milieu)が重要とされている[27,28]が，その詳細は不明である。

著者らは，細胞同士の直接接触が環境因子のひとつであるとして以下の実験を行った[29]。ラット新生児心筋細胞をホスト心筋(CM)とし，GFP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，共培養実験系を作製した。GFP-BMCとCMとの間に隔壁をおいたdouble chamber培養では，GFP-BMCに特に変化を認めなかった。これに対し，GFP-BMCとCMを混合した共培養系では，あるGFP-BMCは，2日後からCMと同期収縮を開始するものが現れた。また，免疫組織染色では，myosin heavy chain-slow(1日後から)，コネキシン43と心房性ナトリウム利尿ペプチド(ANP)(2日後から)，トロポニンI(4日後から)が経時的に発現し漸増した。5日後にはmyosin heavy chain-slow陽性細胞はおよそ2.5%になった。この結果，幹細胞の心筋分化には，ホストの心筋細胞との直接接触が重要な役割を果たしていることが明らかになった。また，循環血液中のヒト骨髄細胞が心筋細胞に分化することが報告されている[30]。2002年に，細胞融合(cell fusion)の問題が報告された[31]。この報告は，ES細胞とGFPマウス由来骨髄細胞との共培養により，一見GFPを発現した細胞が分化増殖するようにみえるが，その細胞の核内にはES細胞由来のDNAも含んでいたとしている。しかし，細胞融合の割合が低いため，この現象は十分には解明されていない。Ohらは，成人心臓からの幹細胞抗原-1を発現する心臓前駆細胞が，ホスト細胞との融合があってもなくても，ほぼ同等に心筋へ分化することを報告した[32]。融合が生理的過程として起こっているのであれば，今後の研究が必要となるであろう。

おわりに

心臓への細胞移植について種々の研究がなされており，その中で用いる細胞種の一つとして骨髄幹細胞がある。また，従来からの外因性細胞移植のみならず内因性細胞移植による心筋再生も注目されている。さらに，心筋への分化における環境因子に関しても検討されている。骨髄幹細胞は，倫理的側面を含み臨床応用が行いやすい細胞種であり，今後さらに検討が進むものと考えられる。

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お知らせ

広島がんセミナー・鳥取バイオサイエンス振興会 国際シンポジウム

Cancer and Epigenetics

—Basic Research and Clinical Implication—

日時　2004年10月30日(土)—31(日)
場所　広島国際会議場
(広島市中区中島町1-5　広島平和記念公園内
TEL 082-242-7777)

Symposium

1. To be announced
Jean-Pierre Issa (University of Texas M D Anderson Cancer Center, USA)
2. Studies of epigenetic regulation in imprinted domains in cancer
Mitsuo Oshimura (Tottori University, Tottori)
3. The role of histone modification in the imprinted gene
Satoshi Fujii (Hiroshima University, Hiroshima)
4. Role of histone modifications in tumor suppressor gene silencing in cancers
Yutaka Kondo (University of Texas M D Anderson Cancer Center, USA)
5. The epigenetic hypothesis of cancer
Andrew P. Feinberg (Johns Hopkins University, USA)
6. Role of methylated DNA-binding proteins in transcription and genome stability
Mitsuyoshi Nakao (Kumamoto University, Kumamoto)
7. Function and regulation of the AML 1 transcription factor complex
Issay Kitabayashi (National Cancer Center Research Institure, Tokyo)
8. Methylation pressure in neuroblastomas with poor prognosis
Toshikazu Ushijima (National Cancer Center Research Institute, Tokyo)
9. Epigenetic regulation of human genome universally involves CTCF/BORIS binding regions
Victor V. Lobanenkov (NIH-NIAID, USA)
10. Genetic and epigenetic regulation of cell cycle genes in human aging
Hidetoshi Tahara (Hiroshima University, Hiroshima)
11. Histone deacetylases-Novel targets for cancer therapy-
Eric Verdin (University of California, San Francisco, USA)
12. Chemical genetic approach to cancer therapy
Minoru Yoshida (RIKEN Wako Institute, Saitama)

主催　財団法人広島がんセミナー
財団法人鳥取バイオサイエンス振興会
参加登録　事前登録要(参加費当日支払　5000円)
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最近の話題から

ここまで進んだ補助人工心臓

中谷武嗣
国立循環器病センター部長

人工心臓には、患者さんの心臓を取り去って人工心臓に置き換える「全置換型」と、心臓はそのまま残しながら、心臓の働きを助ける「補助人工心臓」があります。補助人工心臓には「体外設置型」と「体内埋め込み型」がありますが、最近は体内に埋め込んで使用するタイプの研究開発が積極的に進められ、2004年4月から、体内埋め込み型補助人工心臓に健康保険が適用されています。ここでは、新型の補助人工心臓について紹介します。

新型補助人工心臓とは

●外出も可能な体内埋め込み型

人工心臓の開発研究は、1950年代後半から進められてきました。現在は、重症の心臓病の治療には心臓移植が行われますが、移植のための臓器の不足が問題であり、心臓移植を受けるまでの〝つなぎ〟の治療として補助人工心臓が位置づけられています。

開発当初は全置換型が主流でしたが、その後、補助人工心臓で十分に心臓の働きが助けられることがわかり、現在はこちらの開発が中心に行われています。

しかし、体外設置型では、大きな装置が必要であり、その装置につながれるため、患者さんは病院内は歩くことができても、外に出ることはできません。そこで近年積極的に開発研究が進められているのが、体内に埋め込むタイプの補助人工心臓です。

●主として左心室の働きを補助する

心臓は、肺で酸素を取り込んだ血液を「左心室」から全身に送り出し、戻ってきた血液を「右心室」から肺に戻す循環の働きをする臓器です。このうち左心室は、血液を全身に送り出すために大きな力を必要とします。ところが、心臓の筋肉（心筋）が障害される「心筋症」のような病気になると、心臓の収縮力が弱まり、弱い力で血液を全身に送ろうとするために、左心室は徐々に大きくなります。そして、大きくなると、さらに収縮力が落ちてしまうという慢性的な悪循環に陥ります。このような変化は数年かけて進行することが多く、最後は心臓が伸びきった風船のようにな

なかたに・たけし
1951年生まれ。神戸大学医学部卒業。専門は人工臓器、補助循環、心臓移植、組織および細胞移植、心臓血管外科

り、十分に機能しなくなる、重症の「心不全」となります。

補助人工心臓は、このような状態の患者さんに対して、主に左心室の働きを助けるために使用します。

体内埋め込み型の仕組み

体内に「血液ポンプ」を埋め込み、「左心室」に管を入れて、血液をポンプ内に取り込み、ポンプの拍動によって、大動脈へと送り込みます。つまり、心臓内で行われる左心室から大動脈への血液の送り出しを、心臓の外部で行うことになります。拍動数はポンプに流れ込む血液の量に応じて変化しますが、通常は1分間に70～80回です。

体内には血液ポンプと駆動装置を埋め込み、電源や制御装置は体外に設置します(左の囲み参照)。電池は4～5時間有効で、取り換えることができます。睡眠時はコンセントにつないで充電することができます。

また、装置の耐久性は良好ですが、使用期間が長くなれば、新しい装置との交換が必要となります。

治療を受けられる場合

埋め込み型の補助人工心臓による治療で健康保険が適用されるのは、「拡張型心筋症」か「拡張相肥大型心筋症」で、「心臓移植」待機中の患者さんです。

また、使用するためには、「心臓以外の臓器に大きな障害がない」「装置を埋め込むだけのスペースがある体格である」などの条件を満たすことが必要です。

補助人工心臓の効果

体内埋め込み型の補助人工心臓の効果は以下のようなものです。

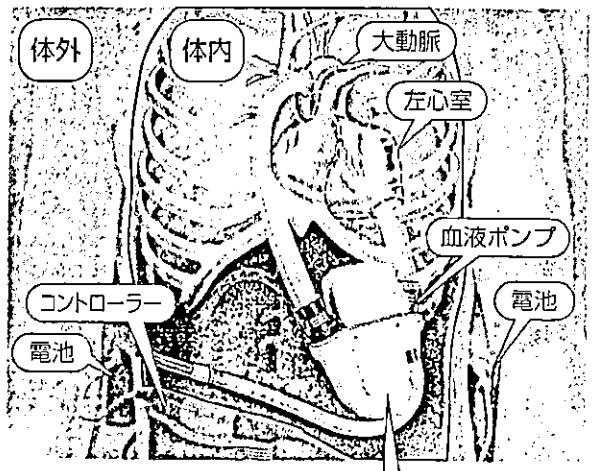
▼延命効果……米国で慢性心不全の患者さんを、補助人工心臓による治療と薬物治療に分けて、効果を比較した研究が行われました。その結果を1年生存率で比較すると、薬物治療では25%でしたが、補助人工心臓をつけた場合は52%でした。

