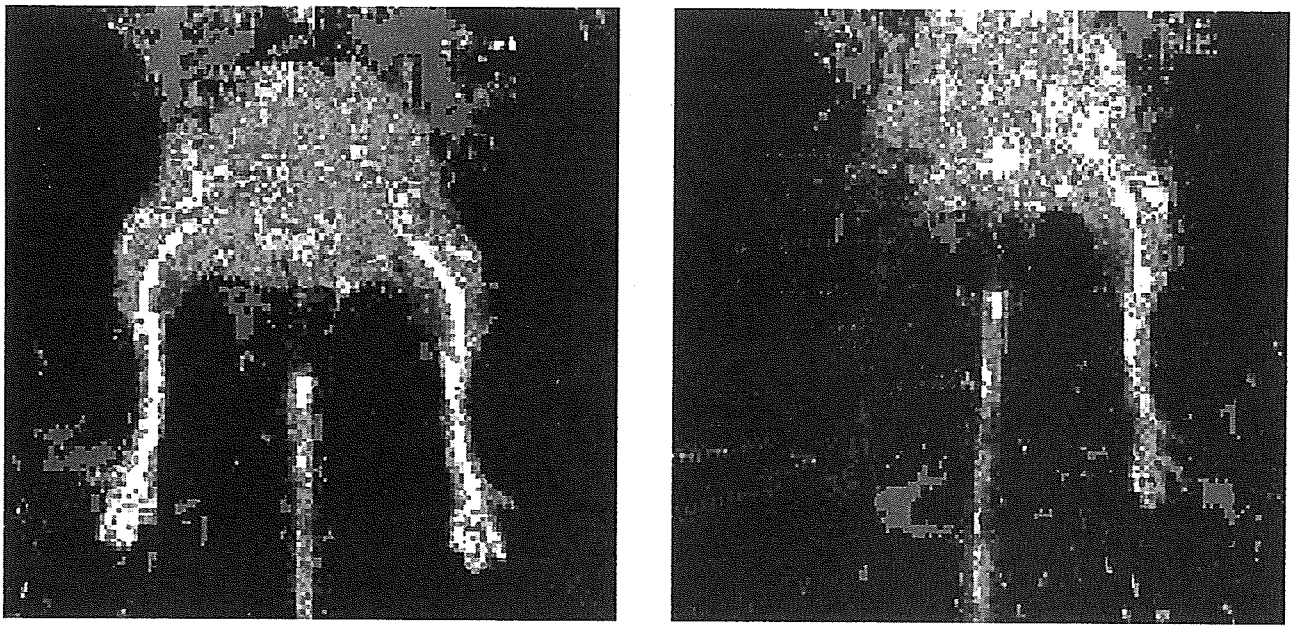

図 2. レーザードップラーによる血流測定

組織学的解析

摘出標本のpreparation

虚血モデル作製後21日目に股関節からマウス両下肢を離断し、上部・中部・下部に分けた後20%中性緩衝ホルマリンにて固定後、50倍希釈のK-CX脱灰液〔株式会社ファルマ〕で一夜浸漬し、浸漬後に 3 時間流水で水洗いし、脱灰を行った。これをエタノールで脱水処理してパラフィン包埋し、短軸方向に3 μmの厚さに薄切し、スライドガラスにのせ24時間42℃で風乾した。

HE染色

脱パラフィン後、ヘマトキシリンで1分染色し流水にて10分間洗浄した後、0.5%エオジン溶液で 2 分染色した。染色後脱水、透徹および封入し光学顕微鏡で観察した。

免疫染色及び微小血管数の比較

脱パラフィン後、クエン酸溶液に浸し、電子レンジで 6 分間 2 回加熱し組織上のホルマリン架橋をはずした。PBSで洗浄後、0.3%過酸化水素水混メタノールに浸し室温にて30分間インキュベーションし内因性ペルオキシターゼ処理を行った。蒸留水および PBS で洗浄後、blockace〔大日本製薬株式会社〕5倍希釈液を用いて60分間室温でインキュベーションし blocking 処理を行った。一次抗体反応は抗CD31抗体（CD31 Rabbit antibody）〔SPRING BIOSCIENCE〕を50倍希釈して4℃で 1 晩行った。PBSで洗浄後、二次抗体反応は抗rabbit IgG抗体〔VECTOR LABORATORIES〕を200倍希釈で、60分間室温で行った。PBSで洗浄後、ABCキット〔VECTOR LABORATORIES〕を用い、ビオチン・アビジン反応を行った。PBSで洗浄後、DABタブレット〔WAKO〕をPBS40mlで溶解し過酸化水素水20 μLを加えた後浸透し発色させた。蒸留水洗浄後、ヘマトキシリンで核染色を行った。蒸留水洗浄、脱水、透徹、封入し、微小血管を数え各群を比較した。

統計方法

統計分析はエクセル統計2006ソフト〔株式会社　社会情報サービス〕を用いて行った。測定される変数の変化は対 t 検定を用いて分析し、評価した。結果はmean±SEM（標準誤差）として表し、P値が0.05未満だったとき、統計学的に有意であると考えた。

