Cytokine Gene Expression After Myocardial Infarction in Rat Hearts
Possible Implication in Left Ventricular Remodeling

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Background—A large transmural myocardial infarction may initiate structural and geometric changes in the left ventricle that are commonly referred to as remodeling. Progressive, adverse remodeling of the myocardium may lead to ventricular dilatation and congestive heart failure. Recent studies have highlighted the effects of some cytokines on immune-mediated myocyte injury, postischemic myocardial inflammation, and cardiac function. However, studies of the involvement of cytokines in remodeling of the heart are few.

Methods and Results—In a rat model of myocardial infarction, progressive dilatation of the left ventricular cavity and lack of appropriate hypertrophy of the surviving myocardium were confirmed by transthoracic echocardiography. The relative expression of mRNA for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the infarcted and noninfarcted myocardium of these rats, as well as in a group of sham-operated animals, was assessed by the technique of quantitative polymerase chain reaction amplification. In the infarcted region, TNF-α, IL-1β, and IL-6 gene expression peaked at 1 week after infarction and decreased rapidly thereafter. In contrast, at 20 weeks after infarction, the gene expression levels of these cytokines remained significantly higher in the noninfarcted than in the infarcted zone or in the myocardium of sham-operated animals. Furthermore, the levels of these cytokines in the noninfarcted region correlated with the left ventricular end-diastolic diameter measured at 8 and 20 weeks after infarction. Among these cytokines, IL-1β expression was highest, and its level correlated well with collagen deposition in the noninfarcted myocardium at 8 and 20 weeks after surgery. At 20 weeks after infarction, immunohistochemical analysis revealed the presence of IL-1β in macrophages, endothelial cells, and vascular smooth muscle cells in the noninfarcted region, whereas no such immunoreactivity was found in the myocardium of sham-operated animals.

Conclusions—These findings suggest the possible involvement of cytokines during the remodeling process of the noninfarcted left ventricular myocardium. (Circulation. 1998;98:149-156.)

Key Words: cytokines ■ remodeling ■ myocardial infarction ■ immunohistochemistry

Ischemic heart disease is the leading cause of CHF in most Western countries.1,2 A large transmural MI may initiate a cascade of progressive structural and geometric changes in the LV that is commonly referred to as remodeling. The remodeling process is believed to serve initially as a compensatory mechanism to maintain cardiac output. However, these architectural changes may eventually contribute to the development of congestive symptoms from afterload mismatch and exacerbation of LV dysfunction.3 The effects of cytokines on immune-mediated myocyte injury and myocardial function have been studied in depth recently. Cytokines such as IL-1β or TNF-α have negative inotropic effects in the isolated perfused heart,4 papillary muscle preparation,5 and cultured myocytes.6 IL-1β activates fibroblasts,7 which might affect the remodeling process of the heart.8 In addition to these humoral effects, these cytokines may cause direct myocyte injury by activating cytotoxic T cells.9 In the present study, the remodeling of the LV in a rat model of MI was monitored with transthoracic echocardiography, whereas the expression of cytokines in the infarcted and noninfarcted myocardium and in the hearts of sham-operated animals was examined by use of quantitative reverse transcriptase PCR and immunohistochemical analysis.

Methods
Experimental MI
Male Wistar rats weighing 250 to 300 g were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) intubated, and ventilated with a volume-cycled small-animal respirator. After the heart was exposed via a left anterior thoracotomy, the left anterior descending coronary artery was ligated proximally with a 7–0 silk suture. Positive end-expiratory pressure was applied to fully inflate the lungs, and the chest was closed in layers. Another group of rats underwent the identical procedure without ligation of the coronary artery. Four animals from each group were killed by...
Cytokines in the Infarcted Heart

Selected Abbreviations and Acronyms

CHF = congestive heart failure
DCM = dilated cardiomyopathy
EDD = end-diastolic diameter
ESD = end-systolic diameter
IL = interleukin
iNOS = inducible nitric oxide synthase
LV = left ventricle, left ventricular
MI = myocardial infarction
PCR = polymerase chain reaction
RWT = relative wall thickness
TNF = tumor necrosis factor

Excision of the heart under pentobarbital anesthesia at 1, 8, and 20 weeks after surgery, respectively.

