Stimulation of Paracrine Pathways With Growth Factors Enhances Embryonic Stem Cell Engraftment and Host-Specific Differentiation in the Heart After Ischemic Myocardial Injury

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Background—Growth factors play an essential role in organogenesis. We examine the potential of growth factors to enhance cell engraftment and differentiation and to promote functional improvement after transfer of undifferentiated embryonic stem cells into the injured heart.

Methods and Results—Green fluorescent protein (GFP)–positive embryonic stem cells derived from 129sv mice were injected into the ischemic area after left anterior descending artery ligation in allogenic (BALB/c) mice. Fifty nanograms of recombinant mouse vascular endothelial growth factor, fibroblast growth factor (FGF), and transforming growth factor (TGF) was added to the cell suspension. Separate control groups were formed in which only the growth factors were given. Echocardiography was performed 2 weeks later to evaluate heart function (fractional shortening [FS]), end-diastolic diameter, and left ventricular wall thickness). Hearts were harvested for histology (connexin 43, α-sarcomeric actin, CD3, CD11c, major histocompatibility complex class I, hematoxylin-eosin). Degree of restoration (GFP-positive graft/infarct area ratio), expression of cardiac markers, host response, and tumorigenicity were evaluated.

Cell transfer resulted in improved cardiac function. TGF-β led to better restorative effect and a stronger expression of connexin 43, α-sarcomeric actin, and major histocompatibility complex class I. TGF-β and FGF retained left ventricular diameter. FS was better in the TGF-β, FGF, and embryonic stem cells–only group compared with left anterior descending artery–ligated controls. Growth factors with cells (TGF-β, FGF) resulted in higher FS and smaller end-diastolic diameter than growth factors alone.

Conclusions—Growth factors can promote in vivo organ-specific differentiation of early embryonic stem cells and improve myocardial function after cell transfer into an area of ischemic lesion. TGF-β should be considered as an adjuvant for myocardial restoration with the use of embryonic stem cells. (Circulation. 2005;111:2486-2493.)

Key Words: infarction ■ growth substances ■ myocytes ■ cells

Myocardial ischemia can cause irreparable loss of viable heart cells.1 Research attention has increasingly focused on a procedure frequently termed myocardial restoration cell transfer.2-6 The reported cell populations and their efficacy are questioned because of impaired in vivo viability of cells after transfer in the area of lesion and the lack of host organ-specific differentiation, also termed plasticity.7

To overcome this obstacle, cardiovascular research increasingly focuses on more primordial and pluripotent cell forms such as embryonic stem cells (ESC).8 Additionally, a wide array of stimulants such as growth factors have been tested on their ability to promote healing, angiogenesis, engraftment, and differentiation.8-15 Evidence exists that ESC have a significant potential to differentiate to more committed progenies and to self-renew multiple times.16,17 Kehat et al18 have reported ESC differentiation along the myocardial pathway and have been able to stimulate this process. Symes19 has achieved high yields of differentiated early ESC, so-called premature cardiomyocytes, at a purification level of ≈70%.

A group of growth factor proteins seems to be involved in both cardiac organogenesis in the early embryonic stages and inhibition of cell death. Vascular endothelial growth factor (VEGF) has been reported to enhance functional improvement of postinfarcted mouse hearts by transplantation of ESC-differentiated cells.9 Recombinant insulin-like growth...
factor I (IGF) has a stimulatory effect on embryonic myogenic cell cultures and on cell survival in tissue areas of reduced nutrient supply. IGF binding protein affects proliferation of porcine embryonic myogenic cells during critical periods of muscle development that may affect ultimate muscle mass achievable postnatally. Members of the transforming growth factor-β (TGF-β) superfamily, namely, TGF-β and bone morphogenenic protein-2, applied to undifferentiated murine ESC, upregulated mRNA of mesodermal and cardiac-specific transcription factors. Embryoid bodies generated from stem cells primed with these growth factors demonstrated an increased potential for cardiac differentiation with a significantly higher occurrence of beating areas and enhanced myofibrillogenesis. Fibroblast growth factor (FGF) has been linked to the expression of intermediate filaments and desmin in the cytoplasmic and cytoskeletal compartments, suggesting that FGF plays a role in cardiomyocyte differentiation during the early stages of development. It is also known to be an integral part of, or to exist in association with, cardiac gap junctions and may comodulate intercellular communication. Finally, recent studies in mutant mice point to the critical role of TGF-β in signaling and autocrine or paracrine regulation of cardiomyocyte proliferation and differentiation.

