骨髄間葉系幹細胞を用いた心筋再生の現状と展望

Present and future prospect of cardiomyocyte regeneration using bone marrow mesenchymal stem cells

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◎1999年に著者らが骨髄間葉系幹細胞から心筋細胞が誘導できると報告して以来[1],多くの研究がなされ現在に至っている.その後,いくつかの研究室でも同様に心筋細胞が得られることが確認された.骨髄細胞が自己の細胞であり,骨髄移植などの経験もあったことから,その後,臨床例も含めて骨髄細胞を直接あるいは骨髄単核球成分にした後に心筋内に注入する方法も行われている[2,3].しかし,いかなる方法が患者に有用であるか,科学的にもっとも優れているかを検証しながら基礎研究,臨床研究を進めていかねばならない.本稿では,骨髄細胞を用いた心筋再生の現状を述べることとする.

Key word 骨髄間葉系幹細胞,SP細胞分画,骨髄単核球

臓器移植患者における心臓の解析

女性ドナーより心臓移植を受けた男性患者が別の原因で死亡した症例で心臓を解析したところ,心筋細胞にY染色体を有する細胞が存在することが報告され,実際に生体でも骨髄由来の細胞が心筋細胞にも分化するのではないか考えられるに至った[4].著者らも,GFPトランスジェニックマウスの骨髄を,致死量の放射線を照射したC57/BL6マウスに骨髄移植をし,心筋梗塞を作製したモデルで解析した結果,心筋梗塞中心部および心筋梗塞境界領域にGFP陽性の心筋細胞が観察された(図1).これらの結果は骨髄細胞の一部が心筋細胞に分化することを示している.それではいかなる細胞がいかなる条件で心筋細胞に分化するのであろうか.

その後の研究で,胚性幹細胞と骨髄細胞を共培養すると,まれな頻度ではあるが,細胞融合することを報告している[5].すなわち,染色体が4倍体の細胞が出現するというものである.細胞融合の存在は研究者に幹細胞が本当に分化するのか,それともみかけだけの分化をするのか,大きな波紋を投げかけた.実際に特殊な肝細胞障害マウスモデルでは生体内での細胞融合が確認された例も存在する[6].しかし,細胞融合の頻度はそれほど多いものではないことも判明し,幹細胞研究の方向はさらに進むこととなった.

造血幹細胞を単離する方法として,Hoechst33342というDNA結合色素を用いる方法が開発され,SP細胞分画とよばれる分画が盛んに利用されるようになった[7].SP細胞分画(図2)は骨髄細胞のみならず,多くの組織中にも存在し,幹細胞単離の方法として注目されるに至った.しかし,SP細胞分画は*in vivo*では細胞増殖するが,*in vitro*では細胞増殖しない(しにくい)こともあり,完全な解明にはなお時間を要する.骨格筋細胞中のSP細胞を用いて細胞移植を行うと,骨髄が完全に再構築できたとの報告があり,組織幹細胞が血液幹細胞になるのではないかと報告され[8],一時話題をさらったが,その後の研究で骨格筋中のSP細胞は骨髄からきた細胞の混入であることが報告され[9],細胞の可塑性は慎重に判断されなければならないことがわかった.

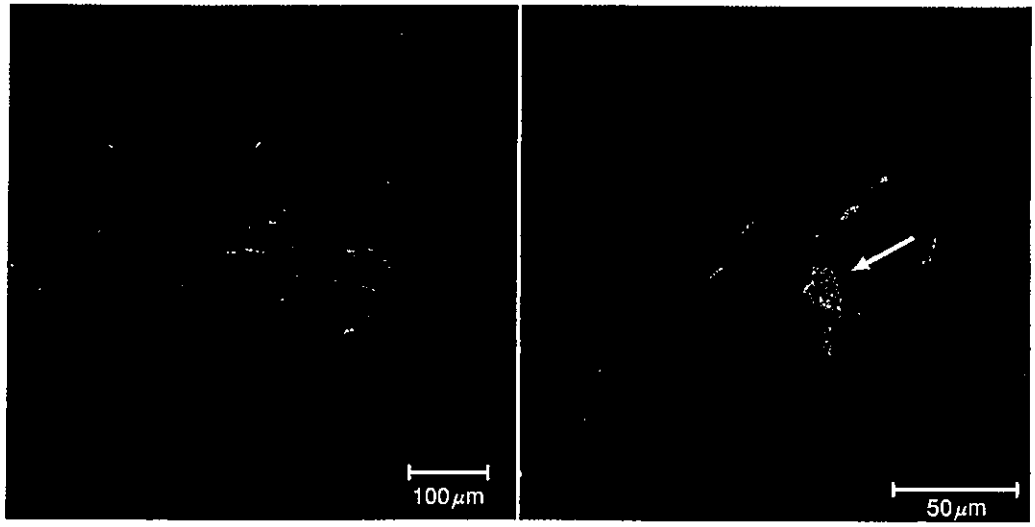

図 1

GFP トランスジェニックマウスの骨髄細胞を健常マウスに骨髄移植し，心筋梗塞を作製した．左は心筋梗塞作製部，右は梗塞部の核大像を示す．赤はアクチニン，緑は GFP，青は TOTO3 染色で核を示す．矢印は，GFP 陽性，アクチニン陽性の再生心筋を示す．

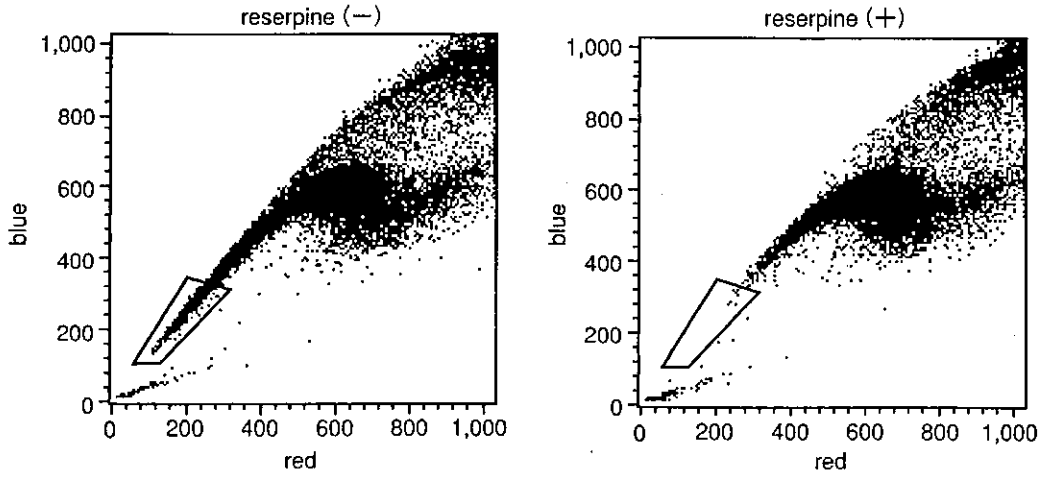

図 2　SP 細胞分画

骨髄や各種組織を Hoechst33342 dye で染色し，blue と red の 2 波長で展開させた FACS 解析を示した．四角で囲んだ領域が SP 細胞分画である．SP 細胞分画はレセルピン前投与で完全に消失する特徴がある．

