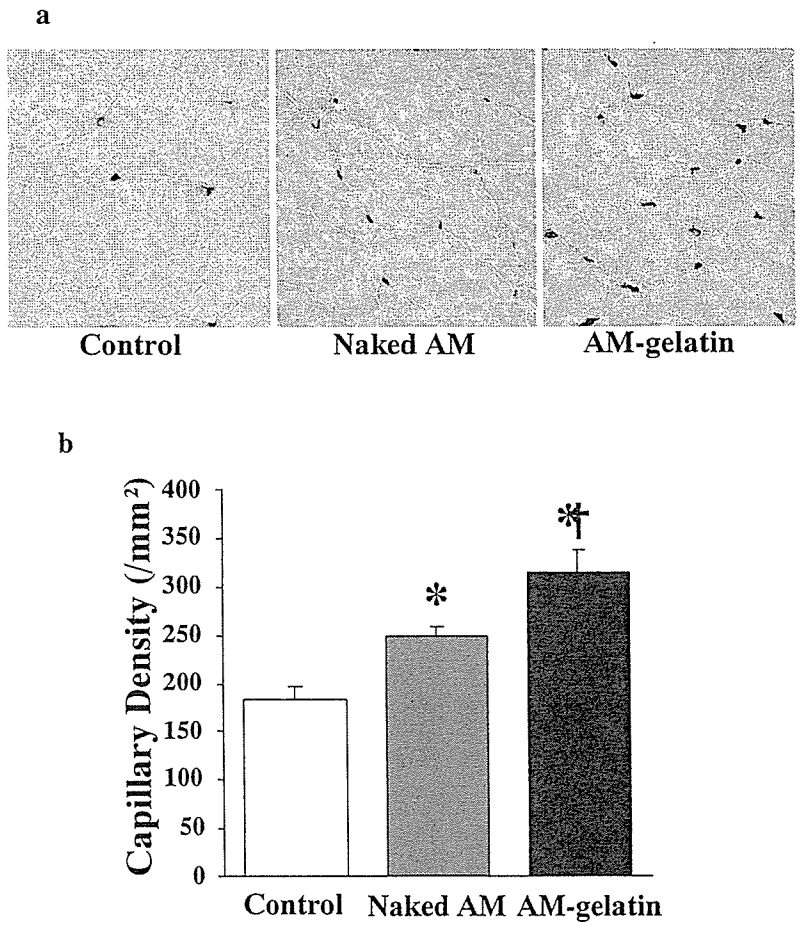

Fig. 3a, b. **a** Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. Magnification x200. **b** Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean ± SEM. *P < 0.05 vs Control group; †P < 0.05 vs Naked AM group.

In vitro transfection

We examined the usefulness of gelatin as a nonviral vector for in vitro gene transfer (Nagaya N, et al. 2003). Here we demonstrate that EPCs have a phagocytosing action which allows nonviral gene transfer into EPCs. To produce ionically linked plasmid DNA-gelatin complexes, we prepared positively charged biodegradable gelatin. Positively charged gelatin was readily complexed with negatively charged plasmid DNA after 24-hr incubation. Then, EPCs were cultured with green fluorescent protein (GFP) plasmid DNA-gelatin complexes. Interestingly, fluorescence microscopy revealed that GFP was expressed in EPCs after a 72-hr incubation period. Quantitative analyses confirmed a high incidence (approximately 70%) of GFP expression in EPCs. Transmission electron microscopy demonstrated that EPCs were phagocytosing DNA-gelatin complexes after 12-hr incubation (Fig.4**a**). These results suggest that EPCs phagocytose plasmid DNA-gelatin complexes in co-culture, which allows nonviral, highly efficient gene transfer into EPCs. A number of DNA particles labeled by rhodamine B isothiocyanate (RITC) were incorporated into gelatin. RITC-labeled DNA particles were gradually released from gelatin within EPCs through gelatin degradation (Fig.4**b**). After 72-hr incubation, RITC-labeled DNA particles released from gelatin were distributed in the cytoplasm, but not the nucleus, of EPCs (Fig.4**c**). These results suggest the ability of EPCs to take up DNA-gelatin complexes and dissolve the gelatin, freeing the DNA into EPCs. Unlike EPCs, neither human mature pulmonary artery endothelial cells (HPAECs) nor human umbilical vein endothelial cells (HUVECs) phagocytosed DNA-gelatin complexes.

