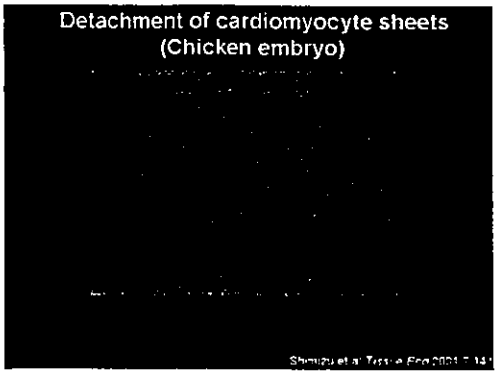


図12

と接着のプロテインが切断されるため、感染に弱くて再接着しにくい皮膚しか取れない。ところが、私たちのグループでは非常に感染に強い皮膚が取れる。同様に網膜もつくることができる。角膜の上皮、内皮、これはもう目が見えなくなった人たちのために非常に朗報となる。

それから、心筋の組織、これはこういう細胞シート同士を重ねることによって構造的に結合するだけではなくて、機能的にも結合し、それぞれのシートが動いて拍動しているが、重ねることによって同期して動く。まさに機能が結合することが実現したといえる（図13）。

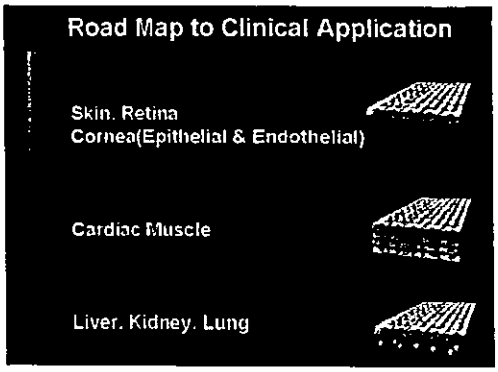
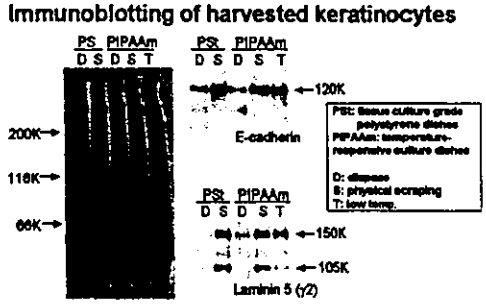


図13

例えば肝臓は血管の内皮細胞と肝実質細胞が積層化されている肝小葉構造となっている。この共培養素で肝細胞は高度な機能を長期に維持されており、肝臓の細胞だけを培養しても１週間くらいしか培養できない。現在、肝細胞の長期培養ができないため。薬の毒性評価には、動物を丸ごと使わなければならない。ところが、肝実質細胞の上に血管の内皮細胞をのせた、またに肝小葉構造をつくると、肝臓の細胞を数ヶ月にわたって生かすことができるようになる。

このように細胞シートと細胞シートを重ねて構造ができると同時に、重ねることによって、そこに機能連結が起きる。まさにサイトカインを介したコミュニケーションが起きるのだということを私たちは示してきている。これはケラチノサイトという皮膚の表皮の培養結果である。通常、皮膚の表皮は、培養し、それをDispaseという酵素ではがす。Dispaseではがしたとき、120Kに現れる、細胞と細胞のジャンクション・プロテインのE-cadherinがDでかなり破壊されているのがご覧頂けると思う。それに対してスクレープ（S）といって、かき取ったときにはちゃんとこのたんぱく質が残っている。温度ではがしたとき（T）には、かき取ったのと同じようにcadherinが残っている。それから、Lamininという接着タンパクがあるが、Dispaseで処理する（D）と消えてきているが、温度ではがしたとき（T）には残っている（図14）。

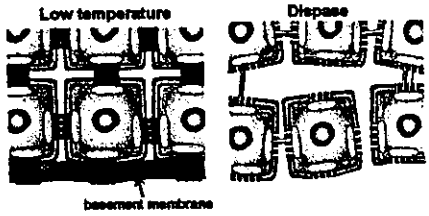


Junction and Adhesive Proteins remained intact in the cell sheets harvested by lowering temperature.

図14

バイオ皮膚をつくるベンチャーは世界中でたくさん興っているが、すべてDispaseを利用して皮膚を利用しているのが現状である。一方で外科医たちからは、例えば10枚のせたときに生着するのは2～3枚で、あとは脱落していって、生着率が極めて低いことを指摘している。同時に、非常に感染に弱い。そのことは私たちの研究でDispaseで処理したためにジャンクション・プロテインがやられているためであることが示された（図15）。

それから、この基底膜になっているタンパク、Laminin 5とかファイブロネクチンから成っているタンパクが破壊されているのではないかということがまさに証明できた。私たちの作った皮膚はジャンクション・プロテインがきっちりと残っていることが証明され、現在、東京女子医科大学の形成外科で臨床が開始されている。



Schematic drawing of harvested keratinocyte sheets

図15

角膜移植への朗報

角膜は周知のように、日本では1年間に2万人くらいの患者が移植を必要としている。累計で十数万人が角膜移植を待っているのだ。ところが、実際には1,600～1,700くらいの角膜移植しかできていない。これはドナー不足のためである。私たちとしては、一個の角膜を一人にいれるという医療から、一個の角膜を数十個、数百個にしてしまえば一つのドナーから多数の患者を治療できると考える。1mmとか2mmの組織の切片から1個を作れないかということを、まさに最近、大阪大学眼科の西田講師らとの共同研究で成功している。これは、大阪大学の田野教授、西田博士と、そのグループと一緒にやっている。角膜は三層からできており、角膜上皮、実質、内皮であり、角膜の表皮だけかえれば治療できるというケースがある（図16）。

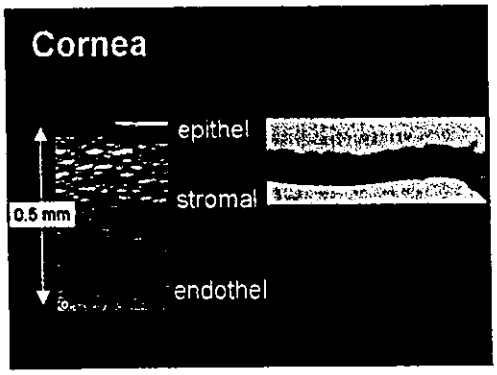


図16

そういう人に対しては、濁ったところを取り出して、温度応答性表面で作った角膜の表皮を、片側がノリ（接着タンパク質）になっているので、のせて治療している。動物レベルではかなりできるようになってきており、1mmとか2mmから1個の角膜ができることから、患者の両親とか、兄弟から細胞が取れる場合や、本人の片目から取れる場合は、培養して増やして移植することができる。今までとは全然違う移植の新しい幕開けが起きるのではないかということで、共同研究を進めている。

網膜という光を感知する場所では、細胞が極性を持った構造となっていて、培養皿の上でグロース・ファクターを上から入れてもうまく極性が出てこない。多孔膜の上に温度応答性表面をつくっておいて、下側からグロース・ファクターを入れると極性がきれいに作

れる（図17）。

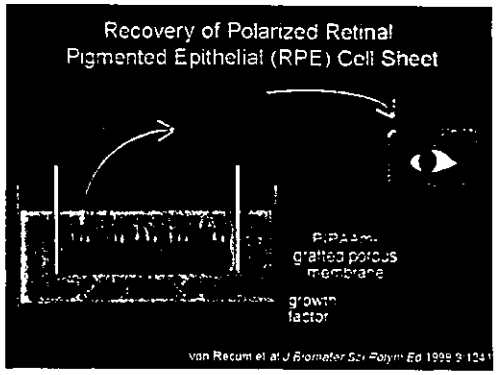


図17

このような状態にして温度ではがして、このシートを移植することもできるようになってきた。これはRPEセルという網膜色素上皮だが、ウサギの目にヒヨコから取った網膜色素上皮を移植したものである。図18のように、完全に接着している。

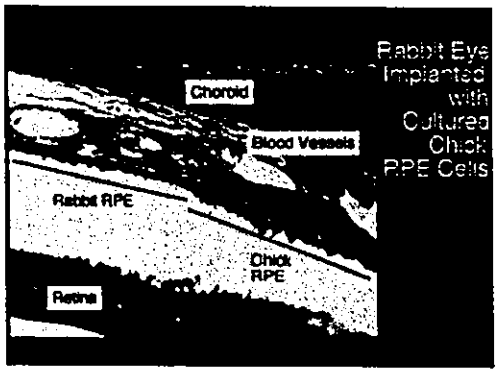


図18

肝細胞の長期培養の革命的成功

細胞が簡単に移植できるのだということは、シートの片面にメリがついていることに起因しているわけである。肝臓の細胞の上に血管の内皮細胞がのっている肝小葉構造を実際肝臓は持っている（図19）。

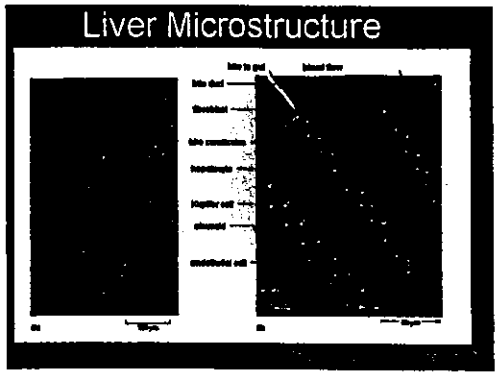


図19

この構造によって肝臓は高機能を維持しているのが、私たちは肝実質細胞とこの内皮細胞を二重構造にして肝小葉モデルを作製し、長期培養を可能にした。

通常、肝細胞と、内皮細胞を単にまぜて培養皿の上にばらまけば一緒に培養できるのだからいいではないかと思うかもしれない。ところが、一緒にまぜて培養しても共培養はできない。両方の細胞が生きるということはないのだ。共培養は、細胞の集団と別の細胞の集団をつくっておいて、重層化させることができる（図20）。

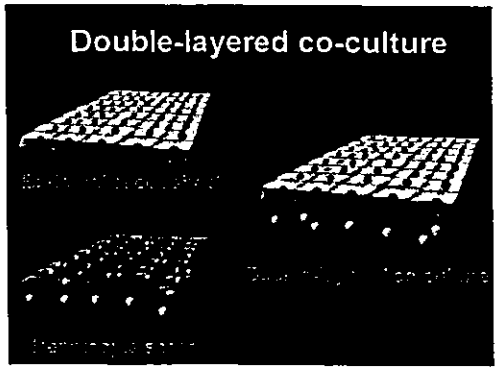


図20

これは共焦点レーザー、顕微鏡を用いて、縦型に切断した画像をコンピュータでつくるわけだが、肝細胞が放出しているアルブミンを緑に染めている。肝細胞がアルブミン産生を

し、赤いところがF-actin．だから細胞全体である。内皮細胞と肝細胞が二重層になっていて、肝細胞はアルブミンを長期に産生していることがこの図で理解できると思う（図21）。

