Canine Neutrophil Activation by Cardiac Lymph Obtained During Reperfusion of Ischemic Myocardium

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Cardiac lymph from a canine model of myocardial ischemia and reperfusion was examined for evidence of chemotactic activity. Lymph was continuously collected from awake animals before and during a 60-minute coronary artery occlusion and up to 6 hours after the initiation of reperfusion. It was assessed for the ability to activate the following proinflammatory functions in neutrophils isolated from the blood of healthy dogs: 1) morphological changes characteristic of chemotactic stimulation, which were assessed by phase contrast microscopy, 2) orientation of canine neutrophils in a gradient of cardiac lymph, which was assessed in Zigmond chambers, 3) the binding of monoclonal antibodies reactive with CD11b and CD18 adherence glycoproteins, which was assessed by flow cytometry, and 4) adherence of canine neutrophils to monolayers of canine jugular vein endothelium, which was assessed in vitro by a visual assay. Lymph samples collected after 1 hour of reperfusion in animals demonstrating ECG evidence of ischemia and histological evidence of infarction exhibited significant stimulatory activity for each of the functions tested. Shape change-inducing activity was evaluated at more frequent intervals than other functions and was found to peak at 1 hour after initiation of reperfusion and to disappear by 6 hours. In addition, the CD11b/CD18 levels on neutrophils isolated from cardiac lymph collected during reperfusion were significantly greater than neutrophils obtained before or during occlusion. Animals that failed to exhibit evidence of infarction also failed to exhibit increased stimulatory activity in lymph collected during reperfusion, and surface levels of CD11b/CD18 on neutrophils collected from reperfusion lymph were not elevated. This study provides direct evidence supporting the hypothesis that chemotactic activity is generated in ischemic and reperfused myocardium. (Circulation Research 1989;65:1751-1762)

The localization of inflammatory cells in ischemic myocardium has been well documented. Indirect experimental evidence suggests that infiltrating leukocytes participate actively in extending myocardial injury, especially in association with reperfusion.\textsuperscript{1-10} Even reversible injury associated with short ischemic intervals may be mediated, in part, by infiltrating leukocytes.\textsuperscript{2} Several mechanisms by which neutrophils may contribute to myocardial damage have been suggested. Histopathologic studies provide evidence that leukocytes can mechanically obstruct capillaries and, thereby, inhibit reperfusion of ischemic tissue.\textsuperscript{5-10} Evidence also exists that the generation of oxygen-derived free radicals and/or the release of proteolytic enzymes by infiltrating neutrophils or monocytes contribute to observed histological or functional derangements of myocardial tissue.\textsuperscript{11-14} Indeed, experimental depletion of peripheral blood neutrophils and anti-inflammatory pharmacological agents have been shown to reduce ischemic injury in animal models.\textsuperscript{1,3-7}

Localized generation of chemotactic activity has been thought to represent an important means by which leukocytes are stimulated during ischemia and reperfusion.\textsuperscript{15-18} Two sources of such activity...
have been proposed: 1) the generation of chemotactic factors by oxidation of plasma lipids and the generation of complement-derived chemotactic factors. Subcellular fractions of cardiac muscle have been shown to activate the classical and alternative complement pathways. In early studies using histological techniques, Pinckard et al and Hill and Ward demonstrated complement activation in ischemic models after 3–6 hours of ischemia. Our laboratory, however, has recently demonstrated in a canine model of myocardial infarction that Clq and radiolabeled neutrophils selectively colocalize within ischemic myocardial segments after ischemic periods of 45 minutes or less. In subsequent experiments, we have shown that proteins of myocardial origin are released into cardiac lymph during ischemia. These proteins are capable of binding Clq and activating the entire serum complement cascade. Although such studies provide strong indirect support for the hypothesis that localized generation of chemotactic activity occurs in ischemic myocardium, we sought to obtain more direct evidence of chemotactic activity by evaluating lymph derived from ischemic tissue.

In the present studies, we have used a model of myocardial ischemia and reperfusion that allows continuous collection from the cardiac lymph duct draining the ischemic area in conscious dogs. Lymph was collected before and during a 60-minute occlusion and for up to 6 hours after initiation of reperfusion. The ability of these lymph samples to activate proinflammatory functions in canine neutrophils was evaluated by use of leukocytes isolated from the blood of the study animal obtained before occlusion or from the blood of healthy donor dogs.

