Cardiac Myocytes Produce Interleukin-6 in Culture and in Viable Border Zone of Reperfused Infarctions

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Background—Previous work from our laboratory demonstrated that interleukin (IL)-6 plays a potentially critical role in postreperfusion myocardial injury and is the major cytokine responsible for induction of intracellular adhesion molecule (ICAM)-1 on cardiac myocytes during reperfusion. Myocyte ICAM-1 induction is necessary for neutrophil-associated myocyte injury. We have previously demonstrated the induction of IL-6 in the ischemic myocardium, and the current study addresses the cells of origin of IL-6.

Methods and Results—In the present study, we combined Northern blot analysis and in situ hybridization to demonstrate IL-6 gene expression in cardiac myocytes. Isolated ventricular myocytes were stimulated with tumor necrosis factor-α, IL-1β, lipopolysaccharide, preischemic lymph, and postischemic lymph. Unstimulated myocytes showed no significant IL-6 mRNA expression. Myocytes stimulated with preischemic lymph showed minimal or no IL-6 mRNA expression, whereas myocytes stimulated with tumor necrosis factor-α, IL-1β, lipopolysaccharide, or postischemic lymph showed a strong IL-6 mRNA induction. Northern blot with ICAM-1 probe revealed ICAM-1 expression under every condition that demonstrated IL-6 induction. We then investigated the expression of IL-6 mRNA in our canine model of ischemia and reperfusion. Cardiac myocytes in the viable border zone of a myocardial infarction exhibited reperfusion-dependent expression of IL-6 mRNA within 1 hour after reperfusion. Mononuclear cells infiltrate the border zone and express IL-6 mRNA.

Conclusions—Isolated cardiac myocytes produce IL-6 mRNA in response to several cytokines as well as postischemic cardiac lymph. In addition to its production by inflammatory cells, we demonstrate that IL-6 mRNA is induced in myocytes in the viable border zone of a myocardial infarct. The potential roles of IL-6 in cardiac myocytes in an infarct border are discussed. (Circulation. 1999;99:546-551.)

Key Words: interleukins ■ myocardial infarction ■ reperfusion ■ myocytes

Reperfusion of infarcted myocardium has been accepted as the treatment of choice for acute myocardial infarction and markedly accelerates the development of an inflammatory reaction in the infarcted tissue. Inflammation plays an important role in the healing of tissue after injury.1–9 However, there is evidence that the accelerated inflammatory response may also extend tissue injury. Clinical and experimental studies showed that the inflammatory response to myocardial infarction is associated with the induction of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which are thought to act in a “cascade fashion.”10–12

Our laboratory has focused on the cellular and molecular basis of the inflammatory reaction resulting from ischemia and reperfusion by using a strategy that integrates the insights from experiments done in cell culture with a disease model of myocardial ischemia and reperfusion in intact animal. Studies in our laboratory demonstrated a potential mechanism of neutrophil-induced myocyte injury. Neutrophils adhere to myocytes in the presence of intracellular adhesion molecule (ICAM)-1 on the myocytes and CD11b/CD18 on the neutrophils13,14 and mediate oxidative cytotoxicity for cardiac myocytes.15 With the use of a canine model in which we cannulated the cardiac lymph duct,16,17 we demonstrated that postischemic lymph contained cytokine activity capable of inducing ICAM-1 expression in cardiac myocytes in vitro. This activity could be abolished by neutralizing antibodies to IL-6.18

In the same model, we demonstrated that reperfusion-dependent induction of ICAM-1 mRNA occurred in the viable myocytes of the border zone of myocardial infarction.19–21

Subsequent studies showed an induction of IL-6 in the myocardium of a canine model of ischemia and reperfusion.
IL-6 mRNA was found in the ischemic area and appeared to peak earlier on reperfusion. These studies demonstrated that IL-6 synthesis is an integral part of the reaction to injury resulting from ischemia and reperfusion and is associated with induction of ICAM-1 on the myocardial cells.\(^2\) IL-6 is produced by a variety of different cell types, including monocytes/macrophages, fibroblasts, endothelial cells, mast cells, neutrophils, keratinocytes, osteoblasts, and many more.\(^2\) In our previous work, we had reasoned that IL-6 mRNA induction was reperfusion dependent because of its association with the reperfusion-induced influx of leukocytes.\(^2\) The current study confirms the induction of IL-6 mRNA in mononuclear cells; however, it also provides the first direct demonstration that cardiac myocytes in vitro and in the viable border zone of a reperfused infarction also produce IL-6 mRNA.

**Methods**

**Isolation of Cardiac Myocytes**

The isolation of cardiac myocytes from healthy mongrel dogs of either sex was performed as described previously.\(^1\) The cells were resuspended with medium A and equilibrated with 95% \(O_2/5\% \ CO_2\). Preparations with a viability of >80% were used in the experiments. Nonmyocyte cells were <10% in all experiments.