GFP 陽性細胞の骨髄移植の研究から，造血幹細胞の可塑性が一時期強調された時期があった．すなわち，血液幹細胞がすべての細胞になりうるのではないかというものである．血液学者の Weissman らは，GFP 陽性の 1 個の造血幹細胞(少数の分化した細胞とともに)を別のマウスに骨髄移植した実験を行った[10]．全身の臓器をくまなく検索し，GFP 陽性の細胞は小脳細胞などのごく一部の臓器で観察されたのみであることを報告した．しかし，彼らの報告は心筋梗塞などの障害のないモデルでの研究であり，組織障害モデルでの研究が求められる．著者らを含めて組織障害モデルでの研究は現在進行中である．

骨髄間葉系幹細胞研究の現状

骨髄間葉系幹細胞は骨髄中に存在するまれな細胞群で，*in vitro* でも骨芽細胞，軟骨芽細胞，脂肪細胞，骨格筋細胞，心筋細胞などに分化する．骨髄の細胞のうち培養皿に接着するものすべてが間葉系幹細胞と考えられている方がいるが，これはよくなされる誤解である．骨髄細胞中で培養皿に接着するもののうち，その多くはマクロファージ

表 1　再生医学の材料としてのES細胞と成体幹細胞の比較

	ES細胞	成体幹細胞
細胞の単離，樹立	すでに確立した方法がある．一度樹立すれば多くの症例で使用可能	単離法はまだ未確立．個々の症例で樹立する必要あり
細胞分裂能	無限に増殖すると考えられる	70継代は可能とされるが，無限かどうかは不明
分化誘導法	胚様体をつくる方法がすでに確立しているが，効率が悪い．特異的な方法はまだ確立していない	特異的な方法はまだ確立していない
腫瘍形成	可能性あり	可能性はない，あるいは低い
拒絶反応	あり	なし
免疫抑制剤の使用	必要	不要
ドナー	ドナーは必要であるが，一度ES細胞を樹立すればその後は必要ない	ドナーは不要であるが，本人の骨髄から採取する必要あり
倫理的問題	慎重な検討が必要	なし
大量生産化	可能，比較的安価	労力と費用がかかる
コメント	工業生産化に向いているが，オーダーメイドの細胞をつくることはできない	オーダーメイドの心筋細胞がつくれるが，時間と経費はかなりかかると予想される

間葉系幹細胞の特徴をES細胞との比較により示した．

と骨髄間質細胞である．骨髄間質細胞は造血系細胞のニッシェ(局所環境)をつくる細胞であり，骨髄間質細胞すべてが多分化能をもつわけではない．間葉系幹細胞はこの分画に含まれるごく少数の細胞で表面抗原などもまったく異なるものである．

骨髄間葉系幹細胞由来の再生心筋細胞の性質に関しては本誌の既刊[11]にも述べているので，詳細は省略するが，概略すれば，胎児期の心室筋型の表現型をとる．また，交感神経，副交感神経の受容体を有し，リガンド刺激にも *in vivo* の心筋と同様の動態をとる[12]．胚性幹細胞から胚葉体形成を経て心筋細胞が分化することは知られているが，その機序は解明されていない(表1)．これと同様に，間葉系幹細胞から心筋細胞への分化の機序は現在解明されていない．著者の考えでは，この細胞は初期設定では骨，軟骨，脂肪細胞などに分化するようにプログラムされており，特殊な状況下において心筋細胞への分化のスイッチがオンになるものと考えている．このため，間葉系幹細胞から骨，軟骨，脂肪細胞などへは，既知の液性因子，細胞外基質などで容易に分化が可能であるし，また長期に継代培養しているだけでこのような細胞が出現してくる．培養心筋細胞上で(間葉系幹細胞ではない)骨髄間質細胞を重層培養した研究で，これらが心筋細胞に分化したと報告されている．しかし，その後の研究でHUVEC細胞や骨格筋芽細胞などの細胞と心筋細胞を共培養すると，細胞融合するとの報告があり，骨髄間質細胞を用いた場合には細胞融合か細胞の重なりを観察したものと考えられる．著者自身は心筋細胞と間葉系幹細胞の共培養では一部の細胞は心筋細胞に分化する可能性があると考えている．

今後は，心筋細胞との共培養の何の因子(細胞増殖因子・接着分子など)が重要であるかを解明する必要があろう．著者の最大の興味のひとつはこの間葉系幹細胞から分化因子の同定にある．また，間葉系幹細胞は大きな塊で移植すると骨や軟骨になることが知られており，心筋内に単なる細胞塊を注射しただけでは再生医学とはいえないであろう．

骨髄間葉系幹細胞の研究はマウスにおいて比較的発展しているが，ヒトの細胞での研究はあまり進んでこなかった．これはヒトの間葉系幹細胞を

細胞株化することがあまり進んでいないことに起因している．すなわち，継代培養しているうちに細胞が老化し，細胞分裂しなくなるためである．これらを克服するための研究も必要である．

