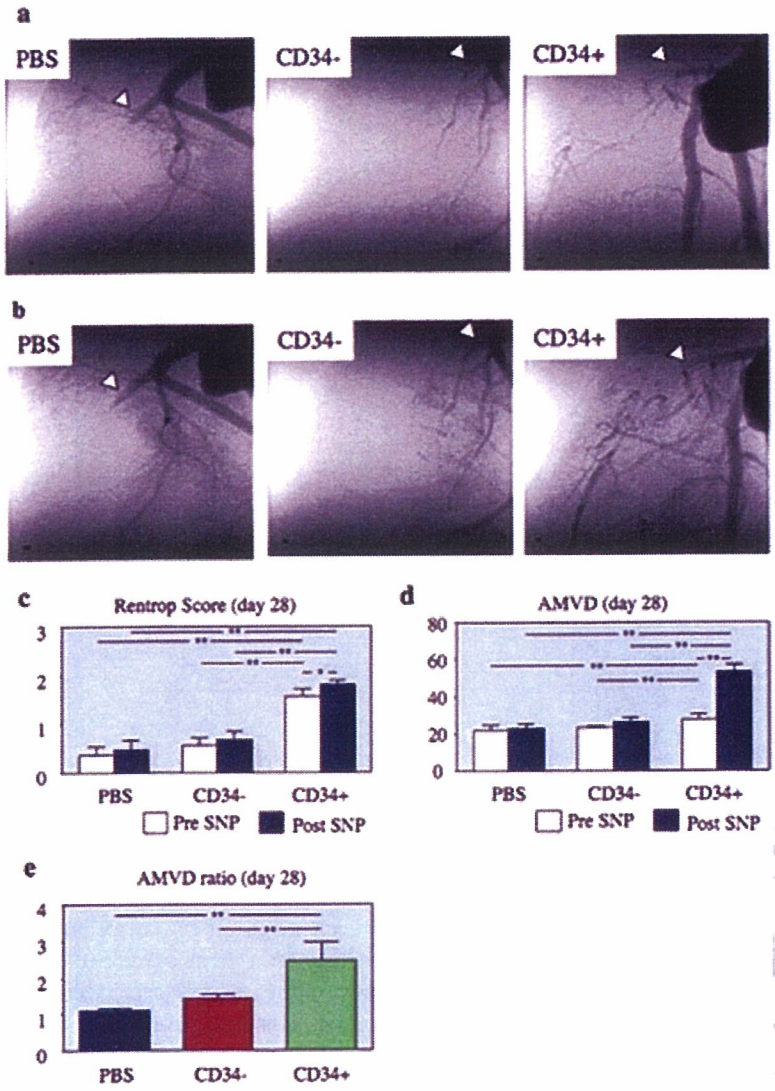

Figure 2. a, Representative microangiographic images pre SNP 28 days after PBS, CD34−, or CD34+ cell transplantation (7.0×7.0 mm). Collateral vessels were better developed in rat receiving CD34+ cells compared with rats receiving PBS and CD34− cells (scale bar, 100 μm). b, Representative microangiographic images post SNP at day 28 (7.0×7.0 mm). Augmentation of collateral microvessel development into distal portion of LAD area was further visualized in rat receiving CD34+ cells compared with rats receiving PBS and CD34− cells (scale bar, 100 μm). c, Rentrop score pre and post SNP at day 28 in each group. *P<0.05; **P<0.01. d, AMVD pre and post SNP at day 28 in each group. **P<0.01. e, AMVD ratio at day 28 in each group. **P<0.01.

pre SNP (post SNP, 53.2±3.8; pre SNP, 27.3±3.2, P<0.05). AMVD post SNP on day 28 was significantly greater in CD34+ cell group compared with CD34− cell and PBS groups (CD34+, 53.2±3.8; CD34−, 26.5±2.0, PBS, 23.0±2.0, P<0.01 for CD34+ versus CD34− and PBS) (Figure 2d). AMVD ratio on day 28 was also significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 2.5±0.5; CD34−, 1.4±0.1, PBS, 1.1±0.1, P<0.01 for CD34+ versus CD34− or PBS). AMVD ratio on day 28 was similar in CD34− cell and PBS groups (Figure 2e).

These results indicate that AMVD analysis may be useful to quantify the effect of therapeutic neovascularization by CD34+ cell transplantation. Similarly as the Rentrop grade examination, AMVD assessment suggests contribution of CD34+ cell transplantation to improvement of collateral vessel function in response to SNP.

