Expression of Tumor Necrosis Factor-alpha–Converting Enzyme and Tumor Necrosis Factor-alpha in Human Myocarditis

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OBJECTIVES
We determined whether tumor necrosis factor-alpha–converting enzyme (TACE) is expressed with tumor necrosis factor-alpha (TNF-alpha) in myocarditis.

BACKGROUND
Tumor necrosis factor-alpha–converting enzyme, which has recently been identified as belonging to the family of metalloproteinase disintegrin proteins, is responsible for the conversion of TNF-alpha precursor to its mature form.

METHODS
We examined TACE and TNF-alpha expressions in endomyocardial biopsy tissues obtained from 14 patients with myocarditis and five control subjects by using quantitative reverse transcriptase polymerase chain reaction and immunohistochemistry.

RESULTS
Expression of TNF-alpha and TACE messenger ribonucleic acid (mRNA) was significantly greater in the myocarditis group than in the control group. A positive correlation was found between TNF-alpha and TACE mRNAs (r = 0.83, p < 0.05). Six patients with severe myocarditis underwent repeat biopsies. Although TNF-alpha and TACE mRNAs were expressed at high levels in the initial biopsies, a marked decrease was noted in the repeat biopsies. The immunostainings for TNF-alpha and TACE were positive in the myocytes and interstitial cells of myocardium obtained from patients with myocarditis. Expression of TACE and TNF-alpha mRNAs was greater in the subgroup in New York Heart Association functional class III or IV than in the subgroup in class I or II. Expression of TACE and TNF-alpha mRNA was correlated positively with left ventricular volume (TNF-alpha: r = 0.85; TACE: r = 0.80) and negatively with left ventricular systolic function (TNF-alpha: r = −0.85; TACE: r = −0.85).

CONCLUSIONS
These findings indicate that the expression of TNF-alpha and TACE may have important implications in the pathogenesis of myocarditis and may influence advanced cardiac dysfunction in myocarditis. (J Am Coll Cardiol 2000;36:1288–94) © 2000 by the American College of Cardiology

Myocarditis has been considered to be a myocardial inflammatory reaction due to infection by a wide range of viruses and autoimmune reactions (1). Several studies have used a molecular approach to demonstrate the relation between pathogenetic factors and viral agents in causation of human myocarditis (2,3). In a murine acute viral myocarditis model, tumor necrosis factor-alpha (TNF-alpha) messenger ribonucleic acid (mRNA) was strongly expressed in infiltrating cells, indicating that these cells mediate the immune responses in viral myocarditis (4). A strong linear relation has also been reported between mortality and TNF-alpha levels in a murine model of congestive heart failure due to viral myocarditis (5).

Other previous experimental and clinical studies have suggested that there is an association between depressed myocardial function and elevated TNF-alpha mRNA and protein levels in the myocardium in human dilated cardiomyopathy (6,7). TNF-alpha is a multifunctional cytokine that mediates various pathologic processes, such as septic shock, inflammation and cachexia (8). Although the major biologic role of TNF-alpha is thought to be a host response against systemic infections, this cytokine has been recently implicated in the pathogenesis of a variety of human cardiac diseases, including myocarditis, dilated cardiomyopathy and congestive heart disease (9–12).

Recently, the protease responsible for the shedding of pro-TNF-alpha was identified and cloned (13,14). Tumor necrosis factor-alpha–converting enzyme (TACE) belongs to the family of metalloproteinase disintegrin proteins (13,14). It has also been suggested that TACE might be an important target enzyme for anti-inflammatory agents in suppression of the final processing stage of TNF-alpha production (13). However, it is uncertain whether TACE is expressed with TNF-alpha in the myocardial tissues in human myocarditis. In this study, we examined the expression of TACE and TNF-alpha in the endomyocardial tissues of patients with myocarditis and control subjects by using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) method and immunohistochemical analysis. We also explored the relation between clinical characteristics and myocardial expression of TACE and TNF-alpha.
Abbreviations and Acronyms

cDNA = complementary deoxyribonucleic acid
CK = creatine kinase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
mRNA = messenger ribonucleic acid
NYHA = New York Heart Association
RT-PCR = reverse transcriptase polymerase chain reaction
TACE = tumor necrosis factor-alpha–converting enzyme
TNF-alpha = tumor necrosis factor-alpha

