Ex Vivo Activated Human Macrophages Improve Healing, Remodeling, and Function of the Infarcted Heart

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Methods and Results—Human activated macrophage suspension (AMS) was prepared from a whole blood unit obtained from young donors in a closed sterile system and was activated by a novel method of hypo-osmotic shock. The AMS (≈4×10^7 cells) included up to 43% CD14-positive cells and was injected into the ischemic myocardium of rats (n=8) immediately after coronary artery ligation. The control group (n=9) was treated with saline injection. The human cells existed in the infarcted heart 4 to 7 days after injection, as indicated by histology, human growth hormone-specific polymerase chain reaction, and magnetic resonance imaging (MRI) tracking of iron oxide–nanoparticle-labeled cells. After 5 weeks, scar vessel density (±SE) (25±4 versus 10±1 per mm^2; P<0.05), myofibroblast accumulation, and recruitment of resident monocytes and macrophages were greater in AMS-treated hearts compared with controls. Serial echocardiography studies, before and 5 weeks after injection, showed that AMS improved scar thickening (0.15±0.01 versus 0.11±0.01 cm; P<0.05), reduced left ventricular (LV) diastolic dilatation (0.87±0.02 versus 0.99±0.04 cm; P<0.05), and improved LV fractional shortening (31±2 versus 20±4%; P<0.05), compared with controls.

Conclusions—Early after myocardial infarction, injection of AMS accelerates vascularization, tissue repair, and improves cardiac remodeling and function. Our work suggests a novel clinically relevant option to promote the repair of ischemic tissue. (Circulation. 2006;114[suppl I]:I-94–I-100.)

Key Words: immune system ■ inflammation ■ myocardial infarction ■ remodeling ■ magnetic resonance imaging

One of the major aims of new cardiology is to optimize the healing processes after myocardial infarction (MI). The damage of MI is often irreversible and progressively leads to left ventricular (LV) remodeling and heart failure. By promoting more effective tissue repair, we could reduce adverse remodeling, heart failure, and death. Injection of autologous progenitor cells early after MI could prevent or reverse cardiac remodeling and dysfunction. However, the efficacy of this strategy in elderly and sick individuals with limited stem cell number and function remain uncertain.

Here, we suggest a novel alternative cell for myocardial tissue repair: ex vivo activated human macrophage obtained from peripheral blood. The rational for this approach is that activated macrophages are a rich source of cytokines, growth, and survival factors and play a key role in wound healing. The processes of MI repair and wound healing have many common components.1 During the proliferative phase of healing, macrophages accumulate in the infarct and regulate granulation tissue formation, and vascularization by releasing growth factors, angiogenic mediators, and proteases.2–6 In addition, macrophages are involved in inflammatory injury suppression,7 myocyte protection,8,9 and regeneration.9,10

In the past few years we have treated >1500 elderly and sick patients with unhealed ulcers and wounds by local injections of human activated macrophage suspension (AMS).11–13 Based on our favorable clinical experience, we hypothesized that the unique wound healing properties of macrophages could be exploited to promote healing and repair of the infarcted myocardium. Thus, the aim of conducting the present study was to test this hypothesis in a rat model of extensive MI.

Materials and Methods

The study was performed in accordance with the guidelines of The Animal Care and Use Committee of Tel-Aviv University and Sheba Medical Center, which conform to the policies of the American Heart Association.

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Preparation of AMS
AMS was prepared as previously described. In brief, a whole-blood unit donated routinely by healthy young donors (age range between 18 to 30 years) was collected into a triple blood-bag system. The unit was then separated into packed red cells, white blood cells (buffy coat), and plasma. The bags containing the plasma and theuffy coat were connected, using a sterile connecting device (Terumo, Tokyo, Japan) to the macrophage preparation system (Teva-Medical, Ashdod, Israel). Administration of CaCl$_2$ to the plasma bag induced coagulation. The serum thus obtained served as an autologous nutritional medium for the AMS. Theuffy coat was treated by hypo-osmotic shock, and isotonicity was re-established after 45 seconds. The cells were sedimented by centrifugation and the supernatant was transferred to an empty bag. The osmotically shock-treated cells were resuspended in the donor serum and transferred into a culture bag containing sterile air. After incubation at 37°C, macrophages adherent to the bottom of the bag were transferred into a culture bag containing sterile air. After incubation at 37°C, macrophages adherent to the bottom of the bag were collected and resuspended to a concentration of 2×10$^6$ cells/mL. The total volume produced from one blood unit was 30 to 40 mL. Purity of cells was assessed by flow cytometry-activated cell sorter (FACS). Monocyte activation was confirmed by enzyme-linked immunosorbent assay (ELISA) kits for interleukin levels in the supernatants obtained from the activated and nonactivated monocyte cultures. Phagocytosis capacity of fluorescent latex beads, by activated and nonactivated monocytes collected from the culture bags, was evaluated by FACS.

