Enhanced Expression of the Apoptosis Inducing Ligand TRAIL in Mononuclear Cells After Myocardial Infarction

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SUMMARY

Tumor necrosis factor (TNF) family proteins including TNF-α and Fas (CD95)-ligand have been implicated in the development of acute myocardial infarction (AMI). We studied whether AMI patients displayed up-regulation of another TNF family member, TNF-related-apoptosis-inducing ligand (TRAIL), on peripheral blood mononuclear cells (PBMCs).

We compared expression of TRAIL on PBMCs from 26 patients in the acute phase of AMI with that on PBMCs from 16 healthy control subjects using flow cytometry and RT-PCR. In addition, expression of TRAIL protein on PBMCs from patients in the acute phase of AMI was also compared with that from the same patients 7 days later. Furthermore, we compared the expression of TRAIL protein on CD4+, CD8+, CD14+, and CD19+ cells from patients in the acute phase of AMI with that from control subjects using flow cytometry. Finally, expression of the TRAIL receptors (TRAILR)-1 and TRAILR-2 in human cardiomyocytes was examined immunohistochemically. Expression of TRAIL protein was significantly higher in the acute phase of AMI than in control subjects. Expression of TRAIL protein was significantly higher in the acute phase of AMI than 7 days later. TRAIL mRNA expression in the acute phase of AMI was higher than in control subjects. Expression of TRAIL protein on CD4+ and CD14+ cells from AMI patients was significantly higher than that from control subjects. Expression of TRAILR-1 and TRAILR-2 in human cardiomyocytes was confirmed immunohistochemically.

TRAIL on infiltrating CD4+ and CD14+ cells may be involved in the induction of cardiomyocyte apoptosis after AMI. (Jpn Heart J 2003; 44: 833-844)

Key words: Acute myocardial infarction, TRAIL, Mononuclear cells, Apoptosis

Necrosis has been thought to be the mode of myocardial cell death after acute myocardial infarction (AMI). However, it was reported that hypoxia induced...
apoptosis with enhanced expression of Fas (CD95) in cultured rat cardiomyocytes and that reperfusion injury induced apoptosis in rabbit cardiomyocytes, and many studies have shown that the induction of apoptosis is involved in myocardial ischemia.\textsuperscript{1,2)}

There are two major pathways for apoptosis induction, that is, a pathway via mitochondrial injury and a pathway via death receptors that reside on cell membranes. In relation to the pathway via death receptors, the relation between the Fas-Fas-ligand system and cardiomyocyte apoptosis after AMI has been primarily reported.\textsuperscript{1,3-5)} Moreover, Jeremias, \textit{et al}\textsuperscript{5)} demonstrated that death inducing ligands (DILs), namely, Fas-ligand, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and TNF-related-apoptosis-inducing ligand (TRAIL) were induced in an ischemic/reperfusion model of isolated rat hearts in Langendorff perfusion and that enhanced cell death after myocardial ischemia was dependent on DILs using cultured rat cardiomyocytes. However, the roles of DILs other than Fas-ligand remain largely unknown in cardiomyocyte apoptosis after AMI.

TRAIL is a 33-to 34-kDa member of DILs, and binds to TRAIL receptors (TRAILRs), belonging to the TNF receptor (TNFR) family consisting of TRAILR-1 (Death receptor-4; DR-4), TRAILR-2 (DR-5), TRAILR-3, and TRAILR-4. The binding of TRAIL to TRAILR-1 and TRAILR-2 results in activation of the caspase cascade and the induction of apoptotic cell death of various tumor cells.\textsuperscript{6,7)} TRAILR is widely expressed in various nonlymphoid as well as lymphoid organs,\textsuperscript{9)} but the expression of TRAILR in human cardiomyocytes has not yet been confirmed.

On the other hand, Varda-Bloom, \textit{et al}\textsuperscript{9)} reported that cytotoxic T lymphocytes activated following myocardial infarction killed healthy myocytes \textit{in vitro}, but the detailed mechanism of their cytotoxicity has not been demonstrated. However, Jeremias, \textit{et al}\textsuperscript{5)} demonstrated that TRAIL induced apoptosis of cardiomyocytes after simulated ischemia. Their model allows specific study of the cardiac process of apoptotic induction since they used the ischemia/reperfusion Langendorff model. However, it is not clear whether human peripheral blood mononuclear cells (PBMCs) take part in the process of apoptotic induction after AMI as mediators of TRAIL.

