Resident Cardiac Mast Cells Degranulate and Release Preformed TNF-α, Initiating the Cytokine Cascade in Experimental Canine Myocardial Ischemia/Reperfusion

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Background—Neutrophil-induced cardiomyocyte injury requires the expression of myocyte intercellular adhesion molecule (ICAM)-1 and ICAM-1–CD11b/CD18 adhesion. We have previously demonstrated interleukin (IL)-6 activity in postischemic cardiac lymph; IL-6 is the primary stimulus for myocyte ICAM-1 induction. Furthermore, we found that induction of IL-6 mRNA occurred very early on reperfusion of the infarcted myocardium. We hypothesized that the release of a preformed upstream cytokine induced IL-6 in leukocytes infiltrating on reperfusion.

Methods and Results—Constitutive expression of TNF-α and not IL-1β was demonstrated in the normal canine myocardium and was localized predominantly in cardiac mast cells. Mast cell degranulation in the ischemic myocardium was documented by demonstration of a rapid release of histamine and TNF-α in the cardiac lymph after myocardial ischemia. Histochemical studies with FITC-labeled avidin demonstrated degranulating mast cells only in ischemic samples of canine myocardium. Immunohistochemistry suggested that degranulating mast cells were the primary source of TNF-α in the ischemic myocardium. In situ hybridization studies of reperfused myocardium localized IL-6 mRNA in infiltrating mononuclear cells and in mononuclear cells appearing in the postischemic cardiac lymph within the first 15 minutes of reperfusion. Furthermore, isolated canine mononuclear cells incubated with postischemic cardiac lymph demonstrated significant induction of IL-6 mRNA, which was partially blocked with a neutralizing antibody to TNF-α.

Conclusions—Cardiac mast cells degranulate after myocardial ischemia, releasing preformed mediators, such as histamine and TNF-α. We suggest that mast cell–derived TNF-α may be a crucial factor in upregulating IL-6 in infiltrating leukocytes and initiating the cytokine cascade responsible for myocyte ICAM-1 induction and subsequent neutrophil-induced injury. **(*Circulation*. 1998;98:699-710.)**

Key Words: cells ■ ischemia ■ reperfusion ■ myocardial infarction ■ cytokines

The association of inflammation with myocardial infarction has been recognized for more than a century[1] and is properly considered part of the healing process. In recent years, the potential role of accelerated inflammation in extension of injury after reperfusion of the infarcted myocardium has been suggested by a variety of experimental studies. Strategies designed to deplete neutrophils,[2] inhibit complement,[3] or block critical adhesion molecules controlling leukocyte trafficking[4,5] have been effective in reducing infarct size.

Our laboratory has concentrated on characterizing the biological basis for inflammatory injury in a canine model of reperfused myocardial infarction. Early in vitro experiments suggested that neutrophil adhesion to cardiac myocytes is dependent on CD18 integrin activation[6] and also on the induction of ICAM-1 on cardiac myocytes.[7] Additional studies showed that Mac-1/ICAM-1–dependent neutrophil adherence to cardiac myocytes activates the neutrophil respiratory burst accompanied by a highly compartmented transfer of reactive oxygen and resultant myocyte oxidative injury.[8] Thus, we postulated that induction of myocyte ICAM-1 was an essential factor for neutrophil-induced cardiac injury.

We developed a canine model of a chronically cannulated cardiac lymph duct to obtain cardiac extracellular fluid under conditions in which the inflammatory mediators associated with acute surgery have dissipated. After reperfusion of the infarcted myocardium, we demonstrated the rapid appearance in cardiac lymph of activity capable of stimulating ICAM-1 expression on isolated cardiac myocytes.[9,10] The observation that an anti–IL-6 antibody neutralized the synthesis of ICAM-1 in cardiac myocytes stimulated by postischemic cardiac lymph suggested that myocyte ICAM-1 induction was mediated by IL-6.[9] These findings led to in vivo

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Selected Abbreviations and Acronyms

DAB = diaminobenzidine
ICAM-1 = intercellular adhesion molecule-1
IL = interleukin
TNF-α = tumor necrosis factor-α

experiments on ischemic and reperfused myocardium that demonstrated ICAM-1 mRNA induction in the viable myocytes on the border of infarcted tissue.[10,11] IL-6 mRNA induction was seen (with an earlier peak) in the myocardium in the same ischemic segments in which ICAM-1 mRNA is found.[12] The early IL-6 induction and subsequent ICAM-1 upregulation was dependent on reperfusion of the ischemic myocardium. Because leukocyte influx is similarly dependent on reperfusion, we have postulated that leukocytes participate in the cascade leading to myocyte ICAM-1 induction.[12]

Recent findings indicate that mast cells can influence biological responses through the production of cytokines. Gordon and Galli[13] identified mouse mast cells as the first example of a cell type that contains stores of preformed TNF-α. They suggested a potential role of mast cell activation, in part through the release of TNF-α, in influencing the recruitment and function of additional effector cells.[14] Furthermore, Ito and colleagues[15] recently presented evidence suggesting that porcine cardiac mast cell degranulation occurs after intracoronary infusion of C5a.

The present study was designed to investigate the role of resident cardiac mast cells in myocardial ischemia/reperfusion as a potential source of preformed TNF-α. Our data suggest that mast cell degranulation and TNF-α release initiate a cytokine cascade involving IL-6 induction in infiltrating mononuclear cells and subsequent ICAM-1 induction in cardiac myocytes.

Methods

Ischemia/Reperfusion Protocols

We used a chronic canine model of myocardial ischemia and reperfusion.[10,12] This model includes placement of a hydraulically activated occluder on the left circumflex coronary artery and cannulation of the cardiac lymph duct. After surgery, the animals were allowed to recover for 72 hours before occlusion. Ischemia/reperfusion protocols were performed in awake animals as described.[10] The coronary artery was occluded by inflation of the coronary cuff occluder until mean flow in the coronary vessel was zero, as determined by the Doppler flow probe. At the end of 1 hour, the cuff was deflated and the myocardium was reperfused. Reperfusion intervals ranged from 1 to 3 hours. During the experiment, the cardiac lymph was collected in 30-minute intervals. The samples were centrifuged, and the supernatant was collected and immediately frozen at −80°C. The pelleted cells were fixed in 4% paraformaldehyde and used for histological studies. After the reperfusion periods, hearts were stopped by the rapid intravenous infusion of 30 mEq of KCl and removed from the chest for sectioning from apex to base into 4 transverse rings ≈1 cm thick. The posterior papillary muscle and the posterior free wall were identified. Tissue samples were isolated from infarcted or normally perfused myocardium on the basis of visual inspection. Myocardial segments were fixed for histological analysis. Duplicate samples were also processed for blood flow determinations with radiolabeled microspheres as previously described.[10] Samples described as ischemic were all from areas in which ischemic blood flow was <25%. Samples of control tissues were taken from the anterior septum and had normal blood flow during coronary occlusion.