骨髄単核球成分の心筋内注入はいかなる意味があるか

循環器領域では国内外を問わず，冠動脈内・心筋内腔からカテーテルを用いて・あるいは手術時に心筋内に注射することにより骨髄単核球成分を移植している．この治療法自体は著者は血管新生の効果があると推測している．骨髄血を FACS 解析すると，その成分は赤血球系の細胞と白血球系の細胞を合わせて，99.9％の細胞が血球系の細胞である．このうち，セルセパレーターで回収される骨髄単核球分画はそのほとんどが中等度に分化した血球系の細胞であり，間葉系細胞は全骨髄中よりは多いが，基本的にはごくわずかな比率であろう．未分化な血球成分は血管新生因子を多量に含有しているので，血管新生の可能性はあるであろう．未分化な造血幹細胞あるいはそれに近い分画は骨髄中のニッシェが必要なため，心筋局所に長期にとどまる可能性は低いものと推測される．実際に骨髄移植をした際にドナー細胞はそのほとんどが骨髄にホーミングしてしまい，局所にはとどまらない．著者の考えでは，局所に注入した少量の間葉系幹細胞がかりに心筋になったとしても，それ自体が心機能を改善するほどのものではないと推測している．もちろん，血流改善に伴う冬眠心筋を活性化し，心機能を二次的に改善することを否定するものではない．したがって，血流改善と心筋再生は一体のものなければならない．心筋再生が全体的に重要である症例では，この方法のみで心機能の回復は難しいものと考えられる．

今後心筋再生研究に求められるもの

著者が考える間葉系幹細胞を用いた心筋再生に関しもっとも重要なものは，第 1 に短時間で全骨髄から間葉系幹細胞を単離する方法の開発，第 2 に多分化能を喪失させることなく長期間培養する方法の確立，第 3 に生体内にある物質あるいは人体に無害な物質により心筋細胞に分化させる方法を確立することである．カテーテルや手術で骨髄を心臓に直接注入する方法は派手であり，人目を引く研究であるが，間葉系幹細胞を用いた心筋再生は地味で時間のかかる研究である．しかし，著者はこれらの研究こそ 21 世紀をブレークスルーできる重要な研究であると考えている．近年，再生医学を志す多くの研究者が増えたことは喜ばしいことである反面，こうした地道な研究を行う研究者はそれほど増えていない．地に足のついた基礎研究を行う若い研究者が増えることを願ってやまない．

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Cardiomyocytes undergo cells division following myocardial infarction is a spatially and temporally restricted event in rats

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Abstract

Dividing cardiomyocytes are observed in autopsied human hearts following recent myocardial infarction, however there is a lack of information in the literature on the division of these cells. In this study we used a rat model to investigate how and when adult mammalian cardiomyocytes proliferate by cell division after myocardial infarction. Myocardial infarction was induced in Wistar rats by ligation of the left coronary artery. The rats were sacrificed periodically up to 28 days following induced myocardial infarction, and the hearts subjected to microscopic investigation. Cardiomyocytes entering the cell cycle were assayed by observation of nuclear morphology and measuring expression of Ki-67, a proliferating cell marker. Ki-67 positive cardiomyocytes and dividing nuclei were observed initially after 1 day. After 2 days dividing cells gradually increased in number at the ischemic border zone, reaching a peak increase of 1.12% after 3 days, then gradually decreasing in number. Dividing nuclei increased at the ischemic border zone after 3 days, peaked by 0.14% at day 5, and then decreased. In contrast, Ki-67 positive cells and dividing nuclei were limited in number in the non-ischemic area throughout all experiments. In conclusion, mitogenic cardiomyocytes are present in the adult rat heart following myocardial infarction, but were spatially and temporally restricted. (Mol Cell Biochem **259**: 177–181, 2004)

Key words: cardiomyocyte, cell division, Ki-67, regeneration, myocardial infarction

Introduction

Up until recently, it was generally believed that the mammalian adult cardiomyocyte population did not contain stem cells, and that terminally differentiated mammalian adult cardiomyocytes could not proliferate and regenerate following tissue injury [1, 2]. Many mammalian adult tissues can regenerate, including adult skeletal muscle, skin, small intestine, liver, and neurons, which recruit mitotically active stem cells that contribute to a stable population [3–7]. Heart regeneration is observed in some species, such as the zebra fish [8], however mammalian heart injury resulting in necrotic tissue and scar formation is thought not to be repaired [9–12]. When cardiomyocytes are severely injury, they respond to an increase in workload by hypertrophying, not by regenerating. When the injured heart is overworked, cardiac hypertrophy results in heart failure. Severe heart failure is an intractable disease, and heart transplantation is the only effective treatment.

A recent study reports that dividing cardiomyocytes can be observed in the human heart following recent acute myocardial infarction [13]. These results are in contrast to previous observations of mammalian hearts [9–12]. The reaction of cardiomyocytes to acute myocardial infarction involves

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several interacting cascades of cellular and molecular responses that include cytokine, chemokine, direct contact signaling, hormonal signaling, extracellular matrix remodeling, and compensatory adaptation of cardiomyocytes. These signaling responses result in cardiomyocyte hypertrophy and are usually considered as a compensatory adaptation. The idea that adult cardiomyocytes retain mitogenic potential is revolutional. It is important to confirm whether terminally differentiated cardiomyocytes can proliferate by cell division and if so, when and how they divide after myocardial infarction. These questions were addressed in the present study using an experimental model for myocardial infarction in rats.

Materials and methods

Animal experiments

Male Wistar rats (n = 44) weighing 200–250 g were used for the study. Left coronary artery (LCA) ligation was performed as described previously [14]. Briefly, after administration of anesthesia (pentobarbital 50 mg/kg, given intraperitoneally), rats were intubated by tracheotomy, and ventilated with room air using a Harvard rodent respirator (model 683, Harvard Apparatus). The tidal volume was 2.0–2.5 ml and the respiratory rate was 95–99 breaths per min. Left thoracotomy was then performed. The proximal portion of the LCA was surgically occluded and the thoracotomy was closed. Rats were sacrificed periodically up to 28 days. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committees of Keio University and conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry

Hearts were removed, fixed overnight in 10% phosphate-buffered formaldehyde, and embedded in paraffin. Sections cut to a thickness of 4 μm were stained with hematoxylin and eosin. Connective tissue was visualized by using Azan staining.

Antigen demasking of formalin fixed paraffin embedded tissue sections was achieved by microwave heating sections in citrate and EDTA buffer. The endogenous activity of peroxidase was blocked by a 10 min pre-incubation of sections in 10% hydrogen peroxide. For the detection of Ki-67, the samples were exposed for 1 h at 37°C to rabbit polyclonal anti Ki-67 (PRO229, YLEM). Control sections were incubated with nonimmune mouse immunoglobulin. Antibody binding was detected using the Vectastain ABC 'Elite' avidin/biotin/peroxidase kit (Vector Laboratories) and this was followed by incubation with diaminobenzidine (DAB, Dako S 3000). Sections were then counterstained with Mayer's hematoxylin, (Burlingame, CA, USA). The ratio of immunopositive cells, compared to the total number of nucleated cells stained with haematoxylin, was determined by counting cells using the light microscope.

