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Cytokine mRNA Expression in Postischemic/ Reperfused Myocardium

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While the role of cytokines in mediating injury during hind limb skeletal muscle ischemia followed by reperfusion has recently been described, the role of cytokines in myocardial infarction and ischemia/reperfusion have remained relatively unexplored. We hypothesize that cytokines play an important role in the regulation of postischemic myocardial inflammation. This study reports the temporal sequence of proinflammatory cytokine gene expression in postischemic/reperfused myocardium and localizes interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)-protein by immunostaining. Rats were subjected to either permanent left anterior descending (LAD) occlusion or to 35 minutes of LAD occlusion followed by reperfusion and sacrificed up to 7 days later. Rat-specific oligonucleotide probes were used to semiquantitatively assess the relative expression of mRNA for TNF-α, IL-1β, IL-2, IL-6, interferon-γ (IFN-γ), and transforming growth factor-β1 (TGF-β1) utilizing the reverse transcriptase-polymerase chain reaction amplification technique. Increased cardiac mRNA levels for all cytokines except IL-6 and IFN-γ were measurable within 15 to 30 minutes of LAD occlusion and increased levels were generally sustained for 3 hours. During early reperfusion, mRNA levels for IL-6 and TGF-β1 were significantly reduced compared with permanent LAD occlusion. In both groups, cytokine mRNA levels all returned to baseline levels at 24 hours, while IL-1β, TNF-α, and TGF-β1 mRNA levels again rose significantly at 7 days only in animals with permanent LAD occlusion. Immunostaining for IL-1β and TNF-α protein revealed two patterns of reactivity: 1) microvascular staining for both IL-1β and TNF-α protein only in postischemic reperfused myocardium in early post-reperfusion time points; and 2) staining of infiltrating macrophages in healing infarct zones which was most prominent at 7 days after permanent LAD occlusion. These results provide evidence for local expression of cytokine mRNA in postischemic myocardium and suggest that regulation of local cytokine release is altered during the postischemic period. (Am J Pathol 1995, 146:419–428)

In acute coronary occlusion leading to permanent myocyte injury, complex local interaction exists between endothelial cells, accumulations of infiltrating leukocytes, and tissue-based monocytes and myocytes. Recent studies have highlighted the adaptive and maladaptive effects of growth factors, a variety of vasoactive substances, and cytokines produced within the myocardium during various forms of myocardial stress and injury.[1–4] These substances, produced and released by either myocardial cells and/or inflammatory cells, are likely to influence the myocardial inflammatory response and possibly mediate or regulate acute myocardial injury.[5–8] Although the precise mechanisms of tissue injury following ischemia with reperfusion remain controversial,[9] recent studies support the hypothesis that postischemic reperfusion activates interconnected inflammatory cascades.[10–14]

At this point, relatively little is known of the signaling molecules that might be released during and after myocardial injury or stress. It has been elegantly demonstrated that during reperfusion following hind limb ischemia, increased circulating levels of tumor necrosis factor-α (TNF-α), interleukin–1 (IL-1), and IL-6 appear in plasma.[13] Hind limb skeletal muscle injury as well as secondary remote pulmonary injury appear to be TNF-α and IL-1-dependent[12] and likely require

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tissue expression of β2 integrins and selectins.[11–13] The role of cytokines in myocardial infarction and ischemia/reperfusion have remained relatively unexplored. The purpose of this study was to use the rat model of left anterior descending (LAD) occlusion to determine both the pattern of proinflammatory cytokine gene expression after permanent coronary artery occlusion or transient coronary artery occlusion followed by reperfusion and to localize myocardial TNF-α and IL-1β protein.

Materials and Methods

Surgical Preparation and Experimental Design

This study conformed to the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by The Johns Hopkins Animal Care and Use Committee. The surgical protocol has been described in detail elsewhere.[10,15,16] Female Sprague-Dawley rats (225 to 300 g body wt) were used for all phases of this study and were anesthetized with sodium methohexital (35 mg/kg body wt ip). Intermittent positive pressure ventilation was given with 95% O_2-5%O_2. The hearts were exposed through a left intercostal thoracotomy and the pericardium was opened. The left coronary artery was ligated within 2 to 3 mm left of the proximal aorta. For permanent occlusions, the chest was closed. For reperfusion, the 2-0 suture thread was released after 35 minutes of LAD occlusion. In both models, myocardial ischemia was confirmed by regional cyanosis. In the permanent LAD occlusion model, 3 animals each were sacrificed at times 0, 15, 30, 60, 180 minutes, 24 hours, and 7 days after occlusion. In the ischemia/reperfusion model, 3 animals each were sacrificed at 15, 30, 60, 180 minutes, 24 hours and 7 days after reperfusion. Therefore, in group 2 animals with 35 minutes of LAD occlusion followed by reperfusion, sacrifice at 15 minutes represents 50 minutes in the permanent occlusion animals (group 1). Two transverse slices were dissected into right and left ventricle specimens, snap-frozen, and stored at −70 C. For control purposes, a piece of psoas muscle was also dissected, snap-frozen and stored at −70 C.

