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重症心不全に対する幹細胞による心筋再生療法の開発

Development of regeneration of myocardium using stem cells for profound heart failure

Keywords
細胞移植
骨髄単核球細胞
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Summary

Researches of cell-based therapy for profound heart failure are expanded to the regeneration of myocardium. Bone marrow cells are advantageous for exogenous cell transplantation because of autologous source, capability of regeneration of myocardium and induction of angiogenesis. Endogenous-stem cell therapy might be a promising strategy for treating failing heart.

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はじめに

重症心不全に対して各種治療が行われるが，広範囲に心筋障害をきたした症例においては，自己心のみによる回復は困難である。このような症例に対しては，心臓移植や人工心臓の適応が考慮される。心臓移植は，国際レジストリーではすでに6.6万例以上施行されており，50％生存率は9.4年に達している。また，わが国においても臓器移植法施行後，すでに27例の心臓移植が行われ,良好な成績を示している。しかし，心臓移植では適当なドナー心が必要であり，その施行数には限界がある。また，人工心臓は，拍動型に加え最近では連続流ポンプも導入され，補助期間および生活の質も向上し，心臓移植の適応とならない患者に対するdestination therapyとしても認められるようになってきた。しかし，感染症や血栓塞栓症など，いまだ解決すべき

問題が残されている。

自己組織を用いた重症心不全治療法として，自己骨格筋を用いる心筋形成術の開発研究が行われ，臨床応用もされたが，補助能力に限界があり普及するに至らなかった。その中で，Marelliらのグループは，骨格筋細胞を心筋へ移植する研究を開始し，心臓への細胞移植が注目されるようになった[1]。

その後，各種の細胞を用いた研究が行われてきたが，本稿では，我々の研究してきた骨髄細胞による心筋再生療法を中心に述べる。

外因性細胞移植(exogenous cell transplantation)

骨髄には多くの造血幹細胞のみならず間葉系幹細胞（mesenchymal stem cell：MSC)があり，このMSCから骨，軟骨，脂肪細胞が分化誘導される。1999年になると，5-azacytidine処理を行うことにより，骨髄細胞から心筋細胞が分化誘導されることが報告された[2][3]。さらに2000年には，環境因子（cardiac milieu）が，骨髄細胞の心筋への分化に重要であることが示された[4][5]。そこで，環境因子として細胞の直接接触を検討した[6]。ラット新生仔心筋細胞をホスト心筋（CM）とし，GFP（green fluorescent protein）遺伝子組み換えマウス由来骨髄細胞（GFP-BMC）を移植細胞として，共培養実験系を作製した。CMとGFP-BMCとの間に隔壁を置いたdouble chamber培養では，GFP-BMCに変化を認めなかった。これに対し，CMとGFP-BMCを混合した共培養系では，GFP-BMCの一部が2日後からCMと同期収縮を開始した。また，免疫組織染色により検討すると，1日後にはmyosin heavy cahin-slowが，2日後にはコネキシン43と心房性ナトリウム利尿ペプチド（ANP）が，4日後にはトロポニンIが経時的に各々発現し，さらに漸増した。myosin heavy chain-slow陽性細胞は5日後において約2.5％になった。この検討より，骨髄細胞が心筋へ分化するには，ホストの心筋細胞との直接接触が重要であることが明らかになった。さらに，循環しているヒト骨髄細胞が心筋細胞に分化し得ることも報告された[7]。この共培養の効果に対して，2002年に細胞融合（cell fusion）が問題提起され，Teradaらは ES細胞とGFP-BMCを共培養した場合，GFP発現細胞が分化増殖するようであるが，その細胞核内にはES細胞由来のDNAも含まれていたと報告した[8]。しかし，その後の研究報告では，細胞融合の割り合いは低いとされている。また，我々の実験で示されたように，共培養においては心筋細胞の特性を経時的に獲得しており，融合のみとは考えられない。また，成人心臓からの心臓前駆細胞は，ホスト細胞への細胞融合があってもなくてもほぼ同様に心筋細胞に分化することが報告されており[9]，さらに検討が必要である。

骨髄細胞による心筋再生療法の有効性について，拡張型心筋症に対する効果の研究は少ない。そこで，ラットを用い，心機能改善効果を検討した[10]。ラットドキソルビシン不全心モデルを作製し，骨髄単核球細胞（bone marrow mononuclear cell：BMMNC）移植群，生理食塩水注入群，sham手術群の3群において，移植4週間後に心エコーおよびLangendorff灌流装置にて心機能を測定し，合わせて心重量と腹水量を測定した。その結果，BMMNC移植群は，心エコー検査において心筋壁厚が有意に維持され，心筋壁厚/内腔比も高値であった。また，Langendorff灌流装置による検討では，他群に比しdeveloped pressureが有意に高値で，end-diastolic pressureは低値であった。心重量も有意に大きく，腹水量は有意に少量であった。この結果，ラットによるドキソルビシン心筋症モデルにおいてBMMNC移植は有効であることが示され，臨床応用の可能性が示唆された。

また，骨髄細胞による血管新生に関し，Asaharaらは，1997年に末梢血に骨髄由来の血管内皮前駆細胞（endothelial progenitor cells：EPC）があり，このEPCが虚血心筋における脈管新生（vasculogenesis）と血管新生（angiogenesis）に貢献していると報告した[11]。さらに，Shintaniらは，BMMNCが虚血肢における血管新生をもたらすことを報告した[12]。このBMMNCによる血管再生療法は，わが国において注目され，積極的に臨床応用が行われ，その安全性と有効性が示された。さらに，血管新生に骨髄細胞からのサイトカイ

ンが大きな役割を果たしている可能性が示された[13]。また，虚血心筋に対する外科的注入も報告されている[14]。

しかし，このBMMNCの血管新生効果の評価が難問であった。そこで，我々は，ブタ心筋梗塞モデルに対する骨髄細胞移植の効果について，心筋コントラストエコー法により検討した[15]。まず，NIBSブタの左前下降枝を結紮し，心筋梗塞モデルを作製した。1ヵ月後，骨髄細胞を梗塞部へ直接注入し，myocardial contrast intensity（MCI）を測定した。移植1ヵ月後に犠牲死させ，組織学的に毛細血管密度（capillary density：CD）を測定した。その結果，MCIとCDに正の相関を認めたが，毛細血管の多くは直径10μm以下であった。移植梗塞部のMCIとCDは，非移植梗塞部よりも有意に増加した。これらより，骨髄細胞移植は，心筋梗塞部位での血流を改善し，さらに，心筋コントラストエコー法によりこの血流改善効果が非侵襲的にベッドサイドで評価し得ることが示された。

BMMNCは，細胞移植の細胞源として，採取法が確立されており，さらに培養する必要がなく用いやすい。しかし，十分な細胞数を得るには全身麻酔が必要であり侵襲が大きい。これに対し，MSCは増殖能力が高く，骨髄細胞を少量採取し，生体外で培養し，必要量が得られてから移植することが可能となる。

そこで，MSC移植による血管再生効果を，BMMNC移植と比較検討した。ラットの左総腸骨動脈の結紮・切除により下肢虚血を作製した後，同数のMSCあるいはBMMNCを移植し，未治療群と比較した。移植3週間後，移植両群は，未治療群より有意な血流増加を認めたが，MSC群がより高度な血流改善を示した。また，毛細血管数も，MSC群はBMMNC群より増加していた。さらに，両群とも移植局所に移植細胞由来と考えられる血管内皮細胞を認めたが，その数はMSC群で有意に多かった。また，MSC群では移植細胞からの血管平滑筋と壁細胞への分化を認めた。さらに，BMMNCと比較しMSCでは，vascular endothelial growth factor（VEGF），hepatocyte growth factor（HGF），adrenomedullin（AM）などの血管新生因子が多量に分泌していた[16]。また，低酸素状態について検討すると，*in vitro*でMSCは管腔を形成した。さらに，無血清培地培養下における低酸素状態では，MSCはBMMNCに比べてアポトーシスは少なかった。したがって，MSCは低栄養および低酸素状態においてより高率に生存し得ることから，MSC移植はBMMNC移植と比較して，同等以上の血管再生作用があると考えられた。

