Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy

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Zhang, Hao, Shafie Fazel, Hai Tian, Donald A. G. Mickle, Richard D. Weisel, Takeshiro Fujii, and Ren-Ke Li. Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. Am J Physiol Heart Circ Physiol 289: H2089–H2096, 2005; doi:10.1152/ajpheart.00019.2005.—We evaluated the impact of donor age on the efficacy of myocardial cellular therapy for ischemic cardiomyopathy. Characteristics of smooth muscle cells (SMC), bone marrow stromal cells (MSCs), and skeletal muscle cells (SKMCs) from young, adult, and old rats were compared in vitro. Three weeks after coronary ligation, 3.5 million SMCs (n = 11) or MSCs (n = 9) from old syngenic rats or culture medium (n = 6) were injected into the ischemic region. Five weeks after implantation, cardiac function was assessed by echocardiography and the Langendorff apparatus. In the in vitro study, the numbers and proliferation of MSCs from fresh bone marrow and SKMCs from fresh tissue but not SMCs were markedly diminished in old animals (P < 0.05 both groups). SKMCs from old animals did not reach confluence. After treatment with 5-azacytidine (azacitidine), the myogenic potential of old MSCs was decreased compared with young MSCs. In the in vivo study, both SMC and MSC transplantation induced significant angiogenesis compared with media injections (P < 0.05 both groups). Transplantation of SMCs but not MSCs prevented scar thinning (P = 0.03) and improved ejection fraction and fractional shortening (P < 0.05). Load-independent indices of cardiac function in a Langendorff preparation confirmed improved function in the aged SMC group (P = 0.01) but not in the MSC group compared with the control group. In conclusion, donor age adversely impacts the efficacy of cellular therapy for myocardial regeneration and is cell-type dependent. SMCs from old donors retain their ability to improve cardiac function after implantation into ischemic myocardium.

ACUTE MYOCARDIAL INFARCTION and the ensuing ischemic cardiomyopathy remain the number one cause of mortality in the developed world. Myocardial cellular therapy has been introduced into the clinical arena as a novel therapeutic intervention that is capable of halting the progression of congestive heart failure. In the setting of acute myocardial infarction, a readily accessible autologous source of cells that requires minimal ex vivo manipulations is required for timely delivery to the ischemic myocardium. Bone marrow mononuclear cells or circulating progenitor cells are easily obtained from patients at the time of acute myocardial infarction and can be safely delivered by intracoronary infusion with the use of catheter-based techniques (1, 2, 19, 21, 24). The preliminary evidence suggests that such therapy is associated with improved cardiac function at follow-up, consistent with the preclinical studies. Diminished functional capability of the cells isolated from patients with advanced age and comorbidities has been recently reported (6, 20) and may have limited the functional benefits of these cells in the clinical trials.

In the setting of chronic ischemic cardiomyopathy, which will be the focus of the present report, different delivery methods and different cells may be required. Intramyocardial delivery into the affected area can be achieved by transendocardial, transepicardial, and transvenous routes. In general, cells of myogenic origin, which retain the distinct capability to remodel the surrounding tissue, are regarded as prime candidates for cell therapy candidates in chronic ischemic cardiomyopathy (9, 10, 12, 23). The three major cell types investigated thus far are skeletal muscle cells (SKMCs) (12), smooth muscle cells (SMCs) (9, 10), and plastic adherent bone marrow cells or marrow stromal cells (MSCs), induced toward a myogenic fate (22, 23).

The impact of donor age on these three cell types, which have been used for myocardial repair in ischemic cardiomyopathy, has not been previously investigated. The in vitro growth properties of these cells, which may predict their in vivo beneficial effect, may be age dependent and may also depend on the cell type in older animals.

We hypothesized that the in vitro yield and characteristics of SMCs would be better preserved than SKMCs and MSCs in aged animals and that the in vitro measurements would predict in vivo performance of the implanted cells in halting the progression of chronic congestive heart failure after experimental myocardial infarction.

METHODS

Experimental Animals

The Animal Care Committee of the University Health Network approved all experimental procedures according to the Guide for the Care and Use of Laboratory Animals (published by the National Institutes of Health, Publication No. 85-23, Revised 1996). All animals were male syngenic Lewis rats obtained from Charles River Canada (Quebec, Canada). To evaluate age-related changes on the in vitro characteristics of the various cell populations, rats ranging in age (2 wk old, 5 mo old, 10 mo old, and 2 yr old) were used. To investigate the effects of donor age on the in vivo effects of the implanted cells, 2-yr-old rats were used as donors and 2-mo-old animals served as recipients. We (4, 9, 10, 22, 23) have previously reported that the implantation of a variety of cell types derived from young donor animals improved regional and global function in young

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recipient animals after cardiac injury. Therefore, we did not repeat those studies and did not include a group of animals that had young cells implanted into young recipient animals.

