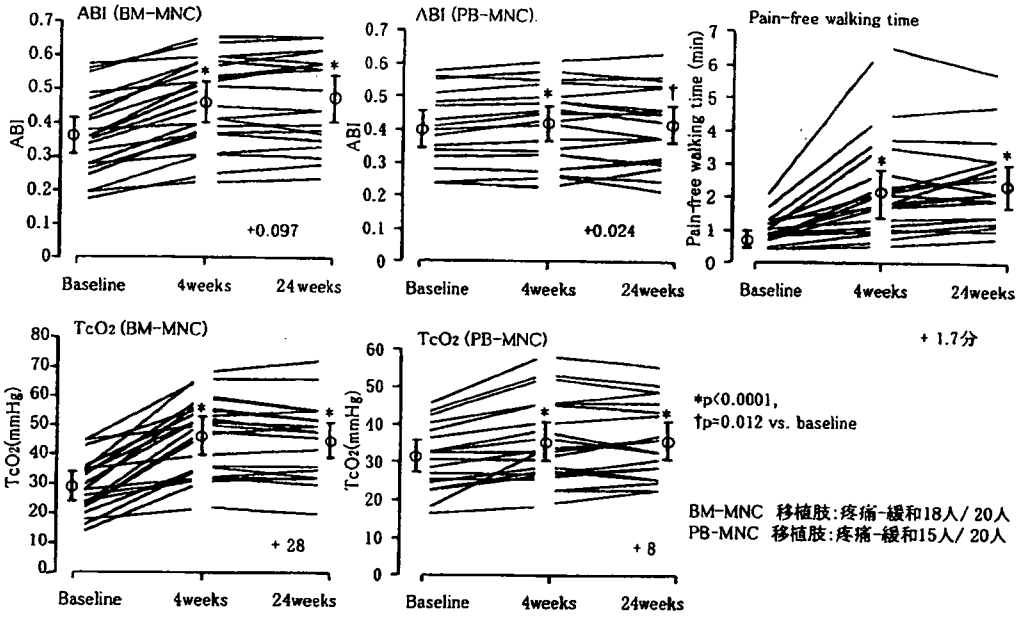


図 11
虚血下肢に対する骨髄単核球細胞(BM-MNC)と末梢血単核球細胞(PB-MNC)移植の上肢/下肢血圧比(ABI),トレッドミルによる下肢痛出現までの歩行時間,組織酸素分圧($TcPO_2$)への効果

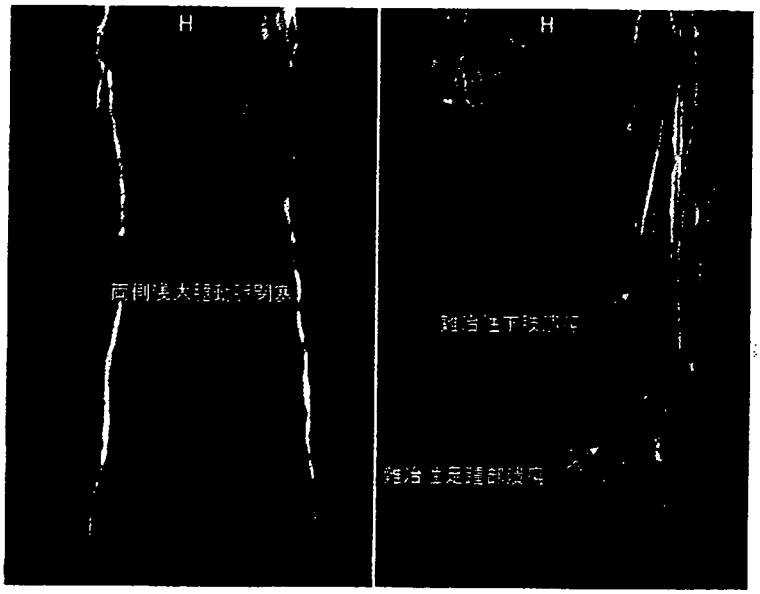
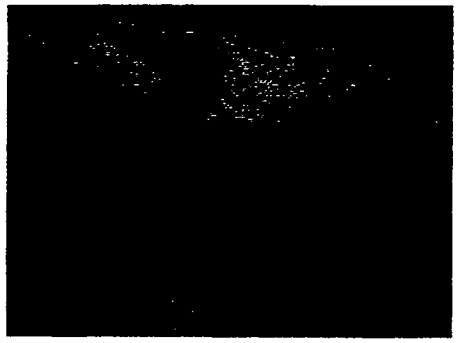


図 12

1. MRSA 感染を伴う下肢・足踵部潰瘍



2. 細胞移植後，肉芽組織の著明な増生

3. 2回の細胞移植後，ABI，TCO_2の改善と難治性足踵部潰瘍が治癒

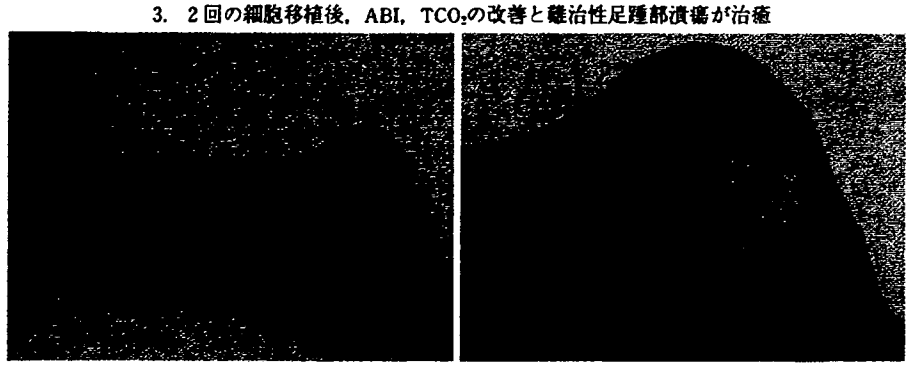


図 13

めて高度先進医療の保険適用を関西医科大学，久留米大学，自治医科大学の3施設に許可した．

症例(図12)は長い糖尿病歴があり，糖尿病性腎症による腎不全(血液透析中)を有するASOの80歳の男性である．右下肢はすでに切断術が施行され下肢MRA上，両側浅大腿動脈の閉塞，右前後脛骨動脈・腓骨動脈の閉塞とMRSA感染を有する難治性下肢・足踵部潰瘍が存在した(図13)．左下肢のABIは0.48，TCO_2は20mmHgであった．この症例に2回の骨髄単核球細胞移植を行い，ABIは0.60，TCO_2は44mmHgに改善し，下肢・足踵部潰瘍は治癒し歩行可能とまでなった．このように重症虚血下肢への骨髄単核球細胞移植は現在本邦で24大学病院を中心に250人以上のno option虚血下肢で苦しむ患者が治療を受けている．

われわれが行っている重症虚血下肢への血管新生療法の適応基準を示す．

①末梢性血管疾患(慢性閉塞性動脈硬化症・バージャー病)：Fontaine分類Ⅲ度およびⅣ度(ABI<0.6を目安)，安静時痛または虚血性潰瘍・壊死を有する患者で，内科的・外科的に血行再建術の適応がなく，将来切断が予想される患者．

②性別：男性および女性(妊娠中および妊娠の可能性のある女性を除く)

③年齢：原則として20歳以上80歳以下

④適応除外事項：

過去3ヵ月以内にアルコールもしくは薬物依存の既往のある患者，悪性新生物を有する患者および3年以内にその既往のある患者，別途規定の諸検査により悪性腫瘍の可能性があると判断された患者，未治療の糖尿病性網膜症を有する患者，インフォームド・コンセントを得られない患者，その他，主治医が不適当と判断した患者．

≪トピックス≫

心筋梗塞への再生医療 Update
――骨髄単核球を利用した細胞治療の現状

五十殿弘二　浅田　聡　高橋知三郎
辰巳哲也　松原弘明*

要旨

- 心筋梗塞への再生医療として，末梢血単核球あるいは骨髄単核球を利用した血管新生治療が臨床応用されている．
- 急性心筋梗塞(AMI)のPCI治療後に骨髄単核球を冠動脈から注入する血管新生治療が，欧米で2001年ごろからスタートした．
- 初期のオープンラベル臨床試験では半年後の心機能が10%前後と改善し世界中の注目を浴びたが，最近の二重盲検試験では有意な改善がみられないとの報告もあり，適応症例の選択が必要になった．
- 造血性サイトカイン(G-CSF)をAMI後に投与して，心機能を改善させる臨床試験も実施されている．
- 一方，陳旧性心筋梗塞(OMI)への骨髄単核球の直接心筋移植は，有効例が多く報告され，開胸・カテーテルを利用した再生医療が期待されている．
- ヒト心筋からの多能性幹細胞も分離され，低心機能の重症心筋梗塞への移植もまもなくである．
- 心筋梗塞への再生医療の，最新の臨床試験の成績を中心に述べ，将来展望についてもふれてみたい．

骨髄細胞による血管新生と心筋再生❷

骨髄細胞中には，造血系や間葉系幹細胞が含まれる．血管内皮細胞は造血系・間葉系幹細胞の両細胞群から分化可能とされる．造血系・間葉系幹細胞を含む骨髄単核球移植は虚血下肢や心筋において血管新生を誘導するが，新生血管のすべてが移植骨髄細胞から派生した(vasculogenesis)ものではなく，移植細胞から分泌されるVEGF，bFGFなどの血管内皮増殖因子が血管新生(angiogenesis)に大きな役割を果たしている．虚血下肢への骨髄単核球移植による血管新生細胞治療の有効性は国際的に承認され，本邦だけでなく世界中において実施されている[1]．

