precisely represent the production or consumption of endostatin in the local coronary and cranial milieus and could have been influenced by acute ischemic events because several angiogenesis-related factors have been known to be influenced by the time course of tissue ischemia/necrosis.[8,9] In the present study, we obtained more site-specific samples from the CS and the LV that could represent the actual dynamics of endostatin in the coronary circulation. In our study, CS levels of endostatin were significantly elevated in patients with CHD. This finding suggests that the production of endostatin, which is considered as an antiangiogenic growth factor, in the heart may be associated with the pathology of CHD. So, we calculated the difference between the CS and LV (CS − LV), which may represent the production or consumption of endostatin across the coronary circulation to evaluate the factors affecting the level of endostatin. The importance of determining the actual dynamics of several substances in the coronary circulation has been stressed recently.[10,11] To our knowledge, this is the first study to investigate the dynamics of endostatin in chronic CHD within the coronary circulation.

In patients with CHD with total (or near total) coronary occlusion, the CS − LV values of endostatin were significantly higher than in those without such occlusion, whereas the presence of CHD risk factors (hypertension, diabetes, dyslipidemia, smoking, and obesity), LV systolic dysfunction, and the greater extent of coronary atherosclerosis were not associated with CS − LV values of endostatin. These results may indicate that in patients with severe coronary stenosis requiring collateral growth, endostatin could be produced significantly within the coronary circulation, and it may have possible effects in regulating coronary collateral vessels. In this study, we divided patients with collaterals according to the collateral flow grade. We found that incomplete filling of coronary collateral vessels led to more production of endostatin within the coronary circulation. A similar result has been shown in pericardial levels of endostatin, which were significantly reduced in patients with well-developed coronary collateral vessels.[4] To explain this finding, there are 2 possible hypotheses. First, the presence of endostatin may impair coronary collateral growth. A similar observation supporting this hypothesis was found in diabetic patients who exhibited impairment of collateral vessel development in the heart, mostly due to reduced expression of vascular endothelial growth factor and alterations in the biology of endothelial progenitor cells.[12,13] Genetic and environmental factors may lead to the production of endostatin in the heart accompanied with the reduction of angiogenic growth factors. Second, endostatin may have a regulatory effect on the development and maintenance of coronary collaterals. Several angiogenic growth factors (e.g., vascular endothelial growth factor) induce new blood vessel formation in response to stimuli such as hypoxia.[14–18] In contrast, the maintenance of endothelial quiescence is believed to be due to the presence of antiangiogenic growth factors.[14] Angiogenic and antiangiogenic growth factors often coexist in tissues in which angiogenesis is executed.[14,15,18] Thus, the status of endothelial cells is determined by a balance between these positive and negative factors.[19] Unlike inappropriate angiogenesis, such as tumor angiogenesis, antiangiogenic growth factors such as endostatin may have an important role in appropriate (physiological) angiogenesis. Further studies are needed, such as those concerning the expression site of endostatin in heart tissue, time course of the production of endostatin during collateral vessel formation, and its interaction with other angiogenesis-promoting growth factors.

This study has some limitations. We have no direct evidence that endostatin is associated with coronary collateral growth. Further experimental studies should be considered. We cannot exclude the time course influence of endostatin by myocardial ischemia because the design of this study was cross sectional. However, to our best knowledge, no studies have specifically addressed the time course of endostatin after acute myocardial ischemia, unlike several other angiogenesis-related growth factors.[8,9] In addition, it was somewhat difficult to evaluate the systemic endostatin in our study because of the possible influences of systemic vascular diseases, the coincidental complication of neoplasms and chronic inflammatory disorders, although the CS − LV value of endostatin was not influenced.

