Increased mRNA expression of tumour necrosis factor-α and its converting enzyme in circulating leucocytes of patients with acute myocardial infarction

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ABSTRACT

Tumour necrosis factor-α (TNF-α) plays an important role in myocardial damage in acute myocardial infarction (AMI). It has recently been discovered that TNF-α-converting enzyme (TACE) cleaves precursor TNF-α into its mature form. However, it remains unknown whether TNF-α expression is related to TACE expression in circulating leucocytes in AMI. Blood samples were obtained from 37 patients with AMI within 24 h of onset and eight healthy controls. Plasma TNF-α levels were measured by ELISA. Total mRNA was then extracted from circulating leucocytes, and the expression levels of TACE and TNF-α mRNAs were determined by reverse transcriptase-PCR. Plasma TNF-α levels were significantly higher in patients with Killip's classes III and IV AMIs (17.1 ± 5.0 pg/ml, n = 11) than in those with Killip's classes I and II AMIs (13.7 ± 4.2 pg/ml, n = 26), or controls (13.0 ± 1.7 pg/ml, n = 8) (P < 0.05). There was a significant increase in expression (arbitrary units) of TACE and TNF-α mRNAs in circulating leucocytes obtained from patients with Killip's classes I and II AMIs [TACE/glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 2.770 ± 0.303; TNF-α/GAPDH, 2.123 ± 0.475] compared with controls (TACE/GAPDH, 1.498 ± 0.209; TNF-α/GAPDH, 1.283 ± 0.274) (P < 0.01). This increase was even greater in patients with Killip's classes III and IV AMIs (TACE/GAPDH, 3.086 ± 0.354; TNF-α/GAPDH, 2.808 ± 0.422) (P < 0.01). Moreover, there was a significant positive relationship between these mRNA expression levels (r = 0.60, P < 0.01). The TACE–TNF-α system in circulating leucocytes is stimulated and may have a negative impact on clinical outcome in AMI.

INTRODUCTION

Several previous studies have shown that circulating tumour necrosis factor-α (TNF-α) levels are elevated in patients with acute myocardial infarction (AMI) [1–3]. In experimental animal models of AMI, the use of a monoclonal antibody against TNF-α markedly reduced myocardial infarct size [4], and other studies have suggested that TNF-α may induce structural and geometric left ventricular remodelling after the onset of AMI [5,6]. Furthermore, TNF-α is primarily synthesized and released not only from activated leucocytes, but also from the ischaemic myocardium itself [4]. It has also been reported that plasma obtained from acute-phase AMI patients stimulates the production of cytokines from cultured leucocytes [7]. These reports suggest that the...
necrotic myocardium itself produces pro-inflammatory cytokines locally, and primes the systemic inflammatory response through the cytokine network, resulting in further exacerbation of myocardial damage after AMI.

TNF-α-converting enzyme (TACE) has been purified and cloned [8,9]. This enzyme is a metalloproteinase disintegrin that specifically cleaves precursor TNF-α to the mature form, which may cause various physiological changes. An experimental study has shown that in a TACE knock-out mouse model various cell types, such as monocytes, T-cells, neutrophils and endothelial cells, loose TNF-α processing activity [9], which suggests that TACE is essential for the production of TNF-α. However, the pathological role of the TACE–TNF-α system in AMI has not been clearly understood.

The aim of the present study was to determine whether an inflammatory response as represented by the TACE–TNF-α system is activated in circulating leucocytes immediately after the onset of AMI. We therefore examined plasma TNF-α levels, and mRNA expression levels of TACE and TNF-α in circulating leucocytes using real-time quantitative reverse transcriptase-PCR (RT-PCR) in patients with AMI and control subjects. We then investigated the relationship between these expression levels and clinical severity in this disorder.

METHODS

Subjects
We studied 37 consecutive patients with AMI who were admitted to Iwate Prefectural Ohfunato Hospital within 24 h of the onset of AMI. Patients with non-cardiac diseases, such as malignant tumour, chronic renal failure treated by haemodialysis or infectious diseases (including pneumonia and sepsis), were excluded from the study. The patient group comprised 23 men and 14 women with a mean age of 68.9 ± 10.7 years. The diagnosis of AMI was based on a history of prolonged ischaemic chest pain, typical electrocardiographic changes and asynery in left ventricular wall kinesis detected by transthoracic echocardiography. Patients with AMI were divided into four classes according to Killip’s classification [10]. Classes I, II, III and IV comprised 17, nine, eight and three patients respectively. Anterior AMI was diagnosed in 22 patients, and inferior AMI in 15 patients. We administered thrombolytic therapy to 13 patients within 6 h of onset. Mean left ventricular ejection fraction measured by transthoracic echocardiography at admission was 53 ± 17%. In 35 patients whose creatine kinase (CK) levels peaked after admission, the median plasma CK level was 2425 (range 562–6100) units/l. Of the total AMI sample, four patients died due to multiple organ failure caused by refractory congestive heart failure (CHF) and two patients died due to cardiogenic shock within 24 h of admission. Eight healthy volunteers (five men and three women, mean age 65.0 ± 6.3 years) were also recruited as controls.