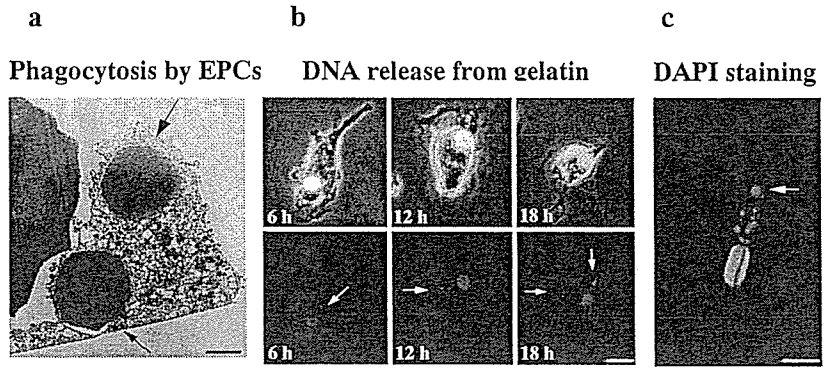

Fig. 4a, b, c. Ex vivo gene transfer into EPCs based on phagocytosing action. **a** Transmission electron microscopy revealed that EPCs had phagocytosed GFP DNA-gelatin complexes (arrows). **b** Time-course of changes in DNA-gelatin complexes within EPCs. RITC-labeled DNA particles (red, arrows) were released from gelatin through its degradation. **c** After 72-hr incubation, RITC-labeled DNA particles released from phagocytosed gelatin (arrow) were distributed in the cytoplasm, but not the nucleus, of EPCs. The nuclei of EPCs were identified by DAPI staining. Scale bars: 10μm (**a**); 5μm (**b** and **c**)

When EPCs were cultured with AM plasmid DNA-gelatin complexes, intense immunostaining for AM was observed in EPCs impregnated with AM DNA-gelatin (Fig.5**a**). After 72-hr incubation with AM DNA-gelatin complexes, EPCs markedly secreted AM into the culture medium (10-fold increase compared to EPCs alone, Fig.5**b**). AM overproduction lasted for more than 16 days after gene transfer. Finally, we examined the effects of AM gene transfer on EPC proliferation in vitro using MTS assay. Proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. In addition, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. This can be explained by recent findings that AM inhibits cell apoptosis through the PI3K/Akt pathway (Okumura H, et al. 2004). Thus, transfection of AM gene may strengthen therapeutic potential of EPCs.

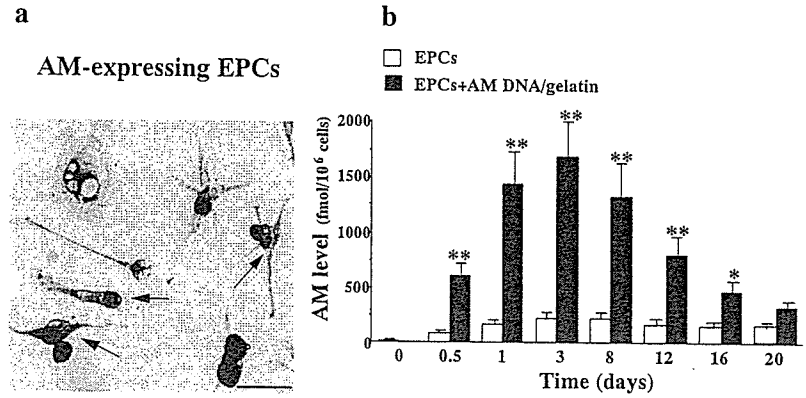

Fig.5a, b. a Immunohistochemistry for AM in EPCs after gene transfer. **b** Time course of AM secretion from EPCs during coculture with AM DNA-gelatin complexes. Each bar represents the mean ± SEM. *P < 0.05; **P < 0.001 versus non-transduced EPCs.

Hybrid cell-gene therapy

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (Nagaya N, et al. 2003). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in co-culture, which allows nonviral gene transfer into EPCs with high efficiency. Third, transplanted gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy: nonviral, noninvasive, and highly efficient gene targeting into the vasculature. These benefits may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by the progression of pulmonary hypertension, ultimately producing right ventricular failure and death. Median survival in

patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus, novel and effective therapy is desirable for the treatment of pulmonary hypertension. Dysfunction of the endothelium may play a role in the pathogenesis of pulmonary hypertension such as PPH. Thus, pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension.