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Letter to the Editor

Serum erythropoietin level as a marker of limb ischemia

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Abstract

Bone marrow implantation (BMI) has been utilized for the treatment of limb ischemia, however, serum markers have not yet been reported to express the degree of limb ischemia. We analyzed the serum levels of several cytokines including erythropoietin (EPO) in the treated legs and the contralateral ones in 11 patients with limb ischemia treated with BMI. The EPO level in the pre-treated legs in the 5 patients with arteriosclerosis obliterans revealed a good correlation with ankle–brachial pressure index. The EPO level, but not the levels of TNF-α, VEGF, and bFGF in the pre-treated legs was significantly higher than that in the contralateral legs in the 11 patients, and the EPO level decreased in 4 weeks after BMI. The serum EPO level may express the degree of limb ischemia presumably through the reactive production of EPO in ischemic tissue.

Keywords: Erythropoietin; Limb ischemia; Bone marrow implantation

1. Introduction

The discovery of endothelial progenitor cells [1] has led to cell therapy for the treatment of tissue and organ ischemia such as bone marrow implantation (BMI) [2]. The production of vascular endothelial growth factor (VEGF) by muscles is induced through hypoxia inducible factor-1 (HIF-1) [3], and erythropoietin (EPO) is also one of the cytokines regulated by HIF-1 [4]. We supposed that serum levels of VEGF and EPO may reflect the degree of limb ischemia. We then investigated the change of serum cytokine levels in the bilateral femoral vein before and after BMI.

2. Methods

The entry criteria for patients to be enrolled in the present study was that they had symptoms of chronic limb ischemia, including severe intermittent claudication, rest pain or non-healing ischemic ulcers, but were not considered to be suitable candidates for non-surgical or surgical revascularization. Among the 19 patients treated with BMI from December, 2001 to April, 2004, 8 patients were excluded from the study as follows: 3 patients underwent hemodialysis and EPO administration, 1 treated for upper limb ischemia, 1 with limb ischemia caused by hypereosinophilia, 2 without complete blood sampling, and a patient with an exceptionally high EPO level as a result of severe anemia (Hct 25.3%). Finally, 11 patients were studied, i.e., 5 with arterial sclerosis obliterans (ASO), 4 with Buerger's disease, and 2 with thromboembolism. Bone marrow mononuclear cells were implanted into the ischemic leg by means of an intramuscular

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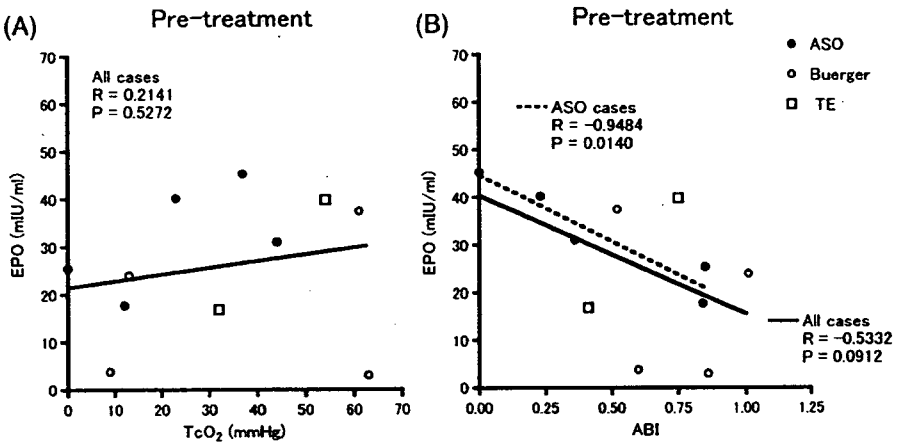

Fig. 1. The correlation between physiological parameters of limb ischemia [TcO_2 or ABI] and the serum level of EPO before treatment. Panels A and B show the correlations of TcO_2 and ABI to the serum EPO level before treatment, respectively. The solid and broken lines indicate regressions in the total cases ($n=11$) and the patients with ASO ($n=5$), respectively. TcO_2: tissue oxygen pressure, ABI: ankle–brachial pressure index, EPO: erythropoietin, ASO: atherosclerosis obliterans.

injection. Clinical data was obtained weekly for the first month of the treatment. Ankle–brachial pressure index (ABI) and transcutaneous oxygen pressure index (TcO_2) were monitored.