Materials and Methods
Preparation of Animal Model
Thirty-three healthy mongrel dogs (15–25 kg) of either sex successfully completed surgery and were entered in the study. Each animal was anesthetized with sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air by a respirator (Harvard Apparatus, South Natick, Massachusetts). A midline thoracotomy provided access to the heart and mediastinum. By use of techniques previously described, cannulation of the cardiac lymph duct was then performed. Briefly, injection of 0.05–0.2 ml of 0.5% Evans blue subepicardially into the posterolateral wall of the left ventricle allowed lymphatic vessel definition. The largest vessel was selected and cannulated (PE-10–90) at a site proximal to the cardiac lymph node and 2–5 cm from the base of the heart. Accessory noncardiac lymphatic vessels and tracheobronchial lymphatic connections in this region were ligated. Subsequently, a hydraulically activated occluding device and a Doppler flow probe were secured around the circumflex coronary artery just proximal or just distal to the first distal branch. Choice of location depended on the proximity and anatomical arrangement of lymphatic vessels adjacent to the coronary vessels so that subsequent dissection would not damage the lymphatic system. To prevent damage of lymphatic vessels in proximity to the coronary vasculature, dissection of adipose tissue adjacent to the coronary vessel at two sequential 3–4-mm sections was carefully performed with subsequent placement of the occluding device and flow probe. In animals selected for experimental assessments of cardiac lymph, intact lymphatic vessels draining the regions of ischemic myocardium were identified by injecting Evans blue (0.05 ml) into the free wall of the left ventricle after the occluder and flow probe were in place. The appearance of Evans blue in the cardiac lymph cannula confirmed the patency of the lymph vessel architecture. Cannulas were also placed in the right and left atria to allow for blood sampling as needed.

Cardiac Lymph Collection
After 48–72 hours of postoperative recovery, animals were placed upright in a nylon mesh sling and allowed to accommodate to the surrounding environment. The electrocardiogram as well as tracings of mean and phasic coronary blood flow were monitored continuously in each animal. Before occlusion, blood was drawn from the right atrial catheter into citrate-phosphate-dextrose (CPD) buffer (0.14 ml/ml blood) to be used for the isolation of peripheral blood neutrophils. Cardiac lymph was collected into 1.5-ml polypropylene tubes containing either 20 IU of preservative-free heparin or 100 µl of 350 mM sodium EDTA. Lymph samples collected in heparin were immediately centrifuged at 6,500g for 1 minute at room temperature, and the supernatant was decanted to a second tube and placed immediately on ice. Lymph samples collected into EDTA were centrifuged at 300g for 5 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in Dulbecco's phosphate buffered saline (D-PBS) and immediately placed on ice. Lymph samples were continuously collected over 30-minute intervals for 1 hour before occlusion, during the occlusion interval, and up to 6 hours during reperfusion. Before occlusion, each animal received Talwin (pentazocine lactate) 0.1–0.2 mg/kg i.v. or s.c. for analgesia. Coronary artery occlusion was achieved by inflating the coronary cuff occluder until mean flow in the coronary vessel was zero, as determined by the Doppler flow probe. Flow was monitored throughout the occlusion interval to be assured that mean coronary flow remained zero. At the end of 1 hour, the cuff was deflated, and the return of coronary flow was immediate. Hyperemic flow was observed during early reperfusion (5–10 minutes) but subsequently diminished to within 5% of preocclusion control flow values. The presence of ischemia was documented by ST segment elevation on electrocardiographic tracings during occlusion and by ventricular ectopy during reperfusion.
Twenty-four hours after occlusion, animals were killed, and their hearts removed for histological assessment of infarct size.

Of the 33 animals that entered the study, 14 animals are included in the data presented. Two animals experienced sudden death in their cage after surgery but before completion of the study. Five animals experienced ventricular fibrillation during occlusion or reperfusion and were excluded. Four animals were excluded because of a malfunctioning occluder and/or flow probe. An additional five animals were excluded because of a lymph cannula that malfunctioned before study completion. Finally, three animals were excluded that had excessive lymph flow from their cannulas (exceeding 12 ml/hr), which either represented flow from collateral lymphatic vessels exclusive of the heart or represented a flow rate that effectively diluted the lymph to the extent that the presence or absence of chemotactic activity could not be accurately determined. Each animal included in the study exhibited interruption of coronary blood flow during occlusion and return of blood flow on release of occlusion, and each provided from 1 to 4 ml lymph per hour throughout the reperfusion collection period. Eight animals exhibited electrocardiographic evidence of ischemia and subsequently demonstrated histopathological evidence of infarction and were included in the group designated “infarction.” Six animals, despite occlusion, failed to exhibit ischemic ECG changes and also failed to demonstrate histopathological evidence of infarction. These animals were included in the “no infarction” group. Lymphatic flow was not different in the two groups.