結　果

マウス下肢虚血モデルにおける経時的血流変化

マウス下肢虚血モデルの術前および術直後のレーザードップラーの写真を図 2に示した。無

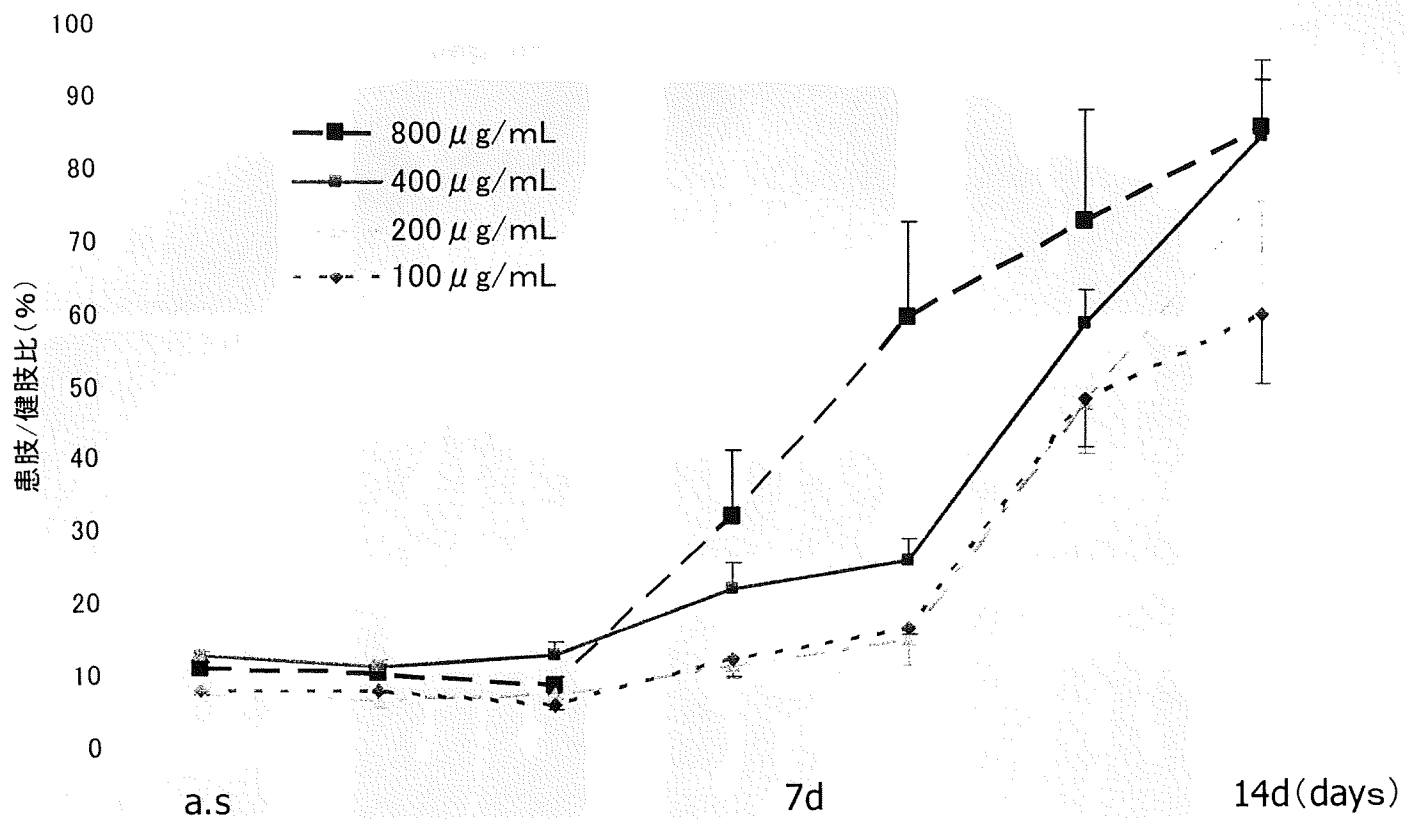

図3. フィブロネクチン単独投与における下肢血流変化

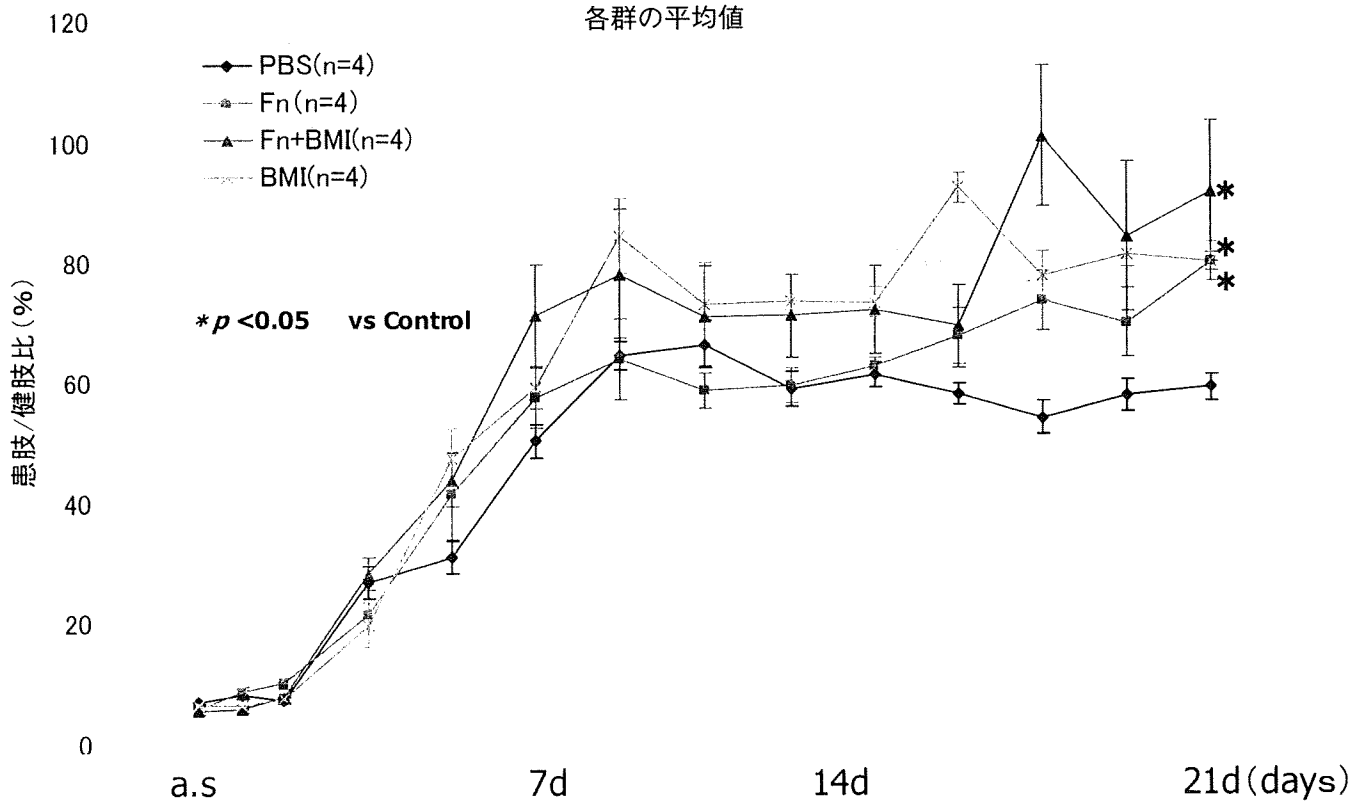

図4. 各治療群における下肢血流変化

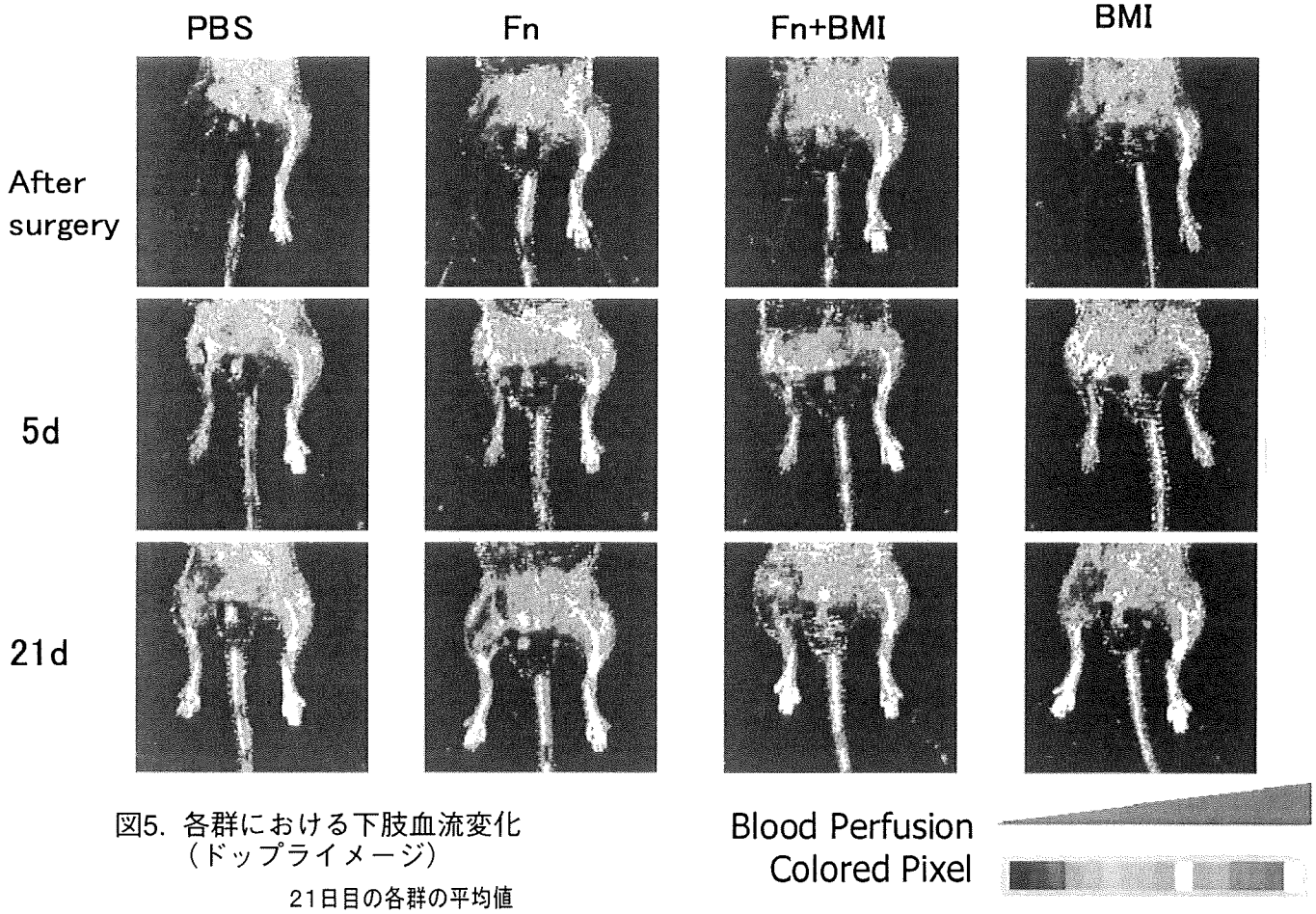

図5. 各群における下肢血流変化
（ドップライメージ）

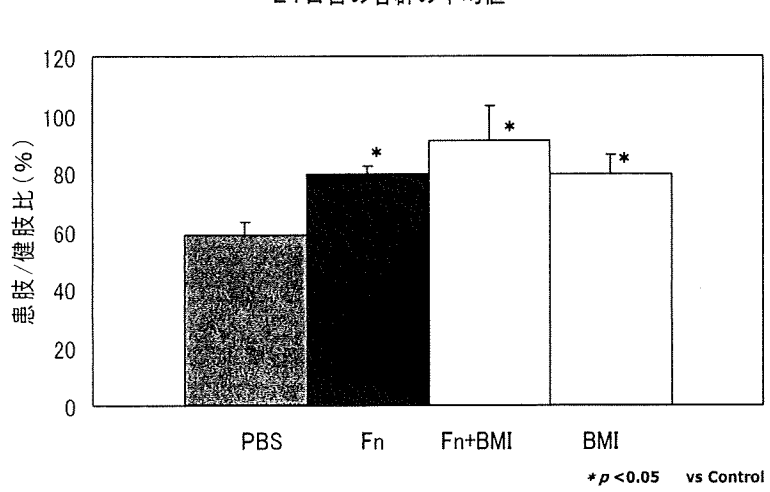

図6. 21日目における各群の平均値

処置の右下肢に比べ、動脈を結紮した左下肢では大腿部および下腿部の血流が低下している。

図 3, 4 はレーザードップラーによる血流変化を定量化し健肢との比によって経時的に示したものであり、図 5 は図 4 における術後、移植後5日、21日のドップラー画像を示したものである。患肢/健肢血流比は術後0.05（<5%）まで低下し2週間でコントロール群においても0.6（60%）まで回復する。

フィブロネクチンを100μg/ml、200μg/ml、400μg/ml、800μg/mlの濃度で単独投与行った場合（図 3)、有意差は見られないものの、400μg/ml以上の投与で血流が改善する傾向にあった。フィブロネクチンの生理的血漿中濃度は約300μg/mlであるため、以降の実験はフィブロネクチン濃度を400μg/mlに調整して進めることとした。

治療群（フィブロネクチン単独投与群、骨髄細胞移植群および併用群）とコントロール群を比較した場合、両群は術後3日目より血流の改善が認められ、治療群は術後5日目にコントロール群よりも血流が回復した。コントロール群は移植後14日目以降、大きな血流改善はみられないものの、治療群については移植後14日目以降も血流改善がみられた。移植後21日目において、治療群とコントロール群に有意差が認められた（図 6)。

