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CLINICAL STUDIES

HEART FAILURE

Inducible Nitric Oxide Synthase and Tumor Necrosis Factor-Alpha in Myocardium in Human Dilated Cardiomyopathy

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Objectives. **We examined the mRNA expression and protein localization of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-alpha) in myocardial tissue obtained from patients with dilated cardiomyopathy (DCM).**

Background. **The etiology of DCM is unknown, but viral infection or autoimmune abnormalities that induce cytokine expression have been proposed as pathogenetic factors. Nitric oxide (NO), synthesized by nitric oxide synthase (NOS), has negative inotropic and cytotoxic effects on cardiomyocytes. Cytokines such as TNF-alpha are potent stimulators of iNOS expression. Expression of iNOS leads to excessive production of NO in the myocardium and may modulate cardiac contractility and ventricular morphology.**

Methods. **We examined the mRNA expression and protein localization of iNOS and TNF-alpha in myocardial tissue obtained from 24 patients with DCM, 20 patients with hypertrophic cardiomyopathy (HCM) and 15 control subjects, using the reverse transcriptase-polymerase chain reaction method and immunohistochemical studies. We then compared the differences in clinical characteristics between DCM patient subgroups with and without myocardial iNOS expression.**

Results. **Messenger RNA expression of iNOS and TNF-alpha was observed, respectively, in 13 (54%) and 18 (75%) patients with DCM. Gene expression of TNF-alpha was consistently detected in endomyocardial tissue from patients with DCM and INOS expression. Inducible NOS protein was evident only in cardiomyocytes, whereas TNF-alpha was apparent in both cardiomyocytes and endomyocardial endothelium. Neither mRNA expression nor protein localization of iNOS or TNF-alpha was observed in cardiac tissue obtained from patients with HCM or control subjects. Patients with DCM and iNOS mRNA showed a lower left ventricular ejection fraction ($p < 0.01$) and a higher left ventricular volume ($p < 0.05$) than the negative DCM group.**

Conclusions. **Inducible NOS was consistently coexpressed with TNF-alpha in myocardial tissue obtained from a subgroup of patients with DCM and advanced left ventricular dysfunction.**

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Nitric oxide is produced from the terminal guanidino nitrogen atom of L-arginine by the nitric oxide synthase (NOS) family of enzymes. There are two types of NOS: constitutive, Ca^{2+}/calmodulin-dependent NOS and Ca^{2+}/calmodulin-independent NOS, the latter being induced by a number of inflammatory cytokines. Cytokines, such as interleukin-1_{beta} and tumor necrosis factor-alpha (TNF-alpha) have been shown to cause expression of iNOS in cardiomyocytes obtained from animals (1–3). Recent studies (4–6) have suggested that generation of nitric oxide (NO) in the endocardium and myocardium regulates cardiac function in a paracrine and autocrine fashion. Depressed myocardial contractility in animals and humans occurs in conditions associated with elevated plasma levels of cytokines, such as septic shock (7). It has been suggested (8,9) that profound cardiac dysfunction can be attributed to excessive NO production resulting from cytokine-induced expression of iNOS in myocardial and vascular tissues.

De Belder et al. (10) observed an increase in the enzyme activity of Ca^{2+}-independent NOS, but not Ca^{2+}-dependent NOS, in the myocardium of patients with DCM. However, it is not clear whether iNOS activity in myocardial tissue in patients with DCM is directly reflected by mRNA expression, and the relation between expression of iNOS and cytokines has not been clarified. We used the polymerase chain reaction (PCR) method to examine the mRNA expression of iNOS and TNF-alpha in endomyocardial biopsies obtained from patients with DCM or HCM and control subjects. The localization of iNOS and the cytokine protein in the myocardial tissue was also studied by immunohistochemical analysis. Furthermore, we explored the differences in clinical characteristics and prognosis between the DCM patient subgroups with and without myocardial iNOS.

Methods

Subjects. We examined endomyocardial biopsies obtained from 24 patients with DCM, 20 patients with HCM and 15

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Abbreviations and Acronyms

DCM = dilated cardiomyopathy
HCM = hypertrophic cardiomyopathy
IL = interleukin
iNOS = inducible nitric oxide synthase
NO = nitric oxide
NOS = nitric oxide synthase
PCR = polymerase chain reaction
SSC = sodium citrate/sodium chloride
TNF-alpha = tumor necrosis factor-alpha

control subjects. *The DCM group* included 19 men and 5 women (mean age 58 years, range 19 to 76). In terms of the clinical severity of congestive heart failure, 3 patients were in New York Heart Association functional class I; 16 were in class II; and 5 were in class III. The mean ± SD values for left ventricular ejection fraction and cardiac index were 31 ± 9% and 2.8 ± 0.8 liter/min per m^2, respectively.

Digitalis and diuretic drugs, including furosemide and spironolactone, were administered orally in 22 and 21 patients, respectively. Seven of the 24 patients were treated with a beta-adrenergic blocking agent, and 5 received nitroglycerin (Table 1). *The HCM group* included 14 men and 6 women (mean age 48 years, range 20 to 65). They had no significant systolic ventricular function and no signs or symptoms of heart failure. Dilated cardiomyopathy and HCM were diagnosed according to the criteria of the World Health Organization/International Society and Federation of Cardiology Task Force (11). Patients were excluded if there was any angiographic evidence of coronary heart disease, ischemic changes during exercise testing, systemic hypertension, concomitant systemic or ventricular function or excessive alcohol consumption. A subject group with myocardial disorder suspected on the basis of premature beats and minor echocardiographic abnormalities underwent endomyocardial biopsy. Neither the resulting pathologic findings nor close clinical examination showed evidence of myocardial disease, and these subjects were designated as the control group with normal cardiac function and morphology. None of the patients or control subjects had any medical history of serious infectious illness (i.e., pneumonia, sepsis or myocarditis). The study protocol was approved by our hospital ethics committee, and written informed consent was obtained from all subjects.