**Isolation of Canine Neutrophils**

Venous blood samples anticoagulated with CPD were sedimented with 6% dextran in saline. Leukocyte-rich plasma was centrifuged at 300g for 5 minutes at 4°C, and leucocytes were suspended in D-PBS, pH 7.4, containing 0.2% glucose. Cells were then layered over a solution of 10 parts of 6% dextran in saline. Final suspensions were washed and resuspended in D-PBS. Neutrophils in the cell button were washed and resuspended in D-PBS. Final suspensions contained more than 95% neutrophils of which more than 99% were viable as determined by trypan blue dye exclusion.²¹

**Shape Change Assay**

Suspensions of neutrophils were exposed to various samples of cardiac lymph or control solutions (positive control: zymosan-activated dog serum [ZADS]; negative control: D-PBS) for 5 minutes at 37°C and then fixed in 1.5% glutaraldehyde, as previously described.³²,³³ The percentage of cells assuming spherical, ruffled, and bipolar configurations was determined visually by phase contrast microscopy. Shape change assays were performed with limiting dilutions of cardiac lymph to determine peak chemotactic activity (i.e., the highest dilution effecting maximal shape change) as well as to determine the optimal concentration of lymph to be used in additional assays described below.

**Orientation and Migration Assay**

Plexiglas orientation chambers were assembled and calibrated as previously described by Zigmond.³⁴ Glass coverslips (22 mm x 40 mm) were pretreated with 5% human serum albumin for 2 minutes and then rinsed with D-PBS. Canine neutrophils (10⁷ in D-PBS) were allowed to attach to the center of the coverslips for 10 minutes at 21°C before the coverslips were inverted over the chambers. The stimulant well adjacent to the bridge was loaded with 100 μl of 1% ZADS (positive control), cardiac lymph, or D-PBS (negative control), and 100 μl D-PBS was injected into the opposite well. Cells in each of 10 x 40 fields over the bridge were observed by phase microscopy at 5-minute intervals after loading the chambers. The direction of orientation and/or locomotion was morphologically determined; the front of locomoting cells was identified by its lamellipodium, and the rear was identified by the knob-like uropod or by the presence of retraction fibers. Accurately orienting cells were scored as those moving into the 150° sector toward the stimulant well.

**Immunofluorescence Flow Cytometry**

Indirect immunofluorescence assessment of the surface expression of CD11b/CD18 subunits on intact canine neutrophils (either peripheral blood neutrophils or neutrophils isolated from cardiac lymph) was performed by using saturating concentrations of murine monoclonal antibodies (MAbs) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G.³⁵ Washed, surface-stained cells were fixed in 1% paraformaldehyde and analyzed in a flow cytometer (FACScan, Becton Dickinson Laboratory, Lincoln Park, New Jersey). Fluorescence intensity was expressed as the mean fluorescence channel minus background fluorescence of cells exposed to X63 immunoglobulin G1 (nonbinding control). The murine MAbs used for these studies included LM2/1 and TS1/18, immunoglobulin G1 antibodies (obtained from T. Springer, Harvard University) specific for the α- (CD11b) or β- (CD18) subunits, respectively, of the human CD11b/CD18 glycoprotein family. In preliminary experiments, these MAb reagents were shown to bind to canine neutrophils; these findings are consistent with a previous report.³⁶