▼生活の質の向上……体力が戻り、楽に動

治療の対象

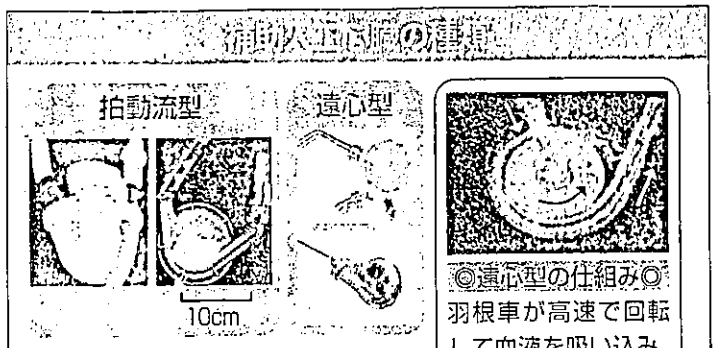
- ①「拡張型心筋症」「拡張相肥大型心筋症」で、心臓移植待機中の患者さん
- ②心臓以外の臓器に大きな障害がない
- ③装置を埋め込むのに十分な体格

拍動流型補助人工心臓



上図の赤い部分は体外。左上腹部に装置を埋め込み、左心室の先端に管を入れて血液をポンプ内に導き、電気で拍動させ大動脈に送る。

拍動流型補助人工心臓の内部。通常1分間に70～80回拍動する。



拍動流型のうち、写真左が健康保険適用になった装置。写真右の拍動流型は、臨床試験が終わったところ、遠心型は臨床試験の準備段階。

◎遠心型の仕組み◎
羽根車が高速で回転して血液を吸い込み、送り出す。血液は拍動によって送られるのではなく、継続して流れる。弁がないので拍動流型より小型。

けるようになります。一般に、重症の心不全で薬物治療を行っている場合、「体が非常にだるく、ベッドの上でも動くことができない」などの状態になりますが、補助人工心臓をつけると、そのだるさやつらさが解消されます。あまり激しい運動はできませんが、通常のデスクワークなどはできるようになります。

▼心臓機能の回復……補助人工心臓をつけていることで、自分の心臓の負担が減り、機能が回復した例があります。

生活上の注意点

▼抗血小板薬と抗凝固薬の服用……補助人工心臓を入れると、血栓ができやすくなりますので、この2種類の薬を服用して血栓を予防します。ただし出血した場合、血が止まりにくくなりますから、けがなどをしないように注意する必要があります。

▼感染症に注意……人工物を体内に入れているため、感染症の治療が困難になります。感染症にかからないよう、小さな傷などでも速やかに対処してください。また、入浴の際、湯船につかることはできません。装置の挿入口を覆ってシャワーを浴びます。

今後の課題と展望

さらに進む研究開発

体内埋め込み型の補助人工心臓で現在使われているのは、「拍動流型」といわれる装置です。このほかに、さまざまなタイプの開発が進められており、実用可能になれば、患者さんに合った安全性の高いもの、体格に合ったものが選べるようになるでしょう。例えば「遠心型」は、逆流防止弁がないため小型で、体の小さい人も使用できるようになることが期待されます（上の囲み参照）。ほかに、制御装置まで埋め込むタイプの研究も進められています。

将来は治療の選択肢になることも

日本では、94年からこれまで約20人の患者さんが今回健康保険に採用された体内埋め込み型補助人工心臓を設置し、使用期間は平均1年くらいで、最長3年でした。補助人工心臓を長期間使用する一因として、心臓移植を必要としている人に対する臓器の不足が挙げられます。臓器提供に関する意思表示カードの普及率が低く、心臓移植が少ないことが、日本の心不全治療の課題の一つといえるでしょう。

現時点では、補助人工心臓は、心臓移植までのつなぎの治療です。しかし、ヨーロッパには補助人工心臓を6年使い続けている例もあり、将来的には、心臓移植か補助人工心臓による治療かを選択できるようになることが期待されます。

（この内容は5月31日に放送されたものです）

写真資料協力：エドワーズライフサイエンス株式会社、サンメディカル技術研究所、ソラテック、テルモ株式会社（五十音順）

特集 XII

Brain & Heart attack —脳虚血性疾患と心臓血管系の関連—

補助人工心臓，心臓移植時の Brain attack

Brain attack in the patients with ventricular assist systems and after heart transplantation

国立循環器病センター臓器移植部
Office of Heart Transplant Project, Department of Organ Transplantation, National Cardiovascular Center

中谷 武嗣（部長） *Nakatani Takeshi*
花谷 彰久 *Hanatani Akihisa*

KEY WORDS

- 補助人工心臓
- 免疫抑制剤
- 抗血小板療法
- PT-INR

SUMMARY

補助人工心臓および心臓移植は，末期心不全患者に対する強力な治療選択であるが，それぞれ血栓塞栓症および感染症対策と，拒絶反応の制御が患者管理において重要である。補助人工心臓施行例における補助期間は，体外設置方式においても1年を越えるようになってきたが，死因としては脳障害および感染症が大部分を占めている。このため，ワルファリンおよび抗血小板剤による強力な抗凝結療法が行われるが，脳障害，特に脳出血を引き起こすと早期にPT-INRの是正を行わなければ致命的となる。また，感染症から引き起こされる脳出血もあり，補助人工心臓システムの皮膚貫通部における感染管理が重要である。心臓移植においては免疫抑制療法が行われるが，主要免疫抑制剤であるシクロスポリンおよびタクロリムスに脳症を起こす可能性があり，注意が必要である。

はじめに

末期心不全に陥った症例に対しては，心臓機能置換としての補助人工心臓（VAS）あるいは心臓移植の適応が考慮される。両者ともに強力な治療選択枝であり心臓ポンプ機能の代行は可能であるが，VASにおいては血栓塞栓症や感染症，心臓移植においては拒絶反応の管理が大きな問題となる。本稿においては，Brain attackの観点から両者について自験例を中心に述べる。

補助人工心臓(VAS)における Brain attack

1．VASシステムおよび抗凝結療法[1]

現在わが国で用いられる主なVASとしては，体外設置方式の東洋紡型およびゼオン型と，体内収納方式のNovacor®およびHeartMate®がある。HeartMate®以外のVASの血液接触面は，smooth surfaceとなっており，抗凝結療法として，ワルファリンおよびヘパリンによる抗凝固療法と，抗血小板療法の併用が行われる。これに対し，HeartMate®はrough surfaceとしているが抗血栓性に優れており，通常は抗血小板療法のみが行われる。

われわれはこれまで主に東洋紡型VASを使用してきた。当初ワルファリン（PT-INRの目標値：2～3）あるいはヘパリンによる抗凝固療法による管理を行ったが，早期に血液ポンプ内に血栓形成を認めることが多く，頻回の血液ポンプ交換を行わざるを得なかった。装着術後早期の血栓形成においては，白色血栓が多く，白血球数1万/㎣以上および血小板数10万/㎣以上になった場合に発生しやすかった。そこで，現在ではsmooth surfaceである東洋紡型およびNovacor®における抗凝結療法として以下のように行っている。

経口摂取が開始された段階で，ワルファリンを開始し，当初の目標PT-INRは2とし，その後3～4を目標値とする。経口摂取ができない場合には，経鼻胃管からの投与を試みるが，経口や経鼻胃管からの投与が早期に行えない場合には，外科的出血が落ち着いた段階で低分子ヘパリンを投与する。なお，PT-INRが目標値以下の場合は，表1に示すようにワルファリン増量による調整と，低分子ヘパリン（フラグミン®）を併用する。また，PT-INRが目標値以上の場合は，ワルファリンの減量，休薬と凍結血漿の投与を表1のプロトコールに従って行う。なお，経口摂取不良となった場合，発熱，疼痛などにより鎮痛解熱薬を投与した場合，および感染症を伴った場合には，PT-INRの上昇に注意する必要がある。併用する抗血小板療法としては，経口摂取が開始された段階で，外科的出血を考慮したうえで，アスピリン81mg®：1錠/日で開始する。また，血小板数が10万/㎣を超えた場合にはできるだけ早期に投与を開始する。投与開始4日から1週後に血小板機能検査（ずり応力下血小板血栓形成能）を行い，必要に応じ投与量の追加あるいは減量を行う。その後，適宜，血小板機能検査を行い，アスピリンの投与量を調整する。

表1　左心補助人工心臓装着患者における抗凝固療法

PT-INR	ワルファリン	フラグミン®（単位/kg/時間）	凍結血漿
＜2	増量	10	(−)
2～2.5	増量	7.5	(−)
2.5～3	増量	5	(−)
3～4	【目標域】	(−)	(−)
4～5	減量，休薬	(−)	(−)
5～5.5	休薬	(−)	(考慮)
5.5＜	休薬	(−)	投与

2．国立循環器病センターにおけるVAS適応患者におけるBrain attack

当センターにおける慢性心不全急性増悪例に対するVAS適応例は66例（HeartMate® VE 3例およ

表2　国立循環器病センターにおける慢性心不全急性増悪例に対する補助人工心臓装着例の成績

結果	症例数（例）	補助期間（日）	平均（日）	＞1年（例）
心臓移植	17	39～993	450	10
離脱	8	90～310	149	—
補助中	15	20～1,089	524	8
死亡	26	7～1,245	345	9
計	66	7～1,245	389	27

