Evolving Technology

Injectable bioartificial myocardial tissue for large-scale intramural cell transfer and functional recovery of injured heart muscle

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Objectives: Most tissue-engineering approaches to restore injured heart muscle result in distortion of left ventricular geometry. In the present study we suggest seeding embryonic stem cells in a liquid matrix for myocardial restoration.

Methods: Undifferentiated green fluorescent protein–labeled mouse embryonic stem cells (2×10^6) were seeded in Matrigel (B&D, Bedford, Mass). In a Lewis rat heterotopic heart transplant model an intramural left ventricular pouch was fashioned after ligation of the left anterior descending coronary artery. The liquid mixture (0.125 mL) was injected in the resulting infarcted area within the pouch and solidified within a few minutes after transplantation (37°C). Five recipient groups were formed: transplanted healthy hearts (group I), infarcted control hearts (group II), matrix recipients alone (group III), the study group that received matrix plus cells (group IV), and a group that received embryonic stem cells alone (group V). After echocardiography 2 weeks later, the hearts were harvested and stained for green fluorescent protein and cardiac muscle markers (connexin 43 and α-sarcomeric actin).

Results: The graft formed a sustained structure within the injured area and prevented ventricular wall thinning. The inoculated cells remained viable and expressed connexin 43 and α-sarcomeric actin. Fractional shortening and regional contractility were better in animals that received bioartificial tissue grafts compared with control animals (infarcted, matrix only, and embryonic stem cells only: group I, 17.0% $\pm$ 3.5%; group II, 6.6% $\pm$ 2.1%; group III, 10.3% $\pm$ 2.2%; group IV, 14.5% $\pm$ 2.5%; and group V, 7.8% $\pm$ 1.8%).

Conclusions: Liquid bioartificial tissue containing embryonic stem cells constitutes a powerful new approach to restoring injured heart muscle without distorting its geometry and structure.

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The sequence of events after severe myocardial ischemia caused by obstruction of coronary flow can have detrimental effects on cardiac structure and function.[1] Myocardial cell necrosis is an irreversible process that can ultimately lead to heart failure. Innovative tissue-engineering attempts to reconstitute organ function after a severe insult promise to diversify our approach to this condition but are associated with significant limitations. A major limitation is the distortion of cardiac geometry and structure, a crucial determinant of proper hemodynamic function. The

scaffold's physical condition, its in vivo kinetics, and its suitability as an adequate microenvironment for the inoculated cells represent another limitation. Finally, sensitive primordial cells with questionable potential to survive in an area of a lesion constitute a restriction of utmost significance.

The heart constitutes a complex helical structure[2] with significant local asymmetry and anisotropy. Variable portions of the left ventricle display distinct mechanical performance and microstructure. The contractions of the particular elements of all synchronized portions of the ventricle have to be orchestrated for maximal hemodynamic output. The vast array of biodegradable materials designed for implantation into the injured ventricular wall were not destined to achieve such a task.[3,4] Furthermore, most of the used biomaterials constitute single-component isotropic matrices, in which cells are seeded according to the rules of gravity, resulting in inhomogeneous distribution and therefore inconsistent performance throughout the graft; the periphery of the graft that lies in culture medium or borders the host tissue is privileged in its blood or nutrient supply, as opposed to the core of the graft, which is exposed to severe undersupply conditions.[5] The natural sequence of events is loss of viable donor cells and therefore loss of function. As a result, the eventual improvement of cardiac function after replacement of portions of either ventricle is frequently ascribed to secondary angiogenesis activity triggered by the implanted grafts without clarity as to which mechanisms mediate such a response (inflammatory or a targeted paracrine effect).[6] A homogeneously populated graft that would align itself to the intricate geometry of the remodeling tissue after infarction and would not add to the load of the diseased area has not yet been introduced.

Moreover, the optimal type of cell to support and retain the injured region of the heart is still controversial. Common sense implies that the ideal cell source would be one's own body to maximize the likelihood of survival and engraftment of the cells. There is a rich body of work with autologous bone marrow stem cells and autologous myoblasts with variable and questionable success. The lacking peripheral plasticity of the former and the limited intercalation of the latter with their host counterparts restricts their potential for large-scale sustained myocardial restoration significantly.