Immunohistochemistry and Histology

For histological study of cardiac tissue, sections taken from endocardium to epicardium were fixed in 4% phosphate-buffered forma-

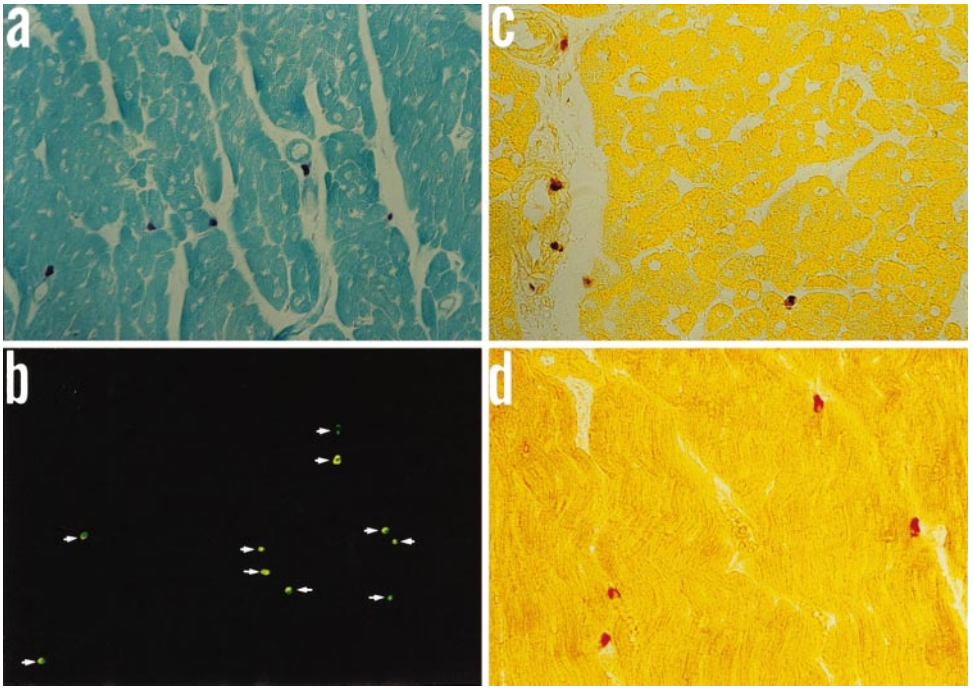

Figure 1. Canine heart contains sizable mast cell population. a, Control canine heart stained with toluidine blue and counterstained with fast green (×400). A significant number of metachromatic cells are seen. b, Control canine heart stained with FITC-avidin to identify resident mast cells (arrows). c, Histochemical staining for tryptase (×400). d, Staining for chymase (×400). Note that mast cells are frequently perivascular.

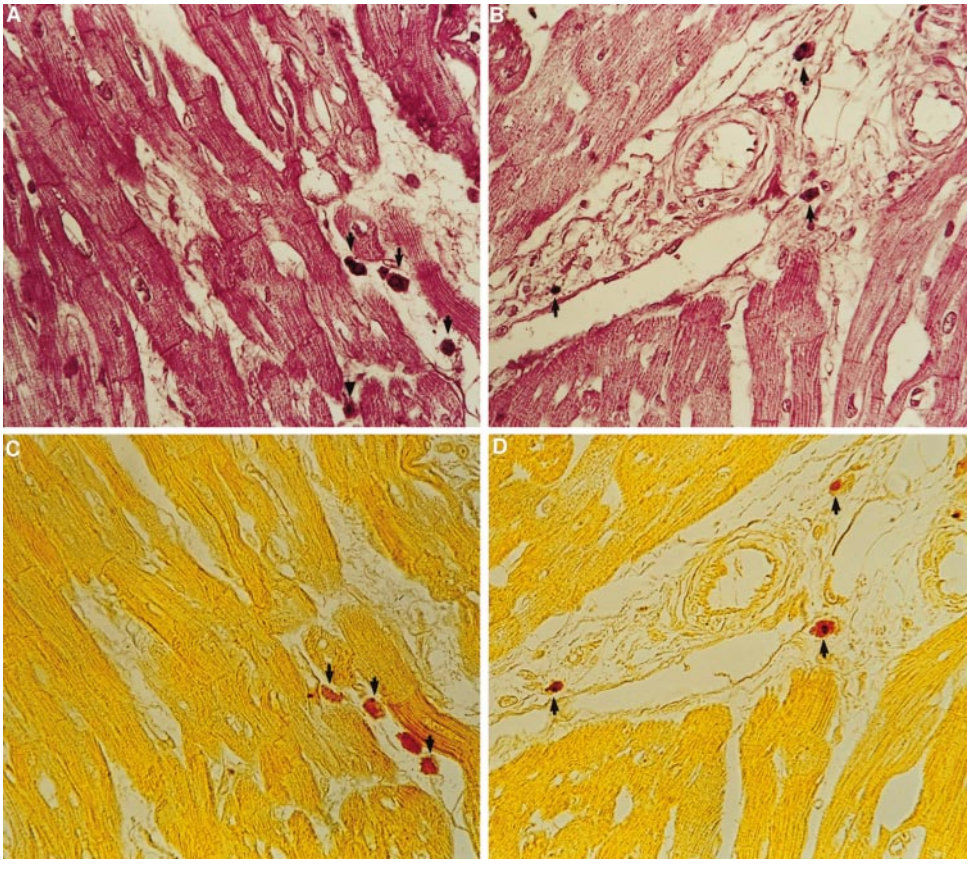

Figure 2. Cardiac resident mast cells contain preformed TNF-α. Serial 5-μm sections of control canine heart were used for histochemical and immunohistochemical staining. A and B, Several TNF-α immunoreactive cells are identified (arrows). C and D, Serial sections stained for tryptase to identify mast cells (arrows) demonstrating TNF-α immunoreactivity.

lin, 2% paraformaldehyde, and Carnoy's, Mota's, or B*5 fixatives and embedded in paraffin. Sequential 2- to 5-μm sections were cut by microtomy. Sections were immunostained for TNF-α adjacent to serial sections stained for tryptase with an enzymatic stain as described by Caughey et al.[16] Chymase activity was detected as previously described by Seppa.[17] Immunostaining was performed with the ELITE rabbit or mouse kit (Vector Laboratories) according to the manufacturer's instructions. The following primary antibodies were used: rabbit polyclonal antibody to human TNF-α (Genzyme) known to cross-react with canine TNF-α,[18] polyclonal antibody to human IL-1β (Genzyme) known to cross-react with canine species,[19] and the neutrophil-specific monoclonal antibody SG8H6.[20] Antibody was detected with a peroxidase-based system using DAB (Vector Laboratories) as a substrate. Slides were counterstained with eosin. Appropriate controls were performed with rabbit or mouse serum substituted for the primary antibody. Fluorescent labeling of mast cells with FITC-avidin was performed as previously described by Bergstresser and colleagues.[21] Double-fluorescent staining was done by fluorescent immunohistochemistry using a rhodamine-labeled anti-rabbit IgG as a secondary antibody and counterstaining with FITC-avidin.

Histamine Assay

Histamine in lymphatic drainage was measured by competitive immunoenzymatic assay[22] (AMAC Inc).

TNF-α Bioassay

Cardiac lymph samples were assayed for TNF-α activity by use of the WEHI 164 subclone 13 fibroblast cytotoxicity assay, as previously described.[23] The WEHI 164 cells are very sensitive to the lytic effects of both murine and human TNF-α, detecting as little as 2 pg/mL. The cytotoxic effect of canine rTNF-α on these cells was recently demonstrated.[24] WEHI cells (5×10^5/mL) were cultured in 96-well microtiter plates (Costar) with test samples and 1 μg/mL actinomycin D (Carbiochem, Boehring Diagnostics). After 20 hours' incubation at 37°C, 180 μL of supernatant in each well was replaced with 180 μL of fresh culture medium with 1 μg/mL of actinomycin D. A 5-mg/mL MTT (20 μL; Sigma) solution was added to all wells, and the plates were incubated at 37ºC. After 4 hours of incubation, 150 μL of supernatant was removed and discarded from all wells, and 100 μL of a 0.04N HCl/isopropanol solution was added to each well to dissolve the crystals characteristic of this particular assay. Plates were wrapped in aluminum foil and stored overnight in a dark moist area at room temperature. The level of lysis was determined with a microELISA Autoreader (550 nm). Units of activity were calculated according to internal rTNF-α standards (Genzyme).