Rat Model of MI and Cell Delivery
Male Sprague-Dawley female rats (≈250 grams; Harlan; Jerusalem, Israel) were anesthetized with a combination of 90 mg/kg ketamine and 10 mg/kg xylazine, intubated, and mechanically ventilated. The chest was opened by left thoracotomy, the pericardium was removed, and the proximal left coronary artery was permanently occluded with an intramural stitch. The ischemic area was identified visually on the basis of pale color and segmental akinesis. One minute after coronary artery occlusion, rats were randomized to either 2 injections of 50 µL of AMS (total ~2×4×10$^6$ cells) or saline using a 27-gauge needle.

Cell Labeling for In Vivo Tracking of AMS by MRI
To track the injected cells in vivo, AMS cells were labeled with a magnetic resonance (MR) contrast agent, Endorem (Guerbet, Villepinte, France), an iron oxide nanoparticle solution provided with a total iron (Fe) content of 11.2 mg/mL, and the transfection agent PLL (Poly-l-Lysine, catalogue No. P1524; Sigma-Aldrich; molecular weight >388 000 and cell culture grade). The labeled and control cells were injected into the infarcted hearts of another group of rats (n=8) 1 minute after coronary artery ligation. From day 1 and every 4 days up to 14 days after cell delivery, the rat chest area was scanned using a 0.5-T GE iMRI machine with a specially constructed 50-mm transverse slice. The ischemic area was identified visually on the basis of pale color and segmental akinesis. One minute after coronary artery occlusion, rats were randomized to either 2 injections of 50 µL of AMS (total ~2×4×10$^6$ cells) or saline using a 27-gauge needle.

Echocardiography to Evaluate Remodeling and Contractility
Transtracheal echocardiography was performed on all animals within 24 hours after MI (baseline echocardiogram) and 5 weeks later. Echocardiograms were performed with a commercially available echocardiography system (Sonos 7500; Phillips) equipped with 12-MHz phased-array transducer (Hewlett Packard, Andover, Mass). We measured LV anterior wall thickness; maximal LV end-diastolic dimension; minimal LV end-systolic dimension and area in the short axis view by 2-dimensional imaging; and fractional shortening (FS) as a measure of systolic function, which was calculated as FS (%)=[[(LVIDd−LVIDs)/LVIDd]×100, where LVID indicates LV internal dimension, s is systole, and d is diastole. All measurements were averaged over 3 consecutive cardiac cycles and were performed by an experienced technician who was blinded to the treatment group.

Histological Examination
At 5 weeks after injection, animals were euthanized with an overdose of phenobarbital. Hearts were sectioned into 4 transverse slices parallel to the atrioventricular ring. Each slice was fixed with 10% buffered formalin, embedded in paraffin, and sectioned with a microtome (5-µm-thick). Serial sections were stained with hematoxylin and eosin and immunolabeled with antibodies against α-smooth muscle actin (α-SMA) isofrom (Sigma-Aldrich), and ED1, a marker for tissue-resident macrophages, CD 68, human monocyte and macrophage lineage antigen (DakoCytomation), and HLA-DR (DakoCytomation). Neovascularization in the infarcted and peri-infarcted myocardium was assessed on representative slides obtained from mid-heart transverse section, immunostained with α-SMA antibodies (Sigma-Aldrich) to localize pericytes and arterioles. Five consecutive adjacent fields were photographed from each section at a magnification ×200, and the vessels were counted.

