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Stem cells: where we stand

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Stem cells are capable of indefinite self-renewal and can give rise to all tissues derived from the three primary germ layers. Embryonic stem cells and, to a lesser extent, bone marrow–derived cells, share these properties, while skeletal myoblasts should be considered as precursor cells for myotubes, because of their restricted commitment to this specific skeletal muscle lineage. Evidence for the differentiation of bone marrow–derived stem cells into cardiomyocytes is inconclusive, and available data indicate that the major effect of the transplantation of these cells is to increase angiogenesis, probably through secretion of angiogenic growth factors rather than conversion into an endothelial-like phenotype. To date, clinical trials have documented the feasibility and safety of transplantation of bone marrow cells and skeletal myoblasts, with the caveat of a proarrhythmic risk associated with the latter. However, efficacy data can only be generated by randomized trials, some of which are already under way. Initial findings suggest that bone marrow cells may be particularly suitable for increasing angiogenesis in patients with acute myocardial infarction or intractable ischemia, whereas myoblasts may be more effective in improving function in the setting of advanced ischemic heart failure.

Keywords: stem cell; hematopoietic progenitor cell; skeletal myoblast; transdifferentiation; myocardial regeneration; myotube; angiogenesis; clinical trial; therapy

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Myocardial regeneration consists of the repopulation of irreversibly damaged muscle with new contractile cells to restore functionality in the necrotic areas, thereby improving global heart function. Ideally, these cells come from the spared peri-infarct myocardium. Until recently, it was thought that adult mammalian cardiomyocytes were terminally differentiated and, therefore, could not divide. This dogma has been recently revisited in light of pathological studies performed in patients with ischemic and dilated cardiomyopathies, which showed that some cells can actually reenter a mitotic cycle.1,2 However, the magnitude of this self-repair mechanism is by far too limited to compensate for the massive loss of cardiomyocytes resulting from a large infarct. Although intellectually attractive, genetically induced conversion of in-scar fibroblasts into myogenic cells has little clinical applicability, and the only practicable perspective is an exogenous supply of cells for effecting repopulation of injured areas.

Ideally, exogenous cells should satisfy the following criteria: (i) be easy to collect and expand; (ii) form stable intramyocardial grafts; (iii) be able to electromechanically couple with host cardiomyocytes so as to beat in synchrony with them; and (iv) be devoid of arrhythmogenic and oncogenic effects. It is important
to note, however, that the “ideal” cell fitting this description is not yet available for clinical use. Historically, the proof-of-principle was established by experiments using fetal (and neonatal) cardiomyocytes. Experiments with these cells have consistently shown that they were able to successfully engraft into infarcted myocardium, express gap junction proteins (thus allowing them to couple with host cardiomyocytes), survive for long periods of time, and improve left ventricular function.\(^8\)\(^{-}\)\(^10\) Overall, this improvement in both regional and recipient cells does not preclude the grafted myoblasts isoforms.\(^7\) Interestingly, the lack of gap junctions with composite fibers coexpressing fast and slow myosin environmental being the emergence of a population of a phenotypic change induced by their new myocardial environment being the emergence of a population of composite fibers coexpressing fast and slow myosin isoforms.\(^7\) Interestingly, the lack of gap junctions with recipient cells does not preclude the grafted myoblasts from promoting improvement in both regional and global left ventricular function.\(^8\)\(^{-}\)\(^10\) Overall, this improvement appears to be sustained over time, is almost linearly correlated with the number of injected cells (hence the importance of large-scale production and of means of reducing the high early posttransplantation cell death rate), and is additive to the protection afforded by angiotensin-converting enzyme inhibitors.

These experimental data provided the rationale for three recently published phase 1 clinical trials.\(^11\)\(^{-}\)\(^13\) carried out in cardiac surgical patients with severe left ventricular dysfunction, postinfarction discrete akinetic and nonviable scars, which are the target for cell injections, and an indication for coronary artery bypass surgery in remote ischemic areas. Skeletal myoblast transplantation has also been performed as a standalone procedure using an endoventricular catheter (this experience has not been published yet). Finally, a recent porcine study (chronologically posterior to the human trial) used iron oxide-loaded myoblasts and magnetic resonance imaging to document the accuracy of these targeted catheter-based implantations into infarcted myocardium.\(^14\) However, as the assessment was performed only 90 minutes after transplantation, the long-term retention patterns and functionality (ie, the ability to differentiate into myotubes) of cells injected through endoventricular catheters remains to be determined.

Overall, the first conclusion drawn from these initial studies is that the procedure is feasible, ie, that it is possible to grow several hundred million cells from a small muscular biopsy within 2 to 3 weeks under Good Manufacturing Practice conditions, and that the final cell yield can be surgically injected into multiple sites across and around the scar without any procedural complications. A second important finding is that myoblast transplantation is potentially proarrhythmic. In our phase 1 study, 4 patients out of 10 developed sustained episodes of ventricular tachycardia and the endoventricular catheter-based trial had to be temporarily stopped because of 2 sudden deaths presumably due to arrhythmia. This concern, however, should be tempered by the finding that in patients with a history of myocardial infarction associated with severe left ventricular dysfunction or congestive heart failure (ie, patients very similar to those comprising the cell transplant population), the risk of postbypass ventricular tachycardia has been reported to increase up to 30%.\(^15\) Nevertheless, even though it is difficult to conclusively establish a causal relationship between ventricular arrhythmias and cell grafting in the absence of control groups and in a patient population prone to develop this type of events because of the underlying cardiac disease, safety requirements make it necessary to consider this relationship as more than likely (in-
deed, only the results of the ongoing randomized trials will allow to assess whether this assumption has to be revisited or not. The mechanism of these allegedly transplantation-related arrhythmias remains elusive, but could be related to differences in electrical membrane properties between donor and recipient cells. Indeed, our observations that action potential durations of engrafted myoblasts are almost ten times shorter than those of host cardiomyocytes is consistent with the creation of microreentry circuits. As long as the potential proarrhythmic risk associated with myoblast transplantation has not been fully elucidated, we believe it is safer to recommend the implantation of an internal defibrillator, the legitimacy of which is reinforced by the fact that the characteristics of this cell transplant patient population closely match those of the second Multicenter Automatic Defibrillator Implantation (MADIT II) trial. Implantation of defibrillators in our ongoing placebo-controlled randomized trial has an additional interest, since interrogation of the Holter function of the device provides a reliable means of quantifying the incidence of arrhythmias if any, as well as their elective schedule of onset, (incidentally, very few implanted patients in our phase I study have yet been shown to fire appropriate shocks).

It is clear that these pilot trials, by virtue of their design (small patient populations, absence of control groups, confounding effect of associated revascularization), do not allow definite conclusions pertaining to efficacy. Nevertheless, our recent finding that approximately 60% of initially akinetic segments recovered some degree of systolic thickening following myoblast implantation is promising and prompted us to launch a large-scale multicenter randomized study with contractility of the grafted regions as the primary end point. The additional, even if limited, reports of the presence in human hearts of myotubes in the injected areas lend further support to a potential link between engraftment of cells and improvement in function.

The mechanisms of this improvement remain, however, unsettled. A limitation of infarct expansion could conceivably result from a scaffolding effect of the implanted myoblasts. This mechanism is currently supported by experimental evidence of favorable changes in left ventricular wall stress and relaxation. However, it is doubtful that such an effect can develop at a late stage, i.e., once remodeling has been completed. A second possibility is that engrafted cells directly contribute to contractility. In spite of the observation that these cells retain cross-striations suggestive of persistent functionality, this hypothesis is, at first glance, challenged by the fact that in-scar myotubes do not express gap junction proteins and are thus expected to remain electrically insulated from the surrounding normal myocardium. This, in turn, makes it difficult to understand how they could synchronously contract and thus contribute to improving function. However, findings from our electrophysiological studies also suggest that grafted myotubes are able to elicit action potentials followed by active contractions in response to a strong depolarizing current. It is thus conceivable that in areas where grafted and recipient cells are in close contact, the former could be excited by electrical currents fired by the neighboring cardiomyocytes and directly channeled through cell membranes, i.e., bypassing the classic gap junction pathway. Finally, it is also conceivable that the benefits of myoblast transplantation are not directly related to their intrinsic contractile properties, but rather to the release of growth factors capable of mobilizing a resident pool of cardiac stem cells, thereby promoting endogenous regeneration and/or rescue of reversibly damaged recipient cardiomyocytes. Identification of these putative mediators is currently under way (our preliminary studies indicate, for example, that insulin-like growth factor-I (IGF-I) is released by human myogenic cells) and their effects on native cardiac cells, vessel growth, and extracellular matrix are being investigated.

Interestingly, the clinical benefits resulting from the transplantation of fetal tissue and Langerhans islets in patients with Parkinson’s disease and diabetes, respectively, provide indirect support for a paracrine mechanism of action of injected cells, inasmuch as these benefits are directly related to the release of dopamine or insulin, respectively, from the grafts. Clarification of the mechanisms by which myoblast transplantation favorably affects the function of the infarcted myocardium has not only a theoretical interest, but practical implications as well. Thus, if the primary effect of transplantation is to alter remodeling, it should be performed at a relatively early stage of the disease, within a given time window that needs to be specified. Conversely, if improved function is the result of additional contractile cells replacing the scar area, engraftment would theoretically be successful at any time point after infarction.

Regardless of the mechanism by which skeletal myoblasts improve postinfarct function, the lack of synchronous electromechanical coupling of these cells with host cardiomyocytes other than occasionally remains a major limitation. As previously mentioned, only fetal cardiomyocytes have been shown, so far, to
express junction proteins and thus really integrate into the host tissue. The recognition that “true” allogenic cardiac cells cannot be easily used clinically justifies the search for an alternative cell type capable of acquiring “cardiac-like” characteristics. These cells would need to have a plasticity enabling them to either jump across their lineage boundaries to become cardiomyocytes or, more realistically, differentiate into cardiomyocytes from an initially uncommitted state. And this is where the story of stem cells in the treatment of heart failure really begins.

**STEM CELLS: IN QUEST OF THE HOLY GRAIL?**

Broadly speaking, stem cells can be defined by two major properties: self-renewal and the capacity to generate various types of differentiated cells. From a practical standpoint, stem cells can be divided into two main categories: (i) embryonic stem cells, which are totipotent, ie, which can generate all differentiated cell types in the body, and (ii) tissue-specific stem cells, which are multipotent, ie, which can only generate the cell types occurring in a particular tissue in embryos and, in some cases, adults. A typical example of this second category is bone marrow–derived hematopoietic stem cells. These tissue-specific stem cells were long considered unable to generate other cell types than those of the tissue in which they normally reside.

However, this concept of lineage restriction was recently challenged by several studies that showed that adult stem cells of a given tissue could in fact differentiate into cells of other tissues, both in vitro and after in vivo transplantation. This process, termed transdifferentiation, enables grafted cells to overcome their developmental restriction program following exposure to signals originating from a novel environment. In the setting of heart failure, this transdifferentiation potential has mainly been recognized in bone marrow–derived cells.

**Bone marrow stem cells**

Over the past few years, transplantation of bone marrow–derived cells has raised growing interest because, like myoblasts, they can be used as autografts, but, unlike myoblasts, their plasticity allows them to change their phenotype in response to organ-specific cues. These cells could thus specifically convert into cardiac and/or endothelial cells following engraftment into myocardial tissue, thereby resulting in true regeneration of postinfarction scars. These conclusions were derived from several experimental studies, mostly performed in rodents and frequently entailing cell injections shortly after the acute ischemic injury. Observations that bone marrow cells were able to colonize infarct areas, undergo phenotypic changes leading to the expression of cardiomyogenic and endothelial markers, and subsequently improve both perfusion and function, have led several overenthusiastic investigators to claim that these cells were the “magic bullet.” As a result, a burst of clinical studies was generated, in a totally disorganized fashion. It is therefore high time for a critical review of the literature and to highlight several key clinical aspects that need to be addressed to avoid the whole issue being jeopardized because of uncontrolled trials lacking robust and sound preclinical foundations.

**Selection of cell type**

The bone marrow is a complex medium that comprises two distinct populations: hematopoietic stem cells and mesenchymal stem cells. Our current ignorance regarding the most suitable bone marrow cells for myocardial regeneration has led some groups to advocate the use of total, unFractionated bone marrow in the assumption that this mix would necessarily contain the “good” cells. This approach immediately gained wide clinical acceptance because of its simplicity: the bone marrow is aspirated, washed of red blood cells, and extemporaneously reinjected without an intervening period of culture (at most, the aspirate is only expanded for a few days). While this use of unpurified bone marrow may be effective, one may speculate about the advisability of administering a treatment without knowing which of its components has the expected therapeutic effect. Of note, a recent study from our laboratory using a sheep model of myocardial infarction has failed to document any benefit of transplantation of total bone marrow into postinfarction scars.

This concern can be addressed by sorting subpopulations on the basis of specific surface markers. The first option consists in selecting hematopoietic progenitors, with the caveat that we do not yet know which population should then be targeted. Some studies have reported successful results with CD34+ cells, whereas others suggest that the CD34– fraction is more suitable for engraftment. CD133+ progenitors, which are more immature than CD34+ cells, and are thus credited with greater plasticity, have also been used clinically although, in a rat model of chronic myocardial infarction, we failed to show that they were functionally more effective than skeletal myoblasts (unpublished data). Endothelial progenitor cells have also been proposed for inducing angiogenesis, but recent data showing
that most of these cells express markers of monocyte-to-macrophage differentiation have raised concern over their use in atheromatous patients because of the role of these cells in plaque instability. This concern is echoed by yet unpublished observations that, in hypercholesterolemic mice, injection of bone marrow mononuclear cells into an ischemic hindlimb result in the expected increase in angiogenesis and perfusion, albeit at the cost of a marked proatherogenic effect.

A second option consists in selecting mesenchymal (or stromal) cells, which can usually be isolated in vitro through their adhesive properties and which, under appropriate culture conditions, generate various mesodermal-type progenies, such as adipocytes, chondroblasts, osteoblasts, and skeletal myoblasts. Catherine Verfaillie and colleagues recently identified a fraction of these cells capable of acquiring the phenotypic characteristics of cells outside the mesoderm lineage, which they called “multipotent adult mesenchymatous cells” (MAPCs). However, the expectations raised by the potential therapeutic value of these cells should be tempered by the following considerations: (i) they are difficult to grow in a consistent fashion; (ii) they cannot be isolated with specific markers (they are only identified by a negative staining for the most common surface antigens); (iii) and there are no available data showing that they can functionally repopulate a damaged myocardium following transplantation in vivo. Finally, it has been suggested that mesenchymal stem cells exhibit an immune privilege that makes them suitable for use as an allogenic off-the-shelf product in emergency situations like acute myocardial infarction. The clinical relevance of this scenario remains to be validated by long-term studies to check that differentiation of these cells in the recipient tissue is not associated with expression of antigens triggering a rejection process that would compromise sustained engraftment.

