Activated macrophages decrease rat cardiac myocyte contractility: importance of ICAM-1-dependent adhesion

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Simms, Matthew G., and Keith R. Walley. Activated macrophages decrease rat cardiac myocyte contractility: importance of ICAM-1-dependent adhesion. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H253–H260, 1999.—Macrophages are found in the heart as part of the inflammatory response. To determine whether macrophages could contribute to myocardial dysfunction, rat ventricular myocytes were isolated and cocultured with elicited peritoneal macrophages in media containing tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, or endotoxin for 4 h. Cardiac myocytes were electrically stimulated, and fractional shortening was determined using videomicroscopy. When myocytes alone or myocytes in coculture with macrophages separated by a membrane were challenged with TNF-α, lipopolysaccharide, or IL-1β, fractional shortening did not decrease. When macrophages were allowed to contact myocytes, fractional shortening decreased from 20.1 ± 0.9% in unchallenged macrophage-myocyte co cultures to 15.5 ± 0.9, 16.3 ± 0.8, and 15.8 ± 0.6% when challenged for 4 h with TNF-α, endotoxin, or IL-1β, respectively (P < 0.05). Myocytes had a mean adherence of 4.2 ± 0.2 macrophages after TNF-α challenge compared with 2.6 ± 0.3 for controls (P < 0.05). The number of adherent macrophages was associated with the decrease in fractional shortening. Anti-intercellular adhesion molecule-1 (ICAM-1) reduced macrophage adherence and prevented the decrease in fractional shortening. This decrease was also prevented by desferoxamine, superoxide dismutase, and nitro-L-arginine methyl ester. This suggests that activated macrophages adhere to myocytes via ICAM-1, and adherent macrophages decrease their contractile function via TNF-α, oxygen free radicals, and nitric oxide.

ischemia-reperfusion; sepsis; tumor necrosis factor-α; intercellular adhesion molecule-1

MACROPHAGES ARE FOUND in the heart after ischemia-reperfusion (3, 14), in inflammatory cardiomyopathies (1, 6, 20), after orthotopic heart transplantation (28), and in animal models of sepsis (6, 20). Activated polymorphonuclear neutrophils can kill myocytes (8) and contribute to decreased myocardial contractility after ischemia-reperfusion (34) and in models of sepsis (12). However, mononuclear leukocytes, not neutrophils, are the predominant leukocyte population within the heart in a number of inflammatory states (1, 6, 32, 37). Whether the macrophage subset contributes is not known. Macrophages release tumor necrosis factor-α (TNF-α), nitric oxide (NO), and reactive oxygen intermediates, all of which can contribute to decreased cardiac myocyte contractility (4, 9, 30, 35). Therefore, intramyocardial macrophages could conceivably contribute to myocardial depression, although very little is known about the interaction of macrophages with cardiac myocytes.

Our goal was to determine whether macrophages could alter cardiac myocyte function. To address this issue, we first cocultured cardiac myocytes and macrophages with various inflammatory mediators and measured cardiac myocyte contractile function. We also examined macrophage adhesion to myocytes and the role of receptor-mediated adhesion in myocyte contractile dysfunction. Finally, we investigated the potential mechanisms whereby adherent macrophages could mediate myocyte contractile dysfunction, including TNF-α, NO, and reactive oxygen intermediates.

METHODS

This study was approved by the University of British Columbia Animal Care Committee and adheres to Canadian and National Institutes of Health guidelines for animal experimentation.