METHODS

Subjects. We examined 20 endomyocardial biopsy tissues obtained from 14 patients with myocarditis by right ventricular endomyocardial biopsy. This group included 10 males and four females (mean age 37 ± 5 years, range 13 to 73 years). Myocarditis was diagnosed on the basis of initial symptoms (e.g., upper respiratory illness, fever and chest pain), and clinical findings included tachycardia, arrhythmias, murmurs, rubs, cardiomegaly, elevated erythrocyte sedimentation rate, leukocytes, cardiac enzyme, C-reactive protein and electrocardiographic changes (e.g., conduction disturbances, ST-T segment and Q wave abnormalities), in conjunction with morphologic evidence of inflammatory infiltrate in the biopsy materials. Myocarditis was histologically diagnosed according to the Dallas criteria (15). Each biopsy sample was examined by three investigators who had no knowledge of the clinical features and results of RT-PCR and immunohistochemistry. Patients with a pathologic diagnosis of idiopathic myocarditis were carefully selected for this study. Specific types of myocarditis, such as rheumatic, septic, mycotic, eosinophilic types and myocarditis associated with collagen disease, sarcoidosis or another known etiology, were excluded from this study. In addition, all patients with myocarditis underwent coronary angiography at the time of each endomyocardial biopsy to exclude ischemic heart disease and other secondary cardiac diseases. The initial endomyocardial biopsies were performed two to 12 days (mean 8 ± 1 days) after admission. Six of the 14 patients in New York Heart Association (NYHA) functional class IV at admission underwent repeat biopsies at 28 to 62 days (mean 35 ± 5 days) after admission to confirm the extent or completion of healing. Echocardiography determined left ventricular ejection fraction and diameter at the time of each endomyocardial biopsy. Serial peripheral blood samples were taken from patients with myocarditis between one and 14 days after admission for measurement of cardiac enzymes and inflammatory variables. Control myocardial tissue samples were obtained by endomyocardial biopsy from five subjects (three men and two women; mean age 48 ± 15 years) suspected of having a cardiac disorder on the basis of premature beats and echocardiographic changes, such as slight ventricular wall thickness. The resulting pathologic findings and close clinical examination showed no evidence of myocardial disease and medical history of infectious illness (e.g., myocarditis, sepsis and pneumonia), and these subjects were designated as the control subjects with normal cardiac function and morphology. These study protocols were approved by our hospital’s Ethics Committee, and written, informed consent was obtained from all subjects.

Positive control cells for TACE and TNF-alpha mRNA. Peripheral human monocytes were prepared from normal donors. The monocytes were washed and resuspended in AIM-R medium (GIBCO BRL, Gaithersburg, Maryland) supplemented with 2 mg of anti-human LeuTM-4 (CD3) (Beckton Dickinson, San Jose, California). They were then allowed to adhere to tissue culture flasks for 48 h at 37°C.

Extraction of total RNA. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method from endomyocardial tissues and cultured monocytes and treated with RNAse I (GIBCO BRL) (16). TagMan glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents were used for fluorogenic detection of human GAPDH transcript (Perkin Elmer Applied Biosystems Division, Foster City, California).

Oligonucleotides of primers and probes. Published complementary deoxyribonucleic acid (cDNA) sequences for human TNF-alpha (17), TACE (13) and GAPDH (18) were used for construction of primers and probes. The sequence of primers and their associated fluorogenic probes was designed using applications-based primer design software (Primer Express version 1.0, Perkin Elmer Applied Biosystems Division). The following primers and probes were used for relative quantification of targeted gene expression—for TNF-alpha: forward primer 5'-CTT CTC TTT CCT CTT CCT GAT GTG GG-3', reverse primer 5'-GGT GGT TAT TTC TCA GCT CCA-3' and probe 5'-CAG GCA GTC AGA TCA TCT TCT CGA AC-3'; for TACE: forward primer 5'-ACC TGA AGA GCT TGT TCA TCG AG-3', reverse primer 5'-CCA TGA AGT CCG ATA GAT GTC-3' and probe 5'-TTG GTG GTA GCA GAT CAT CGC TCC T-3'; for GAPDH: forward primer 5'-GAA GGT GAA GTG CGG AGT-3', reverse primer 5'-GAA GGT GAT GGG ATT TC-3' and probe 5'-CAA GCT TTC CGT CAG CC-3'. The PCR products of TNF-alpha, TACE and GAPDH were amplified at the sizes of 266, 190 and 226 base pair, respectively.