We present cell-based gene delivery into the pulmonary vasculature. EPCs are mobilized from within the bone marrow into the peripheral blood in response to tissue ischemia or injury, then migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ. These findings raise the possibility that transplanted EPCs may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. AM is a potent vasodilator peptide which also inhibits cell apoptosis and enhances endothelial cell differentiation. Thus, we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats.

GFP-expressing EPCs (1×10^6/rat) were intravenously administered into rats with monocrotaline-induced pulmonary hypertension. Three days after transplantation, GFP-expressing EPCs were incorporated into the walls of pulmonary arterioles in monocrotaline rats and composed pulmonary vasculature. Transplanted GFP-expressing EPCs were distributed on the walls of pulmonary arterioles and capillaries. AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses of rat and human CD31 demonstrated that the transplanted EPCs were of endothelial lineage and constituted vessel structure similar to rat endothelial cells. However, transplanted EPCs were rarely distributed to other tissues such as cardiac ventricles, kidneys, aorta, and brain (data not shown).

Next, we examined the effects of AM-expressing EPCs on pulmonary hemodynamics. Monocrotaline rats received intravenous injection of 1×10^6 EPCs, 1×10^6 AM-expressing EPCs, or culture medium. Pulmonary hypertension developed three weeks after monocrotaline injection. Mean pulmonary arterial pressure was not strikingly decreased in the EPC group

(-14%), but was significantly lower in the AM-EPC group (-29%) compared to that in the CONTROL group. Pulmonary vascular resistance was significantly lower in both the EPC group (-16%) and AM-EPC group (-39%) than that in the Control group. Importantly, the AM-EPC group showed significantly greater improvement in pulmonary vascular resistance than the EPC group. Finally, we examined the effects of hybrid cell-gene therapy on survival in MCT-injected rats. AM-expressing EPCs were used immediately after 72-hr incubation with AM DNA-gelatin complexes. Kaplan-Meier survival curves demonstrated that MCT rats transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone.

These findings suggest that gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may apply for intractable cardiovascular diseases including ischemic heart disease. Thus, genetic manipulation of stem cells opens new avenues for regenerative medicine.

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A Novel Micro-Angiography Detecting Angiogenesis, Application for Autologous Bone Marrow Mononuclear Cells Transplantation in the Patients with Critical Limb Ischemia

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Summary. Conventional Anigiographic Findings in Autologous Bone Marrow Mononuclear Cells Transplantation for Critical Limb Ischemia: Bone marrow mononuclear cells have many of the characteristics of stem cells for mesenchymal tissues, and secrete many angiogenic cytokines. We performed autologous transplantation of bone marrow mononuclear cells in six patients with critical limb ischemia due to Buerger disease, who were not candidates for catheter or surgical revascularization. Leg pains at rest and skin ulcers improved after bone marrow transplantation in all patients, although significant collateral developments after the therapy by conventional angiography could not be observed. Autologous transplantation of bone marrow mononuclear cells including stem cells improved critical limb ischemia due to Buerger disease. Neovascularization after therapeutic angiogenesis might be quite small and could not be visualized by conventional angiography.

Novel Micro-angiograpy:We developed in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera). We visualized mid-zone collaterals after femoral arterial exfoliation with and without therapeutic angiogenesis in rabbit ischemic limbs and assessed the radio-absorptions in a clinical setting. The micro-angiography clearly demonstrated mid-zone collaterals after the treatment with a diameter of down to 50μm, but the conventional angiography did not. The sum of ra-

dio-absorptions for 10 seconds in clinical settings was 300 mSv. The newly developed in-house micro-angiography could illuminate micro-vessels with a diameter of down to 50μm in clinical settings safely and could be useful in the evaluation of therapeutic angiogenesis.

Keywords. Micro-angiography, Angiogenesis, Autologous bone marrow mononuclear cells transplantation, Critical limb ischemia, Buerger disease

Introduction

Endothelial progenitor cells (EPCs) possess the ability to mature into cells that line the lumen of blood vessels(Asahara T, et al. 1997). Therapeutic angiogenesis could be induced by the transplantation of bone marrow mononuclear cells including EPCs. Several studies demonstrated that therapeutic angiogenesis using autologous bone marrow mononuclear cells transplantation (BMT) was effective for ischemic vascular diseases although conventional angiography could not precisely detect developed collaterals after therapeutic angiogenesis(Iba O, et al. 2002, Inaba S, et al. 2002, Shintani S, et al. 2001,Tateishi-Yuyama E, et al. 2002). We developed an in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera).