RNA Isolation and Polymerase Chain Reaction Amplification

Total cellular RNA was extracted from heart sections at times 0, 15, 30, 60, and 180 minutes as well as 24 hours and 7 days after permanent occlusion or 35 minutes of LAD occlusion followed by reperfusion. The methods used in the current study have been described elsewhere.[17–19] In brief, approximately 100 mg of cardiac muscle were homogenized with 800 μl RNAZOL STAT (Teltest, Friendswood, TX) in a 1.5-ml microfuge tube, after which 80 μl chloroform was added. After vigorous vortexing the mixture underwent centrifugation and the aqueous phase transferred to a new microfuge tube containing an equal volume of isopropanol and the RNA recovered by precipitation by chilling at −20 C for 1 hour, 2 μg of total RNA were subjected to first-strand cDNA synthesis in a 20 μl reaction containing 50 mmol/L Tris-HCl (pH 8.3 at 42 C), 20 mmol/L KCl, 10 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 1 mmol/L of each dNTP, 20 μg/ml oligo-dT(15) and 20 U AMV reverse transcriptase (Boehringer-Mannheim, Indianapolis, IN) for 40 minutes at 42 C. After completion of first-strand synthesis, the reaction was diluted to 100 μl with distilled water and 5 μl was used for each polymerase chain reaction (PCR). PCR reactions (in a volume of 50 μl) contained 200 μmol/L of each dNTP, 1 μmol/L of each specific primer, buffer as supplied with the Taq polymerase (Boehringer-Mannheim) and 2.5 U Taq polymerase (Boehringer-Mannheim). The primers were designed to amplify a product of between 250 and 500 nucleotides in length (Table 1) and were also designed to cross introns to avoid confusion between cytokine mRNA expression and genomic contamination. The PCR reaction was performed at 3 different cycle numbers to ensure it was performing in the linear range at which there is a fixed relationship between input RNA and densitometric readout. The optimal cycle number for the monokines [TNF-α, IL-1β, IL-6, and transforming growth factor-β1 (TGF-β1)] was 25 and for the lymphokines interferon-γ (IFN-γ) and IL-2, 35. A 9600 thermal cycler (Cetus Corp, Emeryville, CA) was used. Our PCR negative control was without cDNA (H_2O) and our positive PCR control was a known positive cDNA. Our cDNA negative control contained no RNA (H_2O), while the positive cDNA control was a known positive RNA. In addition, the amount of PCR product was determined by comparison of signal density to that of standard curves from simultaneously amplified serial dilutions of a positive control for the cytokine of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Generation of these standard curves ensured a fixed relationship between the initial RNA input and the densitometric read-out.[18,19]

A portion of the PCR reaction product (25%) was electrophoresed through a 1.2% agarose gel and transferred to nitrocellulose. Filters were prehybrid-

Table 1. *Sequences of the Oligonucleotide Primers Used for PCR Amplification for Cytokine mRNAs, Product Sizes Predicted, and the Sequences of the Internal Oligonucleotides Used for Southern Blot Analysis of Amplified Products*

Target	3′ Oligonucleotide	5′ Oligonucleotide	Product size (*NT*)	Probe
GAPDH	CTCAGTGTAGCCCAGGATGC	ACCACCATGGAGAAGGCTGG	508	GTGGAAGGACTCATGACCACAGTCCATGCC
IL-1α	CTTTATCCTACCCATCCGGCAC	CACAGGTAGTGAGACCGACCTC	589	CTAGTGGAACCAGCCCGACATATGATACTG
IL-1β	CTCTGCTTGAGAGGTGCTGATGTAC	GAAGCTGTGGCAGCTACCTATGTCT	520	CTGGAGAGTGTGGATCCCAAACAATACCCA
IL-2	GAGCCCTTGGGGCTTACAAAAAG	CAGGTGCTCCTGAGAGGGATCG	500	GCCAATTCGATGATGAGCACGAACTGTGG
IL-6	CTAGGTTTGCCGAGTAGACCTCATAGTGACC	ATGAAGTTTCTCTCCGCAAGAGACTTCCAG-CCAG	636	GGTCTGTTGTGGGTGGTATCCTCTGTGAAGTCTCCTCT-CCGGACTTGTG
IFN-γ	TCAGCACCGACTCCTTTTCCGCTTCCTTAGGC	GTTACTGCCAAGGCACACTCATTGAAAGCC	413	GACAACCAGGCCATCAGCAACAACATAAGT
TGF-β	GACGTCAAAAGACAGCCACT	GAAGCCATCCGTGGCCAGAT	461	TCTCTGCAAGCGCAGCTCTGCACGGGACAGCAA
TNF-α	GGACTCCGTGATGTCTAAGT	CACGCTCTTCTGTCTACTGA	546	TGAGAAGATGATCTGAGTGTGAGGGTCTGG

ized in 2X SSC containing 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin, 2 mmol/L sodium pyrophosphate, 1 mmol/L ATP, and 50 μg/ml *Escherichia coli* tRNA at 55 C for 3 to 4 hours. Hybridization was in the same buffer containing 0.1% SDS at 55 C for 12 to 14 hours. Oligonucleotide probes (Table 1) internal to the PCR primers were radiolabeled with [^{32}P]ATP by T4 polynucleotide kinase. After hybridization, filters were washed in 6X SSC, 0.1% SDS at 55 C, and finally in 2X SSC at 55 C before autoradiography. The relative radioactivity for bands on autoradiograms was estimated by volume integration by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). The relative intensity of bands for cytokine mRNA was normalized using the intensity of the autoradiogram for the internal control, GAPDH.