**Cell Isolation, Culture, and Identification**

SMCs were isolated from harvested rat aorta by 0.2% trypsin and 0.1% collagenase digestion as described previously (9, 10). SMCs were counted and cultured in Iscove’s modified Dulbecco’s medium (IMDM), with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The purity of the cultured SMC was evaluated with a monoclonal antibody against α-smooth muscle actin (Sigma Aldrich, Toronto, Canada).

MSCs were isolated by flushing the femur and tibia with a 21-gauge needle into 5 ml of IMDM supplemented with 2% FBS. An aliquot of the bone marrow cell suspension was diluted 1:100 for counting, and the cells were cultured in MesenCult medium (Stem Cell Technologies, Vancouver, Canada) or IMDM with 10% FBS and antibiotics to yield MSCs. To assay differentiation toward a myogenic phenotype after treatment with 5-azacytidine, the MSCs were stained with an antibody against desmin (DAKO Diagnostics, Mississauga, Canada) as described previously (22, 23). Five fields of each culture dish were randomly selected, and the number of positive and negative cells was counted.

SKMCs were isolated as we previously described (4). The minced muscle tissue was digested with 0.2% collagenase for 15 min, followed by 0.15% pronase for 15 min. The isolated cells were cultured on laminin-coated dishes in M-199 medium containing 10% FBS and antibiotics.

**Cell Growth Characteristics**

Freshly isolated SMCs, MSCs, and SKMCs were counted, and 1 × 10^6 cells from six rats per group (young, 5 mo old; old, 2 yr old) were seeded into 5-cm dishes. The cell number at 1, 2, 3, and 4 wk of culture was counted, and the cell number from two dishes per time point per animal per cell type was averaged.

** Colony-Forming Unit Assays**

Bone marrow cells were isolated from 2-wk-old and 5-, 10-, and 24-mo-old animals (n = 6 per group). An aliquot of the bone marrow cell suspension was diluted 1:100 for counting, and 1 × 10^6 cells were plated in six-well plates with MesenCult medium (Stem Cell Technologies) for 1 wk. To identify colony formation, the medium was decanted and the cells were washed with PBS. Wright-Giemsa solution (Sigma Aldrich) was added and incubated for 3 min. The colony-forming unit fibroblast (CFU-F) was identified with the positive blue color. The number of CFU-granulocyte-macrophage (CFU-GM) was quantified after cells were plated in MethoCult medium (Stem Cell Technologies). Colonies containing 50 cells or more were counted.

** Coronary Ligation**

Two-month-old rats were anesthetized with ketamine hydrochloride (22 mg/kg) and pentobarbital sodium (30 mg/kg) and maintained with isoflurane (0.5–2%). Through a left lateral thoracotomy, the proximal left anterior descending coronary artery was ligated with a 6-0 polypropylene suture. Myocardial ischemia was confirmed by regional myocardial dysfunction, color change, and electrocardiographic documentation. The incision was closed, and antibiotics (150,000 U/ml penicillin G benzathine and 150,000 U/ml penicillin G procaine) and buprenorphine hydrochloride (0.01 mg/kg) were administered.

**Cell Transplantation**

Cells isolated from 2-yr-old animals were used for transplantation to evaluate potential benefits to infarcted hearts. Because we could not grow enough SKMCs from old donors, only MSCs and SMCs were studied. Before transplantation, the MSCs were treated with 5-azacytidine (final concentration of 10 mmol/l) to induce differentiation toward a myogenic phenotype as described (22). To identify therafted cells in the host myocardium, one-third of the implanted MSCs and SMCs were labeled with bromodeoxyuridine (BrDU) before implantation. A subpopulation of the cells was stained with the monoclonal antibody against BrdU (Sigma Aldrich) to assay the efficiency of labeling.

At 3 wk after anterior descending coronary artery ligation, SMCs or MSCs in suspension (50 μl, 3.5 × 10^6 cells) were injected into the center of the scar. The same volume of culture medium was injected into the scar in the control group. Antibiotics and analgesics were given.