びNovacor®2例を含む）であるが，その成績を表2に示す。平均補助期間は389日と1年以上に及び，27例は1年以上の補助例である。死亡例は26例であるが，平均補助期間は約1年で，死因の内訳は図1に示すように感染症6例，脳出血15例，脳梗塞3例である。しかし，脳出血15例中5例は創部感染症に引き続くもので，剖検においても脳血管に小さな感染性脳動脈瘤を認めた。また，2例は脳梗塞後の脳出血例であった。図2に最近多く用いる東洋紡製左室脱血方式左心補助人工心臓装着44例における累積補助期間を示す。その死因の多くは脳障害に伴うもので，感染症から引き続く脳出血も5例にみられた。このような脳出血例への対応としては，できるだけ速やかにPT-INRの是正を行い，出血に伴う病変が小さい状態でコントロールすることができるかどうかが予後を左右する。最近われわれは，投与早期にPT-INRを是正できる乾燥人血液凝固第9因子複合体製剤を用い，良好な結果を得ている。なお，脳出血における開頭手術は，出血PT-INRが是正され，出血範囲が小さい場合には効果を期待できるが，広範囲に及んだ場合は不良である。

また，感染防止はBrain attack予防にも有効であり，体外設置型における送血および脱血管あるいは体内収納型における駆動チューブの皮膚貫通部のケアに配慮することが重要である。

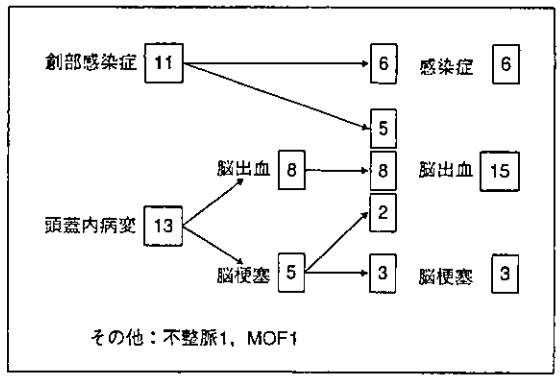


図1　国立循環器病センターにおける左心補助人工心臓装着例における死因

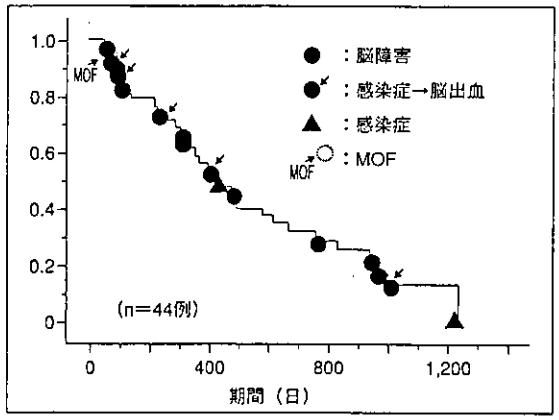


図2　国立循環器病センターにおける東洋紡製左室脱血方式左心補助人工心臓装着例の累積補助期間

心臓移植における Brain attack

心臓移植手術においては，ドナー心臓の吻合部，特に左房および右房（bicaval法では上下大静脈）における血栓形成の危険性は否定できず，抗血小板剤の服用が行われる場合が多い。しかし，移植後において注意すべきものとして，薬剤，特に免疫抑制剤による中枢神経・精神障害があり，痙攣，頭痛および意識障害などの神経学的徴候に画像診断によって，大脳白質の後頭から頭頂部を中心に異常所見を

示す免疫抑制剤関連脳症に注意する必要がある[2]。

最近われわれが経験した症例では，移植後2週間が経過し，シクロスポリン，ミコフェノール酸モフェチルおよびプレドニン®による三者併用による免疫抑制を行いながら，移植病棟でリハビリを開始していた。突然，視野障害と一過性の意識レベル低下が出現し，さらに，全身痙攣，左共同偏視を認めるようになった。頭部CTを施行したところ，後頭葉皮質下に限局したLDAを認め，当初脳梗塞を疑った。2日後にMRI検査を行ったところ梗塞巣はなく，後頭葉白質に浮腫状の変化を認め，シクロスポリンによる可逆性後頭葉白質脳症（RPLS）と考えた。その後シクロスポリンからタクロリムスに変更したところ，1週間後には，MRIにて後頭葉白質の浮腫は改善し，視野障害などの症状も軽快した。1ヵ月後には後頭葉白質の浮腫は消失し，神経障害も認めなくなった。

まとめ

補助人工心臓による補助期間は従来考えられていたより長期になり，体外設置方式においても3年以上の補助例がみられるようになってきた。その死因も大多数はBrain attackに伴うものであり，抗凝結療法および抗感染対策を効果的に行うことが重要である。また，心臓移植においては，免疫抑制剤によるBrain attackとして，基本免疫抑制剤であるシクロスポリンおよびタクロリムスによる脳症が報告されており，注意が必要である。

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用語解説

●補助人工心臓

Ventricular assist system（VAS），自己心の近傍に装着される血液ポンプおよび駆動装置からなる循環補助手段。血液ポンプの設置部位により体外設置方式と体内収納方式があり，最近では完全埋込みシステムの臨床応用も開始されている。

●PT-INR

ワルファリンの薬理効果の指標とされてきたプロトンビン時間（PT）を標準化するために提唱された指標（International Normalized Ratio：INR）。

●シクロスポリンおよびタクロリムス：

三者併用療法に用いられ，ともにTリンパ球内においてカルシニューリンと結合しIL-2産生を抑制する。

Bone Marrow Mononuclear Cell Transplantation Had Beneficial Effects on Doxorubicin-induced Cardiomyopathy

Michiko Ishida, MD,[a,b] Shinji Tomita, MD, PhD, FAHA,[a,b] Takeshi Nakatani, MD, PhD,[c] Shinya Fukuhara, MD,[d] Masaki Hamamoto, MD,[a,b] Noritoshi Nagaya, MD,[e] Yoshinori Ohtsu, MD,[a] Michiharu Suga, MD, PhD,[a] Chikao Yutani, MD, PhD,[d] Toshikatsu Yagihara, MD, PhD,[b] Kazuhiko Yamada, MD, PhD,[a] and Soichiro Kitamura, MD, PhD[b]

Background: Cell transplantation is a promising therapy for treating end-stage heart failure. Bone marrow mononuclear cells (BMMNC) have been used to enhance angiogenesis in ischemic heart disease. However, the effect of BMMNC transplantation in non-ischemic dilated cardiomyopathy is unknown. In this study, we evaluated the efficacy of BMMNC transplantation in doxorubicin-induced cardiomyopathy in a rat model.

Methods: Doxorubicin (15 mg/kg, IP) was introduced into 52 Lewis rats. They were divided into 3 groups at 4 weeks after injection: transplant group (TX, BMMNC [1×10^6] implantation, $n = 18$), control group (CN, saline injection, $n = 18$), and sham group (SH, thoracotomy, $n = 16$). At 4 weeks after surgery, we used echocardiography to measure systolic left ventricular diameter (LVDs), diastolic left ventricular diameter (LVDd), fractional shortening (FS), and left ventricular wall thickness/LVDs. We used a Langendorff apparatus to measure systolic, diastolic, and developed pressures. We used radioimmunoassay to measure circulating atrial natriuretic peptide concentration, and we performed histologic study, including electron-microscopic study.

Results: Left ventricular wall thickness/LVDs in the TX group was the largest of all groups ($p < 0.05$). Systolic and developed pressures in the TX group were the greatest ($p < 0.005$). Systolic left ventricular diameter, FS, and end-diastolic pressure in the TX group were smaller than in the SH group ($p < 0.05$). These cardiac parameters did not differ significantly between TX and CN groups, but secondary changes (decreased heart weight, developed ascites, and increased atrial natriuretic peptide concentration) caused by doxorubicin-induced heart failure were most attenuated in the TX group. In the TX group, vascular density was greatest ($p < 0.05$) in the left ventricular free wall and in the septum. In addition, electron microscopy showed that myocardium in the TX group was most maintained.

Conclusion: Bone marrow mononuclear cell transplantation had beneficial effects in doxorubicin-induced cardiomyopathy.

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Cell transplantation is a promising therapy for end-stage heart failure, and has been investigated rigorously, especially in ischemic hearts.[1] Ischemic cardiomyopathy and idiopathic dilated cardiomyopathy (IDCM) are the major reasons for heart transplantation.[2] In Japan, patients with IDCM occupy 90% of the registration for heart transplantation,[3] and heart transplantation is limited because of the small number of donated hearts. Few reports of cell transplantation in an IDCM model have been published and include studies of fetal cardiomyocytes,[4] heart cells,[5] and skeletal myoblasts.[6]

Bone marrow mononuclear cell (BMMNC) transplantation has been investigated[7-9] and used clinically for

From the [a]Department of Regenerative Medicine and Tissue Engineering, [b]Department of Cardiovascular Surgery, [c]Department of Organ Transplantation, [d]Department of Pathology, and [e]Department of Cardiology, National Cardiovascular Center, Osaka, Japan.