Two recent decisive developments in cell science and microsurgery might help harness the potential of tissue engineering and propel efforts to restore myocardium. Of paramount importance, pluripotent embryonic stem cells (ESCs) isolated from the embryonic trophoblast have become easy to maintain and to purify in a more committed state. The remarkable potential of these cells to self-renew or give rise to a more differentiated progeny translates into high viability and promises survival in the hostile environment of an ischemic lesion. Second, the introduction of heterotopic heart transplant in a small animal[6] facilitates vigorous manipulations and large-scale replacement of heart muscle for the most demanding and complex tissue-engineering approaches, thereby paving the way for innovative experimentation and circumventing cardiopulmonary bypass. Finally, green fluorescent protein (GFP)–based labeling of donor cells has been introduced to identify and track the in vivo fate of donor cells.[7,8] Simple and reliable methods of cell labeling before transplantation should be viewed as an essential part of studies that involve cell or tissue transfer.

Here we introduce a novel approach to promote recovery of injured myocardium: we use a liquid compound that solidifies in vivo for optimal cell seeding and in situ distribution. GFP-labeled ESCs are used as a potential cell substrate in this medium to create bioartificial heart tissue equivalents. We report on the resulting innovative, robust, and nondistorting mass transfer for myocardial repair.

Methods

Animal Care

All surgical interventions and animal care were performed 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.

Animal Groups

Five groups were formed, each including 5 rats. To design a model applicable to the future clinical situation, we chose an immunocompetent syngeneic model that involved Lewis rats as donors and recipients (Harlan Sprague Dawley, Indianapolis, Ind). In the first group an undamaged heart was transplanted; in the second group the donor heart had undergone left anterior descending coronary artery (LAD) ligation before heterotopic transplantation; in the third group matrix alone was implanted in a LAD-ligated heart, which was then heterotopically transplanted; and in the fourth group the bioartificial cell-Matrigel (B&D, Bedford, Mass) compound was implanted after LAD ligation and heterotopic heart transplantation. The last group was composed of animals that received an LAD-ligated heart treated with cell only (without matrix). The animals were killed, and the hearts were harvested 2 weeks later.

EGFP ES Cells

Polypeptide chain elongation factor 1 (pEF-1) a-enhanced green fluorescent protein (a-EGFP), which contains the enhanced *EGFP* gene under the control of human elongation factor 1 (human EF1), a promoter, and a neomycin resistance cassette, was constructed as follows. The promoter region of polycistronic enhanced green fluorescent protein-N3 (pEGFP-N3) (Clontech, Palo Alto, Calif) was removed by cutting out the *Ase*I-*Nhe*I DNA fragment and joining blunt-ended termini. Human EF1a promoter from pEF-BOS (*Hind*III-*Eco*RI DNA fragment) was inserted into the *Hind*II-

*Eco*RI site of the plasmid. D3 ES cells were transfected with pEF-1 a-EGFP by means of 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 Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, 2 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 1× nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, 1000 U/mL leucemia inhibitory factor (LIF) (ESGRO, Temecula, Calif), 100 U/mL streptomycin, and 100 μg/mL penicillin. Cells were collected after trypsinization with ethylendiamine tetraacetic acid and placed in aliquots of the same medium as above for inoculation into Matrigel. One milliliter of growth factor–reduced Matrigel was maintained in one leg of a Cohesion Y-shaped applicator (Cohesion, Palo Alto, Calif), and 1 mL of cell suspension that contained 5×10^6 GFP-labeled mouse ESCs was maintained in the second. At the time of injection, the 2 components are mixed, resulting in a viscous liquid compound that solidifies at 37°C because of this specific physical property of the vehicle.

Myocardial Injury

Rats were preanesthetized in an isoflurane inhalation chamber and received an intraperitoneal injection of Ketanest (Ketamin, Park Davis, Berlin, Germany; 50 mg/kg). Anesthesia was maintained with inhalational isoflurane for the rest of the procedure (Figure 1, *A*). We have used a small animal heterotopic heart transplantation model for the restorative procedure. Briefly, a median thoracotomy was performed in the donor animal, the LAD was ligated while the heart was still beating, and a pale demarcated area was identified as a consequence. The heart was flushed with 5 mL of cardioplegic solution (modified Stanford solution), explanted, and placed on ice. The jelly compound was prepared before the beginning of the procedure and maintained in a Y-shaped applicator (Figure 1, *A*). Two million ESCs were introduced into one of the 2 legs of the applicator, and Matrigel was introduced in the other, so that through application of pressure, the 2 components were mixed in the synthetic tip of the applicator. The compound was injected (0.125 mL) into the demarcated area, and the heart was left on ice. A median laparotomy and preparation of the large intra-abdominal vessels of the recipient followed. The heterotopic transplantation was performed as described elsewhere by using the nonworking heart principle, which involves aortoaortic and pulmocaval anastomoses. In this model the ventricles are reperfused through the coronary circulation; however, they do not eject. This model is frequently used in transplantation science, and despite the nonworking classification, there is still vigorous contractility and fractional shortening present. In various studies this model has been used for long-term viability studies after transplantation by using regular palpation of the intra-abdominal graft as an index of graft integrity.