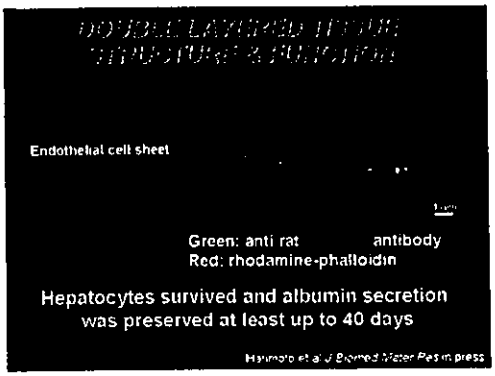


図21

40日後であっても肝細胞は高機能を示している。通常、肝細胞は1週間くらいで死んでしまう。このように数ヶ月にわたって肝細胞を生かすことができるわけだから革命的であるといえよう。

心筋細胞への適応

のせるということは、構造的にのせているだけではなく、機能的に連結しているのだということを意味している。まさにサイトカインを介した細胞集団と細胞集団の間にコミュニケーション場ができて、組織を作り上げていることを意味している。

心筋細胞をbiodegradableな高分子の中に入れて組織をつくろうという試みは世界中でやられているが、目に見えて動くような組織はなかなかできなかった。ところが、私たちは心筋細胞シートを重ねていくことによって心筋組織を作ることに成功した（図22）。

現在、大阪大学第一外科の松田教授、澤助教授たちと共同研究をしており、冠状動脈をくくって心筋梗塞のモデルをつくっている。血

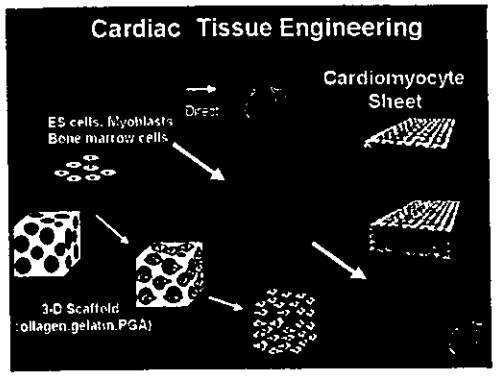


図22

流が閉ざされるから梗塞部分ができる。動きが悪くなってしまった心筋に対し、シートをつくって、貼り付けて治療する（図23）。現在、骨格筋や何かで細胞を取って注射するという治療はもう臨床が始まっている。

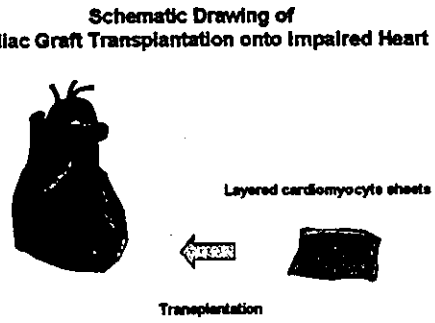


図23

しかし、先程から何度も繰り返しているように、そういう細胞は酵素で処理されるため、機能的にかなり落ちた細胞となり、これを使わざるを得ない。そういう意味ではシートを使うことによって極めて効率的な治療ができるだろうと思う。

われわれは梗塞のモデルつくった。血管を結んで血流を止めて2週間後に梗塞を確かめると、心臓の一部分が動かなくなってきている。この上に心筋のシートを貼り付ける。ちょうど2週間後に見てみると、心筋組織がき

れいに動き始めている。梗塞モデルをつくると、最初の45%まで心臓からの拍出流量が低下し、2層のシートを貼り付けた結果心拍出量は65%まで回復した。つまり、梗塞モデルをつくり、心筋細胞シートを貼り付けることで動かなくなっていた心筋の梗塞部分を治療することができることを示している。

最後に

まさに細胞のシートを使うという技術革新が起きることによって新しい治療が始まる。これから薬という概念、あるいは移植という概念が新しい技術によって大きく変革しようとしていると言っていいと思う。今までの薬とか、今までのバイオロジーとか、今までの医学とか、タテ型学問領域がそのままの延長線上に大きな発展はなく、ヨコ型の集学的なアプローチによって技術革新が起きるのだと思う。私たちの研究所は内科医、外科医、セルバイオロジスト、モレキュラーバイオロジスト、それから高分子学者、機械などのエンジニアも含めて、一緒に研究をやっていけるような体制をつくりながら研究を進めている。

おそらく一般に思われているほど再生医療が医療現場の中で動き始めるのは遅くないのではないかと思う。たぶん10年くらい後を考えていると思うが、かなり前倒しで医療現場の中に入り込んでいくのではないかと思う。現実にもう治療が始まっているところもあり、今後、大きく発展が期待される。

Cutting Edge

細胞シート工学

Cell Sheet Engineering

大和　雅之・岡野　光夫
東京女子医科大学先端生命医科学研究所

Key Words
temperature-responsive, cornea, tissue engineering

■ はじめに

我々は，生分解性高分子製足場を用いることなく，移植に必要な大きさの組織を再構築する新技術の開発を目指して，「細胞シート工学」を提案し，その体系的追求に尽力している。すなわち細胞シート工学とは，細胞－細胞間接着と細胞自身が培養の間に作り出す細胞外マトリックスによりシート状をなす細胞集団すなわち細胞シートを根幹単位として，これを用いて組織構造を再構築する技術の総称である。

■

通常，培養細胞の回収に用いられるトリプシンなどのタンパク質分解酵素は細胞－細胞間接着を破壊してしまうため，培養細胞を細胞シートとして回収することはできない。この問題を解決するため，我々は温度応答性培養表面を開発した。温度応答性培養表面には，温度に応じて親水性・疎水性を大きく変化させる温度応答性高分子が共有結合的に固定化されており，タンパク質分解酵素を用いることなく，温度を下げるだけで培養細胞をまったく非侵襲的に回収することができる。肝実質細胞等の多くの細胞がトリプシン処理により非可逆的に分化機能を消失するが，この方法では分化機能を維持したまま細胞を回収できる。また，温度応答性培養表面を用いて作成した細胞シートは，底面に培養の間に沈着した細胞外マトリックスを接着したまま回収されるため，容易に他の表面に接着する[1]。注射針を用いて細胞懸濁液を組織に注入する細胞移植では，正着率の低さや細胞の散逸が問題になっているが，細胞シート移植ではこのような問題は生じない。

Masayuki Yamato, Mitsuo Okano
Institute of Advanced Biomedical Engineering and Science

表皮細胞シート[2]や角膜上皮細胞シート[3]は，そのまま移植に供して熱傷やスティーブンス・ジョンソン症候群などの角膜上皮幹細胞疲弊症の治療に用いることができ，すでに臨床応用を開始している。従来の角膜移植では縫合が必須であるが，温度応答性培養皿を用いて作製した角膜上皮細胞シートは５分程度で角膜実質層に接着し，縫合の必要がまったくない。また，細胞－細胞間接着が維持されているため，移植直後からきわめて良好なバリア機能を有している。歯周病は高齢化社会における重要な問題の一つであるが，歯周組織の再生はきわめて困難で，いまだ対症療法的な治療しかない。我々は培養系で増殖させた歯周組織（歯根膜）の細胞で細胞シートを作製し，これを病変部位に移植することで歯周組織がきわめて良好に再生することを見出した。この他，細胞シートを積層して心筋梗塞部位に移植する心筋再生技術についても取り組んでいる[4]。

さらに細胞シート工学を再建外科と組み合わせることで，血管系と神経系を有する機能をもつ分厚い組織構造を再構築することができる。たとえば我々は，膀胱の小片から膀胱上皮細胞を採取し，これを温度応答性培養皿上で培養して膀胱上皮細胞シートとして回収し，胃や小腸の上皮組織と置換して膀胱再建をおこなう新手法の開発に取り組んでおり，現在イヌを用いた動物実験をおこなっている[5]。現在臨床で用いられている胃や腸を用いる膀胱再建術では，これらの組織の上皮に起因する合併症が問題となっているが，培養膀胱上皮細胞シートで置換することで，このような合併症をおこさない新しい膀胱再建術が開発できた。

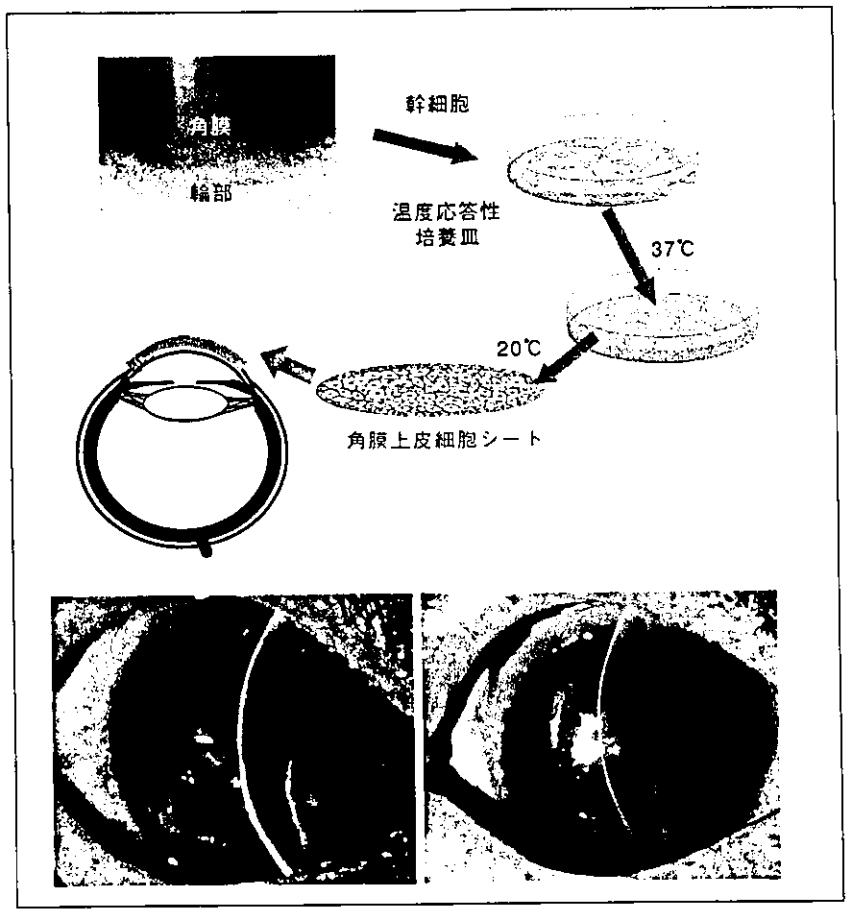


図　細胞シート工学による角膜再生
輪部に局在する角膜上皮幹細胞を単離し，温度応答性培養皿上で培養する。温度を20℃に下げ，細胞シートとして回収し，これを移植に供する。術前（下段左）ではザルツマン変性による角膜上皮幹細胞疲弊症のため重度の角膜混濁と血管の進入が認められるが，術後3ヶ月（右）で透明な角膜が再生している。
（臨床例の写真は大阪大学眼科西田幸二先生の御提供）

■ おわりに

以上概観したように，生分解性高分子製の足場を利用することなく，温度応答性培養皿から非侵襲的に回収した細胞シートを積層することで組織構造を再構築する本技術は，次世代組織工学の中核的技術として大きな期待を集めている。

文　献

1) Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T, Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules. 1: 377-381, 2000.
2) Yamato M, Utsumi M, Kushida A, Konno C, Kikuchi A, Okano T, Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng. 7: 473-480, 2001.
3) Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T, Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface, Transplantation. 77: 379-385, 2004.
4) Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T, Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res. 90: e40, 2002.
5) Shiroyanagi S, Yamato M, Kikuchi A, Yamazaki Y, Toma H, Okano T, Urothelium regeneration using viable cultured urothelial cell sheets grafted on demucosalized gastric flaps, BJU Int. 93: 1069-1075, 2004.