Isolation of Rat Ventricular Myocytes

Male Sprague-Dawley rats (250–300 g) were anesthetized with 3% halothane by inhalation, and 1,000 units of heparin (Organon Teknika) were injected intraperitoneally. Fifteen minutes later, a midline thoracotomy was performed, and the heart was excised and placed in oxygenated (95% O2-5% CO2) HEPES-10 kU/l modified MEM buffer (BiGCO-BRL, Grand Island, NY) at 0°C. Within 2 min, the heart was mounted, via the aorta, on a modified Langendorff apparatus and was perfused with oxygenated MEM at 37°C for 2 min. After blood in the coronary circulation was cleared, the perfusate was changed to 30 ml of recirculating Ca2+-free MEM containing 236 U/ml collagenase (type 2; Worthington Biochemical, Freehold, NJ). At 15 and 20 min, the Ca2+ concentration was increased stepwise to 0.025 and 0.075 mmol/l by adding 75 and 150 µl of CaCl2 (10 mM), respectively, to the recirculating buffer. After a 30- to 40-min period of digestion, the ventricles were removed from the perfusion system and gently teased apart. The tissue, in oxygenated 0.075 mmol/l Ca2+-free perfusion buffer, was then agitated for 2–3 min at 37°C in a water bath. The tissue and dispersed cells in solution were then filtered through a 200-µm nylon mesh. The filtrate was then centrifuged at 500 rpm for 2 min, and the supernatant was replaced with fresh perfusion buffer. The cells were then washed three times at 37°C in MEM containing increasing Ca2+ concentrations (200 µM, 500 µM, and 1 mM), with the cells allowed to settle by gravity for 10 min between each wash. Cells were resuspended in 37°C HEPES-modified medium 199 (M199) buffer (BiGCO-BRL) with 1% BSA (Sigma Chemicals, St. Louis, MO), and viable cell concentration was determined using a hemocytometer. The cells were diluted to a final concentration of 50,000 cells/ml. The cells were loaded into laminin-coated 96-well plates (VWR CanLab) at 100 µl/well.
(5,000 cells/well) and were incubated with 95% O2-5% CO2 at 37°C. At 90 min, the medium was changed to fresh M199 with BSA and was incubated for 24 h. Cells were considered viable if they demonstrated a characteristic rod shape without cytoplasmic blebbing. This morphometric assessment of viability was confirmed in a subset of experiments with trypan blue exclusion. We found that the fraction of viable myocytes was always >85%. Myocyte cultures were then assessed for endotoxin content using the Limulus amebocyte lysate assay (Associates of Cape Cod) and were found to contain no more than 0.03 endotoxin units/ml.

Isolation of Peritoneal Macrophages

Ten milliliters of sodium caseinate were injected intraperitoneally in rats anesthetized using 3% halothane by inhalation. The rats were allowed to recover and 72 h later were again anesthetized using 3% halothane by inhalation. Peritoneal lavage was performed using three aliquots of 30 ml of normal saline containing 1 U/ml heparin at 37°C. Peritoneal lavage fluid was then filtered through sterile gauze in 50-ml tubes. Cells were then centrifuged at 1,700 rpm for 6 min. The pellet was resuspended in four volumes of NH4Cl solution to lyse the red blood cells, centrifuged again, and then resuspended in M199 buffer containing 1% BSA. After a final centrifugation, the pellet was resuspended in M199 buffer with BSA, and the concentration of macrophages was determined using a hemocytometer. The concentration of macrophages was then adjusted to 500,000 cells/ml. The percentage of macrophages was found to be 90–95%, and these cells were viable as indicated by trypan blue exclusion.

Coculture of Macrophages and Cardiac Myocytes

After 24 h of cardiac myocyte incubation, 50,000 macrophages were added to each well of cardiac myocytes (ratio of 10 macrophages/cardiac myocyte) in the 96-well laminin-coated plates for coculture experiments. These cocultures were incubated at 37°C in 95% O2-5% CO2 for 4 h. After the 4-h incubation, fractional shortening of electrically stimulated myocytes and the number of adhering macrophages per myocyte were determined.