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Reprint requests: Dr. Takeshi Nakatani, Department of Organ Transplantation, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, Japan 565-8565. Telephone: +81-6-6833-5012; Fax: +81-6-6872-8160; E-mail: tnakatan@res.ncvc.go.jp

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ischemic heart disease.[10,11] Bone marrow mononuclear cell transplantation is easy for clinical application because of its simplicity and autologous model. Therefore, this method does not involve the problems of ethics and immune rejection. The cells also are suitable cell sources because of their capacity for differentiation to multipotential progenitor cells and secretion of angiogenic growth factors. However, the efficacy of BMMNC transplantation in IDCM has never been investigated.

In this study, we examined the effect of BMMNC transplantation in doxorubicin-induced cardiomyopathic heart failure in a rat model.

METHODS

Animal Preparation

We used adult male Lewis rats (230-270 g). All procedures, approved by the Animal Care Committee of the National Cardiovascular Center, were performed under the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1985). The rats were maintained at 22°C with a 12-hour light/dark cycle and had free access to standard rodent chow and tap water.

Preparation of BMMNC

The rats were anesthetized with IM administration of ketamine hydrochloride (3 mg) and IP injection of sodium pentobarbital (10 mg) and heparin (100 U).[7] After dislocation, both legs were cut and bone marrow was extracted with a 22-gauge needle. The bone marrow cells were transferred to a sterile tube containing phosphate-buffered solution (PBS). The cell suspension was loaded on a Percoll gradient (Lymphoprep, Amersham Biosciences; Piscataway, NJ). The cells were centrifuged at 800*g* for 20 minutes at 4°C. The top 66% of the total volume was transferred into a tube and then washed with PBS to remove the Percoll. The cell pellet was resuspended with PBS to obtain a concentration of 1×10^6 cells in 40 μl.

Generation of Doxorubicin-induced Cardiomyopathy and BMMNC Transplantation

We induced heart failure with doxorubicin as described by Suzuki et al[6] Briefly, we administered doxorubicin hydrochloride (Sigma Chemical; St. Louis, MO) in 6 equal injections (each containing 2.5 mg/kg in 0.5 ml saline, IP) to 52 Lewis rats during a 2-week period at a total dose of 15 mg/kg. At 4 weeks after the final injection, the rats were divided randomly into 3 groups. Under general anesthesia, we intubated and ventilated the rats at a rate of 180 ml/min, with room air supplemented with oxygen (2 liter/min), using a ventilator (Shinano Medical; Matsumoto, Japan). The heart was exposed through a lateral thoracotomy. In the transplant group (TX, $n = 18$), BMMNC (1×10^6/40 μl) were injected into the left ventricular free wall with a 31-gauge tuberculin syringe. To prevent leakage, we sutured the injection site with 6-0 prolene. In the control group (CN, $n = 18$), we injected 40 μl PBS into the same region, and in the sham group (SH, $n = 16$), we performed only thoracotomy. We closed the chest with 3-0 prolene in 3 layers.

Measuring Heart Function

We performed echocardiography just before surgery and at 4 weeks after surgery. We used a Sonos 5500 (Hewlett-Packard, UT) equipped with a 7.5-MHz linear transducer. Each rat was anesthetized using a ventilation mask with 1.5% isoflurane and oxygen at 180 ml/min. The anterior chest wall was shaved, and 2-dimensional images and M-mode tracings were recorded from the parasternal short axis view at the level of the papillary muscles. From the M-mode tracings, we obtained the anatomical parameters in diastole and systole.[12] We measured left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), fractional shortening (FS), and left ventricular posterior wall thickness (LVPW)/LVDs.

At 7 weeks after surgery, we used a Langendorff apparatus to measure heart function.[6] After echocardiography, the rat was anti-coagulated with intravenous heparin injection. A mid-line sternotomy was performed, and the heart was removed. The heart was suspended and perfused with filtered Krebs-Henseleit buffer (in NaCl, 118 mmol/liter; KCl, 4.7 mmol/liter; KH_2PO_4, 1.2 mmol/liter; $CaCl_2$, 2.5 mmol/liter; $MgSO_4$, 1.2 mmol/liter; $NaHCO_3$, 25 mmol/liter; and glucose, 11 mmol/liter; pH 7.4) and equilibrated with 5% CO_2 and 95% O_2 at a pressure of 100 mm Hg. A latex balloon was passed into the left ventricle through the mitral valve and connected to a pressure transducer (Model P231D, Gould Instrument System; Statham, USA), a transducer amplifier (Model AP-641G, Nihon Kohden; Tokyo, Japan), and a differentiator amplifier (Model EQ-601G, Nihon Kohden; Tokyo, Japan). After 20-minute stabilization at a left ventricular end-diastolic pressure of 10 mm Hg, we measured coronary flow in the empty-beating state without pacing. We adjusted the end-diastolic pressure of zero mm Hg by first increasing the balloon volume. We then increased the balloon size by adding water in 20-μl increments until the total volume was 200 μl. We recorded left ventricular systolic and diastolic pressures at each balloon volume without pacing and calculated the developed pressure. We weighed the heart, and after laparotomy collected and measured ascites fluid.

Measuring Atrial Natriuretic Peptide

Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure the circulating atrial natriuretic peptide (ANP) concentration using radioim-

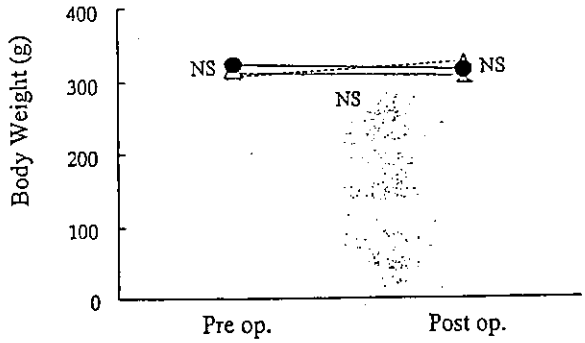

Figure 1. Body weight just before and at 4 weeks after surgery. Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In each group, body weight did not change from before to after surgery. •, transplant group; δ, control group; X, sham group.

munoassay after extraction with Sep-Pak C18 cartridges (Millipore, Waters; Milford, CT).[13] We added and investigated normal rats without any procedures ($n = 5$) for ANP and histologic study as a fourth group.

Histologic Studies

At 4 weeks after surgery, we collected tissue samples (0.5 cm^3) from the injection site (left ventricular free wall) and the remote area (septum) and fixed the samples in neutralized 10% formaldehyde for histologic study. The samples were embedded and cut to yield 6-μm sections, which were stained with hematoxylin and eosin, as described in the manufacture's specifications (Sigma Chemical; St. Louis, MO). Sections also were stained for von Willebrand factor. A pathologist and an orthopediatrician investigated bone formation and tumorigenic formation.

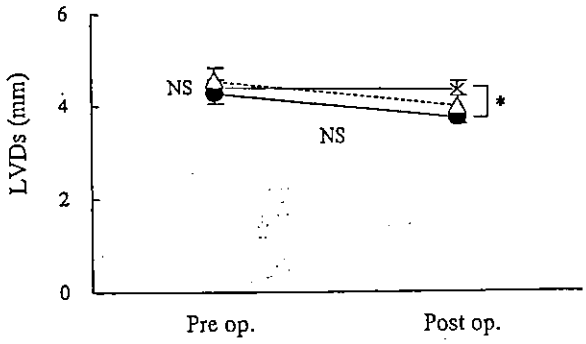

Figure 2. Systolic left ventricular diameter (LVDs) just before, and at 4 weeks after surgery. At 4 weeks after surgery, LVDs was smaller in the transplant group (•) than in the sham group (X), $*p < 0.05$, whereas LVDs in the control group (δ) did not differ from that in the sham group. We found no significant difference between transplant and control groups, and LVDs did not change significantly from before to after surgery in any group.

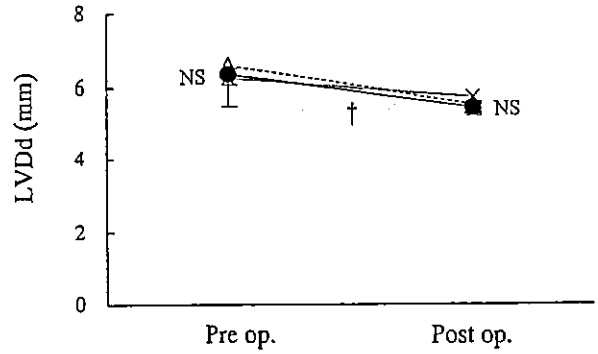

Figure 3. Diastolic left ventricular diameter (LVDd) just before, and at 4 weeks after surgery; LVDd was similar for the 3 groups. In each group, LVDd significantly decreased ($\dagger p < 0.05$). •, transplant group; δ, control group; X, sham group.

