Mesoangioblasts, Vessel-Associated Multipotent Stem Cells, Repair the Infarcted Heart by Multiple Cellular Mechanisms

A Comparison With Bone Marrow Progenitors, Fibroblasts, and Endothelial Cells

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Objective—To test the potential of mesoangioblasts (Mabs) in reducing postischemic injury in comparison with bone marrow progenitor cells (BMPCs), fibroblasts (Fbs), and embryonic stem cell–derived endothelial cells (ECs), and to identify putative cellular protective mechanisms.

Methods and Results—Cells were injected percutaneously in the left ventricular (LV) chamber of C57BL/6 mice, 3 to 6 hours after coronary ligation, and detected in the hearts 2 days and 6 weeks later. Echocardiographic examinations were performed at 6 weeks. LV dilation was reduced and LV shortening fraction was improved with Mabs and BMPCs but not with ECs and Fbs. Donor cell colonization of the host myocardium was modest and predominantly in the smooth muscle layer of vessels. Capillary density was higher in the peripheral infarct area and apoptotic cardiomyocytes were fewer with Mabs and BMPCs. Mabs and BMPCs, but not Fbs or ECs, promoted survival of cultured cardiocytes under low-oxygen in culture. This activity was present in Mab-conditioned medium and could be replaced by a combination of basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, and hepatocyte growth factor (HGF), all of which are produced by these cells. Conditioned medium from Mabs, but not from Fbs, stimulated proliferation of smooth muscle cells in vitro.

Conclusions—Mabs appear as effective as BMPCs in reducing postinfarction LV dysfunction, likely through production of antiapoptotic and angiogenic factors. (***Arterioscler Thromb Vasc Biol.*** **2005;25:692-697.)**

Key Words: stem cells ■ mesoangioblasts ■ myocardial infarction ■ angiogenesis ■ myogenesis

In the past 2 years, a flurry of studies reported the effect of different cell types transplanted in animal models of myocardial infarction (MI) and some pilot clinical studies have also appeared.[1,2] Most studies basically agree on beneficial functional effects, but those that have explored the underlying mechanisms are controversial. When reported, the yields of replacement of cardiomyocytes lost after the ischemic injury differs largely among studies and routes of cell administration, ranging from ≈50%[3] to as low as 0.02%.[4] Recently, serious doubts have been cast on the cardiomyogenic potential of hematopoietic stem cells, the most widely used cell type for cardiac repair after MI in animals and humans.[5,6]

Reduction in left ventricular (LV) dilation and improvement in fractional shortening have been shown after administration of different cell types, including skeletal muscle satellite cells,[7,8] smooth muscle cells,[9] fetal cardiomyocytes,[10] embryonic stem cells,[11] endothelial progenitor cells,[12] and bone marrow-derived, hematopoietic, and mesenchymal[3,13] stem cells. The benefits observed have been ascribed mostly to replacement of dead cells (contractile and vascular structures) but also to angiogenic growth factors produced by transplanted cells.[14]

Recently, we described a novel type of vessel associated stem cell that can differentiate into different mesoderm cell types and was therefore named "mesoangioblast" (Mab).

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Mabs efficiently differentiate into endothelium, smooth, and cardiac muscle in vitro,[15–17] and we tested their potential to restore cardiac function after experimental MI in the mouse. We reasoned that a better understanding of the performance of different cell types and of the putative mechanisms for the reported benefits could come from a direct comparison of different cells in the same experimental model of MI.

To this aim, we administered 4 different cell types a few hours after coronary ligation in mice and we evaluated their contribution to regeneration of myocardium and blood vessels, their production of growth factors, and their long-term effects on left ventricular remodeling and function.