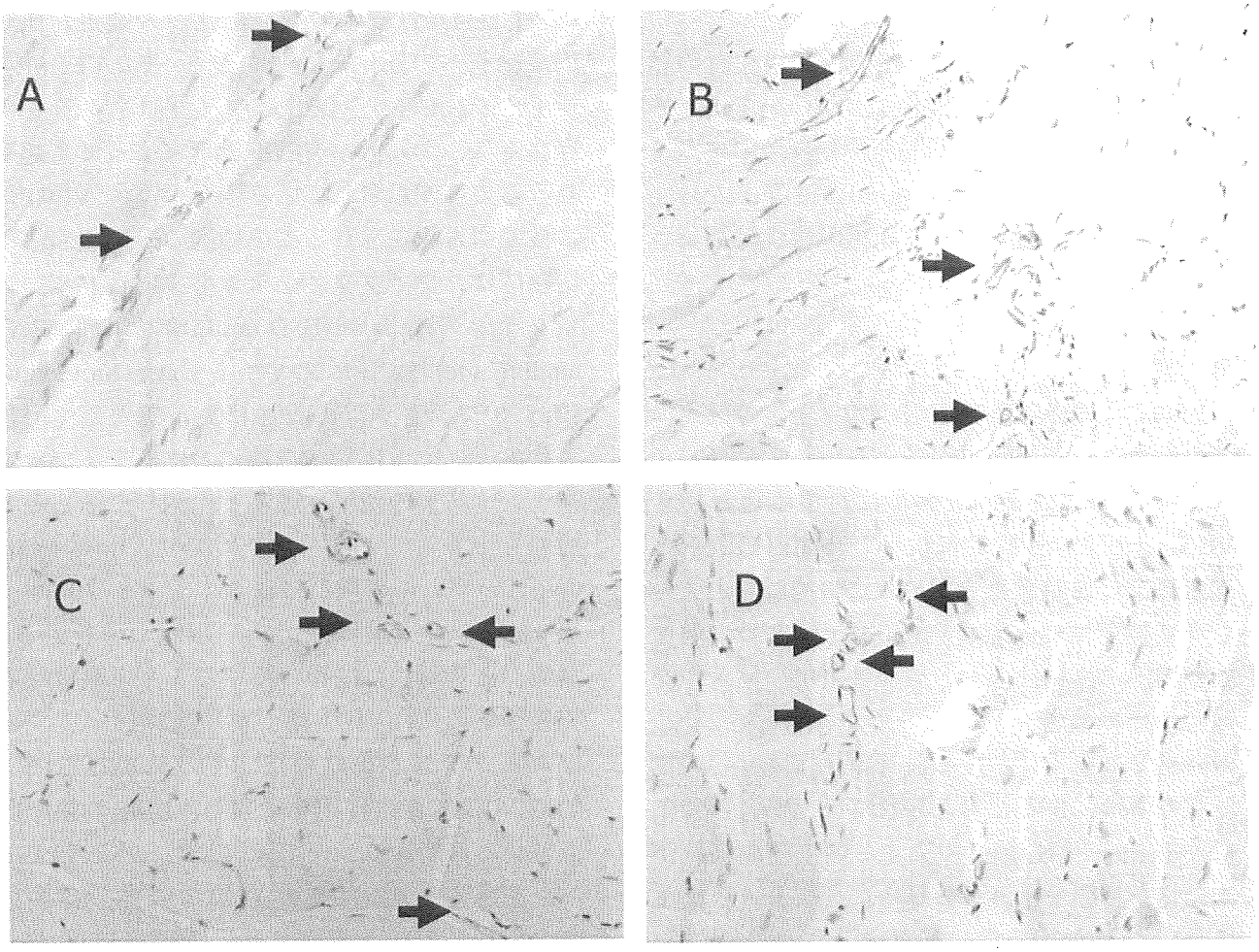

図7. CD31陽性細胞の免疫染色（A:PBS, B:Fn, C:Fn+BMI, D:BMI, ×400）

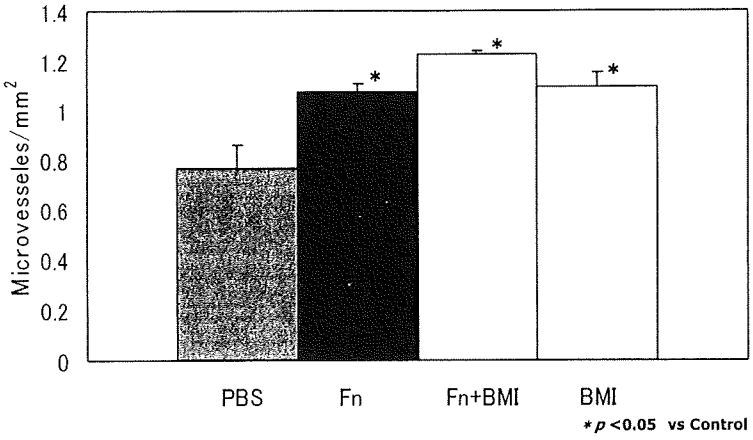

図8. 微小血管のカウント

また、治療群での比較では、移植後21日目の患肢/健肢比において、有意差は認められなかったものの、フィブロネクチン単独投与群0.79±0.02、骨髄細胞群0.79±0.03、併用群0.91±0.12と併用群が最も血流改善がよい傾向がみられた。(図4、5、6)。

微小血管数の比較

モデル作製後21日目の組織における微小血管密度を比較するために、血管内皮細胞に対するマーカーのひとつである抗CD31抗体による免疫染色を行った。

コントロール群、治療群いずれにおいても、虚血肢の筋肉内にCD31陽性細胞を認めた。CD31陽性細胞は骨格筋細胞間に浸潤するように散在し、微小血管と思われる管腔構造を形成するものも観察された(図7)。

この微小血管数を面積あたりの血管数で比較すると、治療群はコントロール群に比べ有意に血管数が多かった。また、併用群と骨髄細胞群には有意差は認めないものの併用群の血管数が最も多い傾向がみられた（図8)。

安全性

図には示していないがHE染色での検討の結果、局所の浮腫や組織壊死などについてはフィブロネクチン単独投与群と非投与群の間に差を認めず、少なくとも800μg/mlの濃度までのフィブロネクチン投与による組織障害性は観察されなかった。

考　察

BMIによる血管新生療法の効果発現のメカニズムには未だ不明な点が多い。Isnerらは1994年に、ウサギ下肢虚血モデルに対する組み換えVEGF蛋白を経動脈的に投与し、血流の改善と虚血部での血管新生を認めたことを報告した[1]。さらに彼らは1996年にVEGF遺伝子をヒト虚血肢筋肉内に直接注入し、虚血性潰瘍の治癒や安静時疼痛の改善を認めたことを報告した2。この時点では成人における血管新生は既存の内皮細胞の増殖によると考えられていたが、1997年にAsaharaら[6]が骨髄に由来する内皮前駆細胞が未分化な状態で血液単核球中に含まれ、末梢血に動員され血管発生に関与することを報告し[7,8]、BMIによる血管新生療法が研究されるようになった[23-25]。本邦では重症虚血肢患者に対するBMIの臨床応用が2000年より始まり、2002年にTateishiら[9]により重症虚血患者に対するBMIの有効性と安全性が報告される一方、効果発現が症例や基礎疾患により異なり、現段階では治療効果の予測が付け難い点が難点とされている。極めて有効である症例がある一方で、効果が不十分な症例も少なくなく、効果発現のメカニズムの解明とともに治療効果の向上にむけた取り組みが必要である。

フィブロネクチンは血漿中に約300μg/mlの濃度で含まれる糖たんぱく質であるが、同時に細胞外マトリックスとして組織内に広く分布し、細胞接着、分裂、細胞遊走といった様々な機能を担う多機能蛋白質である。VEGFと特異的に結合するHeparin-II Domainを有し、VEGF/フィブロネクチン複合体は内皮細胞の増殖過程におけるもっとも重要な因子のひとつである[26]。フィブロネクチンはVEGFによって誘導されるCD34陽性細胞の虚血部位への遊走、血管内皮細胞への分化を促進する[27]。またVEGFR-2を介したErkのリン酸化はフィブロネクチンの存在下に、より低いVEGF濃度によって惹起されうることが示されている[21,26]。さらにVEGFによって引き起こされる内皮細胞の遊走能および増殖能は、フィブロネクチンあるいはフィブロネクチンのHeparin-II Domainサイト（VEGF binding site)およびRGDサイト（integrin binding site）のフラグメント投与によって著しく増強される[21,27,28]。HUVEC培養におけるフィブロネクチンコートの使用は濃度と比例して細胞の増殖能も上がることも示されており、in vitro /in vivo　いずれの実験系においてもフィブロネクチンがVEGFと共同して内皮細胞の分化誘導、増殖、血管新生に関わっていることを裏付ける報告がある[29-32]。我々は、本研究においてフィブロネクチンが虚血骨格筋内で発現しているVEGFの血管新生作用を増強し、BMI効果を向上させる可能性について検討した。本研究で用いた虚血肢モデルは無治療においても側副血行路の発達により2週間で患肢/健肢血流比は0.6前後まで回復しこれは4週間目においても変わらない。また、治療群においては、2週間目以降も血流の改善がみられ、3週間目から4週間目でプラトーに達する。したがって本研究は3週間までの血流回復の速さと、術後3週間での最終的な血流回復値によって評価した。治療群（フィブロネクチン単独投与群、BMI群および併用群）とコントロール群の両群は術後3日目より血流の改善が認められ、治療群は術後5日目にコントロール群よりも血流が回復している。術後早期（術後5日目まで）の血流回復は骨髄細胞を移植された群（BMI群および併用群）において高く、フィブロネクチン単独投与群は術後早期の評価ではコントロール群と同じ程度の回復に留まっている。この時点では一見単独ではもちろん、BMIとの併用によってもフィブロネクチン投与の効果はないように見える。しかし術後7日目以降、BMI群の回復率はプラトーに達し、逆にフィブロネクチン単独投与群において血流回復率は徐々に上昇し始め、最終的に

BMI群とフィブロネクチン単独投与群とで同様の血流比（0.8前後）となった。フィブロネクチン投与/BMI併用群は術後後期になっても変わらず回復し続け、最終的に患肢/健肢血流比は正常の1.0前後を示した。このことは単独移植された骨髄細胞のほとんどが7日目までにアポトーシスに陥り死滅するとしているこれまでの報告を裏付け、かつ今回併用投与されたフィブロネクチンが何らかの形でBMIの効果を持続・増強させている可能性を示唆する。

さらにフィブロネクチン単独投与群においても一定の血流改善効果が得られたものの、フィブロネクチン単独投与群ではBMI群との併用に比べ十分な血流改善は得られず、血管数、大腿直筋の組織学的変化、いずれをとってもフィブロネクチン単独投与/BMI併用群において最も良好な結果を得た。またBMI群において、術後早期における血流回復が併用群とほぼ同等に観察された一方、フィブロネクチン単独投与群では術後早期の血流回復は認められなかった。このことは移植細胞が、特に移植後早期において何らかの役割を担っていることを示唆するものであり、骨髄細胞がアポトーシスに陥り移植局所から消滅するまでの間、局所に留まり何らかの刺激作用を発現している可能性も示唆される。