The purpose of the present study was to evaluate the clinical effects and conventional angiographic findings on BMT for critical limb ischemia, and to validate the usefulness and safety of the novel micro-angiography technique for the evaluation of therapeutic angiogenesis.

Methods

Patients

Patients qualified for autologous BMT if they had chronic critical limb ischemia including rest pain and/or non-healing ischemic ulcers for a minimum of 4 weeks without evidence of improvement in response to

conventional therapies and were not optimal candidates for surgical or catheter revascularization. Buerger's disease was diagnosed by segmental occlusion of small- and medium-sized arteries, absence of atherosclerosis, and corkscrew collaterals circumventing the occlusion in angiogram and the exclusion of autoimmune diseases such as scleroderma or systemic lupus erythematosus, hypercoagulable states, diabetes, or acute arterial occlusion secondary to embolism. Patients with retinopathy and/or malignancy were excluded. Although 30 patients with atherosclerotic peripheral artery disease were candidates for BMT, they were excluded from the present study due to their systemic atherosclerotic complications. Six patients with Buerger's disease were recruited for the present study. All patients had legs pain at rest and five patients had foot ulcers. Written consent was obtained from all participants of this study. This clinical trial of autologous BMT for the treatment of patients with critical ischemia was approved by the Medical Ethics Committee of the National Cardiovascular Center.

Autologous BMT

Bone marrow fluid (700-800ml) was collected from the iliac bone under general anesthesia. The harvested bone marrow fluid was diluted with RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) containing heparin, then stored in a sterile pack from the Bone Marrow Collection Kit (Baxter, IL, USA). The mononuclear cell fraction was prepared with a Fresenius AS104 (AMCO, USA). The injection volume was 0.5ml and injections were spaced 2-3cm apart, using a 1ml syringe and a 27-gauge needle. Leg pains were measured by a visual analog pain scale and foot ulcers were evaluated by area and appearance.

Novel micro-angiography

The in-hospital micro-angiographic equipment consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera) (Fig.1).

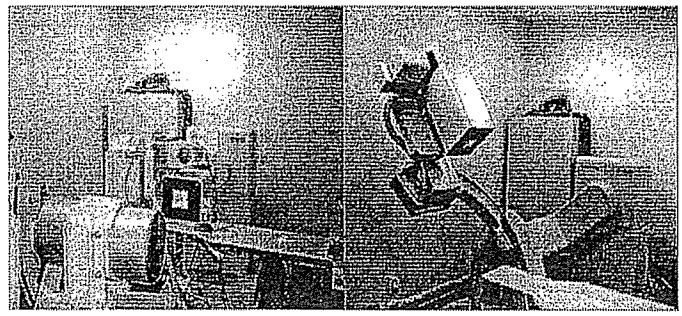
a  b

Fig. 1a, b. The micro-angiographic equipment that we developed. High-voltage power X-ray source **a** and a detecting system with a high spatial resolution(25μm) and high sensitivity (100 times of CCD camera) **b.**

Limb ischemia models in rabbits were made by ligating the femoral artery and treated by fibroblast growth factor 4 (FGF-4) genes incorporated to gelatin hydro gel (GHG). One month after the treatment, we evaluated collateral micro-vessels by using conventional and micro-angiographic systems. The approach was via the left femoral artery so that the catheter was located in the abdominal aorta. A 5ml bolus of Iodine contrast medium (300mg/ml) was injected at 3ml/sec using an auto-injection system. Imaging was recorded using a digital source in 1000 x 1000 pixels. The sum of radio-absorptions for 10 seconds in clinical settings was studied.

Results

Autologous BMT for Critical Limb Ischemia

The number of transplanted bone marrow mononuclear cells were one to five multiplied 10^9. Rest pains decreased or disappeared in one month after BMT (Fig.2) and Skin ulcers improved in one to three months after BMT in all patients (Fig.3).