Serum was obtained from bilateral femoral veins before and 4 weeks after BMI to measure cytokine levels. The serum level of EPO was determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). For the measurement of VEGF, TNF-α, and bFGF, a cytometric bead assay system was utilized (Human Angiogenesis Kit, BD Biosciences, San Diego, USA).

3. Results

TcO_2 measured in the pre-treated legs, but not ABI, improved by BMI ($P<0.05$) 4 weeks after treatment ([TcO_2] basement: 30.6±20.7 mm Hg, 4 weeks: 42.4±16.5 mm Hg, $P=0.0466$; [ABI] basement: 0.585±0.312, 4 weeks: 0.611±0.327, $P=0.4248$). The serum EPO level in the femoral vein did not correlate with ABI ($R=-0.5332$, $P=0.0912$) or TcO_2. The patients with ASO ($n=5$) revealed a significant correlation of the EPO level and ABI ($R=-0.9484$, $P=0.0140$) (Fig. 1). No correlations were observed between the levels of other cytokines and the physiological parameters (data not shown).

As shown in Fig. 2A, the EPO level was higher in the pre-treated legs than in untreated legs before treatment ($P<0.01$), therefore the laterality of the EPO levels may reflect the degree of leg ischemia in individuals. The laterality of the EPO level disappeared 4 weeks after BMI (Fig. 2B). The EPO level decreased in the treated legs (Fig. 2C), but not in the untreated legs (Fig. 2D), after the treatment. No significant change was observed in the levels of other cytokines (data not shown). There was no difference in hematocrit levels measured before and after BMI (basement: 36.67±3.69%, 4 weeks: 37.67±4.38%, $P=0.3784$).

4. Discussion

TcO_2 reflects peripheral blood-flow, while ABI is affected by stenosis in relatively large arteries. BMI introduces small vessel angiogenesis, which may improve TcO_2 but not ABI. On the other hand, the total amount of produced EPO in the ischemic loci is virtually prescribed by the degree of the ischemia and the volume of EPO-producing tissue, which may correlate to ABI. The elevated level of EPO decreased after BMI through the improvement of limb ischemia. A study will follow to observe the change of EPO level in patients with limb ischemia treated with conventional therapies such as bypass operation and percutaneous transluminal angioplasty.

Beyond the erythropoietic effect, EPO also expresses a tissue-protective effect in an autocrine/paracrine manner in the cardiovascular and the central nervous system in physiological situations, as well as in several cancers in pathological situations [5,6]. The plasma levels of EPO, thrombopoietin, GM-CSF, and soluble kit-ligand were reported to increase in the mouse ischemic hindlimbs [7]. Ischemic tissues may regulate neovascularization through the direct release of these hematopoietic cytokines in the ischemic loci. The observations in the present study may support the findings above in a clinical situation. The reason is not known why the VEGF level did not reflect ischemia.

The significant laterality of the femoral vein EPO level and the decrease after treatment may, at least, refer to the clinical importance of the level of this unique cytokine, EPO, for the estimation of tissue ischemia.