Histological Evaluation of Capillary Density

Histochemical staining for isolectin B4 was performed to identify capillaries in ischemic myocardium 4 weeks after cell transplantation (Figure 3a). Histological capillary density was significantly greater in CD34+ cell group than in CD34− cell and PBS groups. Histological capillary density in CD34− cell group was not significantly different from that in PBS group (CD34+, 711±15; CD34−, 365±23; PBS, 294±17/mm^2, P<0.01 for CD34+ versus CD34− and PBS) (Figure 3b).

Correlation Between SRM and Histological Assessments

To confirm whether AMVD is precise assessment of vascular development by CD34+ cell transplantation, we investigated correlation between AMVD and histological capillary density on day 28. AMVD pre SNP did not significantly correlate with histological capillary density (R=0.16, P=0.42), however AMVD post SNP closely correlated with histological capillary density (R=0.82, P<0.0001) (Figure 3c).

These results suggest that AMVD post SNP may be accurate and useful for precise evaluation of collateral and vascular formation following SCTx.

Discussion

Many investigators have demonstrated efficacy of various stem/progenitor cell transplantation against ischemic disease

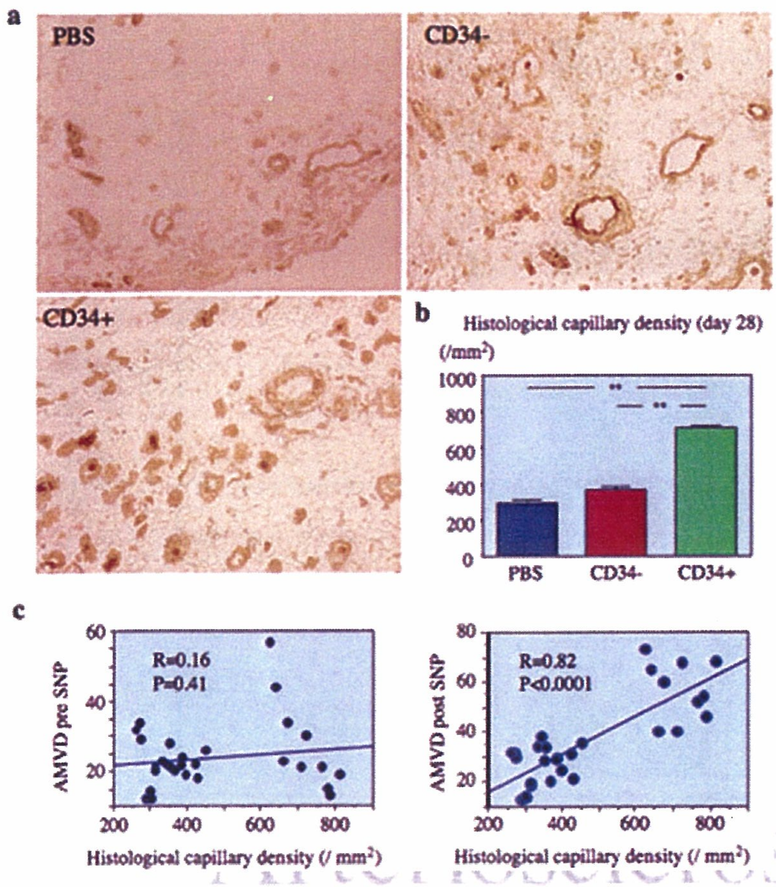

Figure 3. Histological and physiological evaluation of myocardial neovascularization after MI. a, Representative immunostaining for isolectin B4 in each group at day 28 (×200). b, Histological capillary density in rats receiving CD34+ cells, CD34− cells, or PBS at day 28. Ischemic neovascularization was significantly enhanced after CD34+ cell transplantation. $^{**}P<0.01$. c, Correlations of AMVD pre SNP or post SNP with histological capillary density 4weeks after the treatment. AMVD post SNP, but not pre SNP, closely correlated with histological capillary density.

such as MI and limb ischemia in vivo.[30,31] Although immunodeficient rats/mice provide enormous information about regenerative property of human stem/progenitor cells in the animal models of tissue ischemia, the vasculogenic/angiogenic effect of the human cells has been mainly evaluated by histological assessments because of technical limitation for physiological examinations in small animals.[15] Recently, Toyota et al[32] reported critical role of VEGF for coronary collateral growth by using micro CT. However, the micro CT can be performed only for postmortem examination, ie, not for fast beating hearts, and the spatial resolution of this method was 18 μm, which is 3 times larger than that in our novel SRM system and is not considered to be ideal for visualization of the collateral vessels.