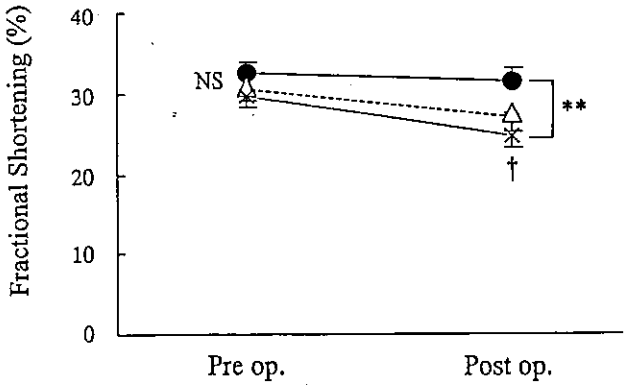

Figure 4. Fractional shortening (FS) just before and at 4 weeks after surgery. The FS was larger in the transplant group (•) than in the sham group (X), $**p < 0.01$, whereas FS did not differ between the control (δ) and sham groups. We found no significant difference between transplant and control groups. In the sham group, FS significantly decreased at 4 weeks after surgery ($\dagger p < 0.05$).

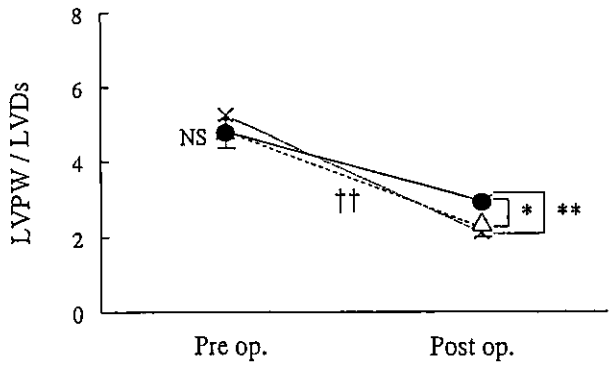

Figure 5. Left ventricular posterior wall thickness (LVPW)/LVDs just before and at 4 weeks after surgery. The LVPW was the greatest in the transplant group (•, $*p < 0.05$, $**p < 0.01$) at 4 weeks after surgery, although it decreased significantly in all groups ($\dagger\dagger p < 0.01$). X, sham group; δ control group.

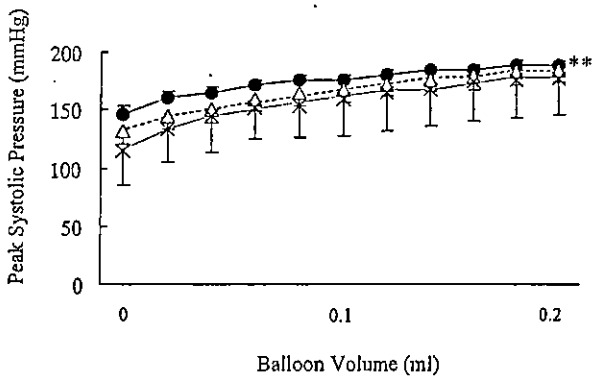

Figure 6. Changes in peak systolic pressure: heart function measured using a Langendorff apparatus at 4 weeks after surgery. Peak systolic pressure was greatest in the transplant group (•), $^{**}p < 0.005$. X, sham group; δ control group.

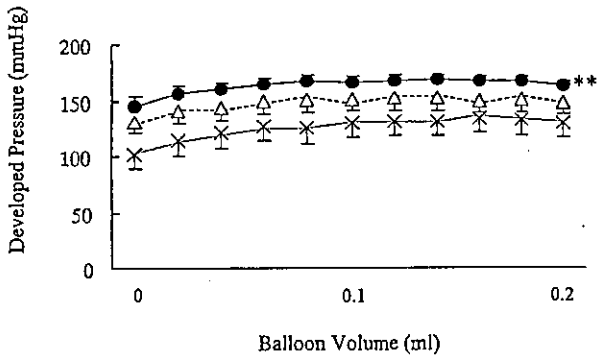

Figure 7. Developed pressure was greatest in the transplant group (•), $^{**}p < 0.0,001$. X, sham group; δ control group.

Measuring Vessel Numbers

An observer masked to the treated groups used light microscopy at ×10 magnification to investigate positive vessel staining for von Willebrand factor in the left ventricular free wall (transplant area) and in the septum (remote area) of all groups. Ten high-power fields in each area were selected randomly, and the number of vessels in each was averaged and expressed as the number of vessels per high-power field (HPF).[14]

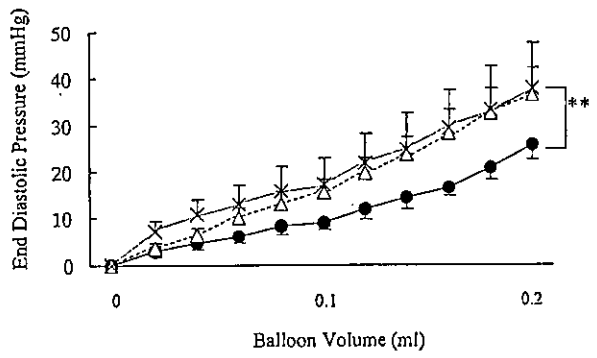

Figure 8. End-diastolic pressure was smaller in the transplant group (•) than in the sham group (X), $^{**}p < 0.0001$. End-diastolic pressure did not differ between the control (δ) and sham groups or between the transplant and control groups, $p = 0.06$.

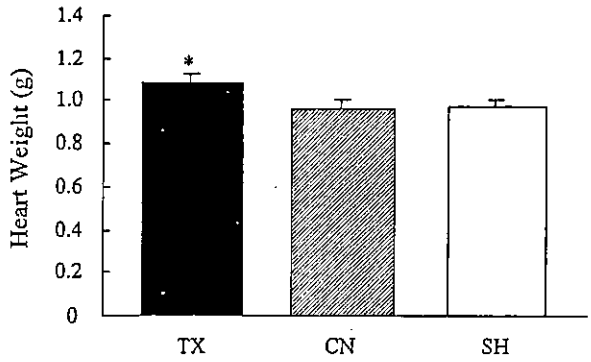

Figure 9. Heart weight at 4 weeks after surgery was greatest in the transplant group (TX),$^{*}p < 0.05$. SH, sham group; CN, control group.

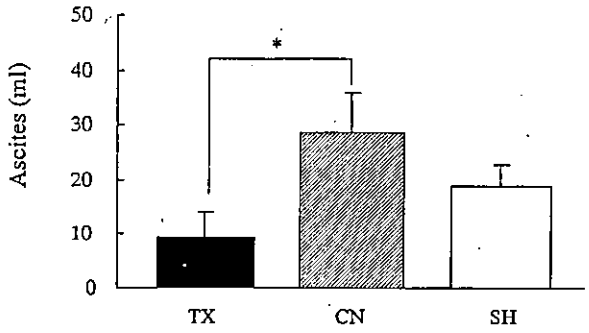

Figure 10. The amount of ascites at 4 weeks after surgery was less in the transplant (TX) group than in the control (CN) group,$^{*}p < 0.05$. SH, sham group.

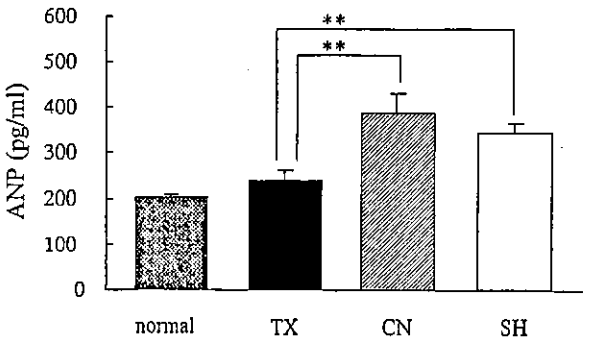

Figure 11. Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure circulating atrial natriuretic peptide (ANP) concentration by radioimmunoassay. Blood was sampled in normal rats using the same method as that used in controls (CN). The ANP concentration in the transplant group (TX), which did not differ from that in the normal rats, was significantly less than that in CN and sham (SH) groups, $^{**}p < 0.01$).