Materials and Methods

An expanded Materials and Methods can be found online at http://atvb.ahajournals.org. Briefly, cell types administered to infarcted mice were mouse Mabs, primary mouse embryonic fibroblasts (Fbs), and an embryonic stem cell-derived endothelial cell (ECs) line 44B, labeled in various ways. Three to 6 hours after coronary artery ligation (CAL), 5×10^5 cells were slowly introduced into LV chamber through percutaneous injection. Echocardiography was performed on conscious mice, 6 weeks after cell transplantation. Polymerase chain reaction (PCR) was used to detect green fluorescent protein (GFP) in organs, blood, and heart. TdT reaction was used to quantify number of apoptotic cardiac myocytes in vivo and in vitro after hypoxia. Neonatal cardiomyocyte cultures to test protective effect of different factors produced by stem cells were prepared and cultured as previously described. Immunohistochemistry and confocal microscopy were used as previously described to detect engrafted cells 2 days and 6 weeks after transplantation.

Results

Overall, 42 mice were available for analysis at 2 days and 78 mice at 6 weeks CAL. Reasons for exclusion were lack of evidence of injury (n=5) or premature death (n=38) (not significant different between groups).

Echocardiography

Six weeks after CAL, echocardiographic examination showed LV dilatation and systolic dysfunction in CAL control group compared with sham-operated: LV end-diastolic diameter increased by 43% ($P<0.001$) (Figure 1A), and shortening fraction decreased by 45% ($P<0.001$) (Figure 1B). LV anterior wall end-diastolic thinning and a decreased systolic thickening was present in CAL controls versus sham-operated (0.79±0.05 mm versus 0.93±0.03 mm, $P<0.05$, and 1.17±0.08 mm versus 1.53±0.06 mm, $P<0.01$, respectively), whereas no difference was found in posterior wall thickness.

In mice treated with Mabs, LV dilation was attenuated: LV end-diastolic diameter in CAL+Mab group was significantly smaller than in CAL control group (3.11±0.11 mm versus 3.84±0.24 mm; $P<0.05$) (Figure 1A). Shortening fraction was less decreased in CAL+Mab group, being 42±2% versus 30±4% in CAL controls ($P<0.001$) (Figure 1B). LV wall motion a/dyskinetic index in mice treated with Mabs was 35% lower than in CAL control group ($P<0.05$) (Figure 1C). Similar benefits were found in bone marrow progenitor cells (BMPCs), but not in Fbs or in embryonic stem cell-derived ECs (Figure 1A through 1C).

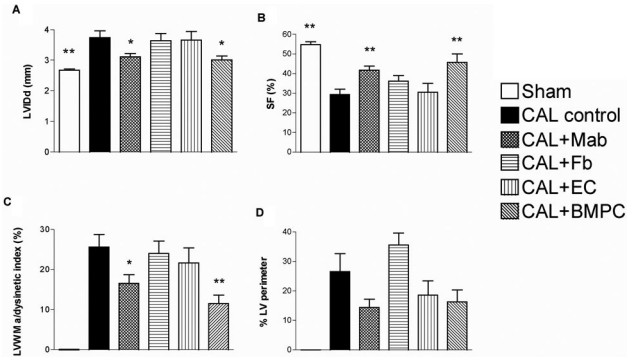

Figure 1. Echocardiographic analysis of infarcted hearts after different treatments. Values of left ventricular (LV) internal diameter (A), shortening fraction (B), and of left ventricular wall motion (LVWM) akinetic/dyskinetic index (C) of mice after sham operation or after left coronary artery ligation (CAL). Histological quantification of infarct size (D). Groups are labeled as follows: sham=sham-operated, untreated mice (n=12); CAL control=CAL untreated or receiving phosphate-buffered saline injection (n=15); CAL+Mab=CAL receiving mesoangioblast injection (n=17); CAL+Fb=CAL receiving fibroblast injection (n=7); CAL+EC=CAL receiving endothelial cell injection (n=7); and CAL+BMPC=CAL receiving bone marrow progenitor cell injection (n=7). Number of mice actually analyzed for histological infarct size was overall 55, because 10 were used for real-time PCR. *$P<0.05$ versus CAL control; **$P<0.01$ versus CAL control.

Heart rate during echocardiographic examination did not differ among groups, and absolute values ranged from 511 to 811 bpm, with group means ranging from 639 to 698 bpm.