Determination of Infarct Size and Histological Analysis

The LV and septum were separated from the right ventricle and weighed. The LV was cut into six transverse slices from apex to base. The first, third, and fifth slices were fixed in 10% formalin, and the remaining three were used for RNA preparation. The former three slices were embedded in paraffin and cut into 4-μm sections that were mounted onto slides and stained with Sirius red F3BA (0.1% solution in saturated aqueous picric acid) to allow a clear discrimination between cardiomyocytes and collagen matrix. The endocardial and epicardial circumferences of the infarcted and noninfarcted LV were outlined with a digital image analyzer (LUSEX3, Nikon). The infarcted mean circumference (mean of endocardial and epicardial circumferences) of the infarcted and noninfarcted interventricular septum at a ×400 magnification. Three sections per animal and 20 fields per section were scanned and computerized with a LUSEX3 digital image analyzer on the basis of the red staining of the collagen. Volume collagen fraction was calculated as the sum of all connective tissue areas divided by the total area of the image. Perivascular collagen was excluded from this measurement. It has been shown that the total volume fraction determined by this morphometric approach is closely related to hydroxyproline concentration of the ventricle.

Echocardiographic Studies

The evolution of the LV dimensions and function in vivo was followed by transthoracic echocardiography performed at 0, 1, 4, 8, and 20 weeks after surgery (Hewlett-Packard) with a 7.5-MHz sector transducer. M-mode echocardiograms, guided by two-dimensional long-axis images, were obtained through the anterior and posterior LV walls at the level of the papillary muscles and recorded at a paper speed of 100 mm/s. The LV EDD and ESD were measured from the M-mode tracings according to the American Society for Echocardiology leading-edge method. The LV posterior wall thickness (PWT) was measured at end diastole. For each measurement, data from at least three consecutive cardiac cycles were averaged. LV fractional shortening (FS) and RWT were calculated according to the following formulas:

\[
\text{LV FS} (\%) = \frac{(\text{EDD} - \text{ESD})}{\text{EDD}} \times 100
\]

\[
\text{RWT} = 2 \times \frac{\text{PWT}}{\text{EDD}}
\]

RNA Preparation and First-Strand cDNA Synthesis

Infarcted hearts at 1, 8, and 20 weeks after surgery were sectioned into noninfarcted and infarcted areas by visual inspections; beginning 1 week after coronary ligation, the infarcted area becomes pale and can be easily distinguished from normal myocardium. The border zone was included in the infarcted area. Total RNA was isolated by use of the guanidinium thiocyanate/phenol/chloroform/isoamyl alcohol procedure. Total RNA (10 μg) was subjected to first-strand cDNA synthesis in a 40-μL reaction mixture containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 1 mmol/L dNTP (Perkin-Elmer Cetus), 0.825-optical density random hexamers (Pharmacia LKB Biotechnology Inc), 40 U of RNAasin (Promega Corp), and 200 U of murine leukemia virus reverse transcriptase (Gibco BRL). The reaction mixture was incubated at 37°C for 60 minutes, heated to 70°C for 5 minutes to denature the reverse transcriptase, then cooled on ice for 3 minutes. Water (60 μL) was added to each sample, and the samples were stored at −20°C.