骨髄造血系幹細胞からの心筋細胞分化は，現在では否定されている．まれに観察されたとしても既存心筋細胞との融合現象であろう．骨髄間葉系幹細胞にはMAPCと呼ばれる多能性幹細胞群が存在し，心筋細胞に分化可能とされる．骨髄中に存在するとされる間葉系多能性幹細胞(MAPC)の存在数の低さを考えると，陳旧性心筋梗塞(OMI)や急性心筋梗塞(AMI)への骨髄単核球移植による心臓ポンプ機能の改善効果は心筋再生によるものとは考えにくく，移植細胞からの血管新生誘導因

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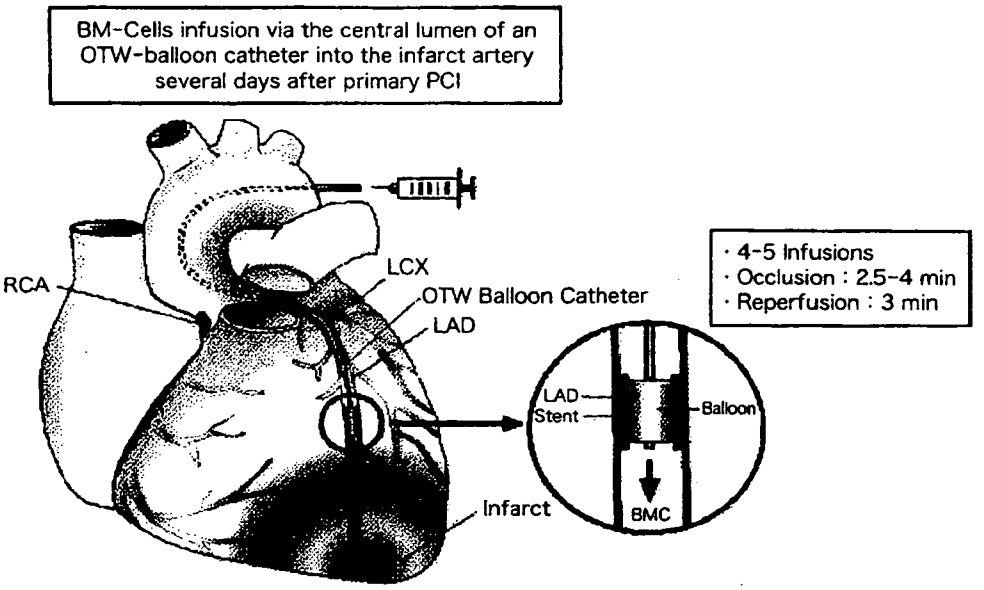

Fig. 1. AMI への末梢血単核球移植

PCI にて閉塞部再疎通に成功した，ST 上昇型 AMI 症例を対象とする．再疎通した冠動脈にバルーンを挿入し，約 5 億個の末梢血単核球分画を低圧膨張させたバルーン先端より 3 回に分けて 2 分かけて注入する．

子や心筋保護因子の分泌などの関与と考えるのが正しいであろう．

AMI に対する骨髄単核球を利用した血管新生治療

AMI の際には急性期 4〜7 日目をピークとして骨髄から末梢血に血管内皮前駆細胞が動員されることや，幹細胞の homing factor である stromal cell-derived factor 1 (SDF-1) が心筋に発現し，SDF-1 を導入した線維芽細胞を移植しておくと梗塞心に骨髄幹細胞の homing が促進され，血管新生効果と心機能改善効果が増強されることが示されている[2,3]．

最近，ST 上昇型 AMI の PCI 再灌流成功後に骨髄単核球細胞または末梢血内皮前駆細胞を採取し，さらに梗塞責任冠動脈より低圧バルーン拡張カテーテル先端より注入移植する (Fig. 1) ことで，心筋血流分布，冠予備能や左室駆出率が 10% 前後改善されるという興味ある結果が報告された[4〜6]．最初の報告では 6ヵ月後の心臓収縮機能の有意な改善 (10% 前後，$p<0.001$) が発表され[4〜6]，世界中の注目が集まり，症例数を増やして randomized 研究が実施された (Table 1)．

このうち，double-blind (骨髄採取を全例に実施し細胞・生食投与した二重盲検 2 アーム) は，Frankfurt 大学 (ドイツ，REPAIR-AMI 研究)[7]，Leuven 大学 (ベルギー，ASTAMI 研究)[8] の二つの臨床研究だけであるが，前者は対照群と比較して 2.5% の EF 増加 (左室造影で評価)，後者は有意差なし (MRI 評価) と報告している．ただし，REPAIR-AMI では PCI 5 日目以降の移植や EF<49% の症例で 7.5% の EF 増加とその効果は倍増していることが報告され，心機能低下例や心筋リモデリング開始時への移植が有効であることは興味深い．

ASTAMI 研究は PCI 翌日に細胞移植を行っているので，REPAIR-AMI の移植タイミングのデータと合致する．二重盲検ではないが，randomized trial デザインでなされた同様の臨床試験では，有意差なし (MRI 評価) と報告されている[9]．骨髄単

Table 1. AMIへの冠動脈を介した骨髄単核球移植の大規模臨床成績

study	improved	not improved	design
Dusseldorf (Strauer et al)	regional LV-function infarct size	LVEDV	
Frankfurt (TOPCARE)	regional LV-function global EF Infarct size	LVEDV	
Hannover (BOOST-1)	regional LV-function global EF Infarct size	LVEDV	randomized
Spain	regional LV-function global EF	LVEDV	
Belgium (Janssens et al)	infarct size	regional LV-function** global EF LVEDV	randomized double-blind
Frankfurt (REPAIR-MI)	global EF+2.5%* (better Tx>5 days, EF<49%)	LVEDV	randomized double-blind
Norway(ASTAMI)		global EF Infarct size LVEDV**	randomized

*positive, **negative.

核球の調整方法がREPAIR-AMIとは異なり，回収細胞数や細胞の遊走能の違いが，臨床結果の違いを説明するともいわれている．

AMIに対するこれら三つの臨床試験の結果を受けて発表されたeditorialコメントは，再生医療におけるプラセボを用いた二重盲検試験の重要性がクローズアップされたとともに，AMIへの骨髄単核球を使った血管新生治療の有効性には否定的な意見が述べられている[10]．しかしながら，筆者はPCI後の心機能低下症例への適応については，有用性が再検討されるべきではないかと考えている．広範囲梗塞などのショック症例は，これらの臨床試験では除かれている．まさに，これらの重症AMI症例が，再生医療の対象になるのではないかと考えている．これら三つの試験の結果を参考にして，患者病態を考慮したAMIに対する，骨髄単核球を使った新たな臨床試験が欧州を中心に進んでいる(BOOST-2 Trial)．

AMIに対する末梢血単核球を利用した血管新生治療◎

AMIの際には急性期4〜7日目をピークとして，骨髄から末梢血に血管内皮前駆細胞が動員されることが室原博士のグループから報告された[2]．われわれはブタの慢性狭心症モデルに末梢血由来単核球を，カテーテルで心内膜側から心筋内へ移植すると，局所血流が改善するとともに，低下した心筋壁運動が改善することを報告した[11]．

これらの報告をもとに，われわれは左前下降枝LADに限局したST上昇型AMIのPCI再灌流成功後に，末梢血由来単核球を責任冠動脈から注入する血管新生の臨床研究を，奈良県立医科大学との共同研究にて2004年2月より開始した(Fig. 2)．細胞移植時期はAMI後3日以内であるが，非細胞治療群に比較して(4.5% EF改善)，末梢血単核球の注入群では12%ものEFの有意な改善がみられている．REPAIR-AMI試験と同様に，移植前の心機能のわるい症例のほうが改善度が高く，適応症例の選択が必要と考えられる．

PCIにて閉塞部再疎通後の急性心筋梗塞に対して骨髄や末梢血単核球の移植を行うことで心機能がよくなるメカニズムとしては，移植細胞から放出されるVEGF，FGF，IGF，PDGFなどの因子(それ以外の未知因子？)により，虚血心筋部位での血管新生促進や虚血心筋細胞への抗アポトーシ

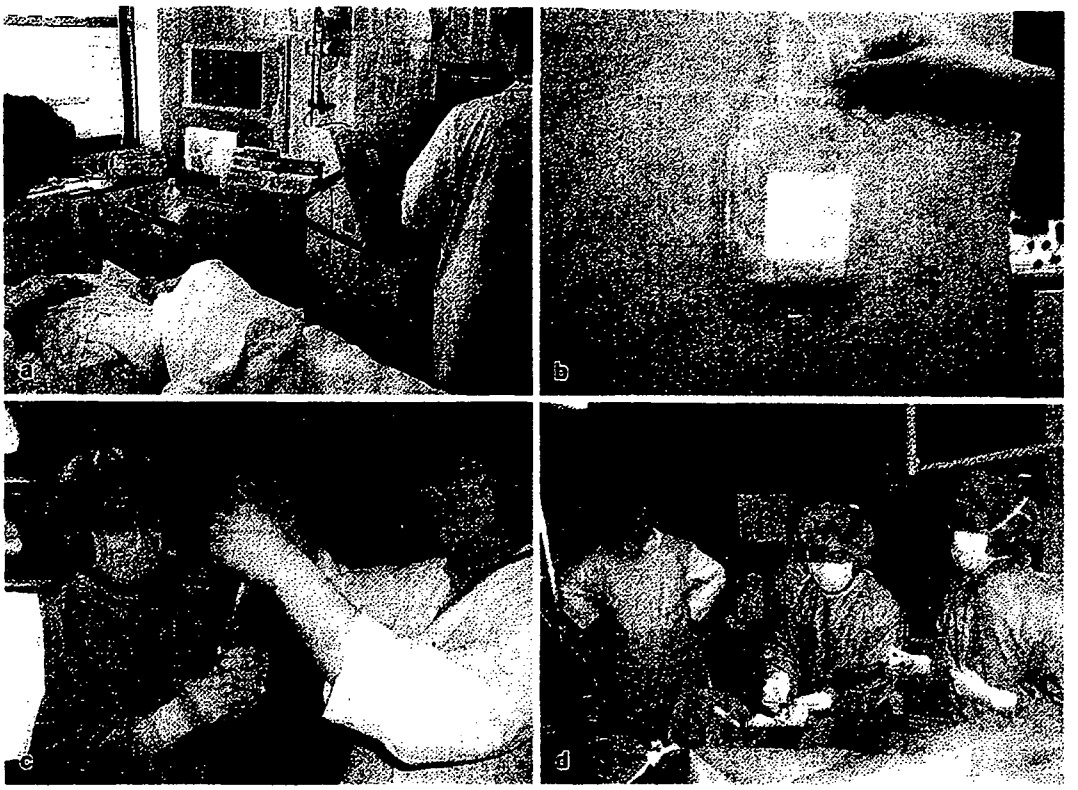

Fig. 2. AMI への末梢血単核球移植の実際
末梢血を患者大腿静脈より採取し(a)，単核球分画を血球分離器にて分離する(b)，再疎通した冠動脈にバルーンを挿入し，約 5 億個の細胞を低圧膨張させたバルーン先端より細胞を 3 回(1 回 2 分間注入，2 分間バルーン圧減圧)に分けて注入する(c，d)．評価項目として移植後 3ヵ月，6ヵ月後の心機能を，LVG，MRI，心エコーで評価する．