Riboprobe Preparation

Digoxigenin-labeled probes were prepared by in vitro transcription from a linearized template according to the method used by Boehringer Mannheim in the Genius RNA probe labeling kit. A 216-bp fragment of canine IL-6 cDNA was obtained from the published sequence by PCR amplification and was subcloned into the PCR plasmid (Invitrogen) so that the use of SP6 polymerase would result in the generation of single-stranded antisense (3′-5′) and the use of T7 polymerase would result in the generation of the sense (5′-3′) RNA probe. Before beginning the transcription reaction, we linearized the DNA templates by digestion with restriction enzymes that cut downstream of the insert to avoid transcription of undesirable plasmid sequences. The template (1 μg) was incubated in 20 μL of 1×NTP mixture (1 mmol/L ATP, 1 mmol/L GTP, 1 mmol/L CTP, 0.35 mmol/L digoxigenin-UTP, and 0.65 mmol/L UTP), T7 or T3 polymerase (2 U/μL), and DEPC-treated water for 2 hours at 37°C. Both RNA probes were precipitated with glycogen and sodium acetate, washed with 70% ethanol, and resuspended in DEPC-treated

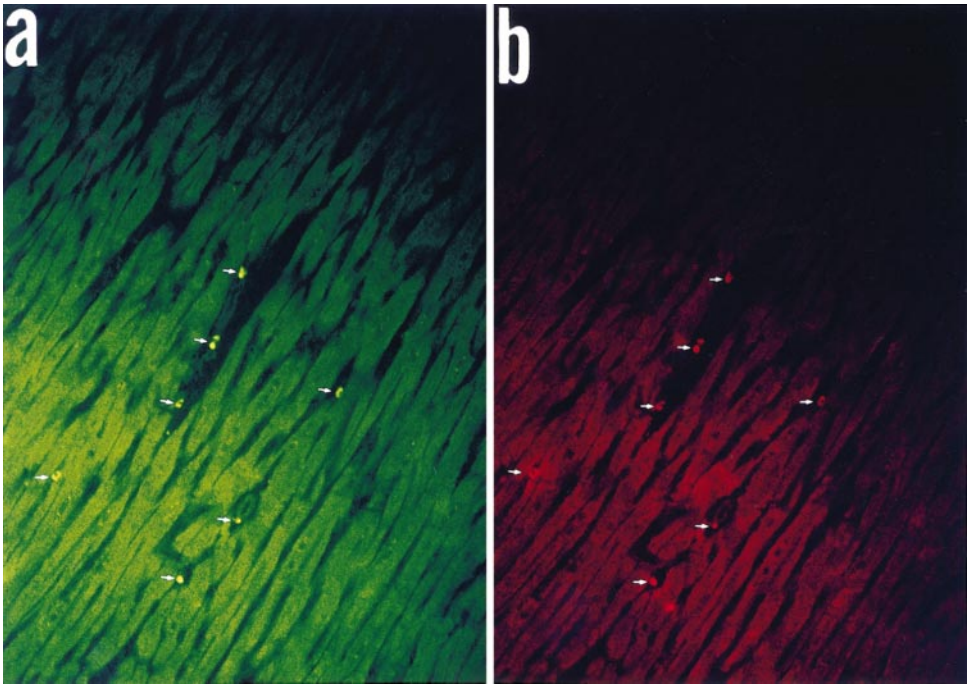

Figure 3. TNF-α in canine heart is localized predominantly in mast cells. Dual fluorescence combining FITC-avidin staining to identify mast cells (a) and immunohistochemical staining for TNF-α with a rhodamine-labeled secondary antibody (b).

water. Both probes were verified by hybridization and detection on a Southern blot (both positive) and a Northern blot (antisense positive) on nylon membrane.

In Situ Hybridization in Tissue Sections

Paraffin-embedded samples fixed with 2% paraformaldehyde were sectioned and deparaffinized by standard protocols and probed with the IL-6–specific digoxigenin-labeled riboprobes. Immunological detection used an alkaline phosphatase–labeled anti-digoxigenin antibody (Boehringer Mannheim) and nitro blue tetrazolium staining of the alkaline phosphatase reaction as previously described.[11] In additional experiments, in situ hybridization was followed by immunostaining with the mouse monoclonal antibody SG8H6, which specifically stains neutrophils.[20] The antibody was detected by use of a peroxidase-based system with 3-amino-9-ethylcarbazole as a substrate.

Mononuclear Cell Isolation and Stimulation

Canine mononuclear cells were isolated by use of a Ficoll/hypaque gradient and resuspended in PBS without calcium and magnesium. For incubation experiments with cardiac lymph, aliquots of lymph were obtained before coronary occlusion and during reperfusion. Mononuclear cells were incubated for 2 hours at 37°C in the presence or absence of recombinant human TNF-α (200 U/mL) (Genzyme). The postischemic cardiac lymph used for these experiments was collected during the first 2 hours of reperfusion. Blocking studies were performed with the addition of the polyclonal neutralizing antibody to human TNF-α IP-300 (7 μL/mL) (Genzyme), which is known to cross-react with canine species. After incubation, a 500-μL aliquot of cells from each tube was removed and fixed in 50% ethanol for 15 to 30 minutes, then resuspended in 75% ethanol and stored at −20°C. The stored cells were subsequently used for in situ hybridization studies. Analysis of IL-6 induction in isolated canine mononuclear cells was performed as follows. Random fields from slides stained for IL-6 were examined with a light microscope at ×400. Five hundred cells from each slide were counted, and the percentage of IL-6 positive cells was calculated. Subsequently, numbers of IL-6–positive cells from different incubation conditions were normalized on the basis of the percentage of positive cells seen in the control sample (medium=1). Eight consecutive experiments were used for quantitative analysis, and ANOVA was used to assess the statistical significance of the findings, followed by Student's *t* test with Bonferroni correction for multiple comparisons.

Statistical Analysis and Sampling

The statistical significance of rising levels of histamine and TNF-α in cardiac lymph was assessed by ANOVA. This was followed by a Student's *t* test, corrected for multiple comparisons (Bonferroni). In histological studies, each experiment and time point was analyzed as a function of time of reperfusion after 1 hour of occlusion. The findings described all occurred in at least 5 consecutive experiments, and degranulation was observed only in ischemic segments. Induction of IL-6 in mononuclear cells was analyzed as described above.

Results

Characterization of Canine Cardiac Mast Cells

Samples from control canine heart were stained with toluidine blue (Figure 1a) and FITC-avidin (Figure 1b), demonstrating a sizable canine cardiac mast cell population, located primarily along vessels. Two populations of mast cells were apparent: 1 resembled "typical" or connective-tissue–type mast cells, which stained with toluidine blue regardless of fixation, and the other population resembled "atypical" or mucosal-type mast cells and exhibited metachromasia only after fixation in Carnoy's or Mota's fixative.[25] Histochemical techniques were used to identify tryptase and chymase activity and demonstrated the presence of tryptase and chymase in most canine cardiac mast cells (Figure 1c and 1d).