Overview

血管再生医工学

Tatsuya Shimizu ◎ 清水達也
Mitsuo Okano ◎ 岡野光夫
東京女子医科大学先端生命医科学研究所

近年，欠損あるいは傷害を受けた組織・臓器に対する新たな治療法として再生医療が注目されている．すでにさまざまの組織・臓器に対する再生の研究が行われており，一部臨床応用されるに至っている．なかでも四肢末梢動脈や冠動脈の狭窄・閉塞に伴う虚血性の疾患に対する血管新生療法は国内でも数多く臨床応用されており，その有効性が示されつつある．一方，これら毛細血管レベルの再生に対し，組織工学的手法を用い3層構造を有する血管組織の再生も追究され，先天性の心血管疾患に対する臨床応用が始まっている．さらに，肝臓や心筋を含む多くの組織再生の研究においては，そのスケールアップを目的に再生組織内にいかに血管のネットワークを形成するかが課題となっており，新たな組織工学的手法の開発が期待されている．

このような背景から，血管の再生は再生医療においてきわめて重要なものとなっているが，実際，人体において血管は酸素・栄養・代謝物など生命維持に必要な物質の輸送経路として人体に欠かせない組織である．その全長は地球の2周半，約10万kmにも及び，すべての臓器において血管のネットワークが形成されている．直径約3cmの大動脈に始まり，末梢組織では数μmの毛細血管となる．構造的には，単層の内皮細胞からなる内膜—多層の平滑筋細胞と，弾性線維からなる中膜—線維芽細胞と，弾性・膠原線維からなる外膜という3層を有する血管から，分岐を繰り返して1個の内皮細胞とそれを覆う周皮細胞のみからなる毛細血管へと至る．また，末梢組織へ向かう動脈と心臓へ戻る静脈とでは，壁厚や弾性線維などの構造に相違が認められる．これは，血管内を流れる血流のメカニクスに起因すると考えられる．機能的には動静脈が心臓から拍出される血液を末梢組織まで循環するための通路として血圧など血行動態の維持に寄与し，毛細血管はさまざまの物質交換が行われる場として組織代謝の維持に貢献している．

以上のような特徴を有する血管の再生において，まず重要となるのは血管の細胞ソースである．これに関しては，近年の幹細胞生物学の目覚ましい発展が大きく貢献している．細胞ソースとして骨髄単核球細胞や血管内皮前駆細胞はすでにその採取法が確立し，虚血

Overview

性疾患に対する血管新生療法や再生血管移植に臨床応用されている．また，ES細胞からは血管内皮細胞のみならず平滑筋細胞の分化誘導も実現しており，将来的な利用の可能性も示されている．次に，これらの細胞が血管を再構築するにあたっては種々の増殖因子が必須である．生体内においては移植した細胞そのものあるいは周囲の組織からもこれらの増殖因子が分泌されるが，さらに外部から増殖因子やその遺伝子を投与することにより血管新生を促進できるものと考えられる．近年のドラッグデリバリーシステム(DDS)の発展により，これらの蛋白や遺伝子投与の有効性も向上しており，細胞との組み合わせによりさらに血管新生を加速することも可能であろう．一方，口径の大きな血管の再生に関しては，細胞の足場として，細胞外マトリックスの代わりに生体吸収性の生体材料を用いる組織工学的手法が必須となっている．より細胞接着の良い材料，生体血管と同様のコンプライアンスを持つ材料が追究されている．組織工学の研究では，血管のバイオメカニクスを考慮した設計の重要性が示され，またバイオリアクターを用いた拍動下での血管組織の環流培養により2,000 mmHgに耐える血管組織も再生されており，工学的アプローチの重要性が増している．

前述したように，血管組織はそれぞれの部位において異なる構造と機能を有するため，これまでの血管再生研究は毛細血管～細血管を対象とした場合と，中～大血管を対象にした場合とで異なるアプローチで異なる研究者が行ってきたが，再生医療という学際的な研究領域において，それぞれの知識や技術に接点も生まれ共同研究も増えつつある．また，今後は細～中血管の再生や毛細血管網を伴った組織・臓器の再生が新たな再生医療のターゲットとなりつつあり，将来的には統合的に毛細血管網から太い血管まで伴った組織・臓器の再生が必要になってくると予測される．そういった観点からも血管の再生に関わる研究者が相互理解を含め，連携・融合していくことが肝要である．

本特集では上記の内容をふまえ，血管再生の細胞ソース，増殖因子とデリバリー法，組織工学的な血管新生，太い血管の再生，また再生血管のバイオニクスといった内容で各専門家による研究・臨床の最先端の知見が紹介されている．本特集が読者の血管再生医療に関する現状と全体像の把握の一助となるとともに，本研究フィールドにおける連携・融合への端緒になれば幸いである．

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Powerful and Controllable Angiogenesis by Using Gene-Modified Cells Expressing Human Hepatocyte Growth Factor and Thymidine Kinase

Yasuyo Hisaka, MS,* Masaki Ieda, MD,† Toshikazu Nakamura, PHD,‡ Ken-ichiro Kosai, MD, PHD,§ Satoshi Ogawa, MD, PHD,† Keiichi Fukuda, MD, PHD*†

Tokyo, Osaka, and Fukuoka, Japan

OBJECTIVES This study investigated the possibility of achieving angiogenesis by using gene-modified cells as a vector.

BACKGROUND Although gene therapy for peripheral circulation disorders has been studied intensively, the plasmid or viral vectors have been associated with several disadvantages, including unreliable transfection and uncontrollable gene expression.

METHODS Human hepatocyte growth factor (hHGF) and thymidine kinase (TK) expression plasmids were serially transfected into NIH3T3 cells, and permanent transfectants were selected (NIH3T3 + hHGF + TK). Unilateral hindlimb ischemia was surgically induced in BALB/c nude mice, and cells were transplanted into the thigh muscles. All effects were assessed at four weeks.

RESULTS The messenger ribonucleic acid expression and protein production of hHGF were confirmed. Assay of growth inhibition by ganciclovir revealed that the 50% (median) inhibitory concentration of NIH3T3 + hHGF + TK was 1,000 times lower than that of NIH3T3 + hHGF. The NIH3T3 + hHGF + TK group had a higher laser Doppler blood perfusion index, higher microvessel density, wider microvessel diameter, and lower rate of hindlimb necrosis, as compared with the plasmid- and adenovirus-mediated hHGF transfection groups or the NIH3T3 group. The newly developed microvessels were accompanied by smooth muscle cells, as well as endothelial cells, indicating that they were on the arteriolar or venular level. Laser Doppler monitoring showed that the rate of blood perfusion could be controlled by oral administration of ganciclovir. The transplanted cells completely disappeared in response to ganciclovir administration for four weeks.

CONCLUSIONS Gene-modified cell transplantation therapy induced strong angiogenesis and collateral vessel formation that could be controlled externally with ganciclovir. (J Am Coll Cardiol 2004;43: 1915–22) © 2004 by the American College of Cardiology Foundation

Growth factors isolated recently, including vascular endothelial cell growth factor, fibroblast growth factor, angiopoietin, and hepatocyte growth factor (HGF), have been found to induce strong angiogenesis (1–5). A number of studies have reported induction of angiogenesis and collateral vessel formation by gene therapy with these factors in both animal experiments and clinical trials. Plasmid or viral vectors have been used in these therapies (2,6,7), but the adenovirus vector entails some serious problems, such as allergic reactions or difficulty with repeated treatment, despite sufficiently high transfection efficiency. Moreover, although plasmid vectors have recently been used in clinical settings, have not been associated with allergic reactions, and could be used repeatedly, their transfection efficiency has been low and has varied with the tissues injected or the patient. These gene delivery methods have the common drawbacks of not being able to choose the target cells and to selectively eliminate the transfected cells once they acquire the character of abnormal growth. Thus, new methods that would provide ideal gene delivery systems have long been awaited.

Regeneration therapy has recently been performed in many tissues and organs. Various types of cells regenerate from embryonic or adult stem cells, and these cells would be transplanted into patients. Rapid and sufficient establishment of angiogenesis and collateral vessel formation to promote the survival and function of the transplanted cells are especially important in terms of blood supply. We investigated regeneration of cardiomyocytes from adult stem cells and concluded that blood vessel formation into transplanted cells is crucial to their survival (8). Because angiogenic gene therapy with plasmid vectors has been insufficient to induce the rapid and powerful angiogenesis required for transplantation of the regenerated cells, a new method has been needed to address this problem.

In the present study, NIH3T3 cells were permanently transfected with a novel angiogenic human HGF (hHGF) and thymidine kinase (TK) of herpes simplex gene and then used as a gene therapy vector. Their effect on blood flow,

From the *Institute for Advanced Cardiac Therapeutics, †Cardiopulmonary Division, Department of Medicine, Keio University School of Medicine, Tokyo; ‡Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, Osaka; and the §Cognitive and Molecular Research Institute of Brain Disease, Kurume University School of Medicine, Fukuoka, Japan. This study was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, and by Health Science Research Grants for Advanced Medical Technology from the Ministry of Welfare, Japan.

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Abbreviations and Acronyms

DMEM	= Dulbecco's modified Eagle's medium
EGFP	= enhanced green fluorescent protein
ELISA	= enzyme-linked immunosorbent assay
hHGF	= human hepatocyte growth factor
IC_{50}	= 50% (median) inhibitory concentration
LDPI	= laser Doppler perfusion image
RT-PCR	= reverse transcription-polymerase chain reaction
SMA	= smooth muscle actin
TK	= thymidine kinase
vWF	= von Willebrand factor

angiogenesis, and collateral formation was investigated in a murine ischemic hindlimb model (9–11). In this paper, we report that gene-modified cells expressing hHGF and TK induced strong angiogenesis and collateral vessel formation, and that they were easily controlled externally with ganciclovir.

METHODS

Cell culture. The NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and penicillin (100 μg/ml), streptomycin (250 ng/ml), and amphotericin B (85 μg/ml).

Stable transfection of hHGF and TK genes. The complementary deoxyribonucleic acid (cDNA) of the hHGF and TK genes was inserted into the pUC-SRα and pGK expression vector plasmids, respectively (10–13). pPUR and pcDNA3.1/Hygro(+) are selection plasmids that confer puromycin resistance and hygromycin resistance, respectively. After co-transfection of pUC-SRα/hHGF and pPUR into the NIH3T3 cells, using the Effectene Reagent (QIAGEN GmbH, Hilden, Germany), the puromycin-nonresistant cells were removed with puromycin (3 μg/ml), and the hHGF-producing cells were clonally selected (NIH3T3 + hHGF). pGK/TK and pcDNA3.1/Hygro(+) plasmids were then similarly co-transfected into the NIH3T3 + hHGF cells; the hygromycin-nonresistant cells were removed with hygromycin (200 μg/ml); and both hHGF- and TK-producing cells were clonally selected (NIH3T3 + hHGF + TK).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of hHGF messenger ribonucleic acid was analyzed by RT-PCR using the primers that specifically detect human but not mouse HGF, as previously described (14).