Measurement of Cardiac Myocyte Fractional Shortening

Fifteen minutes before the 4-h time point, 2 µl of trypsin (0.5% wt/vol) were added to each well to cleave the myocytes off the laminin-coated bottom. Preliminary experiments demonstrated that this concentration of trypsin did not alter cell viability and cleaved >95% of the adherent myocytes from their attachments to the bottom of the well. Specially designed platinum electrodes were then lowered in each well in the 96-well plate, and the cardiac myocytes were electrically stimulated (Grass S48 stimulator, West Warwick, RI; 45 V, 2.2-ms duration, 25-Ω resistance) while being recorded by videomicroscopy (Sony SLV-760HF; Fig. 1). This electrical stimulus was chosen from preliminary threshold experiments as two times the minimum electrical stimulus required to maximally contract the cardiac myocytes. Still frames of systolic and diastolic myocytes were captured for computer analysis from the video recording, and myocyte fractional shortening was then measured using Scion ImagePC (Scion, Frederick, MD).

ELISA for ICAM-1 Expression

The cardiac myocyte culture without added macrophages was challenged with human recombinant TNF-α (R&D Systems, Minneapolis, MN) in concentrations of 0–50 ng/ml for 4 h. Intercellular adhesion molecule-1 (ICAM-1) expression was then quantified by ELISA. Briefly, after the 4-h incubation, cells were fixed using 4% paraformaldehyde (100 µl/well) for 10 min at room temperature and then were washed two times with 1× PBS and stored at 4°C until the ELISA. Plates were then washed two times with 1× PBS and blocked by adding 250 µl/well of 5% normal goat serum (Dako Diagnostics Canada, Mississauga, ON) in PBS for 1 h at 37°C. Next, 100 µl/well of diluted 1:5,000 rabbit anti-rat ICAM-1 (gift from Dr. P. Ward, University of Michigan) in PBS was added, after the 1× PBS wash, and was incubated for 1 h at 37°C. The wash was then repeated, and 100 µl/well of a biotinylated goat anti-rabbit antibody complex (Dako Diagnostics Canada), diluted 1:5,000 in PBS, was added and incubated at 37°C for 30 min. A chromogen substrate (OPD; Dako) was then added, and the reaction was halted with 0.5 M H2SO4. Plates were then read at 490 nm using a microplate reader (Rainbow

Fig. 1. Diastolic (left) and systolic (right) videomicroscopic images of a typical cardiac myocyte. Fractional shortening is determined as the difference between diastolic length and systolic length, divided by diastolic length. Dotted line (left) indicates measurement of myocyte length by computer.
Fractional shortening of myocytes. We studied 1) cultures of cardiac myocytes alone, 2) cultures of cardiac myocytes with macrophages added in a culture well insert (0.45-µm pores so that myocytes and macrophages were cultured in the same well but the cells were kept separated), and 3) cocultures of cardiac myocytes and macrophages in which cell interaction was not prevented. TNF-α, lipopolysaccharide (LPS), interleukin (IL)-1β, or control saline was added to each well at the start of the cardiac myocyte-macrophage coculture. The final concentration of TNF-α in the wells was 20 ng/ml, chosen to elicit maximum effect based on initial dose-response experiments. In these experiments, TNF-α added to myocyte-macrophage cocultures in concentrations of 5, 10, 20, 50, and 100 ng/ml decreased fractional shortening from control by 3.2, 15.4, 16.1, 14.2, and 12.4% and increased 5, 10, 20, 50, and 100 ng/ml decreased fractional shortening added to myocyte-macrophage cocultures in concentrations of (LPS), interleukin (IL)-1β, or control saline was added to each well at the start of the cardiac myocyte-macrophage coculture. The final concentration of TNF-α in the wells was 20 ng/ml, chosen to elicit maximum effect based on initial dose-response experiments. In these experiments, TNF-α added to myocyte-macrophage cocultures in concentrations of 5, 10, 20, 50, and 100 ng/ml decreased fractional shortening from control by 3.2, 15.4, 16.1, 14.2, and 12.4% and increased adherence from a control of 1.89 to 2.84, 3.13, 2.96, 3.71, and 3.70%, respectively. The final concentration of IL-1β was 20 ng/ml, and the final concentration of endotoxin was 10 µg/ml. At the end of a 4-h incubation, one microscope field was randomly selected in each well and was electrically stimulated one time. Generally, one to four myocytes were present in each field. Diastolic length and fractional shortening were determined for each experiment.