The protocol was approved by our hospital ethics committee, and written informed consent was obtained from all subjects or their relatives.

Extraction of total RNA and measurement of plasma TNF-α levels
Peripheral blood samples (10 ml) were taken just after admission, within 6 h of thrombolytic therapy and prior to the commencement of intensive treatment. Immediately, total RNA was extracted from circulating leucocytes in 1 ml blood samples using a commercial kit (QIAamp RNA Blood Mini Kit; Qiagen GmbH, Hilden, Germany). The remainder of each sample was collected into a sterilized tube containing EDTA, and the plasma was separated by centrifugation (2500 g) for 5 min at 4 °C. The yield of total RNA and plasma was stored at −80 °C until analysis. Plasma concentrations of TNF-α were measured using a commercially available ELISA kit (BioSource Europe, Nivelles, Belgium).

Oligonucleotides of primers and probes
The previously published cDNA sequences for human TNF-α [11], TACE [8] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [12] were used for construction of primers and probes. The sequences of primers and their associated fluorogenic probes were designed using applications-based primer design software (Primer Express version 1.0; PerkinElmer Applied Biosystems Division, Foster City, CA, U.S.A.). The primers and probes were used for relative quantification of targeted gene expression as follows. For TNF-α: forward primer, 5′-CTT CTC CCT CCT GAT CGT GG-3′; reverse primer, 5′-GCT GGT TAT CTC 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 GTT CCG ATA GAT GTC-3′; and probe, 5′-TTG GTG GTA GCA GAT CAT CGC TTC T-3′. For GAPDH: forward primer, 5′-GAA GGT GAA GGT CGG AGT-3′; reverse primer, 5′-GAA GAT GGT GAG ATT TC-3′; and probe, 5′-CAAT ACC TCC GTG TCT CAG CC-3′. The PCR products of TNF-α, TACE and GAPDH were amplified to the sizes of 266, 190 and 226 bp respectively.

Quantitative RT-PCR
We analysed TACE and TNF-α mRNA expression levels using a real-time quantitative RT-PCR method as described previously [13,14]. The cDNA was synthesized and amplified from total RNA and 10-fold serial dilutions of human control RNA (PerkinElmer Applied Biosystems Division) by RT-PCR using the TaqMan EZ RT-PCR kit (PerkinElmer Applied Biosystems Division).
Tumour necrosis factor in acute myocardial infarction

Figure 1  TACE/GAPDH and TNF-α/GAPDH mRNA levels in control subjects and patients with AMI within 24 h of onset (classified according to Killip’s class)

Results are expressed as the means ± S.D.

RESULTS

Plasma TNF-α levels were significantly higher in patients with Killip’s classes III and IV AMIs (17.1 ± 5.0 pg/ml, n = 11) than in those with Killip’s classes I and II AMIs (13.7 ± 4.2 pg/ml, n = 26) and control subjects (13.0 ± 1.7 pg/ml, n = 8) (P < 0.05). However, no significant difference was seen between Killip’s classes I and II AMI patients and control subjects. In contrast, there was a significant increase in mRNA expression levels (arbitrary units) of TACE and TNF-α in circulating leucocytes obtained from Killip’s classes I and II AMI patients compared with control subjects (TACE/GAPDH, 2.770 ± 0.303 versus 1.498 ± 0.209, P < 0.01; TNF-α/GAPDH, 2.123 ± 0.475 versus 1.283 ± 0.274, P < 0.01; Figure 1). These high levels were more pronounced in Killip’s classes III and IV AMI patients (TACE/GAPDH, 3.086 ± 0.354; TNF-α/GAPDH, 2.808 ± 0.422; ANOVA, P < 0.01 versus controls for both variables; Figure 1). Moreover, expression levels were higher in subjects who subsequently died than in surviving cases (TACE/GAPDH, 3.165 ± 0.423 versus 2.806 ± 0.304, P < 0.05; TNF-α/GAPDH, 2.952 ± 0.268 versus 2.205 ± 0.513, P < 0.01; Figure 2).