In the present in vivo study, we injected undifferentiated and labeled ESC into infarcted mouse myocardium, added growth factors to promote engraftment and differentiation, and comparatively evaluated the restorative effect of donor cells and the functional improvement of the recipient heart.

Methods

Animal Care

All surgical interventions and animal care were provided in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication No. 78-23, revised 1978), and the Guidelines and Policies for the Use of Laboratory Animals for Research and Teaching of the Department of Comparative Medicine, Stanford University School of Medicine.

Enhanced Green Fluorescent Protein ESC

pEF1 α-enhanced green fluorescent protein (EGFP), which contains EGFP gene under the control of human EF1 promoter and a neomycin resistance cassette, was constructed as follows. The promoter region of pEGFP-N3 (Clontech, Palo Alto, Calif) was removed by cutting the Asel-Nhel DNA fragment and joining of blunt-ended termini. Human EF1α promoter from pEF-BOS (HindIII-EcoRI) DNA fragment was inserted into the HindIII-EcoRI site of the plasmid. D3 ESC were transfected with pEF1 α-EGFP by electroporation and selected in the presence of G418. One clone that brightly expresses EGFP was chosen and used for the experiments.

The clone was adapted to feeder-free conditions and maintained on the gelatin-coated dish in DMEM supplemented with 15% fetal calf serum, 2 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 1X nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, 1000 U/mL ESGRO, 100 U/mL streptomycin, and 100 μg/mL penicillin. Cells were collected after trypsinization with EDTA and placed in aliquots of the aforementioned medium for intramyocardial injection 1 hour later.

Animal Groups and Myocardial Injury Model

We injected ESC originating from 129sv mice into 3 groups of BALB/c recipient hearts (n=5), i.e., a group for every growth factor, a control group that had undergone left anterior descending artery (LAD) ligation and ESC transfer without growth factor, and finally a control group with LAD ligation but without cell transfer or growth factors. Furthermore, 3 groups of animals underwent surgery and received the growth factor only, without cell injection, along with a control group that underwent LAD ligation and received a 25-μL injection of saline.

To assess the restorative and tumorigenic potential of ESC grafts, we performed hematoxylin-eosin (H&E) and trichrome stains. These
also allowed for identification of vessels and measurement of their cross-sectional area. Trichrome and H&E stains were used to estimate the extent, distribution, structure, and kinetics of ensuing scar after the infarction and injection of cells, the mode of their organization, vessel count, and cellular type. H&E was helpful for evaluating cellular atypia and nuclear polymorphism, regarded as indicators of tumor formation. The trichrome and H&E findings were expressed in a scale ranging from 0 (vessels absent, no scar tissue, no cellular atypia, no nuclear polymorphism) to 4 (dense vascularization of the optical field corresponding to the GFP-positive graft, extensive scarring, severe cellular atypia and chaotic pattern, severe nuclear polymorphism).

To identify quality and quantity of cellular (inflammatory/immune) response to the injected cells, we stained the same sections for CD3, which indicates presence of T-lymphocytes, and CD11, which is expressed by activated dendritic cells, which are antigen-presenting cells. To assess the presence of MHC molecules on the GFP-positive cells, we stained for MHC class I, which is expressed by all adult cells. We also report on the distribution of the MHC signal (Texas red–conjugated secondary antibody, BD Pharmingen, San Diego, Calif) and its behavior over time.

**Morphometry**
Sections were obtained at 5 different levels of the harvested heart. The infarct was identified as a dark area with few cells that contained the injected GFP conglomerate. To quantify the extension of expression of specific markers, 5 random sections of the GFP-positive graft were photographed and evaluated with the use of Spot advanced software, version 3.4.2 (Diagnostic Instruments Inc). The marker-positive area and the area occupied by the GFP graft on every section were placed in relation and provided an area ratio, which was expressed as a percentage.