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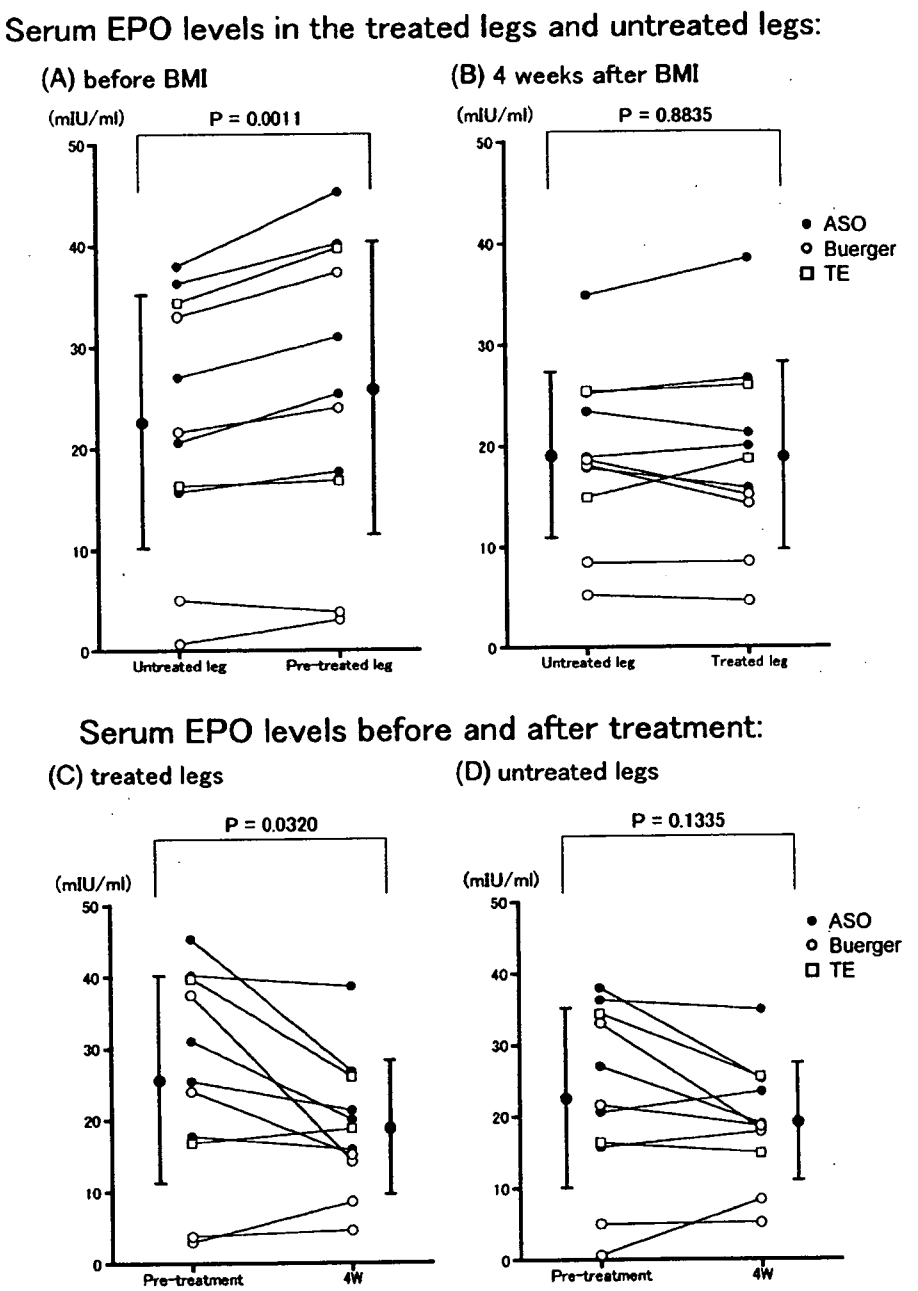

Fig. 2. The laterality of femoral vein EPO levels before (A) and after (B) treatment, and the change of EPO levels in the treated legs (C) and the contralateral legs (D) by the treatment. EPO: erythropoietin.

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特集 サイトカインと透析療法

Ⅷ 透析患者の幹細胞治療

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津田 一郎* 川村 明夫*

要旨 当院では閉塞性動脈硬化症（ASO）や糖尿病性血管障害を伴った血液透析患者を中心に，末梢血幹細胞による血管再生治療を行ってきた．119例に行い，65例は救肢された．壊疽が指趾を越えたり，病態が急速に増悪する症例の成績は不良であった．透析患者では，より早期に治療を行うべきである．

〈key point〉

Ⅰ．末梢血幹細胞による血管再生治療とは

ここでの重要なポイント
- 四肢末梢血管障害の血液透析患者に，末梢血幹細胞を局所注射して末梢血管の再生をはかる．
- CD34陽性細胞を末梢血から採取する方法（PBSCC）を行った．