Scale-up
Regardless of the type of bone marrow–derived stem cells selected, progenitor cells are only present in minute amounts in the peripheral blood and their percentage in the bone marrow itself is hardly any greater (1% to 2% of the total bone marrow cell population). Not unexpectedly, the degree of engraftment and the related improvement in outcome parameters are directly related to the number of injected cells. To achieve clinically meaningful benefits, it is thus mandatory to scale-up the number of stem cells, which poses a real challenge. One possibility is to try to expand them in vitro, but the risk is then that they might loose, at least in part, their pluripotentiality. Another, more conceptually attractive approach consists in mobilizing progenitors endogenously by cytokines like granulocyte colony-stimulating factor (G-CSF) and/or granulocyte macrophage colony-stimulating factor (GM-CSF). However, the efficacy of this strategy, first demonstrated in mice, could not be duplicated in primates. In addition, safety concerns were raised about the effects of such mobilization, and the associated leukocytosis, in patients at the acute phase of myocardial infarction. It is hoped that this as yet unsettled issue will be clarified by the results of ongoing clinical trials.

Fate of engrafted cells
The changes undergone by grafted cells upon exposure to their new myocardial environment are still largely unknown. In most cases, their lineage-unrelated progenitors endogenously by cytokines like granulocyte colony-stimulating factor (G-CSF) and/or granulocyte macrophage colony-stimulating factor (GM-CSF). However, the efficacy of this strategy, first demonstrated in mice, could not be duplicated in primates. In addition, safety concerns were raised about the effects of such mobilization, and the associated leukocytosis, in patients at the acute phase of myocardial infarction. It is hoped that this as yet unsettled issue will be clarified by the results of ongoing clinical trials.
of the transplanted cells, but are instead solely related to the secretion of angiogenic growth factors.\(^{30,37}\) Furthermore, it is also important to mention that interventions targeted at inducing cells to express a cardiomyogenic phenotype may be difficult to implement clinically. Thus, bone marrow mesenchymal cells have been shown to express cardiac-specific markers only if exposed during culture to 5-azacytidine.\(^{38}\) However, this demethylating agent may cause unregulatable genomic changes and, therefore, is unlikely to be approved for human application. Alternatively, conversion of graft ed cells to a cardiomyocyte-like phenotype appears to require cocultures or coimplantations with fetal cardiac cells,\(^{39,40}\) which is consistent with the previous observation that direct cell-to-cell contact is an effective means of inducing the targeted differentiation of cells with a plasticity potential.\(^{41}\) However, these manipulations are likely to be equally difficult to implement in clinical practice. Of note, unprocessed mesenchymal cells have been reported to improve function in a porcine model of myocardial infarction, but although the engrafted cells seemed to have converted to myogenic cells, none of them expressed markers specific to a cardiac phenotype.\(^{42}\) Since then, the same group has reported on the ability to track these magnetically labeled mesenchymal cells with magnetic resonance imaging following their endocardial delivery, but in the only animal that was assessed at 4 weeks after transplantation, only one fourth of the original injection sites could still be detected, and postmortem histology failed to provide any evidence for “transdifferentiation” of the engrafted cells.\(^{43}\)

**Role of host environment**

That host environment has a profound influence on the fate of engrafted cells is demonstrated by the homing phenomenon. Thus, the intravenous injection of radioactively labeled endothelial progenitors does not result in any noticeable engraftment in normal myocardium and it is only in the case of myocardial infarction that the cells are found homing in the border zone,\(^{44}\) thus suggesting that damaged tissue emits signals that act as sensors for circulating bone marrow cells. The occurrence of homing has been further supported by recent observations of cardiac chimerism in sex-mismatched heart transplant recipients in whom recipient cells, presumably of bone marrow origin, have been detected in the donor allograft.\(^{45}\) However, these cells are only found in minimal amounts and their functional contribution is unlikely to be clinically relevant. This assumption is further supported by experimental findings showing that repopulation of infarcted mouse hearts by hematopoietic progenitors from syngeneic animals only yields 0.02% and 1% to 2% cardiomyocytes and endothelial cells of donor origin, respectively.\(^{46}\) However, recognition that local signals can drive the fate of implanted cells towards a given lineage may be a double-edged sword inasmuch as grafting cells into fibrous scars could merely convert them into fibroblasts, which has indeed been observed and is exactly opposite to the intended goal. This assumption is indirectly supported by our failure, in the previously mentioned study in sheep,\(^{24}\) to show that in-scar transplantation of fresh unpurified bone marrow yielded any discernible benefit in terms of systolic or diastolic function, compared with controls injected with culture medium. In keeping with these data, there was no histological evidence for any conversion of the grafted cells into cardiomyocytes or endothelial cells.

Taken together, these data suggest that bone marrow cells may be electively useful, primarily for inducing angiogenesis, when transplanted into ischemic—and thus still living—tissue that harbors the appropriate signals for inducing lineage switching and/or reversibly injured cells that could be rescued by the increased vascularization triggered by the graft-secreted angiogenic growth factors. Conversely, skeletal myoblasts appear to be better suited for inducing myogenesis following engraftment into postinfarction fibrous scars, and restoring, at least partially, their functionality. Should this paradigm be validated by further studies, its clinical correlate would be that bone marrow–derived cells are best indicated in the setting of *acute coronary syndromes*, whereas skeletal myoblasts remain more effective cell substitutes at the late stage of *chronic heart failure*.

**Analysis of clinical trials**

As indicated above, in spite of the many still unresolved issues, the potentiality of bone marrow cells immediately generated much enthusiasm, which promptly translated into a flurry of clinical studies. Some of these studies involved intraoperative injections of total unfractionated bone marrow or CD133+ progenitors,\(^{28}\) concomitantly with coronary artery bypass grafting. In most trials, however, cell delivery was achieved percutaneously, using three different approaches. In patients with acute myocardial infarction, bone marrow was injected directly into the culprit coronary artery shortly after it had been reopened by balloon angioplasty and stenting. In one study,\(^{48}\) the injectate consisted of overnight-cultivated mononuclear cells; in another study,\(^{49}\) either 3-day cultured endothelial progenitors collected from peripheral blood or mononuclear cells extemporaneously harvested from...
the bone marrow itself were delivered intracoronarily to the infarcted myocardium. In spite of the paucity of preclinical data, it was assumed that proximal balloon occlusion along with ischemia-related changes in vessel permeability would facilitate transendothelial passage, although one cannot exclude that such trafficking is irrelevant if the primary mechanism of action is the release of growth factors. In patients seen at a later, more chronic stage, cells were delivered transendocardially or transvenously through a coronary sinus catheter. This device has an extendable needle, which, under echocardiographic guidance, perforates the venous wall, and a microcatheter is then advanced into the postinfarction scar tissue where cells are dropped. Finally, in patients with refractory ischemia, mononuclear cells were injected through an endoventricular catheter after electromagnetic mapping, with the primary objective of increasing angiogenesis and relieving ischemic symptoms. This approach was supported by a porcine study showing the efficacy of transendocardial delivery of bone marrow cells in improving collateral perfusion and function.

Although these trials, as well as those still under way, should be considered as preliminary, several clinically relevant lessons can nevertheless be drawn. First, the procedure appears technically feasible regardless of the route of cell delivery. Second, the precise phenotypic characterization of the injected cells is overall rather poor and usually limited to the identification of CD34+ progenitors, the percentage of which was not unexpectedly found to be very low (no more than 2%). Third, the technique, on the whole, appears to be safe. There were no apparent complications following iliac crest biopsies, nor have any posttransplantation arrhythmias been documented. However, these trials have not attempted to increase the number of progenitors by in vivo pharmacologic manipulations, and ongoing studies, which now involve cytokine-induced stem cell mobilization, will need close monitoring of their safety record. Fourth, all these studies have reported improved perfusion and function following bone marrow cell transplantation, to an apparently similar level whether cells are collected from bone marrow or peripheral blood and, more surprisingly, whatever the number of injected cells, which is strikingly variable from one trial to the other and even within the same trial (9 to 28 × 10^6 in the study of Strauer et al.48 and 10 to 245 × 10^6 in that of Assmus et al.).49 However, in the absence of concurrent control groups and randomization, the robustness of these data is arguable and only phase 2 studies designed and powered to show efficacy, if any, will allow a definite conclusion. Importantly, this efficacy is not only dependent on cell type, but also on the method for transferring them. Preclinical data are still scarce in this respect and additional animal experiments are required to assess which of the percutaneous approaches (intracoronary, transvenous, endoventricular) is the safest, the easiest to implement clinically, and the most effective in optimizing cell functionality, retention, and long-term engraftment.

**Embryonic stem cells**

These totipotent cells are conceptually attractive because their capacity to generate all differentiated cell types should allow them to truly regenerate infarcted myocardium. These cells can be derived from fertilized oocytes that are no longer targeted for childbearing. This involves differentiating them into cardiomyocytes prior to engraftment into injured areas. However, several major issues remain to be addressed before considering potential clinical applications. These include: (i) identification of the factors committing embryonic stem cells to a cardiomyogenic lineage and the phenotypic characterization of the resulting cells; (ii) demonstration of their engraftment potential into infarcted myocardium along with establishment of electrical junctions with host cardiomyocytes and functional efficacy; (iii) checking that this engraftment is not associated with an uncontrolled proliferation leading to teratomas; and (iv) assessment of their immune potential, although the latter problem could theoretically be overcome by obtaining cells after nuclear transfer into enucleated recipient oocytes (therapeutic cloning).

Meanwhile, preliminary experimental studies have yielded encouraging results. Transplantation of mouse embryonic stem cells into infarcted areas in immunosuppressed rats has been associated with differentiation into cardiomyocytes, increased angiogenesis, and improved survival and left ventricular function for up to 32 weeks after transplantation. Notwithstanding the ethical and political debate around embryonic stem cells, it thus seems important to pursue research in this direction to avoid missing a potentially effective means of achieving cell replacement therapy.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The long-standing belief that infarcted tissue is irreversibly lost is now challenged by the observation that endogenous regeneration can occur to some extent, even if at low frequency. This finding has stimulated
research into cell replacement therapeutic strategies based on either replacement of scarred tissue by precursor cells (skeletal myoblasts) or attempts at regenerating it by bone marrow–derived stem cells. The validity of this approach is now supported by a large number of experimental studies. Nevertheless, several key problems remain, chief among which: (i) the determination of the optimal cell type in relation with the objective and, therefore, the patient population (ie, angiogenesis in ischemic patients vs myogenesis in those with heart failure), (ii) the development of means of enhancing cell survival and long-term engraftment, possibly by concurrent induction of gene- or cell-based angiogenesis, (iii) the selection of the most appropriate method of cell delivery, and (iv) the potential applications of cell therapy in nonischemic cardiomyopathies. In the meantime, clinical studies should be carefully designed to assess the safety and efficacy of cell therapy, taking great care that their methodology complies with the guidelines adopted for drug trials, as this is a prerequisite for them to yield clinically meaningful data. We will probably have to wait another 3 to 5 years before knowing whether the high expectations currently raised by myocardial cell replacement therapy are indeed borne out by actual outcomes in patients.

THREE KEY QUESTIONS

In spite of all the remaining unanswered questions regarding the techniques and the efficacy of myocardial cell replacement therapy, the excitement generated by the potential uses of skeletal myoblasts and stem cells continues unabated and work on clinical applications is proceeding apace. The Expert Answer section of this issue of *Dialogues* examines some of the most pressing questions posed by myocardial regeneration in the setting of myocardial infarction and heart failure. Implied in the phrasing of the first question, “Myogenesis: where and how can we get skeletal myoblasts?” is the word “quickly,” as the current delay for obtaining myoblasts from the patient’s own muscle tissue is 3 to 4 weeks. Jacques P. Tremblay discusses the prospects of avoiding this delay by obtaining “off-the-shelf” immediately available myoblasts from donors or derived from embryonic or adult stem cells. Charles E. Murry and Hans Reinecke are asked, “How can cellular grafts be kept alive and synchronized with the rest of the heart?” and show that heat shocking graft cells will improve survival, while genetic modification may enhance synchronization of the grafted cells with the host myocardium. Stefanie Dimmel and Andreas M. Zeiher discuss the methods for stimulating neovascularization in the ischemic heart in reply to the question “Endothelial cells versus angiogenic factors: which is the best strategy for stimulating neoangiogenesis?” The authors suggest that the future lies in the combination of gene therapy (recombinant growth factors) and cell therapy (endothelial progenitor cells).
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EMBRYOGENESIS AND MUSCLE REPAIR

The skeletal muscle is formed by the migration of myoblasts from the somites. These cells eventually fuse to form muscle fibers. However, some of the myoblasts remain as quiescent mononuclear cells located near the muscle fibers under the basal lamina surrounding each muscle fiber. These cells are called satellite cells (Figure 1). Following the embryological development of the muscle, damage to the muscle fibers induces the activation of these satellite cells and their proliferation as cells that are named again myoblasts. These cells not only repair the muscle fibers, but reform new satellite cells, available for later repair.

USE OF MYOBLAST TRANSPLANTATION TO TREAT MUSCULAR DYSTROPHIES

The muscle fibers of patients affected by Duchenne muscular dystrophy (DMD) are frequently damaged during normal muscle activity because...
of the absence of the dystrophin protein, due to a mutation of the coding gene. Thus, satellite cells in the dystrophic muscles are frequently activated to generate myoblasts for muscle repair and regeneration of satellite cells. These frequent cycles of damage and repair eventually lead to the senescence of the patients’ satellite cells. A possible treatment of DMD investigated by my group as well as others is the transplantation in the skeletal muscles of myoblasts obtained by the proliferation of satellite cells isolated from a donor muscle biopsy. The fusion of donor myoblasts with the damaged host muscle fibers enables nuclei containing the normal dystrophin gene to be introduced into these fibers. Moreover, transplantation of normal myoblasts also leads to the formation of new satellite cells, thereby restoring the regenerating capacity of the dystrophic muscle.

USE OF MYOBLASTS TO PREVENT CARDIAC INSUFFICIENCY

Myoblasts were initially injected in the heart in the hope that they would transform themselves into cardiomyocytes. Although this transformation did not occur, the formation of myotubes in the heart increases the viability and the strength of the tissue.