Histology of the Infarcted Hearts

Hematoxilin and eosin staining of hearts of mice euthanized at 6 weeks revealed a scar whose extension averaged 27±6% of LV perimeter (endocardial+epicardial) in CAL controls. Infarct size tended to be smaller (post-hoc comparisons by Dunnett test $P>0.05$) in Mab, EC, and BMPC groups, whereas it was similar to CAL controls in Fb group (Figure 1D).

Homing of Transplanted Cells to the Infarcted Hearts

Two days after transplantation, a limited number of donor Mab could be detected by a number of different methods (fluorescent in situ hybridization, X-gal staining, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, or GFP) in the host myocardium, more concentrated in the area of infarct, but also scattered over the whole section of left and right ventricles (Figure IA through IE, available online at http://atvb.ahajournals.org).

The number of cells detected per section in 10 nonserial sections that spanned most of the infarct from base to apex ranged between 20 and 100, suggesting that many of the injected cells that had entered the coronary circulation (presumably, 10^4 cells) were indeed present in the tissue on day 2. This corresponded to a weak signal consistently detected by PCR revealing the presence of the GFP gene, integrated into the donor cell genome after in vitro transduction with a lentivector expressing GFP (Figure IF). Because of the mode of administration, donor cells should have also been distributed through the general circulation, and we detected the

presence of donor cells in filter organs such as liver or lung (Figure IF). Only some of these organs showed a positive signal, likely caused by dilution of injected cells through the general circulation resulting in a weak signal that in some cases was below the threshold of detection by standard PCR analysis. Similar results were obtained by analyzing individual hearts transplanted with GFP-labeled cells (Figure IG). On the contrary, PCR did not reveal presence of GFP in whole blood at 1 and 2 days after GFP-labeled Mab had been injected into the LV chamber (Figure IH). The cells that had hosted in the myocardium and in other organs survived for weeks in the host since they could still be detected, with a slightly reduced frequency after 6 weeks, suggesting that they had not been counterselected or had experienced a major selective growth advantage. When different types of donor cells were compared for efficiency of engraftment, they all appeared to have colonized the host tissues with similar efficiency.

At 6 weeks after transplantation, GFP-labeled donor cells were located mainly in or around the vessel wall and some in the myocardium. A modest fraction of donor GFP positive cells also coexpressed smooth muscle actin, even though they still represented ≈1% of total smooth muscle actin-positive cells. GFP-positive cells coexpressing platelet-endothelial cell adhesion molecule or MyHC were rare and represented ≈0.01% of total endothelial and myocardial cells. Because of the extremely low frequency of GFP-positive cells, quantification was difficult and statistics irrelevant. Examples of GFP-positive endothelium, smooth muscle, and myocardium are shown in Figure 2A through 2C, 2D through 2F, and 2G through 2I, respectively. Both ECs and BMPCs contributed a larger proportion of differentiated endothelial cells (0.6% of total PECAM-positive cells examined), whereas Fbs did not differentiate into endothelium; all 3 cell types differentiated very rarely into cardiac muscle (Figure 2J to 2L) and more frequently into smooth muscle cells (data not shown).