そこで，心筋梗塞モデルにおいてMSCの経静脈投与の効果を検討した[17]。左冠動脈結紮により作製した心筋梗塞モデルラットの頸静脈からMSCをカテーテルにより移植した。その結果，MSCの一部は梗塞巣周囲に集積し，さらに心筋細胞および血管内皮細胞に分化し，心機能を改善させた。

また，MSC細胞移植の拡張型心筋症への効果をラット心筋症モデルで検討した。近交系ラットの大腿骨より骨髄組織を取り出し，培養皿底面に付着するMSCを分離・培養した。このMSCを，ミオシン投与拡張型心筋症モデルラットの心筋壁内に心外膜より直接注入し，未治療群と比較した。4週間後における心エコーおよび心臓カテーテル検査では，未治療群と比較し，MSC移植群は左室拡張末期圧の有意な低下および左室収縮能の有意な改善を認めた。病理学的検討では，MSC移植群は心筋コラーゲン含量が減少し，さらに心筋壁内で血管内皮細胞や平滑筋細胞に分化し，管腔構造を形成した。また，免疫組織染色にて，心筋内に注入したMSCの一部はトロポニンT，desmin，およびコネキシン43が陽性であった。さらにMSCは種々の血管新生因子やアポトーシス抑制因子を分泌した。したがって，MSCは心筋細胞や血管細胞へ分化し，さらにパラクライン因子として心筋および血管再生に関与することが示唆された。次いで，ブタを用いた前臨床研究を行い，骨，軟骨，脂肪などへ分化しないこと，不整脈が出現しないことなど，MSC移植の安全性を確認した。

以上の結果を踏まえ，虚血性心疾患や拡張型心筋症などによる心不全例で，利尿剤，ACE阻害薬，β遮断薬などの既存の治療が困難な症例を対象に，患者の骨髄液15mLを採取し，体外で培養増殖させ，カテーテルを用いて心内膜側より心筋内へMSCを注入

する臨床試験を計画した。国立循環器病センター倫理委員会に「間葉系幹細胞移植による難治性心不全治療の臨床評価」の実施を申請し，承認された。これまで数例の難治性心不全症例に対して自己MSC移植を行ったが，重度の不整脈などの副作用はなく，順調に経過している。今後，補助人工心臓装着例への応用なども検討している。

内因性細胞移植(endogenous cell transplantation)

Orlicらは，2001年に急性心筋梗塞マウスモデルに顆粒球コロニー刺激因子（G-CSF）と幹細胞因子（SCF）を投与し，心機能の改善および生存率の改善が得られたことから，G-CSFとSCFによる幹細胞の賦活化を報告した[18]。しかし，再生心筋がホストの心筋由来か骨髄由来かは不明であった。我々は，この内因性幹細胞の由来を検討した。放射線照射後のC57b6マウスにGFP-BMCを移植し，キメラマウスを作製した。心筋梗塞モデルでの検討ではG-CSF投与群で心筋梗塞1ヵ月後における生存率の改善傾向を認めた[19]。また，G-CSF群では，心筋梗塞境界部のGFP-BMC数がコントロール群より有意に増加し，そのGFP-BMCのうち約20％がトロポニンI陽性細胞で，ネスチン陽性細胞も多数認めた。さらに，ドキソルビシン投与心不全モデルにおいても同様の結果であった[20]。したがって，骨髄は再生心筋の細胞源の一つで，G-CSFがその効果を増強することが示唆された。また，ヒト心筋細胞を用いた検討において，G-CSFは病的心筋に直接働き，G-CSFレセプターを介してトロポニンI陽性細胞の増殖を増強することが確認された[21]。しかし，骨髄由来の心筋細胞数が少なく，心臓ポンプ機能の改善効果には限界があった[22]。

しかし，内因性幹細胞は，障害された心筋とともに動脈硬化巣にも遊走する可能性があり[23]，心筋障害に対する効果的な内因性幹細胞を用いた治療には，内因性幹細胞の遊走に関する生理学的メカニズムの解明が望まれる。

おわりに

細胞を用いた心筋再生において，骨髄細胞は，自己細胞を用いるため拒絶反応を避けることができ，倫理的問題も少ない。さらに，心筋内に移植することで心筋と血管が同時に再生され得るため，拡張型心筋症などの難治性重症心不全に対する治療として期待される。

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特集「異種移植」

ブタ組織の脱細胞化

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はじめに

一般に組織の置換や欠損部の修復を同種あるいは異種組織移植により行おうとする場合，そのままでは激しい拒絶反応が引き起こされ，組織は壊死・脱落する。この拒絶反応の原因となる同種・異種の主要抗原は主に組織中の細胞膜に存在するため，組織から細胞成分を完全に除去できれば抗原性はほとんど消失すると考えられる。そこで脱細胞化した組織を移植し，これをscaffold（足場）として宿主細胞により組織が再構築されれば，拒絶反応に対する免疫抑制治療の必要なく，場合によっては成長性を期待することもできる新たな再生医療となりえる。

これまで生体組織の脱細胞化法として，急速な凍結・溶解による細胞破壊，界面活性剤や蛋白分解酵素による細胞除去など試みられてきたが，細胞成分の残存，ウィルスを含む病原微生物の存在，組織力学特性の変化，使用薬剤の毒性，組織保存・輸送にかかる費用などの点でそれぞれ一長一短であった。

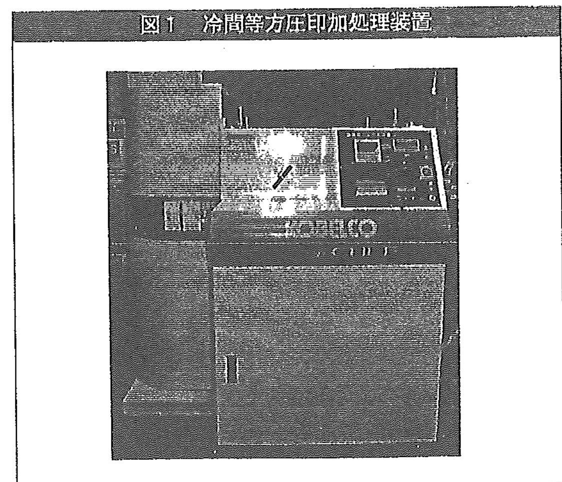
われわれは，上記諸問題をクリアする脱細胞化方法として新たに超高圧処理法を開発し，各種組織においてその移植可能性を検討しているので，ここにその一部を紹介する。

超高圧処理による脱細胞化

生体組織に液体を圧力媒体として温度上昇を抑えながら等方圧力を加えていくと，多くの機能性蛋白は300 MPa程度の加圧により失活・変性し，酵母や芽胞のない細菌も500 MPaで死滅する。さらに600 MPaではHIVなどのエンベロープを有するウィルスまで不活化させることができる[1]。

そこでわれわれは，低温を保ちながら等方圧力を加えることのできる冷間等方圧印加処理装置（図1）を用い，ブタから採取した各種組織を4℃にて980 MPa（10,000気圧）で10分間加圧し，続いてPBSを主体と

図1　冷間等方圧印加処理装置



した洗浄液で2週間震盪洗浄した。この処理により，多くの組織では深部まで十分に細胞成分が除去され，その一方でEVG染色においてコラーゲン繊維やエラスチン繊維はそのまま保存されていることが確認された。また，力学試験を行うと力学特性は処理前と同様に保持され，さらにPCR法にてブタ内在性レトロウィルス(PERV)-DNAが検出されなくなっていた。

以上より，本法は異種組織移植を行うための生体scaffold作製法として有望であると考えられた。

心臓弁

脱細胞化異種心臓弁の先駆例としてCryoLife社のSyner Graft心臓弁があり[2]，2001年に欧州で市販された。しかしその成績が必ずしも満足のいくものでなかったこともあり，現在は販売が中止されている。心臓弁の脱細胞化法としてはHaverichらが界面活性剤であるTriton X-100やトリプシンによる方法を[3]，またInghamらがSDSによる細胞除去の有用性を報告している[4]。