Enzyme-linked immunosorbent assay (ELISA) for hHGF. Production of hHGF was determined by ELISA with anti-human–specific HGF monoclonal antibodies (Institute of Immunology, Tokyo, Japan) (6,15,16).

Ad.CA-hHGF. The adenoviral vector plasmid pAd.CA-hHGF, which is composed of a cytomegalovirus immediate early enhancer, a modified chicken beta-actin promoter, and hHGF cDNA, was constructed by the in vitro ligation method (17). The pAd.CA-hHGF plasmid was partially cut with *Pac*I and then transfected into 293 cells, followed by culture with 0.5% overlaid agarose-α-minimal essential medium (MEM) containing 5% horse serum for 10 to 15 days. Viral plaques, which had been confirmed by restriction enzyme analysis and ELISA for hHGF, were propagated in 293 cells, purified by $CsCl_2$ gradient ultracentrifugation twice, and desalted with a desalting column (18). Viral particles were calculated by means of optical density at 260 nm.

Murine model of hindlimb ischemia. All animal experiments were approved by the Animal Care and Use Committee of Keio University. After anesthetizing male BALB/c nude mice (eight weeks) with diethyl ether, the femoral artery was gently isolated, and the proximal portion was ligated with 7-0 silk ligatures (19,20).

Transplantation of continuously hHGF-producing NIH3T3 cells. The hindlimb ischemic mice (n = 192) were randomly classified into five groups. The control groups received 0.2 ml saline only (n = 14), 500 μg pUC-SRα/hHGF plasmids in 0.2 ml saline (n = 10), 10^9 particles Ad.CA-hHGF in 0.2 ml phosphate-buffered saline (n = 10), or NIH3T3 in 0.2 ml DMEM (n = 14). The experimental group received NIH3T3 + hHGF + TK in 0.2 ml DMEM (n = 144). All injections were given via a 27-gauge needle (21). The numbers of cells transplanted ranged from 10^4 to 10^7. They were injected into two different sites in the ischemic thigh (adductor) skeletal muscles on postoperative day 1. The direction of injection was parallel to the muscle fibers. Angiogenesis and collateral vessel formation were assessed at four weeks.

Laser Doppler blood perfusion analysis. The blood perfusion rate in the ischemic (left leg) and normal (right leg) hindlimb was measured with a laser Doppler perfusion image (LDPI) system (Moor Instruments), as described previously (20,22).

Histopathology. Frozen sections (4 μm) were cut from tissue specimens (23). Immunohistochemical staining for hHGF, endothelial cells, and alpha-smooth muscle actin (SMA) was carried out with anti-human HGF (R&D Systems Inc., Minneapolis, Minnesota), anti-human von Willebrand factor (vWF)/horseradish peroxidase (HRP), and anti-human SMA/HRP (Dakocytomation, Kyoto, Japan), respectively. Sections for staining and counterstaining were incubated with 3,3′-diaminobenzidine tetrahydrochloride and Mayer's hematoxylin solution, respectively. Elastica van Gieson staining was carried out by the standard method. Paraffin sections (3 μm) were cut from tissue specimens, and hematoxylin-eosin staining was carried out by the standard method.

Assay of growth inhibition by ganciclovir in vitro. After seeding cells on six-well plates (10^5 cells/well) and culturing for 24 h, they were exposed to ganciclovir in concentrations ranging from 0 to 10^{-3} g/ml for 72 h (24,25).

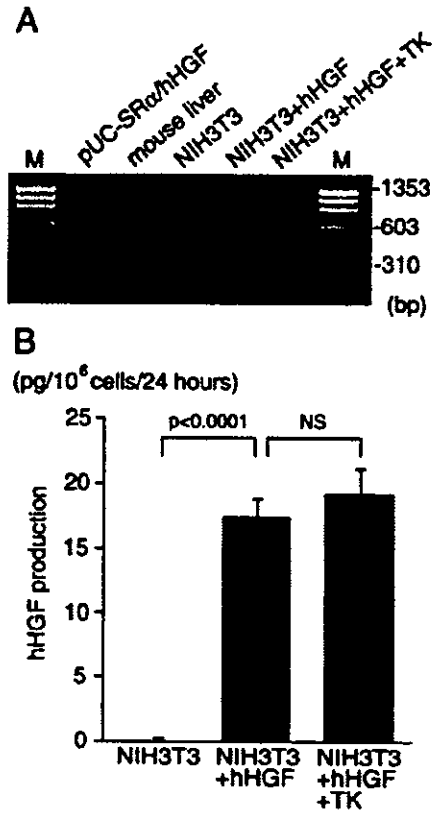

Figure 1. **(A)** Expression of human hepatocyte growth factor (hHGF) messenger ribonucleic acid in the hHGF-transfected NIH3T3 cells. The primer set of reverse transcription-polymerase chain reaction specifically detects hHGF but not mouse HGF. pUC-SRα/hHGF plasmid and mouse liver were used as a positive and negative control, respectively. M = the ΦX174-*Hae*III digest. **(B)** Production of hHGF protein. This ELISA system specifically detects only hHGF because of the lack of cross-reactivity by the antibodies. Data are expressed as hHGF concentrations adjusted for cell number. Both NIH3T3 + hHGF and NIH3T3 + hHGF + thymidine kinase (TK) groups expressed hHGF messenger ribonucleic acid and produced hHGF protein (n = 5).

Detection of ganciclovir-induced apoptosis with annexin V. Annexin V is an early apoptotic marker. The NIH3T3 + hHGF + TK group was exposed to 10^{-7} g/ml ganciclovir for 48 h, and the apoptotic cells were detected with an annexin V-enhanced green fluorescent protein (EGFP) apoptosis detection kit (Medical & Biological Labs Co. Ltd., Nagaya, Japan) (26).

Regulation of transplanted cell growth with ganciclovir in vivo. We investigated the dose-response relationship of growth inhibition by ganciclovir by transplanting NIH3T3 + hHGF + TK (10^7 cells) and administering ganciclovir two weeks later. The transplanted mice received different doses (0, 1, 10, 50, or 80 mg/kg per day) of ganciclovir orally once a day for four weeks.

Statistical analysis. The data were processed using StatView J-4.5 software. Results are reported as the mean value ± SE. Comparisons of values among all groups were performed by one-way analysis of variance. The Scheffe's *F* test was used to determine the level of significance. The probability level accepted for significance was $p < 0.05$.

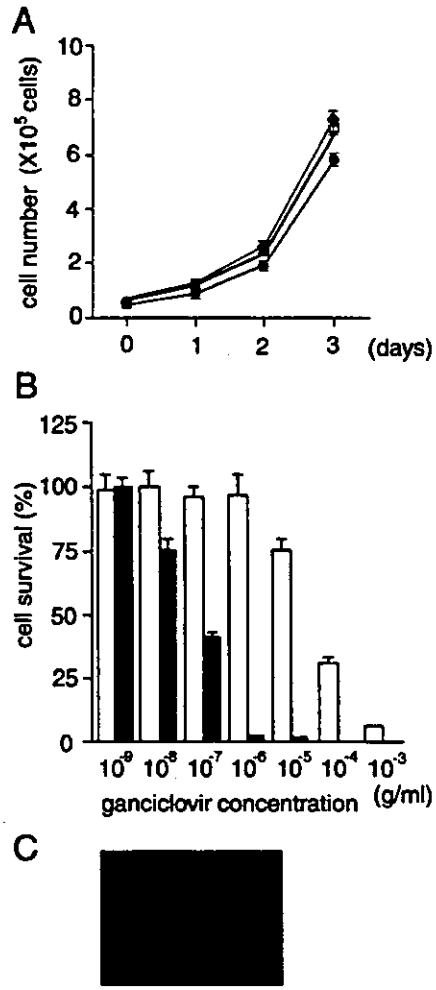

Figure 2. **(A)** Influence of hHGF and/or TK genes on cell growth in vitro. The growth rate of the hHGF-transfected NIH3T3 cells was slightly higher than that of the nontransfected cells, but TK had no effect on cell growth. **(circles** = NIH3T3; **diamonds** = NIH3T3 + hHGF; **squares** = NIH3T3 + hHGF + TK) (n = 3). **(B)** Growth-inhibitory effect of ganciclovir. The IC_{50} of ganciclovir for the NIH3T3 + hHGF + TK group **(solid bars)** was ~1,000 times lower than that for the NIH3T3 + hHGF group **(open bars)** (n = 5). **(C)** Apoptotic cells stained with annexin V-EGFP at the cell membrane after exposure to ganciclovir. Abbreviations as in Figure 1.

RESULTS

Permanently hHGF-transfected NIH3T3 cells produced hHGF protein. The NIH3T3 + hHGF cells were obtained after two weeks of exposure to puromycin, and NIH3T3 + hHGF + TK cells were obtained after two more weeks of exposure to hygromycin. We confirmed that both the NIH3T3 + hHGF and NIH3T3 + hHGF + TK groups expressed hHGF mRNA and then produced hHGF protein at a rate of 17.3 ± 1.4 and 19.1 ± 2.0 pg/10^6 cells per 24 h, respectively (Fig. 1).

Ganciclovir-inhibited cell growth and induced apoptotic cell death. It is well known that HGF regulates cell growth. To determine whether transfection of hHGF affects the growth of NIH3T3 cells, we counted the numbers

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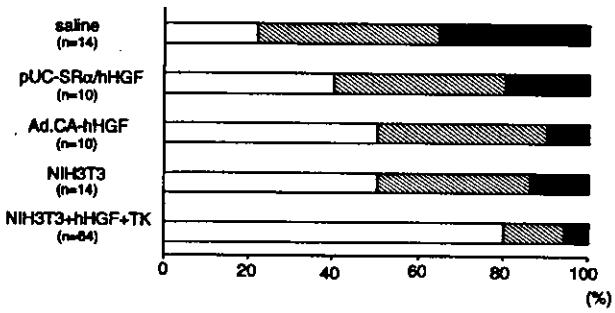

Figure 3. Frequency of necrosis in the ischemic hindlimbs. Severe hindlimb necrosis was significantly reduced in the NIH3T3 + hHGF + TK group. **Open areas** = negative necrosis; **lined areas** = necrosis on toes; **solid areas** = necrosis on foot. Abbreviations as in Figure 1.

of cells in vitro (Fig. 2A). The growth rate of the hHGF-transfected NIH3T3 cells seemed to increase slightly, but the increase was not significant on day 3. Transfection of the TK gene had no effect on their growth rate.

Next, we investigated the growth-inhibitory effect of ganciclovir on these cells (Fig. 2B). The IC_{50} of ganciclovir for the NIH3T3 + hHGF + TK group was ~1,000 times lower than that for the NIH3T3 + hHGF group. These findings confirmed that the TK plasmid genes had been effectively transfected, and that hardly any of the cells that expressed the TK gene survived exposure to ganciclovir at a concentration of 10^{-6} g/ml, which did not affect the control cells.