ス効果が生じる結果，心筋の保護が促されたり，血管新生や線維芽細胞からのコラーゲン産生が梗塞巣の expansion を抑制することで，梗塞心のリモデリングが抑制され心機能が改善する可能性が考えられている[12]．移植骨髄細胞からの心筋再生の可能性についてはいまだ明らかではないが，骨髄間葉系幹細胞からの trans-differentiation や fusion の問題とともに，今後解明されなければならない課題が残っているといえる．

OMI に対する骨髄細胞移植治療◎

ブタ動物を用いた基礎的研究に基づき[13]，われわれはこれまでに，本邦において 4 例の重症狭心症の患者に外科バイパスと併用しない虚血冬眠心筋への骨髄細胞移植のみの治療を行った．提示する症例は 64 歳の男性で，心筋梗塞発症後 8 年を経過し，バイパス手術 2 回，冠動脈形成術 5 回を受けている．CCS class Ⅳの重症狭心症であり，安静時狭心痛が頻発し，1 日 15 回程度のニトログリセリンスプレーを使用している．肋間小切開にて NOGA mapping システムで同定された虚血冬眠心筋に，心外膜側より自家骨髄単核球を 30ヵ所に移植した．経カテーテル的に冬眠心筋に骨髄単核球を移植した部位は，著しく運動低下部位が改善した．14 日以内に狭心痛はまったく消失した．4ヵ月間，週 1 回 24 時間 Holter 心電図フォローした

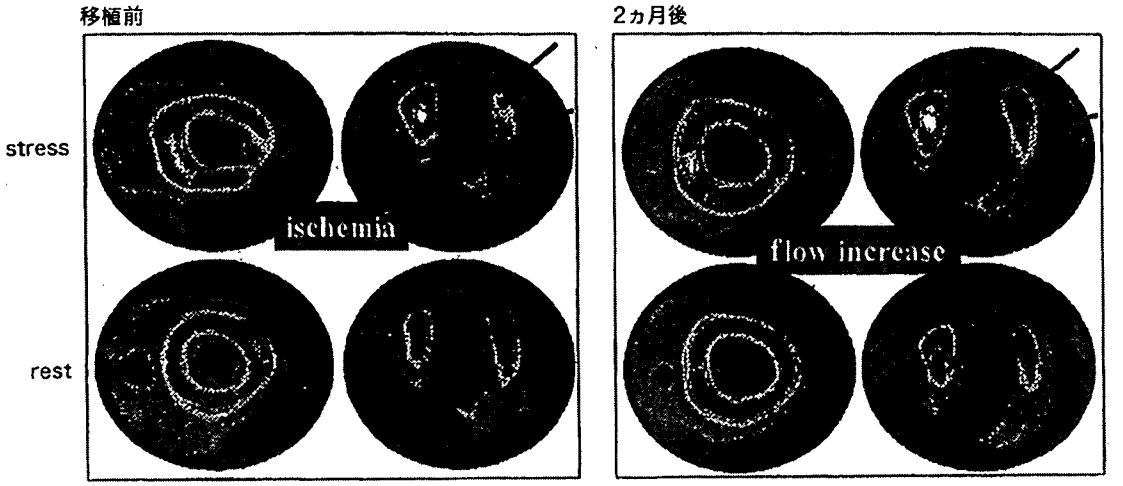

Fig. 3. SPECT-sestamibi 心筋シンチによる狭心症症例への骨髄単核球移植効果の評価
左室下壁側壁の負荷後再分布領域(矢印)は，骨髄単核球移植の3ヵ月後には消失している．

が，不整脈の出現は認めなかった．CPK，toroponin で評価される心筋傷害は最小限であり，4日以内に正常域に復帰した．左室収縮率は 43% から 52% へと増加した．心筋シンチでは負荷後再分布現象は消失し，運動耐容能は3倍も亢進した(Fig. 3)．その他の症例も胸痛の消失，心機能の改善がみられている．特異的な副作用は出現していない．

これまでに報告された虚血性心臓病に対する細胞移植再生医療を，Table 2 に示す．Stamm らは心筋梗塞発症後3ヵ月以内の6人の患者に，他の領域へのバイパス手術と併用して 1.5×10^6個の $AC133^+$ 自家骨髄単核球細胞を梗塞境界領域に移植し，心筋血流分布とともに左室駆出率による心機能の改善がみられたことを報告した[14]．

また Tse らは，8人の狭心症患者に NOGA mapping システムを用いて経カテーテル的に自家骨髄単核球細胞を移植し，われわれと同様に狭心痛の軽減，MRI で評価した心筋血流分布や局所壁運動の改善を報告している[15]．

米国では同じく経カテーテル的に重症の虚血性心不全患者 21 人に，自家骨髄単核球を移植する治療が行われ，安全性とともに虚血部血流増大や心機能の改善が認められている[16]．この成績をベースに米国 FDA は，慢性虚血性心筋症の患者への自家骨髄単核球の心筋内移植治療の臨床応用を 2004 年に許可した．現在，NOGA ナビゲーションのもとでの MYOSTAR カテーテルを用いた二重盲検臨床試験が米国で実施されている．

心筋梗塞に対する骨髄細胞移植治療の将来展望◎

TOPCARE などの初期の臨床成績からは，通常の PCI 後に 10% 前後の心機能や心筋リモデリング改善効果が得られ，インターベンション治療と再生医療の組み合わせが新しい標準治療となる可能性が示唆され，世界中の循環器内科医の注目を集めた．

その後，二つの randomized，double-blind 試験が実施され，最近発表された[7~8]．その結果は期待とは反するものであり，有効であったとしても 2~5% の EF 改善であり，梗塞巣の縮小，リモデリング抑制効果も小さく，骨髄採取の侵襲度を考えると標準治療として拡大する見込みは少ないと思われる．

一方，慢性虚血性心臓病の冬眠領域への骨髄細胞の心筋内移植は，狭心症の軽減も含めて，心機能改善の点では非常に有効との報告が多い．しか

Table 2. OMI への骨髄単核球の心筋直接移植による大規模臨床成績

著者	移植方法	移植細胞	疾患	移植日時	結果	掲載論文
Strauer BE et al	intracoronary transplantation	骨髄単核球 (BMCs)	AMI	5〜9 日	冠血流，心機能の改善	Circulation 106：1913, 2002
Assmus BA et al	intracoronary transplantation	BMCs	AMI	4.3 日	冠血流，心機能の改善	Circulation 106：3009, 2002
Perin EC et al	catheter(NOGA)-based transplantation	BMCs	ICM	—	冠血流，心機能の改善	Circulation 107：2294, 2003
Stamm C et al	transplantation with CABG	BMCs	MI	10 日〜3カ月	冠血流，心機能の改善	Lancet 361：45, 2003
Tse HF et al	catheter(NOGA)-based transplantation	BMCs	AP OMI	—	冠血流，心機能の改善	Lancet 361：47, 2003
Kang HJ et al	intracoronary transplantation	peripheral blood stem cells mobilized with G-CSF	AMI OMI	6 日以後	冠血流，心機能の改善	Lancet 363：751, 2004
Wollert KC et al	intracoronary transplantation	BMCs	AMI	4.8 日	心機能の改善	Lancet 364：141, 2004

しながら，重症疾患患者が多いため，randomized, double-blind 試験は困難と考えられたが，2005 年 3 月より米国で実施されており，その結果が待たれる．

心筋梗塞への心筋再生医療(ヒト心筋由来心筋幹細胞の発見と心筋再生治療)

広範囲の梗塞巣を有する心筋梗塞や心筋破壊の進んだ心筋症では，心筋細胞の移植・補充が心筋収縮能の改善には必要である．心筋再生医療を実施するためには，ヒト心臓から心筋前駆細胞を採取・増殖させ病態心筋へ移植する必要がある．心筋幹細胞マーカーとして c-kit, sca-1, isl-1 が報告されている．われわれは臨床応用を目的に，手術時に得られたヒト心房，肺動脈組織や心筋生検組織から単クローン幹細胞の単離に成功した．

この幹細胞は，無血清培地下で sphere と呼ばれる間葉系幹細胞の表現系を強く呈する浮遊系の細胞塊を形成し，高い増殖能を示した．特異的成長因子の存在で神経細胞，上皮細胞，脂肪細胞に分化可能な間葉系由来の多能性幹細胞であった．電気生理学的にも成熟心筋と同じイオン電流・活動電位をもち，心筋移植後には connectin-43 などの gap junction 蛋白も正常に発現する心筋細胞へ分化しており，心筋創生に向けた探索医療に十分適合した幹細胞ソースである．

虚血心筋に移植されたときには，移植後の生存度が大きな問題となるが，われわれはゲラチンシートを用いて特異的な幹細胞維持因子を除放させ，移植後の生存率が大きく改善することを発見した．このシートとヒト心筋由来幹細胞を用いたハイブリット療法が，現在ではもっとも優れた心筋再生治療と考えられ，現在は OMI のブタモデルにて前臨床試験を京都大学探索医療センターで実施している．

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Concise Report

Local implantation of autologous mononuclear cells from bone marrow and peripheral blood for treatment of ischaemic digits in patients with connective tissue diseases

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Objective. CD34-positive bone marrow mononuclear cells (MNCs) have been successfully used for regeneration of small arteries in Buerger's disease. The objective of this study is to examine the angiogenetic potential of autologous MNCs from bone marrow and peripheral blood implanted into the ischaemic digits from patients with connective tissue diseases.