われわれも当初Triton X-100溶液を用いた心臓弁の脱細胞化を検討していたが，24時間の浸漬処理と2週間の洗浄を行った後も表層から1 mm程度までしか細胞が除去されず，弁基部の組織内部では大部分の細胞成分が残存した。また，洗浄後も組織中にTriton X-100の残留を認め，力学特性においても弁弾性率が有意に増加したため，最終的にこの方法による脱細胞化処理を中止し，新たな脱細胞化法として超高圧処理法の開発へ移行した。超高圧処理を行った弁では組織深部まで細胞が完全に除去され，力学特性への影響も認めなかった。

心臓弁scaffoldを移植する場合，宿主の自己細胞をあらかじめ移植片に組み込んで移植することで抗血栓性や組織の再構築促進が期待される。われわれはブタ同種心臓弁移植実験において，移植予定の宿主ブタから採取した大腿動脈の血管内皮細胞を分離・培養し，十分な細胞数まで増やした後に心臓弁scaffoldへ播種する方法を検討した。心臓弁は複雑な形状であるため細胞浮遊液中への単純な静置では表面全体に均一に播種できないことから，採血管用ローラー攪拌器を改良した2軸回転バイオリアクターと遠心型血液ポンプを利用した循環型バイオリアクターを独自に開発し，順に4時間および2日間の播種・培養を行うことで，心臓弁全体を内皮細胞でconfluentに覆うことに成功した。

この方法を用いて，Triton X-100により脱細胞化したブタ肺動脈弁に宿主内皮細胞を播種してから同所性に同種移植を行い，細胞播種を行わないで移植した脱細胞化弁，凍結保存弁と移植後成績を比較した。細胞播種脱細胞化弁では移植後1カ月の時点で内腔表面が一層の内皮細胞に覆われ，3カ月までに組織内部の再細胞化(平滑筋細胞，繊維芽細胞)が進行した。一方，未播種の脱細胞化弁や凍結保存弁では内皮細胞による被覆までは同様に認められたが，その後の組織内再細胞化が進展せず，また凍結保存弁においては脱細胞化弁と比べて炎症細胞の浸潤が顕著であった[5]。従って，われわれの開発したバイオリアクターによる血管内皮細胞播種が移植弁組織の早期再細胞化に有用であると考えられた。現在，超高圧処理した脱細胞化弁とバイオリアクターによる内皮細胞播種を組み合わせた移植の検討を行っており，将来的には組織の再構築をさらに促進する目的で幹細胞を含めた複数の細胞種を導入したテーラーメード型移植用心臓弁の開発を計画している(図2)。

気管

悪性腫瘍や良性疾患による瘢痕・狭窄などにより気管のlong segmentを切除すると，再建に何らかの補填が必要となるため，人工気管や同種・異種気管の移植可能性が検討されてきた。そして，気管においても凍結保存や界面活性剤などによりドナー細胞(特に上皮細胞，腺細胞)を除去することで，移植時の抗原性が著しく減弱することが各種の動物実験において報告されている[6-8]。

われわれは，超高圧処理により脱細胞化したブタ気管をscaffoldとして移植利用する目的で，同法を他の脱細胞処理法(凍結保存およびTriton X-100処理)と比較検討した。超高圧処理気管は980 MPa(10,000気圧)，

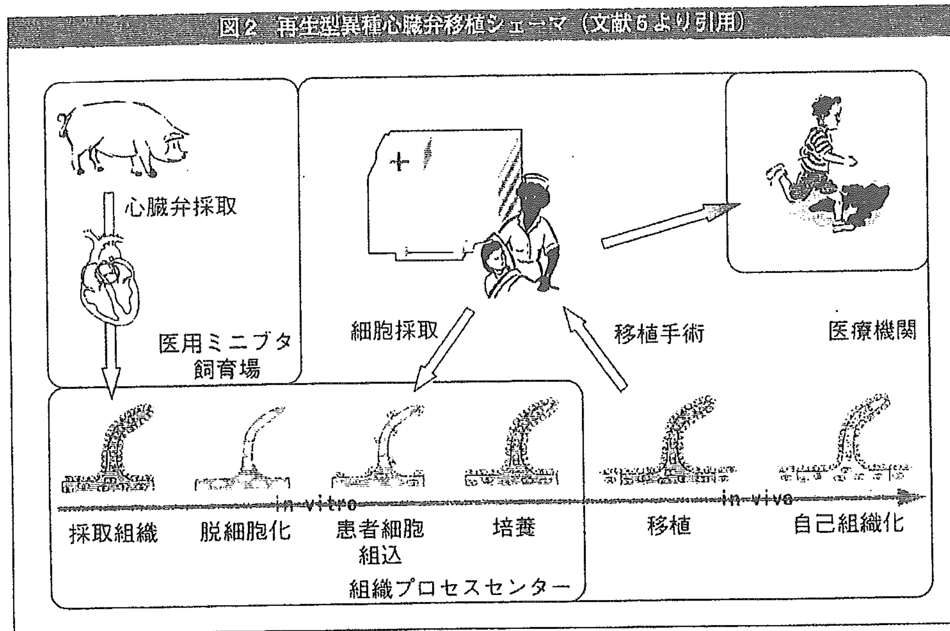


図2　再生型異種心臓弁移植シェーマ（文献5より引用）

4°Cで10分間加圧後，2週間緩徐に震盪洗浄し，その後PBS中に浸漬し4℃の冷所にて保存した。凍結保存気管は液体窒素にて急速冷却後，−80°Cで凍結保存した。Triton X-100処理気管は1% Triton X-100溶液で24時間処理した後，2週間洗浄し，その後PBS中に浸漬し4℃の冷所にて保存した。各処理気管の病理像を比較すると，凍結保存では当然のことながら気管組織内に細胞成分がほぼ完全に残存しており，Triton X-100処理後も浅部組織の細胞成分は消失したものの軟骨内はほとんど除去できていなかった。一方，超高圧処理を行った気管では軟骨部細胞成分の残存が他の処理法に比べ少なかった（図3）[9]。今後，さらに軟骨部細胞成分の除去を徹底するため，超高圧処理の時間や回数，また超音波処理との組み合わせ等を検討する予定である。気管では移植後に管腔構造を保持する必要があるため，気管の圧縮試験を行い各処理気管で軟骨の力学強度を比較した。すると，Triton X-100処理気管は超高圧処理，凍結処理気管と比較して力学強度が約60%にまで低下していた。また，PERV-DNAは超高圧処理気管でのみ除去された。

次いで，ラットを用いた同種気管移植実験を行った。B-Nラットの気管を凍結保存，Triton X-100処理および超高圧処理したところ，Triton X-100処理気管では管腔構造が維持できないほどに軟骨強度が低下したため移植困難と判断し，凍結保存気管と超高圧処理気管をLewisラットへ同所性に移植した。4週間後に摘出し病理検査を行うと，両処理気管とも上皮の再生は比較的良好であったが，軟骨部にはほとんど新たな細胞は認められなかったため，軟骨部の再細胞化を早期に誘導する工夫が必要と考えられた。間葉系幹細胞は軟骨を含む各種細胞への分化が明らかとなっていることから[10]，現在われわれは，宿主の間葉系幹細胞を移植時に気管scaffoldに導入することで軟骨部も含めた早期再細胞化を実現するべく，その至適導入方法を検討

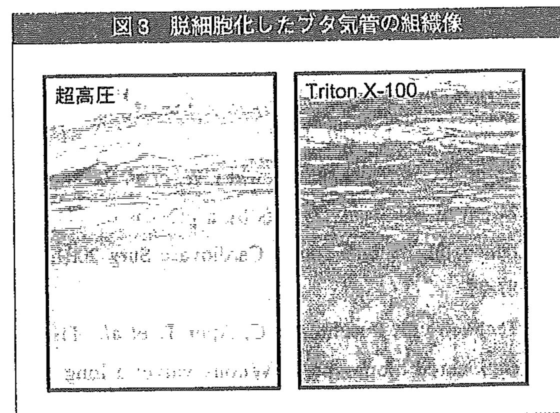


図3　脱細胞化したブタ気管の組織像

している。さらに，超高圧処理したブタ気管scaffoldについても，将来的な臨床応用を目指し同種・異種移植実験を計画している。

おわりに

われわれの開発した超高圧処理法による生体組織の脱細胞化について概説した。同種・異種組織scaffoldの作製法として本法は，組織深部まで脱細胞化を行うことができるとともに，組織の力学特性保持やウィルスを含む滅菌などの点で従来法と比較して優れていると考えられ，さらに保存時に凍結の必要がないため保存や輸送に関してもメリットがあると考える。われわれは，脱細胞化処理した組織scaffoldに宿主の自己細胞を組み合わせた再生型組織移植の臨床化の早期実現を目指し，研究を続けている。

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●特集—「異種移植」

ブタ組織の脱細胞化

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別 刷

Endogenous Bone-Marrow–Derived Stem Cells Contribute Only a Small Proportion of Regenerated Myocardium in the Acute Infarction Model

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Background: Our recent study showed that granulocyte-colony stimulating factor (G-CSF) promoted bone-marrow cells (BMC) to migrate into the infarcted heart and that they differentiated into cardiomyocytes. However, we still do not know to what degree bone-marrow-derived cardiomyocytes contribute to myocardial regeneration after injury. In this study, we verified the proportional contribution of cells from bone marrow (BM) and from non-bone marrow (n-BM) in regenerating neomyocardium after myocardial infarction.