1．治療の成り立ち

末梢血幹細胞移植とは，G-CSF（顆粒球コロニー刺激因子）の投与により血中に造血幹細胞を放出させ，それを用いて骨髄移植の代わりとする治療で，血液内科で行われてきた．造血幹細胞の表面にはCD 34という抗原が発現している．1997年頃より，CD 34陽性細胞の分画中に血管内皮前駆細胞が存在し，これは骨髄に由来し，虚血肢における血管新生に関与することが，実験的に明らかになった[1)~4)]．そこで，CD 34陽性細胞移植による血管再生療法が，まず骨髄単核球を採取筋注することで行われた[4)~6)]．経過は良好で最長14カ月下肢の血流増加は保たれた．しかし，骨髄採取には全身麻酔や自己血輸血が必要で患者への負担が大きいことから，CD 34陽性細胞を先の血液内科と同じ方法で末梢血

CD 34陽性細胞移植

Key words 血管再生治療，末梢血幹細胞，CD 34陽性細胞，血液透析

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PBSCC

から採取する方法（PBSCC）[7]を行った．さらに，採取液からCD34陽性細胞のみを純化[8),9)]しようとすると1回に数十万円ほどの試薬が必要となる．採取液中には赤血球，単核球および血小板が混在しているが，1×10^7個以上のCD34陽性細胞が含まれていれば，採取液をそのまま筋注することにした[10)]．

2. その他の方法

・自己骨髄単核球移植による治療：TACT（Therapeutic Angiogenesis by Cell Transplantation）studyが開始され，高度先進医療として認可された．今後は大規模なclinical trialを行い，より一般的な治療となっていく．

・末梢血単核球移植による治療：下肢切断を勧められた47例の重症下肢虚血患者に施行し，大切断の回避が89％，疼痛，潰瘍の改善が72％に認められた．2005年7月には高度先進医療の認定を受けた．単核球移植により虚血肢の筋組織の再生が促され，血管再生因子の発現を維持している．

・末梢血血管内皮前駆細胞移植による治療：16例に試験治療を開始，14例で1年後追跡検査を終了している．重篤な有害事象はなかった．自覚症状や潰瘍の改善は全例に認めた．

II. 当院での成績

ここまでの重要なポイント

- 重篤な虚血性心疾患と脳血管障害のない，四肢末梢血管血行障害の患者を対象とした．
- 現在までに119例に行い，改善36例，変化なし29例，切断54例であった．

1. 対象と方法

虚血性心疾患
脳血管障害

四肢末梢血管血行障害の患者を対象とした．重篤な虚血性心疾患と脳血管障害のないことを，心エコー，心筋シンチ（BMIPP），脳CTなどで確認した．次いで，G-CSF 5 μg/kg/dayを4日間毎日皮下注した．4日目にPBSCCを，CS-3000またはSpectraを用いて行った．採取されたCD 34陽性細胞数をフローサイトメトリー（FACScan）で計測した．同日中に，採取液を患肢に1カ所0.5～1.0 m*l*ずつ23 G針を用いて筋注した．麻酔は，上肢は全身麻酔，下肢は腰椎麻酔によった．治療効果の判定には，自覚症状，プレチスモグラフ，サーモグラフ，ABI（足関節/上腕血圧比），3 D-CTおよび血流シンチによった．

なお，臨床研究対象者に対する人権擁護上の配慮として，平成13年

表 1　透析，糖尿病と治療成績

	HD(+) DM(+)	HD(+) DM(−)	HD(−) DM(+)	HD(−) DM(−)	計
症例数	61	23	19	16	119
非切断（%）	24 (39.3)	13 (56.6)	13 (68.4)	15 (93.8)	65
小切断（%）	10 (16.4)	3 (13.0)	1 (5.3)	0 (0)	14
大切断（%）	27 (44.3)	7 (30.4)	5 (26.3)	1 (6.2)	40