TISSUE CULTURE OF MYOBLASTS

Techniques to grow myoblasts have been developed long before they started to be used to treat diseases. These techniques have, however, been improved for larger-scale production of purer myoblasts to satisfy clinical objectives. In most of the clinical trials done so far for myoblast transplantation in the heart, a muscle biopsy of 5 to 10 grams was obtained from a large muscle (such as the quadriceps) of the patient. (Figure 2). It may be eventually possible to reduce the size of this biopsy, since only a 1-gram biopsy is routinely used by our laboratory to produce millions of myoblasts for transplantation to DMD patients. This biopsy has to be obtained in a surgery room under as sterile conditions as possible. It is preferable to obtain two separate muscle biopsies, which are then handled separately, in case one of them is contaminated. These biopsies are placed in different bottles with a transport culture medium containing penicillin-streptomycin (100 µg/mL) and fungizone (2.5 µg/L). In our laboratory, we use a modified MCDB120 (without phenol red) culture medium for the transport and proliferation of the myoblasts. The biopsy is immediately sent to the tissue culture laboratory. If shipment is required, the tissue should be maintained at 4°C, but freezing has to be avoided. Shipment can take up to 48 hours without significantly reducing the yield of myoblasts. (Biopsies from patients located far from the culture laboratory can therefore be adequately shipped.) Each muscle biopsy is minced separately using sterile forceps and scissors. The biopsy pieces are rinsed three times with sterile HBSS (Hank’s balanced salt solution). The small pieces of muscle are then placed in a trypsinization unit containing 0.2% irradiated collagenase (Liberase, Roche-Boehringer, Inc) diluted in HBSS. The unit is closed and transferred to a CO₂ incubator at 37°C for agitation using a magnetic stirrer during 60 minutes. The trypsinization unit is then brought back under the sterile hood, the content is aspirated with a disposable sterile pipette and transferred to a sterile centrifugation tube. The tube is closed and centrifuged at low speed (1500 rpm, ie, 200 g). The tube is opened under the hood, and the supernatant is discarded. The pellet containing the muscle fibers is then suspended in HBSS containing 0.125% irradiated trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) and transferred again with a sterile disposable pipette in the trypsinization unit and digested during 45 minutes at 37°C. After that time, the content of the trypsinization unit is transferred again under the hood using a sterile disposable pipette to a centrifugation tube that is capped. After centrifugation at 200 g, the pellet containing the cells (roughly 25 000 cells for a 0.5-g biopsy) is transferred under the hood using a disposable sterile pipette, to five 25-cm² culture flasks containing the MCDB120 culture medium (5000 cells per flask, a total of 10 flasks since there are two different biopsies).

To obtain a higher percentage of myoblasts in the tissue culture, it is recommended to do one or two preplatings. The fibroblasts present among the cells transferred to the tissue culture flask are allowed to attach to the plastic during 1 hour. The nonattached cells are recovered by suction and the operation is repeated a second time. The nonattached cells are then trans-
ferred in tissue culture flasks, which are placed in a 5% CO₂ incubator at 37°C for 24 hours. As mentioned previously, my laboratory is using a modified MCDB120. For the expansion of myoblasts, although the transport medium includes antibiotics, there are no antibiotics present throughout the 3 weeks of culture. This approach has the advantage that any contamination of 1 of the 2 culture batches is rapidly detected. In case of contamination, the culture is completely discarded. In our experience, the main source of contamination is the biopsy itself. The culture medium has to be changed every 2 days to maintain a fresh supply of basic fibroblast growth factor (bFGF) (10 ng/mL). The density of the cells has to be maintained below 60% confluence to prevent the fusion of the myoblasts. Depending on the number of cells required, tissue culture flasks of 25 or 75 cm², cell factories, or rolling bottles, which have a much larger surface, may be used.

The cells are currently harvested with a 0.1% trypsin/0.02% EDTA treatment. However, it may be interesting in the future to develop a method that would better preserve the cell surface proteins, as the removal of integrins or of receptors for growth factors may perhaps be involved in the high percentage of cell death observed in the first days following transplantation.

**Percentage of myoblasts**

A small sample of each batch is kept to count the cells with a hemacytometer. A small sample of cells in each batch is also labeled with an anti NKH-1 antibody (Coulter Inc) coupled to phycoerythrin to estimate the percentage of myoblasts by immunofluorescence. The percentage of labeling is evaluated by counting 500 cells under the fluorescence microscope.

**Myogenicity test**

A sample of 200 000 cells from each batch is placed in a well from a 6-well plate and grown in 10% serum for 5 days to allow fusion. At that time, 500 nuclei will be counted and the myogenicity, ie, the percentage of nuclei within myotubes, is established. If it is less than 25%, the myoblast population is enriched again by preplantings.

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**Figure 2.** The myoblasts are currently obtained from biopsies of an adult muscle (usually the patient’s own muscle). The main steps involve fragmentation of the biopsies and digestion with collagenase, then trypsin (details in the text).
Enrichment of myoblasts by preplating

If the percentage of myoblasts is lower than 80% (based on the NKH-I flowcytometry results) or the myogenicity is less than 25%, the myoblast population is enriched by a series of 1-hour preplatings until 80% of the cells are myoblasts. For preplating, the cells are detached with irradiated trypsin. The trypsin is neutralized by adding culture medium containing 15% fetal calf serum (FCS). The cells are added to a 75-cm² culture flask. This flask is placed in the CO₂ incubator at 37°C for 1 hour. The cells that are not attached are collected. A small sample of cells in each batch is labeled with an anti-NKH-1 antibody as previously described. The percentage of labeling is evaluated by counting 500 cells under the fluorescence microscope. If the percentage of myoblasts is still less than 80% the preplating procedure is repeated.

Bacterial test, endotoxin test, and mycoplasma tests

A sample of the myoblasts from each batch is kept and tested for potential bacterial and mycoplasma infections. The bacterial test is an hemoculture test. Mycoplasma infections are detected by Hoechst 33258 staining according to the method of Chen (1977). The presence of endotoxins is assessed with the limulus amebocyte lysate (LAL) test, which is the standard Food and Drug Administration (FDA) approved test.

IS THERE AN AGE LIMITATION FOR GROWING MYOBLASTS FROM A MUSCLE BIOPSY?

Blau's group has reported that there was a progressive reduction in the proliferating capacity of the myoblasts with aging. However, Vilquin, who was involved in the production of the myoblasts in the clinical trial conducted by Menasché's group, told me that they did not experience any difficulty in proliferating the myoblasts of patients more than 70 years old (personal communication).

FUTURE PROSPECTS: DEVELOPMENT OF IMMUNOLOGICAL TOLERANCE AND THE USE OF STEM CELLS

Currently, myoblasts are produced from a biopsy of the patient's own muscle. This is to avoid two problems: (i) the necessity of using a sustained immunosuppression to avoid the rejection of the transplanted cells; and (ii) the possibility of transmitting diseases from a donor. This approach, however, is impractical because it requires separate handling of cultures for each individual patient. This is possible in clinical trials, which involve at most a few hundred patients, however, eventually thousands of patients could benefit from such a treatment. Thus it would be better if one lot of myoblasts could be used for many patients. This would reduce the work not only for producing the myoblasts but also for testing each lot for its sterility, viability, and myogenicity. This would eventually permit to have off-the-shelf, immediately available cells for the treatment of patients within days of their infarct rather than waiting the 3 to 4 weeks currently required for the production of cells from their own muscle biopsy. I can foresee two solutions to this problem. The first is to produce a very large number of myoblasts from a given cadaveric organ donor. I think that it could be possible to produce 10^10 myoblasts from 1 gram of tissue obtained from a young donor. This large lot of cells could be produced in a large bioreactor and divided up for immediate administration or frozen for later use. The complete lot would be tested only once for sterility, viability, and myogenicity. The only remaining problem is how to avoid the rejection of such allogenic myoblasts? There is currently important progress being done in the development of immunological tolerance. It is indeed possible to transiently block the immune response to permit the transplantation of bone marrow stem cells (that would be obtained from the same donor as the muscle biopsy). Such transplantation permits the establishment of chimerism of the hematopoietic stem cells and a permanent immunological tolerance towards the allogenic myoblasts. Indeed, the development of hematopoietic chimerism includes the dendritic cell population, which migrates into the thymus and participates in the negative selection of lymphocytes, which could react with the donor myoblasts. Impossible dream? No, it is the very near future since our laboratory has already been able to obtain a sustained immunological tolerance permitting the transplantation of allogenic myoblasts into the muscle of dystrophic mdx mice (manuscript in preparation). The transplantation of these allogenic myoblasts formed muscle fibers expressing dystrophin that remained in place more than 100 days later.

An alternative to obtaining the myoblasts and bone marrow cells from the same donor may eventually be to obtain both types of cells from a common stem cell. The first possibility is of course embryonic stem (ES) cells (Figure 1). This type of cell proliferates indefinitely, thus the production of very large numbers of stem cells is not a problem. Moreover, these ES cells have already been shown to differentiate in
hematopoietic stem cells and into myoblasts. Thus both types of cells derived from a common donor could be used to develop immune tolerance and heart repair (Figure 3). If the use of embryonic stem cells remains an ethical problem in the future, it may also be possible to derive the hematopoietic stem cells and the myoblasts from stem cells obtained from adult tissues such as the MAPCs (multipotent adult progenitor cells) or the bone marrow or muscle side population (SP cells).

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How can cellular grafts be kept alive and synchronized with the rest of the heart?

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Although infarct size limitation remains a highly desirable goal, it has been extremely difficult to achieve clinically. A fundamental problem is the fact that ischemic myocardium dies quite rapidly. Infarcts achieve ≈80% of their potential size within 3 hours of coronary occlusion. Thus, myocardial infarction and subsequent heart failure are likely to remain major health problems. Cellular cardiomyoplasty has been explored as a strategy for repairing the infarcted heart. Quite naturally, most studies have used myocytes, reasoning that they would be best suited for the task of replacing the lost cardiomyocytes and restoring systolic wall motion. Here, we will focus on the use of cardiac and skeletal myocytes to achieve this ambitious goal.

How can the grafted myocytes be kept alive?

Cardiomyocytes

Ideally, dead myocardium should be replaced by living cardiac tissue and, therefore, cardiomyocytes should be a first choice for cardiomyoplasty. Initial studies generated significant excitement after demonstrating that cardiomyocytes from fetal mice formed viable grafts after injection into normal myocardium. In order to restore systolic function, it seems likely that grafts need to replace a substantial fraction of the lost myocardium. Reasoning that more grafted cardiomyocytes would give rise to larger grafts, we performed a dose-escalation study in injured rat hearts using neonatal cardiomyocytes. Disappointingly, all grafts were small (<2% of the left ventricular mass), and there was no increase in graft size with increasing cell dose. This indicated that cell death was likely limiting the amount of new myocardium formed. Poor survival of cardiomyocytes in injured hearts was seen by other investigators as well. In fact, depending on the transplantation protocol, injury (cryoinjury, infarct) and animal model (mouse, rat, rabbit, dog, pig), cell survival after transplantation may range anywhere from 0% to ≈20%.

The next question becomes, “why do the cells die?” Information on mechanisms of graft cell death is currently limited, but it appears likely that ischemia plays a major role. Cell survival is better in normal hearts than acutely injured hearts, and vascularized granulation tissue supports cell survival better than acutely necrotic myocardium, although not as well as normal myocardium. The dilemma here is that cardiomyocytes are extremely sensitive to ischemic injury, exactly the reason they die in the ischemic heart in the first place. Similarly, an old

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Keywords: myocardial infarction; cell transplantation; cardiomyocyte; skeletal myoblast; cell death; cell survival; electromechanical coupling

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infarct scar represents a relatively ischemic tissue and, therefore, one might expect poor survival of this highly metabolic cell type after transplantation. We sought to protect the neonatal cardiomyocytes to better withstand environmental stresses after grafting into cryoinjured rat hearts. Only modest benefits in survival were noted when the cytoprotective kinase, Akt (protein kinase B), was overexpressed in the graft cells. However, significantly better results were obtained when cardiomyocytes were heat shocked the day before grafting (Figure 1), ie, a 54% reduction in cell death was observed.3 Heat shock is thus a simple and effective approach to enhancing graft cell survival.

**Skeletal myocytes**

Because of some of the limitations of cardiomyocytes, our group and others have studied skeletal muscle as a repair cell for the infarcted heart. Before describing these studies, it is worthwhile to review a few points of basic skeletal muscle biology. Mature skeletal muscle fibers originate from undifferentiated, mononucleated progenitor cells, which are termed myoblasts. When local growth factors become depleted, myoblasts withdraw irreversibly from the cell cycle, activate expression of muscle-specific genes (eg, actins, myosins, creatine kinase) and fuse to form multinucleated cells called myotubes. Myotubes undergo progressive maturation and hypertrophy to form differentiated myofibers characteristic of adult skeletal muscle. Not all myoblasts fuse into myotubes; however. Rather, some become quiescent stem cells, or satellite cells, residing in close apposition to the muscle fiber. Satellite cells can reenter the cell cycle in response to muscle injury and are responsible for the ability of skeletal muscle to regenerate. In contrast to cardiac myocytes, skeletal muscle cells are among the most ischemia-tolerant in the body and, consequently, are capable of forming large grafts in the injured heart. When injected into acutely cryoinjured myocardium, skeletal myoblasts proliferate for up to 3 days and then differentiate to form multinucleated myotubes, eventually forming hypertrophic myofibers.6

Despite their hardiness, a significant fraction of the skeletal myocytes will undergo cell death due to limited resources (eg, nutrients, oxygen) in the graft bed. Promising strategies to improve survival of grafted myoblasts include again heat shock prior to grafting7 and enhancement of vascularization in the graft bed, eg, by the use of vascular endothelial growth factor (VEGF).8 The latter is based on the hypothesis that the cellular cardiomyoplasty effect could be reinforced by improved graft survival resulting from an improved blood supply to the graft through both vasodilation and enhanced angiogenesis induced by VEGF. This would be particularly beneficial in the early stage after cell transplantation, when graft cells are subjected to various pathological processes caused by environmental stress, such as ischemic and mechanical injury known to result in both the necrosis and apoptosis of grafted myoblasts.7,9 Given that angiogenesis takes a minimum of several days to vascularize an infarct, and that cell death is most exten-

**Figure 1.** Heat shock protects grafted cardiomyocytes. Neonatal cardiomyocytes were heat shocked at 43°C for 30 min and 1 day later 5x10⁶ cells were grafted immediately after cryoinjury. (A) Western blotting showed marked upregulation of Hsp70. (B) Heat shocked cardiomyocytes showed a significant reduction in TUNEL staining 20 h after grafting into acutely necrotic myocardium.