Methods: Eight C57BL/6 mice were irradiated (900 cGy), and green fluorescent protein (GFP) mouse-derived BMCs (GFP-BMC, 1×10^6 cells) were injected. Four weeks later, the left descending coronary artery was ligated. Recombinant human G-CSF (200 μg/kg/day, 8 days) was injected. At 4 weeks after ligation, hearts were fixed for histology. We calculated the proportions of cardiomyocytes derived from BM and n-BM after taking the chimeric rate into consideration.

Results: The chimeric rate was 54.6% ± 5.9%. At the infarcted border area, the total cell number was 1000.3 ± 56.5/mm^2, and mobilized BM-derived GFP-BMC was 103.3 ± 13.1/mm^2. After compensation with the chimeric rate, we found BM-derived troponin I-positive cells at 23.9 ± 4.1/mm^2, nestin-positive cells at 12.9 ± 2.6/mm^2, and Ki67-positive cells at 18.3 ± 2.6/mm^2, respectively. We found significant differences in the contribution of troponin I-(6.7% ± 1.7% vs 93.3% ± 1.7%), nestin- (2.4 ± 0.5 vs 97.6 ± 0.5), and Ki67-positive (3.9 ± 1.0 vs 96.1 ± 1.0) cells derived from BM and n-BM.

Conclusions: Bone marrow was one of the origins of regenerated cardiomyocytes; however, the contribution of cells from BM was very small compared with those of n-BM origin in the infarction model. J Heart Lung Transplant 2005;24:67–72. Copyright © 2005 by the International Society for Heart and Lung Transplantation.

Several reports suggest that bone marrow can provide stem cells capable of myocardial regeneration.[1-3] More recently, the possibility of self-renewing adult myocytes was reported.[4] Granulocyte-colony stimulating factor (G-CSF) treatment has been shown to improve post-infarct cardiac dysfunction.[5] Our recent study showed that G-CSF promoted bone-marrow cells to migrate into infarcted myocardium and that they differentiated into cardiomyocytes.[6] However, we still do not know to what degree bone-marrow-derived cardiomyocytes contribute to regeneration of injured myocardium.

This study answers this question by verifying the proportional contribution of cells from bone marrow and from non-bone-marrow (host myocardium) in regenerating neomyocardium after acute myocardial infarction.

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MATERIALS AND METHODS

Subjects

All animals received humane care in compliance with the *Principles of Laboratory Animal Care*, formulated by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). All procedures were approved by the Animal Care Committee of the National

Table 1. Comparing the Contribution of Bone-marrow–Derived Cells with Non–bone-marrow–Derived Cells

Actual cell number of respectives.

Total cell number = total = A + B = X + Y

A = Total GFP-positive cells

B = Total GFP-negative cells = total − A

C = GFP-positive and troponin I–positive cells

D = GFP-negative and troponin I–positive cells

E = Bone-marow–derived and GFP-negative cells = A ÷ R − A

F = GFP-negative cells and troponin I–positive cells = E × C ÷ A

X = Bone-marrow–derived and troponin I–positive cells = C + F

Y = Non–bone-marrow–derived and troponin I–positive cells = D − F

The percentage of the contribution of bone marrow to form troponin I–positive cells at the infarcted border area = X ÷ (X + Y) × 100 (%).

GFP, green fluorescent protein; R, Chimeric rate.

Cardiovascular Center, Osaka, Japan. We purchased C57BL/6 mice from a licensed vendor. Dr. Okabe provided transgenic mice expressing green fluorescent protein (C57BL/6Tg14[act-EGFP] OsbY01; GFP mouse).[7] Animals were housed in an air-conditioned room with free access to food and water at all times.

Bone-marrow Cells of GFP Mice (GFP-BMC)

GFP mice were anesthetized with diethylethanol.[6] The bone-marrow plugs of the femora and tibiae each were flushed using a 27-gauge needle and a syringe filled with phosphate-buffered saline solution (PBS). The bone-marrow cells were suspended in a tube containing PBS and centrifuged at 1,000g for 5 minutes. This cell pellet was then resuspended in PBS to make a cell density of 1×10^7 cells/2 ml. The cell suspension was preserved in ice until use.

Myocardial Infarction

Eight C57BL/6 mice at 8 weeks of age were irradiated (900 cGy), and GFP-BMC (1×10^6 cells) were injected through a tail vein.[6] At 4 weeks after transplantation, splenectomy was performed. Two weeks later, the left descending coronary artery was ligated. Recombinant human G-CSF (200 μg/kg/day, Chugai; Tokyo, Japan) was injected intraperitoneally for 3 days before ligation and for 5 days after ligation. At 4 weeks after ligation, the mice were killed, and their hearts were collected. They were washed at once in cold PBS to remove residual blood and clots and fixed with 4% paraformaldehyde for histologic study.[8]

Immunohistochemistry

We performed immunostaining as described in our previous study.[6] Briefly, we incubated the heart sections with antibodies at 4°C overnight and identified cells as follows: Mature cardiomyocytes were identified by a mouse monoclonal antibody against cardiac-specific troponin I (TnI; Hytest, 4C2; Euro, Finland). Stem cells were recognized by a mouse monoclonal antibody against nestin (BD Biosciences, USA).[9] Cell proliferation was identified using a rabbit polyclonal antibody against Ki67 (DAKO, CA, USA).[4] Primary antibodies were detected with a goat anti-mouse immunoglobulin-G antibody or anti-rabbit immunoglobulin-G antibody (Alexa Fluor 568, Molecular Probes, Wako; Osaka, Japan). The cells then were evaluated and photographed with a FLUOVIEW FV300 confocal laser scanning microscope (Olympus; Tokyo, Japan).

Nuclei of the cells in each section were labeled with 4′,6-diamidino-2-phenylindole (DAPI).[10] Briefly, DAPI (Sigma; St Louis, MO) was added at a final concentration of 50 μg/ml and washed with PBS. We viewed DAPI-labeled migrated cells under fluorescent microscopy with appropriate excitation (330 nm) and emission (450 nm) spectra.

Quantitative analysis

We calculated the relative contribution of bone marrow to several protein-specific cells at the infarction border (most distant from the center of scarred myocardium and adjacent to normal myocardium). Regarding TnI, for example, we counted raw numbers as follows: total number of nuclei of all cells (total); GFP-positive cells (A); GFP-negative cells (B); TnI-positive cells, GFP-positive and TnI-positive cells (C); GFP-negative and TnI-positive cells (D); and chimeric rate (R), Table 1, Figure 1. Using all these raw data, we calculated bone-marrow–derived and GFP-negative cells (E):

$$\text{Bone-marrow total cells} \times R = A$$

$$\therefore \text{Bone-marrow total cells} = A/R$$

$$\text{Bone-marrow–derived and GFP-negative cells} = A/R - A = E$$

We calculated the proportion of the TnI-positive cells derived from bone marrow (X) and those derived from non–bone marrow (Y) and made pie charts. We also calculated the proportions of 2 derivations of nestin- and Ki67-positive cells using this method.