TNF-α and Not IL-1β Is Constitutively Expressed in Control Canine Heart and Localized Predominantly in Mast Cells

TNF-α immunoreactivity was present in many resident cells in the canine myocardium (Figures 2 and 3). The positively labeled cells were predominantly perivascular and resembled metachromatically granulated mast cells in number and mor-

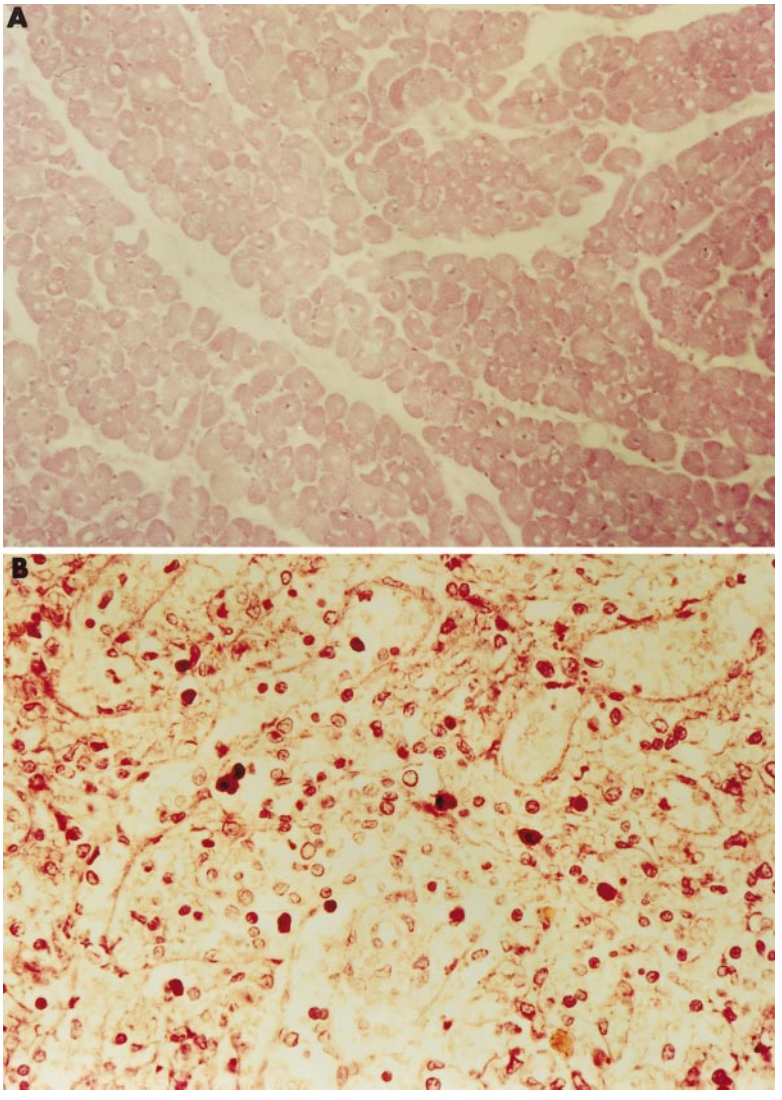

Figure 4. IL-1β is not constitutively expressed in canine heart. A, Peroxidase-based immunohistochemistry shows no IL-1β immunoreactivity in control canine heart. B, Section from spleen of an endotoxin-stimulated animal was used as a positive control.

phology. Comparison of adjacent 5-μm sections from control canine heart revealed that TNF-α was localized predominantly to mast cells (Figure 2A, 2B, 2C, and 2D), identified by their unique granule content of the neutral protease tryptase (Figure 2C and 2D). Double-labeling studies combining fluorescent immunocytochemistry for TNF-α and FITC-avidin counterstaining showed granular cytoplasmic TNF-α immunoreactivity to be confined almost exclusively to FITC-avidin–labeled mast cells (Figure 3). At the antibody concentration used in these studies (Figures 2 and 3), no TNF-α staining was detected in any other cell type found in the myocardium. Similar immunohistochemical experiments using an antibody to IL-1β demonstrated no IL-1β immunoreactivity in the control canine heart (Figure 4).

Histamine Release in the Postischemic Cardiac Lymph During Reperfusion

Histamine concentrations in the cardiac lymph were measured in samples collected from 8 ischemia/reperfusion experiments (Figure 5). Histamine concentration in the preischemic cardiac lymph was 2257$\pm$239 pmol/L. Significant elevations of histamine levels in the postischemic cardiac lymph were noted in 7 of 8 experiments of coronary occlusion (0 to 30 minutes: 2.15$\pm$0.31-fold increase; $P<0.05$, n=8; histamine concentration 4646$\pm$824 pmol/L; range, 1184 to 9986 pmol/L).

Histochemical and Immunohistochemical Evidence of Mast Cell Degranulation

Sections from ischemic and reperfused myocardium were stained with FITC-labeled avidin. As demonstrated in Figure 6A, mast cells in the ischemic and reperfused myocardium showed significant degranulation. In contrast, mast cells in normally perfused segments appeared to be fully granulated (Figure 6B). Degranulating mast cells were absent in the control canine heart.

Immunostaining for TNF-α in ischemic sections after 1 hour of coronary occlusion and 3 hours of reperfusion showed that TNF-α immunoreactivity was localized predominantly in mast cells. No significant IL-1β expression was noted in the ischemic heart in early reperfusion. Some of the TNF-α–positive cells in the ischemic segments showed evidence of degranulation, whereas all the mast cells in the normally perfused tissue samples appeared to be fully gran-

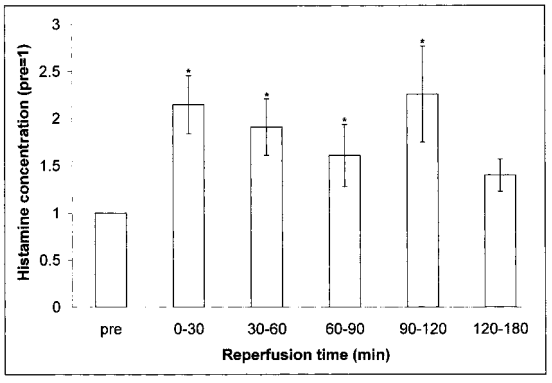

Figure 5. Kinetics of histamine release in postischemic cardiac lymph. Histamine concentration in cardiac lymph was measured in 8 consecutive experiments of experimental canine myocardial infarction. Concentrations from each experiment were normalized (pre=1). A significant early increase in histamine concentration was noted (0 to 30 minutes, 2.15±0.3-fold increase; *$P<0.05$ vs preischemic lymph). Number of experiments of ischemia/reperfusion: n=8 for pre, 0 to 30 minutes, and 30 to 60 minutes; and n=6 for 60 to 120 minutes and 120 to 180 minutes.

ulated. Figure 7 shows a transmyocardial section spanning the region from the endothelial surface to 400 μm into the wall. The mast cells in the subendocardial area show evidence of TNF-α egress (degranulation), whereas those from the midmyocardium (well outside the infarct zone) are not degranulated. In the area of degranulation only, leukocyte and endothelial staining is seen (Figure 8); the potential significance of the latter staining will be discussed below.

Presence of TNF-α Activity in the Cardiac Lymph After Ischemia/Reperfusion

TNF-α bioactivity was measured in lymph samples collected during the ischemia/reperfusion experiments, and the values were expressed as a percentage of the baseline value (Figure 9). TNF-α bioactivity in the preischemic cardiac lymph was 2.6±1.17 pg/mL. Release of TNF-α bioactivity in the cardiac lymph was noted in all experiments and peaked in the first 30 minutes of reperfusion (0 to 30 minutes, 7.94±3.11-fold increase; $P<0.05$, n=8; range, 0.26 to 31.5 pg/mL). A second peak in the third hour after reperfusion was noted in 2 experiments.