**Measurement of Left Ventricular Function**

Echocardiography was performed before cell transplantation. The left parasternal images were taken in the right lateral decubitus position with the use of a 15-MHz linear transducer (Sequoia C256 and 15L8 probe; Acuson, Mountain View, CA). The two-dimensional and M-mode images at the midpapillary level of the left ventricle (LV) were stored. The LV wall thickness and cavity diameters were measured outside the infarct area in both diastole and systole. For each measurement, three consecutive cardiac cycles were traced and averaged by an experienced examiner in a blinded fashion. Animals with ejection fractions (EFs) lower than 50% and fractional shortening (FS) lower than 30% were randomly divided into three groups: SMC transplantation (n = 11), MSC transplantation (n = 9), and medium injection (control, n = 6) groups. Five weeks after cell transplantation, echocardiography was performed again as described.

The rats were then euthanized. The hearts were quickly excised and perfused in a Langendorff apparatus as described previously (18). Peak systolic pressure (PSP) and end-diastolic pressure (EDP) were measured at various ventricular volumes starting at 0.04 ml and increasing in 0.02-ml increments until EDP was over 30 mmHg. Developed pressure was calculated as the difference between the PSP and EDP at each balloon volume (Ponemah Physiology Platform; Gould Instrument Systems, Valley View, OH). Hearts were then arrested with potassium chloride and fixed at a ventricular pressure of 30 mmHg.

**Histology**

Three random animals per group were euthanized for histological studies. The fixed hearts were cut into 3-mm-thick sections perpendicular to the long axis. The infarcted areas (two sections from each heart) were embedded in paraffin, and each section was cut into five specimens (10 specimens per rat). Two specimens from each rat were used for hematoxylin and eosin staining according to the method of the Pathology Department of Toronto General Hospital. Two specimens from each rat were also used for identification of implanted cells at the transplanted area. The specimens were incubated with the antibody against BrdU and the polyclonal antibody against myosin heavy chain (Santa Cruz Biotechnology, Santa Cruz, CA). To measure vascular density, three specimens from each section of each rat were used for a blood vessel density study (6 specimens 3 mm apart per rat with 3 rats per group). Five fields in each specimen were randomly chosen (90 fields per group), and their vascular densities were counted and expressed as blood vessels per 0.2 mm^2 by a blinded observer at ×200 magnification. We have previously validated this approach by using microsphere perfusion measurements (8).

**Statistical Analysis**

All data are expressed as means ± SE. SPSS software package for Windows (version 10.0; SPSS, Chicago, IL) was used for analysis. Comparisons of CFU numbers at different ages were performed by
one-way ANOVA, and comparisons between groups were performed by two-way ANOVA. Comparisons of vascular density and echocardiography data were performed by the unpaired *t*-tests. Langendorff function data were evaluated by analysis of covariance. *P* < 0.05 was considered statistically significant.

RESULTS

**In Vitro Studies**

_Morphology and growth characteristics of cultured aged cells._ SKMCs from 5-mo-old donors were easily isolated from muscle biopsies, established rapidly proliferating colonies, and fused to form multinucleated myotubes, a hallmark of SKMCs (Fig. 1A). With the use of the same harvest technique, however, very few SKMCs could be isolated from the 2-yr-old donors (Fig. 1B). The isolated old SKMCs had very limited proliferative capacity in vitro and never achieved confluence (Fig. 2, A and D). The SKMCs from old donors did not fuse to form elongated myotubes.

SMCs from young (5 mo old; Fig. 1C) and old (2 yr old; Fig. 1D) donors were morphologically similar. They rapidly proliferated in culture (Fig. 2, B and D), forming a confluent monolayer of spindle-shaped cells; 73 ± 5.3% and 71 ± 6.6% of cultured SMCs from young and old donors stained with smooth muscle actin, respectively (Fig. 3, A and B).

MSCs from both young (5 mo old) and old (2 yr old) donors formed small, plastic adherent colonies that subsequently underwent proliferation (Fig. 2, C and D). Morphologically, the MSCs from young (Fig. 1E) and old (Fig. 1F) donors appeared similar. To assay the myogenic potential of MSCs, the cells were treated with 5-azacytidine and probed for expression of the myogenic progenitor marker desmin. The percentage of cells that became desmin-positive after treatment with 5-azacytidine was 14.8 ± 4.6% in the young cell population (Fig. 3C) and 6.2 ± 3.1% in the old cell population (Fig. 3D), which was significantly different (*P* = 0.02).

Quantification of the proliferative capacity of the three primary cell cultures demonstrated that the growth rates of the three cell types isolated from young and old donors were significantly different (Fig. 2). After 1 wk, cultured SMCs from 2-yr-old rats proliferated more extensively and more rapidly than did either MSCs (*P* < 0.01) or SKMCs (*P* < 0.01) from the same animals.