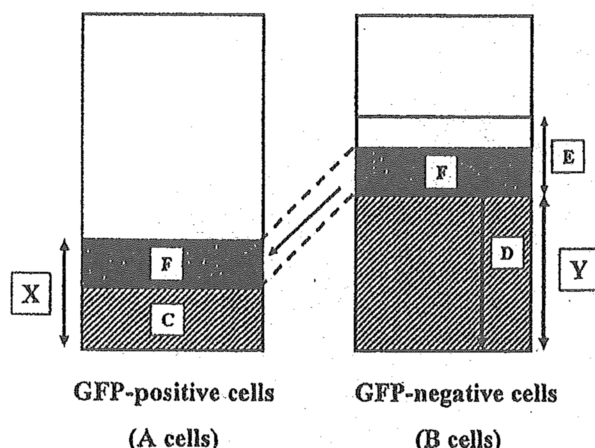

Figure 1. The contribution of cells of bone-marrow origin and those of non–bone-marrow origin to constitute certain protein-positive cells. Actual cell numbers are as follows: A, the number of green fluorescent protein (GFP)–positive cells. B, the number of GFP-negative cells. C, The number of GFP-positive and certain protein-positive cells. D, the number of GFP-negative and certain protein-positive cells. E, the number of bone-marrow–derived and GFP-negative cells. F, the number of GFP-negative and certain protein-positive cells. X, the number of certain protein-positive cells derived from bone marrow. Y, the number of certain protein-positive cells derived from non–bone-marrow. E, F, X, Y were calculated using the equations in Table 1.

RESULTS

Migration of Bone-marrow–Derived GFP-positive (BMD-GFP) cells.

All values were expressed as means ± SE. We compared BMD-GFP cell migration into the infarction border with the center of the infarcted area (Figure 2). The total cell number at the infarction border was 1000.3 ± 56.5/mm^2. Bone-marrow-derived GFP cells constituted a relatively large number of cells at the infarction border, 103.3 ± 13.1/mm^2 (Figure 3). The migrated BMD-GFP cells were spindle shaped or cylindrical shaped. The BMD-GFP cells differentiated into TnI-, nestin-, and Ki67-positive cells (Figure 4).

Contribution of Bone Marrow

We calculated the contribution of bone-marrow-derived TnI-, nestin-, and Ki67-positive cells at the infarction border. The chimeric rate was 54.6% ± 5.9%. After compensation for the chimeric rate, the number of bone-marrow-derived and TnI-positive cells was 23.9 ± 4.1/mm^2, the number of nestin-positive cells was 12.9 ± 2.6/mm^2, and the number of Ki67-positive cells was 18.3 ± 2.6/mm^2. We found large differences in the contribution of TnI- (6.7% ± 1.7% vs 93.3% ± 1.7%), nestin- (2.4% ± 0.5% vs 97.6% ± 0.5%), and Ki67-positive (3.9% ± 1.0% vs 96.1% ± 1.0%) cells between

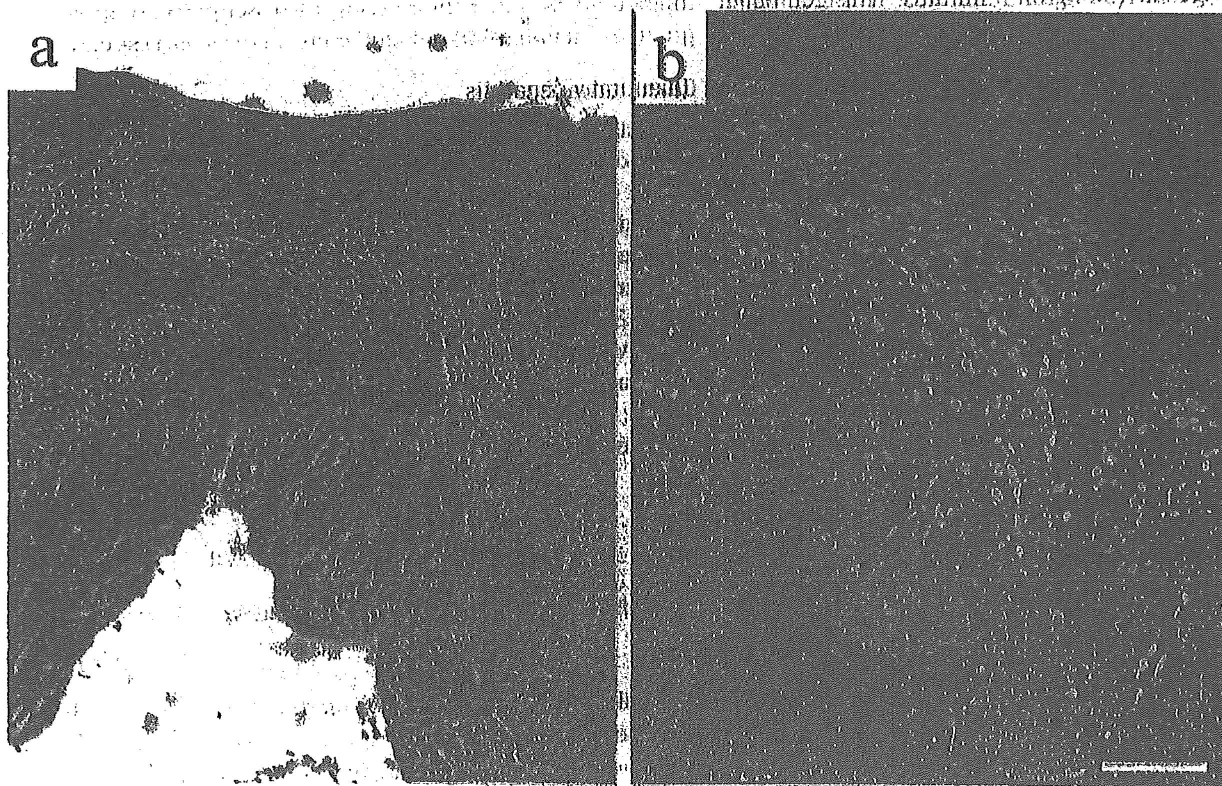

Figure 2. The bone-marrow–derived green fluorescent protein (BMD-GFP) at the infarcted border area. The BMD-GFP migrated into the infarcted border area under fluorescent microscopy (a, hematoxylin–eosin; b, GFP). Scale bar represents 200 μm.