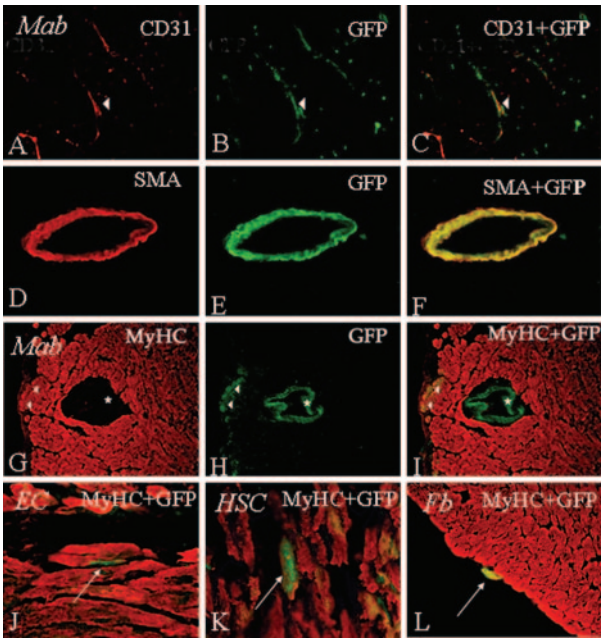

Figure 2. Immunofluorescence analysis of infarcted hearts 6 weeks after injection in LV chamber of Mabs (A to I), ECs (J), BMPCs (K), and Fbs (L). A donor-derived EC, double-stained by an anti-CD31 antibody (red in A, arrowhead) and anti-GFP antibody (green in B, arrowhead) appears orange in (C). The anti-smooth muscle actin–positive muscular layer of a small artery (red in D) is also stained by anti GFP antibody (green in E) and appears in large part orange in (F). G to I, An area of myocardium close to the infarcted area, stained with anti-myosin heavy chain (MyHC, MF20) antibody (red in G), where few subepicardial cardiomyocytes (arrowheads) are also stained by the anti-GFP antibody (green in H), which also stains the smooth muscle layer of a large artery (*). The cardiomyocytes appear orange in (I), whereas the artery (that is not stained by MF20) does not. Isolated, double-labeled cardiomyocytes (orange in the merged figures) were also rarely detected in hearts injected with ECs (J), BMPCs (K), and Fbs (L).

Angiogenesis in the Infarcted Heart and Angiogenetic Factors Produced by Donor Cells

The overall low level of differentiation of engrafted cells by any of the donor cell population could not explain the dramatic improvement detected by echocardiography with Mabs and BMPCs. We thus reasoned that stimulation of host angiogenesis by donor cell-released factors might be responsible for some of the functional benefit. Figure 3A shows that capillary density in the peri-infarct area was increased by ≈30% in Mab or BMPC groups, but not in controls, Fb, or EC groups. A micro-array analysis of gene expression in Mab[16] revealed that several growth factors are expressed at high level in these cells: vascular endothelial growth factor B (VEGFB), basic fibroblast growth factor (bFGF), FGF7, platelet-derived growth factor AA (PDGF AA), hepatoma-derived growth factor, and stromal derived factor 1. We thus analyzed by reverse-transcription PCR the expression of these factors in the different cell types used in this study. Figure 3B shows that bFGF is expressed almost exclusively in Mab, insulin-like growth factor (IGF)-1 is expressed in all cell types except Fbs and hepatocyte growth factor (HGF); stromal derived factor is expressed in all cell types except ECs; PDGF AA is expressed in Mabs and Fbs only; and FGF7, hepatoma-derived growth factor, and VEGFB are expressed in all cell types, although the latter more robustly in Mab. Overall, these data indicate that several growth factors that may stimulate survival and/or proliferation of heart cells are produced by the cell types that induced a functional improvement. Proliferation of endothelial cells in vivo was shown by nuclear labeling with Ki67 in cells positive for *Griffonia Simplicifolia* lectin within the area of infarction (Figure 3C and 3D).

Apoptosis of Cardiac Myocytes In Vivo and In Vitro

Apoptosis of cardiac myocytes was decreased in vivo and in vitro by Mabs and BMPCs, but not by Fbs (Figure II, available online at http://atvb.ahajournals.org).

Identification of Active Factors That Prevent Cardiomyocyte Apoptosis

We used the in vitro low-oxygen assay to test whether factors produced by Mabs may prevent or reduce cardiomyocyte apoptosis. The results obtained by replacing Mab-conditioned medium with purified growth factors known to be produced