**Canine Neutrophil-Endothelial Monolayer Adherence Assay**

Canine jugular vein endothelial cells were obtained by a modification of the method of Ford et al³⁷ and
characterized as previously described.\textsuperscript{38-40} Jugular veins were everted on glass rods and incubated in collagenase solution (50 units/ml) for 10 minutes. Cells were collected by centrifugation and suspended in Dulbecco's modified Eagle's medium (DME) containing 4% fetal calf serum, 4% bovine calf serum, 50 \( \mu \)g/ml endothelial cell growth factor (ECGF, Collaborative Research, Bedford, Massachusetts), 50 \( \mu \)g/ml heparin, 1 mM sodium pyruvate, and antibiotics. Cells were seeded on Primaria flasks (Becton Dickinson). After 2-4 days incubation at 37° C in a \( \text{CO}_2 \) incubator, areas of cells with "cobblestone" morphology were collected by scraping, transferred to gelatin-coated flasks, and grown to confluence. Second-passage cells were obtained by scraping, seeded onto type I collagen-coated (at 5 \( \mu \)g/ml) 25-mm round coverglasses, and grown to confluence. These monolayers were inserted in the adherence chambers, and adherence of isolated canine neutrophils was determined in the absence of shear stress by a visual assay as previously described.\textsuperscript{41} In experiments with stimulated endothelial cells, monolayers were exposed to 2 ng/ml lipopolysaccharide (LPS) for 3 hours at 37° C and rinsed by dipping the coverslip five times in two changes of D-PBS before being inserted into the adherence chambers.

\textit{Histopathologic Examinations}

Twenty-four hours after occlusion-reperfusion experiments, animals were killed, and their hearts were excised, cut into 1-cm transverse sections, and visually inspected for gross defects. Each section was subsequently stained with 2,3,5-triphenyltetrazolium chloride and examined for histopathologic evidence of infarcted tissue.\textsuperscript{42} Infarct size was determined by planimetric analysis and expressed as the percent of total cross-sectional area of the left ventricle.

\textit{Statistics}

Pooled data are presented as the mean±SEM. In the shape change assay and in assays of CD11b/CD18 expression, statistical differences between data from infarction and no infarction groups were assessed by repeated-measures analysis of variance and trend analyses.\textsuperscript{43} In the adherence assay, within group and between group comparisons were made with Student's paired and nonpaired \( t \) test, respectively.

\textit{Results}

\textit{Histopathologic Analysis}

In the group of eight animals with ECG evidence of ischemia during occlusion, myocardial infarctions were demonstrated ranging in size from 3.6% to 10.4% of the total cross-sectional area of the left ventricle. Mean infarct size was 6.9±1.1%. In a second group of six animals, no ischemic changes on ECG were demonstrated during occlusion. Four animals failed to show any area of infarction. In the two remaining animals, small infarctions measuring 1% or less of the total cross-sectional surface area of the left ventricle were noted on the posterior papillary muscle.

\textit{Shape Change–Promoting Activity in Cardiac Lymph}

Previous studies have documented that human or canine neutrophils undergo rapid alterations of cell morphology in response to chemotactic stimuli in vitro and in vivo.\textsuperscript{32,33,44} Most cells assume the polarized configuration characteristic of neutrophils migrating in a chemotactic gradient\textsuperscript{45} with ruffles on one pole and a uropod on the other. Preliminary studies were performed to assess the morphological response of canine neutrophils exposed to ZADS, a source of complement-derived chemotactic factors. As shown in Figure 1, as little as 0.2% ZADS was

\begin{figure}
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\caption{Graphs showing morphological responses of canine neutrophils suspended in dilutions of zymosan-activated dog serum (ZADS). In dose-response experiments (panel A), cells were incubated in phosphate buffered saline (PBS) or 0.1-1.0% ZADS for 5 minutes at 37° C before fixation in 1.5% glutaraldehyde. Time-response studies (panel B) used cell suspensions incubated with 0.5% ZADS for 1-5 minutes at 37° C before fixation. Results of studies performed on neutrophil suspensions from five separate dogs are shown.}
\end{figure}
Neutrophil Chemotactic Activity During Ischemia/Reperfusion

**FIGURE 2.** Photomicrographs showing morphological response of canine neutrophils to cardiac lymph. Peripheral blood canine neutrophils were suspended in lymph for 5 minutes at 37°C and then fixed in a final concentration of 1.5% glutaraldehyde. Panel A: Effects of preischemic cardiac lymph from a single animal (see Figure 3) showing the spherical shape of unstimulated neutrophils. Panel B: Effects of reperfusion cardiac lymph from an infarcted animal (1-hour sample, see Figure 3) showing the characteristic bipolar configuration of stimulated cells. Photomicrograph, ×40 phase contrast objective.

sufficient to stimulate canine neutrophils isolated from healthy control dogs to assume this bipolar shape. Maximal responses were evident with 0.5–1.0% ZADS (Figure 1A). In kinetics experiments using 0.5% ZADS, a maximum percentage of cells was bipolar after a 2–5-minute incubation interval (Figure 1B).