Immunohistochemistry

To localize cytokine protein and macrophages within the myocardium, a colloidal gold procedure with silver enhancement was used. The primary reagents used were rabbit anti-human TNF-α (Endogen code:300A451, Boston, MA), rabbit anti-human interleukin-1β (UBI catalog no. 06-220 Lake Placid, NY), and mouse anti-rat monocytes and macrophages (ED1) (Serotec Harlan Bioproducts for Science, code no. MCA 341, Indianapolis, IN). The secondary reagent used for cytokine localization was a biotinylated goat anti-rabbit IgG (heavy and light) (Vector Laboratories, catalog no. Ba-1000, Burlingame, CA), and for the macrophage marker, a biotinylated F(ab′) goat anti-mouse Fc (Jackson Immunoresearch Laboratories, code no. 115-066-071, West Grove, PA). The tertiary used was a streptavidin albumin, colloidal gold (Sigma S-4275, St. Louis, MO) and the appropriate dilution was incubated for 1 hour. After the tertiary incubation, slides were washed in TBS pH 8.2, 3 to 5 times for at least 2 minutes for each wash and then fixed in 2.5% glutaraldehyde for 15 minutes. Silver enhancer solution A (S-5020) and B (S-5145) (Sigma silver enhancement kit SE-100), were mixed 1:1. Incubation times for these experiments were 12 to 16 minutes. Slides were then rinsed thoroughly in distilled water and fixed in 2.5% aqueous sodium thiosulfate for 2–3 minutes. Slides were then washed, counterstained, and mounted.

Two blinded trained observers reviewed the sections. At least two sections from each animal were immunohistochemically stained and assessed for each primary reagent. To verify the specificity of the cytokine staining, the following control experiments

were done: 1) identification of a microvascular pattern of staining within the myocardium of rats treated with lipopolysaccharide (LPS) 0.25 mg/kg/d for 14 days), 2) preabsorption of the primary antibody with corresponding peptide antigen (1 nmol peptide/ml of diluted antiserum), 3) substitution of the primary antibody with nonimmune mouse serum, and 4) omission of primary antibody. Immunoreactivity of the right ventricle served as an internal control for non-ischemic/reperfused myocardium in each animal.

Statistics

Comparisons of cytokine mRNA levels values at different time points in group 1 and group 2 animals versus control heart samples were made with the means of normalized OD readings (3 animals performed twice), using analysis of variance followed by a Fisher's PLSD. A P value of 0.05 was considered statistically significant. All statistical analyses were performed using Statview 4.01 (Abacus Concepts, Berkeley, CA).

Results

The mortality rates for the permanent LAD occlusion group and the reperfusion group were not significantly different (10% versus 14%, respectively).

RNA Isolation and PCR Amplification

Figure 1 shows representative autoradiograms highlighting mRNA expression for TNF-α, IL-1β, IL-6, and TGF-β1, IL-2, and IFN-γ. Figure 2, A to F, is a graphic representation of the time course of relative levels of IL-1β, TNF-α, IL-6, IL-2, TGF-β1, and IFN-γ mRNA (OD cytokine band/OD GAPDH) in rats subjected to permanent LAD occlusion (group 1) and 35 minutes LAD occlusion followed by reperfusion (group 2). The data were then normalized by assigning an artibrary number of 1 to the sample which demonstrated peak expression of each cytokine and then means and standard errors were calculated. Each PCR reaction was performed at least twice and three animals per time point were utilized.