Myocardial tissue samples. Five right ventricular endomyocardial biopsies were obtained from each subject with a bioptome (Type A, Yufu Corp., Tokyo, Japan) by the femoral vein approach. Four tissue samples were fixed in 10% formalin, embedded in paraffin and cut into 4-μm sections. Each section was stained with hematoxylin and eosin and was also used for

Table 1. Clinical Characteristics and Results of Reverse Transcriptase-Polymerase Chain Reaction and Immunohistochemical Analysis in 24 Patients With Dilated Cardiomyopathy

Pt No.	Age (yr)/ Gender	NYHA	Treatment		LVEF (%)	PCWP (mm Hg)	CI (liter/min per m^2)	PCR Result		Immunohist	
			ACEI	BB				iNOS	TNF-Alpha	iNOS	TNF-Alpha
1	19/M	I	+	+	37	10	3.4	−	−	−	−
2	28/M	I	−	+	46	8	2.4	−	+	−	−
3	37/M	II	−	−	44	9	4.7	−	−	−	−
4	41/M	III	+	−	33	8	2.5	−	+	−	+
5	43/M	III	+	−	13	15	3.8	+	+	+	+
6	52/M	III	+	−	27	11	2.8	−	+	−	−
7	54/M	II	−	−	43	16	2.5	−	+	−	+
8	54/M	III	−	−	43	16	2.5	−	+	−	+
9	57/M	II	−	−	28	8	3.3	+	+	−	−
10	60/M	II	−	−	45	2	2.0	−	−	−	−
11	60/M	II	−	−	24	14	4.0	+	+	−	−
12	61/M	II	−	+	35	4	2.0	+	+	+	+
13	65/M	II	−	−	31	6	2.0	+	+	+	+
14	66/M	II	−	−	19	16	2.2	+	+	+	+
15	69/M	III	+	−	30	6	2.6	+	+	+	+
16	69/M	II	−	−	22	3	2.9	+	+	+	+
17	70/M	II	+	+	17	7	3.6	−	−	−	−
18	73/M	II	+	+	23	9	3.7	+	+	+	+
19	75/M	II	+	−	36	13	2.1	−	−	−	−
20	64/F	II	+	+	22	11	2.1	+	+	−	+
21	67/F	II	−	−	30	7	2.2	+	+	−	−
22	68/F	II	−	−	32	20	2.5	−	−	−	−
23	73/F	II	+	+	30	12	2.9	+	+	+	−
24	76/F	I	+	−	39	13	2.9	+	+	−	+

ACEI = angiotensin-converting enzyme inhibitor; BB = beta-blocker; CI = cardiac index; F = female; Immunohist = immunohistochemical analysis; iNOS = inducible nitric oxide synthase; LVEF = left ventricular ejection fraction; M = male; NYHA = New York Heart Association functional class; PCWP = pulmonary capillary wedge pressure; Pt = patient; TNF-Alpha = tumor necrosis factor-alpha; + = positive; − = negative.

immunohistochemical analysis. The remaining tissue was immediately frozen at −80°C and used for reverse transcriptase-PCR analysis.

Positive control cells for iNOS and TNF-alpha mRNA. Human aortic vascular smooth muscle cells were isolated from an aortic medial layer (Kurabou Corp., Osaka, Japan) and seeded in culture flasks (Becton) in a humidified atmosphere of 5% CO_2–95% air at 37°C. The culture medium (RPMI-1640, Gibco) was changed every 3 days. The cells reached confluence after 7 days, after which the medium was replaced with fresh medium containing IL-1_{beta} (10 ng/ml), and the cultured vascular smooth muscle cells were incubated for 6 to 48 h. For measurement of concentrations of nitrite and nitrate, the final products of liberated NO, the culture medium was measured by mixing 500 μl of medium with an equal volume of Griess reagent. Absorbance at 550 nm was measured, and nitrite and nitrate concentrations were determined using a curve calibrated from sodium nitrite standards. Vascular smooth muscle cells in which significant NO production was confirmed by an increase in nitrite and nitrate levels were used as positive control cells for detection of iNOS mRNA.

Peripheral blood monocytes were prepared from normal blood donors. After centrifugation, the monocytes were washed and resuspended in AIM-R medium (Gibco) supplemented with 2 mg of anti-human LeuTM-4 (CD3) (Becton Dickinson). The monocytes were allowed to adhere to the tissue culture flasks for 48 h at 37°C in a humidified atmosphere of 5% CO_2–95% air. They were then used as positive control cells for the detection of TNF-alpha and beta-actin expression.

Preparation of total RNA. After the endomyocardial tissues and cultured positive control cells were homogenized in a denaturing solution of guanidium thiocyanate, total RNA was extracted with phenolchloroform and recovered with isopropanol (12). The purity of the extracted RNA was estimated by determining the ratio of absorbance at 260 nm to that at 280 nm. The RNA extract was diluted to 200 ng/ml with double-distilled water.

Oligonucleotides. The following PCR primers were used to amplify cDNA: for iNOS (13): sense, 5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′ and anti-sense, 5′-GGT GCT ACT TGT TAG GAG GTC AAG TAA AGG GC-3′; for TNF-alpha (14): sense, 5′-GCC TGT AGC CCA TGT TGT AG-3′ and anti-sense, 5′-AAT GAT CCC AAA GTA GAC CTG CCC-3′; and for beta-actin (15): sense, 5′-CCT GGC ACC CAG CAC AAT GA-3′; and anti-sense, 5′-TTG GGA AGG TTG GAT GTT CG-3′. The probes used for Southern blot hybridization were for iNOS: 5′-AGG CCC TGT GAC CTC AGA TAA TCG AGA GCT GGC TCC ATC CTT AAG TTC TGT GCC GGC AGC-3′; for TNF-alpha: 5′-TTT GCG GTG GAC GAT GGA GGG GCC GGA ATA GTA ATA CTC CTG CTT GCT GAT CCA CAT CTG-3′; and for beta-actin: 5′-TTT GCG GTG GAC GAT GGA GGG GCC GGA ATA GTA ATA CTC CTG CTT GCT GAT CCA CAT CTG-3′.