Localization of IL-6 mRNA in Mononuclear Cells Infiltrating the Ischemic and Reperfused Myocardium

In situ hybridization for IL-6 mRNA was performed in sections of canine myocardium obtained after occlusion and reperfusion experiments (Figure 10). By examining samples obtained after 1 hour of occlusion and 1 hour of reperfusion,

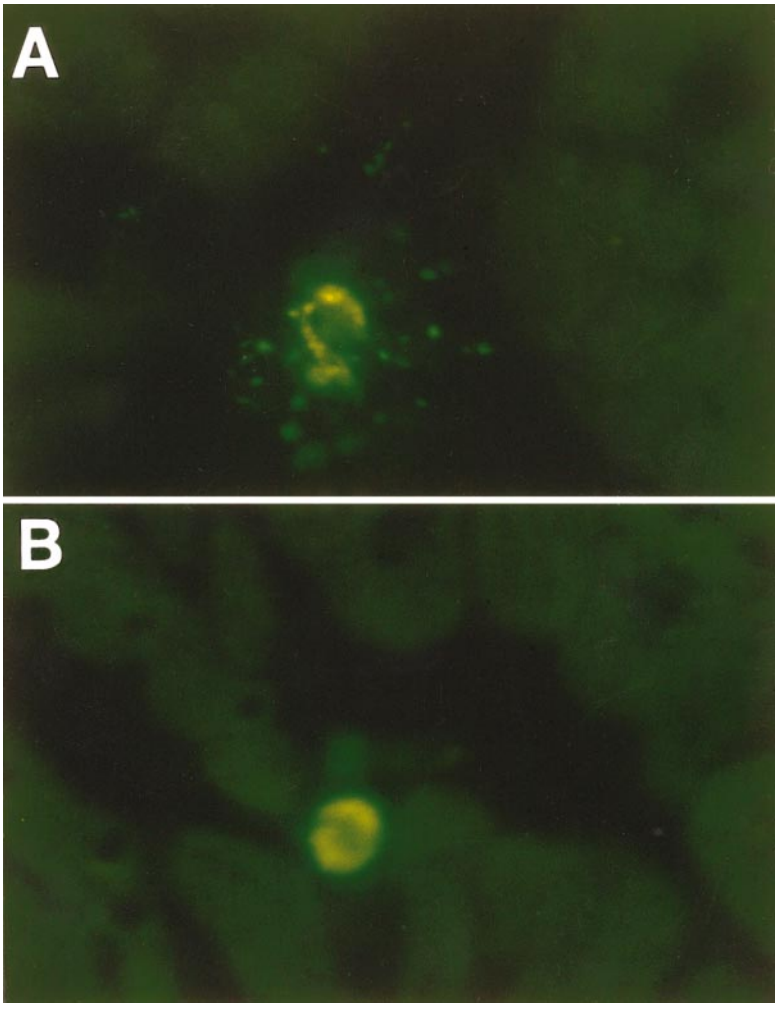

Figure 6. Mast cell degranulation in ischemic myocardium. Samples obtained from animal exposed to 1 hour of coronary occlusion and 3 hours of reperfusion. Sections were stained with FITC-labeled avidin to identify mast cells (×1000). Degranulating mast cells were found in ischemic myocardial segments (A), whereas mast cells in control segments appeared fully granulated (B).

Figure 7. TNF-α immunoreactivity in ischemic and reperfused myocardium. Transmural 300-μm segment from 1 hour ischemia and 3 hours reperfusion, spanning from injured subendocardial area to normal epicardial region (top, ×100). Note that significant mast cell degranulation is found only in cells located in injured subendocardial area (B) (bottom, ×400). Mast cells in epicardial region appear fully granulated (A) (bottom, ×400). Staining of endothelium and infiltrating cells is also noted in area of mast cell degranulation.

we demonstrated that IL-6 mRNA was localized predominantly in infiltrating mononuclear leukocytes. Minimal staining was observed in endothelium, smooth muscle cells, and myocytes. There was no staining in normally perfused samples. Serial sections incubated with the sense riboprobe demonstrated no staining.

Samples of cardiac lymph taken in the first 15 minutes of reperfusion were studied by in situ hybridization, and the isolated leukocytes demonstrated IL-6 mRNA expression (Figure 11). No staining was seen in cardiac lymph–derived leukocytes obtained before coronary occlusion. In both Figures 10 and 11, the IL-6–positive cells showed morphological characteristics of mononuclear cells. This was confirmed by the absence of staining for the neutrophil-specific antibody SG8H6 (Figures 10 and 11) in the leukocytes staining for IL-6 mRNA in both tissue and lymph.

Postischemic Lymph Induction of IL-6 mRNA Is Inhibited by an Anti–TNF-α Antibody

Canine mononuclear cells were incubated with preischemic and postischemic cardiac lymph. After a 2-hour incubation with a 1:10 dilution of the cardiac lymph, the cells were fixed in ethanol and in situ hybridization for IL-6 was performed. Cells incubated with recombinant human TNF-α were used as a positive control and demonstrated significant induction of IL-6 (2.29±0.39-fold, $P<0.05$ compared with mononuclear cells incubated with medium, n=8), which was inhibited with addition of a TNF-α antibody. The cardiac lymph from 8 consecutive experiments of myocardial ischemia was used. In 7 of 8 experiments, incubation with postischemic cardiac lymph significantly increased the number of IL-6 mRNA–positive cells (1.96±0.29-fold, $P<0.05$ compared with control mononuclear cells, n=8). In contrast, incubation with preischemic cardiac lymph did not induce IL-6 mRNA in mononuclear cells (0.89±0.19; $P>0.5$, n=8). Addition of a neutralizing antibody to TNF-α markedly reduced the percentage of mononuclear cells showing IL-6 mRNA expression (1.11±0.24, $P<0.05$ compared with mononuclear cells stimulated with postischemic cardiac lymph, n=8) (Figure 12). In 2 experiments, inhibition of IL-6 induction after addition of the antibody to TNF-α was partial (<50% reduction in the number of IL-6–positive cells), and in 1 experiment, no inhibition was noted.

Discussion

In this report, we investigated the "upstream" components of the cytokine cascade ultimately responsible for ICAM-1 induction in the reperfused myocardium. The ability of postischemic cardiac lymph to induce ICAM-1 in myocytes was neutralized by an antibody to IL-6,[9] and IL-6 was shown to be a potent inducer of ICAM-1 on cardiac myocytes. In contrast, ICAM-1 expression in canine endothelial cells stimulated with postischemic cardiac lymph was not neutralized by anti–IL-6 antibody,[9] and IL-6 does not stimulate endothelial ICAM-1 in culture. The following observations led us to hypothesize the presence of other cytokines in the cardiac lymph that must be of importance in acute inflammation after reperfusion of the ischemic myocardium.