Macrophage adherence to myocytes. Cocultured myocytes and macrophages were challenged using TNF-α, IL-1β, LPS, or vehicle. TNF-α and IL-1β were added at a concentration of 20 ng/ml whereas LPS was added at 10 µg/ml at the start of the 4-h coculture. Next, the number of adherent macrophages to myocytes was counted. In additional experiments to determine the role of adhesion molecules expressed by cardiac myocytes, either antibodies to ICAM-1 or an IgG control was added at 200 µg/ml, immediately before stimulation. The number of macrophages adherent to myocytes after 4 h was counted.

Fractional shortening of cardiac myocytes under these experimental conditions, fractional shortening did not change in 4-h cardiac myocyte cultures exposed to tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), or interleukin (IL)-1β. Similarly, when contact between myocytes and macrophages was prevented by culture well inserts, challenge with TNF-α, LPS, or IL-1β did not alter contractility. However, in cocultures activated by TNF-α, endotoxin, or IL-1β where macrophages could contact myocytes, fractional shortening significantly decreased (*P < 0.05). Nos. in bars are n of myocytes sampled.
length) was not significantly altered by TNF-\(\alpha\) (61.39 units of length) or LPS (68.82 units of length) so that the decrease in fractional shortening was due to a change in systolic contractility. In these experiments, the number of adherent macrophages was significantly greater in co-cultures challenged with TNF-\(\alpha\), LPS, and IL-1 (2.8 \pm 0.2, 3.0 \pm 0.2, and 2.6 \pm 0.2 macrophages per myocyte) than in unchallenged co-cultures (1.8 \pm 0.1 macrophages per myocyte; \(P < 0.05\)). Thus macrophage adhesion is important in decreasing myocyte function.

Anti-ICAM-1 prevented increased adherence of macrophages to cardiac myocytes induced by TNF-\(\alpha\), IL-1\(\beta\), and LPS in macrophage-myocyte co-culture (Fig. 3). IgG control showed similar results as with no antibody. ELISA for ICAM-1 showed that, when myocytes were challenged with human recombinant TNF-\(\alpha\), the expression of ICAM-1 on cardiac myocytes was dose-dependent, reaching a plateau at a dose of 20 ng/ml of TNF-\(\alpha\) (Fig. 4).

To determine if ICAM-1-mediated adhesion played a role in causing the decrease in cardiac myocyte function induced by activated macrophages, we added either anti-ICAM-1 or IgG control to co-cultures. Anti-ICAM-1 prevented the decrease in myocyte fractional shortening induced by TNF-\(\alpha\), LPS, or IL-1\(\beta\) challenge, whereas IgG controls were similar to having no antibody (Fig. 5).

We then examined the role of oxygen free radicals, NO, and peroxynitrite. As in previous experiments, TNF-\(\alpha\) challenge of macrophage-myocyte co-culture significantly decreased cardiac myocyte fractional shortening (Fig. 6). This decrease was completely inhibited by desferoxamine, SOD, and L-NAME. Urate did not prevent the decrease in fractional shortening to a statistically significant degree. Urate was sampled at 10 times the concentration and 10 times less than the concentration with or without TNF-\(\alpha\) challenge. When unchallenged, concentrations of urate had no significant effect on fractional shortening (19.77 \pm 1.35\% compared with...
18.22 ± 1.51, 21.07 ± 0.97, and 20.22 ± 1.48%). However, when challenged, urate had an effect starting at the normal concentration (20.03 ± 0.90% compared with 17.45 ± 1.69%, P < 0.05). Beyond that, the effect reached a plateau (19.81 ± 1.02%). The number of adherent macrophages did not decrease to account for improved fractional shortening with desferoxamine, SOD, or L-NAME.