Polymerase chain reaction procedure. Reverse transcriptase-PCR was performed with a RNA-PCR kit (GeneAmp, Perkin Elmer-Cetus Corp.). The cDNA was synthesized from 200 ng of total RNA by reverse transcription at 42°C for 30 min using random hexamers and then amplified with 45 cycles of PCR using a temperature cycler (Hybain, Middlesex, UK). Polymerase chain reaction amplification of cDNA was performed as follows: denaturation for 1 min at 94°C, followed by 45 cycles of denaturation for 94°C at 30 s, annealing for 1 min at 55°C, extension for 1 min at 72°C and final extension for 5 min at 72°C.

Southern blot hybridization. Polymerase chain reaction products (10 μl) were electrophoresed on a 3% NuSieve/1% Seakem agarose gel (FMC Bioproducts). The DNA bands were denatured and transferred onto a nylon membrane (Hybond-N, Amersham, UK) using 0.4 mol/liter of NaOH as the denaturing buffer and 20 × sodium citrate/sodium chloride (SSC) (0.3 mol/liter NaCl and 0.03 mol/liter sodium citrate at pH 7.0) as the transferring buffer. The DNA bands were fixed on the membrane by an ultraviolet linker (Spectromics). The membrane was then prehybridized in a solution of 50 mmol/liter Tris-HCl, 1 mol/liter NaCl, 10% dextran sulfate, 1% sodium dodecyl sulfate and sheared salmon sperm DNA at 65°C for 2 h. Hybridization was performed in the same solution with a [^{32}P]-5′-end-labeled probe prepared with a kit (Megalabel, Takara, Kyoto, Japan). After incubation for 16 h at 65°C, the membrane was washed three times at room temperature for 10 min and then twice at 65°C for 10 min in 2 × SSC containing 0.1% sodium dodecyl sulfate. The membrane was then autoradiographed at −80°C for 24 h.

Immunohistochemical analysis. Immunohistochemical analysis was performed on paraffin sections to examine the source of iNOS and TNF-alpha protein production. Anti-mac NOS (Tranduction Laboratories) and anti-human TNF-alpha (Genzyme Corp.) were used as primary antibody. To test for nonspecific staining by the secondary antibody, distilled water was substituted for the primary antibody on control sections from patients with DCM.

The tissue sections were deparaffined by xylene and rehydrated in 95% ethanol. Endogenous peroxidase activity was quenched in 2% H_2O_2 in 60% methanol for 30 min. The tissue sections were washed for 20 min in phosphate-buffered saline (pH = 7.4). The appropriate normal serum was applied to each section for 20 min. The excess serum was then blotted off; the primary antibody was added; and the sections were left for 8 h at 4°C. All primary antibodies were diluted in phosphate-buffered saline. The sections were washed three times in the buffer, incubated with biotinylated secondary antibody for 10 min and washed in the buffer and then incubated with a peroxidase conjugated avidin-biotin complex (Histofine SAB-PO kit, Nichiren Corp., Tokyo, Japan) for 5 min. The sections were washed three times in phosphate-buffered saline. Diaminobenzidine tetrahydrochloride was used as chromogen to visualize the presence and distribution of iNOS and TNF-alpha.

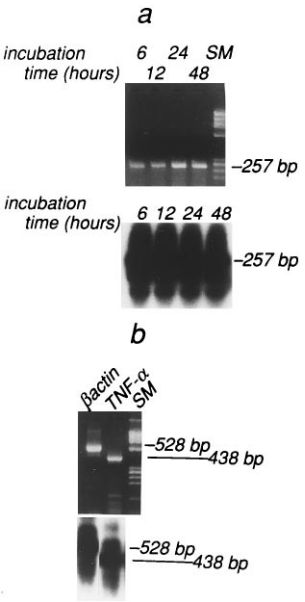


Figure 1. a, Reverse transcriptase-PCR and Southern blot hybridization show the expression of iNOS mRNA in human vascular smooth muscle cells. The vascular smooth muscle cells were incubated with IL-1_{beta} for 48 h and amplified with cDNA for use as an iNOS probe. **b,** Reverse transcriptase-PCR and Southern blot hybridization show the expression of TNF-alpha in cultured human monocytes. The monocytes were incubated with CD3 for 48 h, amplified with 528- and 438-base pair (bp) cDNA and hybridized with cDNA for use as beta-actin and TNF-alpha probes. SM = size marker.

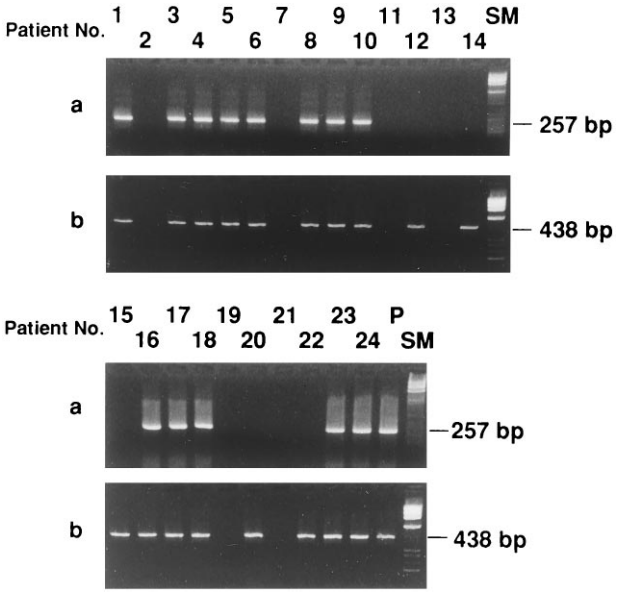


Figure 2. Reverse transcriptase-PCR of iNOS (**a**) and TNF-alpha (**b**) mRNA in endomyocardial biopsies from 24 patients with DCM. Inducible NOS mRNA was expressed in 13, and TNF-alpha mRNA in 18, of the 24 patients. bp = base pair; P = positive control cells; SM = size marker.