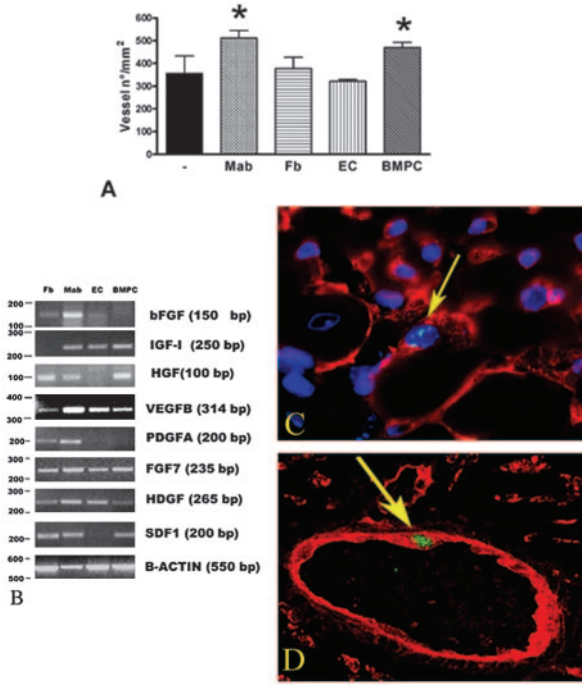

Figure 3. A, Micro-vessel density in the border zone of infarcted hearts from mice injected with Mabs or Fbs, embryonic stem cell-derived ECs, BMPCs, or from CAL control (−). Density was detected by counting CD31-positive microvessels 6 weeks after CAL (*$P<0.05$). B, Reverse-transcription PCR for different factors expressed by Fbs, Mabs, ECs, and BMPCs. Factors analyzed are basic fibroblast growth factor (bFGF), insulin growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor B (VEGFB), platelet-derived growth factor A (PDGF AA), fibroblast growth factor 7 (FGF7), hepatoma-derived growth factor (HDGF), and stroma-derived factor-1 (SDF1). Beta actin was amplified for normalization. The size of amplified fragments is reported in parenthesis. Ki67 nuclear antigen, expressed on all proliferating cells during late G_1, S, and G_2 phases of cell cycle, is shown (green) in a nucleus (blue) indicated by an arrow in (C). The Ki67-positive nucleus belongs to an endothelial cell identified by *Griffonia simplicifolia* lectin I staining (red). D, Another Ki67-positive nucleus in ECs from a larger blood vessel.

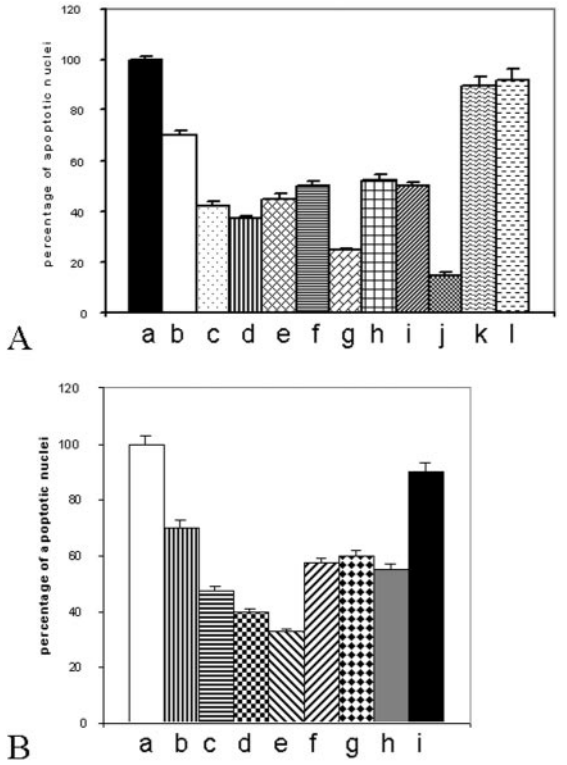

Figure 4. A quantitative analysis of hypoxia-induced apoptosis in neonatal mice cardiomyocytes in culture. A, Percentage of apoptotic nuclei, relative to cardiomyocytes exposed to hypoxia in low-serum (2% horse serum) medium (a); in Fb-conditioned medium (b); in Mab-conditioned medium (c); in 2% horse serum + bFGF (10 ng/mL bFGF) (d); + HGF (10 ng/mL) (e); + IGF-1 (10 ng/mL) (f); + bFGf and HGF (g); + IGF-1 and bFGF (h); + IGF-1 and HGF (i); + bFGF, IGF-1, and HGF (j); + VEGFB (10 ng/mL) (k); and + PDGF AA (10 ng/mL (l). B. Percentage of apoptotic nuclei, relative to cardiomyocytes exposed to hypoxia in low-serum (2% horse serum) medium (a); in Fb-conditioned medium (b); in Mab-conditioned medium (c); in Mab-conditioned medium + anti PDGF AA neutralizing antibody (d); in Mab-conditioned medium + Flt-IgG (soluble ligand for VEGFB) (e); in Mab-conditioned medium + anti-bFGF neutralizing antibody (f); in Mab-conditioned medium + anti–IGF-1 neutralizing antibody (g); in Mab-conditioned medium + anti HGF neutralizing antibody (h); and in Mab-conditioned medium + anti-bFGF + anti–IGF-1 + anti-HGF neutralizing antibodies (i).