Competitive PCR

Competitive PCR was performed by titration of sample cDNA with known amounts of nonhomologous TNF-α, IL-1β, IL-6, and GAPDH-MIMIC standard produced with the use of the Clontech PCR MIMIC construction kit. Briefly, a sense primer (A) and an
antisense primer (B) for rat TNF-α,17 rat IL-1β,18 rat IL-6,19 and rat GAPDH20 were synthesized with the use of the published cDNA sequences. The actual sequences of the oligonucleotides were as follows: TNF-α A, 5'-ATGACCGAGAACATGGATCGCAG-3'; TNF-α B, 5'-CCAAATCGTAGCTGGGCAGCCT-3'; IL-1β A, 5'-ATGCGAACCTGCTGAACTCAAC-3'; IL-1β B, 5'-CAGCACAGTGTAGAATTCACCCCTT-3'; IL-6 A, 5'-CCAGTTGCTCTTTGGAATGTG-3'; IL-6 B, 5'-ATTTCCTGACCACTTGAAG-3'; and GAPDH A, 5'-TTCCTGTCAGTGCAGTGC-3'; and GAPDH B, 5'-TAGGAACACGGAGGCCATGCGAC-3'.

Composite primers comprising the TNF-α, IL-1β, IL-6, and GAPDH gene-specific primers described above with v-erb B oncogene-specific 20-nucleotide base sequences at the 3' end (upstream, CGCAAGTGAAATCTCCTCCG; downstream, TCTGTCAATGACTGCAGAA) were used to construct fragments of the v-erb B oncogene with TNF-α, IL-1β, IL-6, and GAPDH-specific sequences at the 5' end of each strand. These TNF-α, IL-1β, IL-6, and GAPDH-MIMIC sequences were amplified by use of the noncomposite TNF-α, IL-1β, IL-6, and GAPDH-specific primers described above, and the molar quantity produced was determined. Ten micrograms of total RNA was used as template for cDNA synthesis. Two percent portions of the cDNA were then amplified in the micrograms of total RNA was used as template for cDNA synthesis. The actual sequences of the oligonucleotides were as follows: TNF-α A, 5'-ATGACCGAGAACATGGATCGCAG-3'; TNF-α B, 5'-CCAAATCGTAGCTGGGCAGCCT-3'; IL-1β A, 5'-ATGCGAACCTGCTGAACTCAAC-3'; IL-1β B, 5'-CAGCACAGTGTAGAATTCACCCCTT-3'; IL-6 A, 5'-CCAGTTGCTCTTTGGAATGTG-3'; IL-6 B, 5'-ATTTCCTGACCACTTGAAG-3'; and GAPDH A, 5'-TTCCTGTCAGTGCAGTGC-3'; and GAPDH B, 5'-TAGGAACACGGAGGCCATGCGAC-3'.

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\[
\text{Target/Internal Control} = \frac{I_T}{I_C} \times \frac{C_C}{C_T}
\]

where \( I_T \) and \( I_C \) represent the intensity of the PCR product from the target and the internal control, respectively, and \( C_C \) and \( C_T \) represent the dCTP content in the PCR product from the target and the internal control. The amount of target molecule was determined as the point of an equimolar ratio between the internal control and the target (Figure 1). The amounts of TNF-α, IL-1β, and IL-6 were divided by those of GAPDH to correct the efficiency of cDNA synthesis.

### Immunohistochemistry

Heart sections were embedded in OCT compound tissue medium (Miles Inc), snap-frozen on dry ice, and stored at −70°C. Tissues were sectioned on a cryostat at 6 μm. The sections were fixed for 10 minutes in 4% paraformaldehyde at 4°C. The primary antibodies used consisted of hamster monoclonal anti-mouse IL-1β (Genzyme Corp) at a concentration of 50 μg/mL and mouse monoclonal anti-rat macrophage (clone Ki-M2R, BMA) diluted to 1:50. Incubation with the primary antibody was performed at 4°C overnight. Biotinylated goat anti-hamster IgG (Cedarlane) diluted to 1:100, biotinylated goat anti-rabbit IgG (DAKO) diluted to 1:300, and biotinylated rabbit anti-mouse IgG (DAKO) diluted 1:300 were used as secondary antibodies. Incubation with secondary antibodies was performed at room temperature for 30 minutes. After incubation with avidin-biotin–horseradish peroxidase complexes (Vector Labs), peroxidase was visualized by 3',3'-diaminobenzidine followed by incubation with diaminobenzidine enhancing solution (Vector Labs). Counterstaining was performed with methyl green. Omission of the primary antibody and preabsorption of anti-mouse IL-1β with recombinant rat IL-1β (provided by Otsuka Pharmaceutical Co, Tokushima, Japan) served as controls to verify IL-1β staining. The primary antibody was omitted as a control for the staining of macrophages.