Echocardiography

Rats were transferred in a portable anesthesia chamber and kept under 2% inhalational isoflurane anesthesia for the duration of the procedure, which took place immediately before the animals were killed. The abdomen was shaved, and the animals were placed in a recumbent position. We used the Acuson Sequoia C256 echocardiography system (Acuson, Mountain View, Calif) with a 15.8-MHz probe. We obtained the following measurements: end-systolic diameter (ESD) and end-diastolic diameter (EDD) in a cross-section, ESD and EDD at 2 different sites of a longitudinal section of the heart (basal and apical), posterior wall thickness and septal wall thickness, and calculated fractional shortening (FS), which was defined as follows:

$$FS = (EDD - ESD)/EDD.$$

Organ Harvest, Tissue Storage, Immunofluorescence, and Confocal Microscopy

Hearts were excised and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 2 hours and cryoprotected in 30% sucrose overnight. Tissue was embedded in OCT medium and sectioned at 5 μm on a cryostat. Serial sections were stained with hematoxylin and eosin (H&E) or Masson trichrome or used for immunohistochemistry. For immunohistochemistry, sections were blocked and incubated with primary antibody for 30 minutes to 1 hour at room temperature. Primary antibodies against cardiac, immunologic, and GFP proteins were used. These included rabbit anti-connexin-43, mouse monoclonal anti-sarcomeric actin, mouse monoclonal anti-smooth smooth muscle actin (Sigma, St Louis, Mo), goat anti-GFP antibody (Rockland, Gilbertsville, Pa), and rabbit anti-GFP Alexa-488–conjugated antibody (Molecular Probes, Eugene, Ore). After brief washing with PBS, sections were incubated with secondary antibodies for 30 minutes to 1 hour at room temperature. Texas red–conjugated secondary antibodies or streptavidin–Texas red was used against the cardiac marker primary antibodies. Goat anti-GFP antibody was recognized by a fluorescein isothiocyanate–conjugated secondary antibody. After brief washing with PBS, sections were mounted with Slowfade antifade reagent with DAPI (Molecular Probes). Stained tissue was examined with a Leica DMRB fluorescent microscope and a Zeiss LSM 510 two-photon confocal laser scanning microscope.

Connexin 43 and α-sarcomeric actin were used to identify differentiation when expressed on donor GFP-positive cells. Trichrome and H&E stains were used to estimate the extent, distribution, structure, and kinetics of ensuing scarring 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 as indicators of tumor formation. The trichrome and H&E observations were expressed on an ordinal 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). Therefore the latter findings are to be regarded as semiquantitative and are represented in the graphs as scale of the individual event.

Morphometry

Sections were obtained at 5 different levels of the harvested heart. The infarct was identified as a dark area that contained the injected GFP conglomerate. For all morphometric evaluations, the focused microscopic field was photographed with an adapted camera (Diagnostic Instruments Inc, Burlingame, Calif). The total GFP-positive area was measured and related to the infarction area at low magnification. Five random section of the GFP-positive graft were photographed and evaluated with Spot advanced software, version 3.4.2 (Diagnostic Instruments Inc), to quantify the extent of ex-