_Effect of rat age on bone marrow colony-forming cells._ The number of colony-forming cells in the bone marrow cell preparations from the different animal age groups was quantified with the use of CFU-F (Figs. 4 and 5) and CFU-GM assays (Fig. 5). The number of CFU-F decreased from 29.6 ± 5.2 per 10,000 cells in the 2-wk-old group to 5.8 ± 2.7 per 10,000 cells in the 24-mo-old group (*P* < 0.001, Fig. 5A). Similarly, the number of CFU-GM declined from a maximum of 22.7 ± 3.1 per 10,000 cells in the

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**Fig. 1.** Photomicrographs of cultured skeletal muscle cells (SKMC, A and B), smooth muscle cells (SMC, C and D), and bone marrow stromal cells (MSC, E and F) isolated from 5-mo-old (young) and 24-mo-old (old) donor rats (×100). When young SKMCs reach confluence, some cells fuse and form myotubes (indicated by arrows).
5-mo-old group to 5.5 ± 2.1 per 10,000 cells in the 24-mo-old group (P < 0.001, Fig. 5B).

In Vivo Studies

LV function. Before cell transplantation (3 wk after anterior descending coronary artery ligation), there were no differences in heart function by echocardiography among the three groups (Fig. 6). Five weeks after cell transplantation, EF and FS were markedly improved in the SMC transplantation group (change: EF, 9.0 ± 2.2%, P = 0.019; FS, 5.1 ± 2.8%, P < 0.001) (Fig. 6, A and B). Aged MSCs treated with 5-azacytidine did not improve EF or FS [change: EF, −2.5 ± 5.5%, P = not
significant (NS); FS, 0.3 ± 2.4, P = NS). There was no difference in end-systolic and end-diastolic dimensions among the three groups. However, the thickness of the LV anterior wall was greater in the SMC transplantation group than in the MSC and medium-injected groups (P = 0.035 and P = 0.016, respectively) (Fig. 6, C and D).

In the Langendorff mode, we did not detect a significant difference in EDP among the groups. The developed pressure, however, was significantly greater in the SMC group than in the MSC or control groups (P = 0.01 vs. control) (Fig. 7). To evaluate load-independent indices of cardiac function, we standardized the EDP in each group and measured PSP. Generated pressures under standardized EDP demonstrated that the SMC group performed best (P < 0.001 vs. control); however, there was no difference between the MSC group and the control group (P = 0.4).

Histological characteristics and vascular density. The in vitro BrdU labeling efficiency was similar between SMC and MSC groups (79 ± 4.9 vs. 83 ± 2.2%, P = NS). BrdU-labeled cells were readily detected in the infarct region of SMC and MSC transplantation groups (Fig. 8, A and B). No such cells were identified in the control hearts. Transplanted SMCs formed tissue in the center of the scar that stained positively for smooth muscle actin (Fig. 8C). Some transplanted MSCs stained positively for myosin heavy chain (Fig. 8D).

The SMC group had the highest vascular density (SMC vs. MSC, 8.7 ± 0.9 vs. 4.8 ± 0.6 blood vessels/0.2 mm², P = 0.003 among the three groups (P = 0.003 SMCs vs. MSCs). When compared with the control group, the vascular density was greater in the two cell-transplanted groups (control, 1.8 ± 0.3 blood vessels/0.2 mm², P < 0.001).

DISCUSSION

The major findings of our report in a rodent model are that 1) age has an effect on the potential benefits of myocardial cellular therapy, 2) the impact of age is cell-type dependent, and 3) in vitro growth characteristics predict in vivo performance of the cells.
Both MSCs (2) and SKMCs (12, 13) have been used in the clinical setting for implantation into ischemic cardiomyopathic hearts. We were unable to establish primary SKMC cultures from older animals, which have also been suggested in clinical studies (7); however, both aged SMCs and MSCs were successfully cultured and transplanted into the infarct scar of syngenic rats. By echocardiography we found that the transplantation of SMCs from older animals improved systolic function and maintained wall thickness. The transplantation of MSCs from older animals reduced but did not prevent the deterioration of systolic function seen in the control animals. MSCs, unlike SMCs, did not maintain wall thickness. The ex vivo Langendorff measurements supported the in vivo echocardiographic findings. Specifically, a load-independent index of systolic function (PSP at varying EDP) was best preserved in the SMC transplant group, and we did not detect a difference between the MSC and the media-injected controls. Our results would suggest that implantation of SMCs is likely to provide superior performance in older patients who require cellular therapy to prevent congestive heart failure, because the ability of SMCs to prevent cardiac dilatation and failure was not affected by the aging process. In both older and younger patients, SMCs can be easily obtained from small segments of the saphenous vein or radial artery, which are discarded at the time of coronary bypass surgery.