The sections were also stained with propidium iodide (10 μg/ml) and an antibody to muscle specific actin, Ab-4 (Clone HHF35, NeoMarkers). Fluorescein isothiocyanate–conjugated anti-mouse IgG was used as a secondary antibody. Specimens were observed with a confocal microscope (LSM510, Carl Zeiss).

Ki-67 labeling index and mitotic index

Cells entering the cell cycle were measured by labeling with the nuclear antigen Ki-67. The fraction of cardiomyocyte nuclei undergoing mitosis was determined by double staining with the anti-sarcomeric α-actin antibody and propidium iodide. The mitotic index was calculated [13].

Results

Azan staining was initially performed on all samples along the short axis of the heart to demonstrate the orientation of ischemic border zone. Figure 1 is a representative photograph of the heart 7 days after myocardial infarction and shows the ischemic border zone and non-ischemic zone.

Ki-67 positive cells

Expression of Ki-67 is required for cells to progress through the cell cycle and undergo cell division. Expression of Ki-67 is first apparent in G_1 phase, increases in late S and G_2 phases, persists during prophase and metaphase, and decreases in anaphase and telophase. The characteristics of Ki-67 make it an appropriate marker for the whole cell cycle. All cells that express Ki-67 go on to divide.

Immunostaining for Ki-67 was performed using specimens from the ischemic border zone and non-ischemic zone. Figure 2 shows representative photographs of Ki-67 positive cardiomyocytes at the ischemic border zone. Figure 3 shows the time course of change in the percentage of Ki-67 positive cells with sarcomere formation at the ischemic border zone and non-ischemic zone. Ki-67 positive cardiomyocytes were not observed at day 0 or 0.5, but gradually increased at the ischemic border zone from day 2, peaking by 1.12% at day 3, then gradually decreased. In contrast, there were very few Ki-67 positive cardiomyocytes in the non-ischemic zone.

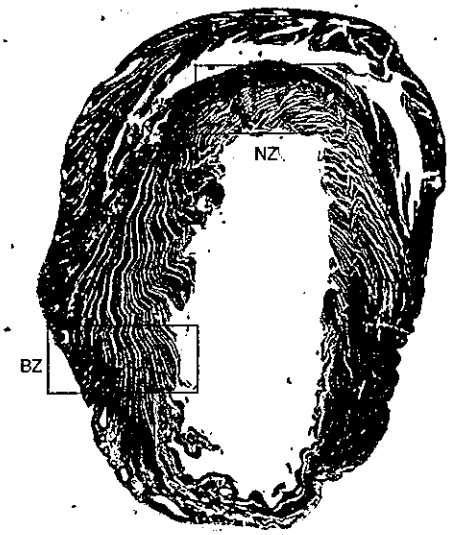

Fig. 1. Azan staining of the whole heart (short axis view) following myocardial infarction. Surrounded area was defined as ischemic border zone and non-ischemic zone. (X1.2).

Nuclear division and cytokinesis

Double immunostaining was then performed with propidium iodide and muscle specific actin. Nuclear division of the cardiomyocytes was observed by confocal microscopy. Figure 4 shows nuclear division and cytokinesis of the cardiomyocytes at the ischemic border zone. Figure 5 shows the time course of change in the percentage of dividing nuclei. Dividing nuclei in cardiomyocytes were first observed at day 0.5, increased at the ischemic border zone from day 3, peaked by

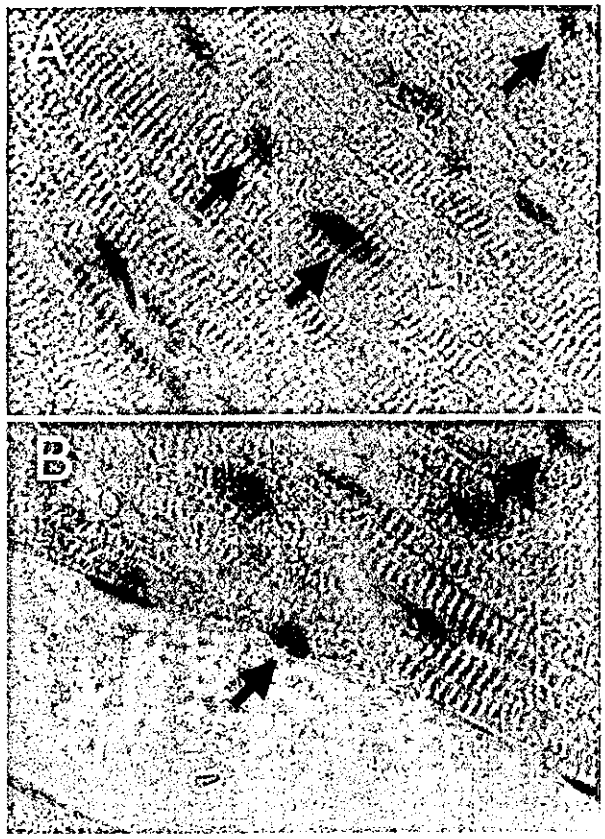

Fig. 2. The immunostaining of Ki-67 positive cardiomyocytes at the ischemic border zone. Ki-67 positive cells were observed at the border zone of the ischemic area 3 days after myocardial infarction. Sarcomere formation indicates that these cells are cardiomyocytes. (Counterstained by Hematoxylin, X132).