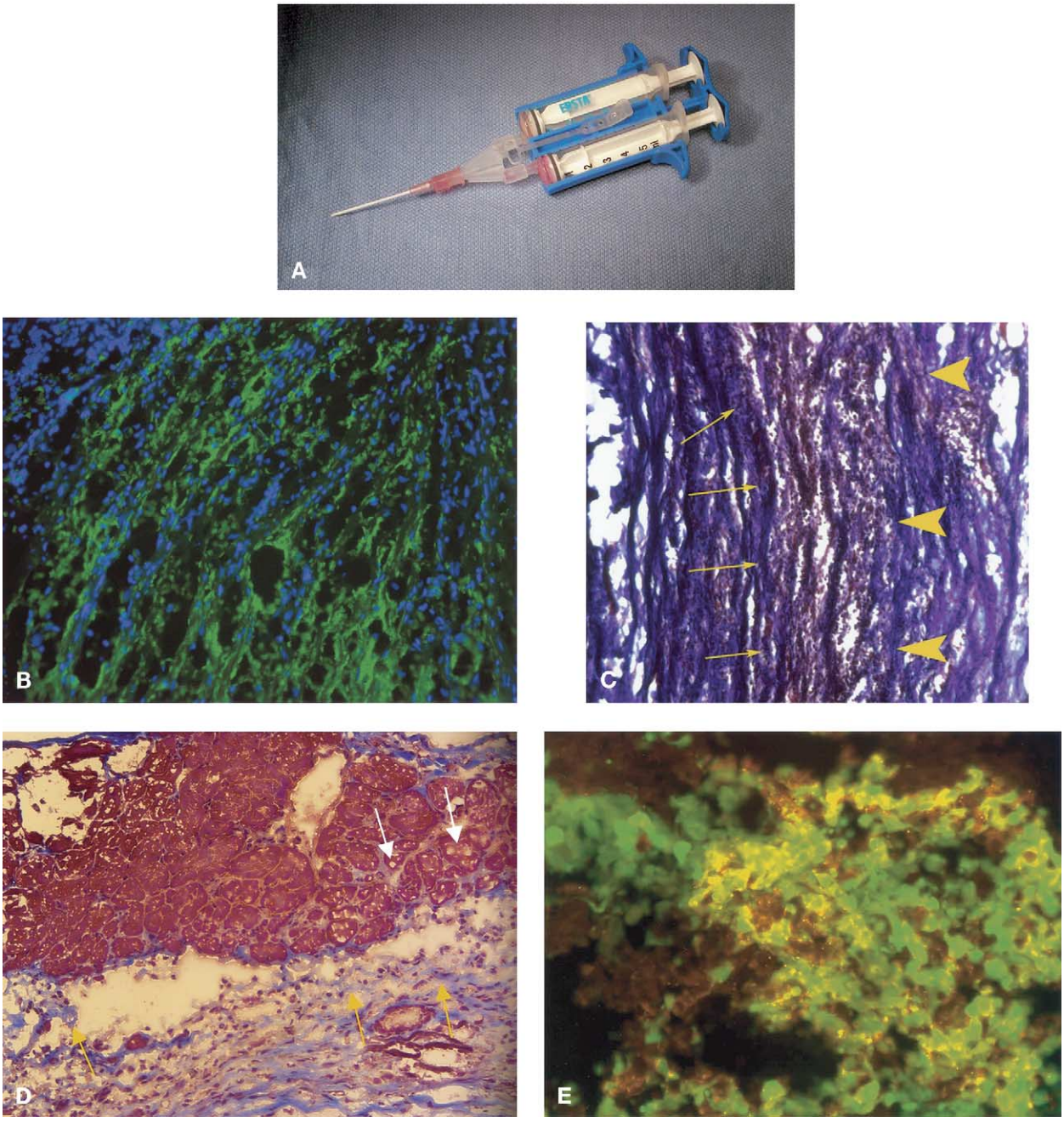

Figure 1. A, The Y-shaped applicator containing the cell suspension in one leg and Matrigel in the other. Injection results in immediate homogenous mixing of the 2 compartments. B, Dense populations of GFP-positive donor cells form robust grafts within injured myocardium as they colocalize with the blue-staining DAPI nuclear signal. C, Intramural injection of a bioartificial, ESC-containing matrix results in homogenous support of injured myocardium, with alignment along the collagen fibers. Smooth intermingling of donor *(red)* and host *(blue)* collagen structures between the epicardial *(arrowheads)* and the endocardial *(arrows)* portion of the recipient myocardium is seen. The gaps between the collagen fibers are filled with donor cells in serial alignment. A significant inflammatory response with massive cell infiltration, foreign-body reaction, and consequent diminishing of the grafted structure was not observed. D, Matrigel injection without cells also provides structural support without distortion of the left ventricular wall architecture. The *white arrows* point at signs of severe ischemia, such as vacuolization and disintegration of cellular structures. As opposed to the group that received matrix and cells, the Matrigel–host tissue contact features disruptions, discontinuity, and gap formations around collagen fibers *(yellow arrows)*. E, An in vivo differentiating progeny of GFP-positive donor cells expresses connexin 43.

pression of specific markers. The quotient of the marker-positive area and the area occupied by the GFP graft on every section provided an area ratio, which was expressed as a percentage. High magnification was chosen for this portion of the morphometry (×400) to avoid missing marker-expressing cells.