Enhanced green fluorescent protein fluorescence was detected at the membranes of NIH3T3 + hHGF + TK cells after ganciclovir exposure (Fig. 2C), indicating that cell death was attributable to apoptosis.

Human HGF-producing cell therapy augmented angiogenesis and collateral vessel formation. To evaluate whether transplantation of hHGF-producing cells improves the perfusion of ischemic hindlimbs, we first determined the rate of necrosis of the ischemic hindlimb. Necrosis was rated on a three-grade scale. The rate of necrosis of the foot and toes in the saline group was 35.7% and 42.9%, respectively. The rates in the pUC-SRα/hHGF group were 20% and 40%, respectively, and in the Ad.CA-hHGF group 10% and 40%, respectively. These therapeutic approaches were effective in comparison with the saline group, but they were not sufficient to fully prevent the necrosis. To further ameliorate limb necrosis, we examined angiogenic gene-modified cell transplantation therapy. The NIH3T3 (10^7 cells) group had rates of 14.3% and 35.7%, respectively, suggesting that the vector cell transplantation itself might improve perfusion of the ischemic limb to some extent. In contrast, the rates in the NIH3T3 + hHGF + TK (10^7 cells) group were 5.8% and 14.5%, respectively (Fig. 3). The rate of necrosis was surprisingly reduced in the NIH3T3 + hHGF + TK group, indicating that transplantation of hHGF-producing cells might be one of the most effective methods of improving limb ischemia.

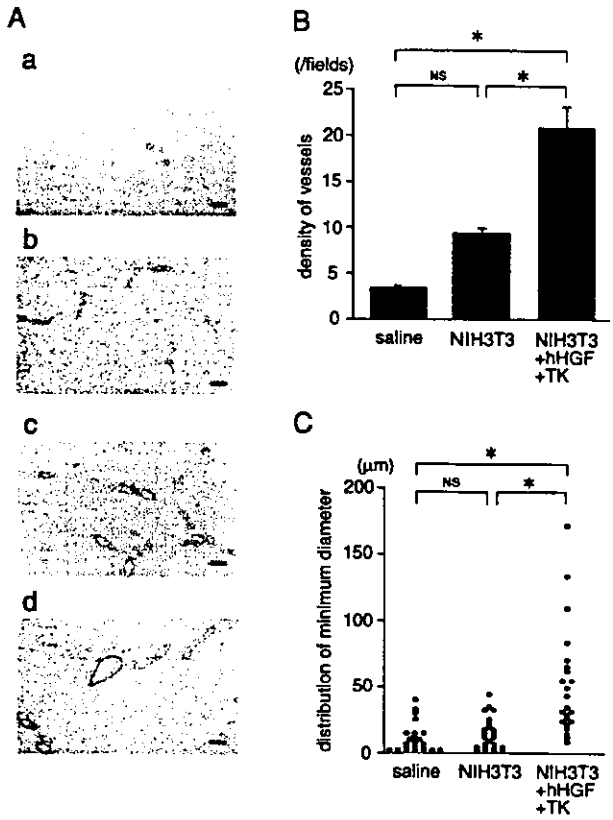

Figure 4. **(A, panels a to d)** Immunohistochemical staining for von Willebrand factor in the triceps muscle of the left calf revealed the presence of numerous vessels. Vessels were larger and more numerous in the NIH3T3 + hHGF + TK group **(panels c and d)** than in the saline **(panel a)** and NIH3T3 groups **(panel b)**. **Scale bars** = 100 μm. **(B)** The number of vessels was determined by observation of 20 random fields from 10 mice (2 fields per mouse; $^*p < 0.01$). **(C)** Distribution of the minimum diameters of the von Willebrand factor-positive vessels ($n = 25$; $^*p < 0.0001$). Abbreviations as in Figure 1.

Vessel density and size. Immunostaining clearly revealed the presence of numerous vessels in the NIH3T3 + hHGF + TK group (Fig. 4A, panel c) and a lower number of vessels in the saline (Fig. 4A, panel a) and NIH3T3 (Fig. 4A, panel b) groups. Quantitative analysis revealed that the vessel density in the ischemic region was significantly higher (Fig. 4B), and the minimum diameter of the vWF-positive vessels was significantly greater (Figs. 4A, panel d, and 4C) in the NIH3T3 + hHGF + TK group.

Vessel maturation. Maturation of the vessels was investigated by staining three consecutive frozen sections of ischemic skeletal muscle. Amazingly, most of the vessels in the NIH3T3 + hHGF + TK group were vWF/α-SMA–double positive (Figs. 5A, panels a and b, and 5B). However, there was no increase in elastic fiber-positive cells, as compared with the saline and NIH3T3 groups (Figs. 5A, panel c, and 5B). These findings showed that NIH3T3 + hHGF + TK cell transplantation strongly induced angiogenesis not only at the capillary level but also at the microvessel (arteriole) level, and it caused angiogenesis at the large blood vessel level.

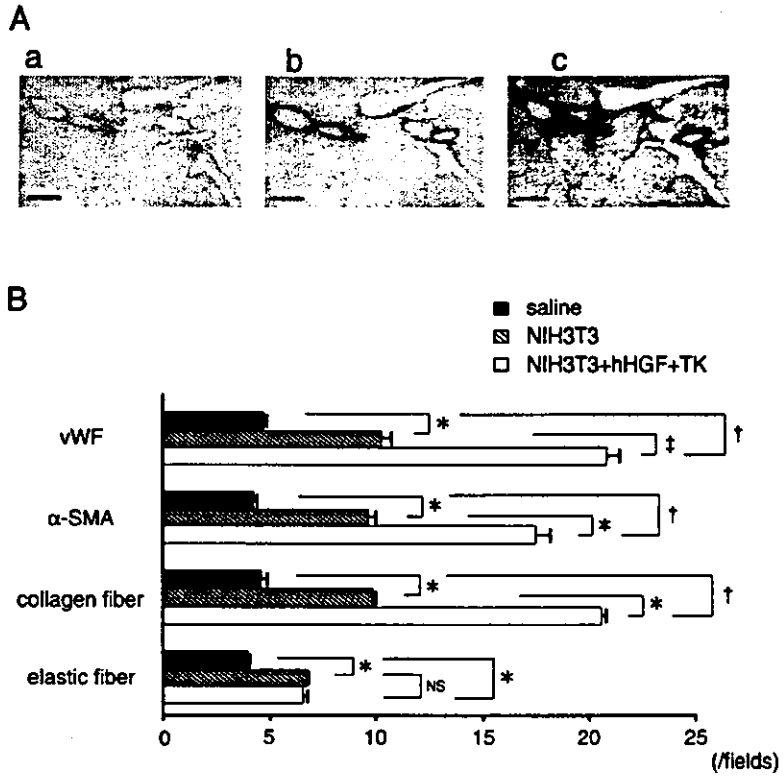

Figure 5. (A, panels a to c) Three consecutive frozen sections of NIH3T3 + hHGF + TK transplanted muscle. **(panel a)** Immunohistochemical staining for vWF and **(panel b)** α-smooth muscle actin (SMA) and **(panel c)** elastica van Gieson staining. **Scale bars** = 100 μm. **(B)** Maturation of vessels was compared by using three consecutive frozen sections. Most of the von Willebrand factor (vWF)-positive vessels in NIH3T3 + hHGF + TK transplanted mice also stained with α-SMA (n = 20; *p < 0.05, †p < 0.001, ‡p < 0.01). Abbreviations as in Figure 1.

Laser Doppler blood perfusion. The LDPI analysis was performed to study subcutaneous blood perfusion. Representative images are shown in Figure 6A, and quantitative analysis of blood perfusion is shown in Figure 6B. No blood perfusion was observed in the hindlimb immediately after femoral artery ligation (Fig. 6A, panel a). Perfusion of the proximal part of the thigh had recovered at four weeks in the saline and NIH3T3 groups, but perfusion distal to the heel joint had markedly decreased (Fig. 6A, panels b and c). In the NIH3T3 + hHGF + TK (10^4 cells) group, perfusion of the ischemic limb almost recovered to the control (nonischemic) level, but perfusion distal to the heel was slightly decreased compared with the control level (Fig. 6A, panel d). In the NIH3T3 + hHGF + TK (10^7 cells) group, perfusion of the ischemic limb was 118.1% (i.e., much greater than that in the control hindlimb) (Figs. 6A and 6B, panel e). To adjust the recovery of blood perfusion in the ischemic limb to the appropriate level, we transplanted NIH3T3 + hHGF + TK (10^7 cells), monitored the LDPI level, and began giving ganciclovir when blood perfusion reached the control level (two weeks). This method enabled us to adjust the blood perfusion rate in the ischemic limb to the same level as in the control limb (Figs. 6A and 6B, panel f).

When the NIH3T3 + hHGF + TK cells were transplanted into the normal nonischemic limb, the blood perfusion increased more than that in the control limb. Up to six weeks after transplantation, no evidence of angiosarcoma or hypervascular tumor was observed in the transplanted limb or other parts of the body (data not shown).

In vivo production of HGF protein. Immunohistochemical staining demonstrated the production of hHGF protein in transplanted NIH3T3 + hHGF + TK cells, but not in transplanted NIH3T3 cells (Fig. 7A).

Cell regulation with ganciclovir and TK. Figure 7B shows a quantitative analysis of the inhibitory effect of ganciclovir on blood perfusion. At a concentration of 50 mg/kg/day of ganciclovir, the blood perfusion was adjusted in the ischemic limb to the same level as in the control limb, and no significant side effects were produced. Histologic examination revealed the natural history of the transplanted cells (Fig. 7C, panels a to c). The transplanted cells formed a mass between the skeletal muscles, which gradually increased in size but did not infiltrate into the skeletal muscle. Two weeks after transplantation of the NIH3T3 + hHGF + TK cells, we began giving ganciclovir orally every day for two to four weeks and then examined tissue samples (Fig. 7C, panels d to f). The NIH3T3 + hHGF + TK cells gradually underwent apoptosis, and by four weeks, no transplanted cells could be detected. The surrounding muscle cells and the generated vessels were unaffected by ganciclovir.