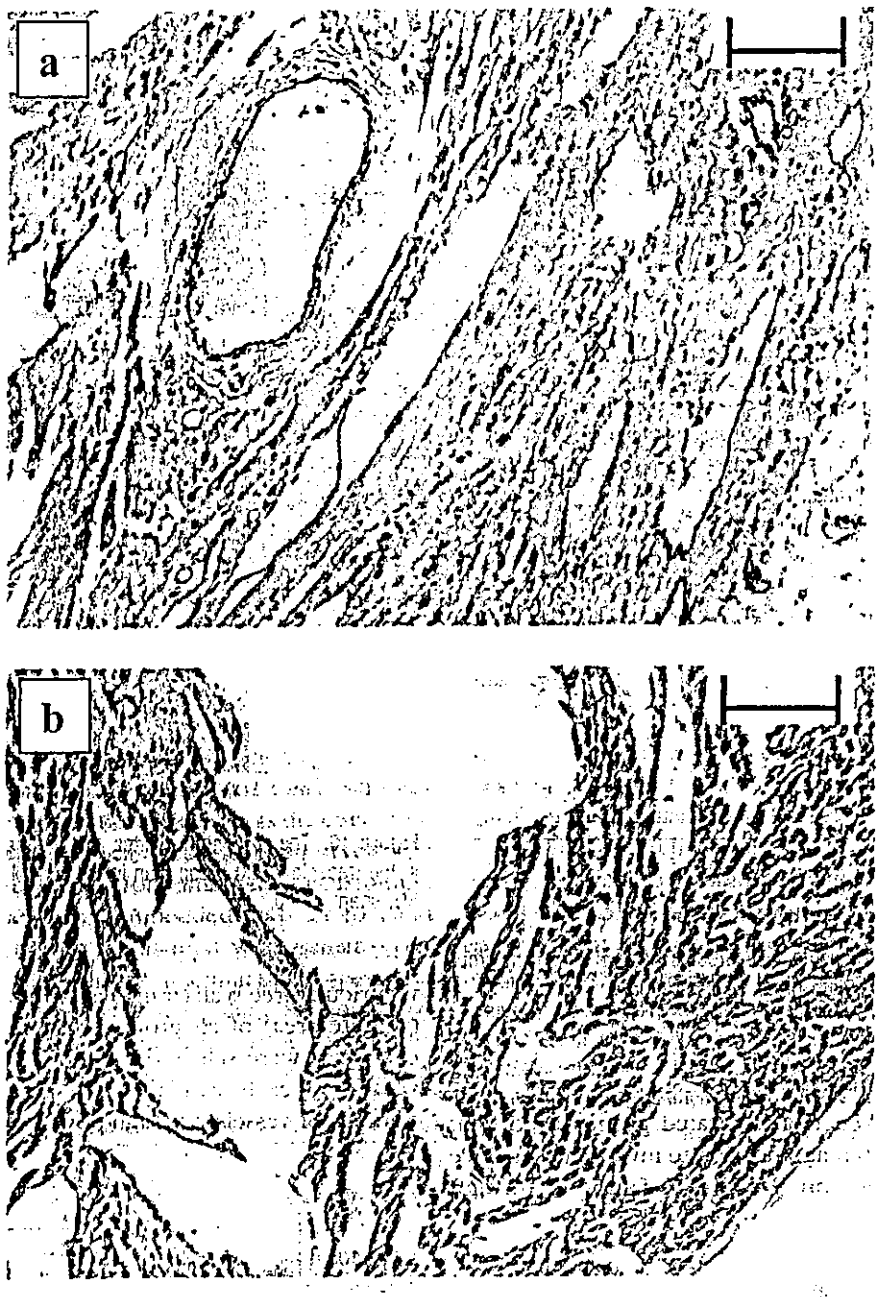

Figure 12. Vessels of the left ventricular free wall (transplant area) stained with von Willebrand factor. a, the transplant group (magnification, ×200); b, the control group (magnification, ×200); and c, the sham group (magnification, ×200). The bar indicates 100 μm. We observed more vessels in the transplant group compared with the control and sham groups. The diameter of most vessels was <50 μm.

Electron Microscopic Study

The samples (n = 2 in each group) taken from the injection site were fixed with 3% glutaraldehyde in 0.1 mol/liter cacodylate buffer (pH, 7.2) for 2 hours at 4°C.[15] These samples were then washed several times with the same buffer and post-fixed with 20% osmium tetroxide for 2 hours at 4°C. After this double fixation, the specimens were washed with 0.1% sodium acetate, stained en bloc with 2% uranyl acetate, washed again with 0.1% sodium acetate, dehydrated through a graded ethanol series, and finally embedded in Spurr's low viscosity resin. Representative areas of each lesion were sectioned at approximately 1 μm thick and stained with toluidine blue solution. Selected areas were trimmed further for thin sectioning and stained with 30% uranyl acetate in 30% ethanol, followed by treatment with Reynolds' lead citrate. The ultra-thin sections were

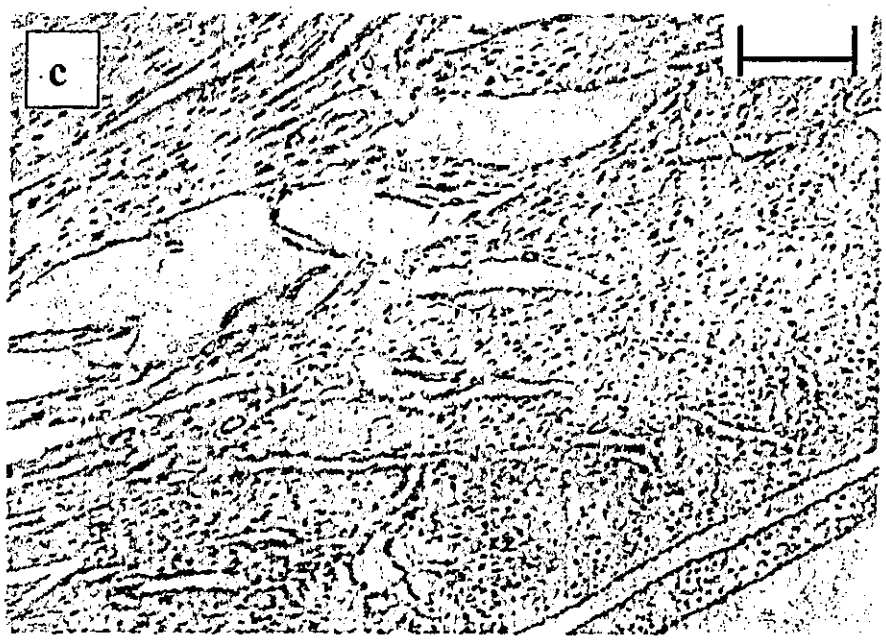

Figure 12. (Continued).

mounted on Veco-R-300 grids, and examined under a Hitachi H-600 electron-microscope operating at 100 kV.

Two pathologists, masked to the treated groups, evaluated and scored cardiotoxicity induced by doxorubicin.[16] The inflammatory change (infiltrating cell number) and vascularity (vessel number) were also counted at ×1,000 magnification. Severity degree was scored as zero = none, 1 = mild, 2 = moderate, and 3 = severe. Eight characteristics of cardiotoxicity were scored with the degree.

Statistical Analysis

All data were expressed as mean ± standard error. In body weight, heart weight, echocardiographic data, ascites, and ANP concentration, we measured used non-repeated analysis of variance to compare groups, followed by Bonferroni's multiple comparison test. We used Student's paired *t*-test to compare the data before and after surgery in each group. We analyzed the data that we measured with the Langendorff apparatus using repeated analysis of variance for comparing among groups, followed by Bonferroni's multiple comparison test. We considered $p < 0.05$ as a significant difference.

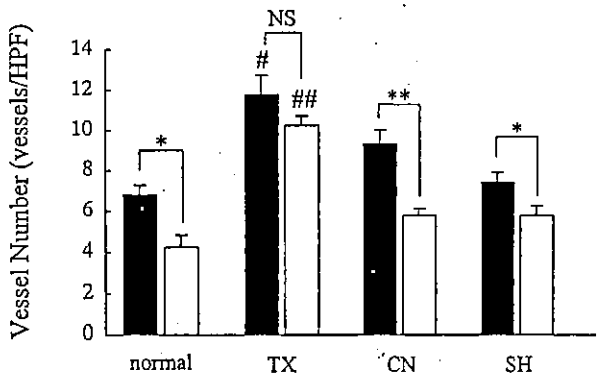

Figure 13. Vessel numbers in the left ventricular free wall (solid bar, transplant area; #$p < 0.05$) and in the septum (open bar, remote area; ##$p < 0.0001$) were greatest in the transplant group (TX). In the other groups, but not int the TX group, the number of vessels in the left ventricular free wall was greater than the number in the septum (*$p < 0.05$, **$p < 0.005$). CN, control group; SH, sham group.

Table 1. Electron Microscopic Findings

Group	TX	CN	SH
Number of animals	2	2	2
Electron microscopic findings			
1. Loss of myofibrils	1	1	1
2. Fragmentaion and sparsity of myofibrils	0	1	1
3. Proliferation of mitochondria	1	2	3
4. Degeneration of mitochondria	1	2	3
5. Widening of intercalated disc	0	0	1
6. Dilatation of endoplasmic reticulum and T tubules	1	2	2
7. Interstitial fibrosis	0	0	0
8. Lipofuschin deposits	0	0	0
Total score	4	8	11
Vessel number	5	3	2
Infiltrating cell number	1	0	0
Irregularity of nucleus	−	−	+

Severity degree: 0, none; 1, mild; 2, moderate; 3, severe.
Total score is sum of scores from 1 to 8.
Infiltrating cell number and vessel number: count number in the field of magnification (×1,000) −, none; +, yes.