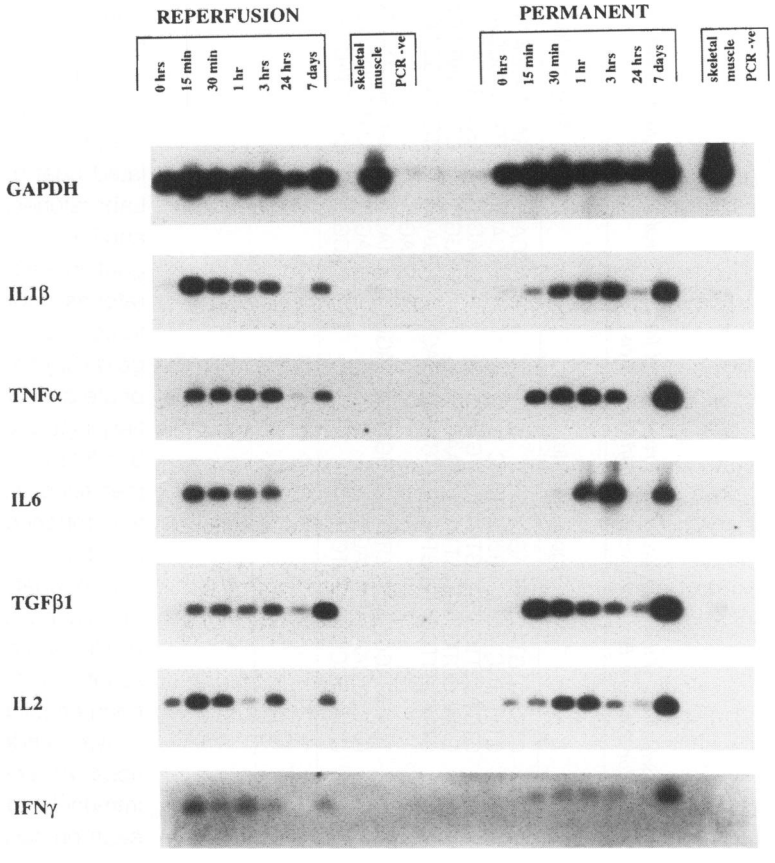


Figure 1. *Cytokine mRNA expression in samples of myocardium and skeletal muscle obtained in rats subjected either to permanent LAD occlusion and sacrificed at 15, 30, 60, 180 minutes, 1 and 7 days; or 35 minutes LAD occlusion followed by reperfusion and sacrificed at 15, 30, 60, 180 minutes, 1 and 7 days after reperfusion. Time 0 = control sample of normal rat left ventricle. PCR-ve = PCR negative control.*