Peripheral blood neutrophils isolated from blood of the experimental animal obtained before occlusion were incubated with cell-free cardiac lymph samples collected sequentially before experimental occlusion, during the 60-minute occlusion interval, and at various intervals during reperfusion. As shown in Figures 2 and 3, low percentages of cells became bipolar when incubated in preocclusion cardiac lymph samples. In most animals, a somewhat higher level of shape change–promoting activity was apparent in the lymph immediately after surgical insertion of the lymph cannula, but this activity largely diminished or disappeared during the 48-hour interval before coronary artery occlusion. Several animals demonstrated a persistently elevated level of activity in preocclusion lymph samples that required serial dilutions of lymph to eliminate the background activity. (All preocclusion, occlusion, and reperfusion samples were then evaluated at this dilution). Minimal or no elevation of activity was detected in lymph collected during coronary artery occlusion, but in each experimental animal in which successful myocardial infarction was achieved, a substantial increase in activity was evident within the first 30 minutes of reperfusion. In these animals, shape change–eliciting activity reached peak levels in lymph samples obtained 1–2 hours into reperfusion (Figures 2 and 3). Thereafter, activity gradually diminished; samples collected greater than 5 hours into reperfusion demonstrated activity comparable with baseline preischemic values. Among experimental animals in which significant infarction did not occur despite coronary artery occlusion, essentially no increase in shape change–promoting activity occurred in cardiac lymph collected during reperfusion.

**Orientation of Canine Neutrophils Exposed to Cardiac Lymph**

To demonstrate that shape change–promoting activity in reperfusion cardiac lymph was truly chemotactic in nature, lymph samples, in which sufficient lymph was available, were incorporated into the stimulant compartment of chambers designed to assess orientation in a chemotactic gradient. Preliminary studies that used 1% ZADS to produce the gradients demonstrated that a high proportion of isolated canine neutrophils assumed a bipolar shape, oriented, and migrated toward the ZADS gradient within 5–10 minutes after loading the chamber (Figure 4). By 30 minutes after establishing the gradients, greater than 90% of cells demonstrated bipolar morphology. Of these, more than 50% were oriented and/or migrating toward the gradient source and less than 5% were directed away from the gradient. At later time intervals, dissipation of the chemotactic gradient was apparent as approximately equivalent proportions of cells were identified orienting toward, away from, or perpendicular to the gradient.

As shown in Figures 4 and 5, reperfusion lymph obtained from four successfully infarcted animals demonstrated a capacity to elicit orientation equivalent to that of 1% ZADS. In contrast, preocclusion lymph as well as reperfusion lymph samples obtained from five unsuccessfully infarcted animals demonstrated minimal chemotactic activity. Less than 10–20% of cells exposed to these sam-
CD11b/CD18 on Neutrophils Exposed to Cardiac Lymph

Preliminary studies using MAbs reactive with human CD11b (LM2/1) or CD18 (TS1/18) were performed to document binding of these reagents to canine neutrophils and to determine if enhanced expression of CD11b/CD18 was induced by exposure to ZADS (Table 1). As shown by flow cytometry, each of these MAbs identified determinants on canine neutrophil surfaces. Binding was enhanced 250–300% over baseline values on exposure to 1% ZADS for 15 minutes at 37°C. These findings are consistent with the conclusion that increases in CD11b/CD18 subunits on canine neutrophil surfaces are inducible by chemotactic stimuli. The surface expression of CD11b and CD18 on peripheral blood neutrophils exposed to cardiac lymph was then assessed. Sufficient lymph was available for this study from five of the dogs with documented myocardial infarction. Cells exposed to lymph samples taken before occlusion demonstrated an increase in CD11b/CD18 expression when compared with unstimulated (baseline) values of peripheral blood neutrophils (an average increase of 52% for CD11b and 47% for CD18). However, cells exposed to lymph collected at 1-hour intervals during the first 3 hours of reperfusion demonstrated an additional increase in the level of CD11b/CD18 expression when compared with preischemic values (Figure 6). In contrast, among each of six animals in whom infarction was not achieved despite coronary artery occlusion, lymph samples collected at the same time intervals elicited minimal or no increase in surface CD11b or CD18 on canine neutrophils (Figure 6). Differences between infarction and no infarc-

FIGURE 3. Graph showing neutrophil shape change–promoting activity in canine cardiac lymph. Evaluations were made in lymph samples collected before occlusion (PRE), during coronary artery occlusion (OCCL), and during reperfusion up to 6 hours. Results of studies in eight animals fulfilling ECG evidence of ischemia and histological criteria of infarction are shown along with those in six animals in which there was no evidence of ischemia or infarction. Results are expressed as the percent of peripheral blood canine neutrophils demonstrating a bipolar shape after incubation in lymph for 5 minutes at 37°C.