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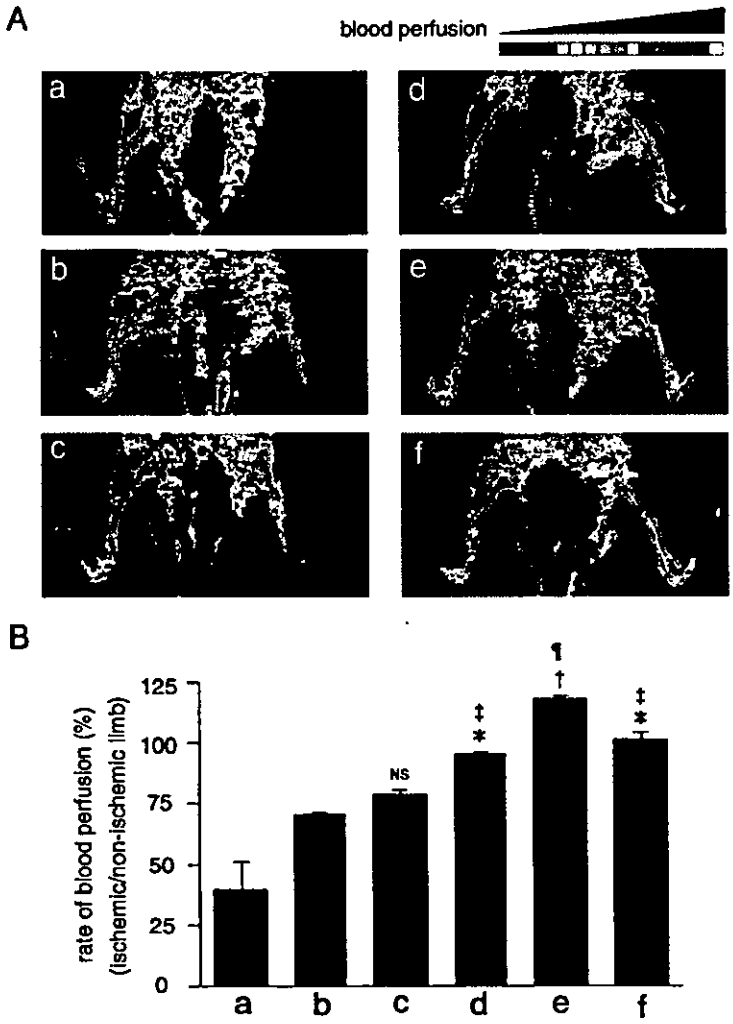

Figure 6. **(A)** Representative laser Doppler perfusion images. **(B)** Quantitative analysis of the rate of blood perfusion of the ischemic/nonischemic limb. **Panel a** = Control mouse on postoperative day 1; **panels b to f** = four weeks after treatment (**panel b** = saline injection; **panel c** = NIH3T3 transplantation [10^7 cells]; **panel d** = NIH3T3 + hHGF + TK transplantation [10^4 cells]; **panel e** = NIH3T3 + hHGF + TK transplantation [10^7 cells]; **panel f** = beginning two weeks after transplantation of NIH3T3 + hHGF + TK (10^7 cells), ganciclovir (50 mg/kg/day) was administered orally for four weeks. Oral ganciclovir administration adjusted the blood perfusion rate of the ischemic limb to the same level as that of the nonischemic limb (eight mice/group). *$p < 0.01$, †$p < 0.001$ vs. saline, ‡$p < 0.05$, ¶$p < 0.01$ versus NIH3T3. Abbreviations as in Figure 1.

DISCUSSION

In this study, we assessed angiogenic gene-modified cell transplantation therapy with fibroblasts permanently transfected with hHGF and TK genes in a murine hindlimb ischemia model. This therapy had the following merits: 1) it induced angiogenesis and collateral vessel formation more effectively than with plasmid and viral vectors. 2) The combination of TK and ganciclovir allowed the angiogenesis to be adjusted by monitoring LDPI. 3) This therapy could be stopped at any time desired for any reason. 4) There was no possibility of the hHGF gene being expressed in nontarget organs or nontarget cells as a result of leakage or dispersion of the vectors. If the plasmid vector was integrated into the genome and neoplastic transformation occurred, it would be difficult to control cell growth. 5) The angiogenic effect can be easily predicted, because the transfection efficiency of the gene is always 100%. 6) The cell vector will be much more effective in patients who require rapid angiogenesis, because plasmid or viral vectors require a week for maximal expression, and the duration of maximal expression is short.

Angiogenic gene-modified cell transplantation therapy has several drawbacks. One is that once the cells are transplanted into patients, their growth cannot be controlled. To solve this problem, we double-transfected the cells with the TK gene, and the results confirmed that permanently transfected cells could be killed with ganciclovir after the establishment of angiogenesis and collateral vessel formation. The finding that the IC_{50} of ganciclovir for the TK-transfected cells was 1,000 times lower than that in the nontransfected cells indicated that this system might be capable of being used in clinical settings.

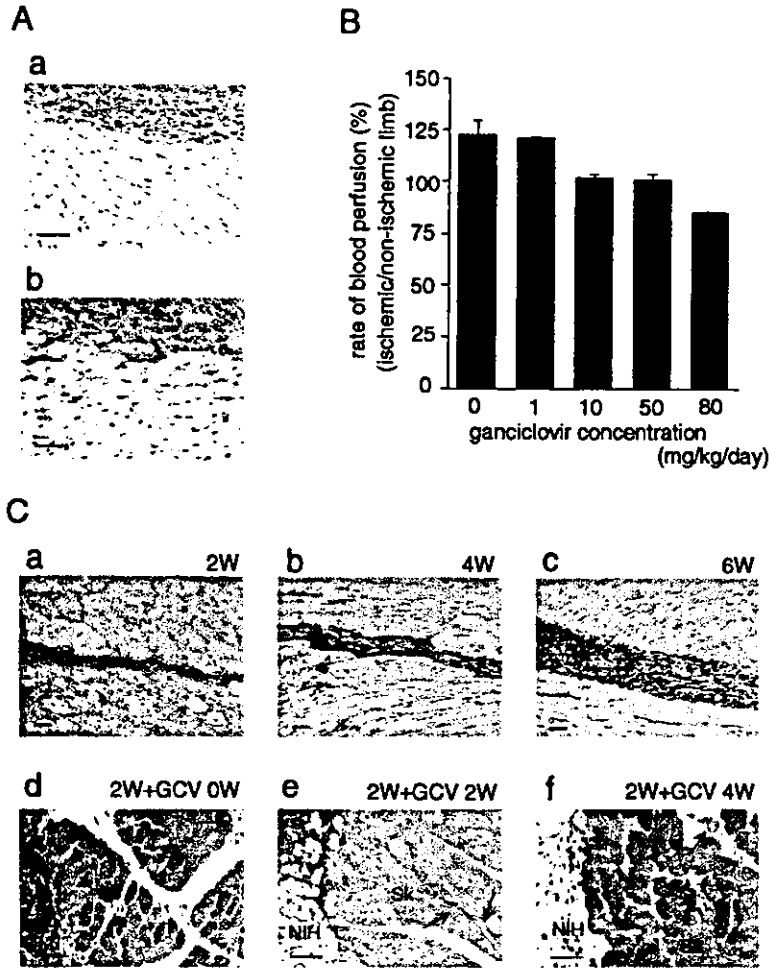

Figure 7. **(A)** Immunohistochemical staining for hHGF in transplanted NIH3T3 cells **(panel a)** and NIH3T3 + hHGF + TK cells **(panel b)** in the skeletal muscle. **Scale bars** = 50 μm. **(B)** The NIH3T3 + hHGF + TK (10^7) cells were transplanted, and two weeks later, various concentrations of ganciclovir were administered for another four weeks. **(C)** Hematoxylin-eosin staining. **(Panels a to c)** The natural history of the transplanted NIH3T3 + hHGF + TK (10^7) cells is shown. **(Panels d to f)** Beginning two weeks after transplantation, ganciclovir (50 mg/kg/day) was administered orally for two to four weeks. The cells had completely disappeared after four weeks of ganciclovir treatment. **Arrows** indicate the microvessels. **Scale bars** = 100 μm. Abbreviations as in Figure 1.

We used NIH3T3, a fibroblast line derived from fetal NIH/Swiss mice, for the following reasons: 1) the transfection efficiency of the plasmid is high; and 2) their growth rate is relatively high in vitro, making it easy to expand the cells. However, their growth rate in vivo is not as high as that of carcinoma cell lines, probably because NIH3T3 cells have a mechanism of growth inhibition by cell-cell contact. To apply this method in clinical medicine, the selection of a human cell line will be requried. Considering the time and cost for preparation of the cells, an autograft might require a long time and be expensive. It took at least two months to prepare the hHGF- and TK-double-transfected cells, and a number of additional experiments were needed to confirm their effectiveness and safety. We think that allograft cells should be used to prepare gene-modified cells. In view of the time, cost, effectiveness, and safety of the cells, allografts would be much better than autografts.

Regenerative medicine has recently been the subject of investigations in many fields, and a number of regenerative cells have been established. The authors have reported that regenerative cardiomyocytes can be generated from marrow mesenchymal stem cells, and transplantation of the regenerated cells will be examined in various organs. One of the reasons why we are considering angiogenic gene-modified cell transplantation therapy is the need for a rapid blood supply to the transplanted cells. To achieve that goal, we can co-transplant target organs with these gene-modified cells in combination with the regenerated cells. Once the blood supply has become established, the angiogenic cells are no longer needed, and they can be eliminated by ganciclovir.

Bone marrow mononuclear cells have recently been used to induce angiogenesis as a means of treating arteriosclerosis obliterans (27). Although bone marrow mononuclear cells contain endothelial cells, the population of endothelial progenitor cells is <1%. The effectiveness of this therapy may be explained not only by the presence of endothelial

progenitor cells but also by the fact that bone marrow mononuclear cells produce various cytokines and angiogenic growth factors. The advantage of angiogenic therapy with bone marrow mononuclear cell autografts is that the cells do not undergo immunorejection. The drawback of this therapy is that the cells may contain a variety of types of cells, such as osteogenic or chondrogenic stem cells, or induce inflammation by secreting cytokines. Using angiogenic gene-modified cells avoids the problem of transplanting different types of cells; however, the efficiency and safety of this procedure needs to be fully investigated before clinical application can become a reality.

Acknowledgments

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Reprint requests and correspondence: Dr. Keiichi Fukuda, Institute for Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kfukuda@sc.itc.keio.ac.jp.