### Statistical Analyses

Echocardiographic measurements are reported as mean±SD. Measurements of cytokine gene expression were normalized by assigning an arbitrary number of 100 to the peak expression of IL-1β from which means and SEs were derived. One-way ANOVA with Fisher’s protected least significant difference test was used for statistical comparisons. A value of P<0.05 was considered significant.

### Results

#### Infarct Size and Chamber Dimensions

There were no significant differences in infarct size within groups over time (Table). The LV mass indexed for body weight was significantly increased in the MI group (overall mean: sham, 1.83 g/kg; MI, 2.32 g/kg; *P<0.05). A similar pattern was observed in the right ventricular mass indexed for body weight.

#### Figure 2. Graphs showing serial changes in LV geometry and function before and 1, 4, 8, and 20 weeks after surgery in sham-operated rats (n=12) and in rats with MI (n=12). Left, Progressive increase in LV diastolic internal dimension (EDD) after MI. Middle, Fractional shortening (FS) shows progressive impairment in rats with MI. Right, Marked decrease in RWT indicates a disproportionate increase in chamber dimension after MI. *P<0.05 compared with controls; #P<0.05 compared with preoperative baseline; ##P<0.05 compared with 1 week after MI.
Echocardiographic Study
Compared with sham-operated animals, rats with MI exhibited progressive LV dilatation. Significant differences in LV ESD and EDD were already measurable between the two groups at 1 week and continued to widen up to 20 weeks (Figure 2, left). LV enlargement was accompanied by a marked decrease in fractional shortening (Figure 2, middle). Similarly, RWT was markedly decreased in the rats with MI throughout the observation period (Figure 2, right). Thus, the increase in LV cavity size was disproportionate relative to the thickness of the surviving myocardium, suggesting a lack of appropriate hypertrophy and a progressive afterload mismatch.

PCR Analysis of Cytokine Genes
The evolution of the relative levels of TNF-α, IL-1β, and IL-6 mRNA in both groups of rats is shown in Figure 3. The gene expression level in the sham-operated rats rose slightly at 1 week and returned to near baseline thereafter. In the rats that underwent coronary ligation, the gene expression level in the infarcted region rose steeply at 1 week after surgery (TNF-α, 32±5%; IL-1β, 100±17%; and IL-6, 31±4%), also falling to near baseline at 20 weeks (4±1%, 7±2%, and 4±1%, respectively). In contrast, in the noninfarcted region, the cytokine gene expression levels rose moderately at 1 week and at 20 weeks remained significantly higher than those measured in both the infarcted zone and the myocardium of the sham-operated rats (16±4%, 47±8%, and 15±4%, respectively). Furthermore, this rise in cytokine expression levels in the noninfarcted heart correlated with the EDD at 8 and 20 weeks after surgery (TNF-α, r=0.743; IL-1β, r=0.719; and IL-6, r=0.713; Figure 4).

Histological Study
Compared with control animals, a 3.63-fold increase in LV collagen density was observed in MI rats at 20 weeks after surgery (Figures 5, bottom panel; and 6, left panel). LV collagen density at 8 and 20 weeks after surgery correlated well with IL-1β mRNA expression level (r=0.86; Figure 6, right panel). No significant correlation was found between collagen density and TNF-α or IL-6 mRNA expression level.