Morphometric evaluation. Morphometric investigation was performed on paraffin sections. These were measured using a microscope equipped with a ×40 objective and ×10 ocular lesions in which a micrometer was settled. Hematoxylin and eosin-stained sections were used for measurement of the fiber diameters and inflammatory cellular infiltrates. The diameter was evaluated on cross sections of myofibers in the nuclear region. Thirty profiles were measured in each case, and the results were expressed as mean value and standard deviation. The number of inflammatory cellular infiltrates (infiltration of macrophage, lymphocyte and pan-leukocyte) into myocardial tissue per field was counted in randomly selected fields (n = 30). The degree of interstitial fibrosis was evaluated using an image analyzer (IBAS-2000, Zeiss, Oberkochen, Germany). The ratio of fibrotic area to total myocardial area was measured on Azan-stained section.

Plasma TNF-alpha concentration. Plasma levels of TNF-alpha in patients with DCM were determined by using a commercially available enzyme-linked immunosorbent assay kit (TNF-EASI, Medgemix, Fleurus, Belgium). Blood samples were obtained from the femoral vein at the time of endomyocardial biopsy and immediately centrifuged and stored at −80°C until assayed. The plasma level in age-matched healthy subjects was <5 pg/ml (n = 10).

Statistical analysis. Results are expressed as mean value ± SD. Differences between groups of patients with or without iNOS expression were analyzed by unpaired *t* test. Results were considered statistically significant at $p < 0.05$.

Results

Positive control cells. Interleukin-1_{beta} stimulated nitrite and nitrate production from cultured vascular smooth muscle cells in a time-dependent manner. Nitrite and nitrate in the cultured medium increased from 1.7 mmol/liter at 6 h to 3.0 mmol/liter at 12 h, 3.9 mmol/liter at 24 h and 4.1 mmol/liter at 48 h. Reverse transcriptase-PCR revealed a single band corresponding to the size of iNOS cDNA (257 base pairs) after stimulation with IL-1_{beta} (Fig. 1a). Tumor necrosis factor-alpha and beta-actin mRNA were expressed in cultured monocytes (Fig. 1b).

Messenger RNA expression. Expression of iNOS mRNA was detected in 13 (54%) of the 24 patients with DCM, whereas expression of TNF-alpha mRNA was detected in 18 (75%) (Fig. 2, Table 1). TNF-alpha mRNA was coexpressed with iNOS mRNA in all 13 patients with iNOS expression (Table 1). Expression of iNOS mRNA alone was not observed in any of the myocardial specimens. TNF-alpha mRNA was detected in five biopsy specimens without iNOS coexpression.

Southern blot hybridization confirmed the presence of both iNOS and TNF-alpha in the PCR product. Neither myocardial iNOS mRNA nor TNF-alpha mRNA was expressed in any of the 20 patients with HCM or the 15 control subjects (Fig. 3 and 4).

Immunohistochemical analysis. Immunohistochemical analysis was performed to identify the source of iNOS and TNF-alpha protein in the myocardial tissue. Immunostaining of iNOS protein was localized to abundant cardiac myocytes in the subendocardium and was distributed patchily and evenly on cytoplasm of myocytes in eight patients with DCM (Fig. 5a).

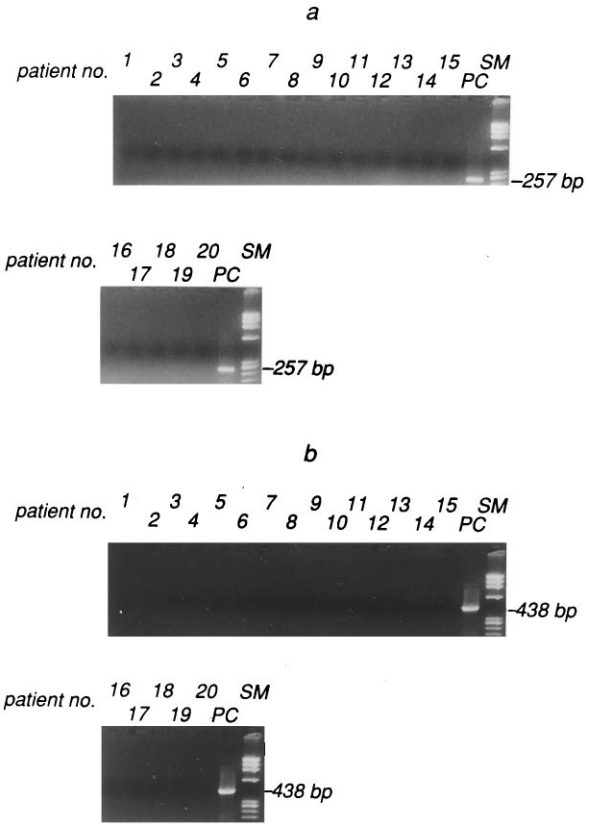


Figure 3. Reverse transcriptase-PCR of iNOS **(a)** and TNF-alpha **(b)** mRNA in endomyocardial biopsies from 20 patients with HCM. As shown, no expression of iNOS and TNF-alpha mRNA was detected in these samples. PC = positive control cells; other abbreviations as in Figure 1.

Inducible NOS immunostaining was consistently detected in the myocardial tissue obtained from patients with DCM who showed positive myocardial iNOS mRNA (Table 1). In seven of eight patients with DCM in whom myocardial iNOS protein was positive, myocardial TNF-alpha was concomitantly present (Table 1). Tumor necrosis factor-alpha immunostaining was localized not only in cardiac myocytes but also in endomyocardial endothelium (Fig. 5c). This pattern was observed consistently in 12 biopsy tissues from patients with DCM who showed positive myocardial TNF-alpha protein. All these samples expressed TNF-alpha mRNA. No significant immunostaining was observed in the infiltrating macrophages or lymphocytes in the myocardium. Testing for nonspecific staining by the secondary antibody alone was carried out on control sections from patients with DCM. Figure 5, b and d, shows an absence of nonspecific myocardial immunostaining with secondary antibody alone in the myocardial tissue obtained from patients with DCM. Neither iNOS nor TNF-alpha immunostaining was present in any of the specimens from the HCM or control groups.