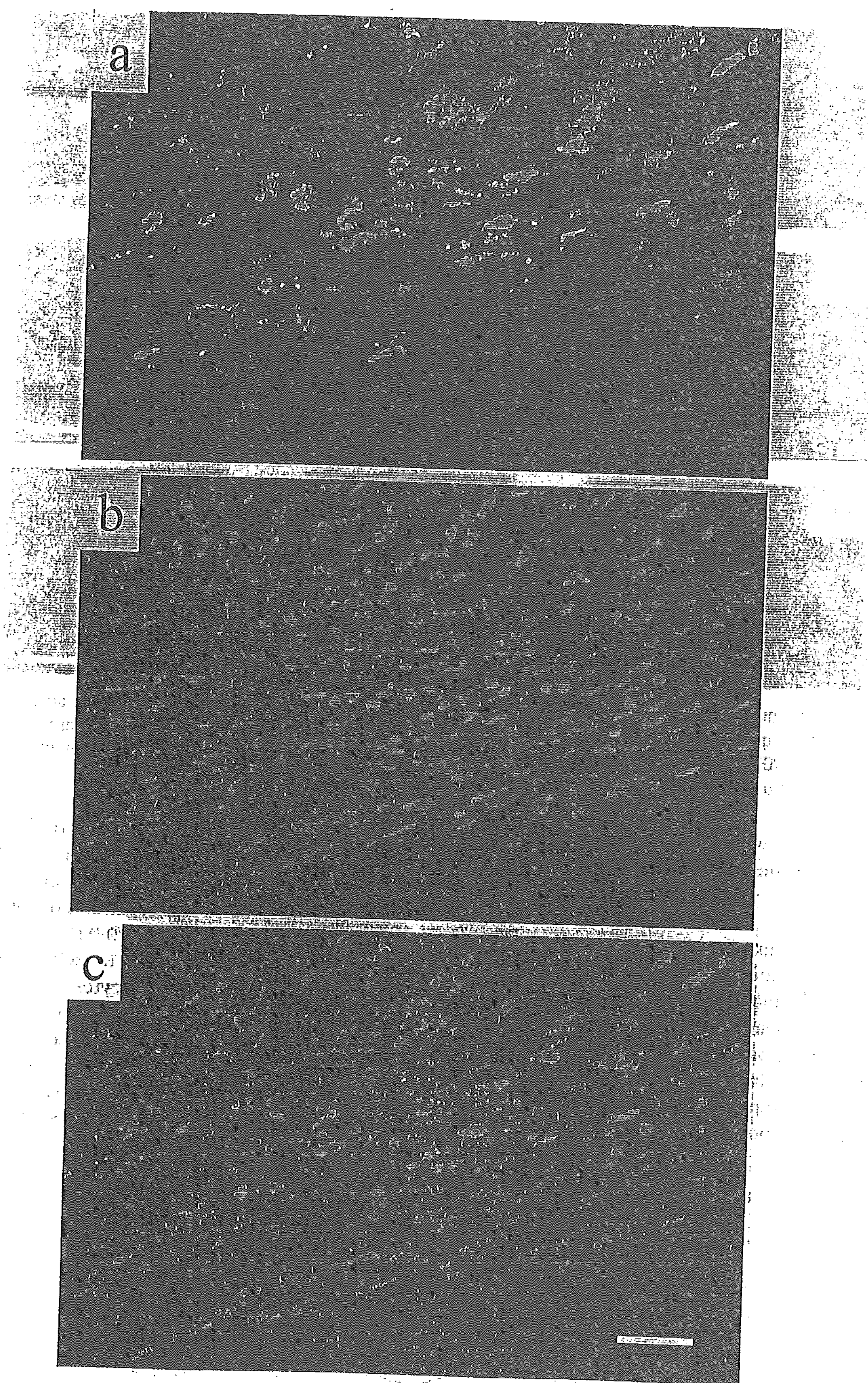

Figure 3. The distribution of bone-marrow–derived green fluorescent protein (BMD-GFP) at the infarcted border area. The total number of cells was that of the 4′,6-diamidino-2-phenylindole–positive nuclei. Many BMD-GFP migrated into the infarcted border area under fluorescent microscopy (a, GFP; b, DAPI; c, merged image). Scale bar represents 10 μm.

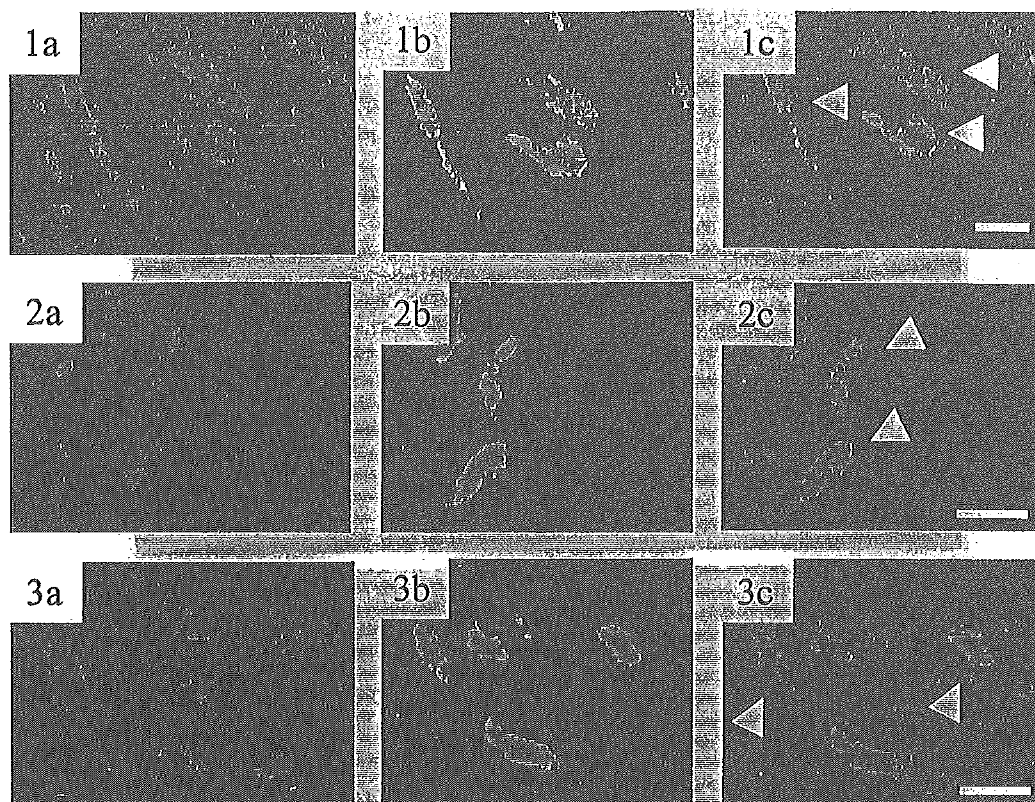

Figure 4. Several differentiations of bone marrow–derived green fluorescent protein (BMD-GFP) at the infarcted border area. Each picture is a red (a), green (b), and merged (c) picture under fluorescent microscopy. Yellow cells (arrowheads) indicate BMD-GFP expressing a certain protein. Troponin I–positive BMD-GFP (1c), nestin-positive BMD-GFP (2c), and Ki67-positive BMD-GFP (3c) were observed at the infarcted border area. Scale bar represents 30 μm.

those of bone-marrow origin and those of non–bone-marrow origin, respectively (Figure 5).

DISCUSSION

Recently Anversa's group reported that adult cardiomyocytes re-entered the cell cycle in the injured heart[4] and that G-CSF promoted the migration of primitive cells into the infarcted heart.[5] This finding suggested that the injured heart may possess self-renewal ability or that a chemokine may be able to draw primitive cells into the infarcted heart. The primitive-cell origin was uncertain. We confirmed that bone marrow was one of the origins of these migrated cells and that they differentiated into cardiomyocytes after myocardial infarction.[6] In several recent studies, host cells have been shown to contribute to some degree to the regeneration of neomyocardium in transplanted hearts (0.04%–16%).[11-13] However, we still do not know to what degree bone-marrow–derived cardiomyocytes contribute to regenerating neomyocardium in myocardial infarction. Therefore, in this study, we investigated this issue using GFP-tagged bone-marrow cells in the infarction model.

After myocardial infarction, necrosis of cardiomyocytes results in cardiac dysfunction. Some believe that

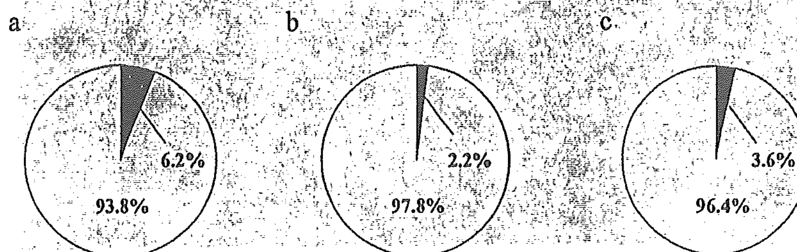

Figure 5. Pie charts of the contribution of bone-marrow cells and non–bone marrow cells (black, bone-marrow origin; white, non–bone-marrow origin). The percentage of cells of bone-marrow origin was very small compared with that of cells of non–bone-marrow origin in 3 immunohistochemistry expression (a, troponin I; b, nestin; c, Ki67).

neocardiomyocytes could compensate for this loss of myocytes and improve cardiac function. Orlic et al[5] suggest that G-CSF induces stem cells to mobilize into areas of myocardial infarction. Our findings clarify the proportional contribution of cells from these 2 sources. The proportion of bone-marrow-derived cardiomyocytes was only 6% at the border zone. Only 2% of the nestin-positive cells (stem cells) were of bone-marrow origin. In contrast, 98% of those cells were derived from non-bone marrow, maybe host myocardium. Ninety-six percent of Ki67-positive cells were derived from non-bone-marrow cells. Although we showed that bone marrow was 1 of the origins of neomyocardium,[6] their relatively minor contribution suggests that bone-marrow-derived cardiomyocytes may not be a major factor in the support of cardiac function after infarction, at least not directly. Instead, these observations suggest that G-CSF could directly affect the host myocardium to mediate the preservation of cardiac function. We now are investigating this direct effect of G-CSF on host myocardium in another study.