The fractional shortening of unchallenged cocultures was also examined to control for direct effects of the inhibitors. When fractional shortening of the unchallenged rat cardiac myocyte coculture was determined along with exposure to either SOD, L-NAME, urate, or desferoxamine, no decrease in shortening was observed compared with controls. Myocyte viability was not altered between groups. When challenged with LPS, anti-TNF-α prevented the decrease in fractional shortening (Fig. 7; P < 0.05).

**DISCUSSION**

Taken together, these observations demonstrate that activated macrophages can contribute to cardiac myocyte contractile dysfunction. Adherence of activated macrophages to ICAM-1 expressed on activated cardiac myocytes appears to be an important step in mediating the observed contractile dysfunction. TNF-α plays a role by contributing to macrophage activation and adhesion and, possibly, by mediating part of the effect of adherent macrophages. Both NO and oxygen free radicals contribute to the myocardial depressant effect of adherent macrophages.

Although neutrophils have been the focus of much attention in ischemia-reperfusion and sepsis (7, 8, 12, 15, 31, 34), macrophages may also play an important role. Macrophages and macrophage-derived mediators have been implicated in myocardial dysfunction in a...
number of myocardial inflammatory states (1, 3, 5, 6, 10, 14, 20, 28). Macrophages and mononuclear leukocytes can dominate the myocardial leukocytes infiltrating the myocardium in cardiomyopathies and myocarditis (1, 6, 32) and during rejection of orthotopic heart transplants (5, 10). In some animal models of septic myocardial dysfunction (24), macrophages are the predominant leukocyte subset in the heart (37). Recent results suggest that, after ischemia-reperfusion, neutrophils may not be the only leukocyte subset contributing to myocardial damage and dysfunction (21). In patients who underwent orthotopic heart transplantation, a significant increase in macrophage infiltration of the heart was observed along with a corresponding increase in TNF-α production by the heart (10). This observation points out that macrophages are particularly interesting because they could contribute to both mediator and leukocyte-induced myocardial depression.

Macrophage release of proinflammatory cytokines may be important in mediating myocardial dysfunction (9, 24, 30, 33). However, it is interesting to note that, over the short time course of our observations, TNF-α alone did not decrease fractional shortening, consistent with previous observations (22). TNF-α administered alone in whole animal models (24, 33) or as one of the mediators released after endotoxin infusion (16) results in a decrease in ventricular contractility within 4–6 h. TNF-α may act synergistically with IL-1β (22) and appears to decrease myocardial contractility by activation of leukocytes and other mediator pathways (9, 30). A number of investigators have shown that part of the effect of proinflammatory cytokines in vitro is due to stimulation of NO production in cardiac myocytes (4, 9, 26). Similarly, in isolated working hearts, Schulz et al. (30) demonstrated that TNF-α and IL-1β contribute to decreased contractile function via NO and peroxynitrite (38). Antibody to TNF-α decreases the functional impairment induced in sepsis models and by ischemia-reperfusion (13). Ischemia-reperfusion upregulates expression of mRNA for the proinflammatory cytokines TNF-α and IL-1β in the heart (17). In isolated rat hearts, myocardium releases significant amounts of TNF-α after ischemia-reperfusion (14), commonly associated with the accumulation and activation of macrophages. TNF-α may contribute to further leukocyte accumulation by stimulation of chemokine production (23) and upregulation of leukocyte adhesion molecules on cardiac myocytes (19). Thus cytokine mediators released from macrophages can contribute to myocardial dysfunction in myocardial inflammatory states. Other studies suggest a role for leukocytes in mediating myocardial depression during myocardial inflammatory states (8, 24), similar to the role of leukocytes in causing other organ system dysfunction. In animal models of sepsis, leukocytes are slowed and retained in myocardial capillaries (11). This is associated with capillary endothelial and cardiac myocyte damage, myocardial edema (33), and decreased contractility (12). Similarly, leukocytes decrease contractility in animal models of ischemia-reperfusion (15, 34). Activated neutrophils have been identified to be particularly important (34). However, the role of the monocyte-macrophage population of leukocytes has not been extensively investigated.