**Statistical Analysis**
All results are expressed as mean±SD. Multilineage differences between groups were analyzed by 2-way ANOVA. If a significant F ratio was obtained, a Bonferroni post hoc test was used to specify pairwise differences. Statistical analyses were performed with StatView 5.0 (SAS Institute), and significance was accepted at \( P<0.05 \). Comparison between 2 groups at a time was performed with the use of the \( t \) test for independent variables, and significance was assumed at \( P<0.05 \).

**Results**

**Extent of GFP Graft and Scale of Myocardial Restoration**
The engrafted cells formed a GFP-positive, voluminous graft in all animals (Figure 1A). The ratio of restored area/infarcted area ranged between 30% and 90%, with the highest restoration potential in the TGF group (82.1±12.5%) (F=20.428, \( P<0.0001 \)) (Figure 2A) compared with all other groups. These changes observed in regard to wall thickness coincided with increasing cellular atypia and graft septation in the majority of treated hearts. The cells formed dense conglomerates, which in many cases did not allow for distinguishing individuals cells. The volume of these grafts did not decrease significantly at 4 weeks after cell transfer (online-only Data Supplement Figure, from parallel studies not yet published). This was often possible via comparison with the corresponding H&E-, trichrome-, or otherwise-stained section or by colocalization studies using GFP and the Texas red signal of the individual cell marker, such as connexin 43.

**Engraftment and Differentiation**
The present study was not designed to provide proof of cell fusion between donor and recipient cells. Even though

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**Figure 1.** A, Injected cells assemble, forming an easily identifiable graft within scar tissue (arrows; magnification ×50). B, Connexin 43 is expressed primarily at contact sites between donor (arrows) as well as between recipient (arrowhead) cells (confocal; magnification ×630). There is evidence of connexin 43 expression at the connections between donor and recipient cells (small curved arrows). C, Donor cells assume myofibrillar phenotype within injured tissue and display typical striation pattern when costained with α-sarcomeric actin (arrows; confocal; magnification ×630). D, H&E staining (magnification ×400) shows epithelioid and myofibrillar forms in random arrangement, from an area corresponding to the GFP-positive donor graft (specific staining to identify cell types was not performed, but nuclear polymorphism and cellular atypia are identifiable at various sites of the graft). Growth factor administration did not trigger hypertrophy or change of morphological patterns.
The expression of connexin 43 (area ratio) was strongest in the TGF group at 2 weeks after cell transfer \((F=28.708, P<0.0001)\) compared with all other groups (Figure 2A). The \(\alpha\)-sarcromeric actin expression was more pronounced in the TGF group \((F=91.104, P<0.0001)\) compared with all other groups (Figure 2A), followed by the VEGF group. MHC class I was expressed more extensively in the TGF group \((F=91.104, P<0.0001)\). CD3-positive cell population was less in the FGF group \((P=0.0001)\) compared with all other groups. CD3-positive cell population was less in the FGF group \((P=0.0001)\) compared with all other groups. FGF was associated with fewer CD11c cells within the graft \((P=0.0001)\). The VEGF group. MHC class I was expressed more extensively in the TGF group \((P=0.0001)\). Scar formation was significantly less in the TGF-treated group \((F=14.26, P<0.0001)\). All groups treated with growth factors displayed less nuclear (nucl.) polymorphism compared with the group treated with ESC only \((F=6.044, P<0.0059)\). Vascularity was highest in the TGF group \((P=0.0001)\). C, FS was highest in the TGF-treated group \((F=7.932, P<0.0005)\). All groups treated with cells displayed better cardiac function than animals that had undergone LAD ligation only \((P<0.0005)\) for growth factor–treated groups; \(P=0.0082\) for ESC-only group). D, EDD was larger in the untreated group \((F=4.337, P=0.0109)\). The groups treated with ESC did not differ from each other. Posterior wall thickness (PWT) and septal wall thickness (SWT) were similar in all groups.