by Mabs indicate that bFGF, HGF, or IGF-1 alone reduced apoptosis and in combination prevented it. In contrast, VEGFB and PDGF AA had no effect (Figure 4A). Consistently, neutralizing antibodies to PDGF AA or a soluble VEGF receptor added to the conditioned medium of Mabs did not interfere with the activity of Mab-conditioned medium, whereas neutralizing antibodies against bFGF, HGF, and IGF-1 abolished the antiapoptotic activity of Mab-conditioned medium in a cooperative fashion (Figure 4B). Immufluorescence analysis revealed an accumulation of bFGF and IGF1 around grafted mesoangioblasts 2 days after transplantation (Figure III, available online at http://atvb.ahajournals.org), suggesting that these cells release these factors also in vivo.

Proliferation of Smooth Muscle in Response to Factors Released by Mesoangioblasts

Detailed results and Figure IV are available online at http://atvb.ahajournals.org.

Discussion

Mesoangioblasts Ameliorate Cardiac Function After Coronary Artery Ligation

Data reported in this article indicate that administration of Mabs in the LV chamber improves cardiac function after experimental MI in mice. This finding is in agreement with many recent reports on different types of stem/progenitor cells.[3,6–11,14] In many of these studies, cells were injected intra-myocardially in the border zone of the infarct and not, as in our case, intra-arterially. This route of administration was chosen because cells are administered as close as possible to the coronaries (approximating what can be performed in patients to increase homing in the heart in comparison with the intravenous route[12]) and because the percutaneous injection into LV chamber was far less traumatic than the reopening of chest and the injection within a LV wall of ≈1-mm thickness.

With the exception of 1 report,[3] all the others failed to detect substantial formation of new myocardium from donor cells, suggesting that cardiac transdifferentiation is rare or sometimes below the threshold of detection, whereas contribution of donor cells to neo-angiogenesis appeared as a more common event.[14,18] Transplantation of BMPCs has recently been the subject of several clinical protocols justified by the functional benefit obtained in experimental models. Very recently, the transdifferentiation to cardiac myocytes has been attributed mostly to the nonhematopoietic mesenchymal fraction of BMPCs,[19] although in quantitative modest proportion.

For these reasons we decided to investigate potency to repair infarcted heart of a newly identified mesodermal stem cell, Mab,[15] shown to differentiate in vitro into cardiomyocytes[17] and to ameliorate structure and function of skeletal muscle in a mouse model of muscular dystrophy.[20] Furthermore, we compared the activity of Mabs with that of other types of stem/progenitor cells such as the bone marrow-derived and the endothelial ones, and with a nonstem cell such as primary Fbs that should reveal beneficial effects probably common to all cell types.