Statistics

Descriptive statistics included means and SDs of all measured values. Comparison between 2 study groups was performed with the Student *t* test for independent variables (cinetic studies were performed in separate animal groups) by using Microsoft Excel 2000. Multirange differences were analyzed by means of analysis of variance with the post hoc Bonferroni test. Statistical analyses were performed with StatView 5.0 (SAS Institute, Cary, NC).

Results

Engraftment and Restorative Effect

The cells assembled along the Matrigel fibers in large populations. They were identified as donor cells on the basis of the GFP signal, which was present 2 weeks after heterotopic transplantation and injection of the compound (Figure 1, *B*). The cell-Matrigel mixture aligned itself along the host organ structure and geometry, which it did not distort (Figure 1, *C*). The implanted collagen inserted well into the surrounding scar tissue, perpendicular to the host connective tissue structures. The restorative effect ranged from 30% to 70% of the injured area, with a mean of 51% ± 12%. The native margins of the left ventricular wall were not displaced. Histologically, we found that GFP-positive cells formed islets or conglomerates within the infarcted region. Injection of Matrigel without seeded cells also resulted in structural stabilization and prevented wall thinning (Figure 1, *D*). The majority of cells did not assume a cardiomyocyte-like phenotype but expressed the cardiomyocyte markers connexin 43 and α-sarcomeric actin (Figure 1, *E*). The cellular distribution of connexin 43 was not limited to the contact point between GFP-positive cells but occurred at various points of the cell body. Counting of individual cells was not possible because of large surviving populations and their conglomerate arrangement. There were only a few signs of foreign-body reaction because only a few cells of the lymphocytic phenotype occurred within or around the graft. Furthermore, no signs of cellular migration beyond the borders of the scar were noticed. The inoculated cells remained within the margins of the compound in the infarcted area. Vessels were evident primarily in the periphery of the compound, in the vicinity of the borders of the graft, and less in the core of the graft. However, overall cellular density was uniform throughout the graft. In the control group with LAD ligation and no treatment, an extensive scar formed after 2 weeks. In the control group with heterotopic transplantation of an uninjured heart that received Matrigel without cells, the graft survived well and was contracting before explantation. Histologic studies with H&E have revealed only a minor parenchymal presence of cells of leukocytic origin, without evidence of graft coronary disease (nude donors and recipients).

Echocardiographic Evaluation of Cardiac Function and Contractility

All animals that had undergone LAD ligation displayed severe posterior wall dyskinesia (ie, the left ventricle assumes a dorsal position in the nonworking heterotopic heart transplant model using our protocol). Even though the transplanted heart does not eject, synchronous wall motion was obvious. The FS in the treated and control groups was as follows: group I, 17.0% ± 3.5%; group II, 6.6% ± 2.1%; group III, 10.3% ± 2.2%; group IV, 14.5% ± 2.5%; and group V, 7.8% ± 1.8%). The ESC-treated group showed significantly higher FS and regional contractility compared with the control group with LAD ligation and transplantation only (14.5% ± 2.5% vs 6.6% ± 2.1%; $P = .018$, *t* test for independent variables; Figure 2, *A*). The group with the highest FS was the one treated with Matrigel and cells ($F = 24$; $P < .001$, post hoc Bonferroni test).

Posterior wall thickness was higher in the same group (1.5 ± 0.4 mm vs 0.6 ± 0.15 mm, $P = .01$) compared with that seen in the infarcted control animals (Figure 2, *B*). Septum thickness was also the highest in the ESC-treated group (1.4 ± 0.2 mm vs 0.9 ± 0.2 mm in the infarcted control group; $F = 9.3$; $P < .01$, post hoc Bonferroni test; Figure 2, *B*). Left ventricular dilatation was prevented in the matrix plus ESC–treated group (EDD: 4.1 ± 0.1 mm vs 4.8 ± 0.4 mm in the group that received no compound; $P = .03$, *t* test for independent variables; Figure 2, *C*). The animals that received matrix only also experienced prevention of wall thinning and end-diastolic dilatation. The comparison of wall thickness and left ventricular cavity dimensions did not yield significant differences between groups III and IV. They did not display a significantly better contractility (FS). The group with ESC only, without matrix, did not experience improved wall thickness or FS.