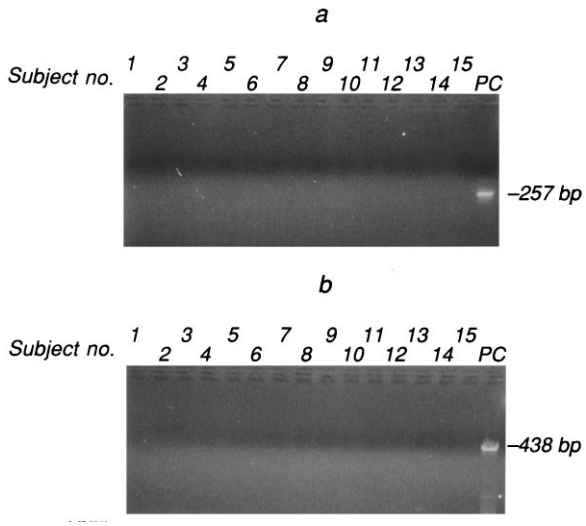
Morphometric analysis. All the endomyocardial biopsy samples were subjected to histomorphometric evaluation. Although cellular infiltration was seen in myocardial tissue from some of the patients with DCM, there was no evidence, such as myocytic necrosis or a significant amount of cellular infiltration, to suggestive active myocarditis. There were no differences in histomorphometric features between the iNOS-positive and iNOS-negative groups (Table 2).

Plasma TNF-alpha concentration. Plasma TNF-alpha levels in the patients with DCM ranged from 2.1 to 77.5 pg/ml (mean ± SD 19.1 ± 18.5). This was significantly higher than in healthy subjects. There was no significant difference between TNF-alpha mRNA-positive and -negative patients (21.1 ± 9.9 vs. 17.3 ± 4.0 pg/ml, $p = 0.71$).

Clinical data. As shown in Table 3, several factors differed between the two groups. In the myocardial iNOS-positive patient subgroup, left ventricular end-systolic diameter was significantly enlarged (57 ± 7 vs. 50 ± 7 mm, $p < 0.05$), and left ventricular ejection fraction was reduced (27 ± 7% vs. 37 ± 9%, $p < 0.01$). However, when the patients with DCM were classified according to the presence or absence of myocardial TNF-alpha mRNA, no significant differences were observed (left ventricular ejection fraction 29.9 ± 8.9% vs. 35.2 ± 10.2%; left ventricular systolic diameter 54.3 ± 7.3 vs. 53.0 ± 8.3 mm).

Eight cardiac events defined as cardiac-related death, pulmonary congestion, ventricular tachycardia and sudden death occurred within the mean follow-up period of 23 ± 8 months in the iNOS-positive group (61%): Six patients had pulmonary congestion; one had ventricular tachycardia; and one died of cardiac-related causes. Two cardiac events occurred within the mean follow-up period of 28 ± 8 months in the iNOS-negative group (18%), both consisting of pulmonary congestion.

Figure 4. Reverse transcriptase-PCR of iNOS **(a)** and TNF-alpha **(b)** mRNA in endomyocardial biopsies from 15 control subjects. No significant expression of iNOS and TNF-alpha mRNA was found. Abbreviations as in Figure 3.