There are several limitations in this study. First, we used cell nuclei to calculate the cell number. Second, we did not investigate the relation between the dose of G-CSF and the magnitude of the contribution of bone-marrow-derived cardiomyocytes. Third, we do not know how G-CSF acts on the myocardium.

In conclusion, we identified the proportional contribution of bone-marrow-derived cardiomyocytes to the regeneration of myocardium after infarction and found it to be minor compared with that of non-bone-marrow-derived cells.

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Clinical research

Favourable clinical outcome in patients with cardiogenic shock due to fulminant myocarditis supported by percutaneous extracorporeal membrane oxygenation

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KEYWORDS
Echocardiography;
Extracorporeal circulation;
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Shock

Aims The clinical outcome of severe acute myocarditis patients with cardiogenic shock who require circulatory support devices is not well known. We studied the survival and clinical courses of patients with fulminant myocarditis supported by percutaneous extracorporeal membrane oxygenation (ECMO) and compared them with those of patients with acute non-fulminant myocarditis.

Methods and results Patients with acute myocarditis were divided into the following two groups. Fourteen patients who required ECMO for cardiogenic shock were defined as having fulminant myocarditis (F group), whereas 13 patients who had an acute onset of symptoms, but did not have compromised, were defined as having acute non-fulminant myocarditis (NF group). In the F group, 10 patients were weaned successfully from percutaneous ECMO. Therefore, the overall acute survival rate was 71%. Patients who were not weaned from ECMO showed smaller left ventricular end-diastolic and end-systolic dimensions, thicker left ventricular wall, and higher creatine phosphokinase MB isoform levels than those who were weaned from ECMO. When compared with patients in the NF group, the fractional shortening in the F group was more severely decreased in the acute phase [F: 10 ± 4 vs. NF: $23 \pm 8\%$ (mean $\pm$ SD), $P < 0.001$], but recovered in the chronic phase (F: 33 ± 7 vs. NF: $34 \pm 6\%$). The prevalence of adverse clinical events in both groups was similar during the follow-up period of 50 months.

Conclusion In patients with fulminant myocarditis, percutaneous ECMO is a highly effective form of a haemodynamic support. Once a patient recovers from inflammatory myocardial damage, the subsequent clinical outcome is favourable, similar to that observed in patients with acute non-fulminant myocarditis.

Introduction

Myocarditis is defined as an inflammation of the myocardium caused by viral, rickettsial, bacterial or protozoal infections, or drug toxicity.[1–3] Its clinical features vary, ranging from asymptomatic secondary to focal inflammation to fulminant fatal congestive heart failure. Moreover, there is a possibility that viral myocarditis may lead to dilated cardiomyopathy, presumably as a consequence of a late immunological response.[2] Patients with fulminant myocarditis often present with cardiogenic shock due to a severe left ventricular dysfunction.

Critically ill patients often require mechanical circulatory support such as a percutaneous extracorporeal membrane oxygenation (ECMO) with a cardiopulmonary bypass. Some studies showed that mechanical circulatory support is effective and can eliminate the need for cardiac transplantation in patients with cardiogenic shock secondary to fulminant myocarditis. These studies further showed an overall survival rate range of 50–70%, in the case of using mechanical circulatory support, is possible either by cardiac recovery or by transplantation.[4–7] These studies showed that the survival rate in the case of using percutaneous ECMO is higher than that in using a ventricular assist device.[6] This result may be due to the quick and easy application of percutaneous ECMO preventing multiple organ failure secondary to haemodynamic deterioration, when compared with a ventricular assist device. McCarthy *et al.*[8] demonstrated that patients with lymphocytic fulminant myocarditis have a better prognosis than those with acute non-fulminant myocarditis, providing important information for a better understanding of the pathophysiology of myocarditis. However, in their study, only two of 15 patients with fulminant myocarditis were treated with mechanical circulatory support. The clinical outcome of critical myocarditis patients with cardiogenic shock who require circulatory support devices is not well known. Thus, in the present

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study, we have focused on the survival and clinical courses of severely ill patients who are under mechanical circulatory support with percutaneous ECMO and compared them with those of patients with acute non-fulminant myocarditis.

Methods

Clinical classification

The diagnosis of myocarditis was made on the basis of the following findings: (i) a recent medical history consistent with the occurrence of a viral infection, (ii) positive findings of inflammation (high fever and increased white blood cell count and C-reactive protein level), (iii) evidence of myocardial damage [significant changes in electrocardiographic and echocardiographic features and elevations of serum creatine phosphokinase (CPK) and its MB isoform (CK-MB) level], and (iv) signs of a recent onset of cardiac dysfunction that were not due to myocardial ischaemia (determined by coronary angiography). Patients who had signs of myocarditis associated with other systemic diseases, such as immunodeficiency, sarcoidosis, collagen diseases, endocrine diseases, drug-induced toxicity, or alcoholism, were excluded. Cardiogenic shock was defined on the basis of the criteria set by the Myocardial Infarction Research Units of the National Heart and Lung Institute.[9]

In the present study, patients with fulminant myocarditis were defined as those who require percutaneous ECMO or a ventricular assist device for cardiogenic shock and do not respond to intensive medical treatments like high doses of intravenous catecholamines or for refractory ventricular tachyarrhythmia. Patients with acute non-fulminant myocarditis were defined as those who had an acute onset of symptoms but did not have compromised haemodynamics following conventional medical treatment.

Details of percutaneous ECMO system

A percutaneous ECMO system is basically a femoro-femora bypass without a reservoir (*Figure 1*). This system is completely preconnected to a compact integrated cardiopulmonary bypass unit consisting of an artificial lung (Kurare Menox EL-4000) and a Sarns Delphin pump (Sarns 3M Healthcare, Ann Arbor, MI, USA). An oxygenator and a centrifugal pump are placed in the body of the compact integrated cardiopulmonary bypass unit as reported previously.[10] Heparin was used for anticoagulation and activated clotting time was maintained between 200 and 300 s.

Study patients

Between January 1993 and December 2001, 27 patients were diagnosed as having acute myocarditis at the National Cardiovascular Centre (Japan). All patients except one had clinical symptoms and signs of acute myocarditis with a distinct onset (from days 2 to 28). The first application of percutaneous ECMO for patients with fulminant myocarditis was in June 1996. The distribution of year when the enrolled patients were admitted was as follows: F group: before 1995, $n = 0$; 1996–98, $n = 5$; 1999–2001, $n = 9$; NF group: before 1995, $n = 2$; 1996–98, $n = 5$; 1999–2001, $n = 6$.

Fourteen patients, whose systemic blood pressure was low [74 ± 15 (mean $\pm$ SD) mmHg] and heart rate was high (134 ± 21 b.p.m.; excluding two patients with cardiac arrest and using temporary right ventricular pacemaker) even after an intensive treatment with inotropic or vasopressor drugs, required percutaneous ECMO (F group) (male, seven; female, seven; mean age, 38 ± 15). The remaining 13 patients whose blood pressure and heart rate were maintained at 118 ± 17 mmHg and 86 ± 21 b.p.m., respectively, were not treated with percutaneous ECMO (NF group) (male, 12; female, one; mean age, 33 ± 18).

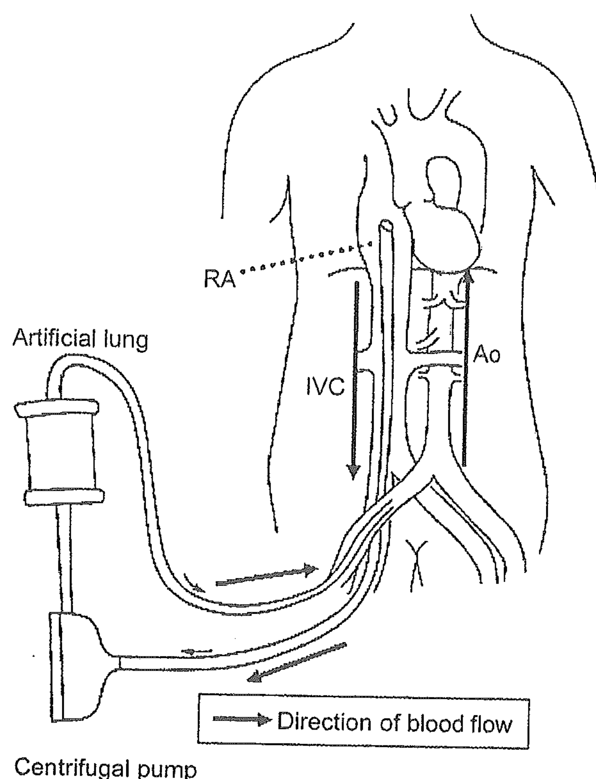

Figure 1 Illustration of ECMO system. RA, right atrium; IVC, inferior venacava; Ao, aorta.

