

Relationship of Aging and Cardiac IL-10

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Abstract: Current therapies for the treatment of myocardial infarction and heart failure include medical, surgical, mechanical assist, and transplantation. These therapies have been based on the dogma that ventricular myocytes themselves are terminally differentiated and, therefore, cannot regenerate. This concept has been recently challenged with stem cell therapy. A potential problem is the ability of cardiac tissue to mobilize, recruit, and transdifferentiate adult stem cells from other tissues. We believe that there is a unique failure of the damaged myocardium to provide the appropriate molecular signals for stem cells engraftment related to age. Our hypothesis is that the overexpression of IL-10 in the aged population reduces cardiac cellular proliferation subsequent to myocardial injury. This hypothesis is sup-

ported by aging models, where elevated levels of IL-10 are associated with reduced healing response to noncardiac tissue injury. We demonstrated an increased cardiac gene expression of IL-10 that may be associated with a reduced proliferative response in the border regions of the infarcted myocardium that are proportional with age. In conclusion, myocardial infarction and heart failure has presented a significant challenge for the clinician to provide reparative therapies. The use of therapeutics to modulate IL-10 and, thereby, optimizing regenerative processes in the injured myocardium may provide a unique means for the cardiac patient. **Keywords:** aging, myocardial regeneration, IL-10, stem cells. *JECT. 2004;36:197-201*

Transplantation of adult stem cells has been proved to be a new method for regeneration of injured myocardial tissue. Recent reports have established that hematopoietic stem cells (HSC) may serve as the stem cell source for regeneration of myocardial tissue and, thus, may become a clinical therapeutic means for the treatment of myocardial infarction to prevent progression to heart failure (1). This may provide a new opportunity for the perfusionist to become an integral member of a team in the application of this technique in the clinical situation. This report provides basic concepts related to stem cell therapy and our data suggests the potential caveats when bridging from the laboratory murine models to the older adult human.

With the population of the United States aging, it is projected that heart failure (HF) will significantly increase in the next two decades. This increase is supported by a report stating over the last 10 years, that annual numbers of hospitalizations due to primary diagnosis of HF have increased from approximately 550,000 to nearly 900,000 and from 1.7 to 2.6 million for HF as the secondary diagnosis (2). These clinical statistics suggest that age is asso-

ciated with basic cellular changes in the myocardium, which contributes to the progression of HF. Clinical data demonstrate that age-related HF is a consequence of ischemic heart disease in about 37 to 39.9% of patients (3). There are 1.1 million myocardial infarctions per year that result in varying degrees of HF and limit individual function and life span. The survival of patients with myocardial infarction is directly related to age (4). In the cited study of 29,285 patients, an individual <55 years old had a risk of death of 4.6%, 65-75 years old had a risk of 16.1%, and >75 years old had a risk of 25.3%. Although not yet proven, this age-related mortality risk in myocardial infarction may be caused by reduced myocardial regeneration, leading to maladaptive remodeling which may be attributable to altered adult stem cell plasticity and cardiac fibroblast function. In summary, these data suggest an immense clinical need for stem cell-mediated myocardial regeneration.

Our overall guiding hypothesis is that one consequence of aging is an increased cardiac gene expression of IL-10 and that may be associated with a decreased proliferative response subsequent to cardiac infarction. The implication of this hypothesis is that the tissue microenvironment may be insufficient to support engraftment of stem cells.

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Therefore, achieving adaptive remodeling, healing, and stem cell engraftment may require optimization of the injured tissue environment.

MATERIALS AND METHODS

Animals

Four- and 16-month-old female C57BL/6 mice purchased from the Jackson Laboratory, Bar Harbor, Maine were cared for in compliance with the "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985) and "Principles of Laboratory Animal Care" (National Society for Medical Research) and with prior approval by The University of Arizona, Animal Review Committee. The mice were conditioned for 2 weeks in the animal facility before the study.