Immunohistochemistry
Because the IL-1β mRNA expression level was the most enhanced, we performed its immunohistochemical analysis in these animals. In the control rats, minimal immunoreactivity was observed by immunohistochemical analysis performed at
1 week to localize IL-1β protein in the heart (Figure 7A). In contrast, in the infarcted region, IL-1β immunoreactivity was found in the infiltrating leukocytes (Figure 7B), and in the noninfarcted zone, it was found in vascular endothelial cells and interstitial cells (Figure 7C). At 20 weeks, IL-1β immunoreactivity remained almost absent in the heart of control rats. In the rats with MI, the infarcted myocardium was replaced by scar tissue with less mononuclear cell infiltration, and immunoreactivity for IL-1β protein was observed in scattered mononuclear cells (Figure 7D). In the noninfarcted myocardium, IL-1β staining was apparent in endothelial cells and vascular smooth muscle cells (Figure 7E) as well as in the interstitial cells that were positive for macrophage marker (Figure 7F and 7G).

Discussion
Ventricular remodeling after MI is a consequence of large infarcts. This complex process is not limited to the areas of infarction. In the present study, we followed the remodeling process in a rat model of post-MI heart failure using serial transthoracic echocardiography. In this model, LV dilatation develops in the first week after MI and continues to progress over the subsequent 20 weeks. In the noninfarcted myocardium, adaptive hypertrophy undergoes a transition, leading to further cardiac dilatation, with a strong tendency toward a decrease in the thickness of the noninfarcted myocardium relative to the LV diameter. The decrease in RWT in rats with MI implies that the limits of compensation have been reached and that there is lack of appropriate hypertrophy of the surviving myocardium.

In acute ischemia leading to permanent myocyte injury, complex local interactions exist among endothelial cells, accumulation of infiltrating leukocytes, and tissue-based monocytes and myocytes. Recent studies have followed the temporal sequence of proinflammatory cytokine gene expression in postischemic/reperfused myocardium. However, those studies addressed the postischemic myocardial inflammation that occurs at a relatively acute stage, and the role of cytokines on LV remodeling has not been thoroughly studied.

Habib et al localized the inducible form of nitric oxide synthase (iNOS) and TNF-α within cardiac tissues in DCM, and they hypothesized that locally produced TNF-α might contribute to the pathogenesis and complications of DCM by inducing iNOS in the heart. Ischemic heart disease is the leading cause of CHF, and we hypothesize that similar inflammatory and immune processes may be implicated at the level of the noninfarcted myocardium. In our model, at 20 weeks, the cytokine expression level in the noninfarcted region was significantly higher than in the control rats and correlated well with EDD. The cause for this cytokine gene upregulation is uncertain, although several factors that cause LV dilatation might contribute to it.

It is becoming increasingly apparent that proinflammatory cytokines play an important role in modulating the function and structure of the heart. Elevated concentrations of TNF-α have been reported in patients with chronic heart failure, and TNF-α has been found to depress the contractility of isolated hamster papillary muscles. Repeated TNF-α infusion
Figure 7. Localization of IL-1β by immunohistochemical analysis in control and infarcted hearts. Magnification ×400; bar=50 μm. A, Absence of IL-1β protein in the heart from a sham-operated rat at 1 week after surgery. B, Presence of IL-1β protein in infiltrating leukocytes of an infarcted zone 1 week after coronary ligation. C, Presence of IL-1β protein in vascular endothelial cells and interstitial cells in the noninfarcted region 1 week after coronary ligation. D, Scar tissue replacement in infarcted territory at 20 weeks after coronary ligation. Immunoreactivity for IL-1β protein is observed in scattered mononuclear cells. E, Positive staining for IL-1β in endothelial cells and vascular smooth muscle cells in the noninfarcted myocardium 20 weeks after coronary ligation. F and G, Positive staining for IL-1β (arrows in F) in interstitial macrophage (arrows in G) in the noninfarcted zone 20 weeks after coronary ligation.
may lead to a permanent decrease in myocardial contractility and may result in DCM.\textsuperscript{33}