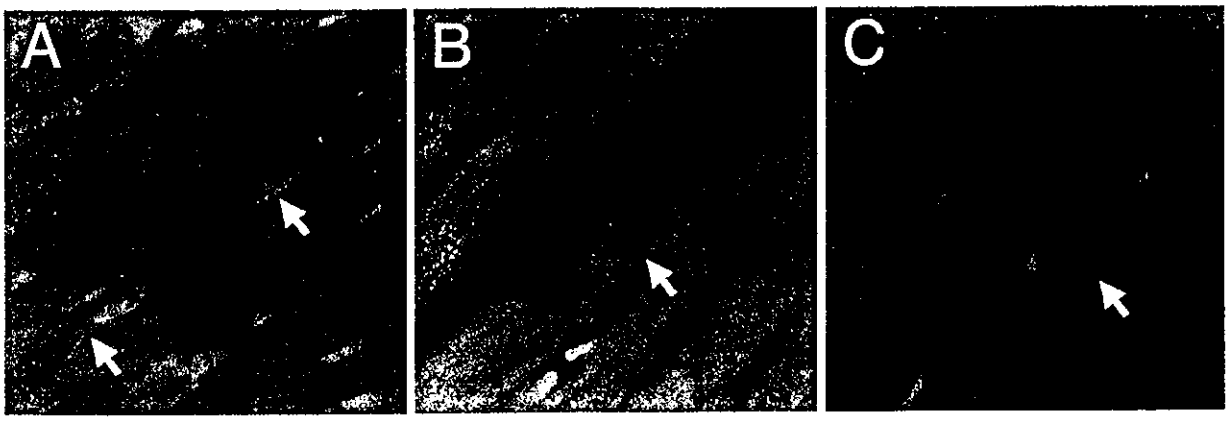

Fig. 4. Double immunofluorescent staining of muscle specific actin and the nucleus. Muscle specific actin was stained with FITC (green), and the nucleus was stained with propidium iodide (red). Arrows indicated the dividing nuclei. (X63).

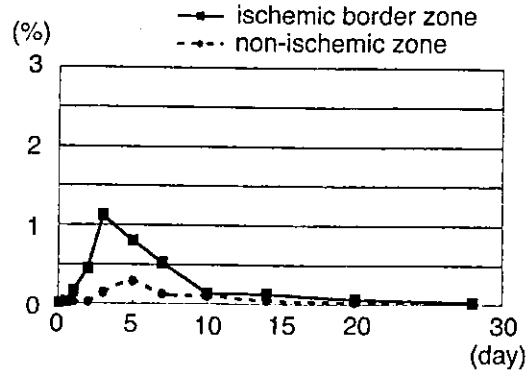

Fig. 3. Time course change in the percentage of Ki-67 positive cardiomyocytes at the ischemic border zone and non-ischemic area. The Ki-67 labeling index is shown. Ki-67 positive cardiomyocytes increased at the ischemic border zone from 2–7 days, then gradually decreased. Very few Ki-67 cells were observed in the non-ischemic area.

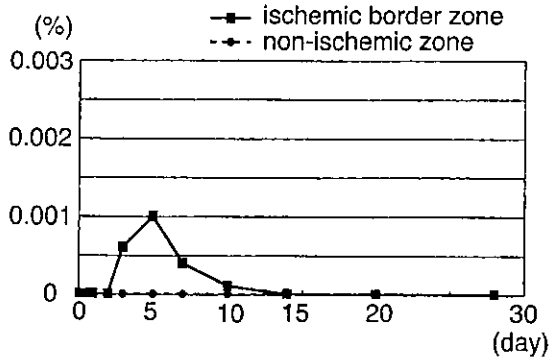

Fig. 6. Time course change in the percentage of cytokinesis at the ischemic border zone and the non-ischemic area. Cytokinesis increased at the ischemic border zone 2–5 days following myocardial infarction, then gradually decreased. Peak cytokinesis was later than the appearance of Ki-67 expression. There was limited cytokinesis in the non-ischemic area.

0.14% at day 5, then decreased. In contrast, dividing nuclei in cardiomyocytes were uncommon in the non-ischemic area in all experiments. Peak nuclear division was observed 2 days following the peak of Ki-67 expression. This may be because Ki-67 is expressed throughout the cell cycle, apart from G_0 phase, while dividing nuclei are restricted to M phase.

Figure 6 shows the time course of change in the percentage of cardiomyocytes undergoing cytokinesis. We found that cytokinesis was uncommon in the experimental myocardial infarction model. Cytokinesis was observed at the ischemic border zone only at day 3, peaked by 0.0011% at day 5, then decreased. Cytokinesis was not observed at the non-ischemic zone. Taken together, the data presented in this report suggests that adult cardiomyocytes have the potential to regenerate following myocardial infarction. This ability is limited to a few days following myocardial infarction, after this time cardiomyocyte regeneration is a rare event.

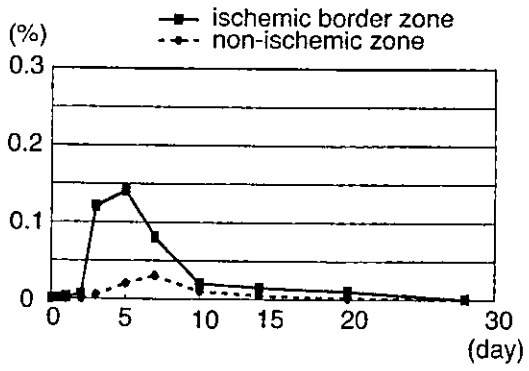

Fig. 5. Time course change in the percentage of nuclear dividing cardiomyocytes at the ischemic border zone and the non-ischemic area. Nuclear division of cardiomyocytes increased at the ischemic border zone 3–7 days following myocardial infarction then gradually decreased. The peak in nuclear division was later than the appearance of Ki-67 expression. There were very few dividing cardiomyocytes in the non-ischemic area.

Discussion

Beltrami reported that human cardiac myocytes divide after myocardial infarction [13]. Although an interesting result of the data was obtained from 13 patients who had died 4–12 days after myocardial infarction, thus making it difficult to assess the reproducibility of these findings. The present study used an experimental model for myocardial infarction in adult rats, and revealed that Ki-67 positive cardiomyocytes and cardiomyocytes undergoing nuclear division were present at the ischemic border zone at peak levels 3 days and 5 days, respectively, following myocardial infarction. After this time cardiomyocytes with these characteristics rapidly disappeared. These findings show that the adult rat cardiomyocyte cell division after myocardial infarction is very restricted spatially and temporally. The finding that Ki-67 positive or dividing cells were observed only at the ischemic border zone, but not the non-ischemic zone, suggests that factors inducing cell division might be caused by regional ischemia or inflammation, and not by myocardial stretch.