1. With regard to the induction of endothelial ICAM-1, most data suggested IL-1 or TNF-α to be the potential cytokine; with respect to the cytokine activity of the cardiac lymph, we demonstrated that the presence of excess IL-1 inhibitory activity precludes its effect in inducing ICAM-1 in cultured endothelial cells.[9] Furthermore, immunohistochemical experiments indicated that IL-1β was not constitutively expressed in the control heart (Figure 4) and was not found in ischemic segments after 1 hour of reperfusion. This led us to postulate that TNF-α was a likely source of cytokine activity

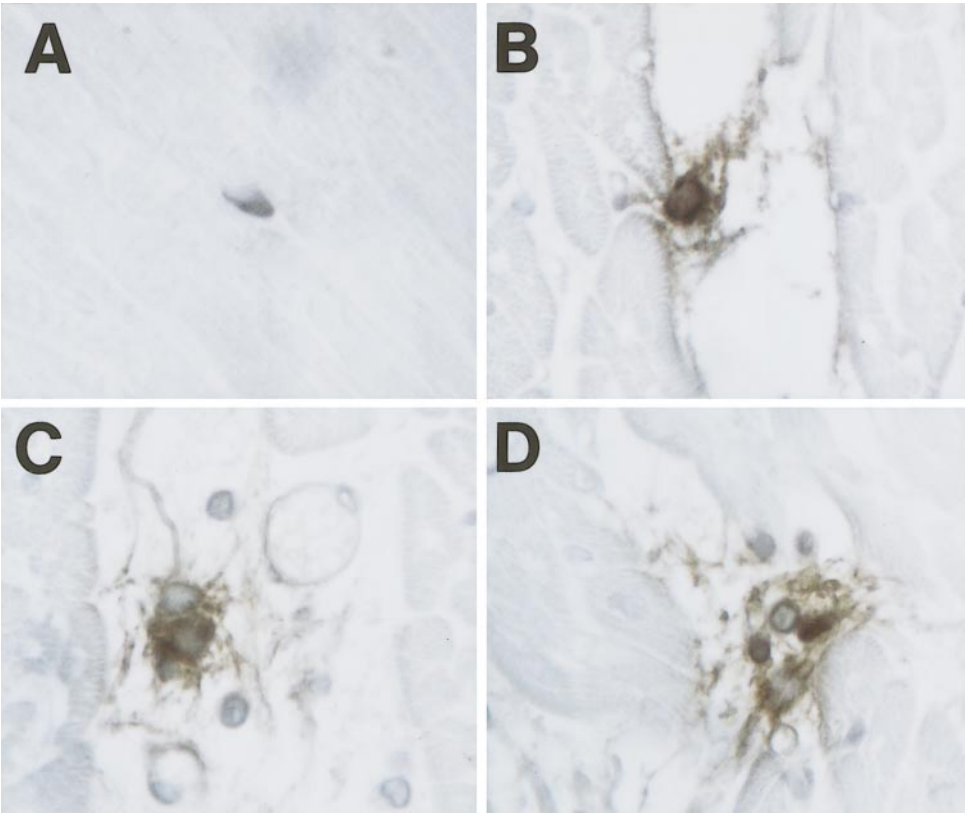

Figure 8. Egress of TNF-α from mast cells in ischemic and reperfused myocardium. Immunohistochemistry with anti–TNF-α antibody in samples from experiments exposed to 1 hour of ischemia and 3 hours of reperfusion. Antibody detection used a peroxidase-based detection system with DAB as substrate. Mast cells in ischemic subendocardial area show significant degranulation (B, C, and D). Note staining of endothelium and infiltrating cells in areas of mast cell degranulation. In contrast, mast cells in epicardial area appear fully granulated (A) ($\times$620).

in the cardiac lymph that might be responsible for induction of ICAM-1 in the endothelium.

2. Because of the relationship between IL-6 and cardiac myocyte ICAM-1 induction, we evaluated the induction of IL-6 and found that it occurred very early on reperfusion. IL-6 mRNA was found after reperfusion of severely ischemic segments only and, during the first 3 hours of reperfusion, was completely dependent on reperfusion of the previously ischemic myocardium. This led us to hypothesize that IL-6 induction is related to the influx of leukocytes into the ischemic myocardium on reperfusion.[12] The data in this study confirm this hypothesis and demonstrate the early induction of IL-6 mRNA in mononuclear cells found in the reperfused myocardium, compatible with the early appearance of IL-6 activity in the cardiac lymph capable of myocyte ICAM-1 induction.[9] The experiments shown in Figure 12 support the hypothesis that preformed TNF-α may be an important upstream cytokine responsible for the rapid induction of IL-6 mRNA in the ischemic and reperfused myocardium.

Potential Role for Mast Cells

The presence of mast cells in the heart has been previously described in several species; in a recent report, Patella et al[26] isolated and characterized human heart mast cells by demonstrating the presence of chymase and tryptase. They also showed that incubation of human heart mast cells with C5a caused a rapid and dose-dependent release of histamine.[26] Furthermore, Ito et al[15] demonstrated in pig heart that cardiac mast cells, when exposed to C5a, rapidly degranulated and released histamine and thromboxane B_2. In previous studies,[27] we have shown the presence of both C5a and thromboxane B_2 in postischemic cardiac lymph. This suggested to us that mast cell degranulation might be an important part of the ischemia/reperfusion process. The discovery that mast cells are an important source of cytokines has suggested new ways in which mast cell activation may influence inflammatory responses.[14,28] Significant evidence in the literature indicates that preformed cytokines, specifically TNF-α, exist in various mast cell populations and that cytokine release can be induced by FcϵRI ligation.[13,14,28] Recently, evidence has arisen that mast cells might also release cytokines in response to anaphylatoxins during a reverse passive Arthus reaction.[29]

Evidence for Degranulation of Cardiac Mast Cells in the Ischemic and Reperfused Myocardium

We used 2 independent methods to assess and characterize mast cell degranulation during ischemia and reperfusion. Each of these methods has limitations that are partially addressed by the other; together, they provide strong evidence for a role of mast cells in the release of TNF-α during the acute inflammatory injury accompanying reperfusion of the ischemic myocardium.

Cardiac Lymph

We have demonstrated a rapid increase in the level of histamine and TNF-α in postischemic cardiac lymph (Figures

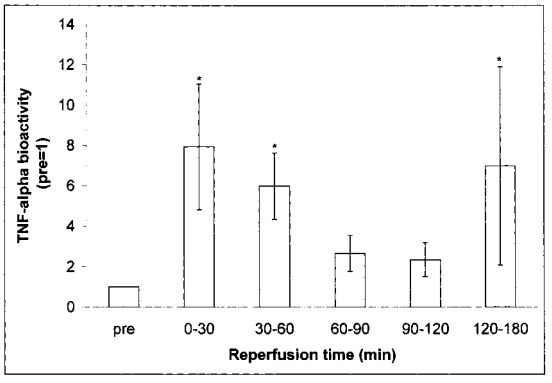

Figure 9. Release of TNF-α bioactivity in canine cardiac lymph after myocardial ischemia and reperfusion. Values are expressed as percentage of bioactivity measured in preischemic cardiac lymph (pre=1). A significant increase in TNF-α bioactivity was noted early after reperfusion (0 to 30 minutes, 7.94±3.1-fold increase; 30 to 60 minutes, 5.99±1.64-fold increase; *$P<0.05$ vs preischemic lymph). Number of experiments used for statistical analysis: n=8 for pre, 0 to 30 minutes, and 30 to 60 minutes; and n=6 for 60 to 120 and 120 to 180 minutes.

5 and 9). These studies used a model developed in our laboratory that allows collection of cardiac lymph from chronically instrumented animals in which all the inflammatory sequelae of the instrumentation surgery have dissipated.[10,30] Although this model has been used extensively in our research, 2 important articles require review here to understand the significance of the present findings. First, in previous experiments,[27] we have demonstrated a prompt rise of thromboxane B_2 during early reperfusion, which may provide additional evidence for mast cell degranulation during the ischemia and reperfusion events. The second important point stems from one of our earliest studies, in which we measured the appearance of creatine kinase and phosphorylase *b* in the cardiac lymph.[31] Because these enzymes egress the infarcted myocardium regardless of the presence of reperfusion, it was of interest that we could measure rises in their activity only on reperfusion of the ischemic myocardium. It became obvious that the absence of perfusion of the ischemic bed markedly diminished its contribution to cardiac lymph so that the time course of creatine kinase or phosphorylase appearance in the cardiac lymph probably represented, at least in part, the washout of previously existing enzymes in the extracellular fluid.[31] Washout of a dye injected into the infarct during occlusion followed a similar time course. This is pertinent to the present experiments because the time course for egress of histamine and TNF-α (as well as thromboxane B_2[27]) is very rapid, with significant activity seen within the first 15 minutes of reperfusion. We would interpret these data as suggesting that at least some of the mast cell degranulation antecedes reperfusion, which suggests that degranulation may be initiated by preexisting autacoids found in the ischemic myocardium.