Methods. Three patients with systemic sclerosis, two with mixed connective tissue disease, and one with CREST syndrome were enrolled who had painful ischaemic digits with necrosis refractory to several vasodilators including intravenous prostaglandins. MNCs obtained from 7 ml/kg bone marrow blood and 400 ml peripheral blood were implanted into 20 different sites in palms and/or soles. The study was performed open-labelled.

Results. Pain in the numeric rating scale improved remarkably up to 1 month after implantation of bone marrow or peripheral MNCs to the same extent, although no significant differences were found in transcutaneous oxygen pressure and thermogram before and after the implantation. Bone marrow MNCs increased blood flow of the hand determined by intra-arterial digital subtraction angiography, while peripheral MNCs did not.

Conclusions. Implantation of autologous MNCs from peripheral and bone marrow into the ischaemic digits was so effective in pain-relief and more clinical trials would be warranted to see whether this could be a new treatment modality for angiogenesis in connective tissue diseases as in Buerger's disease.

KEY WRODS: Connective tissue disease, Bone marrow mononuclear cell, Peripheral mononuclear cell, CD34-positive cell, Angiogenesis.

Introduction

Damage to the vascular endothelium leading to arteriolar stenosis and occlusion are observed in hands and feet from patients with connective tissue diseases. Without successful treatment, ulceration and necrosis often ensue and a mandatory amputation is not infrequent [1]. Vasodilators such as calcium channel blockers and prostaglandins have been used most frequently for their treatment, although with very limited effects, especially in severe cases [2–4].

A group of angiogenic growth factors, such as basic fibroblast growth factor, angiogenic growth factor, and endothelial growth factor were isolated and their amino acid sequences were determined in the 1980s. In the 1990s, therapeutic trials using these angiogenic factors were performed with great success in experimental models of ischaemic heart disease and limb ischaemia [5–7]. It was shown, subsequently, that functional vascular endothelial growth factor (VEGF) was secreted along with angiogenesis in myocardium and skeletal muscles of rats after implantation of adeno-associated virus incorporated with the VEGF gene [8, 9]. On the other hand, autologous bone marrow-derived mononuclear cells implanted locally were shown very efficacious in improvement of limb ischaemia of Buerger's disease in the human clinical trial, termed the TACT study [10].

We performed a therapeutic trial on six patients with connective tissue diseases who had severe ischaemia and necrosis in their fingers and/or toes to examine the angiogenic potential of mononuclear cells (MNCs) from their bone marrow and peripheral blood. The results of 1-yr follow-up are reported here.

Patients and methods

Six Japanese patients were enrolled in this trial: case 1, 49-yr-old woman with systemic sclerosis for 27 yrs; case 2, 64-yr-old man with systemic sclerosis for 9 yrs; case 3, 60-yr-old man with mixed connective tissue disease for 4 yrs; case 4, 51-yr-old woman with systemic sclerosis for 18 yrs; case 5, 47-yr-old woman with mixed connective tissue disease for 11 yrs; and case 6, 72-yr-old man with CREST syndrome for 14 yrs. They all had long-standing intractable digital ulcers and finger necrosis with severe pain despite use of vasodilators including intravenous prostaglandins. According to their previous histories, amputation of the ischaemic digits was highly probable if left untreated.

Under general anaesthesia, 7 ml/kg bone marrow blood was aspirated from iliac bones and MNCs were purified by centrifugation on Ficoll-Hypaque (Axis Shield, Oslo, Norway) as reported previously [11]. Purified MNCs were suspended in 20 ml of RPMI1640 and aliquots of 0.5 ml were implanted intramuscularly at 20 different sites in palms and/or soles. Red cells were recovered and returned to the patients.

Peripheral blood of 400 ml was withdrawn from individual patients and MNCs were purified and implanted in the same manner as for MNCs from bone marrow. MNCs from bone marrow and those from peripheral blood were implanted on the opposite side to compare the difference in effectiveness. The side with more severe lesion received bone marrow MNCs, although the patients were blinded on this.

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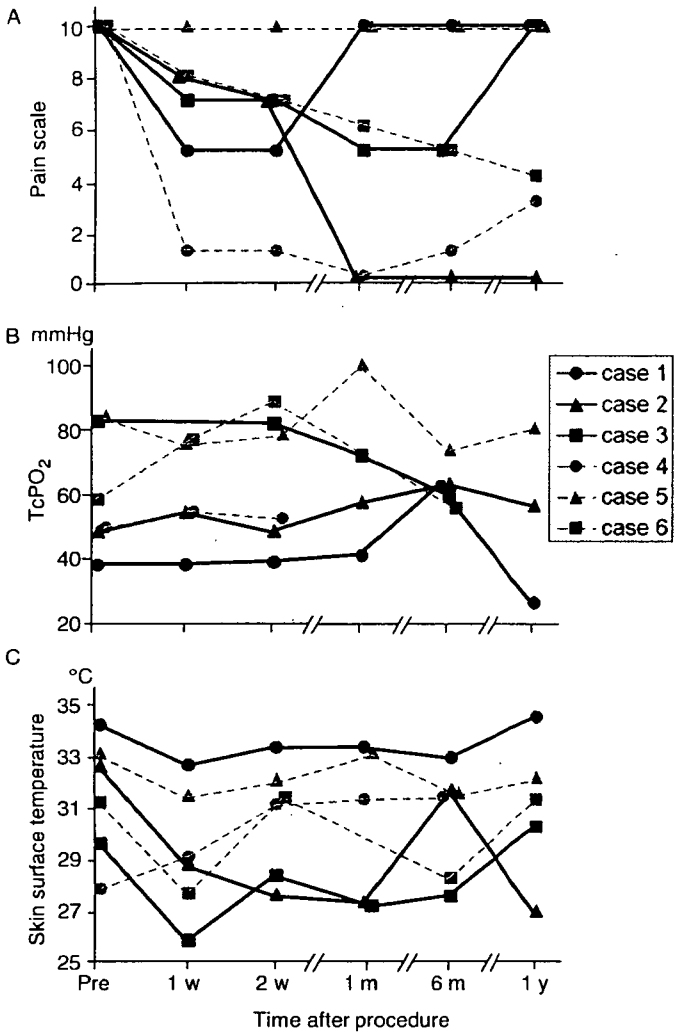

FIG. 1. Changes in clinical parameters between pretreatment and up to 12 months after the local implantation of bone marrow mononuclear cells. (A) Data on the pain scale. (B) Data on the transcutaneous oxygen pressure. (C) Data on the skin surface temperature. Abbreviations: w, week; m, month; y, year.

The evaluation of effectiveness included: (1) numeric rating scale for evaluating the improvement in pain made in 11 levels from 0 to 10 [12]; (2) transcutaneous oxygen pressure ($TcPO_2$) by PO-850 (Sumitomo-Hightechs, Tokyo, Japan) for the assessment of peripheral blood flow; (3) thermography by TH3106ME (NEC San-ei Instruments, Tokyo, Japan) to measure the skin surface temperature on hands and feet; and (4) intra-arterial digital subtraction angiography (IADSA) before and 2 months after the implantation. Items (1)–(3) were performed pretreatment, 1 week, 2 weeks, 1 month, 6 months and 1 yr after the implantation. The assessor was different from the implanter, although the blindness between the two was not complete.

This study design was deliberated and accepted by the Jichi Medical University Ethics Committee. Every patient gave a written consent on the purpose of this clinical trial.

Results

An average of 7.7×10^8 (5.0×10^8 to 10.8×10^8) MNCs was isolated from 7 ml/kg bone marrow blood with an average recovery of 3.5×10^6 (2.85×10^6 to 5.33×10^6) CD34-positive-cells. Likewise, an average of 3.5×10^8 (1.0×10^8 to 4.8×10^8) MNCs was isolated from 400 ml peripheral blood with an average recovery of 0.14×10^6 (0.01×10^6 to 0.37×10^6) CD34-positive cells.

Relief of pain as judged by the pain scale was achieved in all but one patient in a week, and continued up to one month in four patients. However, pain continued at the baseline level throughout the study period in one patient (case 5). Bone marrow and peripheral MNCs brought about the similar level of pain-relief; Fig. 1A depicts changes in pain level on the side of the body where bone marrow MNCs were implanted. Pain-relief remained satisfactory in three patients (case 2, 4 and 6) until 1 yr; however, pain gradually returned to the pretreatment level in two patients (case 1 and 3).

As shown in Fig. 1B, $TcPO_2$ slightly increased in two patients (cases 4 and 6) before 2 weeks after implantation of bone marrow MNCs, although the courses thereafter were not obtained. Case 2 gained a gradual increase in $TcPO_2$ beyond 1 month, and kept increased levels until 1 yr. This might be responsible for the perfect pain control observed in him. $TcPO_2$ measurement on the side implanted with peripheral blood MNCs showed an increase similar to that of bone marrow MNCs (data not shown).

Skin surface temperature judged by thermography did not change remarkably throughout the study period except in one patient (case 4). It decreased and stayed lower than before up to 1 yr in the patient (case 2) who completely lost pain by the end of 1 yr. Fig. 1C shows the thermogram on the side of bone marrow MNCs. The peripheral blood MNCs' side showed similar improvements (data not shown).