Our results extend previous findings by demonstrating that activated macrophages can contribute to myocardial dysfunction when they adhere to cardiac myocytes via ICAM-1. The importance of adherence of macrophages to myocytes was demonstrated by prevention of myocyte dysfunction as follows: 1) when macrophages were separated from myocytes using porous culture well inserts, 2) when increased macrophage adherence was blocked by anti-ICAM-1, and 3) by the observed relationships between macrophage adherence and the decrease in myocyte fractional shortening.

Fig. 7. Fractional shortening of rat cardiac myocytes in coculture with macrophages after 4-h exposure to unchallenged media and with exposure to LPS. When added to coculture, either SOD, L-NAME, urate, or desferoxamine showed no decrease in fractional shortening compared with controls (χ). Anti-TNF-α prevented the decrease in fractional shortening induced by LPS challenge alone († compared with †, P < 0.05). Nos. in bars are no. of myocytes sampled.
ICAM-1 expression on cardiac myocytes is minimal under normal conditions (31) but increases in inflammatory states. We found that TNF-α increased cardiac myocyte ICAM-1 expression in a dose-response manner. This action of TNF-α may be an important step in mediating leukocyte-induced myocardial depression in inflammatory states such as ischemia-reperfusion, cardiomyopathy, posttransplant rejection, and sepsis. Macrophage binding to cardiac myocytes involved ICAM-1.

ICAM-1 expression on cardiac myocytes has been observed after TNF-α, IL-1β, and monocyte chemotactic protein-1 challenge (29). ICAM-1-mediated adherence of neutrophils to cardiac myocytes has previously been observed (7, 31). Adherence of neutrophils to myocytes is also blocked by anti-CD18 identifying the ICAM-1 receptor ligand as a β2-integrin in this setting (7). Cytokine induction of expression of cell adhesion molecules (2, 31) on cardiac myocytes may be an important component in cardiac inflammation.

TNF-α, oxygen free radicals, and NO play a role in mediating the contractile dysfunction induced by activated, adherent macrophages. Adherence could contribute to decreased contractility in at least two ways. First, adherence may simply keep macrophages in very close proximity to cardiac myocytes so that mediator concentrations (for example, TNF-α and IL-1β, as well as oxygen free radicals and NO) are high in the immediate vicinity of the cardiac myocyte. In other tissues, leukocytes can adhere to cells, enveloping a pocket between the two cells that may contain very high concentrations of mediators. Thus, physical proximity may be an important issue. The second possibility is that cardiac myocyte ICAM-1 may act as a receptor that, by itself or in conjunction with costimuli, may result in intracellular signaling leading to decreased contractile function. Oxygen free radicals and NO could mediate part of their effect by direct tissue damage (12), by formation of peroxynitrite (30), or as components of intracellular signal transduction, for which there is growing evidence (29, 36).

It is important to recognize that we used macrophages that had emigrated from the blood to the peritoneal cavity. Emigrated leukocytes express different cell adhesion molecules than the circulating blood population (27). Although peritoneal macrophages may differ from those recruited to the heart during an inflammatory response, we felt that emigrated peritoneal macrophages may better model emigrated intramyocardial macrophages than peripheral blood monocytes.

In summary, activated macrophages adhering to cardiac myocytes via ICAM-1 can contribute to myocardial contractile dysfunction. The effects of adherent macrophages are mediated in part by oxygen free radicals and NO.

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REFERENCES