Groups

Two age groups (4-months- and 16-months-old mice) were studied to compare the effect of age on cardiac cellular proliferation. Study I assessed the rate of proliferation in the border zone of a myocardial infarction (four mice per group) with a BrdU immunofluorescence technique. Study II determined if the expression of the endogenous immunosuppressant, IL-10, is differently expressed by the cardiac fibroblast (CF) between the two age groups (six mice per group), which could account for the decreased rate of myocardial proliferation in the aged heart. CF's were isolated and prepared for RT-PCR to compare the level of IL-10 mRNA expressed by the two age groups. Pilot studies demonstrated the number of animals required for this study.

Coronary Occlusion

General anesthesia was induced and maintained by an intraperitoneal injection of 2.5% Avertin (2.5% 2,2,2-tribromoethanol, 2.5% tert-amyl alcohol in normal saline; Aldrich). Sterility was maintained throughout the procedure. Hair was removed from the animals' neck and chest areas using a depilatory agent. Tracheotomy was performed, and a 24-gauge catheter was inserted into the trachea to allow for artificial respiration. The ventilator (Harvard Rodent Ventilator Model 683) was set to deliver an inspiratory volume of 0.9 mL at FIO₂ of 1.0 at 120 cycles/minute. Epicardial access was accomplished by performing a left thoracotomy. Ligation of the left anterior coronary artery (LAD) 1 mm below the left atrium was performed with an 8-0 prolene ligature. Before tying the suture, it was retracted to occlude the LAD to confirm proper suture location by a blanching in surface color of the anterior aspect of the left ventricle. The chest was closed in three layers, and a 30-gauge needle was attached to a syringe to evacuate air from the chest. Mice were

removed from ventilation and recovered under a warming lamp.

Quantification of Proliferation with BrdU

To establish the proliferative activity in the infarcted, border zone, and noninfarcted sites of the heart, we administered BrdU (50 mg/kg/day, IP). At day 14, the hearts were removed, placed in Histochoice fixative (Ameresco), sectioned, and incubated with antiBrdU antibody (Molecular Probes, A-21303, mouse monoclonal PRB-1, Alexa Fluora 488 conjugate). The proliferating cells labeled with BrdU were compared by confocal microscopy in each treatment group. Corresponding sections from each block were stained with Trichrome to define areas of infarction, border regions, and noninfarcted regions.

Fibroblast Isolation

The CF isolation protocol and DDR methods were kindly provided by Thomas Borg, Ph.D., University of South Carolina by personal communication to our laboratory. The mice were sacrificed using cervical dislocation, and the heart was quickly removed. The apical ventricular portions were minced and rinsed in four washes of warm sterile Krebs-Henslett buffer (KHB). A digestion preparation solution was made by dissolving 0.54 mg Liberase 3 enzyme (Roche Biochemical) in 60 mL warm sterile KHB. Ten mL of digestion solution was added to the heart tissue in a 15 mL conical tube and placed in rotator in the incubator (37°C) for 15 minutes. The tube was vortexed for 30 seconds, the supernatant (containing fibroblasts) was then centrifuged at 1000 rpm for 10 min, and resuspended. The first two digestions were discarded, and the subsequent 10 digestions were retained for adherence of the CF in culture flasks. The isolated fibroblast concentrate was plated in a T75 with 20 mL of DMEM/Hepes with 10% FCS medium. After 2 hours, the adherent cells CF were retained, and the floating cells (noncardiac fibroblast cells) were discarded. This method of isolation has provided fibroblasts of greater than 88% purity using the CF-specific DDR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with flow cytometry. At this time, the adherent primary CF cells were processed for RT-PCR for the *ex vivo* analysis.