**Abbreviations:** Hsp, heat shock protein; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP “nick-end labeling” (a measure of DNA damage).
Coupling of skeletal myocyte grafts

It is without doubt that cardiomyocytes fulfill all the requirements to couple electromechanically with the host myocardium. Indeed, several studies have shown development of intercalated disks, complete with gap junctions, between grafted fetal or neonatal cardiomyocytes and host cardiomyocytes.\(^2\)\(^,\)\(^3\) Importantly, Rubart et al.\(^1\) recently demonstrated that grafted cardiomyocytes exhibit synchronous calcium transients with host cardiomyocytes in normal mouse hearts. The principal impediment to coupling of cardiomyocyte grafts with the host myocardium is formation of scar tissue. When implanted into the injured heart, grafted cardiomyocytes couple well with one another. At early time points, it is possible to identify adhesive and gap junctions between graft cardiomyocytes and host myocytes. As infarct healing proceeds, however, the grafted cardiomyocytes typically become infiltrated by scar tissue. The scar forms a physical barrier between the graft and the host cardiomyocytes, and in our studies, we were unable to demonstrate coupling of graft and host at later time points.\(^3\) Thus, any cell-based therapy that aims to induce such electromechanical coupling will need to address the issue of insulation by scar tissue.

Although there have been reports suggesting transdifferentiation of skeletal muscle cells into cardiomyocytes,\(^9\)\(^,\)\(^17\) previous studies from our laboratory have shown unambiguously that skeletal muscle cells in the heart appear firmly committed to their fate.\(^6\)\(^,\)\(^18\) This is characterized by myoblast fusion and myotube formation, maturation, expression of skeletal muscle specific myosin heavy chains, and the failure to express cardiac markers such as α-myosin heavy chain, cardiac troponin I, and atrial natriuretic factor.\(^18\) Studies with myocardial wound strips showed that the skeletal muscle grafts would contract when exogenously stimulated.\(^6\) The grafts showed the ability to undergo tetanic contraction under high-frequency stimulation, a property not shared by myocardium because of its refractory period after depolarization. As the electrical field stimulation was increased, the skeletal muscle grafts showed increasing twitch tension, indicating recruitment of additional fibers. Fiber recruitment implied that the skeletal muscle grafts were electrically insulated from one another, unlike cardiomyocytes, which are electrically coupled by gap junctions.

These observations led us to explore the expression of the intercalated disk proteins N-cadherin and connexin43 in skeletal muscle. Immunostaining revealed that skeletal muscle grafts in the heart had undetectable levels of N-cadherin and connexin43, indicating that the grafts were not electromechanically coupled with one another or with host myocardium. These findings make it unlikely that skeletal muscle grafts in the heart are electrically excited by the host myocardium. However, when skeletal and cardiac muscle cells were placed in coculture, the cells formed a synchronously beating network.\(^14\) Skeletal myotube contractions could be accelerated by the β-adrenergic agonist isoproterenol, suggesting the cardiomyocytes were the pacemakers. Skeletal muscle contraction was inhibited by the gap junction blocker heptanol, suggesting electrical excitation was mediated through gap junctions. Moreover, synchronous calcium transients and dye transfer were observed, indicating tight coupling between the skeletal and cardiac myocytes. Finally, confocal microscopy revealed the presence of N-cadherin–mediated adherens junctions and connexin43-mediated gap junctions between skeletal muscle cells and cardiomyocytes. These experiments indicate that cardiomyocytes have the capacity to form electromechanical junc-

understand how synchronization may be accomplished, it is first necessary to briefly review normal electromechanical coupling between cardiac myocytes. The heart acts as a functional syncytium, meaning that all the myocytes in the heart act together as an electromechanical unit. This is in contrast to skeletal muscle, where cells are truly syncytial, ie, have fused to form multinucleated fibers, but the individual fibers are insulated from one another. Electromechanical coupling in the heart is achieved by specialized cell–cell junctions, the intercalated disks, which contain adherens junctions and gap junctions for mechanical and electrical coupling, respectively. Adherens junctions are the first to form during development and are composed of N-cadherin molecules anchored in the sarcolemma,\(^10\) providing binding to N-cadherin molecules in neighboring cells. Electrical coupling is achieved by gap junctions. Connexin43 is the major gap junction protein in the mammalian left ventricle.\(^11\) In contrast to heart muscle cells, mature skeletal muscle fibers are electrically isolated from one another, a prerequisite for fine motor control. Interestingly, skeletal muscle cells express N-cadherin and connexin43 as replicating myoblasts and require these proteins for fusion to form myotubes. As the cells differentiate further, however, N-cadherin and connexin43 expression are markedly downregulated.\(^12\)\(^,\)\(^13\)\(^,\)\(^14\)
How can cellular grafts be kept alive and synchronized with the rest of the heart? - Murry and Reinecke

With skeletal muscle cells and to use these junctions to induce synchronous beating in the skeletal muscle. Why does this coupling not occur in vivo after grafting? Skeletal muscle cells in culture are less differentiated than in vivo graft cells, and in culture the cells still have low levels of N-cadherin and connexin43. It appears that this low-level expression is sufficient to permit physiologic coupling. As the graft cells mature in vivo, however, N-cadherin and connexin43 appear to be downregulated to undetectable levels, thereby precluding coupling. We hypothesized that forced expression of adherens and gap junctions in differentiated skeletal myocytes should permit electromechanical coupling with cardiac myocytes. In an attempt to achieve electrical coupling, we overexpressed connexin43 in C2C12 myoblasts (a mouse myoblast line). Indeed, the differentiated myotubes showed expression of the gap junction protein in vitro (Figure 2 A). We then grafted these cells into mouse hearts and 2 weeks later tested for expression of the transgene in vivo. Figure 2 B shows a section of a left ventricle grafted with the C2C12 cells overexpressing connexin43. In some cases, we observed very close apposition of skeletal graft cells and host cardiomyocytes. Immunostaining for connexin43 revealed presumptive gap junctions between the skeletal myotubes and host myocardium. A similar approach by Suzuki’s group showed that connexin43 overexpression in rat L6 myoblasts resulted in enhanced intercellular dye transfer, although this group did not study the cells after differentiation into myotubes, when gap junctions are normally downregulated. Although encouraging, we also have observed reductions in the viability of skeletal muscle grafts that express connexin43 (unpublished observations). It is therefore possible that differentiated skeletal muscle has not evolved mechanisms to accommodate gap junctions without cell injury. The greatest concern for attempts to couple skeletal and cardiac muscle is possible arrhythmogenesis. Skeletal muscle cells have much faster action potentials and fiber conduction velocities than cardiac myocytes. Hence, it is possible that successful coupling of the two muscle types would result in an arrhythmogenic substrate. Nevertheless, the possible gain from coupling the two muscle types justifies additional, careful animal experimentation. Finally, it is clear that separation of muscle grafts from host myocardium by scar tissue is just as problematic with skeletal muscle as it is with cardiac muscle grafts, and would need to be solved before proper coupling could be achieved.

SUMMARY

New muscle tissue can be generated by grafting either cardiac or skeletal myocytes into the injured heart. However, the amount of new muscle tissue is limited by ischemic cell death. Heat shocking the cells prior to grafting protects against ischemic cell death and thus benefits graft cell survival. Moreover, heat shock is simple and feasible in the clinical situation. Angiogenic therapy along with or prior to myocyte grafting has not been explored thoroughly, however, long-term survival of myocyte grafts may benefit from enhanced angiogenesis.
Synchronization between graft and host remains a major challenge for cellular cardiomyoplasty. Both cell types, cardiac and skeletal myocytes, are likely to be separated from the host by scar, thus precluding coupling. Principally, synchronization is likely to occur if grafted cardiomyocytes can be brought into close contact with the host myocardium. In contrast, differentiated skeletal muscle cells do not express the proper molecules to couple to myocardium in vivo. Thus, it appears unlikely that coupling will occur using naïve skeletal muscle cells. Expression of transgenes mediating electromechanical coupling in these cells may prove feasible in the laboratory, but this approach is potentially arrhythmogenic and is certainly quite far from a clinical application.

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Overexpression of connexin 43 in skeletal myoblasts: relevance to cell transplantation to the heart.
Endothelial cells versus angiogenic factors: which is the best strategy for stimulating neoangiogenesis?

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Stimulation of postnatal neovascularization is an important therapeutic option to rescue tissue from critical ischemia. After the discovery of growth factors promoting the migration, proliferation, and tube-forming activity of endothelial cells, recombinant growth factors or genes encoding for growth factors were used to improve tissue neovascularization. Recent findings that endothelial progenitor cells can be isolated from the bone marrow or the circulation open further perspectives for the cell therapy of ischemia. Infusion of these precursor cells was shown to augment neovascularization and improve cardiac function after ischemia. Both strategies have achieved promising effects in small pilot trials. The combination of gene therapy and cell therapy may thus be an important option for the future.

The understanding of the processes underlying the formation of new blood vessels after ischemia has dramatically changed over the past few years. In the past, postnatal neovascularization was believed to be exclusively due to angiogenesis, involving the migration, proliferation, and sprouting of existing “mature” endothelial cells. Angiogenesis is regulated by a variety of growth factors that predominantly increase migratory, proliferative, and matrix degrading activity of the endothelial cells. However, there is increasing evidence suggesting that circulating endothelial progenitor cells (EPCs) home to sites of ischemia and are incorporated into the newly formed blood vessels, thereby contributing to the newly formed capillaries—a process termed vasculogenesis. These findings led to the development of novel therapeutic options based on the use of ex vivo expanded endothelial progenitor cells for the “cell therapy” of ischemia. Experimental as well as clinical data for both strategies—the increase in angiogenesis by growth factors and the use of cell therapeutic approaches—is reviewed in this chapter. At issue is the prospect that the combination of growth factors and cells for combined therapeutic interventions holds promise as an additional future option.

Keywords: angiogenesis; progenitor cell; stem cell; VEGF; vasculogenesis

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thelial marker proteins and tube-forming activity. These initial studies established the markers to define EPCs with angioblast activity, i.e., cells that express CD34 or CD133 and the vascular endothelial growth factor (VEGF) receptor-2 (KDR), analogous to the angioblast in embryonic development. However, peripheral blood-derived mononuclear cells, which contain quite a low number of CD34+ or CD133+ cells, can also be expanded ex vivo to endothelial cells. Moreover, purified monocytic cells, which express the CD14 marker, can also give rise to endothelial cells.

Although these studies convincingly show that endothelial cells can be differentiated out of adult circulating cells, the lineage of differentiation is still unclear. This is illustrated by our recent findings that endothelial cells can be cultivated out of CD14+ monocytic cells, thus confirming previous reports, but also out of CD14− cell populations (S. Dimmeler, personal communication). This may reflect that it not yet possible to define “the” EPC by using currently available surface markers. A functional characterization defined as the capacity of a cell with clonal expansion potential and the capacity to form colonies with endothelial characteristics may currently be the only way to really functionally define EPCs.

Another debated point is the source of circulating endothelial cells. Whereas it has been convincingly shown by various groups that EPCs can derive from the bone marrow, this does not rule out the possibility of other sources, which are not yet defined. Indeed, recent studies suggest that a large proportion of circulating endothelial cells, which are incorporated into ischemic tissue, do not come from the bone marrow (C. Heeschen, personal communication).

Despite the current discussion on the definition and source of EPCs, the therapeutic potential of these cells for improvement of neovascularization in animal models is obvious. Infusion of EPCs derived from mobilized CD34+ cells, bone marrow mononuclear cells, or mononuclear cells augments the neovascularization of ischemic tissue in various animal models. Human mononuclear cell-derived EPCs were shown to improve neovascularization after hind limb ischemia as well as after myocardial infarction. Likewise, purified human CD34+ cells improve neovascularization and cardiac function in nude rats after myocardial infarction. Other bone marrow–derived cells, such as the so-called “side population” (SP) cells, were also shown to be incorporated into capillaries. Finally, initial clinical studies suggest that total bone marrow mononuclear cells and EPCs improve neovascularization and cardiac function in patients after acute myocardial infarction. Moreover, intramuscular injection of bone marrow–derived total mononuclear cells improve peripheral neovascularization in patients with peripheral arterial occlusive disease.

The mechanism by which cell therapy with EPCs improves cardiac function requires further investigation. Numerous studies demonstrated that EPCs home to sites of ischemia and contribute to the newly formed blood vessels. However, cell
transplantation also reduced fibrosis after myocardial infarction in rats\textsuperscript{14} and improved the contractility of the heart in experimental and clinical settings.\textsuperscript{13,16} It is not yet clear whether the improvement of neovascularization is sufficient to achieve these biological effects. One may speculate that the incorporation of EPCs exerts paracrine effects, which affect left ventricular remodeling. In addition, recent experimental studies suggest that EPCs and embryonic endothelial cells can also differentiate to cardiac myocytes.\textsuperscript{18} However, even in vitro, the maximal differentiation rate of EPCs to cardiac myocytes is about 10\%. Thus, the magnitude of these transdifferentiation events in vivo may be below the level required to achieve improvement in contractility solely by cardiac differentiation. The limiting factor of autologous cell therapy using EPCs might be the functional impairment by risk factors of coronary artery disease. Thus, ex vivo cultivated EPCs from patients with coronary artery disease or diabetes showed a significantly reduced migratory capacity and tube-forming activity.\textsuperscript{19,20} Some of the functional defects may be offset by treatment of patients with β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins)\textsuperscript{21} or incubation of the ex vivo cultured EPCs with statins, which improves the migratory response and reduces cellular senescence.\textsuperscript{8,22} However, additional strategies may exert better or synergistic effects. For example, ex vivo transfection of EPCs with telomerase reverse transcriptase (TERT), which prevents replicative senescence by elongation of telomere length, was shown to improve the functional activity of EPCs after hind limb ischemia.\textsuperscript{23} Further strategies may also improve the homing of EPCs by eliciting overexpression of chemotactant factors. Overexpression of SDF-1, which attracts EPCs, may be one such option.\textsuperscript{24}

ANGIOGENIC FACTORS

Angiogenesis is promoted by various growth factors that act on the endothelial cells to promote endothelial cell migration, proliferation, survival, and matrix degradation to enable penetration of the tissue. Several growth factors have been shown to increase angiogenesis in vitro and in vivo (Table I). The most prominent factors used so far are members of the VEGF family. VEGF stimulates VEGF receptors 1 and 2 on endothelial cells, thereby eliciting a proangiogenic response.\textsuperscript{2} The family of fibroblast growth factors (FGF) acts on several cell types, among which endothelial cells, and promotes angiogenesis and arteriogenesis.\textsuperscript{25} Several other factors, such as hepatocyte growth factor (HGF) and erythropoietin (EPO), augment neovascularization after ischemia.\textsuperscript{26,27} Whereas the aforementioned factors predominantly affect the formation of capillaries, other cytokines such as granulocyte macrophage–colony-stimulating factor (GM-CSF) and monocyte chemo-attractant protein–1 (MCP-1) have a proarteriogenic action.\textsuperscript{28}