IL-1\(\beta\) has deleterious effects on cardiac contractility in isolated perfused rat hearts.\textsuperscript{1} IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) increased NO production in rat cardiocytes by induction of iNOS gene expression.\textsuperscript{34} These cytokines also have cytotoxic effects on cultured myocytes.\textsuperscript{35} More recently, IL-1\(\beta\) acting via an NO-independent mechanism has been shown to cause myocyte hypertrophy and downregulation of calcium regulating genes,\textsuperscript{36} and in ischemic cardiomyopathy, alterations in collagen concentration and phenotypes have been demonstrated.\textsuperscript{37} Enhanced type I and type III collagen production by fibroblasts in the noninfarcted myocardium has been reported in the rat heart after MI.\textsuperscript{38} In our model, collagen density of noninfarcted myocardium gradually increased and correlated well with IL-1\(\beta\) expression level. Because IL-1\(\beta\) exhibits mitogenic effects on human fibroblasts in certain circumstances,\textsuperscript{39–41} the upregulation of IL-1\(\beta\) in the noninfarcted myocardium in our model may be partly responsible in this setting for altering the compliance of the myocardium, resulting in exacerbation of heart failure. We found a similar correlation between IL-1\(\beta\) mRNA expression and cardiac fibrosis in a murine model of myocarditis.\textsuperscript{41} However, the effects of IL-1\(\beta\) on fibroblasts may be complex. For example, Palmer et al\textsuperscript{42} reported an antiproliferative effect of IL-1\(\beta\) on cultured rat cardiac fibroblasts. In another study, Thaik et al\textsuperscript{43} found that IL-1\(\beta\) stimulated protein synthesis in cultured rat cardiac fibroblasts, although it inhibited DNA synthesis. Therefore, further studies are needed to define more precisely the effects of IL-1\(\beta\) on the collagen deposition in vivo.

High cytokine levels have been found to activate metalloproteinase under certain conditions.\textsuperscript{44} Cleutjens et al\textsuperscript{45} reported that posttranslational activation of latent collagenase (MMP-1) plays a predominant role at the site of infarction, and this activation, measured by zymography, began at day 2, peaked at day 7, and declined thereafter, somewhat earlier than our findings of increased expression of cytokines in the noninfarcted myocardium; in addition, Cleutjens et al found no changes in MMP-1 activity at remote sites or in sham-operated controls. Therefore, the contribution of extracellular proteolysis seems relatively small in the present study, and the positive correlation between IL-1\(\beta\) expression and collagen deposition suggests that increased production exceeds its degradation.

In addition to necrosis, myocyte loss induced by apoptosis has recently been proposed as an important mechanism in the pathogenesis of CHF.\textsuperscript{46} However, Saraste et al\textsuperscript{47} observed few apoptotic cells in the remote, noninfarcted myocardium, and the contribution of apoptotic myocyte death on interstitial fibrosis in the surviving myocardium remains unknown.

An increasing number of experimental observations suggest that IL-6 is also capable of modulating cardiovascular function, exerting a negative inotropic effect through NO-dependent pathways as well.\textsuperscript{1} Mice with overexpression of both IL-6 and IL-6 receptors have been reported to show constitutive tyrosine phosphorylation of gp130 and to develop cardiac hypertrophy.\textsuperscript{48} Cardiac myocytes are reported to produce IL-6 under hypoxic stress.\textsuperscript{49} Therefore, in the present experiment, IL-6 in the noninfarcted myocardium may have been upregulated by relative ischemia in the hypertrophied myocyte itself.

The mechanisms responsible for the upregulation of cytokine gene expression in the noninfarcted myocardium are not known. Further experiments will be directed toward determining the mechanism of cytokine induction and the physiological role of the intracardiac cytokine system.

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### References

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