IADSA performed before and 2 months after the implantation did not change significantly in five patients. In the remaining one patient (case 6), however, IADSA imaged an increased arterial flow up to the tip of fingers (Fig. 2B) on the bone marrow MNCs' side, where the arterial blood flow was recognized only slightly distal to the arcuate artery before the implantation (Fig. 2A). In case 6, implantation of the peripheral blood MNCs did not increase arterial blood flow to the level that could be visualized in IADSA (data not shown).

Discussion

Remarkable pain-relief was obtained in case 4 immediately after the implantation and pain-relief in satisfactory levels was obtained in the remaining four patients within 2 weeks. However, no changes in the pain level were observed throughout the study period in case 5. The difference between bone marrow and peripheral blood MNCs was not appreciable. $TcPO_2$ did not show any constant trends after the implantation. It was kept at a slightly increased level in case 2 whose pain was dramatically improved, while it fluctuated and became even lower than the pre-implantation level 6 months after the procedure in case 6; his finger pain remained in satisfactory levels.

The surface temperature of fingers and/or toes measured by thermography did not increase significantly; it decreased even in the patient in case 2 who gained remarkable reduction in pain.

A point which came as a surprise to us was no difference in the pain scale, $TcPO_2$, or thermography achieved between MNCs from bone marrow and peripheral blood, in sharp contrast to the results in Buerger's disease in the TACT study. The implantation of CD34-positive bone marrow cells was quite effective in pain-relief and recovery of circulation detected by IADSA in Buerger's disease in comparison with MNCs from peripheral blood [10]. Because the number of CD34-positive cells from bone marrow blood was much higher than that of those from peripheral blood, they did not seem to be the main cell population effective in rebuilding the vasculature of ischaemic digits in patients with connective tissue diseases. The number of circulating endothelial progenitor cells increases in the early stage of systemic sclerosis, although their number in bone marrow decreases and they are functionally impaired [13]. This could be another possibility and needs to be clarified in the future trial.

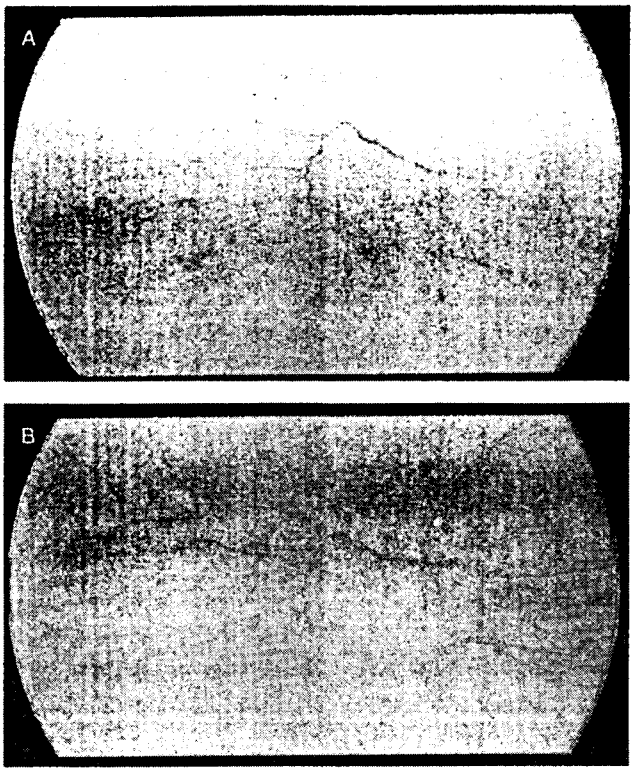

FIG. 2. Images by the intra-arterial digital subtraction angiography (A) before and (B) 2 months after the local implantation of bone marrow mononuclear cells in case 6.

In this study, only one patient accomplished an increased vasculature with collateral vessels detectable by IADSA in the hand of the side where bone marrow MNCs were implanted. However, it was not clear whether collateral vessels were newly developed or merely re-opened to recover from a collapsed state. Tateishi-Yuyama *et al.* [10] reported in the TACT study that collateral vessels were visualized by IADSA in 27 of the 45 (60%) patients with Buerger's disease; we followed the similar procedure, although the implanted cell number was a little lower in this study. New vascular formation and its maintenance would be more difficult in the arterial obstruction due to connective tissue diseases than Buerger's disease.

Pain-relief was not apparently accompanied by concomitant increase in $TcPO_2$ or surface skin temperature. Although the precise mechanism remains to be clarified, it is conjectured that even a small increase in microvasculature around nerve endings might be effective for reduction of the pain induced by ischaemia. Such an increase would be too small to be detected by thermography or arteriography, because a visible increase in IADSA was observed in only one patient (case 6). Such newly developed microvasculature may soon be obliterated, since pain returned to the pretreatment level in two patients 1 yr after the implantation. This contrasted sharply with the results of the TACT study, in which pain-relief lasted much longer and re-vascularization was clearly visible in more than half of the patients [10].

The importance of CD34-positive cells in angiogenesis was not ascertained in patients with connective tissue diseases in the present study. MNCs from bone marrow contained more than 20 times higher number of CD34-positive cells than those from peripheral blood. Despite this fact, pain-relief obtained by the implantation of peripheral blood MNCs was almost in the same level as that of bone marrow MNCs. Hence, CD34-positive cells from peripheral blood might be more efficacious than those from bone marrow. We have not obtained the evidence that the implanted cells were functioning at the site of injection or they were involved in new angiogenesis as endothelial progenitors. It is possible that not cells but various cytokines or pro-inflammatory substrates, released by the implantation of CD34-positive and CD34-negative MNCs, might contribute to pain-relief as well as the generation of microvasculature.

This study was performed in the small number of patients and was not blinded in a strict sense. The data obtained were not necessarily uniform. However, clinical effectiveness of pain-relief was satisfactory and a new clinical trial is now under way in our division to examine the effectiveness of repeated implantations of MNCs.

Rheumatology key messages

- Mononuclear cells from patients' own bone marrow and peripheral blood were locally implanted to facilitate angiogenesis in ischaemic digits.
- The pain-relief was satisfactory, although thermogram and angiogram showed inconsistency.

Acknowledgements

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The authors have declared no conflict of interest.

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Circ J 2007; **71:** 343–347

Angiopoietin-1, Angiopoietin-2 and Tie-2 in the Coronary Circulation of Patients With and Without Coronary Collateral Vessels

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Background The role of the angiopoietin (Ang)/Tie-2 system in coronary collateral growth is not well understood, so the purpose of this study was to investigate and elucidate the relationship of this system to coronary collateral formation in patients with coronary artery disease (CAD).

Methods and Results Fifty-nine patients with CAD were recruited. Blood samples from the left ventricle (LV) and coronary sinus (CS) were obtained during cardiac catheterization, and serum concentrations of Ang-1, Ang-2, and Tie-2 were measured by enzyme-linked immunosorbent assay. Patients were then classified as mild CAD (n=30), defined as ≤90% stenosis of the coronary arterial luminal diameter, or severe CAD (n=29), which was total (or near total) coronary occlusion requiring coronary collateral growth. Ang-1, Ang-2, and Tie-2 in the LV and CS sera were not significantly different between groups. In the severe CAD group, spillover of Tie-2 (CS–LV value) from the coronary circulation was found in comparison with the mild CAD group (3.43±2.22 vs −3.29±1.54 ng/ml, p=0.01), whereas the CS–LV values of Ang-1 and Ang-2 did not differ between groups. Tie-2 production was markedly increased in patients with well-developed collaterals. A positive and significant correlation was found between coronary Ang-2 and Tie-2 levels (r=0.44, p<0.001).

Conclusions Tie-2 is probably produced in the coronary circulation and may induce the development or maintenance of coronary collaterals in CAD patients. Furthermore, the role of Ang-2 in the formation of coronary collaterals may be more important than that of Ang-1. (*Circ J* 2007; **71:** 343–347)

Key Words: Angiopoietin/Tie-2 system; Coronary artery disease; Coronary circulation; Coronary collaterals

Development of coronary collateral vessels is a natural and useful adaptation to coronary arterial narrowing and/or occlusion, and it is able to preserve the function of ischemic myocardium! Growth of coronary collateral vessels is usually believed to depend on the transformation of pre-existing arteriolar connections to mature vasculature (arteriogenesis) and occasionally on sprouting of new vessels from neighboring blood vessels (angiogenesis)![1–3] Several angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor and placenta growth factor, are closely associated with coronary collateral growth![1,3,4] but as coronary collateral vessels disappear immediately after revascularization, the maintenance mechanism of these collaterals is also important in chronic coronary artery disease (CAD). Maintenance of collateral vessels seems to depend on elevated shear stress as well as some growth factors![5] A relationship between serum levels of VEGF in the coronary circulation and the growth of coronary collateral vessels has previously been reported![6–8] but it is not an exclusive one. Other growth factors and/or the cooperation of several growth factors may regulate the development and maintenance of coronary collateral vessels.

Recently, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and their receptor Tie-2 have been identified[9–11] and it appears that the Ang/Tie-2 system regulates the growth, formation, and maturation of new blood vessels. In cardiovascular diseases, upregulation of these factors in patients with acute coronary syndrome (ACS) has been reported![12,13] and in experimental models, the system has been shown to relate to postnatal neovascularization![14,15] However, the dynamics of Ang-1, Ang-2, and Tie-2 in the coronary circulation and their relation to coronary collateralization are uncertain, so we examined their production or consumption within the coronary circulation of chronic CAD patients with or without coronary collateral vessels.