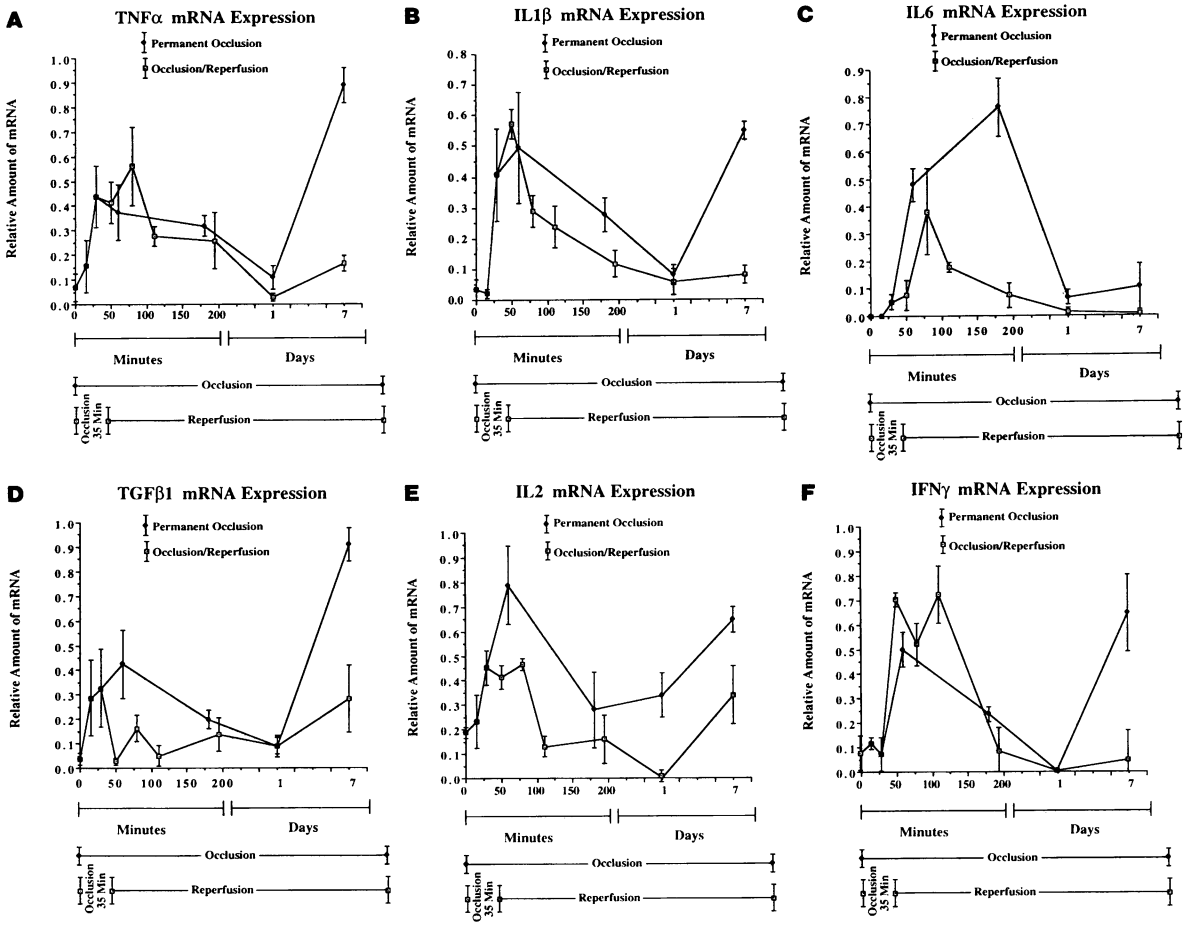
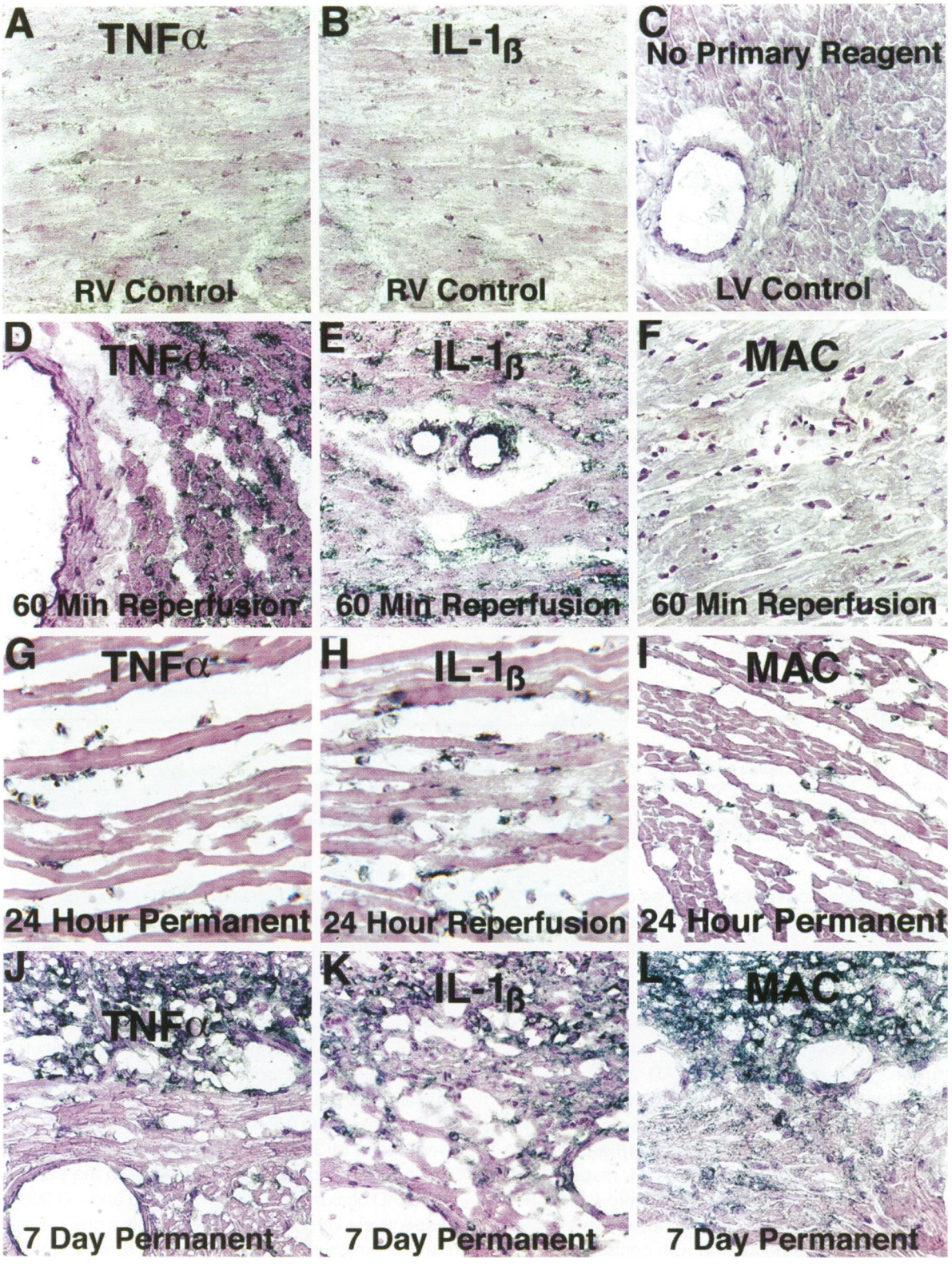


Figure 2. *Time course of relative levels of IL-1β, TNF-α, IL-6, TGF-β1, IL-2, and IFN-γ mRNA (OD cytokine band/OD glyceraldehyde-3-phosphate dehydrogenase band) in rats subjected to permanent LAD occlusion and 35 minutes of LAD occlusion followed by reperfusion. The data were normalized by assigning an arbitrary number of 1 to the sample, which demonstrated the peak expression of each cytokine and then means and standard errors were calculated. Each time point represents the normalized mean and standard error of three animals with each PCR performed twice. Time 0 represents results obtained from the left ventricle of a normal control animal.*