本研究においてBMIの効果発現が術後ごく早期から現れたことは、移植翌日から疼痛の軽減、皮膚温の上昇といった効果がみられるとする臨床報告とも合致しており、血管新生の速度を鑑みても、BMIの効果が、特にごく早期においては血管新生によってのみもたらされるものではないことを支持する結果であった。臨床症例の中でBMIの効果が術後早期のみ一過性に現れ、遠隔期にはその効果が減じたとされる報告があるが、これらもまた、BMIの効果が炎症性サイトカインを通じて発揮されている可能性を支持する。

フィブロネクチンは，オプソニン作用や線維芽細胞、単球・マクロファージ系細胞の遊走促進作用などの生物活性を通じ、炎症過程での初期の局所反応、損傷部位での有害物質の除去、創傷治癒などと深く関係しており、炎症が発生した場合、血中のフィブロネクチン濃度が変動することが臨床的、基礎的に確認されている。また、炎症性サイトカインであるIL-1βの刺激によって線維芽細胞からフィブロネクチンが産生されること[34]や、あるいはIL-1βがヒト単球のIL-6産生を誘導し、IL-6はさらにフィブロネクチンの産生を誘導することが知られている[35,36]。術後ごく早期のBMI群における血流増加、後期におけるフィブロネクチン投与群の血流改善は、BMIによって刺激されたIL-1β、IL-6といった炎症性サイトカインが、一方で局所の一時的な血流増加をもたらし、フィブロネクチン産生を促すことにより血管新生を惹起している可能性も考えられる。

創傷治癒過程においても、フィブロネクチンは創傷内に強発現し、創部への細胞の遊走を促し創傷治癒を助けると言われており、眼科領域においては自己血液より採取されたフィブロネクチン点眼薬による創傷治癒が試みられている[37,38]。今回の研究では潰瘍形成をきたすような虚血肢に対しての実験は行っていないが、BMI療法を必要とする患者の多くが難治性潰瘍を合併していることを考えれば、フィブロネクチンを併用投与薬剤として用いるメリットは少なくないかもしれない。また点眼薬といった形であれ、すでに臨床応用されているといった点で安全性が確立しており、また自己血液からの精製手段が確立している点においても、臨床応用可能な治療法である可能性が示唆される。

本研究の限界として、側副血行路の発達がおこりやすいマウスを実験動物に用いたため、コントロールにおいても14日で虚血がある程度改善してしまい治療効果を明らかな差として示しえなかったこと、また短期間での検討しか行えていないことがあげられる。今後、より虚血の強い脱落肢モデルあるいは大動物を用いた検討を加えていく必要がある。

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追　加

平成16-18年度に総合研究報告書に報告した内容を以下のごとく報告した

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Changes in Angiogenesis-related Factors in Serum Following Autologous Bone Marrow Cell Implantation for Severe Limb Ischemia

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Abstract

Bone marrow mononuclear cell (BM-MNC) implantation (BMI) for critical severe limb ischemia especially for Buerger's disease shows excellent clinical results, but the mechanism of this treatment is still unknown. In this study, we investigated the change of angiogenesis-related factors serum levels after BMI treatment. Twelve patients had undergone BMI treatment: 9 patients had Buerger's disease, 2 patients had arteriosclerosis obliterans (ASO) and 1 had systemic sclerosis (PSS). These patients were taken venous blood from the transplanted limb before and after BMI treatment. Adrenomedulin (AM), soluble vascular cell adhesion molecule-1 (sVCAM-1), and C-reactive protein (CRP) serum levels 24 hours after BMI treatment were significantly increased compared with those before BMI treatment ($P<0.05$). The peak levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF) and nitric oxide (NO) serum levels after BMI treatment significantly increased between 1 week and 3 months after BMI treatment ($P<0.05$). There was no correlation between the numbers of implanted cells and serum levels of measured angiogenesis-related that were significantly increased after BMI treatment. It was

considered that the mechanism underlying BMI treatment involves both early and late phases. The early phase involves the direct action by implanted cells, and the late phase involves indirect paracrine action. In addition, it was considered that BMI treatment is effective when we implant a sufficient level of bone marrow (600ml) to treat severe limb ischemia.

Key words: Angiogenesis-related factors, Bone marrow mononuclear cell implantation (BMI), Buerger's disease, Critical severe limb ischemia, Inflammation, Paracrine action.

Introduction

Recently, the number of patients with peripheral arterial occlusive diseases has been increasing. Symptoms such as intermittent claudication, resting pain, ischemic skin ulcers are observed in these patients, whose quality of life is greatly disturbed by such symptoms. Conventionally, the treatments of peripheral arterial occlusive diseases include pharmacotherapy, percutaneous transluminal angioplasty, and bypass surgery, which are chosen depending on the severity of the symptoms. However, the patients with critical limb ischemia especially Buerger's disease are at risk of amputation.

Endothelial progenitor cells (EPCs) were recently identified in adult peripheral blood[1] and have been shown to participate in postnatal neovascularization after mobilization from bone marrow[2, 3]. In general, these endothelial precursors are characterized by co-expression of three cell surface markers: CD34, an early hematopoietic stem cell marker; CD133, a marker for stem and progenitor cells; and the vascular endothelial growth factor receptor-2 (VEGFR-2/KDR)[4, 5]. It was reported that direct local implantation of autologous bone marrow mononuclear cells (BM-MNCs) containing EPCs augmented neovascularization and collateral vessel formation in the ischemic tissue[6, 7]. Currently, BM-MNC implantation (BMI) treatment is performed for critical limb ischemia especially Buerger's disease, and has been reported to be very effective [8-10].

Initially, the mechanism of BMI treatment was thought to be formation of new vessels from implanted BM-MNCs[11]. However, direct incorporation of implanted cells into newly formed vessels is reported to be relatively rare. Thus, it has been assumed that angiogenic factors secreted by implanted cells are responsible for the efficacy of BMI treatment[12, 13].

There are many experimental reports describing the mechanisms of BMI treatment, but there are few reports using clinical data. Thus, we investigated changes of angiogenesis-related factors before and after clinical BMI treatment in this study. Only cases of successful BMI treatment were included in this study. We investigated changes in vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and granulocyte-colony stimulating factor (G-CSF) before and after BMI treatment. VEGF and bFGF have been reported to be released by BMI treatment[13]. In addition to these growth factors, we investigated the change of Adrenomedulin (AM), nitric oxide (NO), C-reactive protein (CRP), Interleukin-1 β (IL-1 β) and soluble vascular cell adhesion molecule-1 (sVCAM-1). AM was reported to have angiogenic function [14] and antiapoptotic effect[15]. NO is well known as a strong vasodilator[16]. CRP and IL-1 β are inflammatory factors reported to relate to cell transplantation [17]. VCAM-1 is a cell adhesion factor affected by inflammation [18, 19]. However, there are few reports describing the relationship between the number of implanted cells and angiogenic factors based on clinical data in BMI treatment. Therefore, we investigated the correlation between the number of BM-MNCs, CD34-positive cells or CD34/133-positive cells and serum levels of angiogenesis-related factors in this report.

Methods

Patients

Twelve patients, whose BMI treatments were clinically very effective, were selected from 19 patients who had undergone BMI treatment at Shinshu University. Patient ages ranged from 30 to 71 years old, 8 males and 4 females; 9 patients had Buerger's disease, 2 patients had arteriosclerosis obliterans (ASO) and 1 had systemic sclerosis (PSS) (Table 1). After approval by the Shinshu University Ethical Committee, we obtained written informed consent from all patients for the study of angiogenesis-related factors in serum.