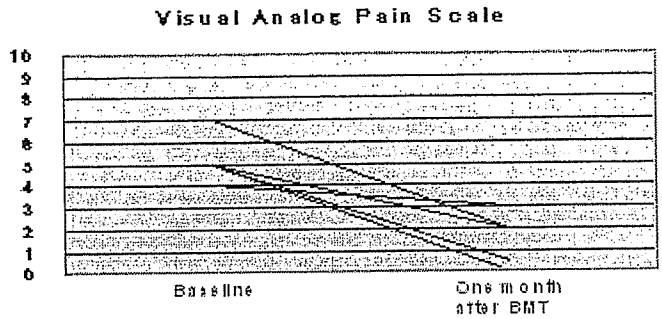


Fig. 2. The Visual analog pain scale in all patients.

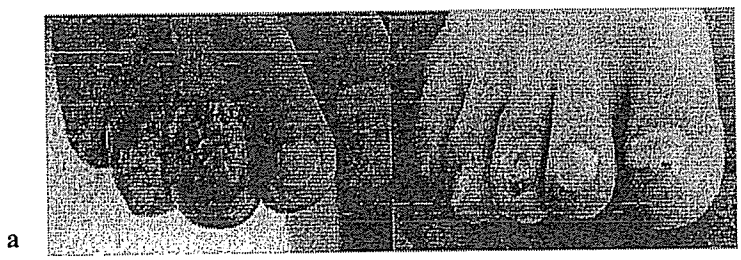
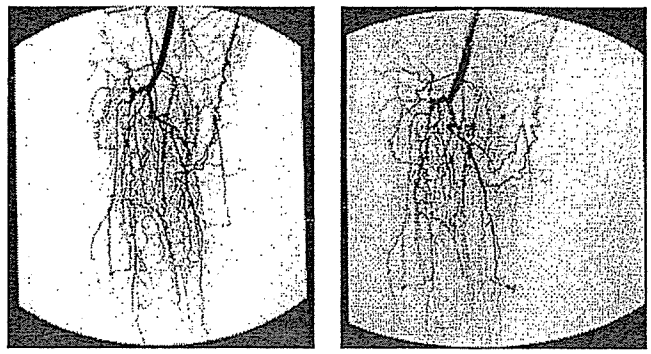


Fig. 3a, b. The skin ulcers in a patient before **a** and one month after autologous bone marrow transplantation **b.**

Conventional angiography was performed before and one month after BMT, but there was no significant changes in any of the patients (Fig.4).

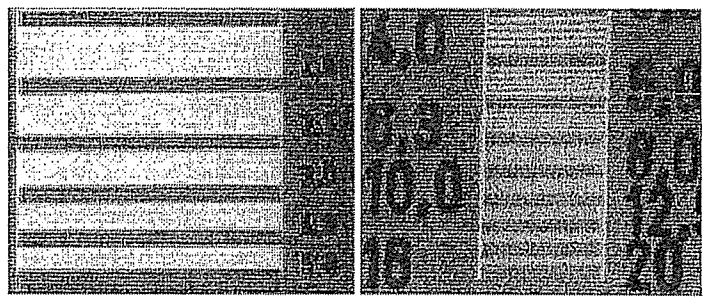


a b

Fig. 4a, b. The conventional angiographic findings in the patient before **a** and one month after autologous bone marrow transplantation **b**.

Novel micro-angiography

The novel micro-angiography can detect to within a limit 50 of μm, although a detection limit of a conventional angiography is 250μm (Fig.5).



a b

Fig. 5a, b. The detection limits on a conventional angiography **a** and the novel micro-angiography **b** using a line chart

Collateral micro-vessels, which were 100-500μm or less in diameter, were demonstrated more clearly in micro-angiography than conventional angiography (Fig.6).

The sum of radio-absorptions at the point of 1m distance from the X-ray source in clinical settings was 300 mSv. for 10 seconds.