Figure 2. A. Restorative effect, expressed as donor graft/infarct ratio (G/I), connexin 43 \((F=28.708, P<0.0001)\), \(\alpha\)-sarcromeric actin (a-sarc) \((F=91.104, P<0.0001)\), and MHC class I expression \((F=91.104, P<0.0001)\), is significantly higher in the TGF-treated group (*) compared with all other groups. FGF was associated with fewer CD11c-positive cells within the graft \((F=15.556, P<0.0001)\). Scar formation was significantly less in the TGF-treated group \((F=14.26, P<0.0001)\). All groups treated with growth factors displayed less nuclear (nucl.) polymorphism compared with the group treated with ESC only \((F=6.044, P<0.0059)\). Vascularity was highest in the TGF group \((P=0.0001)\). C, FS was highest in the TGF-treated group \((F=7.932, P<0.0005)\). All groups treated with cells displayed better cardiac function than animals that had undergone LAD ligation only \((P<0.0005)\) for growth factor–treated groups; \(P=0.0082\) for ESC-only group). D, EDD was larger in the untreated group \((F=4.337, P=0.0109)\). The groups treated with ESC did not differ from each other. Posterior wall thickness (PWT) and septal wall thickness (SWT) were similar in all groups.

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Function
FS was higher in the TGF \((29.6\pm3.4\%)\) and FGF \((27.5\pm2.8\%)\) groups than in the ligated but untreated control group \((17.5\pm2.7\%; P<0.0005)\). The group with ESC without growth factor displayed a better FS \((23.1\pm3.6\%)\) in comparison to the control group treated with LAD ligation only \((P=0.0082)\) (Figure 2C). The highest FS was observed in the TGF-\(\beta\) group \((F=7.932, P<0.0005)\). Echocardiography revealed significantly larger EDDs in the control group that did not receive cells or growth factor and the ESC-only group \((3.29\pm0.41 \text{ mm} \text{ in the TGF group, } 3.55\pm0.42 \text{ mm in the FGF group, } 3.55\pm0.36 \text{ mm in the VEGF group, } 3.48\pm0.42 \text{ mm in the group with ESC only, and } 4.3\pm0.4 \text{ mm in the group with LAD ligation only; } F=4.337, P=0.0109)\). There was no significant difference between the groups treated with growth

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factors in addition to ESC. Posterior and septal wall thickness values were similar in all groups, including the controls (Figure 2D). FGF and VEGF administration together with ESC was not superior compared with administration of ESC alone. When TGF-β was administered without cells, it resulted in inferior FS ($P=0.014$) and larger EDD ($P=0.01$) (Figure 3A) compared with the group that had received TGF-β and cells. Posterior and septal wall thickness values were comparable between these groups (ESC plus growth factors versus growth factors alone; Figure 3C and 3D). TGF-β did not improve cardiac function, EDD, or wall thickness if given alone compared with LAD-ligated controls (Figure 4A to 4D). FGF was also inferior in regard to cardiac function ($P=0.018$) (Figure 3A) and EDD ($P=0.028$) (Figure 3B) when given alone compared with the FGF/ESC combination. FGF also did not improve cardiac function, EDD, or wall thickness if given alone compared with LAD-ligated controls (Figure 4A to 4D). VEGF resulted in similar results both with and without additional cell administration.

**Discussion**

TGF-β enhances the effect of ESC transfer for the restoration of myocardial function. There are significant differences in the degree of functional improvement, expression of cardiac markers, and scar formation among groups that have been treated with distinct growth factors. TGF-β displays the most remarkable restorative and differentiation potential compared...
with FGF and VEGF administration. The angiogenic effect of VEGF, which was not subject to evaluation in the present study, does not seem to affect myocardial function significantly either via combined treatment with ESC or when given alone. The inferior FS and larger EDD in the animals treated with growth factors without concurrent cell administration indicate that growth factors alone were not sufficient to improve heart function after extensive ischemic injury in our model. We cannot exclude an eventually positive result if the induced infarction had been of lesser dimensions or if a higher growth factor dose had been used. Thus, the functional improvement of the recipient hearts in the setting of acute ischemia of the scale induced here cannot be attributed to the paracrine effects of growth factors alone. VEGF is not known as a cardiogenic factor during early organogenesis or as protective against reactive oxygen species or other deleterious conditions within ischemic muscle but rather as an angiogenic mitogen. This could potentially explain why VEGF administration resulted in comparable findings both with and without cells. The present study was not designed to provide insight regarding the interplay of molecular mechanisms involved in growth factor action; hence, the optimal dose and relation of cells/growth factors for myocardial restoration in the clinical setting remain to be studied. For this purpose, more extensive studies on the route and time point of intervention must be performed.