The cell samples were incubated for 60 minutes at 37°C under one of the following conditions: control, TNF-\(\alpha\) (100 U/mL), IL-1\(\beta\) (200 U/mL), lipopolysaccharide (LPS) 50 \(\mu\)g/mL, preischemic lymph (1:10 dilution), or postischemic lymph (1:10 dilution). TNF-\(\alpha\) and IL-1\(\beta\) were obtained from Genzyme Corp. LPS was obtained from Sigma Chemical Co. After the incubation, the cells were spun again, the supernatant discarded, and the cells were immediately processed for RNA isolation or for immunohistochemical studies.

All dishes and instruments used in the isolation process were pretreated with E-Toxase-Clean (Sigma Chemical Co) before sterilization to eliminate LPS contamination. Supernatants and solutions were periodically tested for LPS contamination with the E-Toxase-Clean solution. Before sterilization, the slides were rehydrated and incubated with hydrogen peroxide. Immunostaining was performed with the Elite kit (Vector laboratories), which has a peroxidase-based detection system. For myoglobin, the color reaction was performed with AEC tablets from Vector Laboratories as a substrate without counterstaining.

**Ischemia-Reperfusion Protocols**

Healthy mongrel dogs were surgically instrumented as described in detail previously.\(^16\) The animals were allowed to recover for 72 hours before occlusion. Coronary artery occlusion was achieved by inflating the coronary cuff occluder until mean flow in the coronary vessel was zero, which was determined by the Doppler flow probe. For subsequent blood flow analysis, radiolabeled microspheres were injected into the left atrium 50 minutes after occlusion. After 1 hour of occlusion, the cuff was deflated and the heart was reperfused for up to 24 hours. Lymph samples used for the myocyte experiment were collected from the cannulas in tubes containing 10 U preservative-free heparin within 3 hours of reperfusion. At the end of the reperfusion period, the heart was stopped by infusion of saturated potassium chloride and removed immediately and processed for RNA isolation or immunohistochemistry and in situ hybridization as previously described.\(^2\) Adherent sections were used for blood flow determination with radiolabeled microspheres as previously described.\(^2\) Analysis of RNA, blood flow determination, and histopathology were performed independently in different laboratories and in a blinded fashion. Sections used for studies of infarction demonstrated infarction by histopathology and ischemic blood flow of <20%.

All animal protocols were reviewed by the appropriate institutional review committee and conform to institutional guidelines.

**Northern Blot Analysis**

RNA was isolated from myocytes and myocardial tissue by use of the acid guanidinium phenol chloroform procedure.\(^2\) RNA was electrophoresed in 1% agarose gels containing formaldehyde, then transferred to a nylon membrane (Gene screen Plus; New England Nuclear) by standard procedure.\(^2\) Loading of RNA was monitored with the use of ethidium bromide staining as well as by probing the nylon membranes with glyceraldehyde 3-phosphate dehydrogenase as previously described.\(^2\) Canine IL-6 cDNA and ICAM-1 cDNA were prepared as previously described.\(^4\)

**Immunohistochemical Methods**

Samples were fixed in 4% formaldehyde, dehydrated by incubation in increasing concentrations of ethanol with a standard protocol, and then embedded in paraffin; 3- to 5-\(\mu\)m sections were obtained with microtomy. For immunostaining, the slides were rehydrated and incubated with hydrogen peroxide. Immunostaining was performed with the Elite kit (Vector laboratories), which has a peroxidase-based detection system. For myoglobin, the color reaction was performed with AEC tablets from Vector Laboratories as a substrate without counterstaining.

To define viable from nonviable cells, histological criteria such as contraction band necrosis and disrupted cell architecture were supplemented with specific histological stains. To distinguish further between infarcted and noninfarcted areas, serial sections to those used for in situ hybridization were immunostained for myoglobin with an antimaglobulin monoclonal antibody (rabbit anti-human antimaglobulin antibody; DAKO) and were stained for glycogen with PAS (periodic acid–Schiff).\(^2\)

**In Situ Hybridization**

Riboprobes for canine IL-6 and ICAM-1 were prepared as previously described.\(^2\) Digoxigenin-labeled probes were prepared by in vitro transcription from a linearized template with the Genius RNA probe labeling kit (Boehringer Mannheim Corp) according to the manufacturer’s instructions. A 215-bp [bases 483 to 697 of the published sequence\(^2\)] fragment of canine IL-6 cDNA was obtained with polymerase chain reaction amplification and was subcloned into the polymerase chain reaction 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’) probe. For the ICAM-1 probe, a 150-bp fragment was subcloned in both orientations into PBPhescript II SK+ and PGEM-3 so that use of T7 polymerase would result in the generation of single-stranded antisense (3’-5’) and of the sense (5’-3’) probe.\(^2\) The procedure was the same as described above for the IL-6 riboprobe.