Semiquantification of Age-Related IL-10 mRNA Expression in CF's with RT-PCR

Isolated CF's were processed for determination of differential expression of IL-10. The mRNA was extracted with TRIZOL reagent (Gibco BRL) and mRNA concentrations were determined using an Eppendorf Biophotometer. RNA was reverse-transcribed (RT) from 2-3 mg of total RNA in a final volume of 20 µL RT mixture for 2 hours at 37°C. The RT mixture consisted of 4 µL 5 × RT buffer, 1 µM dNTPs, 20 U RNase inhibitor, 3 µg random

primers, and Superscript RT enzyme. PCR was performed in a 50 μ L reaction volume containing 1ml cDNA, 5 μ l 10 \times PCR buffer, 1ml 10 mM dNTPs, 2 μ l 50 mM MgSO₄, 1 μ L 10 mM of IL-10 or GAPDH primers, and 1 U Taq DNA polymerase. GAPDH was used as an internal standard. The murine IL-10 and GAPDH primers purchased from Integrated DNA Technologies, Coralville, IA were 5'-CCA GGG AGA TTT GAT GA-3' for sense and 5'-GCT GCT ACA AAG GCA GAC AA-3' antisense for IL10 and 5'-GAC TCC ACT CAC GGC AAA TTC-3' sense and 5'-GAC TCC ACT CAC GGC AAA TTC-3' antisense for GAPDH. Linearity of IL-10 was determined before assay using cycles between 24 and 32. A hot start was applied for 4 minutes at 95°C and the cycle (denaturation step at 95°C for 30 sec, annealing step at 60°C for 30 sec, and an elongation step at 72°C) was repeated for 28 cycles. PCR products were then analyzed by horizontal electrophoresis in a 1% agarose gel. The gels were photographed on a UV light source and analyzed with a Bio-Rad GS-800 Calibrated Densitometer.

RESULTS

Figure 1 shows representative BrdU immunofluorescence of cardiac sections of the border region of the site of myocardial infarction. There was much less BrdU staining in the 16 month old mice, which implies that cellular proliferation in this region was markedly decreased when compared to the younger age group. The survival rate after the induction of the myocardial infarction for the 2-week study period was 75% less in the older group as compared to the younger group, and therefore, additional mice were required until achieving a final number of 4 per age group.

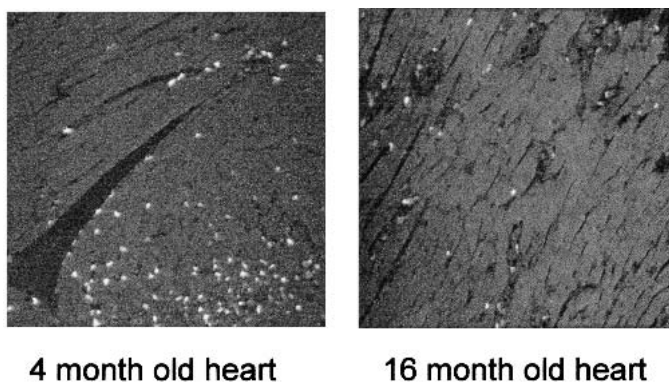


Figure 1. These panels illustrate representative tissue sections of the border regions of the infarcted mouse heart. The fluorescence staining are cells that have incorporated BrdU into their DNA attributable to cellular replication over the 2-week treatment period. The panel on the left demonstrates the proliferative response of the young mouse heart (4 months old) and is contrasted with the proliferative response of the older mouse heart (16 months old). These confocal photographs suggest that there is a lowered rate of cellular proliferation in the old mice after myocardial injury.

Figure 2 demonstrates that the IL-10 mRNA expressed by CF's harvested from 4- and 16-month old mice is much greater (80%) in the older group.

DISCUSSION

Because IL-10 is an endogenous immunosuppressant, the IL-10 overexpression we found the CF's may be a mechanism by which the cellular proliferation in the aged heart is lower compared to the young, as illustrated in Figure 1. Although not proven by this study, it does suggests that adaptive remodeling—the healing processes—post-myocardial infarction may be impaired by the overexpression of IL-10 in the aged. The adaptive remodeling involves the immune system to a great extent where the neutrophils, macrophages, and lymphocytes modulate fibroblast function; it is possible that the immunosuppressive activity of IL-10 could modulate this healing process (5). To compare the age of a mouse to humans, the normal life span of a mouse is 28 months; therefore, the 4-month-old mouse is understood to be juvenile, and the 16-month-old mouse is equivalent to an older adult.