Bone marrow cells implantation

Under general anesthesia, approximately 600mL of bone marrow was harvested from the iliac bone. BM-MNCs were separated from bone marrow and concentrated to a final volume of 50mL using an AS.TEC-204 Cell separator (Fresenius HemoCare, Germany). Then, 0.5mL of BM-MNCs were injected into each of 30-40 sites on the ischemic hindlimb muscle, spaced 1-2 cm apart, using a 26-gauge needle.

Blood sampling

Venous blood samples from the patients were taken from the transplanted limb before BMI treatment, and further samples were taken at 24 hours, 1 week, 2 weeks, 1 month, 3 months and 6 months after BMI treatment.

Measurement of angiogenesis-related factors

Serum levels of human VEGF, bFGF, G-CSF, EGF, and HGF were measured by Luminex ® system. Human Growth Factor Four-Plex antibody Bead Kit (BioSource International, USA) and Human HGF antibody Bead Kit (BioSource International, USA) were used. The serum level of human nitrate/nitrite (NOx) was measured by NO_2/NO_3 Assay kit-FX(r) (Dojindo, Japan). The serum level of human AM was measured by immunoradiometric assay using AM RIA SHIONOGI AM (Cosmic Corporation, Japan). The plasma level of human CRP was measured latex immunonephelometry using CRP LT2 (Wako, Japan). Serum level of human IL-1 β and sVCAM-1 were measured by ELISA assay using Quantikine® Human kit (R&D Systems, USA).

Fluorescence-Activated Cell Sorter

The numbers of CD34-positive cells and CD34/133-positive cells in bone marrow were quantified by FACSCalibur and CELLQuest software (BD Biosciences, CA). BM-MNCs were stained with a fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibody (My10, BD Biosciences, NJ), and phycoerythrin-conjugated anti-CD133 monoclonal antibody (AC133, Miltenyi Biotec, Germany). Positivity for the antigens was determined with FACSCalibur. The samples were subjected to a 2D side scatter-fluorescence dot plot analysis[20].

Statistical Analysis

All statistical analyses were performed using StatView-J software (version 5.0; Abacus Concepts, USA) Changes in the variables measured were analyzed using t-test and the degree of correlation was assessed using Pearson's correlation coefficient. Results were expressed as the mean $\pm$ SD, and were considered significant when the p value was less 0.05.

Results

Changes in VEGF, bFGF, EGF, HGF, G-CSF and NOx serum levels after BMI treatment

VEGF serum levels at 1, 2, 4 weeks and 3 months after BMI treatment were significantly increased compared with that before BMI treatment (Figure 1-A.). However, bFGF, G-CSF, EGF and HGF serum levels after BMI treatment were not significantly

changed compared with those before BMI treatment (Figure 1-B, C, D, E.). NOx serum levels, 2 weeks after BMI treatment, was significantly increased compared with that before BMI treatment (Figure 1-F.). The peak levels of VEGF, bFGF, G-CSF and NOx serum levels after BMI treatment were significantly increased compared with those before BMI treatment, but EGF and HGF serum levels after BMI treatment were not significantly increased compared with those before BMI treatment (Figure 2.).

Changes in AM, sVCAM-1, CRP and IL-1 β serum levels after BMI treatment

AM, sVCAM-1 and CRP serum levels 24 hours after transplant were significantly and transiently increased compared with those before BMI treatment (Figure 3-A, B, C.). IL-1 β serum levels after BMI treatment were slightly higher than those before BMI treatment, but the difference was not significant (Figure 3-D.).

The relationship between the number of implanted cells and serum levels of VEGF, bFGF, G-CSF, NOx, AM, sVCAM-1 and CRP

The numbers of implanted BM-MNCs, CD34-positive cells and CD34/133-positive cells in bone marrow were quantified in each case. The mean volume of harvested bone marrow fluid was 634± 41 ml, and the mean numbers of implanted BM-MNCs, CD34-positive cells and CD34/133-positive cells were $4.03\pm0.70\times10^9$, $4.71\pm1.90\times10^7$, 2.00± 0.76x107, respectively (Table2.). There was no significant correlation between the number of CD34/133-positive cells and serum levels of measured angiogenesis-related factors (VEGF, bFGF, G-CSF, NOx, AM, sVCAM-1 and CRP), which were significantly increased after BMI treatment (Figure 4.). There was no significant correlation between the number of BM-MNCs and serum levels of these factors in this study, or between the number of CD34-positive cells and these factors (Data not shown).

Discussion

In the 1990's BMI treatment was developed as angiogenic therapy, but then it was reported that most of transplanted BM-MNCs in BMI treatment had disappeared from ischemic muscle within 7 days after BMI treatment[16]. And angiogenic factors such as VEGF and bFGF were reported to be increased in ischemic tissue 2 weeks after BMI treatment[13]. It was considered that angiogenic factors are responsible for the efficacy of this treatment. VEGF and bFGF serum levels after BMI treatment were significantly higher than those before BMI treatment in this study in accordance with the findings of previous basic study. VEGF serum levels significantly increased 1 week to 3 months after BMI treatment, while bFGF serum levels were slightly increased 1 week to 3 months after BMI treatment. This showed that VEGF and bFGF were secreted in these patients from 1 week to 3 months after BMI treatment in this study as well as in previous experiments. Therefore, it was considered that paracrine action plays an important role in BMI treatment.

G-CSF is a cytokine that mobilizes CD34-positive EPCs from the bone marrow into the peripheral blood[21]. However, recent study also demonstrated that intramuscular injection of G-CSF induced angiogenesis through mechanisms independent of EPCs mobilization[22]. In this study, G-CSF serum levels after BMI treatment were significantly higher than that before BMI treatment. Although the time of G-CSF peak levels differed in each patient, serum levels of G-CSF, VEGF and bFGF showed a tendency to increase 1 week to 3 months after transplantation. Therefore, it is possible that G-CSF participates in the mechanisms of the effect of BMI treatment through paracrine action along with VEGF and bFGF.

NO plays important roles in vascular biology including regulation of vascular tone and blood

pressure[23], as well as the regulation of angiogenesis[24]. VEGF augments the release of NO from cultured human umbilical venous endothelial cells (HUVECs) and up-regulates the expression of mRNA and protein expression[25, 26]. Furthermore, bFGF increases the production of NO[27]. The release of NO in response to these growth factors is considered critical to their angiogenic actions. Direct in vitro evidence that NO may induce angiogenesis was demonstrated by Papapetropoulos et al [28], while Ziche et al established the first line of evidence that NO can induce angiogenesis in vivo [29, 30]. Murohara et al showed that angiogenesis in the ischemic hindlimb was significantly impaired in endothelial NO synthase (eNOS)-deficient mice compared with wild-type controls evaluated using either laser Doppler flow or capillary density measurement[31]. Amano et al demonstrated that transgenic mice overexpressing eNOS in the endothelium increased new capillary formation in response to tissue ischemia[32]. Namba et al reported that intramuscular injection of bovine eNOS plasmid induced therapeutic angiogenesis in a rat ischemic hindlimb model[33]. Thus, NO is a critical regulatory molecule for angiogenesis in response to tissue ischemia. Akasaki et al. reported that angiogenesis was induced via eNOS combined with thermal therapy in mice with hindlimb ischemia, showing the effect of thermal therapy on the dynamics of EPCs and the involvement of bone marrow-derived cells[34]. In this study, NOx serum level, 2 weeks after BMI treatment, was significantly increased compared with that before BMI treatment, it was suggested that NO participates in the mechanism of BMI treatment. In addition, the timing of the peak level of NOx was consistent with the timing of VEGF, bFGF, and G-CSF peak levels in serum. Therefore, NO might participate in the angiogenic action and the mobilization of blood-derived cells in response to BMI treatment.