We (4, 9, 10, 22, 23) have previously demonstrated that a variety of cell types derived from young animals improved LV function in animal models of LV injury. This may be the first study to demonstrate that some of these same cells derived from older animals are not able to improve ventricular function compared with their media-injected controls.

One of the possible mechanisms by which MSCs, which when immediately plated form fibroblast-like colonies (3), may improve heart function is the ability of the MSCs to undergo differentiation toward a myogenic fate (11, 14, 22, 23). Makino et al. (11) discovered that when MSCs were cultured in the presence of 5-azacytidine, up to 30% of the cultured cells adopted a cardiomyocyte-like phenotype, and these cells beat in synchrony after extended culture. We showed that transplantation of MSCs isolated from young donors improved cardiac function by increasing the developed pressure from 21% to 24% across a wide range of ventricular balloon volumes in a rat cryoinjury model only if these cells were cultured after treatment with 5-azacytidine (22). We therefore hypothesized that the improvement in cardiac function after bone marrow cell transplantation may be directly proportional to the concentration of MSCs in the preparation, as determined by the CFU-F assay, and the myogenic differentiation capability of the MSC after treatment with 5-azacytidine (22). In the present study, we demonstrated that the bone marrow cells from 2-yr-old rats had a nearly sixfold reduction in the number of CFU-F cells than did bone marrow cells from 2-wk-old rats. Furthermore, the cells from old animals were half as likely to differentiate to myogenic precursors as evidenced by desmin staining. In combination, therefore, the same number of bone marrow cells from older animals would contain 12-fold fewer cells that...
could potentially commit to a myogenic lineage. Given that our long-term culture conditions do not favor the growth of bone marrow stem cells, failure to improve cardiac function after transplantation is unlikely related to the decreased number of functional bone marrow stem cells in older animals.

Previous work has demonstrated that the vascular SMCs retain their proliferative capacity in older patients. In fact, smooth muscle hyperplasia occurs in atherosclerotic lesions (5, 16, 17) and in saphenous veins (15) used for coronary artery bypass grafting, which are used in patients with ischemic cardiomyopathy, typically of advanced age. It is unclear how the transplantation of cultured SMCs is able to halt the progression to heart failure and improve systolic function. Although both aged SMCs and MSCs could induce angiogenesis in the scar tissue, only the transplanted SMCs were found to incorporate into new large blood vessels. The process of angiogenesis induced by SMC transplantation likely involves a combination of angiogenic growth factor secretion in addition to other undefined paracrine mechanisms that would involve processes such as beneficial extracellular matrix remodeling. SMCs, by an alteration of the balance of proteolytic enzymes and their inhibitors, may also impact the myocardial cytokine milieu. Another possibility could be that the SMCs are more tolerant to ischemic injury, and their survival rate may be higher in the infarcted heart compared with other aged cells. Although some of these hypotheses remain to be tested, our data indicate that increasing age does not adversely impact the ability of SMCs to improve heart function after myocardial transplantation.

In light of the ongoing clinical trials, the impact of aging on myocardial cell transplantation needs to be further characterized. It is possible that cells from older individuals, because of telomere attrition and other effects of aging, may not survive as long as cells from younger individuals after being implanted. Cells lacking telomerase activity would therefore be poor candidates for myocardial cell therapy. The impact of other patient comorbidities on cell therapeutic strategies should also be examined. A recent study (6) reported the diminished ability of bone marrow mononuclear cells to induce angiogenesis when harvested from patients with ischemic cardiomyopathy. In our study, the cell recipients were young animals. The beneficial effects of cell transplantation may in part be due to the host response to the implanted cells, which may be impaired in older individuals. For instance, the extent of angiogenesis induced by cell transplantation may be significantly attenuated in older recipients because of aging endothelial progenitor cells (20). The impact on myocardial function may also depend on implanted cell engraftment and interactions between host cardiomyocytes and extracellular matrix elements.

In summary, we found that MSCs and SKMCs from older rats did not grow as robustly as their younger counterparts, and the MSCs from older animals had significantly diminished plasticity. MSC transplantation into experimental infarcts did not confer the same degree of benefit as was previously seen in our laboratory. Caution, therefore, must be exercised when extrapolating experimental animal data to design clinical studies of cell therapy.

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