This study was an attempt to demonstrate that TRAIL expressed on PBMCs may participate in the induction of cardiomyocyte apoptosis after AMI as a DIL and that PBMCs may play a role as mediators of TRAIL. Consequently, in this study we investigated the expression of TRAIL on PBMCs from AMI patients using flow cytometry and RT-PCR. Furthermore, to investigate the cell populations positive for TRAIL in AMI, we studied the expression of TRAIL on CD4\textsuperscript{+}, CD8\textsuperscript{+}, CD14\textsuperscript{+}, and CD19\textsuperscript{+} cells. Finally, we examined immunohistochemically the expression of TRAILR-1 and TRAILR-2 in human cardiomyocytes. This
study has some implications for understanding mechanisms underlying apoptosis induction after AMI.

METHODS

Subjects: Twenty-six patients (male, 20 patients; age, 61.2 ± 2.09 years; onset of AMI, 3.1 ± 0.5 days; named “acute phase” of AMI) who had been diagnosed as AMI between April 2000 and March 2001, and 16 healthy volunteers (male, 11 patients; age, 62.1 ± 3.5 years) participated in the first study as control subjects. To examine the expression of TRAIL on PBMCs, blood samples (20 mL) were obtained from AMI patients and control subjects by standard venipuncture techniques. Next, from six patients (4 males, age, 70.8 ± 2.9 years) belonging to the above 26 AMI patients from whom consent for blood sampling could again be obtained 7 days later (named “subacute phase” of AMI), the second blood samples were taken in the same manner. PBMCs from each subject were prepared by Ficoll-Conray centrifugation.

To determine the cell populations positive for TRAIL, double immunostaining for TRAIL and cell type-specific markers was carried out. Six patients (4 males, age, 60.4 ± 7.3 years; onset of AMI, 2.8 ± 0.7 days) who had been diagnosed as AMI between April 2001 and November 2001 and six healthy volunteers (4 males, age, 61.0 ± 6.1 years) participated in the second study as control subjects. Blood samples (25 mL) were drawn from AMI patients and control subjects by the same technique. PBMCs were prepared as described above.

There were no significant differences in age and sex ratio between the AMI patients and control subjects in each study.

All AMI patients underwent coronary angiography (CAG) immediately after admission and subsequently received direct balloon angioplasty or intracoronary thrombolysis. As a result, target lesion revascularization was achieved in all patients. All subjects participated in this study after giving their informed consent. The investigation conformed with the principles outlined in the Declaration of Helsinki.

Immunofluorescence: Indirect staining was done as previously described. Briefly, PBMCs were incubated with mouse antihuman TRAIL monoclonal antibody (mAb) (DAKO Japan Co. Ltd., Kyoto, Japan), washed, and then stained with fluorescein-conjugated goat antirabbit IgG (Rockland, Gilbertsville, PA, USA).

Samples were analyzed on a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company Ltd., Tokyo) using CELL Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The fluorescence distribu-
tion histograms were plotted as the number of cells vs fluorescence intensity on a logarithmic scale.

Double-fluorescence staining was performed as follows: PBMCs were first incubated with rabbit antihuman TRAIL Ab. In a second step, the cells were sequentially incubated with RPE-conjugated mAbs (anti-CD4, anti-CD8, anti-CD14, and anti-CD19) and fluorescein-conjugated antirabbit IgG. Samples were analyzed on a double-color flow cytometer using CELL Quest software. The distribution of fluorescence was dot plotted as FITC-TRAIL vs RPE-conjugated mAbs.

**RT-PCR:** Total RNA was isolated from PBMCs with Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer’s instructions. RNA was reverse transcribed using an RNA PCR Kit (AMV, Takara Shuzo, Tokyo) for PCR with the supplied oligo d(T) primer using a thermal program of 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. PCR reactions were done with Taq polymerase (Takara Shuzo) using the following primers: human TRAIL (forward, 5'-GAGGATCCCATGGCTATTGAT GGAGTCCAG-3'; reverse, 5'-GGATTCGCCACATCTAGAGGCCCGAAA-3'); human G6PD (forward, 5'-CTGGCTATTTCACCACCCAC-3'; reverse, 5'-GGTTCAACCTACAGTTAG-3'), generating fragments of 866 bp, and 151 bp. PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide under ultraviolet light.