Different kinetic profiles of cardiac cytokine mRNA expression were observed in rats with permanent occlusion (group 1) as compared with 35 minutes of LAD occlusion followed by reperfusion (group 2). In group 1 animals, significant elevations (as compared with control hearts) in TNF-α, IL-1β, IL-6, and TGF-β1 were found during the 3-hour period after LAD occlusion. After 15 minutes of LAD occlusion, only TGF-β1 levels are significantly increased ($P = 0.04$). At 30 minutes, peak levels for TNF-α were found ($P = 0.005$); at 1 hour peak levels for IL-1β and TGF-β1 were found ($P = 0.003$ and $P = 0.004$, respectively); and at 3 hours peak levels of IL-6 were found ($P < 0.0001$). While all the cytokine levels were significantly elevated as compared with control heart at 3 hours (TNF-α, $P = 0.02$; IL-1β, $P = 0.01$; IL-6, $P < 0.0001$; TGF-β1, $P = 0.008$), all returned to baseline levels at 24 hours. Evidence for a biphasic process was found in that at 7 days after LAD occlusion, cytokine mRNA levels for TNF-α, IL-1β, and TGF-β1 all rose significantly again from control values ($P < 0.0001$, 0.004, and < 0.0001, respectively).

In group 2 animals, mRNA for TNF-α, IL-1β, IL-6, and TGF-β1 could all be demonstrated after reperfusion. Peak levels of IL-1β were seen at 15 minutes after reperfusion (representing 50 minutes in the permanent LAD occlusion group; $P = 0.008$ compared with control heart). Peak levels for TNF-α and IL-6 were found at 30 and 60 minutes after reperfusion, respectively (representing 65 minutes in the permanent LAD occlusion group) ($P = 0.026$ and < 0.0001, respectively, versus control heart). Unlike group 1 animals in which mRNA cytokine levels remained elevated at 3 hours after LAD occlusion, in group 2 animals all cytokine levels returned to baseline levels by 3 hours and remained so at 24 hours and at 7 days.


A
TNFα
RV Control
B
IL-1β
RV Control
C
No Primary Reagent
LV Control
D
TNFα
60 Min Reperfusion
E
IL-1β
60 Min Reperfusion
F
MAC
60 Min Reperfusion
G
TNFα
24 Hour Permanent
H
IL-1β
24 Hour Reperfusion
I
MAC
24 Hour Permanent
J
TNFα
7 Day Permanent
K
IL-1β
7 Day Permanent
L
MAC
7 Day Permanent

The expression of lymphokines IL-2 and IFN-γ was also examined. IL-2 and IFN-γ were more difficult to detect requiring 35 cycles of amplification. Both were detectable in group 1 and 2 animals early after reperfusion, but the significant peaks were noted at 7 days in the permanent LAD occlusion model (group 1). Hearts from non-operated rats, portions of right ventricle, and skeletal muscle from operated rats all demonstrated low levels of expression of all cytokines.

Immunohistochemistry

Control sections incubated with nonimmune mouse serum as the first antibody, with a primary antibody preabsorbed with homologous antigen or omission of the primary antibody, showed no specific immunoreactivity (Figure 3). Control LPS-treated rats demonstrated diffuse immunoreactivity for both IL-1β or TNF-α protein within the myocardial microvascular bed (not shown). In experimental animals during early postischemia time points, black immunoreaction product indicating either IL-1β or TNF-α protein was limited the microvascular bed of the left ventricular free wall only in reperfused animals at 60 minutes and 3 hours after reperfusion. No specific immunoreactivity was observed in the right ventricle or interventricular septum of these same samples, and no increase in the numbers of infiltrating macrophages were observed at these time points. No specific immunoreaction products were observed in the myocardium at earlier time points of ischemia/reperfusion or at any early time point (up to 3 hours) after permanent LAD occlusion.

During later postischemia time points, small clusters of macrophages were observed infiltrating the edges of myocardium undergoing coagulation necrosis at 24 hours after permanent LAD occlusion, and rare macrophages randomly distributed throughout the left ventricular free wall were noted at 24 hours in the reperfused animals. Double immunostaining demonstrated that individual macrophages were positive for either IL-1β or TNF-α protein. Rare flattened cells immediately adjacent to myocyte sarcolemmal surfaces (which likely represented endothelial cells) also showed immunoreactivity. At 7 days, only animals with permanent coronary occlusion had extensive macrophage infiltration within healing infarcted myocardium. In contrast, at 7 days, reperfused myocardium had only rare macrophages in smaller infarct areas which appeared to contain more organized scar and less mononuclear cell infiltration. Immunoreactivity for IL-1β and TNF-α protein was observed in individual mononuclear cells which stained with the macrophage marker by double immunostaining, most prominently in rats subjected to permanent coronary occlusion. To determine whether cytokine protein and gene expression would be different in reperfused myocardium at a time point when macrophage infiltration predominated, we performed pilot experiments in which rats were subjected to transient LAD occlusion followed by reperfusion and sacrificed at 3 days. At this time point, similar to animals subjected to permanent LAD occlusion at 7 days, extensive macrophage infiltration of reperfused myocardium was noted and cytokine gene expression and IL-1β and TNF-α protein localized to infiltrating macrophages was indistinguishable from animals with permanent occlusion at 7 days (data not shown).