表 2　壊疽の範囲と治療成績（平成 13 年 12 月〜平成 18 年 10 月）

Fontaine	4 壊疽（+） 指趾を越える	4 壊疽（+） 指趾に限局	4 壊疽（−） 潰瘍（+）	3	2	1	計
症例数	20	25	31	19	16	8	119
非切断（%）	7 (35.0)	6 (24.0)	15 (48.4)	17	14	6	65
小切断（%）	0	7 (28.0)	4 (12.9)	1	1	1	14
大切断（%）	13 (65.0)	12 (48.0)	12 (38.7)	1	1	1	40

表 3　切断肢の血管造影所見（33 例）

① 膝窩動脈が途絶していた例…… 4 例
② 足関節付近で前または後脛骨動脈が途絶していた例……16 例
③ 前・後脛骨動脈が下腿から足にかけて細い例…… 3 例（1 例はバイパス例）
④ 造影剤が注入できなかった例……10 例

12 月 27 日に札幌北楡病院医学倫理委員会による審査を受けた．G-CSF の副作用で心筋梗塞や脳血管障害を起こした報告[7),11)]がある．したがって，重篤な虚血性心疾患や脳血管障害がないことを確認すべきであるとされた．

2. 結　果

現在までに 119 例に行った．年齢は 25〜86 歳（平均 65.1 歳）．男性 82 例，女性 37 例で，血液透析中の患者が 84 例（70.6 %）を占めた．平均透析歴は 7.8 年だった．一方，糖尿病合併例は 80 例で，血液透析例 84 例中 61 例（72.6 %）が糖尿病だった．下肢に治療したのが 109 例，上肢が 10 例で，Fontaine 1 度が 8 例，2 度 16 例，3 度 19 例，4 度が 76 例あった．