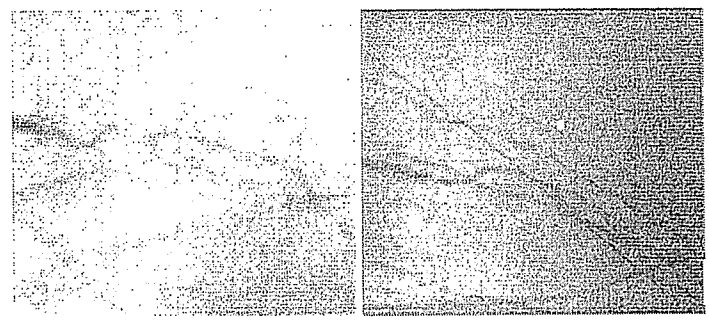


Fig. 6a, b. In 2.5x2.5cm view size, Collateral micro-vessels after therapeutic angiogenesis in the rabbit limb ischemia model. Vessel sizes in the range of 100-500μm or less, were demonstrated in the novel micro-angiography **b** more clearly than in a conventional angiography **a**. The diameter of the line in the micro-angiography is 130μm.

Discussion

Autologous BMT improved chronic severe limb ischemia due to Buerger's disease. Conventional angiography could not disclose developed collateral vessels after BMT. A novel micro-angiography technique could illuminate promoted collateral vessels after therapeutic angiogenesis in rabbit models although a conventional angiography did not. The sum of radio-absorptions in the novel angiography could be accepted in clinical settings.

Autologous BMT and Buerger's disease

Bone marrow harvests need an amount of more than 500ml bone marrow fluid and general anesthesia in therapeutic angiogenesis using BMT. Such factors have practical limitations to select candidates with peripheral artery disease complicated with systemic atherosclerosis and aging for BMT. Buerger's disease is a segmental vasculitis that affects the distal arteries of the upper and lower extremities. It typically occurs in young people. The majority of patients with Buerger's disease have pain at rest and digital

ulcerations and are hard to treat by revasculaizations, including catheter angioplasty and surgical bypass grafting, because of peripheral artery lesions. Patients with Buerger's disease, however, tend to have less systemic atherosclerotic lesions and normal cardiac function. These suggest that patients with Buerger's disease are the ideal candidates for therapeutic angiogenesis using autologous BMT.

Discrepancy between clinical improvements and conventional angiographic findings after BMT

BMT improved critical limb ischemia clinically. Promoted collateral vessels after the treatment were not, however, visualized well by conventional angiography. These vessels are quite small and the detection limit of small vessels by conventional angiography is about 200μm in diameter.

Novel micro-angiography

Recently, synchrotron radiation system characterized by high brightness, monochromatic and collimated nature bypass, revealed micro-vessels in situ. However the high cost of a synchrotron system strictly limits its clinical application (100 million dollars or more). We developed an in-house micro-angiographic system with a relatively low cost of approximately 1million dollars, which consisted of a high-voltage power X-ray source and a detecting system with a high spatial resolution (25μm) and high sensitivity (100 times of CCD camera). We evaluated collateral micro-vessels one month after therapeutic angiogenesis by using the conventional and micro-angiographic system. The in-house micro-vessel angiographic system could detect the micro-vessels more precisely than conventional angiographic system. We thought that the present micro-angiography should be useful for evaluating efficacy of therapeutic angiogenesis in clinical settings.

Conclusions

Conventional angiography failed to disclose the promoted collateral vessels after BMT although BMT improved the critical limb ischemia clini-

cally. The in-house micro-angiographic system could detect the micro-vessels more precisely than conventional angiographic system and the sum of the radio-absorption in the equipment could be acceptable in clinical settings. The novel in-house micro-angiographic system can be useful in the evaluation of therapeutic angiogenesis clinically.

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細胞増殖因子と再生医療

GROWTH FACTOR & REGENERATIVE MEDICINE

編集

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8. VEGF/VEGF-E

末梢動脈疾患

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1 末梢動脈疾患とは

末梢動脈疾患（peripheral artery disease：PAD）とは，末梢動脈の狭窄や閉塞によって，四肢をはじめとする末梢組織に虚血をきたすような疾患を指す。末梢動脈疾患には，閉塞性動脈硬化症，閉塞性血栓血管炎（Buerger病），膠原病に伴う血管炎などが含まれるが，その中でも最も多いのは閉塞性動脈硬化症である。閉塞性動脈硬化症では，末梢動脈の粥状動脈硬化により血管内腔の狭窄が進行し下肢に虚血が生じる。これに伴い，しびれ，冷感，間歇性跛行（後述），疼痛，潰瘍，壊疽などのさまざまな症状が出現する。欧米における罹患率は人口の数%程度とされているが，わが国における確立された疫学データは残念ながら存在しない。自覚症状による病期分類としてFontaine分類が代表的である（表）。Fontaine Ⅰ度の軽症患者に対しては，禁煙指導や糖尿病・高血圧など動脈硬化の危険因子をコントロールしながら経過観察するのが通常である。病状が進行してくると，Fontaine Ⅱ度にみられるような間歇性跛行が出現する。間歇性跛行とは，一定距離の歩行後に下肢の疼痛が出現するが，休息により痛みは消失し，再び歩行可能