Recent studies have demonstrated that bone marrow stem cells can differentiate into cardiomyocytes [15]. We have previously reported that mesenchymal stem cells from bone marrow can be induced to differentiate into cardiomyocytes in vitro, and that these cells express functionally active adrenergic and cholinergic receptors [16, 17]. Another group reported that a heart transplanted from a female donor to a male patient showed chimerism resulting from stem cells migrating from the recipient to the grafted heart [18]. If these findings are true, it is possible that bone marrow derived-stem

cells could mobilize and infiltrate from the circulating blood to the border zone area, and differentiate into cardiomyocytes. On the contrary, we believe that the Ki-67 positive and dividing cells observed in the present study originated from the original cardiomyocytes and not stem cells. Reasons for this include the presence of clear striated myofilaments associated with the regenerating cardiomyocytes, and the fact that the timing of their appearance was too close to the myocardial infarction event to allow differentiation into cardiomyocytes. Previous studies revealed that ES cells require at least 8 days to fully differentiate into cardiomyocytes [19, 20], and bone marrow derived cardiomyocytes (CMG cells) require at least 14 days to begin to beat spontaneously [16].

Tamamori-Adachi *et al.* reported that in neonatal rat cardiomyocytes, cyclin D1 and CDK4 were present predominantly in the cytoplasm, and Rb was in an underphosphorylated state [21]. They constructed a variant of cyclin D1, known as D1NLS, directly linked to nuclear localization signals (NLSs). Co-infection of recombinant adenoviruses expressing D1NLS and CDK4 induced Rb phosphorylation and CDK2 kinase activity, and was sufficient to promote re-entry into the cell cycle, leading to a 3-fold increase in the number of cardiomyocytes after 5 days. Similar experiments were conducted in adult rat hearts to promote re-entry of cardiomyocytes into the cell cycle. Expression of Ki-67, and BrdU incorporation was observed indicating that postmitotic cardiomyocytes have the potential to proliferate providing that cyclin D1/CDK4 accumulates in the nucleus [21]. The above results suggest that an active process must be in place in cardiomyocytes inhibiting re-entry into the cell cycle.

The present study shows that the frequency of cell division in adult cardiomyocytes is a very rare event. We believe that cell division of adult cardiomyocytes following myocardial infarction contributes little to improve cardiac function without molecular intervention. Further investigation is needed to control the cell cycle of postmitotic cardiomyocytes for the next step.

Acknowledgements

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

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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,

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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).

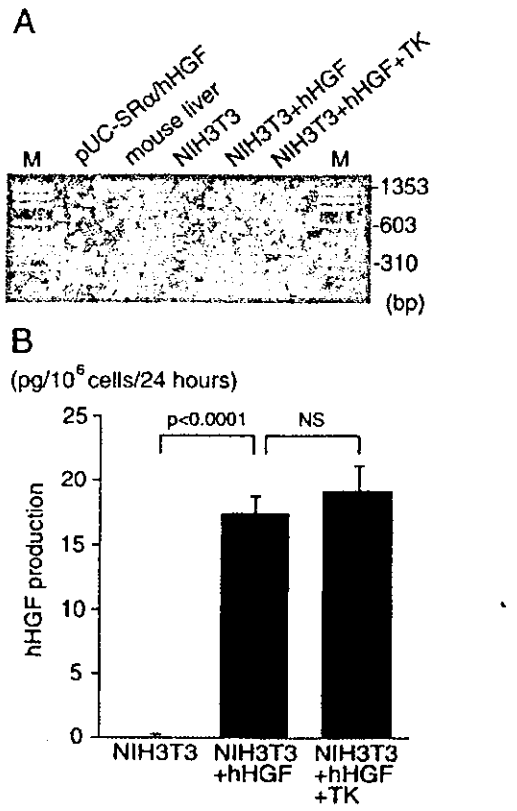

Figure 1. **(A)** Expression of human hepatocyte growth factor (hHGF) messenger ribonucleic acid in the hHGF-transfected NIH3T3 cells. The primer set of reverse transcription-polymerase chain reaction specifically detects hHGF but not mouse HGF. pUC-SRα/hHGF plasmid and mouse liver were used as a positive 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 $p < 0.05$.

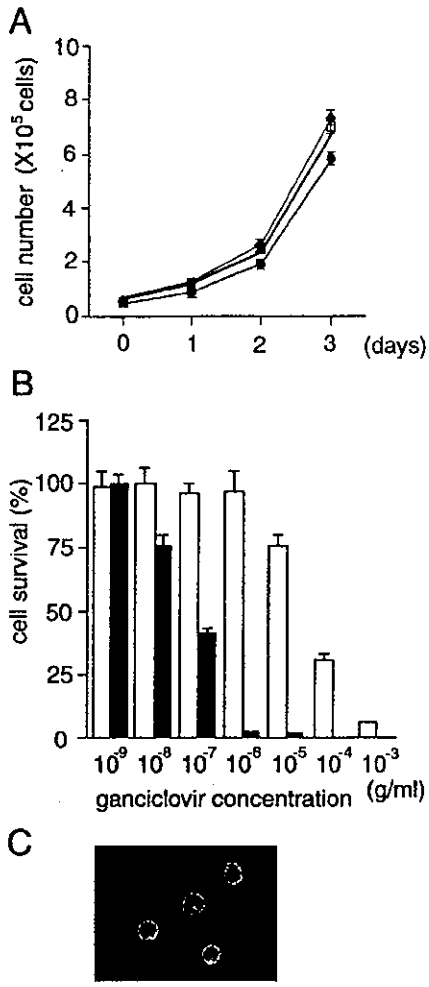

Figure 2. **(A)** Influence of hHGF and/or TK genes on cell growth in vitro. The growth rate of the hHGF-transfected NIH3T3 cells was slightly higher than that of the nontransfected cells, but TK had no effect on cell growth. **(circles** = NIH3T3; **diamonds** = NIH3T3 + hHGF; **squares** = NIH3T3 + 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

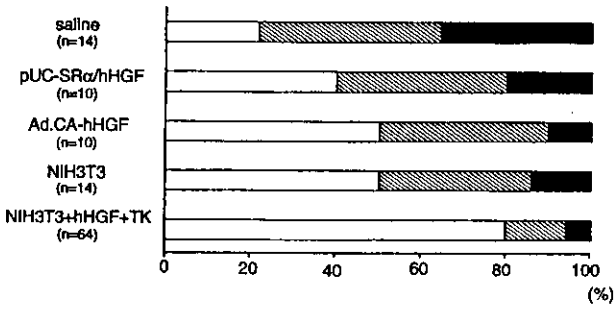

Figure 3. Frequency of necrosis in the ischemic hindlimbs. Severe hindlimb necrosis was significantly reduced in the NIH3T3 + hHGF + TK group. **Open areas** = negative necrosis; **lined areas** = necrosis on toes; **solid areas** = necrosis on foot. Abbreviations as in Figure 1.