The failure of significant regeneration in the adult heart tissues following cardiac injury may not necessarily be attributable to the absence of resident or peripheral stem cells, but the potential problem is the ability of cardiac tissue to mobilize, recruit, and transdifferentiate adult stem cells from other tissues. We believe that there is a unique failure of the damaged myocardium to provide the appropriate molecular signals to activate and promote the recruitment and differentiation of stem cells in a sufficient number to repair injuries compared to other tissues. We

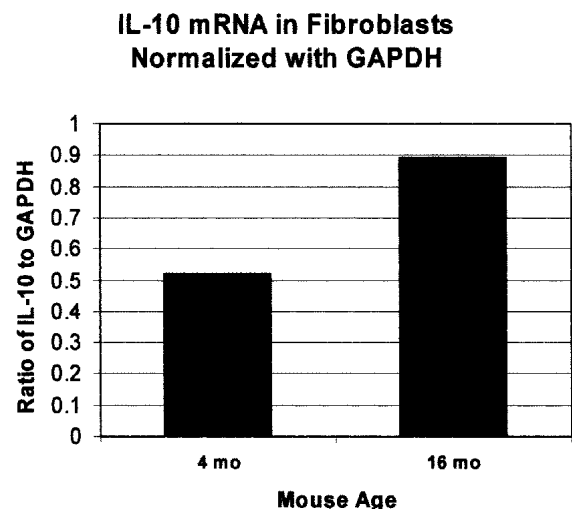


Figure 2. The RT-PCR compares the IL-10 mRNA in CF's isolated from young (4- months-old) and old (16-months-old) mice. Each lane represents a pooled CF mRNA from six mice of the respective age groups. This figure suggests that IL-10 is overexpressed in the aged CF, and as a result, may exert its immunosuppressive activities on the wound healing process in the heart post-myocardial infarction.

have shown that there is a decrease in regional proliferation subsequent to a myocardial infarction in the aged mouse and that the age-related overexpression of IL-10 by the CF could account for this observation. Therefore, the immunosuppressive activity imparted by IL-10 could produce a localized microenvironment at the site of the infarction that reduces the normal wound healing mechanisms.

We have also shown that the CF expresses IL-10 and that this expression is much higher in the aged individual when compared to the younger. Systemically, IL-10 is understood to inhibit IL-2 T-lymphocyte expression, Th1 immune responses, antigen presenting cells, and inflammation processes (5). At the regional level, IL-10 blocks cellular migration and wound healing in response to proinflammatory cytokines and the lack of IL-10 results in an acute inflammation and accelerated wound healing (6,7). IL-10 reduces cytokine, growth factor, and chemokine secretion that are required for cardiac tissue repair (8). More specifically, IL-10 inhibits the overexpression of chemokines (MCP-1, MIP-1 α) and proinflammatory cytokines (IL-1 β , IL-6, TNF- α) in vivo which are required for a normal response to tissue injury. The most telling evidence regarding the effect of IL-10 in the context of cardiac repair is that IL-10 inhibits the infiltration of neutrophils and macrophages toward the site of injury. These effects suggest that IL-10 may play an inhibitory regulatory role in the phase-specific infiltration of neutrophils and macrophages as well as the cytokine production in the inflammatory response necessary for cardiac healing.

The CF secretes numerous cytokines, growth factors, and chemokines and regulate the extracellular matrix composition. CF represent two-thirds of the nonmyocyte cells in the heart (9) and are key cells in the remodeling of tissue following injury—including the heart. CF have been shown to be responsible for cardiac collagen homeostasis and associated with increased myocardial fibrosis related to the aging process (10). There are phenotypic differences of fibroblasts in the heart; namely, CF, myofibroblast (MyoFb), and a minor type of valvular interstitial fibroblast (VIC). The differentiation of CF to myofibroblasts (MyoFb) is a critical step in wound contraction in wound healing in vivo (11). Cytokines, growth factors, chemokines, and inflammatory mediators are released by MyoFb (12). This list of MyoFb-secreted products emphasizes the role of fibroblasts in cardiac wound healing and supports our observation that the aging process may alter the ability of the heart to recruit and support cardiac regeneration subsequent to a myocardial infarction.