Discussion

Injectable bioartificial tissue bears crucial advantages compared with the rigid scaffolds used for myocardial repair. The quality of these scaffolds is measured by their usefulness as ventricular wall support in relation to the goal to be achieved. Thereby specific physical properties, such as porosity, density, and biodegradability, play a critical role. As opposed to rigid scaffolds, a Matrigel-based liquid compound does not have to be fixed to the surface of the heart or at the edges of removed muscle. This aspect prevents necrosis caused by isolation of core portions of the matrix from healing elements and blood supply. It contracts within the scar or injured area perfectly aligned with the surrounding tissue. Being liquid at the time of administration allows for a targeted cell therapy approach, which was not feasible with preformed or precultured solid scaffolds. Through this

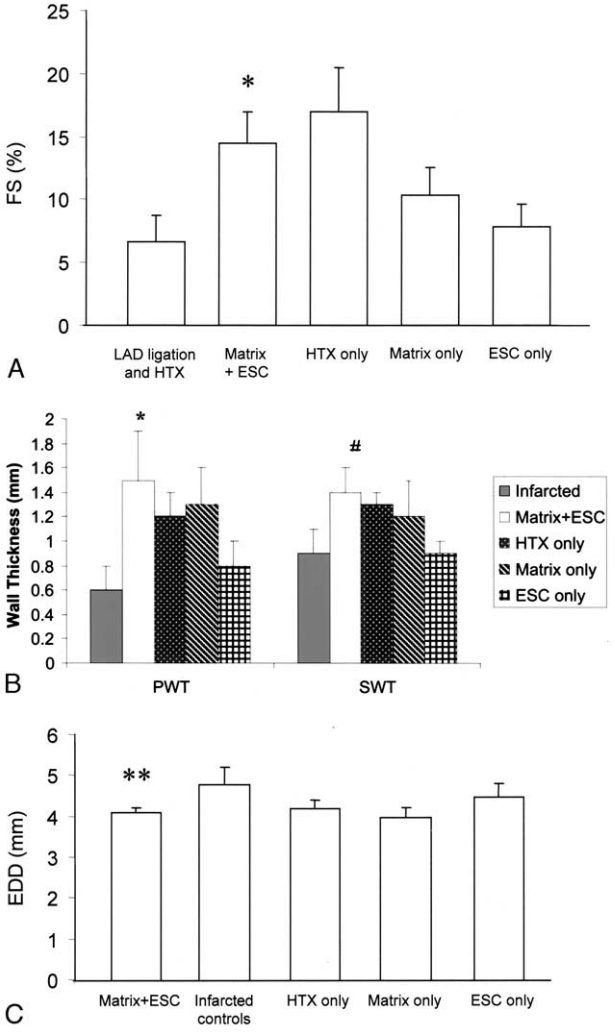

Figure 2. A, FS. In the nonworking but still contractile ventricle of the transplanted heart, FS was the highest in the matrix plus ESC–treated group (F = 24, *P < .001, post hoc Bonferroni test). B, Myocardial wall thickness. Posterior wall thickness *(PWT)* is higher in the matrix plus ESC–treated group of rats than in the infarcted control animals (*P = .01, *t* test for independent variables). Similarly, septal wall thickness *(SWT)* was the highest in the matrix plus ESC–treated group (F = 9.3, #P < .01, post hoc Bonferroni test). C, EDD. Injection of the liquid cell–Matrigel compound prevents dilatation of the severely damaged left ventricle best compared with that seen in the infarcted group (P = .03, *t* test for independent variables); however, the same effect is observed in the matrix-only and ESC-only groups. EDD is represented in millimeters.**

injection route, administration of the bioartificial compound can occur in multiple locations, without increasing the surgical trauma to the heart significantly. In contrast, implantation of solid matrices in multiple locations would result in a severely injurious and lengthy procedure.[9-11] Furthermore, the use of injectable supporting scaffolds would diminish concerns of invasiveness, allowing for port access approaches and targeted transfer at various otherwise unapproachable sites of the diseased heart. This advantage is of paramount importance for the future of the emerging field of tissue engineering in an era of reduction of surgical trauma and associated hospitalization. As to the labeling method of the donor cells, we chose GFP transfection over alternative techniques (the carboxyfluorescein diacetate succinimidyl ester method and the membrane fluorescent intercalated dye pkh26-gl method) because it is clearly superior in terms of staining intensity and duration. The immunogenic or toxic effects of GFP have not been fully addressed.