REFERENCES

1. Isner JM, Pieczek A, Schainfeld R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996;348:370–4.
2. Yukawa H, Takahashi JC, Miyatake SI, et al. Adenoviral gene transfer of basic fibroblast growth factor promotes angiogenesis in rat brain. Gene Ther 2000;7:942–9.
3. Suri C, McClain J, Thurston G, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282:468–71.
4. Morishita R, Nakamura S, Hayashi S, et al. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 1999;33:1379–84.
5. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–95.
6. Hayashi S, Morishita R, Nakamura S, et al. Potential role of hepatocyte growth factor, a novel angiogenic growth factor, in peripheral arterial disease: downregulation of HGF in response to hypoxia in vascular cells. Circulation 1999;100 Suppl II:II301–8.
7. Byun J, Heard JM, Huh JE, et al. Efficient expression of the vascular endothelial growth factor gene in vitro and in vivo, using an adeno-associated virus vector. J Mol Cell Cardiol 2001;33:295–305.
8. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103: 697–705.
9. Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J Virol 1969;4:549–53.
10. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989;342: 440–3.
11. McKnight SL. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res 1980;8:5949–64.
12. Hayashi S, Morishita R, Higaki J, et al. Autocrine-paracrine effects of overexpression of hepatocyte growth factor gene on growth of endothelial cells. Biochem Biophys Res Commun 1996;220:539–45.
13. Adra CN, Boer PH, McBurney MW. Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. Gene 1987;60:65–74.
14. Ueda H, Sawa Y, Matsumoto K, et al. Gene transfection of hepatocyte growth factor attenuates reperfusion injury in the heart. Ann Thorac Surg 1999;67:1726–31.
15. Nakamura Y, Morishita R, Higaki J, et al. Expression of local hepatocyte growth factor system in vascular tissues. Biochem Biophys Res Commun 1995;215:483–8.
16. Yamada A, Matsumoto K, Iwanari H, et al. Rapid and sensitive enzyme-linked immunosorbent assay for measurement of HGF in rat and human tissues. Biomed Res 1995;16:105–14.
17. Mizuguchi H, Kay MA. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum Gene Ther 1999;10:2013–7.
18. Chen SH, Chen XH, Wang Y, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci USA 1995;92:2577–81.
19. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. Am J Pathol 1998;152: 1667–79.
20. Murohara T, Asahara T, Silver M, et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 1998;101:2567–78.
21. Morishita R, Sakaki M, Yamamoto K, et al. Impairment of collateral formation in lipoprotein(a) transgenic mice: therapeutic angiogenesis induced by human hepatocyte growth factor gene. Circulation 2002; 105:1491–6.
22. Shintani S, Murohara T, Ikeda H, et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001;103:897–903.
23. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J Clin Invest 1994;93:662–70.
24. Smee DF, Martin JC, Verheyden JP, Matthews TR. Anti-herpesvirus activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl) guanine. Antimicrob Agents Chemother 1983;23:676–82.
25. Cho HS, Park YN, Lyu CJ, et al. Effects of retroviral-mediated herpes simplex virus thymidine kinase gene transfer to murine neuroblastoma cell lines in vitro and in vivo. Acta Oncol 1999;38:1093–7.
26. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84:1415–20.
27. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85: 221–8.

Research article Related Commentary, page 811

Endothelin-1 regulates cardiac sympathetic innervation in the rodent heart by controlling nerve growth factor expression

Masaki Ieda,[1] Keiichi Fukuda,[1,2] Yasuyo Hisaka,[2] Kensuke Kimura,[1] Haruko Kawaguchi,[2] Jun Fujita,[1] Kouji Shimoda,[3] Eiko Takeshita,[4] Hideyuki Okano,[5] Yukiko Kurihara,[6] Hiroki Kurihara,[6] Junji Ishida,[7] Akiyoshi Fukamizu,[7] Howard J. Federoff,[8] and Satoshi Ogawa[1]

[1]Cardiopulmonary Division, Department of Internal Medicine, [2]Institute for Advanced Cardiac Therapeutics, [3]Laboratory Animal Center, [4]Department of Laboratory Medicine, and [5]Department of Physiology, Keio University School of Medicine, Tokyo, Japan.
[6]Division of Physiological Chemistry and Metabolism, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Tokyo, Tokyo, Japan.
[7]Center for Tsukuba Advanced Research Alliance and Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan.
[8]University of Rochester Medical Center, Center for Aging and Developmental Biology, Rochester, New York, USA.

The cardiac sympathetic nerve plays an important role in regulating cardiac function, and nerve growth factor (NGF) contributes to its development and maintenance. However, little is known about the molecular mechanisms that regulate NGF expression and sympathetic innervation of the heart. In an effort to identify regulators of NGF in cardiomyocytes, we found that endothelin-1 specifically upregulated NGF expression in primary cultured cardiomyocytes. Endothelin-1–induced NGF augmentation was mediated by the endothelin-A receptor, Giβγ, PKC, the Src family, EGFR, extracellular signal–regulated kinase, p38MAPK, activator protein-1, and the CCAAT/enhancer-binding protein δ element. Either conditioned medium or coculture with endothelin-1–stimulated cardiomyocytes caused NGF-mediated PC12 cell differentiation. NGF expression, cardiac sympathetic innervation, and norepinephrine concentration were specifically reduced in endothelin-1–deficient mouse hearts, but not in angiotensinogen-deficient mice. In endothelin-1–deficient mice the sympathetic stellate ganglia exhibited excess apoptosis and displayed loss of neurons at the late embryonic stage. Furthermore, cardiac-specific overexpression of NGF in endothelin-1–deficient mice overcame the reduced sympathetic innervation and loss of stellate ganglia neurons. These findings indicate that endothelin-1 regulates NGF expression in cardiomyocytes and plays a critical role in sympathetic innervation of the heart.

Introduction

Cardiac tissues are extensively innervated by autonomic nerves. The cardiac sympathetic nerve plays an important role in modulating heart rate, conduction velocity, myocardial contraction, and relaxation. Although several molecules that regulate the development of the heart have been well characterized, little is known about the mechanism that regulates sympathetic innervation of the heart. The cardiac sympathetic nerve extends from the sympathetic neuron in stellate ganglia (SG), which is derived from the neural crest (1). Nerve growth factor (NGF) is a prototypic member of the neurotrophin family, members of which are critical for the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems (2, 3). Levels of NGF expression within innervated tissues roughly correspond to innervation density (4). The volume of sympathetic ganglion is reduced by at least 80% at postnatal day 3 in mice with a disruption of the *NGF* gene. In mice that lack the NGF receptor TrkA, no neurons remain at postnatal day 9 (2). Deletion of a single copy of the *NGF* gene results in a 50% reduction in sympathetic neurons (5), while overexpression of NGF in the heart results in cardiac hyperinnervation and hyperplasia in SG neurons (6). These results demonstrate the importance of NGF in the regulation of sympathetic neuron development and innervation.

In pathological states, NGF production in the heart is variable. In ischemic hearts, an increase in cardiac NGF leads to regeneration of sympathetic nerves (7, 8). In a previous experiment, we found that NGF was upregulated in streptozotocin-induced diabetic murine hearts (9). In contrast, it was reported that NGF and sympathetic innervation were reduced in congestive heart failure (10). Despite their importance, the molecular mechanisms that regulate NGF expression and sympathetic innervation in the heart remain poorly understood.

Endothelin-1 (ET-1) is believed to play a critical role in the pathogenesis of cardiac hypertrophy, hypertension, and atherosclerosis. Gene targeting of ET-1 and its receptor endothelin-A (ET_A) resulted in unexpected craniofacial and cardiovascular abnormalities. These phenotypes are consistent with interference of neural crest differentiation. The influence of ET-1 on neural crest development remains undetermined (11–13).

We hypothesized that ET-1 could affect the induction of neurotrophic factors, and that its disruption might contribute to the immature development of neural crest–derived cells. In this study, we found ET-1–specific induction of NGF in cardiomyocytes, iden-

Nonstandard abbreviations used: activator protein-1 (AP-1); angiotensinogen-deficient (mouse) (*Agt*$^{-/-}$); B-type natriuretic peptide (BNP); carboxyl terminus of β-adrenergic receptor kinase (βARK-ct); cardiac-specific overexpression of *NGF* gene under the control of the cardiac-specific α-myosin heavy chain promoter (mouse) (MHC-NGF); CCAAT/enhancer-binding protein δ (C/EBPδ); C-terminal Src kinase (Csk); deletion mutant of EGFR (533delEGFR); dominant-negative mutant adenovirus of extracellular signal–regulated kinase (DN-ERK); dominant-negative mutant adenovirus of p38MAPK (DN-p38); embryonic day (E); endothelin-1 (ET-1); endothelin-A (ET_A); ET-1–deficient (mouse) (*Edn1*$^{-/-}$); extracellular signal–regulated kinase (ERK); growth-associated protein 43 (GAP43); leukemia inhibitory factor (LIF); nerve growth factor (NGF); pertussis toxin (PTX); protein gene product 9.5 (PGP9.5); protein kinase A (PKA); stellate ganglia (SG); tyrosine hydroxylase (TH).

Conflict of interest: The authors have declared that no conflict of interest exists.

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tified the signaling pathways involved, and studied the ET-1–NGF pathway–mediated development of the sympathetic nervous system in the heart. In ET-1–deficient (*Edn1*$^{-/-}$) mice, the sympathetic nerve density and norepinephrine concentration were markedly reduced, and a loss of SG neurons by excess apoptosis was observed due to downregulation of cardiac NGF. Moreover, we demonstrated that the sympathetic nerve retardation was restored in *Edn1*$^{-/-}$ mice, which overexpressed the *NGF* gene under the transcriptional control of the cardiac-specific α-myosin heavy chain promoter (*Edn1*$^{-/-}$/MHC-NGF mice). These findings show that ET-1 is a key regulator of NGF expression in cardiomyocytes, and that the ET-1–NGF pathway is critical for sympathetic innervation in the heart.

Methods

Cell culture. Primary culture for cardiomyocytes and cardiac fibroblasts and the cultures for PC12 cells were as described previously (14, 15).

RNA extraction, Northern blot, and quantitative RT-PCR. RNA and quantitative RT-PCR were performed as described previously (14). To detect the four murine NGF alternatively spliced transcripts, four primer sets were designed as follows: the exon 2–specific forward primer (for transcript a) was 5′-CTCCTAGTGAAGATGCTGTGCC-3′, the forward primer specific to exons 1B–3B (for transcript b) was 5′-AGCGCATCGAGTTTTGGCCTGT-3′, the exon 1A–specific forward primer (for transcript c) was 5′-TGGCTTTTCCTGGCTATGTCC-3′, the exon 3A–specific forward primer (for transcript d) was 5′-AGTGCTTGCCTTATTGGGAC-3′, and the same reverse primer was used in four primer sets, 5′-CTGTGGCTGTGGTCTTATCTC-3′. For Northern blot analysis, 2 μg of poly(A)$^+$ RNA was used. Rat NGF, B-type natriuretic peptide (BNP), and GAPDH cDNA were obtained by RT-PCR from the heart. Rat NGF primers were 5′-GCAGACCCGCAACATCACTG-3′ and 5′-TCTCCAACCCACACACTGACA-3′. The primers and probes for murine NGF were forward, 5′-GCCAAGGACGCAGCTTTCTA-3′; reverse, 5′-GCCTGTACGCCGATCAAAA-3′; and probe, 5′-FAM-CCGCAGTGAGGTGCATAGCGTA ATGTC-TAMRA-3′. Primers for NGF expression in *Edn1*$^{-/-}$/MHC-NGF mice were forward, 5′-GATCGGCGTACAGGCAGAA-3′; reverse, 5′-TGGGCTTCAGGGACAGAGTCT-3′; and probe, 5′-FAM-CGTACACAGATAGCAATG-MGB-3′. Primers for neurotrophin-3 were forward, 5′-AACATAAGAGTCACCGAGGAGAGTACT-3′; reverse, 5′-ATGTC AATGGCTGAGGACTTGTC-3′; and probe, 5′-FAM-CACCCACAGGCTCTCACTGT CACACA-TAMRA-3′. The mRNA levels were normalized by comparison to GAPDH mRNA.