移植した CD 34 陽性細胞数は平均 3.6×10^7 個で，改善が 36 例（観察

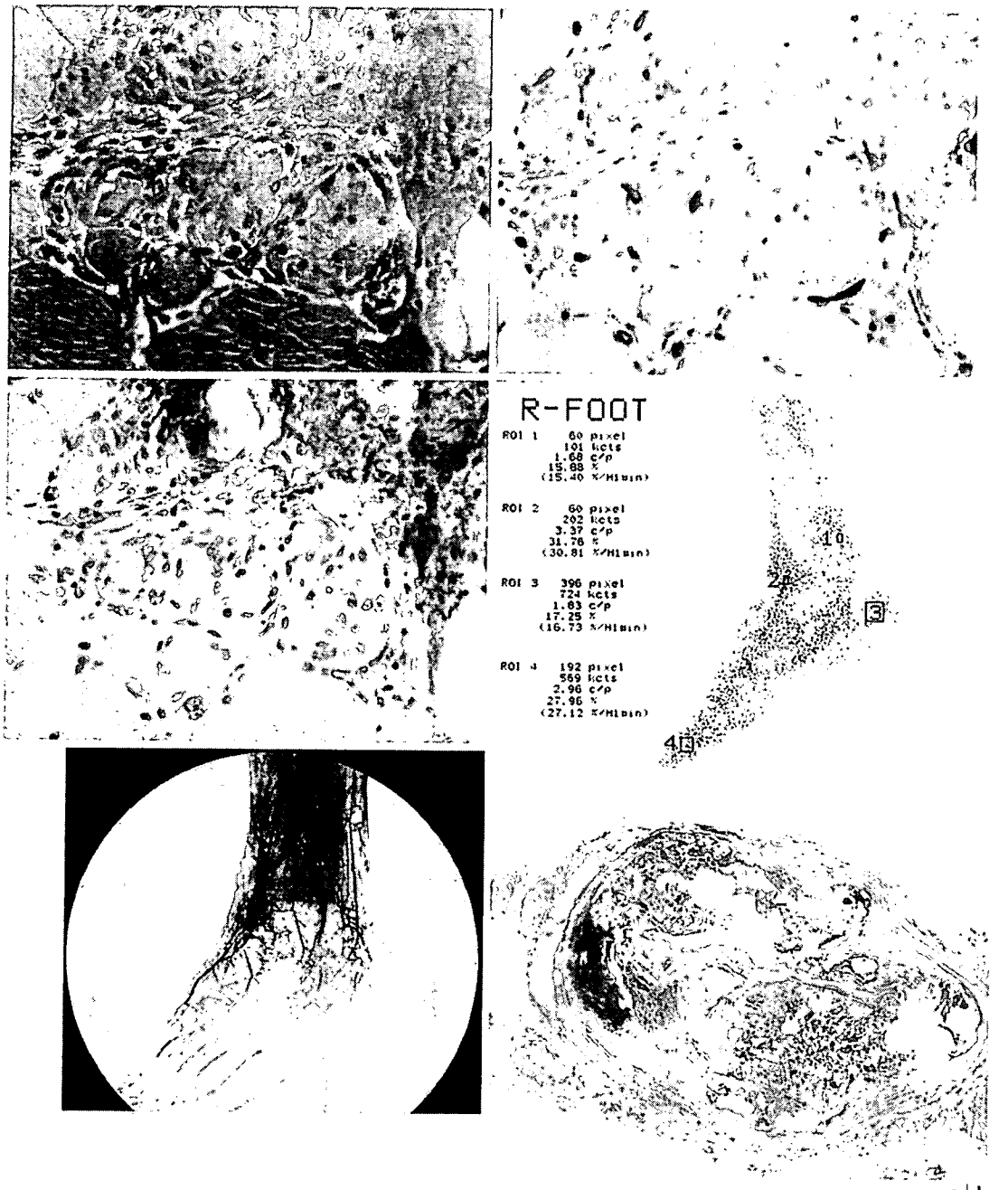

図　症例：59歳，男性

a～c：切断後の組織所見，1カ月後．（×400）．

a：HE，b：CD34，c：Factor Ⅷ

血管様構造の cluster が見られ，注入細胞集塊と考える．CD 34，Factor Ⅷともに一部の細胞に陽性を示す．

d：治療前血流シンチ．

e：切断肢の血管造影．

f：血管壁に著明な石灰化．注入細胞の増殖像なし（後脛骨動脈）．（×40）

期間が1～55カ月），変化なし29例（1～44カ月），切断が54例（0.3～31カ月）であった．切断は48例がFontaine 4度で32例は壊疽を伴っていた．また，1カ月以内に切断となった例は15例あり，9例が糖尿病と壊疽とを合併していた．一方，小切断は14例であった．全症例を通しての救肢率は54.6％だった．治療前での各種検査の結果は，サーモグラフでは118例中81例に低温領域を認めた．プレチスモグラフでは，117例中81例で脈波が消失していた．治療後1カ月での自覚症状とサーモグラフの改善のパターンが似ていた．

救肢率

糖尿病合併

糖尿病と壊疽とを合併した症例は34例（血液透析は29例）あり，1カ月以内切断15例中9例を占めた．34例中25例が切断となり，20例が大切断だった．切断とならなかった9例中4例と，小切断の5例はいずれも壊疽が指趾に限局していた．一方，指趾を越えた16例中11例は大切断となった．

透析（HD），糖尿病（DM）と治療成績（**表1**）をみると，HD(＋) DM(＋)，HD(＋) DM(－)，HD(－) DM(＋)，およびHD(－) DM(－)の順に症例数も切断率も高かった．大切断では，HD(＋) DM(＋)が44.3％，HD(－) DM(－)は6.2％だった．

Fontaine分類

Fontaine分類，壊疽の範囲と治療成績（**表2**）では，症例数はFontaine 4度で，壊疽のない例が31例ともっとも多く，切断率は大小合わせて51.6％だった．大切断率でみると，指趾を越える壊疽，指趾に限局する壊疽，壊疽のない潰瘍，Fontaine 3度の順で高く，糖尿病合併と同様の結果だった．