Discussion

This study is the first to demonstrate induced myocardial gene expression for various proinflammatory cytokines both after permanent LAD occlusion and temporary LAD occlusion followed by reperfusion. Induced cytokine gene expression was clearly demonstrated within heart samples at early time points in both models before significant histological evidence for inflammation is usually apparent. This suggests that cytokine gene expression may be primarily generated by intrinsic myocardial cells in response to ischemia/reperfusion. Immunohistochemical studies

Figure 3. *Control immunohistochemical staining for TNF-α and IL-1β in right ventricular myocardium* (A, B) *and with omission of primary reagent in left ventricular myocardium* (C), *in an animal subjected to 35 minutes of LAD occlusion followed by 60 minutes of reperfusion. No significant immunoreactivity is noted. Sections from left ventricular free wall from the same animal show intense immunoreactivity for TNF-α and IL-1β within the microvascular bed* (D, E). *In addition, immunoreactivity for TNF-α and IL-1β within arteriolar vessels is demonstrated* (D, E). *The large artery* (D) *does not show immunoreactivity for TNF-α. No significant macrophage infiltrates were noted after 60 minutes of reperfusion* (F). *Although photomicrographs* D *to* E *do not exhibit contraction band injury, multifocal zones of contraction band injury were typically found in reperfused myocardium.* G *to* I *represents sections from the border zones of infarct areas in animals subjected to either permanent LAD occlusion for 24 hours* (G, I) *or 35 minutes of LAD occlusion followed by reperfusion for 24 hours* (H). *Individual round interstitial mononuclear cells, as well as scarce flattened cells which appear to line sarcolemmal surfaces of myocytes, show immunoreactivity with TNF-α* (G) *and IL-1β* (H), *respectively. Significant macrophage infiltrates were noted at 24 hours in such border zones in both permanent LAD occlusion* (I) *and in reperfused animals at 24 hours* (*data not shown*). J *to* L *represents sections of healing infarcted myocardium within the left ventricle taken at 7 days from an animal subjected to permanent LAD occlusion. After 7 days of permanent LAD occlusion, the zone of organized infarcted myocardium contains a large number of infiltrating macrophages* (L), *while immunoreactive mononuclear cells for TNF-α and IL-1β are found throughout the infarct zone* (J, K). *In contrast, within zones of organized infarcted myocardium at 7 days after reperfusion, only rare macrophages were noted and no significant immunoreactivity within the myocardium for TNF-α and IL-1β was evident* (*data not shown*).

demonstrated IL-1β and TNF-α protein within the microvascular venous and arteriolar bed in animals with postischemic reperfusion, suggesting an induction of cytokine protein synthesis in postischemic/reperfused myocardium. After cytokine gene levels essentially returned to baseline levels by 24 hours in both models, significant cytokine gene expression for TNF-α, IL-1β, and TGF-β1 was again seen at 7 days, most notably in the permanent LAD occlusion model. Myocardial lesions in the permanent LAD occlusion model at 7 days are composed primarily of infiltrating macrophages and immunostaining for IL-1β and TNF-α demonstrated immunoreactivity for both cytokines in infiltrating macrophages within healing infarcted myocardium. In contrast, in animals subjected to LAD occlusion followed by reperfusion, infarct zones at 7 days postischemia have more collagen deposition and significantly less inflammation. In these animals, significant cytokine gene expression and cytokine protein at 7 days was not demonstrated. Although more late time points will need to be studied in the future, these data suggest a possible role for cytokines in infarct healing and/or myocardial remodeling after infarction, perhaps most prominently when conditions allow for the development of large transmural infarcts.

Cytokines in Early Postischemic Myocardium

The presence of induced cytokine gene expression and cytokine protein within postischemic myocardium represents a myocardial response to injury. Although it is well established clinically that early reperfusion will salvage myocardium compared to permanently occluded coronary arteries, it remains controversial whether reperfusion has detrimental effects on postischemic myocardium,[20,21] and the precise role that cytokines play in myocardial reperfusion remains speculative. In our model, reperfusion was associated with induced cytokine protein localized to the microcirculatory endothelium which may provide an efficient mechanism for induction of endothelial cell adhesion molecule expression. Indeed, in experiments to be described in a subsequent publication, induction of intercellular cell adhesion molecule (ICAM-1) within microcirculatory endothelium was found only after postischemic reperfusion in a similar pattern to that found for TNF-α and IL-1β. It should be noted, however, that in our current study, it was not possible to determine whether the induction of cytokines during the early post-reperfusion period was secondary to a direct effect of reperfusion per se versus an effect related to a smaller infarct size typically found after reperfusion.