Quantitative RT-PCR. We analyzed TACE and TNF-alpha mRNA expression levels using a quantitative RT-PCR method, as previously described (19). The cDNA was synthesized and amplified from total RNA and tenfold serial dilutions of human control RNA (Perkin Elmer Applied Biosystems Division) by RT-PCR using the TagMan EZ RT-PCR kit (Perkin Elmer Applied Biosystems Division). The cDNA products were synthesized at 60°C for 30 min and amplified with 40 cycles of PCR, with each cycle consisting of denaturation at 94°C for 20 s and
Table 1. Clinicopathologic Characteristics and mRNA Expression Levels in Patients With Myocarditis

<table>
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<th>No.</th>
<th>Age (years)</th>
<th>Gender</th>
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<th>NYHA class</th>
<th>Duration* (days)</th>
<th>CK (IU/liter)</th>
<th>LVESD (mm)</th>
<th>LVEF (%)</th>
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*Duration from hospital admission.

Statistical analysis. All data are presented as the mean value ± SEM. Statistically, the difference in TNF-alpha and TACE expression levels between the myocarditis and control groups was analyzed by using the unpaired t test. Comparison of TNF-alpha and TACE mRNA expression levels in serial biopsy samples was analyzed by using two-way repeated measures analysis of variance. Pearson’s correlation coefficients were used to examine the relation between levels of mRNA expression and clinical variables. A value of p < 0.05 was considered statistically significant.

RESULTS

Quantitative RT-PCR. The clinical characteristics, pathologic diagnosis and RT-PCR results of the myocarditis group are shown in Table 1. As shown in Figure 1, the expression of TNF-alpha and TACE mRNA was significantly greater in the myocardial tissues of patients with active myocarditis than in the tissues of control subjects (TNF-alpha/GAPDH ratio 3.70 ± 0.57 vs. 0.04 ± 0.02, p < 0.05; TACE/GAPDH ratio 2.44 ± 0.48 vs. 0.03 ± 0.02, p < 0.05). There was a positive correlation between the TACE/GAPDH ratio and TNF-alpha/GAPDH ratio in the myocarditis group (r = 0.83, p < 0.05). In six patients with severe myocarditis who underwent repeat biopsy, although high levels of both TNF-alpha and TACE mRNA were expressed in myocardial tissues obtained from the initial biopsy when active myocarditis was present, both TNF-alpha and TACE mRNA decreased markedly in the repeat biopsies when cellular infiltration had become reduced and was intimately associated with necrotic myocytes.
Expression of TACE and TNF-alpha in Myocarditis

(TNF-alpha/GAPDH ratio 5.54 ± 0.67 vs. 3.33 ± 0.52, p = 0.03; TACE/GAPDH ratio 4.36 ± 0.29 vs. 1.74 ± 0.17, p < 0.01) (Fig. 1). However, the repeat biopsy levels were still higher in patients with myocarditis than in control subjects (p < 0.05) (Fig. 1).

Immunohistochemical analysis. Immunohistochemical analysis was performed to identify the source of TACE and TNF-alpha protein within myocardial tissues. Immunostaining for both proteins was found in cardiac myocytes and interstitial cells in myocardial tissues obtained from patients with active myocarditis (Fig. 2a, c). Positive interstitial cells for TACE and TNF-alpha were predominantly infiltrating mononuclear cells and macrophages. However, both TACE and TNF-alpha immunostaining was markedly decreased in cardiac myocytes and interstitial cells from repeat biopsy tissues, coinciding with the healing stage (Fig. 2b, d). There was no evidence of nonspecific immunostaining in the myocardial tissues obtained from patients with myocarditis. Neither TACE nor TNF-alpha immunostaining was present in any of the specimens from the control group.