Despite promising reports, it remains controversial how a rigid scaffold inoculated with undersupplied cells would participate in or even improve contractility.[12,13] Even though biocompatible scaffolds are known to ignite some degree of cellular infiltration and angiogenesis, this is most likely the result of a foreign-body reaction, and there is no evidence that the immigrating cells are of a muscular phenotype or capable of engaging in bridging intercalations with host contractile elements.[14,15] We believe that the majority of related studies that claim engraftment and functional improvement of the heart with rigid scaffolds must be viewed with caution as long as they fail to determine the fate of the inoculated cells and propose secondary angiogenic events to be the causative mechanism of functional improvement.

Furthermore, a rigid scaffold lacking self-renewing cells is unlikely to keep pace with the growing host when used for the repair of congenital defects. What happens most likely is a fibrous transformation of the vehicle scaffold, loss of viable contractile elements, and scar formation. Injectable forms of bioartificial tissue might not impair the growth progress because they assume the host geometry and solidify secondarily in a manner that does not impair the elastic properties of the injection site. Moreover, injectable bioartificial tissue infiltrates the target organ structure and can be applied variably at borderline zones between healthy and diseased regions, thereby bridging dead scar with neighboring tissue with retained viability and intact metabolism. This unique property might facilitate migration of primordial cell forms and enhance the healing process. Our data (Figures 2, *B* and *C*) indicate that there is a smooth intermingling of donor and host structures rather than an abrupt transition. However, both the present and other articles in the field report significant improvement of cardiac function. Impressive success with the use of a plethora of cell types raises the suspicion that functional improvement of the heart after injury is not attributable to the specific cell type but rather to the viability support, which is provided by any transfer of viable matter. All myocardial restoration studies share a further flaw: by transplanting tissue, compounds, and cells in a random fashion into the heart muscle without

respecting the asymmetric nature of the myocardium, they do not reproduce cardiac muscle with the highest possible fidelity. Therefore it needs to be pointed out that fibroblasts (noncontractile elements) or Matrigel itself could possibly provide enough structural support to enhance cardiac function. The present study does not escape this critical point either. However, it appears clear to the authors that restorative operations in the future will involve minimal invasive techniques that cause less surgical trauma. Furthermore, a robust and sustained viability support of the scale provided by ESCs cannot be achieved with other types of cells, as shown in a series of studies from our laboratory.[16] The advantage of the principle introduced here (ie, injecting rather than implanting tissue) offers a restorative approach that is less invasive than the methods currently used in the clinic (transplantation and bypass procedures) and the experimental cardiomyoplastic procedures reported thus far. An endoscopic application with the appropriate instruments would be desirable.

The use of injectable bioartificial compounds such as the one used in our study bears a significant limitation: it allows only for large-scale intramural support rather than transmural replacement of the right or left ventricles of the heart. Eschenhagen and coworkers[17] have used preformed Matrigel-based constructs for myocardial restoration that had been solid at the time of implantation. Such constructs would allow for in vitro preconditioning, including endothelialization. Although this approach probably constitutes the ultimate endeavor of tissue engineers, it is certainly unlikely that the injured portion of the heart can be effectively replaced with a nonbeating or nonviable element after the inoculated cells because of poor vascular supply. Finally, the liquid phase of our compound facilitates supplementation of cell suspension with growth factors, healing promoters, and pharmaceutical substances, for example, which could be added to promote engraftment and differentiation of the seeded cell population. Matrigel is considered as basement membrane and generated from Engelbreth-Holm-Swarm (EHS) sarcoma. Matrigel normally contains not only basement membrane components (collagens, laminin, and proteoglycans) but also matrix-degrading enzymes, their inhibitors, and growth factors. Invasion of tumor cells into Matrigel has been used to characterize involvement of extracellular matrix receptors and matrix-degrading enzymes, which play roles in tumor progression. For the present study, we used growth factor/enzyme–reduced Matrigel to minimize the interaction of growth factors and ESCs. The use of the Y-shaped applicator allows for homogenous distribution of any supplemental substance within the cellular or Matrigel component right at the site of instrumentation or surgical intervention. With the advent of tissue engineering, a plethora of appealing materials have emerged that could display a better alternative for restoration of the heart in the future. Matrigel might not be the material of choice, but at the present time, it offers physical advantages over other types of collagens we and others have used in the past. It does not disintegrate before administration (as collagen type I does), it maintains liquid form and solidifies in vivo, and its growth factor–reduced form does not elicit severe inflammatory response.