Growth factors and their corresponding genes have been administered diversely in various clinical studies in the past 10 years. In addition, cell transplantation has gained increasing significance as an alternative therapeutic option for congestive heart failure. Studies with bone marrow–derived stem cells and skeletal myoblasts have been performed in patients for the last 2 years. Therefore, we believe that in the setting of acute and chronic ischemia as well as dilated cardiomyopathy, the strategy introduced in the present study may be suitable for selected patients. Growth factors, trophic factors, or genes may be supplemented with primordial, pluripotent, or committed cells to drive their differentiation to the desired phenotype and enhance their engraftment. In parallel, considerable legislative efforts and scientific collaborations are forming to facilitate the use of human ESC for organ restoration. It is expected that supplementary measures will be introduced to harness the full restorative potential of ESC engraftment.

However, previous studies have not examined growth factors, which are known to be involved in differentiation, angiogenesis, or cytoprotection, in the context of ESC administration for myocardial restoration. For VEGF, TGF-β, and FGF, the directed injection or transfection into injured myocardium has already been reported with the use of either the protein itself or the factor-expressing gene. In regard to the model used in the present study, at 2 weeks the controls had already developed aneurysms and congestive heart failure. In mice, there is significant deterioration in function and geometry 1 week after infarction. Two weeks seems to be an appropriate time point to observe the full spectrum of induced disease in the mouse model. We chose to use the smallest possible amount of cells for myocardial restoration to stay in compliance with eventual clinical studies of the future. Because of the nature of the cells, their availability from human donors might be a controversial issue in their clinical application.

We believe that ESC are a potent tool for the restoration of parenchymatous organs. They have superior robustness compared with other cell types, and they proliferate, depending on their stage of differentiation. Furthermore, they display evidence of expression of organ-specific markers and improved organ function better than any other cell with which they have been compared by us, such as bone marrow–derived stem cells. Two major limitations must be overcome for safe clinical use in the future: tumorigenicity and immunogenicity of ESC in vivo. To suppress tumorigenicity, an ESC population must be selected for in vivo transfer, which is highly purified for the phenotype of cell that is needed (i.e., cardiomyocytes for intramyocardial transfer). We are currently addressing this issue in extensive studies, using ventricular light chain promoters and connexin 43–expression techniques. Our preliminary data indicate a host response to transplanted ESC, comprising infiltration of reporting dendritic cells, MHC class II expression, and inflammatory cytokine production. In our latest experiments we have seen increasing humoral response by thymocytes to recipient serum using fluorescence-activated cell sorter analysis. It is therefore plausible that extensive restorative procedures in the future will demand some mild immunosuppression, eventually as an adjuvant measure for a few days after cell transplantation, to prevent the early beneficial effects of stem cells from being annihilated.