**Immunohistochemistry:** Paraffin sections of the left ventricle from five autopsy cases of myocardial infarction were used for immunohistochemical staining of TRAILR. Both intact and infarcted myocardium were examined. Immunohistochemical staining was achieved by the ABC method using a commercially available kit (DAKO Japan, Kyoto, Japan). Two primary Abs against the TRAILR-2 were used: a goat antihuman TRAILR-2 polyclonal Ab (Chemicon International, Temecula, CA, USA) and a goat antihuman TRAILR1 polyclonal Ab (R&D systems, Research & Diagnostic Systems, Minneapolis, MN, USA). The location of the antigen was visualized using the chromogen 3',3'-diaminobenzidine. After immunohistochemical staining, the sections were counterstained using hematoxylin.

**Statistical analysis:** Data are expressed as the mean ± SEM. The Mann-Whitney U test was performed to compare expression of TRAIL protein on PBMCs from AMI patients with that from control subjects, and to compare the expressions of TRAIL protein on CD4+, CD8+, CD14+, and CD19+ cells from AMI patients with that from control subjects. The Wilcoxon signed-rank test was used to compare the expression of TRAIL protein on PBMCs from patients in the acute phase and expression in the subacute phase of MI. P values less than 0.05 were considered statistically significant.
RESULTS

Up-regulation of TRAIL protein expression during the acute phase in patients with AMI: TRAIL protein on the surface of PBMCs from a patient with AMI and a control subject is shown in Figure 1. Flow cytometry showed that expression of

![Flow cytometry analysis of TRAIL expressed on PBMCs from a patient with AMI and a control subject.](image)

**Figure 1.** A: Flow cytometric analysis of TRAIL expressed on PBMCs from a patient with AMI and a control subject are shown. Expression of TRAIL protein was higher in the AMI patient with AMI than in the control subject. B: Expression of TRAIL protein on PBMCs was significantly higher in AMI patients than in control subjects. C: Detection of TRAIL mRNA expression by RT-PCR. TRAIL mRNA expression is higher in AMI patients than in control subjects. The RT-PCR products of glucose 6 phosphate dehydrogenase (G6PD) are shown as internal controls. **; $P < 0.01$
TRAIL protein on PBMCs was substantially augmented in the acute phase of AMI patients compared with that in control subjects (AMI: 30.1 ± 2.3%, P < 0.01 vs control: 17.1 ± 2.0%).

Expression of TRAIL protein on PBMCs was significantly higher in the acute phase than in the subacute phase of AMI (acute phase: 30.9 ± 4.8%, P < 0.05 vs subacute phase: 21.5 ± 7.4%) which was almost comparable to that of the control subjects (Figure 2).

**Up-regulation of TRAIL mRNA transcripts during acute phase in patients with MI:** RT-PCR was carried out to determine whether the up-regulated expression of TRAIL protein resulted from increased mRNA transcripts. The levels of TRAIL mRNA expression in relation to G6PD during the acute phase in the patients with AMI were higher than those of the control (Figure 1), suggesting that the expression of TRAIL is up-regulated at both the protein and mRNA levels.

**Population of cells positive for TRAIL expression:** Double immunostaining for TRAIL and cell type-specific markers was carried out and analyzed by flow cytometry to determine the cell population positive for TRAIL. A minor proportion of CD4+ T cells (12.8 ± 1.7%) and CD14+ cells (12.5 ± 1.4%) from the healthy controls expressed TRAIL, which was moderately enhanced in both cell types (CD4+: 19.4 ± 2.1%, CD14+: 20.4 ± 1.2%) from AMI patients. In contrast, expression of TRAIL on CD8+ T cells from AMI patients was slightly higher compared with that from control subjects (AMI: 16.3 ± 1.4%, P = 0.08 vs control:

![Figure 2](image_url). Expression of TRAIL protein on PBMCs was significantly higher in the acute phase than in the subacute phase of AMI. In the subacute phase, expression of TRAIL protein on PBMCs decreased relative to the expression level in control subjects. *; P < 0.05
10.1 ± 1.9%), although not statistically significant (Figure 3). CD19+ B cells from the control subjects did not express TRAIL protein (less than 3%), while the expression was unaltered in AMI patients.