AM was identified as a vasoactive peptide in 1993 [35]. Previous studies reported that there were vascular structure abnormalities in homozygous AM knockout mice[36, 37]. A recent study demonstrated that blood flow recovery in ischemic limb and tumor angiogenesis are substantially impaired in heterozygous AM knockout mice[38]. Furthermore, AM has been shown to inhibit vascular endothelial cell apoptosis and induce angiogenesis[39]. Those findings suggest that AM is indispensable for modulating angiogenesis and vasculogenesis. Iwase et al. reported that apoptosis of BM-MNCs occurred in ischemic muscle 24 hours after BMI treatment for rat limb ischemia, and that infusion of AM inhibited apoptosis of BM-MNCs[40]. In addition, they reported that AM significantly enhanced expression of VCAM-1 and intercellular adhesion molecule-1 in HUVECs, as well as facilitating adhesion of BM-MNCs to endothelial cells after BMI. In this study, AM serum levels 24 hours after BMI treatment were significantly increased compared with those before BMI treatment. In addition, sVCAM-1 serum levels, as well as those of AM, 24 hours after BMI treatment were significantly increased compared with those before BMI treatment. It has been reported that VCAM-1 facilitates adhesion of BM-MNCs to endothelial cells[41]. Therefore, these findings suggest that AM inhibited apoptosis of implanted cells that occurred in ischemic muscle 24 hours after BMI treatment and enhanced the expression of VCAM-1.

Tateno et al. reported that CRP, IL-1 β, IL-6, and VEGF levels of plasma in peripheral blood mononuclear cell (PB-MNCs) implantation treatment of the responder group were significantly higher than those in the non-responder group[17]. They also showed that implanted cells stimulated muscle cells to proliferate and induce the expression of angiogenic factors in an experimental model of limb ischemia using IL-1 β-deficient mice. Thus, they demonstrated the relationship between the secretion of angiogenic factors and inflammation. In this study, serum CRP levels rose significantly 24

hours after BMI treatment. AM and VCAM-1 as well as CRP serum levels 24 hours after transplantation were significantly increased compared with those before BMI treatment. Therefore, our findings and those of previous reports showed that the secretion of AM and VCAM-1 is affected by inflammatory factors[42, 43]. Therefore, it was suggested that the secretion of AM and sVCAM-1 were promoted by inflammation, and play an important role in the effect of BMI treatment.

Considering all the data shown in this study, we could divide these findings into the early-phase response group (phase I) and the late-phase group (phase II). The phase I group consisted of AM, CRP and sVCAM-1, which were transiently increased 24 hours after transplantation. The phase II group consisted of VEGF, bFGF, G-CSF and NO, which were increased within weeks or months after BMI treatment. As for phase I, implanted cells and inflammation were considered to promote secretion of AM and VCAM-1. It was considered that this effect of phase I is a direct action of implanted cells, and affects the cellular adhesion of implanted cells. As for phase II, implanted cells and ischemic tissues secrete angiogenic factors such as VEGF, bFGF, NO and G-CSF, which have actions that induce therapeutic angiogenesis. Therefore, it was considered that this effect is an indirect paracrine action induced by the implanted cells.

Regarding the relationship between the number of implanted cells and clinical efficacy, Saigawa et al. reported the MNCs and CD34-positive cells affect the number of EPCs, which play a major role in revascularization. Clinical data suggest that it is likely that there is a direct relationship between the number of mononuclear cells or CD34-positive cells and the clinical efficacy of BMI[44]. Kinnaird et al. reported the relationship between the number of implanted bone marrow stoma cells and the therapeutic effect in a mice hindlimb ischemic model[12]. Nizankowski et al. reported the efficacy and safety of BMI for clinical lower limb ischemia, but the clinical effect did not correlate with the volume of injected CD34-positive cells[45]. In our previous study, we reported the effect of BMI treatment for hand ischemia in peripheral arterial disease patients, and there was no correlation between the number of implanted BM-MNCs, CD34-possitive cells and CD34/133-possitive cells and digital/brachial pressure index[46]. Tateno et al. reported that there was no significant difference in neovascularization between the PB-MNC group and BM-MNC group in a mouse model of limb ischemia[17]. BM-MNCs have been reported to contain many more endothelial progenitor cells than PB-MNCs. However, there was no significant difference in neovascularization between VEGFR2-positive cells and VEGFR2-negative cells after implantation of PB-MNCs in a mouse model of limb ischemia. In addition, Li et al. reported that CD117-positive cells play a key role in therapeutic angiogenesis induced by BMI treatment [47]. In this study, there was no significant correlation between the number of implanted cells (BM-MNCs, CD34-positive cells and CD34/133-positive cells) and serum levels of measured factors, which were significantly increased after BMI treatment. In this study, the mean volume of harvested bone marrow was 634 ± 41 ml, and the mean number of implanted BM-MNCs was $4.03 \pm 0.70 \times 10^9$, CD34-positive cells was $4.71 \pm 1.90 \times 10^7$ and CD34/133-positive cells was $2.00 \pm 0.76 \times 10^7$. We considered that the volume of bone marrow or number of BM-MNCs harvested in this study was much higher than the level needed to relieve hindlimb ischemia in all patients. However, it is considered that more investigation is necessary to determine the optimal number of transplanted cells.

In summary, our findings suggest that secretion of angiogenesis-related factors investigated in this study after BMI treatment plays an important role in the mechanism underlying the effect of BMI treatment. It was suggested that this mechanism

involves early and late phases. The early phase involves direct action by implanted cells, whereas the late phase involves indirect paracrine action. In addition, it was considered that BMI treatment is effective when we implant an adequate volume of bone marrow (600ml) for ischemic limbs, especially for Buerger's disease.

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研究報告書

難治性血管炎に対する血管再生療法の多施設共同研究

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研究要旨

【目　的】

重症虚血状態の難治性血管炎に対して骨髄細胞移植（BMI）が行われてきたが、さらなる治療効果の改善が求められる。我々はBMIによる血管新生において移植骨髄中に含まれる赤芽球とマクロファージが主要な役割を果たしていることを報告した。今回、少量の骨髄細胞からこれらの細胞を培養・増幅し血管再生治療に応用（EVEETA）した第 I/II 相臨床試験の結果につき報告する。さらに当院では難治性血管炎に対するBMI治療の実績が11例あり、BMIとEVEETAを比較検討し、EVEETA の有用性を検証した。

【方法と結果】

対象は標準的治療が無効の難治性血管炎 6 症例。局所麻酔下に少量の骨髄液（平均68.5ml）を採取し単核細胞成分を分離後、細胞プロセッシング室で Flt-3L・SCF・トロンボポエチンの存在下で 7 日間培養し未分化前駆細胞を増幅、引き続き 7 日間エリスロポエチン・SCF・IGF-I の存在下で培養し前赤芽球とマクロファージを増幅した。得られた細胞を虚血下肢に筋注し 5 日間エリスロポエチンを局所に筋注した。移植後、下肢痛の速やかな軽減、難治性皮膚潰瘍の治癒、血管造影所見の改善、組織酸素分圧の上昇を得られた。いずれの症例も有害事象は認めなかった。

【展　望】

体外増幅赤芽球移植による血流改善効果は従来の BMI より強力であり、患者への侵襲も少なく、重症虚血肢に対する次世代血管新生治療として期待できる。

緒　言

重症下肢虚血症例に対する自己骨髄細胞移植（BMI）はこれまで最も広く行われてきた血管新生療法である[1]。当施設においても高度先進医療の認可を受け積極的にBMIを行ってきたが、真に血管再生を必要とする重症患者に対しての効果はまだ改善の余地がある[2,3]。