Even though these responses are not strong enough to affect graft size, they might be progressive in the long term. Supplemental data from concurrent studies in our laboratory that have been performed with the same donor/recipient combination indicate that the graft/infarct area ratio is retained at 4 weeks after cell transfer and that it encompasses 45% to 50% of the infarcted area. Ultimately, we believe that it is critical to provide the ischemic myocardium with cellular support in the early remodeling phase to prevent disadvantageous shear in the border zones and nonischemic extension of the infarct. Our data provide evidence that administration of the cells in the early sensitive phase, after acute ischemia, prevents wall thinning, even as a passive support only, and therefore inhibits the increase of circumferential wall stress and production of reactive oxygen species and attenuates cell death pathways. The frequency of in vivo expression of connexin 43 and α-sarcomeric actin indicates that highly regulative mechanisms at severe undersupply conditions educate stem cells to selectively give rise to more committed progenitors. The phenomenon of target-specific asymmetry operates in the ischemic myocardium to sustain an increasing amount of committed cells. It has been reported that population asymmetry facilitates the response to variable physiological needs. These multiple feedback controls and reciprocal cellular interactions need to be investigated further. Despite the significant degree of cardiac marker expression and its enhancement by primarily TGF-β, within the graft we only rarely observed spindle-like cells, which are always positive for connexin 43 and α-sarcomeric actin. It was not the aim of
this study to seek multilineage differentiation of the donor cells. All of our donor cells did not express myocyte markers. Therefore, it is probable that cell types other than cardiomyocytes will arise from the mother preimplantation stage population that was injected. Growth factors did not appear to have any impact on this pattern compared with the ESC-only group of mice. The better cardiac function of treated versus control animals indicates that the differentiation process may be a causative factor but does not prove electrophysiological coupling and synchronicity of the injected cells and their host environment. Therefore, a direct link between differentiation and functional improvement of the recipient hearts is still unproved.

All models and routes of cell transfer into ischemic myocardium share a common limitation: they are performed in a random fashion with regard to the myocardial microstructure and do not appreciate the unique laminar/fibrillar structure of the heart muscle, which is highly anisotropic.11,12 There is increasing evidence of the importance of functional improvement of hibernating myocardium, border zone viability, and stress amplitudes, which are regulated by the production of reactive oxygen species. Furthermore, the modified law of Laplace for the heart utilizes left ventricular wall thickness as a determinant of left ventricular wall stress. Despite encouraging reports from various groups, including ours, in the heart we may only be maintaining higher left ventricular wall thickness at the site of injection and preventing exponentially occurring myocyte apoptosis, thus escaping the vicious cycle of wall thinning, increased circumferential wall stress, border zone shear, reactive oxygen species, and nonischemic infarct extension. Even though cardiomyocyte/myocyte markers were expressed abundantly on donor cells, we could not prove significant myofiber formation and architectural orientation that resembles the unique myofiber assembly within heart muscle. Therefore, the active participation of injected cells in the syncytial contraction of the myocardial parenchyma remains to be proven. Growth factors may contribute to increasing cellular viability, hence potentiating the aforementioned mechanisms. Ultimately, alternate cell sources are also reported to prevent heart function deterioration early after infarction, and it is possible that a mixture of cells, including endothelial cells or fibroblasts, would also trigger reparative mechanisms. Direct and multiple comparisons of various types of cells in a single study were not within the scope of this work and should be designed separately.

Further studies of ESC differentiation in vitro in the presence of various growth factors must be conducted to evaluate the timeline of phenomena that occur in the process of development. The effect of growth factors on cell signaling, cellular hypertrophy, and cell survival after injection into the area of infarction was not the subject of the present study. It is also possible that systemically or locally administered growth factors mobilize autologous stem cells that might home and repopulate the lesion area. The study of the specific interactions between donor cells and mobilized autologous stem cells of the recipient in myocardial restoration is a complex issue that is unresolved at the present time. Still, the exact microenvironmental interactions between ESC and host cells need to be studied in more detail. It is unknown, for instance, whether ESC express growth factor receptors in early differentiation stages and in which sequence. The present study is limited by the fact that it was not designed to address this question. It presupposes that the injected ESC will express the receptor sequences on their surface while residing in the area of ischemic lesion. Moreover, the injected cell population is a mixed one and is not optimal to isolate the events that are orchestrated by the ischemic microenvironment toward a single cell type. Transplantation of a differentiated, replicating progeny might be the optimal substrate of transfer into the damaged heart. High purification of an ESC-derived population of cardiomyocytes will be at the cost of proliferative capacity and therefore survival of the graft. Postexplantation microarray experiments will facilitate determination of the sequence of developmental steps and help to isolate premature cardiomyocytes in the desired differentiation phase.

References


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