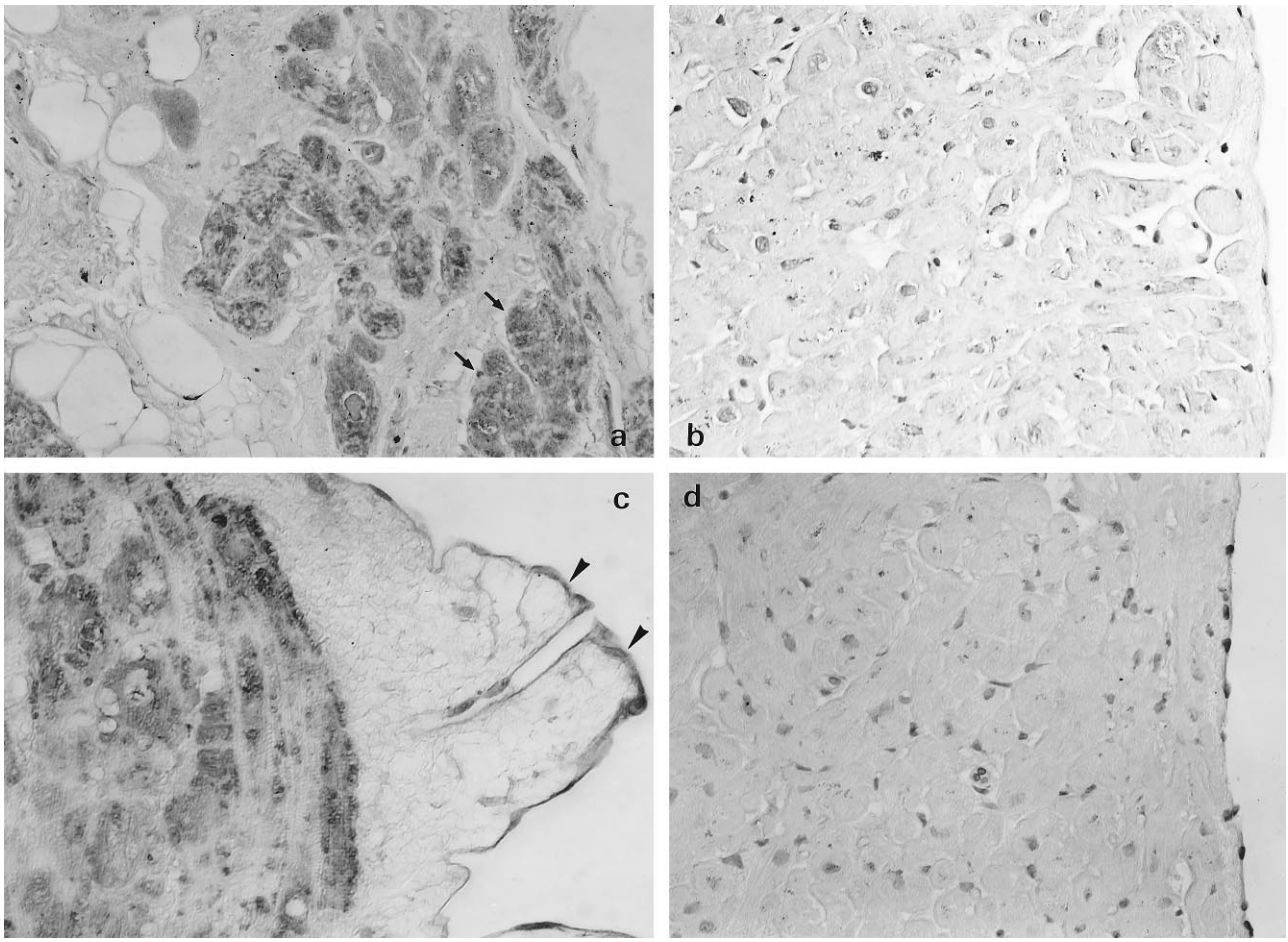


Figure 5. Immunohistochemical analysis of iNOS and TNF-alpha in endomyocardial tissues obtained from patients with DCM (×250, reduced by 5%). **a and c,** Detection of iNOS and TNF-alpha proteins with primary and secondary antibodies. Inducible NOS protein was located in the cardiomyocytes **(a) (arrows),** whereas TNF-alpha protein was also present in endomyocardial endothelium **(c) (arrowheads). b and d,** Results obtained with secondary antibody alone: There was no evidence of nonspecific immunostaining of the secondary antibody in the endomyocardial tissue from patients with DCM.

Discussion

Dilated cardiomyopathy is defined as primary cardiac failure in the absence of coronary, valvular, congenital or pericardial disease. It is characterized by dilation of the left ventricle, either alone or in association with right ventricular dilation, and impaired systolic function (11). Bowles et al. (16) have suggested that persistent or recurrent viral infection is an etiologic factor in DCM. Subsequent studies (17–20) have detected viral genomes in endomyocardial biopsies in 10% to 53% of patients with DCM. Recent studies (21,22) suggest the involvement of an immune dysfunction in the pathogenesis of myocytic damage in DCM. Cytokines such as IL-1_{beta}, interleukin-6 and TNF-alpha induced by viral replication and autoimmune mechanisms have also been proposed as pathogenetic factors (23–25). Tumor necrosis factor-alpha is considered a representative cytokine that strongly induces cellular cytotoxicity, cachexia, fever and shock (26–28). However, the mechanism of cytokine-mediated progression of ventricular dysfunction has not been clarified.

Relation between TNF-alpha and iNOS. In the present study, iNOS mRNA was consistently expressed together with TNF-alpha mRNA in the myocardial biopsies obtained from 13 patients with DCM. Immunohistochemical analysis demonstrated that the protein biosynthesis of iNOS and TNF-alpha was concomitantly observed in cardiac myocytes. In accordance with these observations, Habib et al. (29) recently reported expression of iNOS protein with a high prevalence of TNF-alpha protein expression in cardiac myocytes associated

Table 2. Histomorphometric Endomyocardial Biopsy Results

	iNOS (mean ± SD)		
	Positive (n = 13)	Negative (n = 11)	p Value
Myocyte diameter (mm)	23.3 ± 4.7	25.0 ± 7.7	0.52
Extent of interstitial fibrosis (%)	29.9 ± 13.7	29.1 ± 10.1	0.87
Inflammatory cellular infiltrate ($1/mm^2$)	2.1 ± 1.8	1.8 ± 2.6	0.77

iNOS = inducible nitric oxide synthase.

Table 3. Comparison of Clinical Characteristics of Patients With Dilated Cardiomyopathy With and Without Inducible Nitric Oxide Synthase Messenger Ribonucleic Acid Expression

	iNOS		
	Positive (n = 13)	Negative (n = 11)	p Value
Age (yr)	64.8 ± 8.5	50.7 ± 18	0.02
M/F	9/4	10/1	0.2
NYHA			
I	1	2	
II	10	6	0.3
III	2	3	
CTR (%)	59.0 ± 5.4	57.4 ± 4.9	0.5
Atrial fibrillation	6	6	0.2
ACEI therapy	6	5	0.9
BB therapy	4	3	0.5
LV end-systolic diameter (mm)	57 ± 7	50 ± 7	0.04
LV end-diastolic diameter (mm)	65 ± 7	63 ± 7	0.4
LVEF (%)	27 ± 7	37 ± 9	0.006
PCWP (mm Hg)	9.5 ± 4	11 ± 5	0.5
CI (liters/min per m^2)	2.8 ± 0.8	2.8 ± 0.7	0.9
LV end-diastolic pressure (mm Hg)	14.1 ± 7.0	10.6 ± 5	0.17

Data presented are mean value ± SD or number of patients. CTR = cardiothoracic ratio; LV = left ventricular; other abbreviations as in Table 1.

with DCM. These observations suggest that expression may be closely related to the cytokine in cardiac tissue and that NO may be synthesized by myocardial iNOS. Recent studies (2,3) have shown that cytokines, including TNF-alpha, induce expression of iNOS mRNA and increase NO production in cultured rat cardiocytes. Tumor necrosis factor-alpha has been reported (30) as increasing intracellular levels of tetrahydrobiopterin synthase, which acts as a cofactor in the enhancement of iNOS mRNA and the production of NO. Furthermore, Balligand et al. (31) reported that adult rat ventricular myocytes expressed iNOS significantly after exposure to cytokines such as IL-1_{beta} and TNF-alpha and that a contractile response to isoproterenol in adult rat ventricular myocytes was depressed after pretreatment with these cytokines. These findings suggest that TNF-alpha is a factor in the production of NO in the myocardium and may be involved in the regulation of cardiac function.

Inducible NOS and myocardium. The expression of iNOS mRNA was observed in >50% of our patients with DCM. Haywood et al. (32) similarly observed a slightly higher frequency of myocardial iNOS mRNA expression in patients with DCM and ischemic heart disease. However, Thoenes et al. (33) used immunoblot analysis to detect iNOS protein expression with a consecutive increase in cyclic guanosine monophosphate in autopsy samples from septic failing heart but not in failing explanted hearts from patients with DCM. These differences may be due to the small patient numbers in the respective studies, the heterogeneity of the patients' clinical backgrounds or the different sources of myocardial samples (endomyocardial biopsy vs. explanted heart), as well as the methodology used (PCR vs. immunoblot analysis).