FIGURE 4. Graph showing orientation of canine neutrophils toward gradients of zymosan-activated dog serum (ZADS) or cardiac lymph. Neutrophils (10⁶) were incorporated into chambers as described in “Materials and Methods.” At 5-minute intervals after loading the chamber, the percent cells with a bipolar shape undergoing accurate orientation was determined.

somes demonstrated bipolar morphology, and these cells oriented randomly.

CD11b/CD18 on Neutrophils Exposed to Cardiac Lymph

Preliminary studies using MAbs reactive with human CD11b (LM2/1) or CD18 (TS1/18) were performed to document binding of these reagents to canine neutrophils and to determine if enhanced expression of CD11b/CD18 was induced by exposure to ZADS (Table 1). As shown by flow cytometry, each of these MAbs identified determinants on canine neutrophil surfaces. Binding was enhanced 250–300% over baseline values on exposure to 1% ZADS for 15 minutes at 37°C. These findings are consistent with the conclusion that increases in CD11b/CD18 subunits on canine neutrophil surfaces are inducible by chemotactic stimuli. The surface expression of CD11b and CD18 on peripheral blood neutrophils exposed to cardiac lymph was then assessed. Sufficient lymph was available for this study from five of the dogs with documented myocardial infarction. Cells exposed to lymph samples taken before occlusion demonstrated an increase in CD11b/CD18 expression when compared with unstimulated (baseline) values of peripheral blood neutrophils (an average increase of 52% for CD11b and 47% for CD18). However, cells exposed to lymph collected at 1-hour intervals during the first 3 hours of reperfusion demonstrated an additional increase in the level of CD11b/CD18 expression when compared with preischemic values (Figure 6). In contrast, among each of six animals in whom infarction was not achieved despite coronary artery occlusion, lymph samples collected at the same time intervals elicited minimal or no increase in surface CD11b or CD18 on canine neutrophils (Figure 6). Differences between infarction and no infarction test groups were statistically significant (p<0.05) at 1 and 3 hours of reperfusion with respect to CD11b and at 1 hour of reperfusion with respect to CD18 values.
FIGURE 5. Photomicrographs showing orientation and migration of canine neutrophils toward a gradient of cardiac lymph. Panel A: Phase contrast photomicrograph of cells showing orientation as evidenced by the position of the ruffles and knob-like uropod (arrows). Panel B: Migration toward the gradient source as evidenced by the length of retraction fibers (arrows). Gradient originating from the left.

CD11/CD18 on Neutrophils Isolated From Canine Lymph

In five animals from the infarction group and in three animals from the no infarction group, neutrophils isolated from lymph samples obtained before occlusion and during reperfusion were stained directly with LM2/1 and TS1/18 and examined by flow cytometry (Figure 7). Neutrophils isolated from preischemic lymph samples again demonstrated increased expression of CD11b/CD18 when compared with unstimulated peripheral blood neutrophils (an average increase of 86% for CD11b and 85% for CD18). However, cells isolated from animals with a documented myocardial infarction also demonstrated increased CD11b/CD18 expression during reperfusion when compared with those animals with no infarction. This difference was significant ($p<0.05$) up to 3 hours of reperfusion for CD11b and up to 2 hours for CD18.

Neutrophil Adherence to Canine Endothelium

Preliminary studies demonstrated that the level of adherence of unstimulated and ZADS-stimulated neutrophils to unstimulated canine endothelium was quite low (data not shown). Thus, to enhance the sensitivity of this assay for neutrophil attachment, all endothelial monolayers were incubated with 2 ng/ml LPS (E. coli serotype 055:B5, Sigma Chemical) for 3 hours before the adherence assay. As shown in Figure 8, unstimulated neutrophils exhibited a substantial level of adherence to LPS pre-treated endothelium. ZADS stimulation of neutrophils (1.0% for 15 minutes) produced a significantly higher increase in the level of adherence than observed for unstimulated neutrophils. These results indicate that canine cells respond much like human umbilical vein endothelial cells and human neutrophils to LPS and chemotactic stimulation.41,46-48 By use of this model of adherence in vitro, the stimulatory effects of preischemic and reperfusion cardiac lymph were assessed. Incubation (15 minutes at $37^\circ$ C) of canine peripheral blood neutrophils with reperfusion lymph samples exhibiting peak shape change-inducing activity (infarcted animals) resulted in significantly greater adherence to canine endothelium when compared with cells incubated with preischemic lymph from the same animals (Figure 8). As demonstrated, the effect of reperfusion lymph approached that of 1% ZADS. In contrast, incubation of neutrophils with reperfusion lymph of noninfarcted animals did not increase adherence to endothelium when compared with neutrophils incubated in preischemic lymph of the same animals. The level of adherence for neutrophils incubated with preischemic lymph from both the infarct and noninfarct groups and for neutrophils incubated with reperfusion lymph from the noninfarct group was comparable with the level of adherence for neutrophils incubated with D-PBS.