In spite of early enthusiasm, intravascular therapy with the recombinant angiogenic growth factors basic fibroblast growth factor (bFGF) or VEGF has been ineffective in placebo-controlled trials (for a review see 29). Some of the studies using intravascular protein delivery may have been unsuccessful due to their short half-life. Indeed, in animal studies, repeated or sustained infusion of growth factor protein was required to accelerate collateral growth. In contrast, gene therapy transducing the vascular endothelium and/or myocardium may enable sustained production of the necessary angiogenic proteins. Recent double-blind, randomized placebo controlled trials showed favorable anti-ischemic effects using either catheter-based intramyocardial injection of plasmids encoding VEGF\textsuperscript{30} or adenoviral delivery of FGF-4 via intracoronary infusion.\textsuperscript{31} A potential improvement may depend on type

<table>
<thead>
<tr>
<th>Factor</th>
<th>Predominant target cells</th>
</tr>
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<tbody>
<tr>
<td>VEGF</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>EPCs</td>
</tr>
<tr>
<td>FGF</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>EPCs</td>
</tr>
<tr>
<td></td>
<td>Other cell types (eg, fibroblasts, smooth muscle cells)</td>
</tr>
<tr>
<td>HGF</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Hematopoietic progenitor cells</td>
</tr>
<tr>
<td></td>
<td>EPCs</td>
</tr>
<tr>
<td></td>
<td>Other cell types (eg, monocytes)</td>
</tr>
<tr>
<td>EPO</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Erythropoietic progenitor cells</td>
</tr>
<tr>
<td></td>
<td>EPCs</td>
</tr>
<tr>
<td></td>
<td>Other cell types (eg, neuronal cells)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Monocytes, granulocytes</td>
</tr>
<tr>
<td></td>
<td>Hematopoietic progenitor cells</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocytes/macrophages</td>
</tr>
</tbody>
</table>

Table I. Growth factors used for improvement of neovascularization. 
Abbreviations: see selected abbreviations and acronyms.
of administration (to achieve long-term expression of the gene of interest), dosage, and selection of those patient groups likely to respond to therapy. Moreover, some of the adverse events could be overcome by using better vectors for gene delivery.

THE FUTURE: COMBINATION OF CELL AND GROWTH FACTOR THERAPY?

Cell therapy and gene therapy have proven effective in promoting neovascularization in various animal models. Moreover, some clinical studies have yielded intriguing results. Although definitive proof from large randomized trials is not yet available, both strategies may be able to treat myocardial or peripheral ischemia by improving the formation of new blood vessels. Combination of these two strategies may have additional advantages. Potential mechanisms are reviewed below, see also Figure 2.

Gene delivery by ex vivo transfected progenitor cells?

In vitro transfection of EPCs may reduce the vector load for treated patients, thereby reducing the major adverse effect of gene therapy. Specific targeting of progenitor cells to ischemic tissue may be achieved by catheter-based local delivery (intracoronary or intramuscular) of the cells. Since progenitor cells are integrated into the tissue, the gene of interest may be delivered to the ischemic region in a way that mimics the endogenous biological release that stimulates classic angiogenesis. Initial studies recently showed that transfection of EPCs with VEGF further improves neovascularization after hind limb ischemia. An alternative might be to enhance the endogenous synthesis of angiogenic factors by EPCs by ex vivo stimulation with pharmacological substances.

Mobilization of endogenous bone marrow–derived or tissue resident stem/progenitor cells by systemic application of growth factors?

Several cytokines are used to increase progenitor cells in animal models. Thus, administration of VEGF increases the amount of circulating EPCs. Likewise, treatment of mice with cytokines (granulocyte colony-stimulating factor [G-CSF] and stem cell factor [SCF]), which mobilize hematopoietic stem/pro-
endothelial cells, was shown to enhance cardiac repair. Erythropoietin, which has been used for stimulation of erythropoiesis, also augments circulating levels of EPCs and promotes neovascularization after hind limb ischemia. The limiting factor of systemic application of growth factors obviously is the lack of specific action on the target tissue. Thus, mobilized stem/progenitor cells are likely to home to other organs such as the spleen. Therefore, the majority of the cells are likely to be scavenged before reaching the tissue of interest. Much more work is needed to determine whether selective homing mechanisms exist that would allow targeting of the mobilized cells to specific tissues.

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Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects.

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Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling.
Jeffrey Thorp, a biologist working in the research laboratories of Imperial Chemical Industries in the mid-1950s, was the person who discovered the first fibrate, clofibrate. The therapeutic target was to achieve a delay or reversal of the progress of atherosclerosis by reducing the plasma concentration of cholesterol, which was considered at that time to be a major etiological factor. It was postulated that raised levels of cholesterol were due to disordered intermediary metabolism that was amenable to chemical modulation. Thorp summarized his approach as follows:

“We therefore attempted to modify, even by a small amount, the normal control of lipid metabolism in normal animals on a normal diet. A stringent requirement was that any effect should be persistent, should not merely involve a redistribution of lipids, and should be unassociated with toxicity.”

Thorp considered that there were four theoretical approaches to modifying lipids. He postulated that the biosynthesis of lipids is linked with protein and carbohydrate metabolism through fatty acids, which in turn are a common factor of all lipids (Table I).

He did not favor a direct inhibition of cholesterol synthesis since it played such a pivotal role in cell structure and hormone function. He preferred to modulate fatty acid synthesis, which depends upon a complex interplay of diet, coenzymes, and endocrine control. As in all drug research, defining the medicinal chemistry approach is pivotal. The first chemical series he explored with his collaborator, Dr Waring, was branched-chain fatty acids. This choice was based on some circumstantial clues. Firstly, Thorp knew from his previous work at the MRC Unit in Johannesburg (Director, Dr Leon Goldberg) that a degraded form of estrogen known as Horeau’s acid had been shown, by Horeau, working at the Collège de France in 1947, to lower serum cholesterol in rats (Figure 1, page 156). The second clue was that he knew, from unpublished observations by Nathan and Hogg, of the Upjohn Company, that a structurally related compound to Horeau’s acid (2-methyl-3-ethyl-4(π-anisyl)-3-cyclohexene carboxylic acid) caused a marked reduction in serum cholesterol in rats (at the dose of 0.045 mg/kg) (Figure 1). Thirdly, he was familiar with the work of the French investigators Cottet and Ridel, who were also studying the effects of simplified structures based on dehydrocholic acid, which had marked choleretic properties. They synthesized 80 analogs relating to disubstituted acetic acid, the best series being the phenyl ethyl acetic acid series, which possessed the greatest choleretic activity. They found that α-phenylbutyric acid was a far more potent choleretic than the original steroid metabolites. They administered the phenyl methyl, phenyl ethyl, and phenyl propyl acetate analogs as sodium salts to rats. They also administered 2 g/day of the sodium salt of the phenyl allyl acetate analog to human subjects and claimed a significant reduction in plasma cholesterol levels, but no data are presented in their paper.

Some 400 branched-chain analogs of fatty acids were synthesized by Waring, but only the oxisobutyric acid analogs...
The discovery of the fibrates - Fitzgerald

The compound that gave the best results was chlorophenoxyisobutyrate (CPIB) either as the salt, free acid, or ethyl ester (clofibrate). It is perhaps ironical that this compound was described in two earlier publications. The first was in 1947 by two Italians, Galinberti and Defranceschi, medicinal chemists working at the Carlo Erba pharmaceutical company in Milan. Their chemistry paper does not provide any biological rationale for their project. The second publication was by Julia, in 1956, working on plant growth at the Agricultural Research Institute in Paris. He showed that an analog (m-methoxyphenoxyphenyl acetic acid) had growth-promoting effects on maize seedlings. Whether Thorp was aware of these papers is not recorded. Rather it was the work of the other French group that caused him to focus on branched-chain fatty acids.

The development of clofibrate subsequently followed a somewhat complex path, primarily because its mode of action in lowering serum cholesterol was not understood. Subsequent studies showed that the hypocholesterolemic response in rats was erratic and this was attributed to seasonal variations in the function of the thyroid and adrenal glands. Initial studies in primate species indicated that CPIB enhanced the rise in serum cholesterol induced by a high-fat diet. Hypothesizing that CPIB was acting by modulating adrenocortical steroids, the effects of combining it with androsterone were studied in the rat and then the monkey. Androsterone (0.02% in the diet) significantly potentiated the action of CPIB in lowering cholesterol at all plasma concentrations. Subsequent experiments showed that this potentiation varied according to the season of the year, which was attributed to seasonal variation in thyroid and adrenal function. Despite these ambiguous findings, it was decided to take clofibrate forward for clinical evaluation as monotherapy and also in combination with androsterone.

EARLY CLINICAL OBSERVATIONS

Extensive clinical trials were carried out on clofibrate between 1959 and 1964. The results of studies involving 1300 patients were published in a symposium in 1963. Several important observations were made. Firstly, coadministration of androsterone was not necessary for the hypocholesterolemic effects of clofibrate. Secondly, clofibrate (2 g/daily) had a specific effect on the SF20-400 (ie, very-low-density lipoprotein [VLDL]) hyperlipoproteinemia causing up to a 50% reduction, whereas it had much less effect on the SF0-20 lipoprotein (low-density-lipoprotein [LDL]: Fredrickson Type III lipoprotein class). Thirdly, clofibrate gave a >80% reduction in serum cholesterol without impairing the growth of young rats, which was used as an index of unwanted toxicity.

Table II. Hyperlipoproteinemia phenotypes (Fredrickson classification).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lipid increased</th>
<th>Laboratory definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Triglyceride (greatly)</td>
<td>Hyperchylomicronemia and absolute deficiency of LPL and PHLA</td>
</tr>
<tr>
<td>Type IIa</td>
<td>Cholesterol</td>
<td>LDL increased</td>
</tr>
<tr>
<td>Type IIb</td>
<td>Cholesterol Triglyceride</td>
<td>LDL increased and VLDL increased</td>
</tr>
<tr>
<td>Type III</td>
<td>Cholesterol Triglyceride</td>
<td>β-VLDL, VLDL cholesterol/VLDL triglyceride &gt;0.35</td>
</tr>
<tr>
<td>Type IV</td>
<td>Triglyceride</td>
<td>VLDL increased</td>
</tr>
<tr>
<td>Type V</td>
<td>Triglyceride (greatly)</td>
<td>Chylomicrons and VLDL increased and LPL present</td>
</tr>
</tbody>
</table>

Abbreviations: LDL, low-density lipoprotein; LPL, lipoprotein lipase; PHLA, postheparin lipolytic activity; VLDL, very-low-density lipoprotein.
the effects of clofibrate were maintained on chronic treatment and the agent was very well tolerated. A consistent theme for debate during that symposium was the lack of understanding of its mode of action despite its attractive therapeutic profile.

**INITIAL HYPOTHESES ON THE MODE OF ACTION OF CLOFIBRATE**

Thorp initially postulated that CPIB acid lowered plasma cholesterol in rats and monkeys by an indirect action due to its highly specific binding to specific sites on plasma albumin and prealbumin. His hypothesis was that endogenous androsterone was displaced by CPIB from its plasma protein binding sites, resulting in a greater effect of androsterone on hepatic intermediary metabolism, causing a reduction in the hepatic production of triglycerides. Subsequently, he expanded the protein-binding displacement hypothesis to include displacement of dehydroepiandrosterone sulfate, L-thyroxine, pyridoxal phosphate, and free fatty acids (FFAs). Selective displacement of L-thyroxine into the liver by CPIB was considered to be the most important factor in modulating hepatic metabolism of steroids. Furthermore, seasonal variations in L-thyroxine plasma levels were believed to explain the variable hypcholesterolemic response to clofibrate in rats.

In a masterly review, Steinberg evaluated the various hypotheses on the mode of action of CPIB (Table III).

He rejected the selective rise in hepatic thyroxine (T4) hypothesis because:
- CPIB treatment inhibits cholesterol synthesis, whereas T4 increases it.

However, Steinberg overlooked the fact that Thorp was claiming selective displacement and uptake of thyroxine by the liver, which would have a different effect on metabolism than systemic nonselective uptake of thyroxine.
- CPIB reduces SF20-400 lipoproteins while T4 primarily reduces SF0-20.
- CPIB increases high-density lipoprotein subclass 3 (HDL3), while T4 decreases it.

**Table III.**

<table>
<thead>
<tr>
<th>Hypotheses Proposed for the Hypolipidemic Effect of Chlorophenoxyisobutyrate (CPIB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enhancement of endogenous hormone activity secondary to competition for binding sites on plasma proteins</td>
</tr>
<tr>
<td>a) Androgens</td>
</tr>
<tr>
<td>b) Thyroid hormone</td>
</tr>
<tr>
<td>2. Decrease in rate of free fatty acid (FFA) mobilization</td>
</tr>
<tr>
<td>3. Decrease in rate of hepatic cholesterol biosynthesis</td>
</tr>
<tr>
<td>a) Block between acetate and mevalonate</td>
</tr>
<tr>
<td>b) Block between mevalonate and cholesterol</td>
</tr>
<tr>
<td>c) Diversion of acetoacetyl-CoA to free acetoacetate</td>
</tr>
<tr>
<td>4. Increase in hepatic cholesterol oxidation</td>
</tr>
<tr>
<td>5. Decrease in rate of hepatic release of lipoproteins</td>
</tr>
<tr>
<td>6. Increase in rate of peripheral uptake of lipoproteins</td>
</tr>
</tbody>
</table>

He concluded that the evidence to support the postulated mechanisms 1, 2, 3c, and 4 (Table III) was rather weak. On the other hand, he felt that the cases for mechanism 3b (ie, block of conversion of mevalonate to total cholesterol) and mechanism 5 (decrease in the rate of hepatic release of lipoproteins) were quite strong. He concluded at that time that the final word on the mechanism of action of this drug was not understood, despite the attention the problem has received.

It took another 20 years of research before the current understanding on the mode of action of CPIB was identified (vide infra).