In humans with left ventricular dysfunction, inhibition of NO production within the heart enhanced the positive inotropic action of beta-adrenergic stimulation (34). Furthermore, Studer et al. (35) recently found a close correlation between myocardial iNOS gene expression and cardiac diastolic function in an isolated ventricular muscle strip preparation obtained from human subjects with dilated and ischemic cardiomyopathy. Inducible NOS has a potent and long-lasting effect on NO generation, and NO has cytotoxic effects by virtue of its ability to inhibit mitochondrial respiration (36,37) and to trigger apoptosis (38–40). It may therefore be speculated that the acceleration in NO production due to cytokine-induced iNOS in the myocardium may not only depress cardiac function but also conspire to bring about myocardial degeneration or cell death.

Tumor necrosis factor-alpha and myocardium. Torre-Amione et al. (41) have suggested that the heart itself may be a major source of secretion and target for TNF-alpha in patients with end-stage heart failure due to dilated and ischemic cardiomyopathy. Their study may be consistent with our findings, although our patients showed a higher positive percentage of myocardial TNF-alpha mRNA expression and additional distribution of TNF-alpha protein in endocardial endothelium. Kapadia et al. (42) showed that endotoxin-stimulated feline cardiac myocytes express TNF-alpha mRNA and synthesize biologically active TNF-alpha. Yokoyama et al. (43) reported that the negative inotropic effect of cardiac contractile dysfunction was directly mediated by TNF-alpha in the adult mammalian myocardium and that this might be caused by decreased levels of intercellular calcium during the systolic contraction sequence. If so, it may also be possible that cardiac contractile dysfunction was not only caused by a TNF-alpha–mediated NO-dependent mechanism but was also partially due to the direct effects of TNF-alpha released from the myocardium.

Clinical considerations and study limitations. Ventricular contractility was found to be impaired in patients with DCM, with a poorer prognosis for the iNOS-positive group than for the iNOS-negative group. On the basis of this finding, it may be possible that the progression of ventricular dysfunction and dilation in some patients with DCM may be related to the following pathogenetic mechanisms. The viral or autoimmune abnormalities, or both, associated with depressed host–defense immunity induce a continuous triggering of an immune inflammatory reaction in myocardial tissue, leading to stimulation of cytokine in cardiomyocytes. One or several of these cytokines then induce expression of iNOS mRNA in myocardial tissue, leading to excessive and persistent production of NO in the myocardium. The NO derived from cytokine-induced iNOS would then reduce cardiac contractility by virtue of its negative inotropic properties in an autocrine or paracrine fashion. Furthermore, because NO has cytotoxic effects, the cardiomyocytes lost may be replaced by nonmyocytic tissue, resulting in thinning of the ventricular wall and dilation, which constitutes the ventricular remodeling characteristic of DCM. This hypothesis is supported by our finding of greater abnor-

mality in ventricular systolic function and left ventricular morphology in the iNOS-positive group than in the iNOS-negative group.

However, the present study could not establish a causal role for iNOS in the etiology of DCM. It is clear that further studies will be required to determine the relation between cytokine-induced myocardial NO production and the development or progression of left ventricular dysfunction.

This study did not examine patients with cardiac disease due to different etiologies matched for ventricular function and clinical severity. It therefore remains uncertain whether the observed high incidence of iNOS mRNA expression is specific to patients with DCM. However, preliminary research (35) suggests that the messenger gene can be detected in the myocardium of patients with coronary artery disease. Lewis et al. (44) recently demonstrated that iNOS mRNA was detected in the human cardiac allograft with associated left ventricular contractile dysfunction. In experimental animal models of acute myocardial infarction, iNOS activity has been reported (45,46) to be significantly increased in the infarcted area of myocardium (45,46). Furthermore, although it was not ascertained whether the pathogenesis of DCM is directly related to iNOS expression and TNF-alpha, these observations suggest that myocardial coexpression of iNOS and TNF-alpha may not only be involved in the development or progression of DCM but may also play a much broader pathophysiologic role in heart failure due to various etiologies.

Conclusions. The present study indicated that iNOS mRNA was consistently coexpressed with TNF-alpha mRNA in myocardial tissue obtained from a subgroup of patients with DCM and advanced left ventricular dysfunction.