The immunologic detection of the hybridized probe was done with the Genius detection kit from Boehringer Mannheim, following the guidelines of the company as previously described.\(^2\) The slides were stained with NBT and x-phosphate and then counterstained with eosin.

**Statistics**

Because of the characteristics of the experiments, raw data are presented for each of the pertinent findings along with controls. Each experiment presented was repeated at least 5 times with the same qualitative results.

**Results**

**IL-6 Induction in Isolated Ventricular Myocytes: Northern Blot Analysis**

The top panel of Figure 1 shows Northern blot analysis of mRNA isolated from ventricular myocyte preparations under a variety of experimental conditions. Marked IL-6 mRNA induc-
tion was noted in myocytes incubated with TNF-α (100 U/mL), IL-1β (200 U/mL), endotoxin (50 μg/mL), or cardiac lymph (diluted 1:10) collected during the first hour after reperfusion of a myocardial infarction. In contrast, cardiac lymph collected before the myocardial infarction (preischemic lymph diluted 1:10) demonstrated minimal or no induction of IL-6 mRNA in cardiac myocytes incubated under identical conditions. All samples were incubated at 37°C for 1 hour. Unstimulated control myocytes kept at room temperature for 1 hour never showed IL-6 mRNA expression. However, after incubation at 37°C for 1 hour, a minimal induction of IL-6 was sometimes observed and was always much lower than in the stimulated myocytes (data not shown).

Because of our previous association of IL-6 with induction of myocyte ICAM-1, we also analyzed the same Northern blots for ICAM-1 mRNA induction (Figure 1, bottom). In every case, ICAM-1 mRNA expression matched expression of IL-6 mRNA qualitatively.

**In Situ Hybridization Studies of IL-6 mRNA Expression in Isolated Cardiac Myocytes**

Serial sections through fixed ventricular myocytes treated identically to those used in the Northern blot analysis were examined for IL-6 induction with a sense and antisense riboprobe as described in “Methods.” The unstimulated cardiac myocytes (control) showed no significant staining with the IL-6 antisense probe. Myocytes stimulated with TNF-α, IL-1β, or endotoxin showed a significant amount of staining with the antisense probe. Myocytes stimulated with TNF-α, IL-1β, or endotoxin showed a significant amount of staining with the antisense probe; no staining was seen with the use of the IL-6 sense riboprobe as a control (Figure 2). Incubation of cardiac myocytes with the preischemic cardiac lymph showed little or no staining with the IL-6 antisense riboprobe, but postischemic cardiac lymph collected over the first 3 hours of reperfusion in the same dilution was capable of robust induction of IL-6 mRNA in myocytes (Figure 2).

**In Situ Hybridization Assessment of IL-6 mRNA Induction in Intact Myocardium**

In Figure 3, serial sections are examined from an experiment of 1 hour of ischemia and 3 hours of reperfusion. In Figure 3D, PAS staining demonstrates an area of predominant glycogen depletion adjoining areas of viable myocardial cells with relatively preserved glycogen on the epicardial aspect of the infarct (arrowheads) and in the innermost layers of the subendocardium (arrow). In situ hybridization studies for IL-6 in panel A show significant IL-6 mRNA induction in viable myocardial cells found in the spared innermost layers of the endocardium (arrows) as well as on the epicardial border zone (arrowheads). The presence of this preserved layer at the innermost portion of the myocardium has been previously reported. Higher magnification (Figure 3C) demonstrates that this staining for IL-6...
mRNA is seen in both the myocardium as well as (more intensely) in infiltrating mononuclear cells. Panel E (compared with panel A) demonstrates that ICAM-1 mRNA induction is seen in the same regions of viable myocytes on the border zone of the myocardial infarct. The colocalization of IL-6 mRNA and ICAM-1 mRNA induction is almost complete at 3 hours of reperfusion.

Induction of IL-6 mRNA in myocytes could be demonstrated by 1 hour of reperfusion (Figure 4) and was consistently found in viable areas abutting the area of contraction band necrosis. Note that in addition to the spared subendocardial and epicardial border zone, there is a region (within the infarct) surrounding a small vessel (arrow) that is preserved (retains glycogen) and expresses IL-6 mRNA.

Figure 5 represents serial sections taken from an animal after 1 hour of ischemia and 24 hours of reperfusion. The architectural features are almost identical to those seen at 3 hours of reperfusion, although there is greater infiltration of mononuclear cells into the infarct, and these cells produce IL-6 mRNA as described above (data not shown). The figure again demonstrates the preserved subendocardial cells expressing IL-6 mRNA.