大切断となった下肢の血管造影所見（**表3**）では，膝窩動脈や前・後脛骨動脈の途絶，閉塞例がほとんどで，狭窄例は少数だった．

症例76は細胞治療後1カ月で切断となった．切断肢腓腹筋の観察では，CD 34陽性細胞の集積，Factor Ⅷ陽性の毛細血管の増生があり，細胞治療の効果とされた（**図**）．切断後の血管造影では足関節付近の前，後脛骨動脈の途絶があり，病理学的に著明な石灰化と血栓を認めた．

Ⅲ．末梢血幹細胞治療の適応

ここでの重要なポイント

- 糖尿病や透析を合併し，動脈硬化がベースにある場合は，壊疽が指趾を越えないうちに行ったほうがよい．
- Fontaine分類の比較的軽い例や，症状の安定している例に行うべきである．

適応について室原ら[4]は，閉塞性動脈硬化症（ASO），バージャー病で他の治療に反応しない40～75歳の患者で，除外項目として悪性新生

物，重症の糖尿病性網膜症，虚血性心疾患とした．

切断の危険因子

重篤な虚血性心疾患と脳血管障害のみを除外項目としたところ，切断例が119例中54例に発生した．切断の危険因子は，血液透析，糖尿病，Fontaine 4度および壊疽の合併である．透析と糖尿病の合併例は119例中61例（51.3％）ともっとも多いが，大切断率も44.3％ともっとも高い．一方，SLEのように，両者の合併のない症例の成績は良好だった．動脈硬化による末梢血管の石灰化と，血管炎との違いかもしれない．

壊疽の範囲

壊疽の範囲に注目すると，指趾を越えた場合，20例中13例（65％）が大切断となり，細胞治療による救肢は困難である．一方，Fontaine 4度でも指趾に限局していたり，壊疽がない場合は効果が期待できる．さらに3度以下では，まず大切断になることはないと考えられる．

したがって，糖尿病や透析を合併し，動脈硬化がベースにある場合は，壊疽が指趾を越えないうちに行ったほうがよい．最近，壊疽が指趾に限局していても，受診前1カ月より病態が進行性に増悪してきた症例に行い，1カ月以内に大切断となった．病態進行例は注意を要する．

切断肢の血管造影所見では，前または後脛骨動脈や膝窩動脈といった太い動脈の途絶が多かった．毛細血管の新生を促しても太い血管の途絶があれば，その効果は期待できない．足関節付近での途絶が多く，経皮的血管形成術（PTA）などの付加治療も試みたが，長期成績は不良であった．

おわりに

末梢血幹細胞による治療は，透析患者を中心に約5年間行ってきた．透析例の累積救肢率は31.4％，生存率は35.7％だった．成績向上のためには，Fontaine分類の比較的軽い例や症状の安定している例に細胞治療を行うべきである．

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Summary

Therapeutic angiogenesis using peripheral blood stem cells for hemodialysis patients

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Therapeutic angiogenesis using bone marrow cell transplantation was reported and this therapy was found to be useful for arterio sclerosis obliterans (ASO) treatment. We attempted the same procedure using peripheral blood stem cell collection (PBSCC). One hundred and nineteen patients were included in this study, 84 were hemodialysis patients. They all had peripheral arterial disease (PAD), 109 in the legs and 10 in the arms. Patients were given 5 μg/kg/day G-CSF for four days, subcutaneously. PBSCC was performed on day four. For all patients, 8.3 *l* peripheral blood was treated with an apheresis machine (CS-3000 or Spectra) and 3.6×10^7 CD 34 cells were harvested, along with mononuclear cells, red blood cells and platelets. The CD34 cells were not purified with the magnetic bead method. CD34 enriched fluid (56.9 m*l*) was injected at 114 points in the arm and leg muscle on the same day. Thirty six patients recovered. Elimination of foot ulcers continued over a four years period. The symptoms of the 29 patients did not change but their extremities were maintained. Fifty four patients required amputations because their ulcers became necrotic or infected.

Key words: therapeutic angiogenesis, PBSC, CD 34 cells, hemodialysis

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