Immunohistochemistry

Our initial studies established the presence of preformed TNF-α in cardiac mast cells in normal myocardium in both control hearts (Figures 2 and 3) and control areas of ischemic and reperfused dog heart. Although no method can absolutely rule out the presence of some TNF-α in other cells, the intense staining of TNF-α observed in mast cells (identified as tryptase and FITC-avidin–positive cells) in control myocardial sections in the absence of any other staining certainly suggests that the mast cell must be a highly significant source of preformed TNF-α.

To investigate degranulation, we used the FITC-avidin staining technique as a simple method for identifying mast

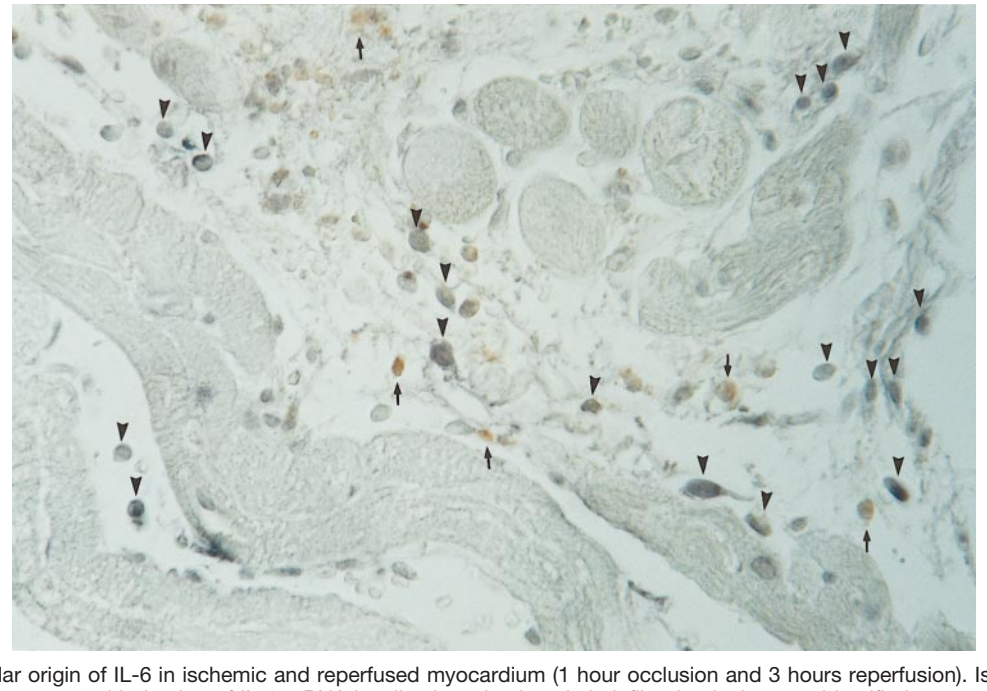

Figure 10. Cellular origin of IL-6 in ischemic and reperfused myocardium (1 hour occlusion and 3 hours reperfusion). Ischemic myocardial segments demonstrated induction of IL-6 mRNA localized predominantly in infiltrating leukocytes identified as mononuclear cells (arrowheads). These cells did not stain with neutrophil-specific antibody SG8H6 (×400). Several neutrophils are also identified (arrows) and do not stain for IL-6.

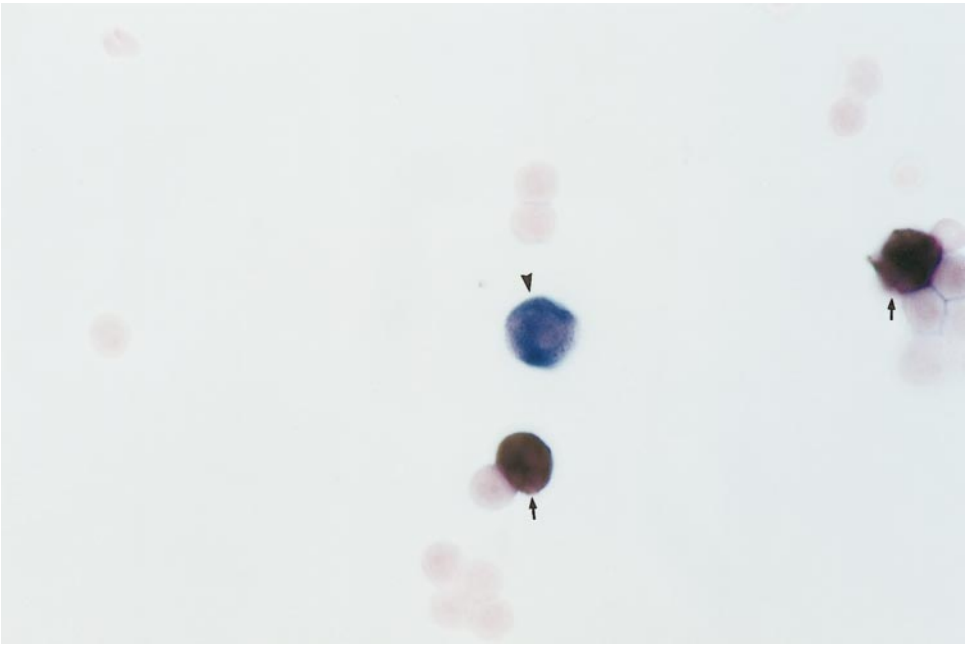

Figure 11. Early appearance of IL-6 mRNA in cardiac lymph–derived leukocytes. In situ hybridization with antisense probe for canine IL-6 (blue) followed by immunohistochemistry with neutrophil-specific antibody SG8H6 developed with DAB (black). Leukocytes obtained during first 15 minutes of reperfusion consistently demonstrated IL-6 mRNA induction and were identified morphologically as mononuclear cells (arrowhead). These cells did not stain with SG8H6, which was used to identify neutrophils (arrows).

cells and immunohistochemical techniques to evaluate TNF-α egress from mast cells. Both of these experiments provided the same information. Degranulating mast cells were seen only in the area of necrosis and the viable area bordering the infarct. In an effort to demonstrate this, Figure 7 shows a transmural segment of myocardium of $\approx$300 μm that spans a subendocardial myocardial infarct with mast cell degranulation and a normal area toward the epicardium with fully granulated mast cells. Infiltrating leukocytes and endothelium in this area are also stained, although much less intensely. In the more external area of the section, where degranulation is not seen, there are no infiltrating leukocytes and the endothelium is not stained. The less intense TNF-α staining of the infiltrating leukocytes and endothelium is most likely a result of binding of the secreted TNF (Figure 8); new synthesis in these cell types cannot be ruled out. It should be emphasized, however, that histochemical determination of mast cell degranulation is not sensitive enough to detect mast cells undergoing a slower degranulation process in cardiac mast cells, for which the term "piecemeal degranulation" has been used.[32] For that, we have also relied on the cardiac lymph studies described above.