In the absence of reperfusion, no significant staining of IL-6 mRNA was detected either at 1 hour, 2 hours (data not shown), or 4 hours (Figure 6) of ischemia. This was compatible with our previous observation of reperfusion dependence of IL-6 mRNA induction.19,22

Discussion

Our study combines in vitro data with isolated cardiac myocytes and in vivo data with our canine ischemia/reperfusion model and clearly demonstrates that myocytes can express message for IL-6 in our experimental setting. Various cytokines as well as postischemic lymph are capable of inducing IL-6 mRNA in the isolated myocytes. To the best of our knowledge, this is the first direct histological demonstration of IL-6 mRNA production in the adult cardiac myocyte, both in vitro and in vivo. These data are compatible with a recent observation with Northern blots,33 suggesting that cultured neonatal cardiac myocytes respond to hypoxic stress by IL-6 production.

Guillen et al10 demonstrated the sequential increase in IL-1β and IL-6 after myocardial infarction, suggesting an inducer function of IL-1β for IL-6. TNF-α is also upregulated in myocardial infarction and may also induce IL-6 production.34,35 In previous work, we have demonstrated that there is a rapid increase in TNF-α in postischemic lymph and that TNF-α is released from preformed stores in cardiac mast cells.36 In our model, we found no evidence for the early presence of IL-1.36 TNF-α is a known inducer of IL-6 in endothelial cells, fibroblasts, and mononuclear cells,36–39 and, in the present study, we could demonstrate that it also induces IL-6 gene expression in cardiac myocytes. We suggested that TNF-α may act as an upstream cytokine inducer of IL-6 in ischemia/reperfusion.
Investigation of IL-6 induction demonstrated reperfusion dependence. Northern blot analysis suggested that IL-6 induction preceded ICAM-1 induction in the reperfused infarct. Because both IL-6 and ICAM-1 induction were reperfusion dependent within the first 6 hours, we postulated that IL-6 production was most likely occurring in infiltrating leukocytes that would not be present in the absence of reperfusion. Indeed, we have demonstrated that mononuclear cells found within postischemic cardiac lymph demonstrate IL-6 mRNA induction within 15 minutes of reperfusion. The current studies demonstrate that mononuclear cells in tissue stained intensely in an in situ hybridization reaction with an IL-6 riboprobe (Figure 3C). However, it became obvious that IL-6 mRNA was being induced (at lower intensity) in cardiac myocytes in the viable border zone.

Relation of Cellular Source of IL-6 to ICAM-1 Induction
The exceptional colocalized induction of IL-6 and ICAM-1 at 3 hours invites consideration of an autocrine mechanism for some of the observed ICAM-1 mRNA induction. It is unlikely, however, that all of the ICAM-1 induction is autocrine. In Figure 5, examination of reperfusion after 24 hours shows that IL-6 mRNA remains confined to cardiac myocytes on the border zone of the myocardial infarction. In contrast, in our previous studies, examination of the reperfused myocardial infarction after 24 hours of reperfusion demonstrated ICAM-1 mRNA in all of the cardiac myocytes. This correlated with our observation that postischemic cardiac lymph retained its ability to induce ICAM-1 for up to 72 hours after reperfusion and was quite high at 24 hours. The induction of ICAM-1 mRNA in the “normal” myocardium might appear to result from IL-6 in the extracellular fluid and blood as observed by us and other laboratories. An autocrine contribution to early ICAM-1 induction in the border zone remains a possibility.

IL-6 Effect on Cardiac Function
Recent studies suggest that “inflammatory cytokines” may exert primary effects on myocardial function. Finkel and his coworkers have demonstrated that IL-6 may act as a nitric oxide–dependent cardiac depressant and may be associated with stunned myocardium. IL-6 has produced a nitric oxide–mediated reduction in calcium flux and contractility in chick ventricular myocytes. IL-6 is a member of a class of cytokines whose receptor mechanisms share the presence of a common protein, gp130. Two members of this family, cardiotoxin and leukemia inhibitory factor, have been shown to induce cardiac hypertrophy. These agents work through the JAK/STAT pathway induced by gp130. Transgenic mice double overexpressing IL-6 and IL-6 receptor also demonstrate myocardial hypertrophy. In addition to their effect on hypertrophy, these cytokines have been shown to be cytoprotective against apoptosis. Cardiotrophin prevents apoptotic cell death induced by serine depletion in neonatal rat cardiac myocytes. Overexpression of IL-6 and IL-6 receptor has been demonstrated to be cytoprotective in cell systems through induction of the antiapoptotic gene bcl-xL, and LIF has been demonstrated to exert an antiapoptotic effect in cardiac myocytes through induction of the same gene. It is interesting to speculate that these 3 responses, reduced contractility, positive protein balance (hypertrophy), and antiapoptosis, might favorably influence myocardial cells and allow them to survive in an area of jeopardy.

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