Table 1 summarizes the results of the 3 methods used to detect the human cells after injection. To track the injected cells in vivo, AMS cells were labeled with a magnetic resonance (MR) contrast agent, Endorem (Guerbet, Villepinte, France), an iron oxide nanoparticle solution, before injection into infarcted myocardium of rats (n=8). Starting at day 1 and every 4 days up to

AMS Characteristics
FACS analysis showed that AMS cell population contained 42.8% CD14 (a marker of human monocytes and macrophages) cells; 36.1% CD15 (a marker of myelomonocytic cells) cells; 0.02% CD34 (hematopoietic and endothelial progenitor cell marker) cells, and 21.1% CD19 (a marker of B cells) cells. By morphometric analysis, the suspension included monocytes (21%), segmental cells (51%), lymphocytes (21%), eosinophils (5%), and apoptotic cells (2%).

Human Cell Tracking in the Infarcted Myocardium

Results
Overall, 44 rats were included in the study. The echocardiography functional study included 30 rats. Within 24 hours, 9 rats died from the surgical procedure used to induce MI (4 from AMS and 5 from the control group) and 2 rats, 1 from each group, died during follow-up. Thus, complete functional analysis was performed in 17 rats treated with AMS (n=8) and saline (n=9). Another group of 16 rats was part of the cell tracking study by histology, PCR (n=8), and MRI (n=8).

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14 days after cell delivery, the chest area was scanned using a 0.5-T GE iMRI machine with a specially constructed animal probe and showed strong positive black signals from hearts treated with labeled AMS cells but not from nonlabeled cells (Figure 1). By conventional human GH gene-specific PCR, the human macrophages were detected in the infarcted hearts (n=8). Strong PCR signals for human GH gene were found only in DNA preparations from 2-day scars. Weak positive signals were observed in DNA from 4- and 7-day scars treated by AMS but not in controls (Figure 2). Two weeks after AMS injection, the PCR was negative in both AMS group and controls. By CD68 (a marker of human monocyte and macrophage) immunostaining, the injected human cells were identified in heart specimens obtained 4 days after injection (Figure 3). However, the human cells were not detected by CD68 and HLA-DR immunostaining in heart specimens obtained at 1, 2, 4, and 5 weeks after injection. Thus, based on PCR, MRI tracking, and histological examination (Table 1), we concluded that the AMS cells existed in the infarcted heart 4 to 7 days after injection.

**AMS Promotes Myocardial Repair**

Five weeks after injection, immunostaining for ED1, a marker of rat tissue macrophages, revealed resident macrophage clusters associated with robust vascularization at the sites of AMS injections (Figure 4). These results suggest that AMS transplantation promotes the recruitment of local monocytes, macrophages, or both into the infarcted heart. Immunostaining for α-SMA showed that AMS promoted myofibroblast accumulation and vascularization in the infarcted myocardium (Figure 5). Vessel density in the scar tissue of

<table>
<thead>
<tr>
<th>Day After Injection</th>
<th>Histology With CD68 and HLA-DR Immunostaining</th>
<th>PCR for Human GH</th>
<th>MRI-Iron Labeled Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>Positive staining</td>
<td>Positive signal</td>
<td>Positive signal</td>
</tr>
<tr>
<td>Day 4</td>
<td>Positive staining</td>
<td>Positive weak signal</td>
<td>Positive signal</td>
</tr>
<tr>
<td>Day 7–8</td>
<td>Negative staining</td>
<td>Very weak signal</td>
<td>Positive signal</td>
</tr>
<tr>
<td>Day 14</td>
<td>Negative staining</td>
<td>Negative signal</td>
<td>Positive signal</td>
</tr>
</tbody>
</table>

Some of the injected human cells survived at least 4 days (confirmed by 3 methods) and no more than 7 days. The persistent positive MRI signals may be related to iron nanoparticles released from dying labeled cells and engulfed by resident macrophages.