Methods

Selection of Subjects

We recruited 59 patients who had undergone coronary arteriography (CAG) for suspected or already diagnosed CAD. Patients with ACS, previous coronary artery bypass grafting (CABG), congestive heart failure, valvular heart disease, and nonischemic cardiomyopathy were excluded to avoid the possible influence of growth factors released from the coronary circulation under these conditions. All patients received standard medical treatment for their clinical con-

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Table 1 Clinical Characteristics and Group Comparisons of the Study Population

	Mild CAD group (n=30)	Severe CAD group (n=29)	p value
CAD risk factors			
Age (years)	66.40±1.77	68.27±1.73	0.45
Male gender	26 (86%)	24 (82%)	0.95
Obesity (BMI ≥25 kg/m²)	5 (16%)	9 (31%)	0.32
Smoker	23 (76%)	23 (79%)	0.94
Hypertension	18 (60%)	23 (79%)	0.18
Diabetes mellitus	9 (30%)	11 (37%)	0.71
Dyslipidemia	13 (43%)	18 (62%)	0.29
Medications			
Statins	15 (50%)	9 (31%)	0.22
ACEIs/ARBs	15 (50%)	12 (41%)	0.68
β-blockers	6 (20%)	10 (34%)	0.33
Laboratory data			
Hemoglobin A1c (%)	6.0±0.32	6.1±0.34	0.30
Total cholesterol (mg/dl)	191.6±6.72	192.2±7.49	0.95
CAD status			
Previous MI	9 (30%)	10 (34%)	0.92
Ejection fraction (%)	57.06±1.97	59.03±2.56	0.54
No. of narrowed coronary arteries ≥2 vessels	14 (47%)	21 (73%)	0.08

Values are mean±standard error of the mean or number of subjects (%). CAD, coronary artery disease; BMI, body mass index; ACEIs, angiotensin converting enzyme inhibitors; ARBs, angiotensin-receptor blockers; MI, myocardial infarction.

ditions and clinical data were collected by chart review. The study protocol was reviewed and accepted by the local ethical committee of Niigata University Graduate School of Medical and Dental Sciences, and informed consent was given by all patients before cardiac catheterization.

Cardiac Catheterization and Blood Sampling Procedures

CAG was performed by the Judkins or percutaneous radial artery technique. Coronary artery lesions were characterized by 3 experienced interventional cardiologists according to the American College of Cardiology/American Heart Association lesion classification scheme.[16] If total or near total occlusion was observed on CAG, the Rentrop grade was also assessed.[17] Grading of collateral filling was as follows: 0=none, 1=filling of side branches only, 2=partial filling of the epicardial segment, 3=complete filling of the epicardial segment. Blood samples from the left ventricle (LV) and coronary sinus (CS) were taken during the catheterization session from all patients. LV samples were obtained with a pigtail catheter. For samples from the CS, we recorded images of CS during the venous phase of left CAG, and then a right Judkins catheter was inserted into the CS via the right jugular vein. Positioning was verified by frontal and lateral fluoroscopy views. All samples were collected into test tubes, centrifuged for 10 min at 3,000 rpm, divided into aliquots, and stored at −80°C until analysis.

Study Groups

CAD was defined as ≥75% coronary stenosis on CAG with chest pain on exertion and included patients who had undergone percutaneous coronary intervention (PCI) at least 6 months earlier. Patients were then classified according to their CAG findings into mild and severe CAD groups. Mild CAD was defined as ≤90% coronary stenosis without coronary collaterals, whereas severe CAD group was defined as ≥99% stenosis and coronary collaterals, requiring PCI or CABG.

Laboratory Measurements

Ang-1, Ang-2, and Tie-2 in both the LV and CS samples were measured by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D system, Minneapolis, MN, USA). The absorbance was measured by optical densitometry with a 450-nm filter.

Statistical Analysis

Normality of distribution of the values of different variables was checked by 1-sample Kolmogorov-Smirnov test. Values are expressed as mean±standard error of the mean for normally distributed variables, or medians with interquartile range for skewed data. Comparisons between groups across a nominal grouping variable with 2 levels were performed by the Student's t-test or Mann-Whitney U-test for normal and skewed data, respectively. Comparisons among 3 groups were performed using the 1-way ANOVA with Tukey's post hoc test or the Kruskal-Wallis test for normal and skewed data, respectively. Comparisons of proportions were performed by chi-square test. Correlations between continuous variables were checked by Pearson's or Spearman's correlation coefficients for normal and skewed data, respectively. Two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS for Windows version 13 (SPSS Inc, Chicago, IL, USA).

Results

The clinical characteristics of the study population at

Table 2 Comparisons of Serum Levels of Growth Factors From the 2 Sampling Sites

Growth factor	Sampling site	Mild CAD group (n=30)	Severe CAD group (n=29)	p value
Tie-2 (ng/ml)	*LV*	28.30±2.28	24.54±2.49	0.27
	CS	25.01±1.70	27.97±1.96	0.25
	CS−LV	−3.29±1.54	3.43±2.22	0.01
Ang-1 (ng/ml)	*LV*	22.00 (12.64:26.13)	15.29 (9.32:21.29)	0.09†
	CS	15.87 (7.63:21.68)	13.14 (6.73:18.23)	0.40†
	CS−LV	−5.13 (−9.69:−0.60)	−2.24 (−5.90:4.28)	0.09†
Ang-2 (pg/ml)	*LV*	321.8±40.91	411.7±70.64	0.27
	CS	327.6±46.01	425.4±78.9	0.28
	CS−LV	5.77±16.05	13.66±28.91	0.81

Values are mean±standard error of the mean or median (interquartile range).
†Mann-Whitney U-test, and otherwise Student's t-test.
LV, left ventricle; CS, coronary sinus; CS−LV, CS minus LV value; Ang, angiopoietin. Other abbreviation see in Table 1.

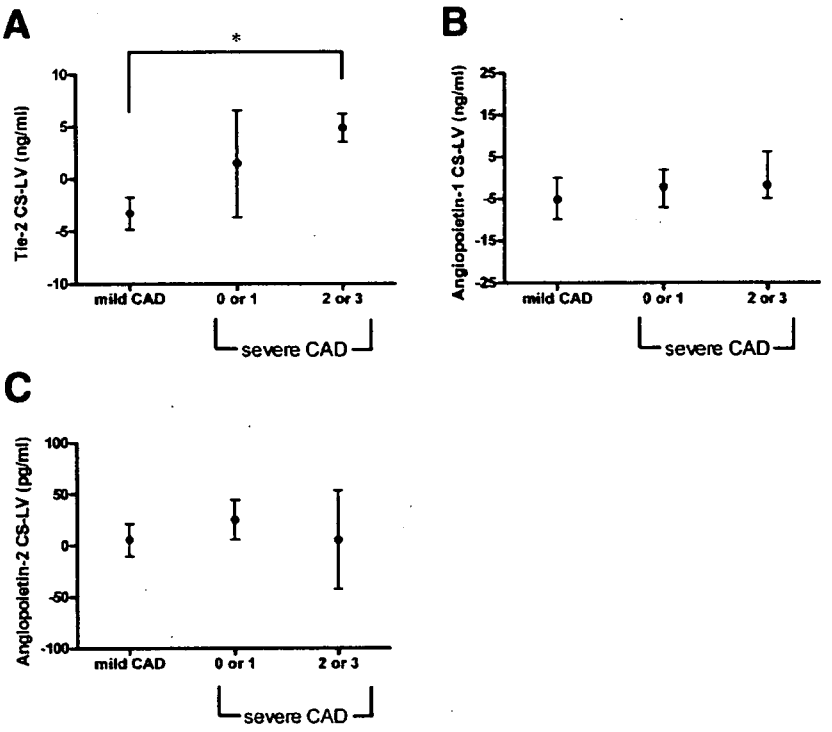

Fig 1. Error bar graphs showing the comparison of mean (median) subtraction values (CS–LV) of Tie-2 (A), angiopoietin-1 (B), and angiopoietin-2 (C) according to collateral flow grade. LV, left ventricle; CS, coronary sinus; CAD, coronary artery disease; CS–LV, CS minus LV value; 0 or 1, Rentrop grade 0 or 1; 2 or 3, Rentrop grade 2 or 3. The bar spins and the black circle within the bar represent mean ± standard error of the mean in the CS–LV values of Tie-2 and angiopoietin-2. The bar spins through the 25th and 75th percentiles, whereas the black circle within the bar represents the 50th percentile (median) in the CS–LV values of angiopoietin-1. *Significant at $p<0.05$.

baseline before CAG are summarized in Table 1. There was a high prevalence of coronary risk factors in both groups. No significant differences in patients' characteristics or laboratory data were observed between groups, nor were no significant differences regarding the history of previous myocardial infarction or left ventricular ejection fraction.

Table 2 shows the serum concentration of the angiogenic growth factors from each sampling site. There were no significant differences between groups in the serum levels of Ang-1, Ang-2, and Tie-2 in either the CS or LV sample. The mean subtraction value (CS–LV) for Tie-2 was significantly higher in the severe CAD group than in the mild CAD group ($p=0.01$). However, the mean (median) subtraction values for Ang-1 and Ang-2 were not significantly different between both groups.

Fig 1 is a comparison of the mean (median) CS–LV values of the angiogenic growth factors according to coronary collateral flow grade. Mean CS–LV values of Tie-2 in patients with grade 0 or 1 (n=12) collaterals and grade 2 or 3 (n=17) collaterals were 1.45±5.11 and 4.82±1.32 ng/ml, respectively. In patients with grade 2 or 3 collaterals, the CS–LV mean value of Tie-2 was higher than in those with mild CAD ($p=0.03$).

Bivariate correlations between growth factors are shown in Table 3. A moderate and significant correlation existed between coronary Tie-2 and Ang-2 ($r=0.44$, $p=0.0008$), but other correlations were not significant.

Table 3 Bivariate Correlations Between the Serum Levels of Growth Factors (n=59)

Comparison	*r*	*p value*
Tie-2 LV & Ang-1 LV	*0.08*	*0.51*
Tie-2 LV & Ang-2 LV	*0.08†*	*0.53*
Ang-1 LV & Ang-2 LV	*–0.11*	*0.38*
Tie-2 CS & Ang-1 CS	*0.08*	*0.51*
Tie-2 CS & Ang-2 CS	*0.44†*	*<0.001*
Ang-1 CS & Ang-2 CS	*–0.14*	*0.29*

†Pearson's correlation coefficient or otherwise, Spearman's correlation coefficient.
Abbreviations see in Table 2.