Discussion

The data in this report clearly support the conclusion that cardiac lymph collected early after reperfusion of ischemic myocardium possesses the ability to activate proinflammatory functions in
canine neutrophils. That each of these functions has been studied extensively in other species, principally humans, provides a basis for understanding the possible role of their activation in the pathogenesis of myocardial injury. Shape change is necessary for cellular locomotion and orientation in a chemotactic gradient and is a consistent manifestation of the neutrophil's response to a wide

**Figure 6.** Graphs showing cell surface expression of CD11b or CD18 on canine peripheral blood neutrophils incubated with preocclusion (PRE) or occlusion (OCCL) cardiac lymph or reperfusion cardiac lymph collected at hourly time intervals. The subunit specific monoclonal antibodies LM2I1 (CD11b) or TS1/18 (CD18) and fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G were used to label neutrophils before evaluation by flow cytometry. Results are expressed as percent change (mean±1 SEM) in mean fluorescent channel from values obtained in preocclusion lymph incubation mixtures.

**Figure 7.** Graphs showing cell surface expression of CD11b or CD18 on canine neutrophils isolated from preocclusion (PRE) or occlusion (OCCL) cardiac lymph or reperfusion cardiac lymph collected at hourly time intervals. Neutrophils were labeled with the subunit specific monoclonal antibodies LM2I1 (CD11b) and TS1/18 (CD18) and fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G and evaluated by flow cytometry. Results are expressed as percent change (mean±1 SEM) in mean fluorescent channel from values obtained in preocclusion lymph cells.
variety of chemotactic stimuli. Orientation of neutrophils toward an increasing concentration gradient of a given substance is a direct demonstration of chemotaxis as shown by the elegant studies of Zigmond and colleagues and others. The activation of shape change and orientation by ischemic cardiac lymph strongly implies the presence of a chemotactic factor(s).

Although cellular locomotion is certainly involved in diapedesis and movement of neutrophils into inflamed tissue, adherence to endothelium is also an early and necessary event in the inflammatory process. Its importance is most dramatically demonstrated in patients genetically deficient in the CD18 family of adhesive glycoproteins in which biopsies of infected tissue reveal a total absence of neutrophils in the face of markedly elevated levels of peripheral blood neutrophils. Chemotactic stimulation of normal human neutrophils not only increases adherence to endothelium but also increases the amount of the CD11b/CD18 heterodimer on the cell surface, an event coordinated with release of a subset of secondary granules. Zymosan-activated human serum stimulates these functional changes in human neutrophils (C.W. Smith et al, unpublished data) with the activity most likely attributable to the chemotactic fragments of CS. Though not proven directly, these functional changes induced in canine neutrophils by ZADS very likely reflect the same or closely analogous molecular mechanisms in canine serum as exist in the human, that is, activation of locomotion and adherence by complement-derived chemotactic factors. Furthermore, the similarities between the results with ZADS and the results with reperfusion lymph suggest that chemotactic factors in the lymph account for the functional changes observed in incubated canine neutrophils.