**Figure 1.** In vivo tracking of AMS cells by MRI. To track the injected cells in vivo, we labeled AMS cells with magnetic resonance (MR) iron oxide nanoparticles. After injections, the chest area was scanned using the 0.5-T GE iMRI machine with a specially constructed animal probe. Imaging sequences showed strong black signal (arrows) from the left ventricular (LV) anterior wall of AMS-treated hearts but not in controls.
AMS-treated animals was greater than controls (25 ± 4 versus 10 ± 1 per mm²; P<0.05). These vessels were functional as indicated by red blood cells in the lumen (Figure 5, upper panel). Myofibroblast accumulation in AMS-treated scars could contribute to scar contraction, thickening and strength. In control hearts, positive α-SMA staining was less extensive and mainly limited to the subendocardium and vessel walls.

AMS Improves LV Remodeling and Function

Serial echocardiography studies, performed before and 5 weeks after injection, showed that AMS injection attenuated the typical course of LV remodeling, scar thinning, and LV dysfunction (Table 2, Figure 6). AMS significantly increased scar thickness (P<0.05), and reduced LV end-diastolic (P<0.05), and end-systolic dimensions (P=0.01), as compared with controls. Additionally, AMS diminished LV end-diastolic (P<0.01) and end-systolic areas (P<0.05), compared with controls. These favorable effects of AMS were associated with improved fractional shortening 5 weeks after MI (P<0.05; Figure 5f).

Discussion

The major new finding of the present study suggests that early after MI, injection of ex vivo activated AMS promotes myofibroblast accumulation, vascularization, and scar thickening. The sum effect of scar thickening is reduction of wall stress (Laplace law), improved stabilization of chamber size, prevention of infarct expansion, and improved post-MI function. Our findings extend the data on the role of activated macrophages in tissue healing and suggest a new approach for improving myocardial repair, particularly in elderly and sick patients. In addition, the present work challenges the dogma that inflammatory cells are always deleterious to the ischemic and infarcted myocardium. Inflammation and collagen synthesis are important steps that affect heart repair after MI.1 In fact, MI patients treated with anti-inflammatory drugs have experienced increased incidence of myocardial expansion, rupture, and death.17

Tissue Repair by Activated Macrophages

Macrophages have key functions in almost every stage of wound healing.18–20 In inflammation, macrophages have 3 major functions: antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors.21 Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation; inhibition of inflammation by removal or deactivation of mediators and inflammatory cells permit the host to repair damaged tissue.21 Activated macrophages are deactivated by anti-inflammatory cytokines such as IL-10 and transforming growth factor-β, and cytokine antagonists that are produced mainly by macrophages.7,21 In addition, macrophages control tissue vascularization after injury by releasing growth factors, matrix metalloproteinases (MMPs), and their inhibitors.22
Macrophages may also promote neovascularization directly by “drilling” into the extracellular matrix. Certain macrophage populations also have the potential to transform into vascular cells. Thus, activated macrophages might modulate local inflammatory response, suppress local injury, and promote tissue vascularization, healing, and repair.

In animal models, depletion of macrophages using antimacrophage serum showed impaired wound healing and decreased matrix production and fibrosis, indicating that macrophages are responsible for laying down matrix. Moreover, Danon et al showed that wound repair was enhanced in old mice by local injection of macrophages derived from young mice.

**Macrophages and Infarct Healing**

Monocytes, macrophages, and their cytokine products have been shown to accelerate vascularization in ischemic tissue, and promote infarct healing, myocyte protection, and possibly regeneration. In a rat model of spinal injury, local implantation of macrophages that were pre-stimulated ex vivo resulted in nerve regeneration and partial functional recovery. These unique regenerative properties of macrophages could contribute to myocardial tissue repair and improve LV remodeling and function. In the present study, AMS injection was associated with improved vascularization, myofibroblast accumulation, scar thickening, and accumulation of resident macrophages, which, in turn, might contribute to infarct healing.

However, the role of macrophages in infarct repair is complex because macrophages produce a wide range of biologically active molecules participating in both beneficial and detrimental outcomes in inflammation. Macrophages could be beneficial in the early stage of infarct healing but deleterious during the late phase of scar formation and LV remodeling. Thus, control of macrophage activity by regulatory feedback is essential for effective healing and repair as well as avoiding excessive fibrosis. In the present study, lifespan of injected macrophages in the scar ranged from 4 to 7 days after injection and effectively improved healing and repair.
Limitations
Macrophages have been implicated in the pathogenesis of atherosclerosis and restenosis and injected AMS could theoretically accelerate atherosclerosis. However, we believe that by local delivery and targeting of macrophages into the necrotic tissue, vascular adverse effects can be avoided. The increase in tissue vascularization could be related to the inflammatory response and recruitment of resident macro-

TABLE 2. Comparison of LV Remodeling and Function Between Activated Macrophage Suspension and the Control Group by 2D Echocardiography, Before (Baseline) and 5 Weeks After Injection.