Discussion

We have demonstrated a significant elevation of Tie-2, but not Ang-1 or Ang-2, in the coronary circulation of CAD patients with coronary collaterals, as well as a significant positive correlation between coronary Tie-2 and Ang-2. It has been recently shown that the peripheral plasma levels of Tie-2 and Ang-2 are significantly higher in patients with ACS than in healthy subjects;[12,13] however, the relation of these growth factors to the presence of coronary collaterals remains unclear. Moreover, that data could not precisely indicate whether production or consumption of these factors occurred across the coronary circulation, because peripheral blood samples were assayed. In the present study, we obtained site-specific samples from the CS and LV which would be representative of the actual dynamics of the growth factors in the coronary circulation. We have previously reported significant changes in the levels of some inflammatory markers within the local coronary milieu in relation to the use of PCI in CAD patients;[8] and to our knowledge, this is the first investigation the dynamics of the angiopoietins and Tie-2 within the coronary circulation, and their relation to coronary collateralization.

Dynamics of Ang/Tie-2 System in the Coronary Circulation

Ang-1 and Ang-2 affect blood vessel formation differently. Ang-1 is an important regulator of endothelial cell survival, and its absence leads to defects in vascular remodeling;[10] whereas Ang-2 promotes the migration of endothelial cells and instability of capillaries.[11] Both Ang-1 and Ang-2 are considered to be essential for postnatal blood

vessel formation[10,11] Furthermore, Ang-1 and Ang-2 exert their biological effects by binding to the same tyrosine kinase receptor Tie-2. Ang-2 can act as a partial antagonist of Ang-1, especially in hypoxic conditions;[15,19,20] however, the regulatory mechanisms for the expression of both Ang-1 and Ang-2 have not yet been completely elucidated.

In our study, we measured the soluble form of Tie-2. The mechanism leading to the formation of soluble Tie-2 is essentially a shedding reaction, which involves limited proteolysis of the extracellular domain of the membrane-bound receptor?[21] It has been shown experimentally in rats that Tie-2 mRNA expression is upregulated in endothelial cells by hypoxia and myocardial ischemia![14,22] At present, however, the physiological role of this soluble receptor is unknown. It has been previously shown that recombinant soluble Tie-2 protein has a inhibitory action on tumor angiogenesis?[23] so further studies are needed to discover if soluble Tie-2 can bind angiopoietins and develop biological activities. In our study, the CS and LV levels of Ang-1, Ang-2, and Tie-2 were not significantly different between the mild and severe CAD groups. It is known that atherosclerosis is a systemic vascular disease and the serum concentrations of growth factors may be influenced by systemic vascular lesions or coincidental complications of neoplasms or chronic inflammatory disorders. Therefore, we calculated the difference between CS and LV (ie, CS–LV), which represents the production or consumption of these factors across the coronary circulation. Although the CS–LV values of both Ang-1 and Ang-2 were not significantly different between patient groups, the mean CS–LV value of Tie-2 was significantly higher in the severe CAD group than in the mild one (as well as being positively signed). In addition, we found that well-developed coronary collateral vessels led to more production of Tie-2 within the coronary circulation. These results imply that soluble Tie-2 may be produced from the coronary circulation, accompanied by an increase in Tie-2 expression, and it may act on the development or maintenance of coronary collateral vessels in severe CAD. Several genetic and environmental factors may regulate the expression of Tie-2 in the heart. Furthermore, it may be possible that the stages of blood vessel formation inducted by Ang-1 and Ang-2 different among patients.

Importance of Ang-2 in the Formation of Coronary Collaterals

Our results also demonstrated a meaningful correlation between the levels of Tie-2 and Ang-2 in the coronary circulation. In human endothelial cells, the expression of Tie-2 has been shown to be stimulated by hypoxia?[22] and the same observation has been made for Ang-2, which is expressed only in sites of vascular remodeling![11] In contrast, Ang-1 is widely expressed by the quiescent vasculature, but not in endothelial cells even if stimulated by hypoxia![10] In an experimental ischemia–reperfusion model, Ang-2 and Tie-2 had a pivotal role in the formation of new capillaries![14] Furthermore, in the presence of VEGF, Ang-2 can stimulate angiogenesis?[24,25] In a clinical study, plasma Ang-2 levels were elevated in patients with ACS and correlated with those of Tie-2![12] Taken together with these previous reports, the correlation found in our study suggests that Ang-2 plays a more important role in the formation of coronary collaterals than Ang-1.

Study Limitations

First, we do not have direct evidence that Ang-2 and Tie-2 play a role in the formation of coronary collaterals. Second, the substances measured in our study are known to be influenced by the time course of myocardial ischemia/necrosis![12] Third, we could not exclude the influence of plaque neovascularization, even slightly, on our results.

Conclusions

We have demonstrated that Tie-2, the receptor for angiopoietins, was significantly elevated within the coronary circulation in CAD patients with coronary collaterals, and that the level of coronary Tie-2 correlated with that of Ang-2. These results suggest that Tie-2, which is produced within the coronary circulation, may play a role in the development or maintenance of coronary collateral vessels and that Ang-2 has a more important role than Ang-1 in the formation of coronary collaterals in CAD patients. In addition, the Ang/Tie-2 system may be a therapeutic target for coronary angiogenesis.

Acknowledgment

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Serum Endostatin in the Coronary Circulation of Patients With Coronary Heart Disease and Its Relation to Coronary Collateral Formation

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The role of endostatin in coronary heart disease (CHD) is not well known. This study aimed to investigate the dynamics of endostatin, an antiangiogenic growth factor, within the coronary circulation and to elucidate its relation to coronary collateral formation in patients with CHD. We recruited 72 subjects with suspected or previously diagnosed CHD. Blood samples from the left ventricular (LV) cavity and coronary sinus (CS) were obtained during coronary angiography, and the serum concentration of endostatin was measured by enzyme-linked immunosorbent assay kits. Patients were then divided into 2 groups: the normal group (n = 15) defined as patients with atypical chest pain and no evidence of organic cardiac diseases and the CHD group (n = 57) defined as patients with ≥75% coronary stenosis at coronary angiography and chest pain on exertion. Endostatin in CS sera was significantly elevated in patients with CHD compared with normal subjects (median 79.7 [interquartile range 46.2 to 130.3] vs median 49.6 [interquartile range 29.1 to 84.5] ng/ml, p = 0.02). Spillover of endostatin (CS − LV value) from the coronary circulation in patients with CHD with severe stenosis was higher than in those with moderate stenosis (28.2 [4.8 to 48.6] vs 7.3 [−37.0 to 25.6] ng/ml, p = 0.01). In addition, endostatin production within the coronary circulation was higher in patients with poorly developed collaterals than in those with well-developed collaterals. In conclusion, endostatin is suggested to be produced from the coronary circulation in patients with CHD and may play an important role in the regulation of the growth of coronary collateral vessels. © 2007 Elsevier Inc. All rights reserved. (Am J Cardiol 2007;99:494–498)

Endostatin, a 20-kDa proteolytic fragment from the C-terminal domain of collagen XVIII, was recently discovered and shown to have an inhibitory effect on tumor growth working as an antiangiogenic growth factor.[1] Endostatin induces antiangiogenesis to suppress endothelial cell proliferation.[1] Collagen XVIII is a component of a basement membrane with structural features of both collagen and proteoglycan and is expressed in several types of tissues, including the heart.[2,3] The precise mechanism of conversion from collagen XVIII to endostatin has not yet been fully elucidated. In cardiovascular diseases, the relation between pericardial fluid levels of endostatin and coronary collaterals has been reported,[4] but very little is known about the function and expression of endostatin in the heart. In addition, the dynamics of endostatin in the coronary circulation and its relation to coronary collaterals remain unclear. In this study, we investigated the dynamics of endostatin (production, consumption, and release) within the coronary circulation in patients with coronary heart disease (CHD) in comparison with normal subjects.

Methods

Selection of subjects: We recruited 72 subjects (57 men; average age 66 years, range 40 to 82 years) who underwent coronary catheterization in our hospital because of suspected or previously diagnosed CHD. Patients with acute coronary syndrome, previous coronary artery bypass grafting, congestive heart failure, valvular heart disease, and nonischemic cardiomyopathy were excluded to avoid the possible influence of growth factors released from the coronary circulation under these conditions. All patients received standard medical treatment for their clinical conditions, and clinical data were collected by chart review. The study protocol was reviewed and accepted by the local ethical committee of Niigata University Graduate School of Medical and Dental Sci-

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Table 1
Clinical characteristics and group comparisons of the study population (n = 72)

Variable	Normal (n = 15)	CHD (n = 57)	p Value
Age (yrs)	63.3 ± 2.4	67.6 ± 1.2	0.12
Men	9 (60%)	48 (84%)	0.03*
Body mass index ≥25 kg/m^2	4 (26%)	13 (22%)	0.75
Smoker	7 (46%)	44 (77%)	0.02*
Hypertension	8 (53%)	39 (68%)	0.27
Diabetes mellitus	2 (13%)	20 (35%)	0.10
Dyslipidemia	7 (46%)	30 (52%)	0.68
Angiotensin-converting enzyme inhibitors/angiotensin receptor blockers	4 (26%)	26 (45%)	0.18
β blockers	2 (13%)	16 (28%)	0.24
HbA1c (%)	5.3 ± 0.1	6.1 ± 0.2	<0.01†
Total cholesterol (mg/dl)	202.4 ± 6.8	191.3 ± 5.1	0.30
High-density lipoprotein cholesterol (mg/dl)	53.2 ± 4.5	47.8 ± 1.8	0.22
Low-density lipoprotein cholesterol (mg/dl)	108.4 ± 6.7	119.1 ± 4.7	0.31
Pulmonary capillary wedge pressure (mm Hg)	6.6 ± 0.6	7.6 ± 0.6	0.38
Ejection fraction (%)	66.8 ± 2.4	57.8 ± 1.6	0.01*

Values are expressed as mean ± SEM and number of subjects (percent).
* Significant at p <0.05.
† Significant at p <0.01.

ences, and informed consent was obtained from all patients before cardiac catheterization.