Comparison of clinical data. When patients with myocarditis were classified into two subgroups according to NYHA functional class on hospital admission, both TACE and TNF-alpha mRNA levels were expressed to a greater extent in the subgroup in functional class III or IV (n = 8) than in the subgroup in class I or II (n = 6) (TACE/GAPDH ratio 3.58 ± 0.56 vs. 0.93 ± 0.19, p = 0.02; TNF-alpha/GAPDH ratio 5.04 ± 0.59 vs. 1.92 ± 0.44, p = 0.01) (Fig. 3). There was a positive correlation between left ventricular end-systolic diameter and expression of both TNF-alpha (r = 0.85, p < 0.05) and TACE mRNA (r = 0.80, p < 0.05). The correlation with left ventricular ejection fraction was negative for both proteins (TNF-alpha: r = −0.85, p < 0.05; TACE: r = −0.85, p < 0.05). The differences in TACE and TNF-alpha mRNA expression between the two

Figure 1. Comparison of TACE/GAPDH (a) and TNF-alpha/GAPDH (b) levels in serial endomyocardial biopsy samples of patients and control subjects. Both TNF-alpha/GAPDH and TACE/GAPDH mRNAs were expressed to a higher level in initial biopsy samples than in repeat biopsy samples (TNF-alpha/GAPDH ratio 5.54 ± 0.67 vs. 3.33 ± 0.52, p = 0.03; TACE/GAPDH ratio 4.36 ± 0.29 vs. 1.74 ± 0.17, p < 0.01). MC = myocarditis.

Figure 2. Immunohistochemical analysis for TNF-alpha and TACE in serial endomyocardial biopsy tissues obtained from patients with myocarditis. Detection of TNF-alpha (a) and TACE (c) proteins with primary and secondary antibodies in the initial biopsy. TNF-alpha and TACE proteins were found in myocytes, infiltrating macrophages and mononuclear cells. Immunostaining of TNF-alpha (b) and TACE (d) was markedly decreased in myocytes and interstitial cells in repeat biopsy. Magnification for a and c, ×230; for b and d, ×240, reduced by XX%.

Figure 3. Comparison of TACE (a) and TNF-alpha (b) mRNA expression according to NYHA functional class. TACE and TNF-alpha mRNA levels were expressed to a higher level in the myocarditis subgroup in functional class III or IV than in the subgroup in class I or II (TACE/GAPDH ratio 3.58 ± 0.56 vs. 0.93 ± 0.19, p = 0.02; TNF-alpha/GAPDH ratio 5.04 ± 0.59 vs. 1.92 ± 0.44, p = 0.01).
subgroups with myocarditis, classified by peak plasma concentration of creatine kinase (CK), are shown in Figure 4. The subgroup with peak plasma CK >500 IU/liter (n = 7) had a significantly higher level of TACE and TNF-alpha mRNA expression than the subgroup with CK <500 IU/liter (n = 7) (TACE/GAPDH ratio 3.84 ± 0.57 vs. 1.04 ± 0.20, p = 0.006; TNF-alpha/GAPDH ratio 5.29 ± 0.62 vs. 2.12 ± 0.42, p = 0.001). There was no statistical relation between TACE and TNF-alpha mRNA expression levels and other clinical variables representing an inflammatory reaction (erythrocyte sedimentation rate, leukocyte and C-reactive protein) and cardiac enzymes (glutamic oxaloacetic transaminase and lactic dehydrogenase).

**DISCUSSION**

In this study, we have demonstrated that both TACE and TNF-alpha mRNAs and proteins are expressed in the myocardium of patients with myocarditis. A positive correlation was found between TACE and TNF-alpha mRNA expression. Interestingly, TACE and TNF-alpha mRNAs were expressed to a high level in advanced cardiac dysfunction in patients with myocarditis. Immunohistochemical analysis revealed that the cellular source of TACE and TNF-alpha proteins was not only infiltrating macrophages and mononuclear cells but also cardiac myocytes. These findings strongly suggest that myocardial expression of TACE and TNF-alpha may be significantly implicated in the myocardial damage that occurs during the inflammatory process associated with myocarditis.