**SUBSEQUENT DEVELOPMENTS IN THE FIBRATE FIELD**

The effects of clofibrate treatment in modifying selected hyperlipoproteinemic phenotypes prompted an intensive search for fibrates that might have greater potency and, hopefully, a greater range of hypolipidemic responders. The search for improved analogs was dominated by concerns about the induction of hepatomegaly, hepatomas, and subsequent carcinoma in murine species. Thorp’s goal was to discover a much more potent fibrate (Figure 2, page 158). Despite Steinberg’s criticism of the T4 displacement hypothesis, Thorp’s primary test for improving fibrates was measurement of T4 displacement from human serum albumin in vitro. This led to the development of two analogs, methyl clofenapate (MCP) and clobuzarit (Clozic). Clinical trials with MCP showed that it had increased potency and lipid-lowering activity (10 mg once/daily) compared with clofibrate. This was attributed to its greater efficacy in displacing thyroxine from human albumin. The second analog, clobuzarit, was also developed as a replacement for MCP—the trials of which had to be suspended because of toxicological findings in rat and mouse. Clobuzarit was less effective than clofibrate in displacing thyroxine from albumin. Clobuzarit had less effect in lowering elevated lipids even than clofibrate, but caused a marked reduction (30%) in assessed...
The discovery of the fibrates

The discovery of the fibrates

plasma fibrinogen levels. This was attributed to a novel anti-inflammatory effect. Clobuzarit was therefore evaluated in clinical trials in rheumatoid arthritis, showing a clear beneficial effect in modifying disease progress.\(^14,15\)

Meanwhile, research in several pharmaceutical companies led to the discovery of other patentable analogs of clofibrate, which were developed commercially (Figure 3). These are still in widespread use for the control of certain hyperlipidemic phenotypes. All these agents reduce plasma triglycerides in Type IIB hyperlipoproteinemia, but the hypolipidemic efficacy in other hyperlipidemic phenotypes varies considerably. Thus, gemfibrozil appears to be more effective than other fibrates in Type III phenotypes,\(^16\) whereas bezafibrate is considered more effective in the Type IIa patients. In a review of the fibrates published in 1994, Gaw et al\(^17\) comment:

> While their clinical efficacy is generally accepted, their mechanism of action has yet to be fully defined and considerable efforts are now being made to examine this issue... the current degree of research activity in the field of the fibric acid derivatives stands as a testament to the clinical value of this group of drugs.

The authors were seemingly unaware of some key papers published between 1990 and 1992 describing the identification of specific hepatic nuclear receptors whose activation resulted in peroxisome proliferation activation, both in rodents and human tissue.\(^18-20\) Activation of these transcription factors by fibrates is now believed to explain both their hypolipidemic and murine hepatoma effects.

**PPARS, HEPATOMAS, AND THE MODE OF ACTION OF FIBRATES**

The initial toxicological studies carried out in rats in the early 60s with clofibrate revealed that chronic treatment caused a marked increase in liver weight. Histological examination showed an unexplained increase in cytoplasmic inclusion bodies subsequently termed peroxisomes. Other fibric acid analogs also caused peroxisome proliferation, but the mechanism was not known. Thorp’s group continued to seek clofibrate analogs that were more potent hypocholesterolemic agents and discovered MCP — methyl...
clofenapate (ICI55695). This compound, which Thorp described as highly “thyroxine-specific” because of its potency in displacing thyroxine binding to human albumin in vitro, caused a 30% reduction in total cholesterol and LDL cholesterol in a single dose of 10 mg/once/day in a Type IIA patient. In doses of 25 mg/kg in rats, MCP caused a doubling of hepatic weight, an increase in mitotic index, and a dramatic increase in intracellular peroxisomes. Prolonged dosing caused hepatic carcinomas, which led to the withdrawal of the compound from clinical studies because it was assumed that the studies in rats were predictive for carcinogenic potential in man. Subsequent studies showed that fibrates varied in their peroxisome proliferation capability depending upon the species, gender, and animal strain.

A breakthrough in understanding the cellular mechanisms whereby fibric acid analogs induce peroxisomes was made by Issemann and Green who were working in the Central Toxicology Laboratories of Imperial Chemical Industry (ICI). Paradoxically, these toxicological scientists worked within half-a-kilometer of Thorp’s laboratory where clofibrate was first discovered 30 years previously. In 1990, they published a seminal paper describing the identification of a receptor in mouse liver, which could be activated by chemicals of widely differing structures, all of which activated nuclear transcription. They proposed the term peroxisome proliferator activated receptor (PPAR). They identified a novel protein (molecular mass 52 000) whose structure had some similarity with related steroid receptors, but was clearly novel. Subsequent studies by other groups revealed that in susceptible species, PPAR activation caused an increase in transcription rate of the PPAR response genes by, firstly, forming a dimer with the receptor coactivator RXR and then binding to DNA regulatory elements (Figure 4). It is now recognized that PPARs play a key role in the intermediary metabolism of dietary fats, especially triglyceride and cholesterol. Three subtypes of PPAR have now been identified termed α, γ, and δ, and three isoforms of PPARγ (ie, 1, 2, and 3) have also been identified. The function of the PPAR isoforms in inducing proliferation of peroxisomes is now much clearer, indicating that marked peroxisome proliferation is mediated by PPARα activation only in the rat and mouse. Recognition of the species-dependent differences in PPAR activation by fibrates took a long time to be appreciated, though Thorp always recognized these differences in species response. Once these differences in species response were understood, the use of fibrates to treat hyperlipidemic phenotypes was accepted. A major contribution to this field was made by the scientists working in the Central Toxicological Laboratories (ICI) summarized in a masterly group of papers. It now seems that the clinically available fibrates have differing binding affinities to the different PPAR receptors (Table IV). It is noteworthy that bezafibrate has an in vitro affinity for the human PPARα receptor equivalent to that of clofibrate, but, in addition, has significant binding to both PPARδ and PPARγ receptors. The clinical relevance of these subtle differences in binding profiles is not established, but the outcome

<table>
<thead>
<tr>
<th>PPAR</th>
<th>EC₅₀ µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofibrate</td>
<td>55</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>30</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>50</td>
</tr>
</tbody>
</table>

Table IV. Human receptor transactivation assay. This assay comprises chimeric receptors in which the peroxisome proliferator activated receptor (PPAR) ligand binding domain is fused with DNA binding domain of the yeast transcription factor Gal 4. The data are expressed as the concentration causing a 50% increase in response.
studies with bezafibrate (Bezafibrate Infarction Prevention [BIP] study) and BEZafibrate Coronary Atherosclerosis Intervention Trial (BECAIT), showing a beneficial effect on morbidity and mortality in patients with clinical coronary artery disease, suggest that its binding to multiple PPARs may be beneficial. Proof of beneficial effects on cardiovascular morbidity was much less well established for the pure PPARα-selective agonist clofibrate, even though it has equivalent hypocholesterolemic efficacy to bezafibrate.

It is now established that selective PPAR activation by fibrates lowers serum triglycerides and raises HDL cholesterol (HDL-C) by increasing the clearance and synthesis of VLDL. PPAR response elements on the apolipoprotein C-III APOC-III gene are inhibited by PPARα agonists, resulting in the reduction of APOC-III, which is a known inhibitor of triglyceride clearance. In contrast to rodents, human apolipoprotein A-I APOA-I gene expression is enhanced by PPARα activation and this causes a rise in HDL-C. Currently, potent, highly selective PPARα agonists such as GW9578 or NS-220 are being developed as agents that reduce the lipid abnormalities that are unresponsive to statins.

However, their role as first-line treatment for most forms of hypercholesterolemia was supplanted by the discovery and development of the β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which have a greater efficacy and have subsequently been shown to cause significant reduction in morbidity and mortality in patients with clinical atherosclerotic disease.

PPARγ agonists were discovered serendipitously by Japanese scientists and led to the development of the hypoglycemic glitazones. PPARγ agonists improve insulin-stimulated glucose disposal as well as inhibiting hepatic glucose production, both in rats and humans. These observations have prompted intense research on the role of PPARγ agonists in diabetes and obesity with compounds such as GW1929 being 100 times more selective for this subreceptor in comparison with the other PPAR isoforms. However, the precise mechanism linking PPARγ activation to improved metabolic profile in diabetic subjects is not fully agreed. More recently, a potent, specific ligand that binds to the PPARγ receptor (GW511516) has been shown to have beneficial effects not only on glucose homeostasis, but also to simultaneously reduce elevated levels of cholesterol and triglycerides in obese diabetic primates. GW511516 has been in clinical trials for some time, but it is not known whether the very attractive profile in diabetic primates is observed in hyperlipidemic diabetic subjects. Ironically, early clinical studies with clofibrate suggested the beneficial effect on glucose control in diabetics, but this was not explored further. Despite these impressive advances in understanding the molecular biology of the PPARs, we still do not understand why Thorp’s original in vitro test measuring displacement of albumin-bound thyroxine predicted the hypolipidemic efficacy in man.

EPilogue

In speculative drug discovery, the lead compound often requires prolonged evaluation, and an early understanding of its mode of action is usually of great benefit. Clofibrate was encountered with significant burdens. There was prolonged debate as to its mode of action and at least half-a-dozen pathways in intermediary metabolism were shown to be modified by clofibrate. Even today it is not fully established that its hypolipidemic effects are mediated solely by PPARα agonism. However, fibrates do not lower plasma cholesterol in PPARα receptor knockout (KO) mice. More importantly, PPAR KO mice do not develop hepatomegaly and hepatomas when chronically dosed with clofibrate. Clofibrate does not cause hepatic enlargement in humans because PPARs are expressed in 10 times lower concentration than in rodents. Thorp recognized the relevance of species differences, but the perception of the carcinogenic potential of fibrates proved to be an insuperable hurdle for the regulatory authorities for about 20 years.

Thorp had a profound understanding of the relevance, not only of lipoprotein phenotypes as atherosclerotic risk factors, but also of the importance of other risk factors such as elevation of the C-reactive protein and fibrinogen. In addition, he developed a uniquely simple methodology for classifying lipoproteins based on their differing particle sizes (SFO-20, S120-400). He measured these in fasting serum using a nephelometric machine that he developed in order to simplify patient subclassification and selection in clinical trials. The technique was based on his knowledge of the physics of the Tyndall effect in that over a certain range of particle sizes, the amount of light scattered when meeting a particle depended on its diameter. The Thorp nephelometer classified hyperlipoproteinemia patients into class small (S), medium (M), and large (L). He validated the nephelometric assessment of lipoprotein phenotype against the gold standard technique of ultracentrifugation. Thorp was widely respected among basic scientists and clinicians in the field of atherosclerosis. He worked closely with Dr Maurice Stone, who had a large cohort of well-characterized patients with lipid abnormalities in his primary health care practice. This cooperation facilitated the early studies, not only with MCP and clofibar, but also on the prognostic value of elevated plasma fibrinogen C-reactive protein levels at a time when these were not recognized as independent atherosclerotic risk factors.
Though the effects of clofibrate were studied in 6 clinical trials, the World Health Organization (WHO) primary prevention trial involving 15,000 healthy subjects sealed its fate. This study lasted 13 years (5 years on treatment and 8 years follow-up).40-42 There were three subgroups in the trial of 5000 subjects each, two high-cholesterol groups which were treated either with clofibrate (1.6 g daily) or olive oil (1.6 g daily) as a “placebo,” though given what we now know about olive oil, this might be regarded more as active “treatment.” There was an excess total mortality in patients on clofibrate, though this was not observed in the other 4 large clinical trials. There was a statistically significant reduction in myocardial infarction rates. No satisfactory explanation for the excess mortality in the WHO trial has emerged, though some commentators have assumed that because of the hepatoma findings in rats, that clofibrate is potentially carcinogenic in humans, despite the fact that the crude and age-standardized cancer incidence in the trial is similar to that in aged-matched similar populations not participating in the trial. Interestingly, the regulatory authorities did not request the withdrawal of clofibrate from the market.

Nevertheless, these results raised a serious debate as to the rationale for reducing plasma cholesterol and by how much 43. Hopefully, the more powerful hypolipidemic statins have now settled the argument in favor of Thorp’s original hypothesis that cholesterol reduction of plasma in patients with atherosclerotic disease would be beneficial.30 Thorp’s seminal contributions to this field have been largely unrecognized. He was an impressive scientific polymath on whose broad shoulders many have subsequently climbed.

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Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium?

J. Leor, M. Patterson, M. J. Quinones, L. H. Kedes, R. A. Kloner

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Leor and colleagues, in this study, demonstrate the feasibility of rat fetal and human myocardial tissue transplantation into the infarcted adult rat myocardium. Fetal myocardial tissue was obtained from human fetuses at 7 to 12 weeks of gestation and from 14-day rat embryos. The ventricular tissue fragments were harvested in culture medium and cultures were examined by differential contrast microscopy for cell growth and contraction. These tissues showed consistent ultrastructural morphology, which was retained in culture over 1 to 4 weeks. The fetal cardiomyocytes were fusiform with large nuclei and cytoplasm filled with short, segmented myofibrils.

Female Sprague-Dawley rats were anesthetized using ketamine-xylaxine anesthesia, and myocardial infarction was induced by 60-min occlusion of the left main coronary artery followed by reperfusion. Fetal myocardial tissue transplantation was performed 7 to 24 days post infarction by direct injection of 2 to 3 myocardial tissue fragments into the myocardial scar. The infarcted rats were then divided into 3 therapy groups: group 1 received human fetal myocyte transplantation as well as an intraperitoneal injection of cyclosporine to prevent tissue rejection; group 2 received rat fetal myocyte transplantation; and group 3 acted as a control with normal saline injected into the myocardial scar. No adverse effects were reported in any of the groups. At 7, 14, 32, and 65 days after cell transplantation, rats were sacrificed and hearts were harvested for electron microscopy and immunohistochemistry. Staining for α-actin was performed, which is known to be present only in fetal cardiomyocytes and absent in adult normal cardiomyocytes. As the authors were only interested in investigating the feasibility of fetal cardiomyocyte transplantation, they did not carry out any hemodynamic measurements or measure infarct size or volume.

Impressively, group 1 rats showed 55% tissue engraftment at day 7 and 100% engraftment at day 14; group 2 rats had an overall engraftment rate of 71% from day 8 to 65; and, as expected, the control group showed no evidence of fetal cells. When analyzing the engrafted heart tissue using electron microscopy, a variety of cell types were revealed—these included mainly fibroblasts and large fusiform cells containing large nuclei and cytoplasm that displayed parallel arrays of filaments forming distinct periodic densities. Some cells developed partial basement membranes, but no cell junctions were seen. α-Actin staining helped in identifying fetal cardiomyocytes grafted into the infarcted adult rat myocardium, but no quantification was made. Control hearts also showed α-actin staining, although this was limited to vessel walls—a characteristic also seen in the experimental hearts.