Reagents. Reagents were supplied by the following sources: Sigma-Aldrich (St. Louis, Missouri, USA) supplied ET-1 (10^{-7} M), angiotensin II (10^{-7} M), phenylephrine (10^{-5} M), BQ123 (10^{-5} M), H89 (2×10^{-6} M), PD98059 (5×10^{-5} M), SB203580 (10^{-5} M), PMA (10^{-6} M), chelerythrine (10^{-5} M), IGF-1 (10 ng/ml), pertussis toxin (PTX; 100 ng/ml), wortmannin (10^{-8} M), KN62 (10^{-5} M), EGTA (4×10^{-3} M), 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) (10^{-4} M), and anti-NGF blocking antibody (1:10,000). Calbiochem-Novabiochem Corp. (San Diego, California, USA) supplied PP2 (10^{-5} M) and AG1478 (5×10^{-7} M). Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) supplied leukemia inhibitory factor (LIF; 1,000 U/ml). Takeda Chemical Industries Ltd. (Osaka, Japan) supplied TAK044 (10^{-6} M).

Adenoviruses. β-Gal (LacZ) and the recombinant adenovirus for the carboxyl terminus of β-adrenergic receptor kinase (βARK-ct) were provided by H. Kurose (University of Tokyo, Tokyo, Japan). Dominant-negative mutant adenoviruses of extracellular signal–regulated kinase (DN-ERK) and p38MAPK (DN-p38) were provided by S. Mitsuyama (Kumamoto University, Kumamoto, Japan). Infection was performed as described previously (16, 17).

Preparation of conditioned medium. Cardiomyocytes were incubated in medium containing serum for 24 hours. The medium was replaced with fresh serum-free medium, and then the cells were stimulated with several factors. After 6 hours, the conditioned medium was collected and assayed.

ELISA for NGF. An ELISA kit was purchased from Promega Corp. (Madison, Wisconsin, USA).

Plasmids and luciferase assay. Luciferase plasmids containing various lengths of NGF promoter were provided by P.F. Johnson (National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, Maryland, USA) (18). C-terminal Src kinase (Csk) and the deletion mutant of EGFR (533delEGFR) were provided by H. Sabe (Kyoto University, Kyoto, Japan) and H. Matsubara (Kyoto Prefectural University of Medicine, Kyoto, Japan) (19).

Differentiation of PC12 cells. Cells were examined under a phase-contrast microscope. The number of differentiated cells was counted in six randomly selected fields. The criterion for distinguishing differentiated from undifferentiated cells was neurite outgrowth greater in length than two cell-body diameters.

Coculture of PC12 cells with cardiomyocytes. PC12 cells (4×10^3/cm^2) transfected with adenoviral LacZ were overlaid onto the cardiomyocytes (4×10^4/cm^2). Cells were fixed and stained with X-gal reagent.

Animals. Edn1$^{-/-}$, angiotensinogen-deficient (*Atg*$^{-/-}$), and MHC-NGF mice were generated as described previously (6, 11, 20). Osmotic minipumps containing BQ123 or candesartan were implanted in the pregnant *Edn1*$^{+/-}$ or *Atg*$^{+/-}$ mice as described previously (12, 21). *Edn1*$^{+/-}$ mice were crossed with MHC-NGF mice to generate *Edn1*$^{+/-}$/MHC-NGF mice, which were in turn crossed with *Edn1*$^{+/-}$ to generate *Edn1*$^{-/-}$/MHC-NGF mice. The Keio University Ethics Committee for Animal Experiments approved all experiments in this study.

Norepinephrine measurement. Norepinephrine concentration was determined by HPLC as described previously (22).

Immunohistochemistry in hearts. To detect nerve fibers in hearts, paraffin-embedded sections were incubated with antibody against tyrosine hydroxylase (TH; Chemicon International Inc., Temecula, California, USA), growth-associated protein 43 (GAP43; Chemicon International Inc.), and protein gene product 9.5 (PGP9.5; Cambridge Bioscience, Cambridge, United Kingdom). Following hybridization with the secondary antibody, sections were incubated with diaminobenzidine. Nerve density was determined using NIH Image, as described previously (7, 8).

Histology in sympathetic ganglia. For whole-mount immunostaining, embryos were fixed with paraformaldehyde and stained with anti-TH antibody as described previously (23). Paraffin-embedded sections were stained with cresyl violet, antibody against TH and Ki-67 (DAKO Corp., Carpinteria, California, USA); neuronal cell apoptosis was detected using ApopTag kit (Chemicon International Inc.) according to the manufacturer's instructions. The total number of neurons and the neuron area were determined as described previously (24).

Statistical analysis. Values are presented as means ± SEM. Differences between groups were examined for statistical significance using Student's *t* test or ANOVA with Fisher's protected least significant difference test. *P* values less than 0.05 were regarded as significant.

Results

ET-1, but not angiotensin II, phenylephrine, LIF, or IGF-1, increases NGF expression in cardiomyocytes. Transcription of the *NGF* gene results in four different sizes by alternative splicing (25). The levels of the four

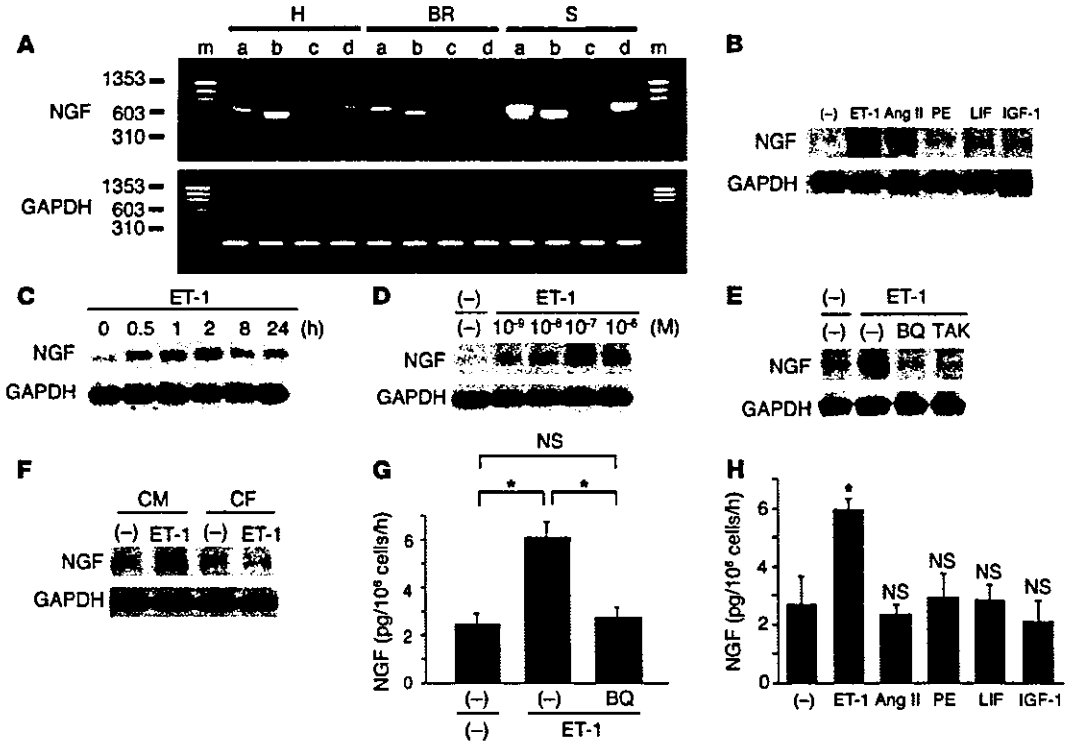

Figure 1

Specific augmentation of NGF expression by ET-1 in cardiomyocytes. (**A**) Gene expression of four NGF alternatively spliced transcripts (a–d) in murine heart (H), brain (BR), and submaxillary gland (S) was determined by RT-PCR. The number of PCR cycles is 35. m, marker. (**B**) Cardiomyocytes were stimulated with ET-1, angiotensin II (Ang II), phenylephrine (PE), LIF, or IGF-1 for 2 hours. Northern blot analysis for NGF was performed. (**C**) Stimulation of cardiomyocytes with ET-1 for specified time intervals. (**D**) Stimulation of cardiomyocytes with various concentrations of ET-1. (**E**) Cardiomyocytes were pretreated with BQ123 (BQ) or TAK044 (TAK) for 30 minutes; then RNA was isolated 2 hours after ET-1 stimulation. (**F**) Induction of NGF expression by ET-1 in cardiomyocytes (CM), but not in cardiac fibroblasts (CF). Results similar to those shown in **A**–**F** were obtained in four separate experiments. (**G**) NGF protein levels in conditioned medium. Augmentation of NGF protein was inhibited by pretreatment with BQ123. ($n = 4$.) (**H**) Secretion of NGF by cardiomyocytes was not induced by angiotensin II, phenylephrine, LIF, or IGF-1 ($n = 4$). *$P < 0.001$. NS, not significant vs. control.

NGF transcripts in the murine heart, brain, and submaxillary gland were determined by RT-PCR using the four primer sets to distinguish each transcript (Figure 1A). All transcripts were detected in the heart. Consistent with a previous study (25), transcript b was the major NGF mRNA species in the heart. Cardiomyocytes were stimulated with various cardiac hypertrophic factors, and NGF expression was ascertained by Northern blot analysis (Figure 1B). Of these factors, only ET-1 augmented NGF expression, which was induced by a 30-minute incubation and peaked after 2 hours in a dose-dependent manner (Figure 1, C and D). Preincubation with BQ123 (an ET_A receptor antagonist) and TAK044 (an $ET_{A/B}$ receptor antagonist) completely inhibited ET-1–induced NGF expression (Figure 1E), indicating that ET_A mediates this induction. To determine the cell type responsible for NGF induction, cardiomyocytes and cardiac fibroblasts were prepared separately (14), and the induction experiments were repeated. We found that NGF induction occurred only in cardiomyocytes (Figure 1F), indicating that the induction process occurs in a cell type–specific manner.

The NGF protein in ET-1–stimulated cardiomyocyte-conditioned medium increased 2.2-fold. BQ123 completely inhibited this augmentation (Figure 1G). Other hypertrophic factors had no effect on NGF expression (Figure 1H).

Intracellular signaling of ET-1–induced NGF augmentation is distinct from the ET-1–induced BNP pathway. The ET_A receptor binds to three types of G proteins: Gs, Gq, and Gi (26). To determine the specific G protein subtype, we pretreated the cells with the Gi inhibitor PTX or the protein kinase A (PKA) inhibitor H89. Contrary to the well-known hypertrophic-signaling pathways, NGF induction was completely repressed by PTX (Figure 2A). To determine whether Giα or Giβγ is involved in this process, we transfected the cells with the βARK-ct adenovirus and stimulated them with ET-1. βARK-ct significantly attenuated induction of NGF (Figure 2B), showing that Giβγ plays an important role in this pathway. Expression of the hypertrophic markers atrial natriuretic peptide (data not shown) and BNP was unaffected in these experiments (Figure 2, A and B).

The Giβγ-mediated pathway is known to activate various signaling molecules in other cell types (16, 27). We examined the effects of various signal transduction inhibitors and found that ET-1–induced NGF augmentation was attenuated by chelerythrine, PMA (long pretreatment), PP2, AG1478, PD98059, and SB203580, but unaffected by wortmannin or KN62 (Figure 2, D–G). Transient stimulation with PMA also induced NGF expression (Figure 2C). BNP was downregulated with PD98059 pretreatment but was unaffected by other