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.

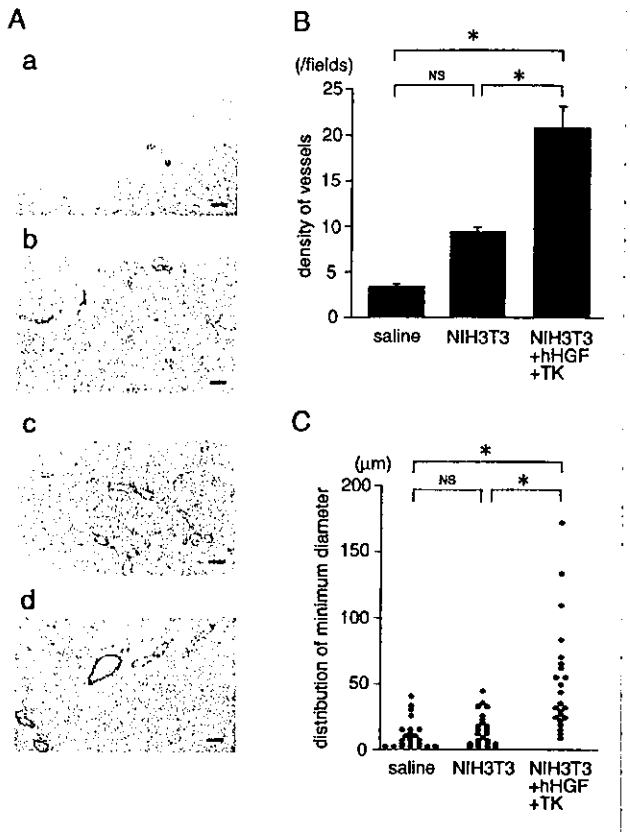

Figure 4. (A, panels a to d) Immunohistochemical staining for von Willebrand factor in the triceps muscle of the left calf revealed the presence of numerous vessels. Vessels were larger and more numerous in the NIH3T3 + hHGF + TK group **(panels c and d)** than in the saline **(panel a)** and NIH3T3 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.

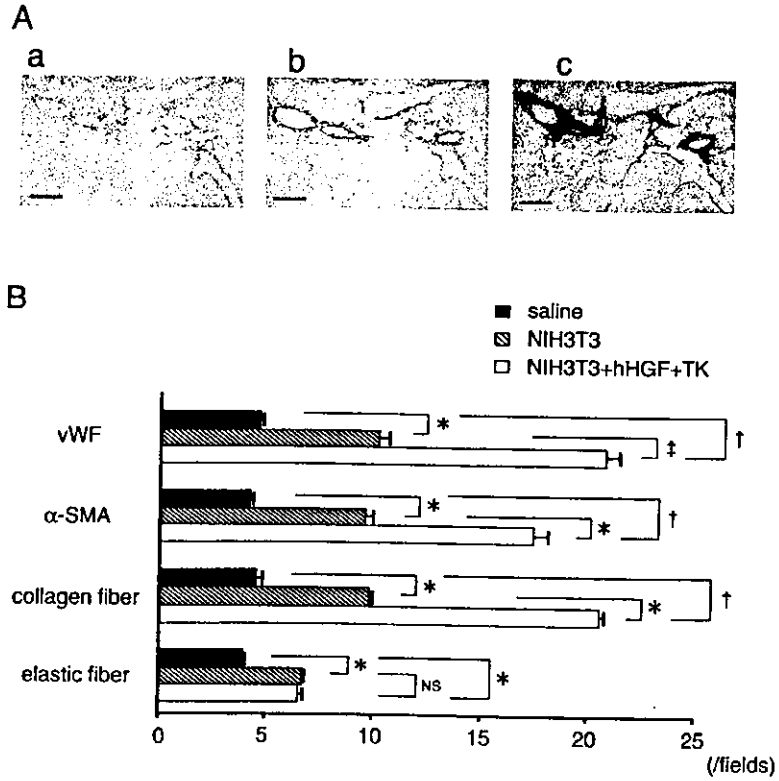

Figure 5. **(A, panels a to c)** Three consecutive frozen sections of NIH3T3 + hHGF + TK transplanted muscle. **(panel a)** Immunohistochemical staining for vWF and **(panel b)** α-smooth muscle actin (SMA) and **(panel c)** elastica van Gieson staining. **Scale bars** = 100 μm. **(B)** Maturation of 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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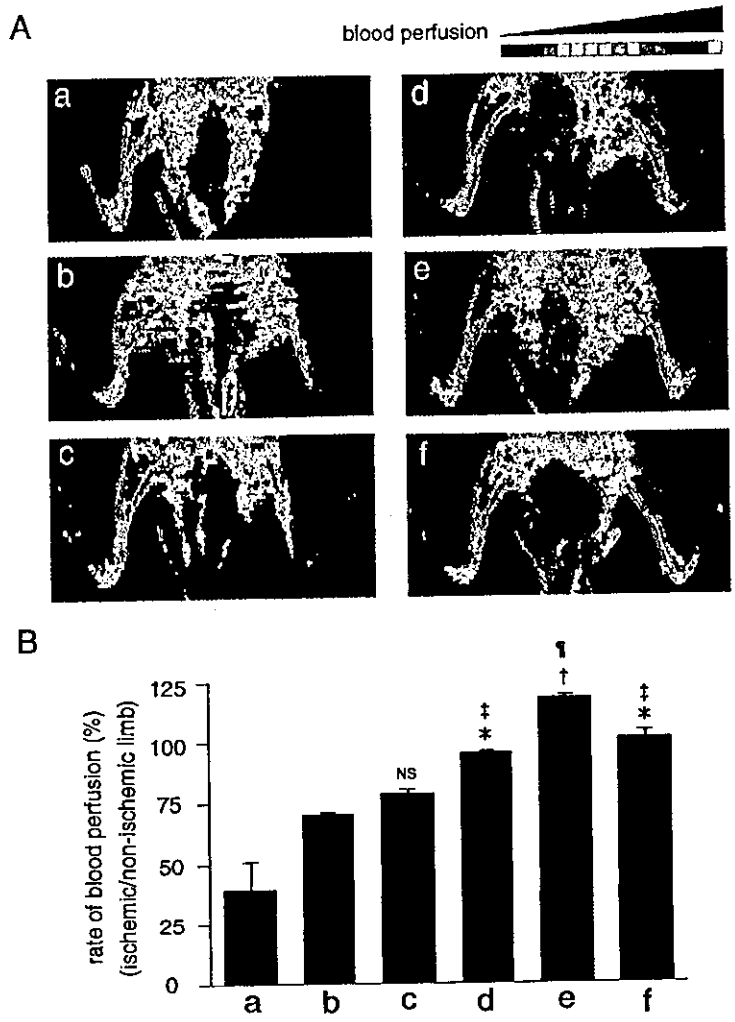

Figure 6. **(A)** Representative laser Doppler perfusion images. **(B)** Quantitative analysis of the rate of blood perfusion of the ischemic/nonischemic limb. **Panel a** = Control mouse on postoperative day 1; **panels b to f** = four weeks after treatment (**panel b** = saline injection; **panel c** = 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.