The study did not measure the degree of engraftment, integration into host tissue, or differentiation into mature cardiomyocytes. The study draws attention to the limitation of use of β-galactosidase–transfected cells, which the authors claim may lead to adverse immune reaction. However, this study clearly demonstrates that fetal cardiomyocytes can be implanted and that they can survive in the disrupted area of myocardial infarction. It also draws attention to the possibility of cardiomyocyte-based gene therapy for introduction of therapeutic proteins into the infarcted myocardium as a therapeutic option.

President Clinton wins a second term in the White House after defeating Republican candidate Bob Dole in the US presidential elections; American football wide receiver Jerry Rice becomes the first player in the NFL to catch 1000 passes, playing for the San Francisco 49ers against New Orleans; and the Stone of Scone, the ancient coronation stone of Scottish kings stolen in 1296 by the English king Edward I, is returned to Scotland.
Skeletal myoblast transplantation for repair of myocardial necrosis

C. E. Murry, R. W. Wiseman, S. M. Schwartz, S. D. Hauschka


This is the first manuscript to demonstrate that skeletal myoblasts can develop into muscle tissue when grafted into injured rat heart. A cell suspension of fibroblasts and myoblasts (≥22% myoblasts as assessed by immunohistochemistry) was initially prepared and cultured from the limb muscles of 1- to 3-day-old Fischer rats. Freeze-thaw injury, using a pre-cooled aluminum rod, was then used to create a disc-shaped region of coagulation necrosis on the anterior surface of rodent left ventricle. Following this, the cells suspended in culture media were injected into the center of the injured myocardium. In group 1, injection immediately followed injury, while in group 2 it was delayed by 1 week. Shams consisted of freeze-thaw injury followed by saline injection. Rats in group 1 were examined between 1 day and 3 months and those in group 2 between 1 day and 7 weeks.

Using superfused myocardial strips harvested from the area of injury, force-voltage, and force-frequency relationships, Lmax, and fatigability were all determined. The force-voltage relationship was staircased, implying continued fiber recruitment and lack of electrical coupling. Such uncoupling could lead to areas of functional block and consequent dysrhythmia complicating the potential therapeutic utility of skeletal myoblasts.

The fatigability test assessed the contractile response to a cardiac-like workload and demonstrated a 53% decline over a 6-minute test period with constant amplitude of stimulation at 1 Hz. In all these assessments, myocardial strips from sham-operated hearts showed little contractile activity.

Donor cells were recognized by fluorescence with the appearance of mature myofibers with sarcomeres by week 2. At all time points after 3 days, embryonic and fast-fiber myosin heavy chain (MHCs) were expressed, whereas cardiac MHC was not seen. Electron microscopy was performed on one heart 2 weeks after myoblast grafting, which identified cells showing changes consistent with developing skeletal muscle. Occasional electron-dense membranes were seen, suggestive of adherens and gap junctions, although this allusion of electrical coupling was not supported by physiological behavior. Stains to identify mitosis suggested it was present until day 3, but only at a low frequency.

A relative paucity in the expression of fast-fiber MHC in group 2 vs group 1 was interpreted as a better conversion to a slow-twitch phenotype. This was also verified by enhanced expression of β-MHC in group 2, suggesting slow twitch fibers develop more rapidly when myoblasts are injected into areas of myocardium where healing is advanced.

This novel study helped pave the way for more detailed experiments, including clinical trials, involving transplantation of skeletal muscle cells into damaged heart. Examining functional as well as anatomical features, this seminal study by Murry and colleagues laid a solid foundation for other investigators entering this exciting field.

1996

Two women are ordained priests in Barbados, the first in the 330-year history of the Anglican Church; Italian film actor Marcello Mastroianni, star of Fellini’s “8½” dies, aged 72; and Kofi Annan, from Ghana, is appointed the UN Secretary-General.
Regenerating functional myocardium: improved performance after skeletal myoblast transplantation


Nat Med. 1998;4:929-933

This is one of the first papers demonstrating that, in intact animals, chronic improvement in systolic and diastolic myocardial performance can be obtained by transplanting autologous skeletal myoblasts into the infarcted myocardium. It is debatable whether adult myocardium can regenerate following infarction; however, skeletal muscle is known to be capable of some repair following injury. On this basis, the authors attempted to transplant skeletal myoblasts into cryoinfarcted myocardium.

Seventeen rabbits were subjected to thoracotomy to implant ultrasonic dimension transducers along the minor axis of the left ventricle. Simultaneously, autologous skeletal myoblasts were harvested from hindlimb soleus muscle. After 5 to 7 days, baseline regional myocardial function was recorded, so that each rabbit could act as its own “control.” Myocardium was infarcted using a cryoprobe applied on the anterior myocardial wall. This resulted in transmural infarction encompassing 30% to 40% of the left ventricle, with complete loss of cardiomyocytes within 2 weeks and formation of a fibrotic scar. One week after cryoinfarct, in 12 of these rabbits, approximately $10^7$ autologous skeletal myoblasts were injected into the infarcted myocardium; 5 rabbits underwent “sham” procedures with injection of serum-free growth medium into the infarcted myocardium. After 3 to 6 weeks, physiological assessment was made and hearts were harvested and processed for histology.

Cryolesion did not change the central hemodynamics, including heart rate, mean arterial pressure, and left ventricular end-diastolic pressure. However, in treated hearts, there was a significant reduction of 49% in preload recruitable stroke work relationship between segmental stroke work and end-diastolic segment length. This reflects improved systolic function and contractility after myoblast transplantation, possibly due to a decrease in regional ventricular dilatation. Also, some rabbits showed an improved diastolic pressure–strain relationship suggestive of decreased stiffness in the scar tissue. However, 5 out of 12 rabbits that underwent myoblast transplantation showed no improvement, possibly due to failure of engraftment, which was confirmed by histology. Light microscopy of the untreated cryoinfarcts showed no differentiated cardiomyocyte survival. Treated cryoinfarcts showed engrafted cells with characteristic striations and peripheral nuclei, and stained positive for myogenin, a factor specific to skeletal myocytes, but not to cardiomyocytes, thus indicating that these striated structures were of skeletal muscle origin. Electron microscopy revealed that the engrafted striated cells were not the expected multinucleated skeletal myotubes, but were single, mononucleated skeletal myocytes connected by intercalated discs, which is characteristic of cardiomyocytes. This finding was confirmed on cross-section histology. The myofibrils showed prominent Z-bands, but lacked A- and M-bands. These ultrastructural observations and the immunohistochemical data are striking. In the absence of cellular connections between graft and intact myocardium, an improvement in contractile function could be due to the mechanical ability of the contractile muscle tissue, thereby improving compliance of the scar. These data provide some basis for attempting autologous myoblast transplantation in myocardial infarction.

1998

Todor Zhivkov, the former dictator of communist Bulgaria, dies, aged 86; President Boris Yeltsin allows the ruble to devalue amid crises in the Russian financial markets; and Libyan leader Colonel Ghadafy accepts a proposal from the British Government to try the two chief suspects of the 1988 Lockerbie bombing in the Netherlands
In this phase 1 clinical trial, the authors highlight the feasibility of autologous skeletal myoblast transplantation in ischemic cardiomyopathy while warning of possible arrhythmias arising in the transplanted hearts.

Ten patients (aged 38 to 73 years) with severe ischemic cardiomyopathy were selected based on criteria of severe irreversible left ventricular dysfunction using echocardiography and \([^{18}F]\)fluorodeoxyglucose–positron emission tomography (FDG-PET) scan. The infarcts ranged from 2 to 13 years of age and involved the posterior or anterior myocardial wall. The infarcted area was angiographically shown to be subserved by a totally occluded and nonrevascularizable coronary artery. Autologous skeletal muscle biopsy was taken from vastus lateralis muscle and myoblasts were harvested in culture. Histology of the muscle revealed normal anatomy. Purity and viability were quantified by flow cytometry after staining for CD56, a marker specific to skeletal myocytes, and exclusion of propidium iodide–stained cells, respectively. All cultures contained at least \(500 \times 10^5\) cells, of which >60% were myoblasts and >90% were viable over 14 to 20 days. All patients underwent coronary artery bypass grafting (CABG). Under cardioplegic arrest, 4 to 8 mL of cell suspension was injected within and around the scar using a 27G needle. There was no perioperative complication relating to multiple injections. Cells were tested for myogenic capacity and were injected into irradiated diabetic SCID mice that showed no macroscopic tumor formation. All patients had an uncomplicated postoperative course except one who died of a mesenteric infarction. Twenty-four-hour Holter ECG monitoring was carried out before and within first 3 postoperative months. Four patients, 3 of whom had preoperative ventricular hyperexcitability, developed clinically tolerable, but sustained, monomorphic ventricular tachycardia by 11 to 22 days and required automated internal cardioverter defibrillator in spite of using antiarrhythmic therapy. Difference in ion channel kinetics in the skeletal muscle or inflammatory reaction following needle punctures could have resulted in arrhythmias. A blinded echocardiographic analysis showed that 63% of the cell-implanted scars had an improved systolic function. One patient was excluded due to poor echo windows, and another died due to stroke at 17.5 months. Follow-up over a period of 10.9 months showed a significant improvement of the mean New York Heart Association (NYHA) class from 2.7 to 1.6 and an increase in ejection fraction from 24% to 32% postoperatively, thus reflecting efficacious revascularization. Although some animal studies have documented improved post–myocardial infarction remodeling following skeletal myoblast transplantation, it is uncertain if this is possible in this case study with already remodeled hearts. Improved kinetics of the transplanted scar on echocardiography could be a mechanical response following contraction of the surrounding myocardium as the skeletal cells do not express connexin43-supported gap junctions with the host cardiomyocytes.

The confounding effect of concomitant CABG may possibly act to cloud interpretation. The extent of improvement suggests that revascularization probably synergized with cell transplantation since it was too great to attribute to revascularization alone. Thus, this preliminary study demonstrates the feasibility and safety of autologous skeletal myoblast transplantation in ischemic cardiomyopathy, albeit with the associated risk of arrhythmias. However, the salutary effects on left ventricular function demand further confirmation in randomized studies.

Cécile de Brunhoff, creator of “Babar the Elephant” dies in Paris, aged 99; US forces enter Baghdad, taking control of the city; and a colossal squid, *Mesonychoteuthis hamiltoni*, weighing 330 pounds and measuring 16 feet, is caught by fishermen in the Antarctic waters off New Zealand.
Cardiomyocytes are thought to be terminally differentiated cells, implying that hypertrophy is the only available form of growth. The authors have challenged this age-old concept that cardiomyocytes are terminally differentiated cells and have outlined several new findings providing an alternative view of myocardial biology that might one day lead to a reconsideration of the therapy of myocardial infarction and chronic heart failure.

Several studies in animal models of myocardial infarction have shown an increase in cell growth in the border zone and in the remote tissue with a preserved blood supply. A 3- to 4-fold increase in growth over normal is observed 1 week after infarction. Also, after myocardial infarction, there is an increase in cyclin E, A, and B, with increased kinase activities, high levels of DNA replication, karyokinesis, and cytokinesis. High-resolution microscopy has demonstrated multiplying myocytes using cell proliferation markers like Ki67 and 5-bromodeoxyuridine. The authors suggest two possible origins for these dividing myocytes. First, these cells could arise from a small subpopulation of replicating myoblasts that asymmetrically and continuously generate new myocytes for normal homeostasis or in response to pathological stimuli. However, there is no evidence of such clonal expansion in vivo in rodents. Second, the dividing myocytes might be amplifying cells derived from stem cells that have expanded and produced a differentiated progeny in response to some stimuli. This could possibly arise either from resident cardiac stem cells or from circulating stem cells that have homed to the heart. Stem-cell specific c-kit-positive (c-kit+) cells have been shown to migrate during fetal growth and form colonies in several organs, including heart. Recent studies have shown that lineage-negative, c-kit-positive (Lin- c-kit+) bone marrow cells can regenerate murine myocardium in vivo. Ventricular remodeling studies in dogs have demonstrated 20% cycling telomerase-competent myocytes expressing Ki67 nucleic protein, suggestive of possible cell multiplication. Also, it is known that the human heart is composed of 80% mononucleated and 20% binucleated ventricular myocytes and that this ratio is unaltered by gender, age, cardiac hypertrophy, or ischemic cardiomyopathy. A positive correlation is seen between myocyte age and expression of the CDK inhibitor p16, a marker of cellular aging. P16 is shown to be present in 10% of myocytes at birth and more than 80% in senescent rats. A similar pattern is seen with telomere length, reflecting a decrease in telomerase activity with increasing age. Young, smaller myocytes may reenter the cell cycle or undergo hypertrophy in response to pathological stimuli, and may be inherently less susceptible to death. This may explain the increased mortality following myocardial infarction in the elderly.

Although implantation of skeletal myoblasts and bone marrow-derived cardiomyocytes have failed to reconstitute healthy myocardium, adult bone marrow cell transplantation could be promising. However, the authors suggest that cardiac stem cell transplantation might be more effective than adult bone marrow cell transplantation. Possibly, the cardiac stem cells could be better programmed, resulting in greater specificity and more competence. The identification, purification, and characterization of the cardiac stem cells and their environmental cues are essential if they are to revolutionize the treatment of myocardial infarction.

The euro (€) replaces national currencies in 12 of the 15 European community member states; heavy downpours outside Sydney, Australia, douse the forest fires threatening the outer suburbs of the city; and the first Taliban and al-Qaeda prisoners are flown from Afghanistan to camp X-ray in Guantanamo Bay, Cuba.
Neovascularization of ischemic myocardium by human bone-marrow–derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function


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As shown by this experiment, cytokine-mobilized autologous bone marrow–derived angioblast transplantation has the potential to neovascularize the infarcted myocardium as a means of reducing ventricular remodeling. Following myocardial infarction, the viable myocardium bordering the infarct undergoes hypertrophy. The capillary network cannot keep pace and is insufficient to support the greater demands of the viable hypertrophied border zone. This subsequently results in apoptosis and progressive infarct expansion, leading to ventricular remodeling and progressive heart failure. The authors postulated that augmentation of vascular bed neoangiogenesis using bone marrow–derived angioblasts might improve cardiac function by preventing the loss of hypertrophied viable cardiomyocytes and ventricular remodeling.