Cardiac Function Amelioration Is Dependent on Multiple Mechanisms

Our results strongly suggest that the benefit is probably multifactorial and depends on host-derived and, to a minor extent, donor cell-derived neo-angiogenesis in peri-infarctual area, decreased apoptotic death of cardiac myocytes, and, to a much lower extent, generation of new contractile elements. Furthermore, in this case, it is likely that fusion is another possible mechanism,[21] even though we did not address this issue here. The simplest underlying mechanism to envision is the release of biologically active growth factors and chemokines by transplanted/progenitor cells that may both promote angiogenesis and reduce host cardiomyocyte death. A microarray analysis on Mabs[16] revealed expression of several growth factors and chemokines. Among these, VEGFB appeared particularly interesting because it is known to promote angiogenesis and further maturation of the vessels at variance with other forms of VEGF. The scenario must obviously be more complex because many other factors such as FGF-7 appear to be produced by all cell types examined, whereas others appear to be produced by some but not all cell types, and still others such as bFGF appear to be produced by Mabs only. In agreement with this hypothesis, a reduced apoptosis was detected few days after CAL around the infarct with Mabs but not with Fbs. The antiapoptotic effect of many growth factors can be investigated in vitro independently from an angiogenetic effect. Mabs dramatically reduced apoptosis in cultured rat neonatal cardiomyocytes exposed to low oxygen, whereas Fbs did so to a limited extent, consistent with the production of some growth factor. Interestingly, conditioned medium from Mabs exerted a similar effect, thus allowing direct investigation on the nature of the active molecules. Replacement of Mab-conditioned medium with bFGF or IGF-1 or HGF, but not with PDGF AA or VEGFB, reduced apoptosis, whereas the 3 active factors together abolished low-oxygen–induced apoptosis in cardiomyocytes. Moreover, neutralizing antibodies against bFGF, IGF-1, and HGF reduced the antiapoptotic effect of conditioned medium whereas their combined activity abolished it, confirming that these 3 molecules, produced by Mabs, prevent low-oxygen–induced cardiomyocyte apoptosis in vitro and most likely exert the same action in vivo, as reported for individual factors in vivo.[22–24] The data reported here prove that they directly prevent apoptosis in vitro and, moreover, have a dramatic synergistic effect, and the immunoreactivity for these factors around engrafted Mabs (Figure III) support this mechanism of action in vivo.

Mabs produce growth factors that are presumably active in promoting proliferation of both endothelial cells and pericytes. Mab-conditioned medium stimulated proliferation of smooth muscle cells but not of endothelial cells, whereas Fb-conditioned medium had no significant effect on either cell type.

The protective effects discussed likely occur within the first days after infarction, and this might explain the smaller infarcts measured at 6 weeks in Mab and BMPC groups (Figure 1D). The functional benefits observed at 6 weeks with Mabs and BMPCs can be, at least in part, the consequence of an early reduction of MI size. The size of the groups and the casual assignment of mice to study treatments greatly reduces the likelihood of selection biases.

Conclusion

Together, these data define a likely mechanism by which different types of stem/progenitor cells elicit a beneficial effect on the infarcted heart. In agreement with previous reports, BMPCs ameliorate cardiac function after MI; Mabs exert the same effect as BMPCs. Mabs are easy to expand in vitro from patients (unpublished results). In contrast, neither Fbs nor ECs have beneficial effects, indicating that not all cell types are equivalent in this function. In all cases, tissue colonization by donor cells is modest and this may depend on hemodynamic (ie, only 2% to 2.5% of cardiac output enters the coronary circulation) and histological reasons. The majority of donor cells differentiate into smooth muscle, whereas production of endothelium and myocardium is extremely rare and certainly insufficient to explain the structural and functional benefits shown by echocardiography.

Production of growth factors has been suggested[13,18,22–24] as a possible alternative phenomenon that may promote angiogenesis and indirectly promote survival of the myocardium in the peri-infarct area, although a direct antiapoptotic effect was also possible.

Here, we show that Mabs produce several growth factors, among which are bFGF, IGF-1, and HGF, that directly prevent low-oxygen–dependent cardiomyocyte apoptosis and also stimulate proliferation of the pericyte/smooth muscle cell of the newly formed vessels. In contrast, they do not promote direct endothelial proliferation. We cannot explain this result, although factors are released by surviving myocardium and/or pericytes/smooth muscle cells, such as VEGFB.[25] Testing the effect of these factors in vivo will require a completely separate study because of the need to compare 3 different candidate factors, both through loss of function modifications of transplanted donor cells and in replacement studies with the purified factors to be administered through

suitable dose regimens and routes. Thus, even if a conclusive demonstration in vivo awaits further experimental in vivo work, the results of the present study reconcile the observed functional benefits after cell transplantation, reported in most studies, with the very rare generation of new cardiac myocytes from donor stem cells.[2,6] They offer for the first time to our knowledge a complete framework that may be useful for further animal studies and set the basis for more focused clinical experimentation.

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