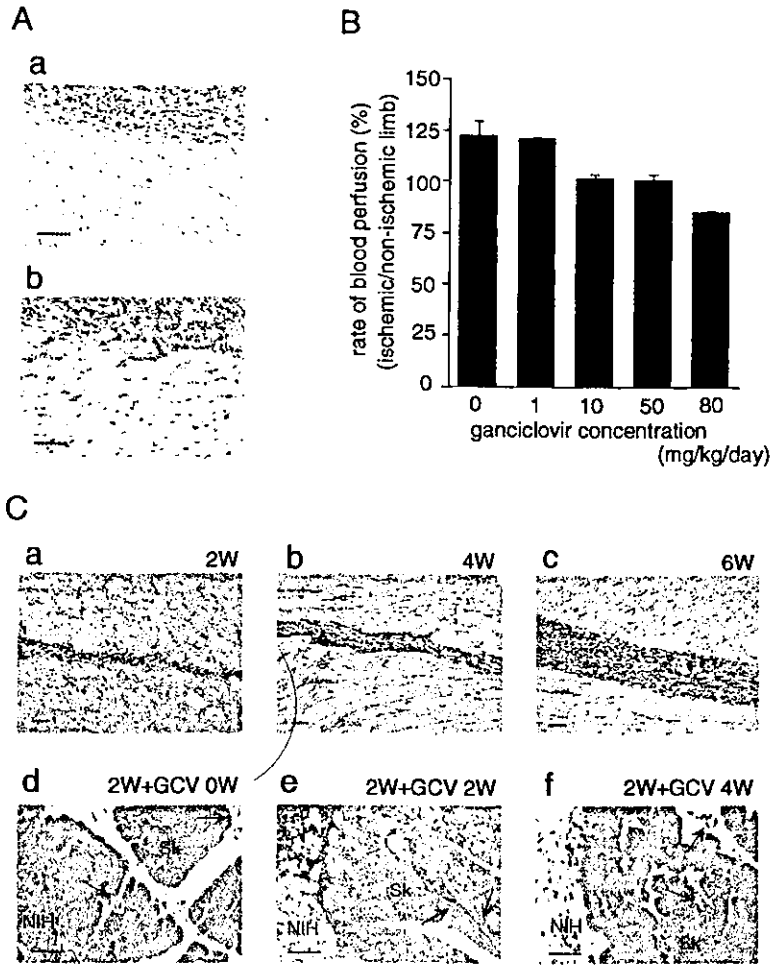

Figure 7. **(A)** Immunohistochemical staining for hHGF in transplanted NIH3T3 cells **(panel a)** and NIH3T3 + hHGF + TK cells **(panel b)** in the skeletal muscle. **Scale bars** = 50 μm. **(B)** The NIH3T3 + hHGF + TK (10^7) cells were transplanted, and two weeks later, various concentrations of ganciclovir 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.

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

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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

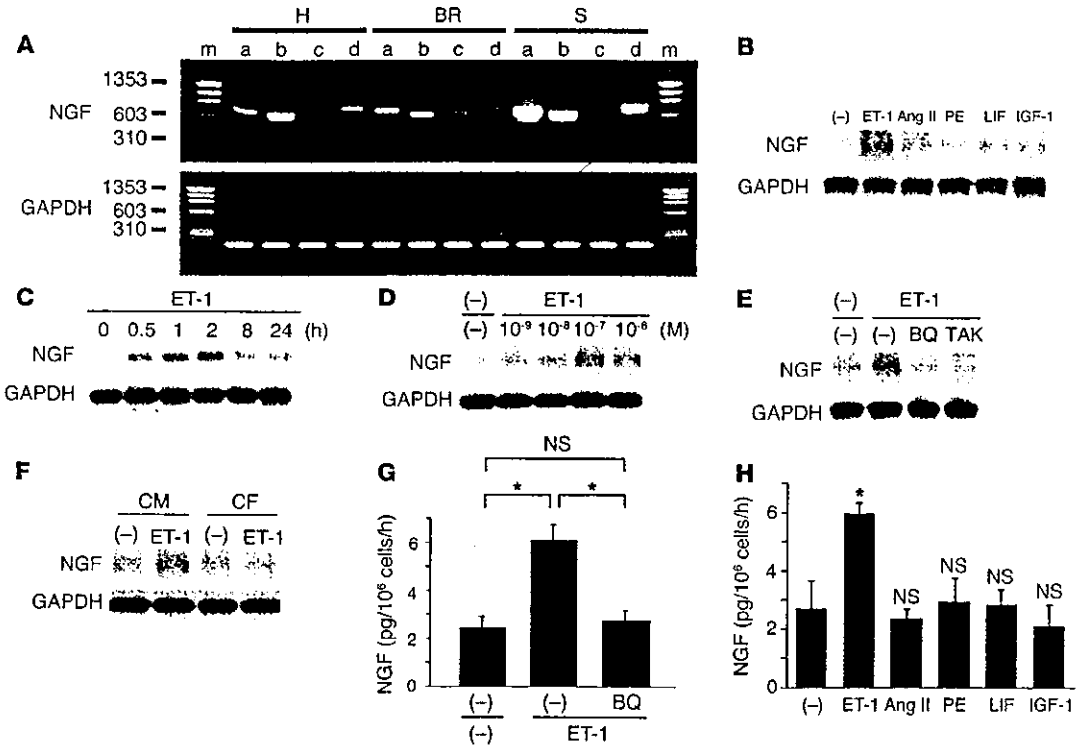

Figure 1

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 $Gi\alpha$ or $Gi\beta\gamma$ 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 $Gi\beta\gamma$ 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 $Gi\beta\gamma$-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