Cardiac catheterization and blood sampling procedures: Coronary arteriography was performed using the Judkins or percutaneous radial artery technique. Coronary artery lesions were characterized by 3 experienced interventional cardiologists according to the American College of Cardiology/American Heart Association lesion classification scheme.[5] If total or near-total occlusion was observed at coronary arteriography, the Rentrop grade was also assessed.[6] Grading of collateral filling was as follows: 0 = none, 1 = filling of side branches only, 2 = partial filling of the epicardial segment, and 3 = complete filling of the epicardial segment. To record pulmonary capillary wedge pressure, right-heart catheterization was performed using the right jugular vein approach. Blood samples from the left ventricle (LV) and coronary sinus (CS) were taken during the catheterization session from all subjects. LV samples were obtained using a pigtail catheter. For samples from the CS, we recorded images of the CS during the venous phase of left coronary arteriography, and a right Judkins catheter was inserted into the CS via the right jugular vein. Adequate position was verified by fluoroscopy of frontal and lateral views. All samples were collected into test tubes, centrifuged for 10 minutes at 3,000 rpm, divided into aliquots, and stored at −80°C until analysis.

Study groups: Patients were divided into 2 groups, according to their clinical status and catheterization data. The normal group was defined as patients who underwent cardiac catheterization due to atypical chest pain and had no evidence of coronary artery narrowing (50%) or LV systolic dysfunction. The CHD group was defined as patients who had 75% coronary stenosis at coronary arteriography with chest pain on exertion, including those who underwent percutaneous coronary intervention ≥6 months earlier.

Table 2
Comparisons of serum levels of endostatin in relation to the sampling site

Substance	Sampling Site	Normal (n = 15)	CHD (n = 57)	p Value
Endostatin (ng/ml)	LV	51.8 (36.3–86.8)	62.7 (38.4–131.2)	0.15
	CS	49.6 (29.1–84.5)	79.7 (46.2–130.3)	0.02*
	CS − LV	5.3 (−29.5–17.4)	16.0 (−14.5–35.4)	0.23

Values are expressed as median (interquartile range).
* Significant at p <0.05.

Measurement of endostatin: Endostatin of both LV and CS samples was measured by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D System, Minneapolis, Minnesota). The absorbance was measured by optical densitometry at 450 nm filter.

Statistical analysis: All statistical analyses were performed with SPSS for Windows version 13 (SPSS, Inc., Chicago, Illinois). Normality of distribution of the values of different variables was checked by the 1-sample Kolmogorov-Smirnov test. Values are expressed as mean ± SEM for normally distributed variables or medians (interquartile range) for skewed data. Comparisons among groups across a nominal grouping variable with 2 levels were performed by the Student's *t* test and Mann-Whitney U test for normally distributed data and skewed data, respectively. Comparisons among ≥3 groups were performed using the Kruskal-Wallis test for skewed data. Comparisons of proportions were performed by the chi-square test. Correlations between continuous variables were checked by Spearman's correlation coefficients for skewed data. A 2-sided p value <0.05 was considered statistically significant.

Results

Patient characteristics: The clinical characteristics of the study population at baseline before cardiac catheterization are listed in Table 1. As expected, a high prevalence of coronary risk factors was found in the CHD group, such as male gender, smoking, and diabetes. In addition, HbA1c levels of the CHD group were significantly higher than those of the normal group. The proportions of patients under medical treatment (such as those being administered angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers or β blockers) were not significantly different between either group. Ejection fraction was significantly lower in the CHD group compared with the normal group (p = 0.01), whereas pulmonary capillary wedge pressure did not show a significant difference.

Serum endostatin level and its relation to clinical characteristics: Table 2 shows the serum concentration of endostatin in relation to the sampling site. In the CHD group, the CS levels of endostatin were significantly

Table 3
Catheterization data and serum concentrations of endostatin in patients with coronary heart disease (n = 57)

	Endostatin (ng/ml)					
	LV	p Value	CS	p Value	CS − LV	p Value
Degree of CS						
<99% stenosis (n = 27)	67.0 (44.7–155.7)		69.1 (44.5–134.5)		7.3 (−37.0–25.6)	
≥99% stenosis (n = 30)	48.4 (32.4–98.9)	0.08	85.9 (53.1–124.9)	0.76	28.2 (4.8–48.6)	0.01*
No. of narrowed coronary arteries						
0 (n = 6)	63.4 (47.8–93.8)		85.9 (40.3–145.6)		−5.4 (−37.3–80.2)	
1 (n = 16)	50.8 (43.3–93.7)		68.8 (58.1–119.4)		28.0 (11.0–42.0)	
2 (n = 14)	130.5 (21.7–160.4)		61.3 (35.6–139.1)		−6.0 (−61.7–18.9)	
3 or left main trunk disease (n = 21)	74.5 (37.3–122.3)	0.98	99.2 (51.0–160.4)	0.80	18.4 (−4.7–61.2)	0.09
Ejection fraction <50% (n = 12)	113.4 (65.6–158.7)		106.1 (55.7–167.6)		11.9 (−15.4–33.3)	
≥50% (n = 45)	49.0 (33.3–123.5)	0.01*	68.4 (46.2–113.5)	0.15	16.4 (−17.1–39.3)	0.86

Values are expressed as median (interquartile range).
* Significant at p <0.05.

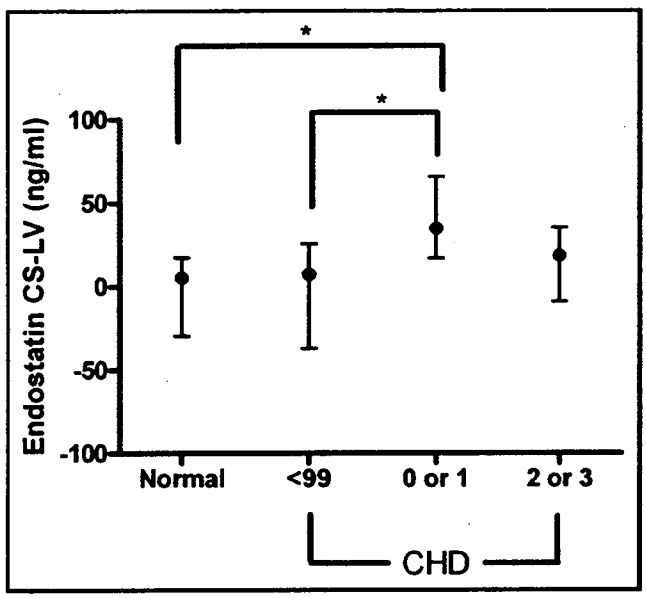

Figure 1. Error bar graphs showing the comparison of median subtraction values (CS − LV) of endostatin according to collateral flow grade. *Bar*, the 25th and 75th percentiles; *black circle within the bar*, the 50th percentile (median). *Significant at p <0.05. <99 = patients with CHD with <99% stenosis in major coronary arteries; 0 or 1 = Rentrop grade 0 or 1; 2 or 3 = Rentrop grade 2 or 3.

higher than those of the normal group. There was no significant difference of serum endostatin levels in LV samples between the 2 patient groups. Also, the median subtraction value (CS − LV) for endostatin was not significantly different between either group. A negative and significant correlation between the LV levels of endostatin and ejection fraction was observed (Spearman's r = −0.306, p = 0.009). However, the correlations between endostatin levels and the other clinical data were not significant.

Serum endostatin levels in patients with CHD: Catheterization data and levels of endostatin in patients with CHD are shown in Table 3. In patients with CHD with severe stenosis, the CS − LV value of endostatin was significantly higher than in those with moderate stenosis (p = 0.01), whereas the number of narrowed coronary arteries and EF did not affect the CS − LV value of endostatin (p = 0.09 and p = 0.86, respectively). The CS − LV value of endostatin was not influenced by gender (p = 0.45) or the presence of hypertension (p = 0.11), diabetes (p = 0.30), dyslipidemia (p = 0.30), smoking (p = 0.25), or obesity (p = 0.22) (data not shown).

Comparison of CS − LV values of endostatin using collateral flow grade: Comparison of median subtraction values (CS − LV) of endostatin according to coronary collateral flow grade in patients with CHD is shown in Figure 1. CS − LV median values of endostatin in patients with grade 0 or 1 (n = 13) collaterals and grade 2 or 3 (n = 17) collaterals were 34.7 (16.9 to 65.6) and 18.4 (−9.1 to 35.2) ng/ml, respectively. In patients with grade 0 or 1 collaterals, the CS − LV median value of endostatin was higher than in those in the normal group and patients with moderate coronary stenosis (p <0.05 for both).

Discussion

In this study, we demonstrated that CS levels of endostatin in patients with CHD were significantly increased compared with normal subjects. Although the dynamics of endostatin within the coronary circulation (CS − LV) were not significantly different between normal subjects and patients with CHD, in those with CHD with total (or near total) coronary occlusion and poorly developed collaterals, the CS − LV median value of endostatin was significantly elevated compared with patients without such occlusions. In contrast, LV systolic dysfunction and the number of narrowed major coronary arteries were not associated with the levels of endostatin within the coronary circulation.

Recently, Panchal et al[4] investigated endostatin levels in the pericardial fluid of patients with CHD and reported its negative association with the presence of coronary collaterals. Also, in patients with symptomatic intracranial atherosclerosis, endostatin has been shown to be associated with a greater extent of the disease and the risk of its recurrence.[7] In most studies, the data obtained from pericardial fluid or peripheral blood samples could not