Expression of TRAILR in myocardium: Immunohistochemical staining using two primary antibodies gave similar results. The cytoplasm of normal myocardium was diffusely positive for TRAILR-1 and TRAILR-2, while the interstitium was negative. In the infarcted myocardium, degenerated myocardial cells showed decreased expression of both TRAILR-1 and TRAILR-2, while necrotic myocardium was negative (Figure 4).

![Figure 3](image)

**Figure 3.** A: TRAIL is expressed on CD4+, CD8+, and CD14+ cells from AMI patients and control subjects. TRAIL is expressed on CD19+ cells with low frequency. B: Expression of TRAIL on CD4+ and CD14+ cells from AMI patients was significantly higher than that of control subjects. *: *P < 0.05
DISCUSSION

The expression of TRAIL and TRAILR on PBMCs and myocardium was examined since cytotoxic T lymphocytes activated after AMI were reported to kill healthy myocytes \textit{in vitro}.\textsuperscript{9} In this study, we showed that expression of TRAIL on PBMCs was substantially augmented in the acute phase of AMI compared with that in the control subjects using flow cytometry and RT-PCR. We achieved target-lesion revascularization in all AMI patients. Leukocytes have been shown to infiltrate into myocardium subjected to ischemia within 2 hours after coronary reperfusion.\textsuperscript{11} Macrophages which infiltrated into the infarcted tissue were frequently seen 2 days after reperfusion therapy.\textsuperscript{12} Expression of TRAIL was also significantly increased, even beyond the 2nd day after the onset of AMI (data not shown).

Furthermore, we demonstrated immunohistochemically TRAILR-1 and TRAILR-2 expression in human cardiomyocytes for the first time. It has been reported that TRAIL induced apoptotic cell death in rat cultured cardiomyocytes after simulated ischemia/reoxygenation.\textsuperscript{5} The data above suggest that TRAIL is related to the induction of human cardiomyocyte apoptosis as a DIL and that PBMCs play a role as mediators of TRAIL after AMI.

Although PBMCs from control subjects expressed TRAIL in this study and TRAIL was reported to be detected in fresh human PBMCs from a significant number of donors,\textsuperscript{13} cardiomyocyte apoptosis of the normal human heart is known to be uncommon.\textsuperscript{14} In an ischemia and reperfusion model, expression of intercellular adhesion molecule-1 (ICAM-1) was detected in cardiomyocytes after 6 hours of reperfusion,\textsuperscript{15} and ICAM-1 production on monocytes in AMI
patients was increased compared with that in control subjects. Furthermore, pretreatment with antibodies against cell adhesion molecules including ICAM-1 reduced not only the infiltration of leukocytes but also the area of infarction in reperfused rat hearts. The same phenomenon was also detected in CD18- or ICAM-1-deficient mice. These data suggest that ICAM-1 appears to play a critical role in apoptotic induction of cardiomyocytes after AMI and would seem to support the hypothesis of the above-mentioned cytotoxicity of PBMCs for cardiomyocytes after AMI and the rarity of cardiomyocyte apoptosis in the normal human heart. Alternatively, a negative regulator such as Fas-associated death domain-like interleukin 1β-converting enzyme inhibitory protein (FLIP) may be involved in the prevention of apoptotic cell death of myocardium. Further examination is needed to address these issues.

Concerning the population of cells positive for TRAIL, expression of TRAIL on CD4+ and CD14+ cells from AMI patients was significantly increased compared with that from control subjects. In contrast, expression of TRAIL on CD8+ cells from AMI patients was slightly higher than that from control subjects, although not statistically significant.