これを克服する目的で、BMIによる血管再生の機序を明らかにした。哺乳類の造血では骨髄で幹細胞から増殖分化した血球が成熟ののちに末梢血管へ動員される。赤血球系細胞は遊走能を持たないため、サイトカインの分泌により新生血管を誘致して末梢へ動員されるという仮説を立て、またこの赤芽球の作用を骨髄外で再現したのがBMIによる血管再生の機序であると考え、これを証明した。すなわち、骨髄細胞のうちVEGF、PLGF等の血管増殖サイトカインを分泌しているのは主に赤芽球であり、BMIによる

血管再生には赤芽球が必須であった。エリスロポエチン（EPO）は赤芽球の生存を高める作用があり、BMIにEPO投与を併用することで血管再生作用を高めることを示した[3]。

BMIでは全身麻酔下に500～1,000ccの骨髄を採取するため、患者への侵襲が大きい。またBMIの血管新生作用に影響する因子には、虚血局所の環境と、移植された骨髄の質があると考えられるが、実際にBMIを施行したASO等の患者骨髄総細胞数およびCD34陽性細胞数は健康人に比し、1/2ないし1/10程度であり、重症虚血患者ほど著しい低形成を示した。これらの問題点を克服するための方策として、少量の自己骨髄から十分量の幼弱赤芽球を体外で増幅する培養技術、すなわち体外増幅自己赤芽球移植による血管新生治療（Ex-Vivo Expanded Erythroblast Transplantation（Autologous）：EVEETA 療法の開発に着手した。

de novoの赤芽球のサイトカイン産生能を明らかにするため、健康人骨髄から多段階比重遠心法によって各種分化段階の赤芽球を分離し、VEGF、PLGF等の血管新生因子を測定した。その結果、超低比重分画から精製した前赤芽球から好塩基性赤芽球にかけての未熟な赤芽球が血管新生因子を圧倒的に活発に産生しており、ヘモグロビン合成の開始後すみやかに産生能が低下することが分かった。従ってこれら未熟な赤芽球を体外培養で大量に入手することを培養法開発の目的とした。健康ヒト骨髄を用いて培養法の試行錯誤を行った結果、TPO等3種の造血因子の存在下で7日間培養（1次培養）することで赤血球前駆細胞が増幅され、さらにこれをEPO等 3 種の造血因子の存在下で 7 日間培養（2次培養）することで、大量の未熟赤芽球が収穫された。

マウス下肢虚血モデルを用いて EVEETA 治療と BMI 治療を比較検討したところ、EVEETA（10^6 cells）はBMI（10^6 cells）に比し有意に下肢血流を改善させ、その効果は10倍量の細胞数を用いたBMI（10^7 cellsを移植）と同等の治療効果を示すほど強力であった。

方　法

平成18年秋に当施設に設置の倫理委員会および臨床研究審査委員会（IRB）から当該第Ⅰ/Ⅱ相臨床試験の認可を受けた。先述の十分な基礎検討のもと、当施設 CPC を利用し、平成19年より体外増幅自己赤芽球移植による血管新生治療（EVEETA study）の第Ⅰ/Ⅱ相臨床試験を開始した（ISRCTN-66803682）。

対象は従来のBMIと同様に末梢性血管疾患（慢性閉塞性動脈硬化症・難治性血管炎 [バージャー病・膠原病に伴う血管炎など]）Fontaine 分類Ⅱb・Ⅲ度およびⅣ度で、著しくQOLが障害されており血行再建術の適応が無く、将来切断が予想される患者、年齢は20 歳以上80 歳未満、患者の人権や安全性に十分配慮し、説明と同意の後に試験への参加を許可した。移植の14日前に患者の両側腸骨より局所麻酔によって約40 mlの骨髄を採取し、速やかに細胞数を計測し、不十分な場合には胸骨より局所麻酔によって不足分の骨髄を採取した。

上述のごとくトロンボポエチン、SCF、Flt3L 等の存在下で 7 日間培養（1次培養）することで赤血球前駆細胞を増幅し、さらにこれをEPO、SCF、IGF-1の存在下で7日間培養（2次培養）して大量の未熟赤芽球を収穫した。細胞に用いた試薬の混入を防ぐために培養細胞を十分に洗浄したのち、各施設へ培養後の細胞を出荷し、移植は細胞を患部に筋肉内注射により投与した。移植当日を含む 5 日間、移植部位局所に EPO 6000 国際単位を連日筋肉内投与した。定法に従い、自覚症状（VAS）・歩行距離・下肢血管造影による血流の評価・皮膚温や組織酸素分圧、皮膚灌流圧、Ankle-brachial pressure index（ABI）等の測定によって、治療効果を客観的に評価した。

結　果

これまですでに7例の末梢血管疾患患者（うち難治性血管炎は6例）に対して EVEETA 治療を施行した（表1下）。EVEETAを施行した難治性血管炎 6 例において平均採取骨髄量は68.5mlであった。採取した骨髄細胞数が少なく十分な

治療前とEVEETA施行4週後の結果

	VAS (mm)	TcO2 (mmHg)	skin ulcer	SPP (mmHg)	DSA	Thermo graphy	総合判定
Case 1 TAO	51→2	25→50	治癒	-	改善	不変	著効
Case 2 SSc	2→0	67→50	-	-	不変	不変	不変
Case 3 TAO	7→6	37→44	-	-	わずかに改善	不変	有効
Case 4 TAO	80→24	43→38	完全に上皮化	13→39	不変	改善	有効
Case 5 SLE	62→13	2→3	完全に上皮化	13→42	不変	改善	有効
Case 6 ASO	31→14	20→9	縮小	22→44	改善	改善	著効
Case 7 TAO	20→11	8→4	縮小	22→25	改善	改善	著効

培養細胞を得られなかった症例もあったが、十分量の細胞数を移植できた症例では、すみやかな下肢痛の軽減及び消失、難治性皮膚潰瘍の治癒、局所組織酸素分圧や皮膚灌流圧の上昇、血管造影での側副血行増加といった非常に良好な結果を得られた。なお、これまで明らかな有害事象は認められていない。

考　察

さらに当院では難治性血管炎対するBMI治療の実績が11例あり、EVEETAを施行した6症例と合わせて表1に示す。全17症例のうち急性期効果は著効例、有効例あわせて15症例と有効率は88%であった。バージャー病症例は全例で有効であった。血管炎症例は5例中3例で有効であった。

移植細胞別に検討してみると、BMI例でも急性期効果は良好であり、大切断術にいたるほどのイベントは認めなかった。しかし中には慢性期再発による再治療例や末梢側切断症例が一部存在した。一方、EVEETA症例では、移植細胞数が少ない症例では治療効果も少ないが、十分量の細胞数を移植できた症例では非常に良好な治療効果が得られた。疼痛改善効果、潰瘍の治癒、血管造影所見の改善といった点ではBMIよりも優れた治療効果を得られている印象がある。さらに注目すべきは症例17である。症例17は過去に他施設でBMIを2回受けているが、全く治療効果なかったが、我々の開発したEVEETA治療には著効した。これはEVEETA治療がBMIに比し非常に強力な治療法であることを示唆するものと考えている。今後再発例、無効例においても積極的にEVEETA治療を検討すべきと思われた。

結　語

難治性血管炎症例に対する細胞移植治療は有効であった。また、体外増幅赤芽球移植による血流改善効果は従来のBMIよりさらに強力であり、患者への侵襲も少なく、重症虚血肢に対する次世代血管新生治療として期待できる。

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