Mononuclear cells were mobilized from a single human donor following recombinant granulocyte colony-stimulating factor and separated into CD34+ and CD34− fractions. CD34+ cells were further separated on the basis of “bright” and “dim” expression of stem cell factor receptor, CD117. CD34+/CD117bright cells coexpressing vascular endothelial growth factor–2 (VEGF 2), Tie 2, AC133, and GATA 2 protein, but not expressing endothelial nitric oxide synthase (eNOS), von Willebrand factor (vWF), E-selectin, and intercellular adhesion molecule (ICAM) were designated as embryonic bone marrow–derived angioblasts. Athymic nude rats underwent myocardial infarction by left anterior coronary artery ligation, followed after 48 h by intravenous injection of 2x10⁸ Dil-labeled human pure CD34+ cells. Sham's were injected with CD34+ cells or saphenous vein endothelial cells or saline. At 2 and 15 weeks, rats treated with CD34+ cells revealed a significant increase in infarct zone microvascularity, cellularity, and numbers of factor VIII+ angioblasts and capillaries, and reduction of matrix deposition and fibrosis in comparison with controls at 2 weeks. Capillaries shown to be of human origin, with endothelial cells coexpressing Dil fluorescence and human CD31, were mainly located in the central infarct zone of collagen deposition. Echocardiographic studies revealed improved systolic function in rats receiving CD34+ cells. Left ventricular ejection fraction significantly improved by 22% at 2 weeks and by 37% at 15 weeks. The mean cardiac index improved significantly. Measurement of human GATA-2 mRNA expression in the bone marrow and heart of infarcted rats collected at 48 h after receiving CD34+ and CD34− cells showed a 3-fold increase in rat myocardium and no such increase in bone marrow in rats treated with CD34+ cells, suggestive of neoangiogenesis. Also, the hearts of rats injected with CD34+/CD117bright/CD117dim cells showed over 3-fold improvement in left ventricular ejection fraction (LVEF) at 2 weeks compared with rats receiving CD34+/CD117dim cells. This indicates that administration of CD34+/CD117bright/GATA-2hi cells may be a necessary requisite and that cell isolation on the basis of phenotypic features alone may not be sufficient. It was also found that injection of CD34+ cells resulted in a 6-fold greater prevention of apoptosis. The scar normal left ventricle ratio was reduced by more than 50% in rats treated with CD34+ cells compared with saline-treated rats, with a significant increase in the viable myocardium confirmed by trichrome staining, thus favoring the hypothesis of myocardial salvage rather than regeneration.

The authors conclude that cytokine-mobilized autologous bone marrow–derived angioblast transplantation offers great potential and, if combined with angiotensin converting-enzyme inhibition or angiotensin II type 1 (AT1) receptor blockade, could further augment postinfarction remodeling.
Pluripotency of mesenchymal stem cells derived from adult marrow


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Cells present within murine or rat adult bone marrow are capable of differentiation into cell types representing all three germ layers. In this study, the first to report this finding, these cells, christened “multipotent adult progenitor cells” or MAPCs, were injected into early blastocysts and implanted into both minimally irradiated and nonirradiated hosts. MAPC cultures were obtained from bone marrow of mice and rats, with those of murine origin most extensively studied in terms of surface receptors, average telomere length, and expression of transcription factors known to maintain an undifferentiated phenotype and pluripotency in embryonic stem cells.

Differentiation was achieved by plating and culturing MAPCs under conditions known to affect lineage determination of embryonic stem cells. For mesoderm, differentiation into cells with an endothelial phenotype was obtained by vascular endothelial growth factor (VEGF). For ectoderm, differentiation into astrocytes, oligodendrocytes, and neurons was obtained by basic fibroblast growth factor (bFGF) and more mature neurons, containing dopamine, serotonin, and gamma-aminobutyric acid (GABA), by the addition of FGF-8 and brain-derived neurotrophic factor. For endoderm, differentiation into epithelioid cells (previously shown to have functional characteristics of hepatocytes) was achieved with FGF-4 and hepatocyte growth factor.

In addition, murine MAPCs were injected into 3.5-day-old blastocysts of C57BL/6 mice, which were transferred to pseudopregnant foster mothers. Chimerism was found in 80% of the resultant offspring derived from blastocysts with 10 to 12 injected MAPCs and 33% of offspring with 1 MAPC injected. Histological evidence of chimerism was seen with the contribution of a single ROSA26-derived MAPC to many somatic tissues as visualized by X-gal staining. To see if murine MAPCs (mMAPCs) given postnataally also engraft, the cells were infused IV into nonirradiated or irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) recipients. Engraftment, assessed by immunofluorescent staining and/or quantitative polymerase chain reaction, was seen in hematopoietic tissues and epithelium of lung, liver and intestines of all recipients. One of the 12 NOD/SCID mice developed a B-cell lymphoma, which, although host-derived and common in aging NOD/SCID mice, did have vasculature that was 40% MAPC-derived. This finding was only discussed in terms of the capability of MAPCs to contribute to neoangiogenesis, overlooking the potential that MAPCs, or perhaps any endothelial progenitor, injected IV may support pathophysiological processes dependent on angiogenesis.

Finally, the bone marrow of primary recipients of mMAPC was infused into secondary recipients. The secondary recipients were irradiated NOD/SCID mice and showed a similar pattern of engraftment to the primary recipients. The findings of this study further challenge preconceptions regarding the capacity of adult progenitor cells to transdifferentiate into tissue of another germ layer. The in vivo studies demonstrate that undifferentiated MAPCs could be used to treat any systemic disease where pathology results from an inability to replace cells of any germ layer. The in vitro studies suggest that ex vivo expanded MAPCs could be engineered in culture to differentiate into a range of cell types that could be targeted to repair specific organs. With access to a patient’s autologous MAPCs, these findings herald an extremely bright future!

Scientists recreate poliovirus using publicly available sequence information and DNA to highlight the potential terrorist risk;

Mathematician Alexandros Yiotopoulos, leader of the Greek November 17 terrorist group, is captured;

and Paul Kagame and Joseph Kabila, presidents of Rwanda and Congo, sign a peace accord ending the 4-year-old war between their countries that has claimed 3 million lives

2002
Can stem cells cross lineage boundaries?

D. J. Anderson, F. H. Gage, I. L. Weissman

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Anderson and colleagues, in this well-structured commentary, address the apparent phenomenon of transdifferentiation and propose that many of the manuscripts reporting such phenomena have technical shortcomings that cloud their interpretation of data and conclusions. The fact that pluripotent embryonic stem (ES) cells can differentiate into cells of every lineage is undisputed. These ES cells further give rise to organ-specific multipotent stem cells, which traditionally were thought to be restricted to the lineage of that particular organ. Recent studies have introduced adult stem cells from one tissue or organ and induced differentiation, both in vitro and in vivo, into cells of other organs. If these findings are accurate, either organ-specific stem cells transdifferentiate upon exposure to a novel environment or the concept of organ specificity is incorrect and what were termed organ specific stem cells are in fact no different from ES cells.

The authors argue that the criteria used to confirm transdifferentiation have been weak. Basic morphology or lineage-specific antibodies have been used to identify lineage-unrelated progeny, in place of the more robust demonstration of multilineage engraftment and functional activity that ensure intralineage conversion. The result is that cells identified as transdifferentiated solely on the basis of protein characteristics may not be truly integrated and/or functional. Even after apparent functional integration, it may remain unclear whether the cells seen are of donor or recipient origin. For example, there may be a recipient hematopoietic stem cell in a donor organ mistaken for a donor stem cell. This mistaken identity is resolvable by using isolated donor stem cells rather than a mixture of donor cells. Demonstration of transdifferentiation from cultured donor-specific stem cells must also be interpreted with caution. Whether the altered environment of cultured cells represents an in vivo phenomenon is questionable. Even using isolated non-cultured cells, the frequency of transdifferentiation, when reported, is extremely low.

Alternative explanations of this phenomenon include the possibility that a tiny subset of the isolated stem cell population is different, but indistinguishable with existing markers. Alternatively, it may be that all donor-specific stem cells do have the potential to cross lineage boundaries, but this occurrence is a rare event, only now identifiable due to improved biotechnology. Finally, it may be that stem cells do have the capacity to switch lineage, but the indigenous cells of the host override this, resulting in its occurrence only at times of host organ injury.

Following their thorough analysis, the authors go on to set criteria they believe necessary to determine true transdifferentiation—the donor population should be prospectively isolated and transplanted without manipulation in culture; transplanted cells should give rise to sustained regeneration of host tissue; and transdifferentiation should be defined molecularly, anatomically, and functionally, with frequency documented.

This commentary not only provides a template for future studies, but also provides an objective and highly critical retrospective analysis of the methods and resulting conclusions of studies that have contributed to the excitement and growth in stem cell research. As the authors point out, transdifferentiation holds many potential clinical rewards, hence it is imperative that the study methodology is wholly accountable.

Former Yugoslavian president Slobodan Milosevic surrenders to Belgrade authorities after being guaranteed a fair trial; a US Navy surveillance plane and a Chinese fighter craft collide mid-air forcing the US plane to make an emergency landing at a military base on the Chinese island of Hainan; and Dennis Tito becomes the first space tourist to visit the International Space Station on an 8-day trip that cost him $20 million
Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI)


Circulation. 2002;106:3009-3017

Although relatively small, this is the largest and most comprehensive study of its kind! Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) reports on findings 4 months after the infusion of progenitor cells derived from bone marrow (BM) or circulating blood (CPCs) into 20 patients with acute myocardial infarction (AMI).

Four days following an AMI, treated initially by primary percutaneous coronary intervention (PCI) with stenting, patients received an intracoronary infusion of progenitor cells. Patients receiving BM progenitor cells had aspirates taken on the morning of infusion. Patients receiving CPCs had blood taken 24 hours after their AMI, from which mononuclear cells were purified and cultured for 3 days. An internal reference group (historical), matched for ejection fraction, infarct location, and creatine kinase (CK)–derived infarct size, was used as control.

Culture and expansion of CPCs resulted in cells showing 90% endothelial characteristics. BM-derived mononuclear cells included hematopoietic progenitors, side population cells, stromal cells, and others, with a mean value of $7.35 \pm 7.31 \times 10^5$ CD34/CD45-positive cells being infused per patient.

Both CPCs and BM cells were infused into the infarct-related artery (IRA) using a balloon catheter to prevent antegrade flow during the 3-minute infusions of 3.3-mL volumes of progenitor cells in suspension. Functional changes were assessed by coronary/left ventricular angiography, coronary flow reserve by flow-wire, stress echocardiography using dobutamine, and [$^{18}$F]fluorodeoxyglucose–positron emission tomography (FDG-PET). Coronary angiography was performed during initial reperfusion and at 4 months' follow-up. Improvements in ejection fraction measured immediately and at 4 months, (from $51.6\% \pm 9.6\%$ to $60.1\% \pm 8.6\%$, $P=0.003$) were significantly greater than those seen in the reference group ($P<0.005$) despite similar baseline values. Compared with baseline, there were significant improvements at 4 months in coronary flow reserve of the IRA and summed wall motion score and FDG uptake within the infarcted segment. Unfortunately, contemporaneous measures were not available for the reference group.

The results should be interpreted with caution given the relatively short follow-up and, more importantly, the lack of a randomized control group. However, as an initial assessment on the feasibility and safety of this procedure, this study is certainly appealing. The intracoronary infusion of progenitor cells was not associated with capillary plugging, since there was no secondary rise in cardiac troponin T or other markers of injury. In addition, despite the small number of observations, the improvement following the infusion of CPCs seems equivalent to that of mixed BM cells, raising the question of whether myogenesis and/or angiogenesis contributed to the improvements seen.

A scientist belonging to the Raelian cult claims that one of its followers has given birth to the world’s first human clone, a girl called Eve; George Roy Hill, director of “Butch Cassidy and the Sundance Kid,” “The Sting,” and “Slaughterhouse Five,” dies at the age of 80; and Chinese archaeologists name a newly discovered species of dinosaur Crichtonsaurus bohlini in honor of the author of “Jurassic Park,” Michael Crichton
Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure


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This open-label, prospective, nonrandomized study investigated the contribution of transendocardial injections of autologous bone marrow mononuclear cells (ABMMNCs) to neo-vascularization. The study included 14 test patients and 7 control patients, all with severe end-stage ischemic heart failure not amenable to revascularization. Ages were 56.9±9.8 years in the treatment group and 64.3±7.2 years in the control group. As part of inclusion criteria, patients had to have a left ventricular (LV) ejection fraction (EF) <40% and chronic coronary artery disease with a reversible perfusion deficit as detected by single photon emission computed tomography (SPECT). Exclusion criteria for the study included the absence of acute myocardial infarction in the 3 months prior to enrolment and no primary hematological disease.

Four hours before injection of cells, bone marrow was aspirated and ABMMNCs were isolated. Characterization of these cells showed only 2.4%±1.3% to be hematopoietic progenitor cells (CD45<sub>lo</sub>CD34<sup>+</sup>), with the largest percent (28.4±10.8) being CD4<sup>+</sup> T cells. Electromechanical mapping (EMM), performed immediately prior to and during the procedure with biplane LV angiography, was matched to areas of reversible ischemia identified by SPECT (SPECT). Exclusion criteria for the study included the absence of acute myocardial infarction in the 3 months prior to enrolment and no primary hematological disease.

One patient in each group died. However, the patient in the treatment group had shown an improvement in cardiac function at the 2-months’ follow-up. No postmortem was performed. 24-Hour Holter monitoring showed no sustained arrhythmias at baseline or after the procedure.

Both treatment and control groups underwent a 2-month noninvasive follow-up that included a treadmill protocol, 2-D Doppler echocardiography, and a resting and stress SPECT scan with quantitative analysis. At this stage, patients in the treatment group experienced fewer anginal symptoms and showed a significant improvement in exercise performance as measured by VO<sub>2</sub>max (P=0.0085) when compared with the control group, which merely showed a trend towards improvement (P=0.08). Cardiac function, measured by EF, improved by 6% in the cell-treated group and actually decreased in the control group. Cardiac geometry, assessed by end-systolic volume, also improved in the cell-treated group, compared with the control group. SPECT scanning revealed no significant change in total reversible perfusion defect or percent of rest defect with scar (50% activity) in the control group, but significant improvement (73% reduction in total reversible defect) in the test group. At 4 months, patients in the treatment group had LV angiograms and EMM repeated. EF was seen to improve from 20% to 29% (P=0.0003), and significant mechanical improvements were seen in the injected segments (P<0.0005).

Identification of areas thought to benefit the most, by a combination of EMM and SPECT, and prospective confirmation of this hypothesis, neatly completes this investigation. The positive results are, however, tempered by the low numbers of patients enrolled and the lack of placebo injections in the control group (under guidance of the ethics committee), which resulted in incomplete blinding.

Gus Van Sant wins the Palme d’Or and best director prize for his film, “Elephant,” at the Cannes film festival; The Blues (Auckland) defeat the Canterbury Crusaders 21-17 to win the 2003 Rugby Super 12 tournament; and Russia celebrates the 300th anniversary of the foundation of St Petersburg
Stem Cells

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selected by Philippe Menasché, MD, PhD
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