Initiation of Mast Cell Degranulation

The data in this article do not speak to the stimulus for mast cell degranulation, but several candidates are obvious. C5a is known to induce degranulation in cardiac mast cells[26] and is present exclusively in the area surrounding the myocardial injury before initiation of reperfusion.[33] In addition, adenosine has been shown to induce mast cell degranulation through an A_3 receptor[34] and would be expected to be increased in ischemic areas. Reactive oxygen has been shown to induce mast cell degranulation[35] and might be an important factor in early reperfusion when production of reactive oxygen is highest. Finally, the C-C chemokine monocyte chemoattractant protein-1 has been shown to be a stimulator of mast cell degranulation[36]; we have shown this chemokine to be induced in the previously ischemic myocardium during reperfusion,[37] but its presence is not important until hours 2 and 3 of reperfusion. In view of the piecemeal degranulation seen in cardiac mast cells in other systems,[32] it is possible that several of these autacoids are important.

Cellular Origin of IL-6

We have previously demonstrated that induction of IL-6 occurs in the ischemic injured myocardium and requires reperfusion of the previously ischemic myocardium.[12] Our present studies showed that IL-6 mRNA could be detected only in SG8H6-negative mononuclear cells (Figure 10). In addition, similar studies in cardiac lymph cells demonstrated the presence of IL-6–positive mononuclear cells in the cardiac extracellular fluid within 15 minutes of reperfusion (Figure 11). These findings suggest a very potent inducing stimulus present from the initiation of reperfusion (because IL-6 induction did not occur in the absence of reperfusion). Our data suggest a major role for TNF-α as an upstream cytokine inducer after myocardial ischemia.

Hypothesized Cytokine Cascade

In summary, the experiments allow us to refine our hypothesis regarding the role of mast cells in the cellular responses to injury governed by cytokine induction. We have demonstrated mast cell degranulation by 2 independent techniques and shown that it is confined to the injured area of the ischemic and reperfused myocardium. This degranulation results in rapid release of preformed TNF-α into the ischemic

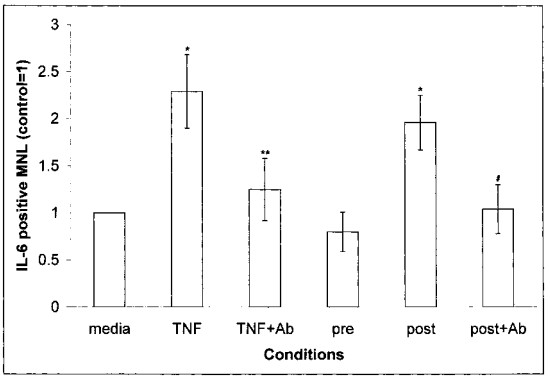

Figure 12. TNF-α is an important upstream cytokine responsible for IL-6 mRNA induction in canine mononuclear cells stimulated with cardiac lymph. Mononuclear cells were fixed and stained for IL-6 mRNA by in situ hybridization after incubation with TNF-α, TNF-α plus a neutralizing antibody to TNF-α, preischemic cardiac lymph (1:10 dilution), and postischemic cardiac lymph (1:10 dilution) with and without TNF-α antibody. Percentage of IL-6–positive cells was counted and normalized (medium alone=1). Incubation of canine mononuclear cells with TNF-α demonstrated a 2.29±0.39-fold increase in number of IL-6–positive mononuclear cells (*P<0.05 vs mononuclear cells incubated with medium only, n=8), which was neutralized with addition of an antibody to TNF-α (**P<0.05 vs TNF-α–stimulated mononuclear cells). Stimulation with early postischemic cardiac lymph caused a 1.96-fold±0.29 increase in number of IL-6–positive cells (*P<0.05 vs control mononuclear cells, n=8). Incubation with a blocking antibody to TNF-α significantly decreased number of IL-6 positive mononuclear cells (PO, 1.96±0.29 vs PO+A, 1.04±0.26; #P<0.05, n=8), suggesting that TNF-α is a significant factor in postischemic cardiac lymph responsible for IL-6 induction in mononuclear cells. Cardiac lymph from 8 consecutive experiments of myocardial ischemia was used for these experiments (n=8). Incubation conditions: medium, TNF-α (200 U/mL); TNF-α+anti–TNF-α antibody; pre, preischemic lymph (1:10 dilution); post, postischemic lymph (1:10 dilution); and post+Ab, postischemic lymph+anti–TNF-α antibody.

area. TNF-α is known to induce IL-6 in endothelial cells,[38] fibroblasts,[39] neutrophils,[40] and mononuclear cells[41–43]; our experiments indicate that the primary cell responsible for IL-6 mRNA expression in early reperfusion of the previously ischemic myocardium is the infiltrating mononuclear cell and suggest that TNF-α released from preformed stores in cardiac mast cells has a crucial role in inducing IL-6 in infiltrating mononuclear cells. As shown in Figure 13, several stimuli pertinent to ischemia may induce mast cell degranulation; it is possible that >1 of these stimuli are important. Histamine may also be an important autacoid in the reaction to injury ensuing on reperfusion of the ischemic myocardium. Histamine has been shown to enhance cytokine-induced IL-6 synthesis via activation of H_2 receptors.[44] In addition, histamine can induce leukocyte rolling in vivo by stimulating surface expression of P-selectin from Weibel-Palade bodies in venular endothelium.[45] The role of P-selectin in leukocyte margination during early ischemia has been proposed by several investigators.[45–47]

Mast Cells in Ischemia and Reperfusion

The role of mast cells in the injury associated with myocardial ischemia and reperfusion was initially suggested by Jolly and coworkers.[48] Furthermore, a growing body of evidence supports a role for mast cells in leukocyte recruitment associated with

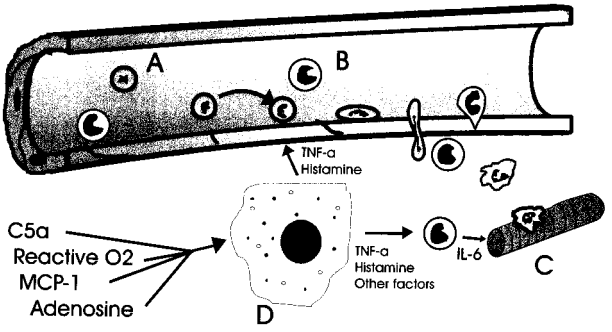

Figure 13. Schematic of cytokine cascade in myocardial ischemia and reperfusion. A, Neutrophil; B, mononuclear cell; C, myocyte; and D, mast cell. TNF-α released from preformed stores in cardiac mast cells plays a crucial role in inducing IL-6 expression in infiltrating mononuclear cells. Subsequently, IL-6 upregulates ICAM-1 synthesis on cardiac myocytes, making them vulnerable to neutrophil-mediated cytotoxic injury. TNF-α may also induce endothelial ICAM-1. Histamine may enhance cytokine-related stimuli and induce surface P-selectin expression on endothelial cells (see text for details).

intestinal ischemia/reperfusion.[49] Recent experiments suggest mast cell degranulation after intestinal ischemia and reperfusion,[50] closely associated with leukocyte rolling and adhesion.[51] Oxidants and anaphylatoxins[49] were suggested as potential factors responsible for mast cell activation. This report seeks to further identify the mechanisms by which mast cells participate in ischemia-reperfusion injury.

Mast Cells in the Healing Phase of a Reperfused Myocardial Infarct

In the companion article,[52] a potential role for mast cells in the later cellular events after myocardial infarction and reperfusion is described. Taken together, these reports suggest that the mast cell may play a critical role in the reaction to myocardial injury.

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Resident Cardiac Mast Cells Degranulate and Release Preformed TNF-α, Initiating the Cytokine Cascade in Experimental Canine Myocardial Ischemia/Reperfusion

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