The probability that the presence of chemotactic activity in cardiac lymph reflects pathological events in ischemic myocardial injury is revealed by the following: 1) Stimulatory activity in reperfusion lymph obtained from dogs with documented infarction was significantly higher than that in the preocclusion and occlusion lymph from the same animals. 2) Stimulatory activity in reperfusion lymph from dogs subsequently found to have minimal or no infarction was not significantly elevated when compared with preocclusion or occlusion lymph. 3) The amount of CD11b and CD18 on neutrophils isolated from reperfusion lymph was significantly elevated in animals with documented infarctions compared with animals without infarction. 4) The temporal appearance of high levels of chemotactic-like activity in cardiac lymph during early reperfusion is consistent with previous pathological events documented in this canine ischemia model. A dramatic increase of creatine kinase and phosphorylase activity has also been detected in cardiac lymph during early reperfusion. Our previous studies in this model have documented C1q fixation in myocardial tissues after only 15–45 minutes of occlusion, as well as colocalization of radiolabeled neutrophils in the ischemic-reperfused myocardial tissue, and efflux of myocardial cell C1q–binding proteins into extracellular lymph. Furthermore, even though a low level of stimulatory activity was detected in preischemic lymph samples, this is believed to be related to tissue injury associated with surgical placement of the occluder and flow probe. The apparent absence of activity in lymph collected during coronary artery occlusion suggests that, because of the absence of coronary flow necessary to generate lymphatic flow, these samples did not contain lymph from the ischemic area but, rather, contained lymph preferentially draining normally perfused myocardial segments. Failure to detect activity in these samples does not suggest that chemotactic factors are not generated during ischemia (i.e., occlusion) but, rather, that their appearance in lymph is dependent on coronary blood flow during reperfusion.

Irreversible myocardial cell injury can occur within 20–30 minutes after occlusion of coronary vessels.
However, it is commonly accepted that thrombolytic therapy may be effective in salvaging ischemic myocardium up to 6 hours after the onset of symptoms. This suggests that processes other than ischemia may influence the extension of myocardial cell death. In the past two decades, considerable evidence points to a possible role for inflammatory reactions.\textsuperscript{1-8,21-24,64,65} Recent work by ourselves\textsuperscript{15,18} and others\textsuperscript{1-3} has suggested a role for inflammatory damage even after relatively short ischemic periods of 90 minutes or less. Evidence for this role has consisted of demonstrations of myocardial salvage when neutrophils are depleted by use of antineutrophil antibodies\textsuperscript{1} or neutrophil filters\textsuperscript{2} or when neutrophils are inhibited by a variety of drugs that alter neutrophil function.\textsuperscript{3-5} In this study, the demonstration of chemotactic-like activity within the cardiac lymph and the observation of enhanced neutrophil CD11b/CD18 expression in cardiac lymph demonstrate that ischemia-related factors directly influence the functional properties of leukocytes after only 1 hour of ischemia. Thus, although inflammatory damage to the myocardium may persist in later periods of reperfusion, these studies suggest that the initial stimulus for inflammation occurs within the first hour and represents a potential mechanism by which augmentation of ischemic damage might be effectuated in later periods.

Our previous findings\textsuperscript{15,18} support the hypothesis that the stimulatory activity detected during early reperfusion is derived in part from serum complement activation initiated in extravascular spaces during experimental occlusion. Data from other laboratories suggest that lipid-derived autacoids may also represent a potential source of neutrophil activation in this setting.\textsuperscript{20,66} The precise molecular characterization of the chemotactic activity described in this study, however, is yet to be determined.

Nonetheless, important implications with respect to therapeutic strategies in ischemic heart disease emerge from the above considerations. Among several neutrophil-mediated mechanisms proposed to be of pathogenic importance in myocardial reperfusion injury (i.e., homotypic aggregation, vascular attachment, oxygen free radical elaboration, and extracellular release of proteolytic enzymes associated with diapedesis of neutrophils), each exhibits a marked dependence on adherence. These functions can be substantially inhibited by incubating cell suspensions with one or more anti-CD11b/CD18 MAbs in vitro.\textsuperscript{41,67} Our findings linking apparent chemotactic activity, enhanced adherence of neutrophils to endothelium, and enhanced neutrophil CD11b/CD18 expression in ischemic/reperfusion cardiac lymph support the rationale that inhibition of one or more leukocyte adherence reactions would protect ischemic myocardial tissue from reperfusion injury. Use of anti-CD11b/CD18 MAbs have recently been shown to alter inflammatory responsiveness in vivo. Anti-CD11b or -CD18 MAbs have been used to prevent or diminish neutrophil-mediated pulmonary or other organ injury in murine, cat, and rabbit models.\textsuperscript{68-72} Of considerable relevance to the present considerations are the recent reports demonstrating that 904, an anti-CD11b MAb, reduced infarct size in an experimental canine model of myocardial ischemia.\textsuperscript{73,74} Additional studies are required to fully define the pathogenic mechanisms by which neutrophil adherence molecules contribute to myocardial injury.

Acknowledgments

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