It has been recently reported that there is regeneration of myocardial tissues following a myocardial infarction including formation of new myocytes from stem cells (13). Regulation of migration and growth of stem cells is most likely directed by cells adjacent to the damaged tissue;

namely, fibroblasts and is documented in wound healing (14). Transplanted hematopoietic stem cells with a fluorescent label have been shown to migrate and engraft into the injured cardiac tissues (13). The molecular mediators that support the migration and differentiation of stem cells may vary related to the degree and type of injury and relate to age (14). Therefore, the aging process may affect the CF function to result in a reduced level of stem cell engraftment and cardiac repair after a myocardial infarction. We are suggesting that the overexpression of IL-10 is associated with this reduced cardiac healing process, as shown in Figure 3.

CONCLUSION

Our results are not conclusive regarding the mechanism of the decreased rate of cellular proliferation in the aged. However, our data are supported by the literature that contends the aged heart is more susceptible to failure after injury. Also, the described role of IL-10 as a suppressive cytokine fits with our model in that it may contribute to inhibition of healing and cellular regeneration in the infarcted heart. Most importantly, current medical therapeutics (diuretics, β -blockers, carvedilol, captopril, biventricular pacing, and AICD) for the treatment of end-stage heart failure (HF) have achieved a prolongation of survival; however they cannot reverse the disease progression (15). Cardiac transplantation and ventricular assist devices are extremely expensive, and thus, are available

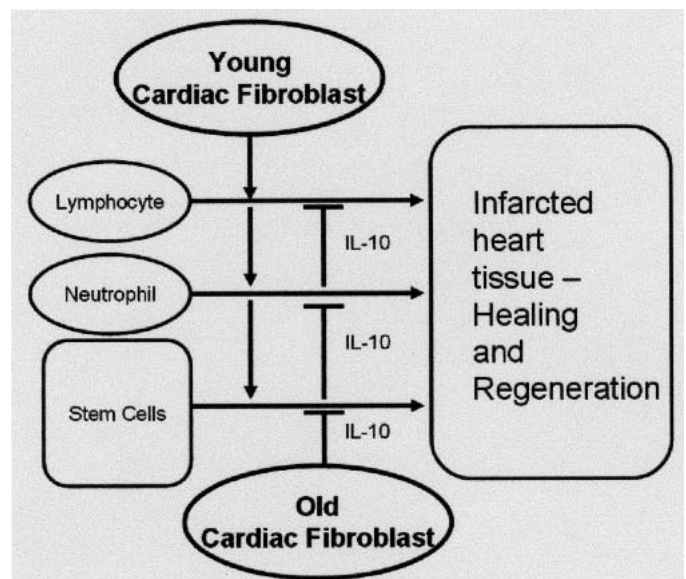


Figure 3. This diagram illustrates a proposed pathway for cardiac healing and cardiac tissue regeneration subsequent to a myocardial infarction. The lymphocyte, neutrophil, and CF's are key sources of growth factors, cytokines, and chemokines to facilitate the cardiac healing. In the old individual, we propose that overexpression of IL-10 by the CF impedes the function of these three secretory cell types plus inhibits the stem cell migration, engraftment, and differentiation into cardiac tissues.

for a very limited number of patients. This report provides a new and provocative suggestion that the overexpression of IL-10 may impede cardiac healing and that possibly, stem cell engraftment, subsequent to a myocardial infarction in the aged may improve progression of disease. Modulation of the CF's expression of IL-10 may, therefore, serve as a means to increase cardiac patients' survival.

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