In addition, there was a significant positive correlation between these mRNA expression levels obtained within 24 h of onset of AMI (r = 0.60, P < 0.01; Figure 3). With respect to the effect of thrombolytic therapy, both TACE and TNF-α mRNA expression levels in AMI patients

Statistical analysis

The results were expressed as the means ± S.D. The ratios of mRNA expression levels of TACE and TNF-α to that of GAPDH were transformed into log10 values for distribution of normal scatter, and expressed as arbitrary units. Comparisons of measurements among the three groups were made using one-way ANOVA followed by Fisher’s test. Pearson’s correlation coefficient analyses were used to assess the relationships between the two measurements. Values of P < 0.05 were considered statistically significant. All data analysis was performed with a commercially available statistical analysis software package (Statview 5.0, Abacus Concepts, Calabasus, CA, U.S.A.).
Figure 2  TACE/GAPDH and TNF-α/GAPDH mRNA levels in patients with AMI divided into those who survived or died during hospitalization
Results are expressed as the means ± S.D.

Figure 3  Relationships between TACE/GAPDH and TNF-α/GAPDH mRNA levels in patients with AMI within 24 h of onset who underwent thrombolysis were not significantly different from those in AMI patients not receiving this therapy (TACE/GAPDH, 2.790 ± 0.308 versus 2.905 ± 0.366, \( P = 0.34 \); TNF-α/GAPDH, 2.197 ± 0.570 versus 2.397 ± 0.544, \( P = 0.30 \)).

DISCUSSION
TACE is essential for the production of a functional enzyme from the precursor TNF-α to the mature form [9,17–19]. However, the role played by TACE in
TNF-α processing in circulating leucocytes obtained from AMI patients and its relationship to clinical parameters have remained unknown. In the present study, levels of TACE and TNF-α mRNA expression in leucocytes in patients with AMI were significantly elevated. Furthermore, a significant relationship was observed between expression levels of the two forms of mRNA with a similar trend in clinical features, suggesting that activation of TACE may play an important role in TNF-α production in AMI. To the best of our knowledge, the present study is the first to demonstrate that increased levels of TNF-α expression are associated with increased TACE expression, at least at a transcriptional level, in circulating leucocytes in patients with AMI.

The present study does not allow for any conclusion to be drawn about the mechanism underlying the upregulation of expression levels of TACE and TNF-α mRNAs in circulating leucocytes in AMI. It is known that in ischaemic myocardial tissue TNF-α is released from various types of cells, including infiltrating macrophages, endothelial cells, smooth muscle cells [20,21] and cardiomyocytes themselves [4,6]. In addition, a previous study has suggested that peripheral blood monocytes also become activated during myocardial ischaemia, which may account in part for the production of TNF-α [22]. It has also been reported recently that the expression of matrix metalloproteinases, including TACE, is regulated by TNF-α in a murine model of retinal neovascularization [23]. Although the present study could not confirm whether increased expression of TACE has a directly causal relationship with the inflammatory process associated with ischaemic or necrotic myocardium due to AMI, consideration of the above reports indicates that it is likely that pro-inflammatory cytokines, such as TNF-α, derived from ischaemic or necrotic myocardium itself, may prime immunoreaction and thus modulate not only local, but also systemic, inflammatory responses through the cytokine network. This would result in increased mRNA expression levels of TACE and TNF-α in circulating leucocytes in patients with AMI.

In the present study we found that expression levels of TACE and TNF-α mRNAs in circulating leucocytes were significantly elevated, especially in severe AMI cases with CHF or cardiogenic shock, and in patients who died. Several previous studies have shown that the production of TNF-α in monocytes and macrophages can be stimulated by various mechanisms, such as hypoxia [24], α-adrenergic receptor stimulation [25] and lactic acidosis [26], which may lead to a more active TACE–TNF-α system in patients with AMI complicated with severe CHF. In addition, it has recently been reported that TNF-α levels were significantly higher in peripheral venous blood compared with coronary sinus blood in advanced CHF, suggesting that widespread organ hypoperfusion caused by decreased cardiac output could be an important stimulus for TNF-α production [27].

The major limitation of our present study is that we did not investigate expression levels of cellular proteins of TACE and TNF-α, which could be determined by flow cytometry. As a consequence we were unable, on the basis of the present results, to determine whether the levels of these two mRNAs in circulating leucocytes directly reflect post-transcriptional or post-translational modulation of TACE and TNF-α in circulating leucocytes. According to a previous study [28], however, stimulated changes in intracellular cytokine mRNA expression, including TNF-α, have shown similar trends to those seen in cellular cytokine protein levels, as determined by flow cytometry in peripheral mononuclear cells.

These findings suggest that local inflammatory response in ischaemic and necrotic myocardium primes systemic immuno-activation. This results in increased cytokine production in circulating leucocytes, which in turn contributes to cardiac dysfunction and injury through the cytokine network. We conclude that the TACE–TNF-α system in circulating leucocytes is activated in the early stages of AMI and may have a negative impact on clinical outcome.

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REFERENCES


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