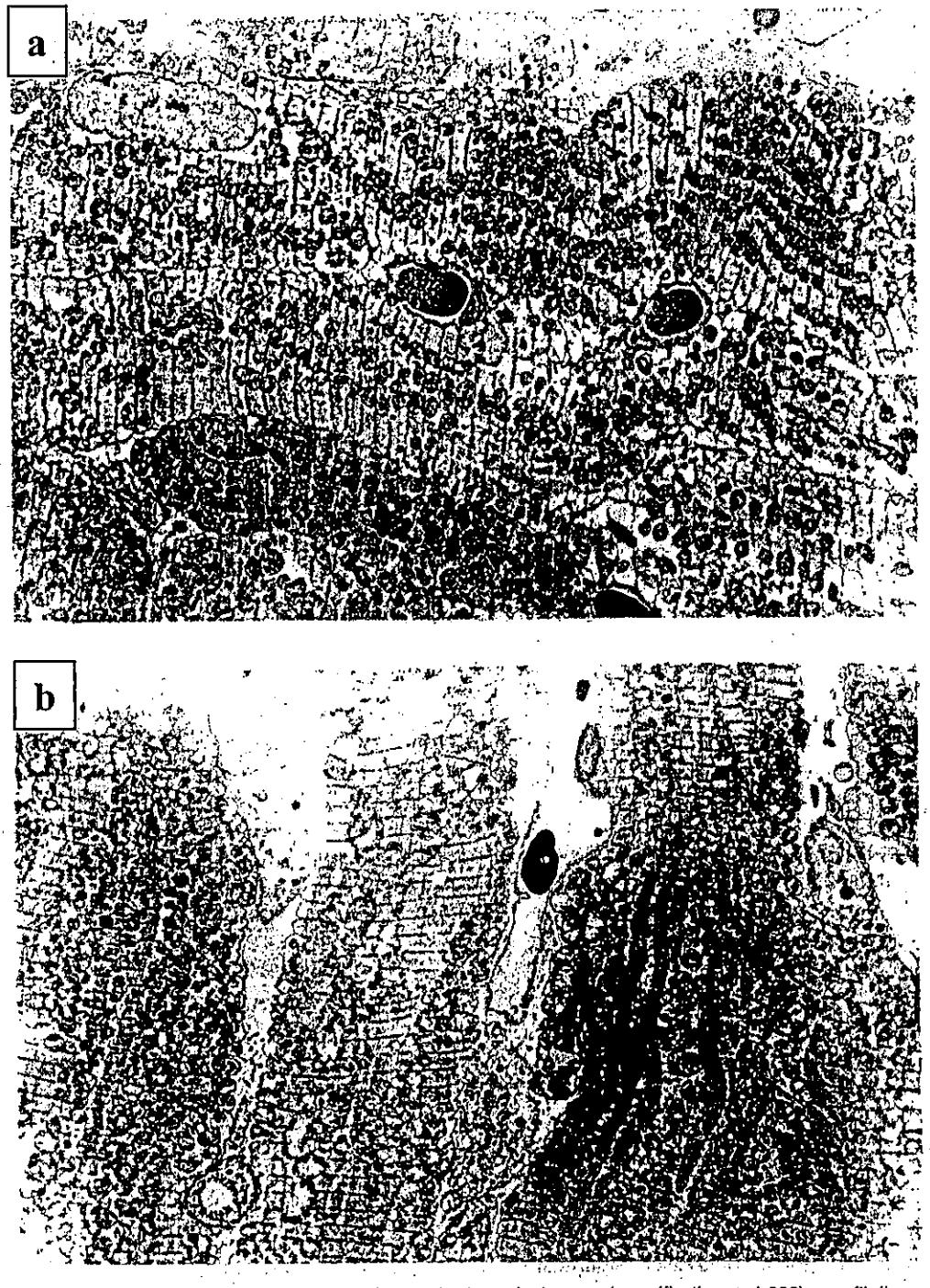

Figure 14. Ultrastructure of myocardium at the injection site. a, the transplant group (magnification, ×1,000): myofibrils were almost well organized. Proliferation of mitochondria was mild. We saw minor change in dilatation of endoplasmic reticulum and T tubules. b, control group (magnification, ×1,000): metamorphic myofibrils were recognized, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was moderate. c, the sham group (magnification, ×1,000): Metamorphic myofibrils were recognized, and endoplasmic reticulum and T tubules were dilated moderately. Mitochondria proliferated severely, and severe degeneration of mitochondria was observed. We found widened intercalated discs and irregular nuclei.

RESULTS

Mortality Rate

No rats died before surgery. During the 4-week period after surgery, the total mortality rate was 11.5% (16.7% in the TX group, 5.6% in the CN group, and 12.5% in the SH group, p = not significant). At 4 weeks after surgery, we used echocardiography and a Langendorff apparatus to measure heart function in

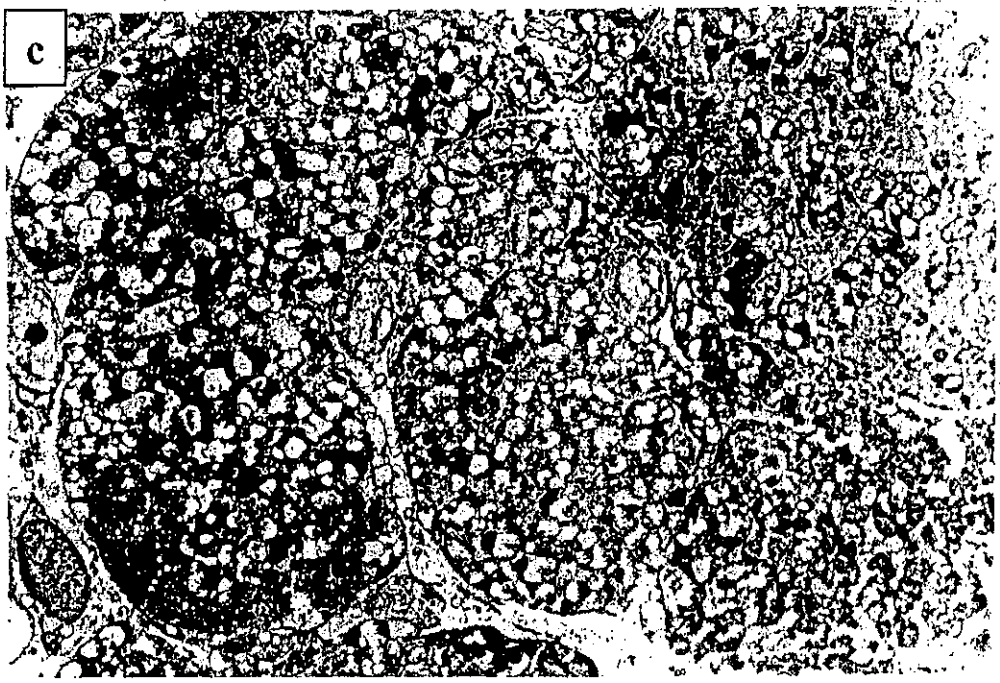

Figure 14. (Continued).

the TX (n = 15), CN (n = 17), and SH (n = 14) groups.

Body Weight

Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In no group did body weight change significantly from just before to 4 weeks after surgery (Figure 1).

Echocardiography

At 4 weeks after surgery, LVDs in the TX group (3.7 ± 0.1 mm) was smaller than that in the SH group (4.3 ± 0.2 mm, p = 0.015), whereas LVDs in the CN group (4.0 ± 0.2 mm) did not differ significantly from that in the SH group (p = 0.2). We found no significant difference between TX and CN groups (p = 0.3). In no group did LVDs change significantly from before to after surgery (Figure 2).

In each group, LVDd decreased significantly after transplantation (p < 0.05, Figure 3). Diastolic left ventricular diameter was similar in the 3 groups.

At 4 weeks after surgery, FS in the TX group (31.4% ± 1.7%) was greater than that in the SH group (24.7% ± 1.6%, p = 0.007), whereas FS in the CN group (27.3% ± 2.2%) was not significantly different from that in the SH group (p = 0.3). We found no significant difference between the TX and CN groups (p = 0.1). In the SH group, FS significantly decreased at 4 weeks after surgery (at 8 weeks after the doxorubicin injection, p = 0.02, Figure 4).

Transplant group LVPW/LVDs (2.9 ± 0.2 mm) was the largest of all groups (SH group, 2.1 ± 0.1 mm, p = 0.005, and CN group, 2.4 ± 0.2 mm, p = 0.04) at 4 weeks after surgery. The CN group did not differ significantly from the SH group (p = 0.3). The LVPW/LVDs decreased significantly in all groups compared with before surgery (p < 0.01, Figure 5).

Langendorff Apparatus

Heart rate during the assessment did not differ among all groups. Systolic pressure in the TX group was greater than that in the SH group (p < 0.0001) and in the CN group (p = 0.0034). Systolic pressure in the CN group was not greater than in the SH group (p = 0.08, Figure 6). Developed pressure in the TX group was greater than that in the SH group (p < 0.0001) and in the CN group (p < 0.0001). Developed pressure in the CN group was greater than that in the SH group (p = 0.0007, Figure 7). End-diastolic pressure in the TX group was less than that in the SH group (p < 0.0001), whereas end-diastolic pressure in the CN group was not less than that in the SH group (p = 0.07). The TX group did not differ from the CN group, with a p value of 0.06 (Figure 8).