The heterotopic rat heart transplantation model introduced by Krupnik and associates[18] is a crucial milestone in the path to large-scale myocardial restorative procedures. The advantages of this model range from fiscal to surgical and are time related. Provided that experienced hands in transplantation microsurgery are involved, the spectrum of surgical manipulations with various scaffolds and cells is almost unrestricted. We chose to injure the heart in an alternative fashion by ligating the LAD and creating a flap on the left ventricular surface, allowing for severe tissue ischemia to take place and simultaneously administering proportionally high amounts of bioartificial compound to restore myocardial structure without distorting the left ventricular wall geometry. An isotropic reconstitution of the myocardial structure is still far from achieved, keeping in mind that the heart is a complex helical structure with high regional variability that cannot be imitated with the currently available scaffolds, neither rigid nor flexible, with optimal fidelity. However, an intramural implantation of jelly compound seems to meet the architectonic criteria of restoration from within more closely. A limitation to our study is that we did not follow the natural course of infarction and scar formation for a longer period of time, up to 8 weeks after LAD ligation. Previous experience in our laboratory has shown severe wall thinning in the area of ischemic injury as early as 2 weeks after injury in rats and 1 week in mice.

A further considerable parameter that is inevitable in this field is the preimplantation labeling of cells. An insufficient labeling before inoculation into the scaffold and transplantation limits the potential of distinguishing cell origin. Nevertheless, a rich body of studies lack efficient cell labeling and tracking methods in the field of tissue engineering. In the present model cells are GFP labeled and possess a strong fluorescent signal 2 weeks after implantation. In vivo studies in our group using mice in a different context demonstrate undiminished GFP fluorescence in the same cell type after 8 weeks. A variety of alternative methods of cell labeling are now also available and have been used effectively on bone marrow stem cells, cardiomyocytes, and ESCs.[19-21]

Immunogenicity of the transplanted ESCs might become a limiting factor in their future clinical use. In preliminary studies (unpublished data) we found that mouse ESCs are incredibly robust after injection into the rat heart. We have carried out several donor-recipient combinations, which all led to the observation that cyclosporine (INN: ciclosporin) or the simple allogeneic setting (mouse major mismatch)

does not lead to a significant difference in engraftment and cell survival. We speculated that ESCs do not have the full spectrum of surface markers to ignite a multifaceted immune response, even though ESCs are known to express small amounts of major histocompatibility molecules under stimulation in the Petri dish. Indeed, cells survived in all animals within the matrix and also in the group receiving ESC injection only. Nude rats did not reveal a significant difference. In a mouse model of ESC transfer, we found increasing amounts of T-lymphocyte infiltration into the graft, but it was not significant enough to reduce the size of the graft, even after 8 weeks (unpublished data).

ESCs and their differentiated derivatives will enter the field of reconstructive surgery and tissue engineering. Identifying the mechanisms that drive the mother cell to produce not only an identical daughter cell but also the cell type that meets the needs of the surrounding conditions will lead to the in vivo enhancement of purification of the transplanted cells, inhibition of tumorigenicity, and improvement of function. We believe that ESCs have the potential to give rise to target organ–specific progeny when injected into this organ. To what extent is not known and is presumably unpredictable without further manipulation. In our mouse experiments we have indices of differentiation, as shown in colocalization studies. In a future study we plan to inject or transfect cell with factors-genes that are known to drive cardiomyocyte differentiation (eg, nFAT), along with the cells and Matrigel.

We did not observe cellular atypia, nuclear polymorphism, or teratoma formation in the present model. This finding does not exclude tumor formation later on in the postimplantation course. These complex interacting phenomena have not been studied in a bioartificial environment with restorative intentions yet and were not subjects of the present study.[22-24] Ongoing studies in our laboratory involve premature cardiomyocytes derived from ESCs. However, only a perfectly (100%) purified population of these cells will prevent tumor formation in vivo.[25,26] This aspect constitutes a limitation in the present study and is the focus of ongoing studies.

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