表　Fontaine分類

グレード	症状
Ⅰ	なし（しびれ，冷感）
Ⅱ	間歇性跛行
Ⅲ	安静時疼痛
Ⅳ	皮膚潰瘍，壊疽

となるような状態を指す。末梢動脈疾患の症状で最も多いのは，この間歇性跛行である。間歇性跛行が軽度の場合，運動療法や抗血小板剤などによる薬物療法を行うが，重症例では狭窄した血管をカテーテルによって拡張する経皮的血管形成術（percutaneous transluminal angioplasty：PTA）や外科手術（バイパス手術）が必要となる。Fontaine Ⅲ～Ⅳ度を重症下肢虚血（critical limb ischemia：CLI）と呼ぶが，このような状態にまで進行すると，安静時にも下肢疼痛が出現し，皮膚の潰瘍や壊疽もみられるようになる。重症下肢虚血を呈する患者では，痛みや壊疽のために運動療法を施行するのは困難で，薬物治療も無効のことが少なくない。また，重症下肢虚血をきたすような血管は動脈硬化性変化が強く，血管形成術やバイパス手術もしばしば困難である。このような重症例に対する治療法として考えられたのが血管新生療法（therapeutic angiogenesis）[1]である。

Ⅱ 血管新生療法とは

血管新生療法は，血管増殖因子やその遺伝子あるいは骨髄や末梢血細胞を用いて血管新生を促進させ，組織虚血の改善を図る治療法で，1994年，米国のIsnerらにより初めて臨床応用された[2]。Isnerらが行ったのは，vascular endothelial growth factor（VEGF）[3]遺伝子を用いた血管新生療法であり，循環器領域における初の遺伝子治療としても知られている。以後，今日までに10年以上が経過し，遺伝子以外にも増殖因子蛋白，骨髄細胞，末梢血細胞などを用いたさまざ

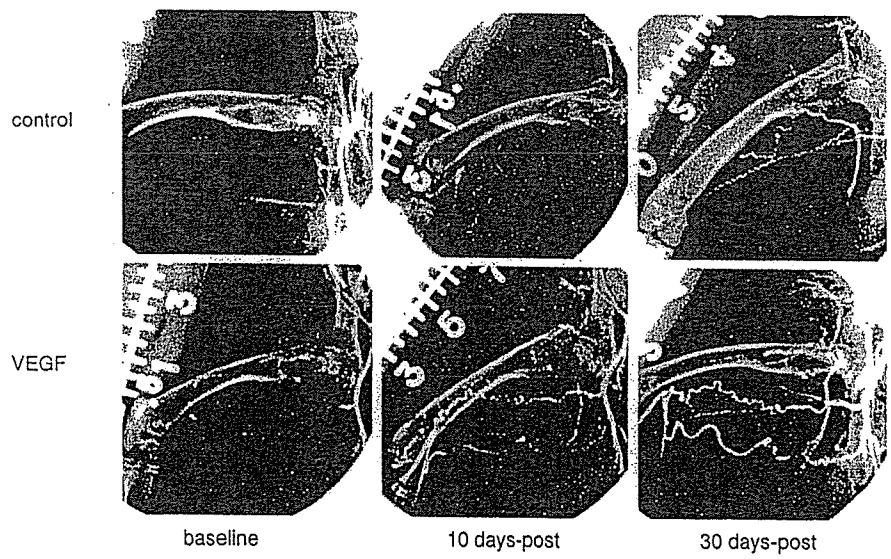

図1　VEGF蛋白投与後の血管造影所見
生理食塩水または組み換えVEGF蛋白を家兎虚血肢モデルの内腸骨動脈内へ選択的に投与し，側副路の発達を比較した。上段の対照群では，治療後30日間において側副血行路に大きな変化は認められない。これに対し，下段のVEGF治療群においては治療から10日間で側副路の著明な改善が認められる。
（文献1より引用）