References

1. Smith JA, Radomski MW, Schulz R, Moncada S, Lewis MJ. Porcine ventricular endocardial cells in culture express the inducible form of nitric oxide synthase. Br J Pharmacol 1993;108:1107–10.
2. Tsujino M, Hirata Y, Imai T, et al. Induction of nitric oxide synthase gene by interleukin-1β in cultured rat cardiocytes. Circulation 1994;90:375–83.
3. Shindo T, Ikeda U, Ohkawa F, Kawahara Y, Yokoyama M, Shimada K. Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. Cardiovasc Res 1995;29:813–9.
4. Brady AJB, Poole-Wilson PA, Harding SE, Warren JB. Nitric oxide production within cardiac myocytes reduced their contractility in endotoxemia. Am J Physiol 1992;263:H1963–6.
5. Brutsaert DL, Andries LJ. The endocardial endothelium. Am J Physiol 1992;263:H985–1002.
6. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. Br J Pharmacol 1992;105:575–80.
7. Kornbluth RS, Edgington T. Tumor necrosis factor production by human monocytes is a regulated event: induction of TNF-α–mediated cellular cytotoxicity by endotoxin. J Immunol 1986;137:2585–91.
8. Anggard E. Nitric oxide: mediator, murderer, and medicine. Lancet 1994; 343:1199–206.
9. de Belder AJ, Radomski MW, Martin JF, Moncada S. Nitric oxide and the pathogenesis of heart muscle disease. Eur J Clin Invest 1995;24:1–8.
10. de Belder AJ, Radomski MW, Why HJF, et al. Nitric oxide synthase activities in human myocardium. Lancet 1993;341:84–5.
11. Report of the WHO/ISFC task force on the definition and classification of cardiomyopathies. Br Heart J 1980;44:672–3.
12. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–9.
13. Charles IG, Palmer RMJ, Hickery MS, et al. Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proc Natl Acad Sci USA 1993;90:11419–23.
14. Shirai T, Yamaguchi H, Ito H, Todd CW, Wallace RB. Cloning and expression in *Escherichia coli* of the gene for human tumor necrosis factor. Nature 1985;313:803–6.
15. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 1985;82:6133–7.
16. Bowles NE, Richardson PJ, Olsen EGJ, Archard LC. Detection of coxsackie-B virus-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. Lancet 1986;1: 1120–2.
17. Archard LC, Freeke CA, Richardson PJ, et al. Persistence of enterovirus RNA in dilated cardiomyopathy: a progression from myocarditis. In: Schultheiß H-P, editor. New Concepts in Viral Heart Disease. Berlin: Springer-Verlag, 1988:349–62.
18. Archard LC, Bowles NE, Cunningham L, et al. Molecular probes for detection of persisting enterovirus infection of human heart and their prognostic value. Eur Heart J 1991;12 Suppl D:56–9.
19. Jin O, Sole MJ, Butany JW, et al. Detection of enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain reaction. Circulation 1990;82: 8–16.
20. Satoh M, Tamura G, Segawa I, Hiramori K, Satodate R. Enteroviral RNA in dilated cardiomyopathy. Eur Heart J 1994;15:934–9.
21. Limas CJ, Goldenberg IF, Limas C. Autoantibodies against β-adrenoreceptors in human idiopathic dilated cardiomyopathy. Circ Res 1989;64:97–103.
22. Caforio ALP, Grazzini M, Mann JM, et al. Identification of α- and β-cardiac myosin heavy chain isoforms as major autoantigens in dilated cardiomyopathy. Circulation 1992;85:1734–42.
23. Lange LG, Schreiner GF. Immune cytokines and cardiac disease. Trends Cardiovasc Med 1992;2:145–51.
24. Henke A, Mohr C, Sprenger H, et al. Coxsackievirus B3-induced production of tumor necrosis factor-α, IL-1β, and IL-6 in human monocytes. J Immunol 1992;148:2270–7.
25. Satoh M, Tamura G, Segawa I, Tashiro A, Satodate R, Hiramori K. Expression of cytokine genes and presence of enteroviral genomic RNA in endomyocardial biopsy tissues of myocarditis and dilated cardiomyopathy. Virchows Archiv 1996;427:503–9.
26. Beutler B, Krochin N, Milsark IW, Luedke C, Cerami A. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science 1986;232:977–80.
27. Urban JL, Shepard HM, Rothstein JL, Sugarman BJ. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. Proc Natl Acad Sci USA 1986;83:5233–7.
28. Kornbluth RS, Edgington TS. Tumor necrosis factor production by human monocytes is a regulated event: induction of TNF-α-mediated cellular cytotoxicity by endotoxin. J Immunol 1986;137:2585–91.
29. Habib FM, Springall DR, Davies GJ, Oakley CM, Yacoub MH, Polak JM. Tumor necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. Lancet 1996;347:1151–5.
30. Nakayama DK, Geller DA, Silvio MD, et al. Tetrahydrobiopterin synthase and inducible nitric oxide production in pulmonary artery smooth muscle. Am J Physiol 1994;266:L455–60.
31. Balligand J-L, Ungureanu-Longrois D, Simmons WW, et al. Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. J Biol Chem 1994;269:27580–8.
32. Haywood GA, Tsao PS, von der Leyen HE, et al. Expression of inducible nitric oxide synthase in human heart failure. Circulation 1996;93:1087–94.
33. Thoenes M, Förstermann U, Tracey WR, et al. Expression of inducible nitric oxide synthase in failing and non-failing human heart. J Mol Cell Cardiol 1996;28:165–9.
34. Hare JM, Loh E, Creager MA, Colucci WS. Nitric oxide inhibits the positive inotropic response to β-adrenergic stimulation in humans with left ventricular dysfunction. Circulation 1995;92:2198–203.

35. Studer R, Hasenfuss G, Kastner S, Drexler H. Functional importance of myocardial gene expression of inducible nitric oxide synthase in ischemic and dilated cardiomyopathy [abstract]. Circulation 1995;92 Suppl I:I-567.
36. Geng YJ, Hanson GK, Holme E. Interferon-γ and tumor necrosis factor synthesize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells. Circ Res 1992;71:1268–76.
37. Stuehr DJ, Nathan CE. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cell. J Exp Med 1989;169:1543–55.
38. Pinsky DJ, Cai B, Yang X, Rodriguez C, Sciacca RR, Cannon PJ. The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor-β. J Clin Invest 1995;95:677–85.
39. Kitajima I, Kawahara K, Nakajima T, Soejima Y, Matsuyama T, Maruyama I. Nitric oxide-mediated apoptosis in murine mastocytoma. Biochem Biophys Res Commun 1994;204:244–51.
40. Packer M. Is tumor necrosis factor an important neurohormonal mechanism in chronic heart failure? Circulation 1995;92:1379–82.
41. Torre-Amione G, Kapadia S, Lee J, et al. Tumor necrosis factor-α and tumor necrosis factor receptors in the failing human heart. Circulation 1996;93: 704–11.
42. Kapadia S, Lee J, Torre-Amione G, Birdsall HH, Ma TS, Mann DL. Tumor necrosis factor-α gene and protein expression in adult feline myocardium after endotoxin administration. J Clin Invest 1995;96:1042–52.
43. Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL. Cellular basis for the negative inotropic effects of tumor necrosis factor-α in the adult mammalian heart. J Clin Invest 1993;92:2303–12.
44. Lewis NP, Tsao PS, Rickenbacher PR, et al. Induction of nitric oxide synthase in the human cardiac allograft is associated with contractile dysfunction of the left ventricle. Circulation 1996;93:720–9.
45. Dudek RR, Wildhirt S, Conforto A, et al. Inducible nitric oxide synthase activity in myocardium after myocardial infarction in rabbit. Biochem Biophys Res Commun 1994;205:1671–80.
46. Wildhirt SM, Dudek RR, Suzuki H, Pinto V, Narayan KS, Bing RJ. Immunohistochemistry in the identification of nitric oxide synthase isoenzymes in myocardial infarction. Cardiovasc Res 1995;29:526–31.