The lack of correlation between our ability to detect mRNA coding for the relevant cytokines and detection of the cytokine protein itself during early postischemic time points in the permanent LAD occlusion model is interesting and suggests that 1) the cytokine is consumed by an autocrine pathway by rapid kinetics; 2) there are increased levels of proteases that are selectively present (or induced) in sufficient levels in the myocardium of permanently LAD occluded animals which result in degradation which leads to failure of the appropriate monoclonal antibody to react; 3) increased stability of the mRNA for reasons not clear at present; 4) there is a block at the translational or post-translational level; 5) there are potential differences in sensitivity between PCR and immunohistochemistry; 6) there is an expected lag in translation of protein; or 7) all or a combination of the above.

Our current study demonstrates that mRNA levels for IL-6 and TGF-β1 were higher in the permanent LAD occlusion model at 60 minutes, and IL-6 continued to be more significantly elevated at 3 hours. Elevated serum IL-6 levels are found after acute myocardial infarction in humans[22] and after coronary artery bypass grafting,[23] the latter study suggesting a possible role for IL-6 in myocardial stunning. In contrast, TGF-β1 may act as a cardioprotectant. Lefer et al[24] have demonstrated that when TGF-β1 was given before or immediately after ischemic injury, there were reduced amounts of generated superoxide anions, maintenance of endothelial-dependent coronary relaxation, and reduced injury mediated by exogenous TNF-α. The rise in mRNA for IFN-γ during reperfusion is also interesting, since IFN-γ and IFN-β cause marked inhibition of cytokine-induced IL-8 gene transcription *in vitro*.[25] Although we did not measure mRNA levels for the rat equivalent of IL-8 in this study (termed ENA78; Strieter et al[26]), IL-8 is a neutrophil chemoattractant and functional stimulant that is induced by IL-1β and TNF-α and may play an important role in recruiting neutrophils to ischemic myocardium.

Possible Effects of Cytokines in Postischemic Myocardium

The concept that cytokine induction may represent a potential mechanism of myocardial protection is supported by studies by Brown et al[27,28] which have shown that rats pretreated with TNF-α or IL-1β within 6 to 36 hours before coronary occlusion were resistant to ischemia/reperfusion injury. Studies have also demonstrated induced heat shock protein formation

in cultured myocytes exposed to cytokines, thus making the cells more resistant to subsequent challenge.[29] While such studies have suggested a possible protective role for cytokines in ischemic injury, other studies suggest a role for cytokines in mediating myocardial injury. Studies by Seekamp et al have shown that during reperfusion following hind limb ischemia, increased circulating levels of TNF-α, IL-1, and IL-6 appear in plasma[13] and tissue injury appears to be TNF-α- and IL-1-dependent.[12] IL-1β and TNF-α have also been shown to have direct negative inotropic effects[5] and promote the induction of ICAM-1 on endothelial cells.[30] Shreeniwas et al[31] demonstrated that hypoxic endothelial cells have increased mRNA for IL-1α, which was followed by induction of ICAM-1 and ELAM-1 and increased neutrophil adherence during reoxygenation.

In addition to the myriad of cytokine effects on endothelium, IL-1β and TNF-α are also potent inducers of ICAM-1 expression within myocytes. Youker et al[32] demonstrated neutrophil adherence to cultured canine myocytes only if the myocytes were previously treated with IL-1β or TNF-α, both of which induced ICAM-1 expression. More recently, Youker et al[33] demonstrated induction of myocyte ICAM-1 gene expression in the viable border zone of reperfused myocardium. Our own studies[34] with human fetal myocytes and others with murine myocytes[35] have shown that myocyte ICAM-1 expression is induced with exposure to IFN-γ or IFN-γ plus TNF-α. High levels of mRNA for both IFN-γ and TNF-α very soon after reperfusion suggest an efficient mechanism by which a large population of myocytes can be induced to express ICAM-1, thereby rendering them both more susceptible to neutrophil-mediated injury and macrophage-mediated removal of necrotic myocytes.

Cytokine mRNA Expression in Late Postischemic Myocardium

In both groups, all increased cytokine mRNA expression fell back to control levels by 24 hours. At day 7 after permanent LAD occlusion, when the early phases of an organizing myocardial infarct are seen histologically, significant increases in mRNA levels for TGF-β1, TNF-α, and IL-1β were again noted. Immunohistochemical studies localized the renewed cytokine production for IL-1β and TNF-α to infiltrating macrophages. In contrast, cytokine gene expression remained at control levels 7 days after reperfusion, which likely reflected both differences in the kinetics of infarct healing and the presence of smaller zones of infarction post-reperfusion.

Conclusions

The role that cytokines play in myocardial ischemia and reperfusion will likely reflect their integration in the complex inflammatory cascade initiated after ischemia/reperfusion. Our current study documents the presence of and time course of gene expression for various proinflammatory cytokines in ischemic and postischemic cardiac tissue. Further investigations designed to specifically block individual cytokines with antibodies or peptides will be useful to determine the role for each cytokine in modulating the postischemic immune response and in the possible modulation of myocardial protection and/or myocardial injury.

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