In our SRM system, monochromatic SR is used as an x-ray source, and high speed and resolution imaging system, which has the potential to visualize blood vessels as small as 20 μm in diameter (spatial resolution: 6 μm), is also used. In the present study, we demonstrated usefulness of the SRM imaging to evaluate therapeutic neovascularization by cell-based therapy in small animals. Similarly as the previous reports,[4,7,8] histological and molecular examinations in this study confirmed endothelial differentiation and therapeutic efficacy of the transplanted CD34+ cells for augmentation of myocardial neovascularization. The SRM examination revealed that diameter of the collateral vessels was generally 20 to 120 μm, which is apparently invisible size in conventional angiography, and the collaterals were better visualized after SNP-induced vasodilatation than pre SNP. In comparison with postmortem studies such as histology, corrosion casts infusion and micro CT, it may be a great advantage of the SRM to elucidate physiology of the microvessels in response to vasoactive agents under the fast beating condition. To our knowledge, this is the first report demonstrating coronary microangiography under fast beating condition in both acute and chronic phases after MI and SCTx. Extent of collateral development was evaluated by conventional Rentrop score and novel assessment of AMVD. Although Rentrop score has been widely used in preclinical and clinical fields,[7] the examination has several limitations: (1) The scoring is semiquantitative; (2) The system is to indirectly evaluate collateral development by grading collateral filling into the occluded coronary artery, and not to directly examine developed vascularization. Therefore, we assessed AMVD to quantitatively and directly evaluate blood vessel development as angiographic vessel density independent of blood flow in the occluded arteries. In the present study, both conventional and novel assessments revealed that collateral development and vascularization in ischemic myocardium was similar in all groups on day 5, but was significantly augmented in CD34+ cell group than other groups on day 28. Interestingly, the intergroup difference in AMVD was observed only post SNP, not pre SNP. Similarly, AMVD post SNP, not pre SNP, closely correlated with histological capillary density, which has been used for morphological evaluation of neovascularization in small animal studies. These results indicate accuracy and usefulness of AMVD post SNP for elucidating preserved vascular volume created by SCTx in fast beating

hearts of small animals, and also suggest that even in SRM with high imaging resolution, SNP infusion may be essential to avoid underestimation of the angiographic vascular density. The correlation between histological capillary density and AMVD post SNP proves the quality of AMVD to identify capillary vascular volume regenerated by SCTx. SNP infusion may increase the diameter of not only already visible vessels but also invisible capillaries (diameter <20 μm) pre SNP up to detectable size, thereby represents significant augmentation of blood perfusion in ischemic myocardium following CD34+ cell transplantation.

Present Limitations and Future Plans

The microangiographic imaging system requires a high shutter speed (short exposure time) to produce sharp and blur-free images of fast-moving hearts. In the current SRM system, the rotating disk X-ray shutter has been developed to produce X-ray pulses with the minimum pulse length of 0.1 ms, because even the beating heart is to remain almost motionless during the exposure time for ideal imaging. However, the exposure time was adjusted to around 2.0 ms in this experiment, because X-ray flux was not sufficient for the 0.1 ms shutter operation. A speed of the coronary arteries in rats is a few μm/ms at the end of diastole, ie, the movement of the arteries in 2.0 ms is several μm in the present system. On the other hand, the limiting spatial resolution of the image detector is approximately 6 μm, when digital images are acquired with a 1024×1024 pixel format, an input field of view of 4.5 mm×4.5 mm and pixel size of 4.5 μm. These facts indicate that the detector's spatial resolution is comparable to the motion blur amount in the present rat heart imaging, however there is still some room for improvement of the image quality. We are planning to develop a new X-ray optical system used for SR to increase the X-ray flux for the 0.1 ms shutter operation. Another limitation of the present study is that despite of the high quality of SRM for visualization of coronary arteries and the microvascular bed of fast beating hearts, we cannot take serial images in each individual at days 5 and 28, because they have to be examined ex vivo not in vivo. Future establishment of in vivo SRM imaging would be also warranted.

Conclusions

The present results indicate that the SRM may be useful to both morphologically and physiologically evaluate therapeutic neovascularization by SCTx in small animals. The novel imaging system may be not only an essential tool in future translational research of stem cell biology but also useful assessment of microvascular beds in small animal models of various diseases such as hypertension, diabetes mellitus, and cardiomyopathy. Further development of in vivo imaging system in future may lead to clinical application of the SRM, which is expected to be useful for assessment of microangiopathy, elucidation of therapeutic neovascularization, and determination of optimal treatment strategies in both preclinical and clinical trials.

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Disclosures

None.

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