CD14+ cells (monocytes) and CD4+ cells may induce cardiomyocyte apoptosis after AMI as mediators of TRAIL. It is also possible that these TRAIL positive cells may play a role in the disappearance of infiltrated cells. Takemura, et al reported that apoptosis was found in infiltrated and proliferated interstitial cells (ie, endothelial cells, interstitial macrophages, myofibroblasts, and infiltrated neutrophils) after AMI. Apoptosis may play a role in the disappearance of infiltrated cells which have completed their role in the course of AMI. Gochuico, et al reported that cultured vascular smooth muscle cells and endothelial cells undergo cell lysis in response to the exogenous addition of TRAIL. Overexpression of TRAIL on PBMCs may induce apoptosis of not only cardiomyocytes and infiltrated cells but also vascular smooth muscle cells and endothelial cells that increase in the infarcted area in the course of AMI, and may be involved in the pathophysiology of AMI. Various antiapoptotic interventions expected to have cardioprotective effects in AMI have been tested experimentally. Yaoita, et al reported that a caspase inhibitor (ZVAD-fmk) is effective in reducing myocardial reperfusion injury, which could be partially attributed to the attenuation of cardiomyocyte apoptosis. However, there was a significant limitation to the use of ZVAD-fmk alone to greatly attenuate infarct size, because neutrophils which escaped apoptosis and acquire a prolonged survival may sustain free radical generating activities. Since a great reduction of the area of infarction induced by ischemia/reperfusion injury was reported to be detected in CD18- and ICAM-1-deficient mice, prevention of the interaction between activated PBMCs with myocardium might be effective as an antiapoptotic intervention after AMI.
It has been shown that apoptosis of cardiomyocytes occurs in the decompensated human heart,\textsuperscript{20,21} and circulating levels of TNF-\(\alpha\) in severe chronic heart failure are elevated.\textsuperscript{22} If TRAIL is involved in the apoptosis induction of cardiomyocytes in the decompensated heart like TNF-\(\alpha\), it will be difficult to discriminate in a strict sense whether the TRAIL upregulation on PBMCs is due to AMI itself or is secondary to cardiac failure after AMI.

Takeda, \textit{et al}\textsuperscript{23} reported that liver NK cells from either interferon \(\text{(INF)-}\gamma\)-deficient or INF-\(\gamma\)-receptor-deficient mice did not express TRAIL at all and administration of INF-\(\gamma\) into INF-\(\gamma\)-deficient mice rapidly induces TRAIL expression on liver NK cells. In this context, INF-\(\gamma\) is essential for expression of TRAIL. In addition, ischemic heart disease causes elevation of circulating INF-\(\gamma\)\textsuperscript{24} These data suggest that AMI at least enhances expression of TRAIL on PBMCs. Further investigation of the mechanism of enhanced expression of TRAIL on PBMCs is necessary.

Other than the Fas-Fas-ligand system,\textsuperscript{1,3-5} TNF-\(\alpha\) has also been examined as a DIL involved in cardiomyocyte apoptosis after AMI. Regarding TNF-\(\alpha\), circulating levels of TNF-\(\alpha\) are already known to be elevated in the acute phase of AMI.\textsuperscript{25,26} Tumor necrosis factor receptor (TNFR)-1 and TNFR-2 are found in human cardiomyocytes.\textsuperscript{27} Krown, \textit{et al}\textsuperscript{28} reported that the concentrations of TNF-\(\alpha\) capable of producing apoptosis in rat cardiomyocytes were within the range found in serum of patients with severe acute myocardial infarction. Consequently, we examined expression of TNF-\(\alpha\) on PBMCs employing the same protocol used in their study. Similarly to that observed with TRAIL, the expression of TNF-\(\alpha\) on PBMC was significantly higher in AMI patients than in control subjects, and the expression of TNF-\(\alpha\) was significantly higher in the acute phase than in the subacute phase of AMI (data not shown). PBMCs also play a role in cardiomyocyte apoptosis after AMI as a mediator of TNF-\(\alpha\).

Kayagaki, \textit{et al}\textsuperscript{29} reported that Fas-ligand is not expressed on peripheral blood T cells, because Fas-ligand could be expressed transiently on T cells upon stimulation but the cell surface expression of Fas-ligand was rapidly down-regulated by shedding mediated by metalloproteinases. Although we failed to detect the expression of Fas-ligand on PBMCs even in AMI patients (data not shown), a more careful method such as metalloproteinase inhibitor would be necessary for addressing these points.

In conclusion, the expression of TRAIL on PBMCs was enhanced in the acute phase of AMI. Expression of TRAILR-1 and TRAILR-2 in human cardiomyocytes was confirmed for the first time. TRAIL appears to be involved in the induction of cardiomyocyte apoptosis. PBMCs, mainly CD4\textsuperscript{+} and CD14\textsuperscript{+} cells, might play some roles as mediators of TRAIL after AMI.
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