Laboratory examination

On admission, blood samples were obtained every 3 h until the peak CPK and CK-MB levels were determined; thereafter, at least every 24 h until the patients recovered. Inflammation indexes (white blood cell count and C-reactive protein level), liver function (total bilirubin, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase levels), and renal function [blood urea nitrogen (BUN) and serum creatinine levels] were also analysed.

Echocardiographic and haemodynamic measurements

Standard two-dimensional echocardiography (SONOS 5500, Phillips) was performed to assess the existence of pericardial effusion and to determine left ventricular end-diastolic dimension (LVDd), end-systolic dimension (LVDs), and wall thickness. These parameters of LV function were measured in the M-mode from the parasternal short-axis view using the leading-edge-to-leading-edge method. Fractional shortening (FS) was also calculated by a standard method.[11] Inferior venacava (IVC) diameters were measured from the long-axis two-dimensional subxiphoid views with the patients in a supine position to 30° upright position.[12] Flow across the valves was assessed by colour Doppler to grade the degree of mitral and tricuspid regurgitation. A 7.5 F Swan-Ganz thermodilution catheter (model: T-157A; Goodtech Inc.) was inserted through the internal jugular vein or the femoral vein to measure cardiac index, pulmonary capillary wedge, and right atrial pressures.

On admission, the data were obtained every 24 h until the patients were weaned successfully from the percutaneous ECMO system. During the period of using ECMO, we also measured LV ejection time corrected for $\sqrt{RR}$ (LVETc). When LVETc improved to >200 m s, ECMO flow rate was gradually decreased to 1.5 L/min, and ECMO was then discontinued if haemodynamics did not deteriorate.[13]

Endomyocardial biopsy and postmortem autopsy

Endomyocardial biopsies were performed via the right internal jugular or femoral veins using disposable bioptomes in surviving patients in stable conditions. Postmortem examination was also performed. At least four specimens were obtained from the right ventricular septum and immediately immersed in 10% formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and examined by a pathologist to determine whether myocarditis was present on the basis of the Dallas criteria.[14]

Follow-up

After discharge, patients visited the hospital every 3–6 months. In the chronic phase (~6–12 months), echocardiography was performed to reassess LV function following myocarditis. The median period of chronic echocardiography was 12 months. Thereafter, follow-up data regarding death and cardiovascular events (e.g. rehospitalization due to congestive heart failure) were obtained from the medical records or telephone interviews of all patients.

Statistical analyses

The values are presented as the mean ± standard deviation (SD) or median (25–75%). The normality of distribution was assessed using the Kolmogorov–Smirnov test. Echocardiography and laboratory findings were compared between the two groups using the Student's *t*-test for normally distributed variables or the Mann–Whitney *U* test for other variables. To compare the proportions of patients, Fisher's exact test was performed. Comparisons of data using all these statistical tests were performed using Sigma Stat version 3.0 (SPSS, Chicago, IL, USA). All statistical tests were two-sided and significance was defined as $P < 0.05$.

Results

Comparisons between patients who were weaned and those who were not weaned from ECMO in the F group

Table 1 shows the summary of the patients' characteristics in the F group. The median time interval to ECMO application from the onset of heart failure was 15 (12–20) h (range: 7–36 h). Among the 14 patients in the F group (on ECMO support), a temporary right ventricular pacemaker was used in four patients (29%). In six patients (43%), intraaortic balloon pumping (IABP) had already been inserted because they had been transferred from other hospitals. Between patients with and without IABP, systolic blood pressure (75 ± 17 vs. 73 ± 15 mmHg), own heart rate (127 ± 17 vs. 138 ± 22 b.p.m.), LVDd (47±8 vs. 46 ± 12 mm), and FS (10±3 vs. 10 ± 5%) were similar before ECMO application. *Figure 2* shows acute changes in LV function before and immediately after the support and at the time of weaning from ECMO. Neither LVDd nor FS changed immediately after the ECMO support.

The median support time for percutaneous ECMO in the F group was 130 (42–171) h (maximum support time, 12 days). Four patients were not weaned from mechanical support and died. In one of them, the support system was changed to a left ventricular assist device (the Toyobo-NCVC-type pump)[15] because of the development of multiple organ failure despite ECMO support. Therefore, the acute survival rate was 71% in the F group.

We then compared the clinical characteristics between patients who were weaned and those who were not weaned from ECMO (*Table 2*). Although systemic inflammation indexes (white blood cell count and C-reactive protein level) and liver function were similar, the peaks of CK-MB level and BUN level differed significantly between patients who were weaned and those who were not weaned from ECMO.

Figure 3 shows echocardiographic measurements for patients in the F group. Patients who were not weaned from ECMO had smaller LVDd (36 ± 10 vs. 50 ± 7 mm, $P = 0.013$) and LVDs (34 ± 10 vs. 45 ± 6 mm, $P = 0.026$) and thicker ventricular wall (15±1 vs. 11 ± 2 mm, $P = 0.023$) than those who were weaned from ECMO. The left ventricular systolic function in patients who were not weaned from ECMO was more depressed than those who were weaned successfully, as shown by the difference in FS (5±4 vs. 11 ± 4%, $P = 0.036$).

Comparison between F group and NF group

All the 13 patients in the NF group survived after the onset of acute myocarditis. IABP was used in one patient in the NF group. Inotropic agents were used under haemodynamic monitoring in five of 13 patients in the NF group and in 14 of 14 patients in the F group ($P < 0.05$). The median doses of dopamine [NF: 0 (0–3.25) vs. F: 5.5 (3–15) μg/kg body weight/min, $P = 0.002$] and dobutamine [NF: 0 (0–3) vs. F: 3 (3–10) μg/kg body weight/min, $P = 0.017$] used were significantly lower for patients in the NF group than for those in the F group. There were significant differences in stroke volume index (NF: 29 ± 12 vs. F: 19 ± 8 mL/beat/m^2, $P = 0.048$), pulmonary capillary wedge (NF: 15 ± 6 vs. F: 23 ± 5 mmHg, $P = 0.013$), and right atrial pressure (NF: 8±4 vs. F: 14 ± 6 mmHg, $P = 0.026$) between these two groups. FS assessed by echocardiography on admission was moderately depressed in patients in the NF group when compared with that in those in the F group (23±8 vs. 10 ± 4%, $P < 0.001$), although peak CK-MB levels and systemic inflammation indexes (e.g. white blood cell count and C-reactive protein level) were similar between these two groups (*Table 3*). Liver and renal functions were preserved in patients in the NF group, whereas these were impaired in patients in the F group.

Follow-up study and clinical course

Endomyocardial biopsy or postmortem examination was performed at 25 (5.75–36.5) days in nine of 14 patients in the F group and at 14.5 (8.5–25.5) days in 12 of 13 patients in the NF group. The percentages of patients positive for myocardial infiltration by inflammatory cells were 78% (seven of nine patients) for the F group and 58% (seven of 12 patients) for the NF group. Moreover, as shown in *Figure 4*, echocardiography performed at the chronic stage (6–12 months) demonstrated that FS reversed dramatically in the F group reaching a similar level to that in the NF group (F: 33 ± 7%, NF: 34 ± 6%), although LVDd did not change throughout the study.

Figure 5 shows the summary of the clinical course. The follow-up period was 50 (40–66) months for the F group and 66 (37–81) months for the NF group. Only one patient in the F group had congestive heart failure 14 months after the onset of acute myocarditis. None of the patients died or received cardiac transplantation in both groups.