<table>
<thead>
<tr>
<th></th>
<th>Activated Macrophage Suspension (n=8)</th>
<th>Control (n=9)</th>
<th>5 Weeks Macrophages vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 5 Weeks</td>
<td>Baseline 5 Weeks</td>
<td>Baseline 5 Weeks</td>
</tr>
<tr>
<td>AW d, cm</td>
<td>0.15±0.01 0.15±0.01</td>
<td>0.9</td>
<td>0.15±0.02 0.11±0.01</td>
</tr>
<tr>
<td>LVEDD, cm</td>
<td>0.72±0.02 0.87±0.03</td>
<td>0.001</td>
<td>0.75±0.02 0.99±0.04</td>
</tr>
<tr>
<td>LVESD, cm</td>
<td>0.51±0.04 0.64±0.04</td>
<td>0.003</td>
<td>0.55±0.04 0.77±0.07</td>
</tr>
<tr>
<td>LVEDA, cm²</td>
<td>0.46±0.02 0.66±0.03</td>
<td>&lt;0.0001</td>
<td>0.45±0.02 0.86±0.05</td>
</tr>
<tr>
<td>LVESA, cm²</td>
<td>0.26±0.04 0.42±0.03</td>
<td>0.002</td>
<td>0.28±0.03 0.58±0.06</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>27±3 30±5*</td>
<td>0.19</td>
<td>27±4 20±4</td>
</tr>
</tbody>
</table>

AW d indicates anterior wall diastolic thickness; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVEDA, LV end diastolic area; LVESA, LV end systolic area; LVFS, LV fractional shortening \([\text{LVIDd}−\text{LVIDs}/\text{LVIDd}] \times 100.\)

Figure 6. Comparison of LV remodeling and function between AMS (red bars) and controls (white bars) by 2-dimensional echocardiography study, before (baseline) and 5 weeks after injection. Compared with controls, AMS improved scar thickening (a); attenuated LV diastolic dilatation (b); LV systolic dilatation (c); LV diastolic area (d); LV systolic area (e); and improved LV fractional shortening (f). At 5 weeks after injection, all differences between groups are significant \((P<0.05).\)
phages triggered by the injection of human AMS into the rat heart. Recent observations have suggested that injection of human endothelial progenitor cells promote wound healing and vascularization by recruitment of local resident monocytes and macrophages.\(^7\) Although the present study did not rule out such a possibility, it should be noted that the healing capacity of macrophages has been proven in syngeneic animal models of wound healing.\(^9\) In addition, in elderly and sick patients with impaired resident macrophage function, injection of allogeneic AMS promotes wound healing and confirms the therapeutic effect of donor AMS.\(^11,12\) Finally, the beneficial effect of allogeneic cells can be explained by the modulation of local immune reactions in response to apoptosis of the transplanted cells.\(^7\) It has been suggested that apoptotic cell ingestion by macrophages induces expression of anti-inflammatory cytokines that could suppress the damage of excessive inflammatory response.\(^7\)

**Summary and Implications**

The present work suggests that early after myocardial injury, activated macrophages may have therapeutic role by promoting myocardial healing and repair. Our findings provide “proof of concept” and could have therapeutic implications for patients with impaired healing and regenerative capacity, such as elderly and diabetic patients. Therapeutic intervention targeting macrophages and their products could open up new avenues for controlling myocardial damage and improving regeneration and repair.

**Acknowledgments**

We thank Parvin Zarlin (immunohistochemistry) and Patricia Benjamin (echocardiography) for excellent technical assistance.

**Disclosures**

J. Leor and D. Danon are named as coinventors on a pending patent filed by Tel-Aviv University that relates to the use of activated macrophages for myocardial repair.

**References**

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