**Myocardial expression of TACE and TNF-alpha.** An animal model of viral myocarditis has demonstrated that proinflammatory cytokines, such as interleukin-1 and TNF-alpha, are implicated in the pathogenesis of myocarditis in the acute phase (4,5). A previous study has reported that the proinflammatory cytokines, especially TNF-alpha, are persistently expressed in myocarditis and dilated cardiomyopathy (11). Kubota et al. (22) and Bryant et al. (23) have described a transgenic mouse model of myocarditis in which TNF-alpha is expressed, particularly in cardiac myocytes. In these models, the mice developed gross lymphocytic infiltration and myocytic necrosis that pathologically resembled human active myocarditis. In addition, biventricular dilation and cardiac contractile dysfunction were noted. Therefore, these reports have strongly suggested that TNF-alpha overexpression is sufficient to cause cardiac morphologic changes and dysfunction, confirming the role of this protein in human diseases, such as myocarditis and dilated cardiomyopathy. However, the mechanism of TNF-alpha production in human myocarditis has not been ascertained. Recently, the protease responsible for the shedding of pro-TNF-alpha was identified and cloned (13,14). This protease—TACE—belongs to the family of metalloproteinase disintegrin proteins (24,25). It has also been suggested that TACE is expressed ubiquitously and that several TACE knock-out cell types lose TNF-alpha processing activity, indicating that TACE is responsible for pro-TNF-alpha shedding in various cell types such as monocytes, T cells, neutrophils, and endothelial cells (13). In this study, TACE mRNA and protein were expressed with TNF-alpha expression in myocardial tissues obtained from patients with myocarditis. The cellular source of TACE and TNF-alpha proteins was found to be not only infiltrating macrophages and lymphocytes but also cardiac myocytes. There was a positive correlation between TACE and TNF-alpha mRNA expression. Although this study could not confirm whether increased expression of TACE has a direct causal relation to the inflammatory process associated with myocarditis, several experimental studies have reported that inflammatory conditions, such as endotoxin challenge and bacterial lipopolysaccharide stimulation on culture monocytes, mediated TACE-like metalloproteinase overexpression, and that metalloproteinase stimulated TNF-alpha production (26,27). Therefore, these findings suggest that TACE may induce TNF-alpha expression in cardiac myocytes and infiltrating cells, such as macrophages and mononuclear cells, during the paracrine or autocrine inflammatory process, or both.

**Comparison between mRNA expressions and clinical variables.** One of the most important findings of this study was a significantly elevated level of expression of TACE and TNF-alpha mRNAs in patients with myocarditis with a greater left ventricular volume and a lower left ventricular ejection fraction. The myocarditis subgroup with high CK levels revealed high expression of TACE and TNF-alpha mRNAs. Although our previous study demonstrated that TNF-alpha mRNA was expressed to a high level with TACE expression in clinically advanced dilated cardiomyopathy (19), TNF-alpha mRNA in patients with severe myocarditis was expressed at more than twice the level seen in patients with advanced dilated cardiomyopathy. Both
Figure 5. The hypothetical role of TACE in myocarditis. First, viral infection or autoimmune dysfunction, or both, occurred in myocardial tissues. These immune responses in infiltrating inflammatory cells (infiltrating macrophages and lymphocytes) and cardiac myocytes mediated expression of TACE and pro-TNF-α. Activated TACE cleaved from pro-TNF-α to its mature form. The mature form of TNF-α induced myocytic injury and left ventricular dysfunction through the paracrine or autocrine process, or both. TACE and TNF-alpha mRNA expression levels had decreased in repeat biopsy samples, but these levels were high as compared with those in control subjects. These findings suggest that the inflammatory process of TACE-mediated TNF-alpha production may induce substantial myocytic injury and impair cardiac systolic function. Studies by Yokoyama et al. (28) and Bozkurt et al. (29) have reported that TNF-alpha has the negative inotropic effects of myocytic injury and left ventricular remodeling in cultured myocytes in the experimental TNF-alpha infusion model. It has also been reported that TNF-alpha-activated cytotoxic T cells may cause direct myocytic injury (30,31). These observations imply that TACE expression may be related to TNF-alpha expression during the inflammatory process of myocarditis. Our speculation about the TACE-activated pathway in myocarditis is shown in Figure 5. Myocardial expression of TACE and TNF-alpha may play an important role in advanced cardiac dysfunction in human myocarditis. We speculate that TACE may be a potentially important factor in the establishment of a new pathologic concept of myocarditis and may also be a novel target for therapeutic intervention in the inflammatory process of myocarditis.

Conclusions. These findings indicate that the expression of TNF-alpha and TACE may have important implications in the pathogenesis of myocarditis and may induce advanced cardiac dysfunction.

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