Heart Weight and Amount of Ascites

The hearts in the TX group were heavier than those in the SH group (p = 0.021) and in the CN group (p = 0.038). The CN group did not differ from the SH group (p = 0.8, Figure 9). The amount of ascites in the TX

group was less than that in the CN group ($p = 0.019$). The TX group did not differ significantly from the SH group ($p = 0.3$, Figure 10).

ANP Concentration

The ANP concentration in the TX group (241.1 ± 19.8 pg/ml), which was not statistically different from that in the normal rats (209.3 ± 9.9 pg/ml, $p = 0.4$), was significantly less than that in the CN group (388.5 ± 41.8 pg/ml, $p = 0.0003$) and in the SH group (344.7 ± 20.4 pg/ml, $p = 0.0,052$, Figure 11).

Histologic Study

In the hematoxylin and eosin staining, the area of the injection was indistinguishable from other areas, but some rats were distinguished with an inflammatory change in the epicardium caused by the injections. We found no cartilage or bone formation at the transplantation sites.

Vessel Number

In the left ventricular free wall (transplant area), the number of vessels in the TX group (11.7 ± 0.98 vessels/HPF, at ×100 magnification) was larger than in the CN group (9.3 ± 0.8 vessels/HPF, $p = 0.039$) or in the SH group (7.5 ± 0.48 vessels/HPF, $p = 0.0007$, Figure 12). In the transplanted area, we saw much smaller vessels. The diameter of most vessels was <50 μm. In the septum (remote area), the number of vessels in the TX group (10.2 ± 0.47 vessels/HPF) was larger ($p < 0.0001$) than the number in the CN group (5.8 ± 0.34 vessels/HPF) or in the SH group (5.8 ± 0.43 vessels/HPF). In normal, CN, and SH groups, the number of vessels in the left ventricular free wall was larger than the number in the septum (normal, $p = 0.018$; CN, $p = 0.003$; and SH, $p = 0.047$; Figure 13). In the TX group, we observed more venules than in the CN and SH groups.

Electron Microscopic Study

Table 1 shows semi-quantitative scoring for electron microscopic findings in the 3 groups. We found a trend for the total score in the TX group to be the smallest of all.

In the TX group, myofibrils were almost well-organized. Proliferation of mitochondria was mild. Minor changes included dilatation of the endoplasmic reticulum and the T tubules (Figure 14a).

In contrast, in the SH and the CN groups, we recognized metamorphic myofibrils, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was severe in the SH group and moderate in the CN group. We found widened intercalated discs and irregular nuclei in the SH group (Figure 14, b and c).

In order, we observed many more vessel in the TX, in the CN, and then in the SH group. The infiltrating cell number was zero or 1 in all groups.

DISCUSSION

We used doxorubicin-induced cardiomyopathy as the model of IDCM. In electron microscopic study, cardiotoxicity was moderate in the hearts of the SH group. We succeeded in creating a heart failure model.

In this study, we showed that BMMNC transplantation had beneficial effects on non-ischemic heart failure, especially for systolic function. The function study, in which we used a Langendorff apparatus, demonstrated the greatest peak systolic pressure and developed pressure (the parameters of systolic function) in the TX group. An increase in systolic pressure without the parallel increase in end-diastolic pressure in the TX group, with increasing balloon volume, suggested that transplantation maintained elasticity instead of stiffness, which also could be changed by inflammation, changes in vasculature, or extracellular matrix. As demonstrated by LVPW/LVDs, transplantation prevented the left ventricular wall from remodeling and may support myocardial reserve for contraction. Although part of the cardiac function data (LVDd, LVDs, FS, and end-diastolic pressure) did not show a significant difference between the TX and the CN groups, it is obvious that results in the TX group were superior to those of the SH group, whereas results in the CN group were similar to those in the SH group.

In addition, secondary changes (decreased heart weight, developed ascites,[17] increased ANP concentration, and destruction of myocardium) caused by doxorubicin-induced heart failure were attenuated by BMMNC transplantation.

Regarding the possible underlying mechanism for improved non-ischemic heart failure after cell transplantation, several paracrine factors released from transplanted cells have been suggested.[4,5] In the ischemic heart model, BMMNC transplantation works as an enhancer for angiogenic ligands beta fibrogenic growth factor (bFGF), vascular endothelial growth factor (VEGF),[9] insulin-like growth factor 1,[18] and angiopoietin 1 and cytokines (interleukin-1β and tumor necrosis factor-α).[8]

In this study, BMMNC transplantation increased blood vessel density not only in the left ventricular free wall (transplant area) but also in the septum (remote area), and we found no significant difference between the 2 areas. In the normal and SH groups, we showed significant difference between the left ventricular free wall and the septum, suggesting that vascular density was originally greater in the left ventricular free wall than in the septum, and the difference was greater in the CN group, suggesting

that the injection itself induced angiogenesis at the injection site.[19] Electron microscopic study showed that the structure of myocardium in the TX group clearly was maintained, with many more vessels than in the SH or CN groups.

Considering previous reports[4,5,8,9,18] and our observations, the possible mechanism by which BMMNC transplantation was beneficial in doxorubicin-induced cardiomyopathy may have been the following: The BMMNC transplantation induced angiogenesis in the whole heart. Microcirculation improved by angiogenesis could contribute to preserving myocardium. Preserved myocardium might have contributed to preventing deterioration of cardiac function.

This study had several limitations. We did not label transplanted BMMNC for identification, because labeling technique may compromise cell function.[20] Our main aim was to verify the efficacy of BMMNC transplantation. Further studies of dose response, fate of transplanted cells, and long-term effect should be conducted.

In conclusion, BMMNC transplantation had beneficial effects in non-ischemic heart failure: doxorubicin-induced cardiomyopathy in rats.

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ORIGINAL ARTICLE

Granulocyte-Colony Stimulating Factor Enhanced the Recruitment of Bone Marrow Cells into the Heart

Time Course Evaluation of Phenotypic Differentiation in the Doxorubicin-induced Cardiomyopathic Model

Objective: We traced and evaluated bone marrow-derived cells after granulocyte-colony stimulating factor (G-CSF) treatment in the doxorubicin-induced cardiomyopathic heart in the time course. *Methods:* C57BL/6 male mice received doxorubicin (15 mg/kg, i.p.). At 1 week after administration of doxorubicin, the mice were irradiated (900 cGy) followed by transplantation of bone marrow cells (BMT) derived from transgenic mice expressing green fluorescent protein (GFP) (1×10^6) via a tail vein (BMT). G-group (n=22) received G-CSF (50 μg/kg/day×8 days, s.c.) after BMT, while C-group (n=17) received saline. At 4 and 7 weeks after BMT, heart sections were fixed to evaluate bone marrow–derived GFP cells (BMD-GFP) with immunostaining for Troponin I (TnI), atrial-natriuretic peptide (ANP), connexin 43, von Willebrand factor, and Ki67. *Result:* There were migrated BMD-GFP in the whole heart of all animals. In the time course, migrated BMD-GFP increased in G-group. At 7 weeks the number of migrated BMD-GFP in G-group (56.2±15.6/HPF) was larger than that in C-group (18.9±10.7/HPF) ($p<0.05$). TnI- and connexin 43-positive BMD-GFP were spindle-shaped. Von Willebrand factor-positive BMD-GFP showed thinner-shape. ANP- and Ki67-positive BMD-GFP showed oval-shape. The numbers of these positive cells derived from BMD-GFP, not different between the 2 groups, did not change from 4 to 7 weeks. *Conclusion:* The migration of BMD-GFP into the heart increased from 4 to 7 weeks after BMT by G-CSF. However, cardiomyocytes and endothelial cells originating from BMD-GFP were very few and neither increased nor changed in their shapes and numbers in the short term. (Jpn J Thorac Cardiovasc Surg 2004; 52: 451–455)

Key words: granulocyte-colony stimulating factor, bone marrow cells, doxorubicin-induced cardiomyopathy, migration, phenotypic change

Yosuke Hisashi MD,[1] Shinji Tomita MD,[1,2] Takeshi Nakatani MD,[3] Shinya Fukuhara MD,[1] Chikao Yutani MD,[4] and Soichiro Kitamura MD.[2]

Cell-based therapy is a promising treatment for end-staged heart failure. In contrast to the exogeneous-cell transplantation, regeneration of myocardium by endogeneous-stem cell was reported.[1] Orlic et al. applied granulocyte-colony stimulating factor (G-CSF) and stem-cell factor to enhance regeneration of myocardium by endogeneous-stem cells.[2] In addition, we proved that a source of cardiac-stem cell was bone marrow in the infarction model.[3] However, the mechanism of endogeneous-stem cells is unknown in detail.

In this study, we traced and evaluated bone marrow-derived cells after G-CSF treatment in the doxorubicin-induced cardiomyopathic heart in the time course.

From Departments of [1]Regenerative Medicine and Tissue Engineering, [2]Cardiovascular Surgery, [3]Organ Transplantation, and [4]Pathology, National Cardiovascular Center, Osaka, Japan.

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Address for reprints: Shinji Tomita, MD, Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.

Subjects and Methods

Animal model. C57BL/6 at 8 weeks (25 g) were purchased from a licensed vendor. All animals received