Preliminary Clinical Measurement of the Expression of TNF-Related Apoptosis Inducing Ligand in Patients With Ankylosing Spondylitis

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It has recently been reported that tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) plays various roles in such autoimmune diseases as diabetes, multiple sclerosis (MS), and systemic lupus erythematosus (SLE). However, it has still remained unclear whether there is a close relationship between TRAIL and ankylosing spondylitis (AS). In this study, we investigated the association between the expression of TRAIL and AS. The specific messenger ribonucleic acid (mRNA) levels of TRAIL in peripheral blood mononuclear cells (PBMCs), serum sTRAIL, and TNF- α concentrations from 60 AS patients, 20 rheumatoid arthritis (RA) patients, and 30 healthy controls were determined by real-time fluorescent quantitative polymerase chain reaction (PCR) and enzyme-linked

immunosorbent assay (ELISA). The results indicated that the expression levels of TRAIL mRNA, and serum sTRAIL were significantly elevated in AS patients, compared with RA patients and healthy controls, and there was a close association between TRAIL mRNA and sTRAIL levels. However, there was no significant difference between human leukocyte antigen (HLA)-B27-positive and -negative AS patients. In HLA-B27-positive patients, TRAIL mRNA and sTRAIL closely correlated with serum TNF- α and C-reactive protein (CRP), but did not correlate in HLA-B27-negative patients. In conclusion, upregulated expression of TRAIL might be somewhat specific for evaluation of AS. *J. Clin. Lab. Anal.* 22:138–145, 2008. © 2008 Wiley-Liss, Inc.

Key words: ankylosing spondylitis; rheumatoid arthritis; TNF-related apoptosis inducing ligand; TNF- α

INTRODUCTION

Ankylosing spondylitis (AS) is a complex, potentially debilitating disease characterized by axial skeletal ankylosis, inflammation at the insertions of tendons, and occasionally, peripheral arthritis. AS was considered a relatively benign form of arthritis; however, it has recently been reported that the rates of pain and disability among AS patients were similar to those among rheumatoid arthritis (RA) patients (1). Furthermore, a great number of AS patients had severe inflammatory symptoms and almost no effective therapy was shown to slow the progression of axial disease. However, anti-tumor necrosis factor (TNF)- α treatment (e.g., with etanercept) is nowadays considered to be a rather effective treatment option for AS (2). Although the etiology and pathogenesis of AS is poorly understood, immune-mediated mechanisms involving human leukocyte antigen (HLA)-B27, inflammatory cellular

infiltrates, cytokines (e.g., TNF- α and interleukin-10 [IL-10]) are thought to play key roles (3).

For 10 years, there have been observations strongly implicating TNF- α in pathogenesis of AS and suggesting the therapeutic potential of TNF inhibitors in the chronic inflammatory disease. An important finding was that AS patients showed significantly higher TNF- α

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serum levels, compared with healthy controls and patients with noninflammatory back pain (4). More recently, increased levels of TNF- α messenger ribonucleic acid (mRNA) and protein have been detected in sacroiliac joint biopsy specimens from AS patients (5). In addition, it has been demonstrated that several TNF-inhibitors show the efficacy in the treatment of AS, such as thalidomide, infliximab, etanercept, and adalimumab (6–7). In recent years, many new members of the TNF superfamily have been identified, but none of these molecules has attracted more attention than TNF-related apoptosis inducing ligand (TRAIL) and its receptors (8). TRAIL, also termed Apo2 ligand, is a new member of the TNF superfamily, discovered by two independent groups, based on its sequence homology to TNF and Fas/CD95L (9,10). Like other members of the TNF superfamily, TRAIL is expressed as a type II membrane protein, including membrane-bound TRAIL (mTRAIL) and soluble TRAIL (sTRAIL), which triggers apoptosis through interaction with death receptors DR4 and DR5 (11,12). It has been known that TRAIL played important roles in so many autoimmune diseases as multiple sclerosis (MS), systemic lupus erythematosus (SLE), and RA (13–15). However, it has still remained unclear whether there is a close association between TRAIL and AS.

In a previous study, we found that the serum level of sTRAIL was elevated in AS patients (16). Here, we went on to detect the mRNA levels of TRAIL in peripheral blood mononuclear cells (PBMCs) from AS patients, RA patients, and healthy controls, to explore the role of TRAIL in the pathogenesis of AS.

MATERIALS AND METHODS

Subjects

This study was approved by the research ethics committee of our institution. After informed consent, the subjects were selected from the in-patient and out-patient clinic of Shanghai Changzheng Hospital, Shanghai, China. The study group consisted of 60 AS patients

(38 HLA-B27-positive: 28 men and 10 women; mean $\pm$ standard deviation (SD) = 30 ± 11 years; 22 HLA-B27-negative: 15 men and seven women; mean $\pm$ SD = 36 ± 13 years), who fulfilled the modified 1984 New York criteria for a diagnosis of AS (17), and were excluded if they had the spondylitis other than AS, clinical or radiographic evidence of complete spinal ankylosis, a history of recurrent infections or cancer, or a serious liver, renal, hematologic, or neurologic disorder. The disease control consisted of 20 patients with RA (11 men and nine female; mean $\pm$ SD = 48 ± 15 years of age), who fulfilled the American College of Rheumatology criteria for RA (18). A group of 30 healthy volunteers (21 men and nine women; mean $\pm$ SD = 33 ± 8 years of age), who had no AS history, AS family history, or other diseases served as controls.

PBMCs Preparation and RNA Extraction

PBMCs were isolated from peripheral venous blood of the subjects by Ficoll-Hypaque density gradient centrifugation. Total cellular RNA was extracted using the Trizol RNA extraction kit (Watson Biotechnologies, Inc., Shanghai, China) in accordance with the manufacturer's instructions. RNA yield and purity were determined spectrophotometrically at 260/280 nm.

Standard Plasmid Construction

For real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), primers and Taqman-MGB probes were designed and synthesized by Shanghai Genecore Biotechnologies (Shanghai, China). The sequences are shown in Table 1. RT-PCR was performed with SuperScript™ Platinum® two-step quantitative RT-PCR kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. PCR products were purified through gel extraction. Ligation of the pGM-T plasmid vector and purified PCR fragment was performed with T4 DNA ligase (Promega, CA). Plasmids were purified on columns with a Qiagen

TABLE 1. Primers and probe sequences of TRAIL and β -actin

Name	Sequence	Amplicon size (bp)
TRAIL		133
Sense-primer	5'-TCCTCAGAGAGTAGCAGCTCACA-3'	
Antisense-primer	5'-TGAATGCCCACTCCTTGATG-3'	
Probe	5'-FAM-TCTTCTCCAACTCCAAGAA-MGB-3'	
β -actin		142
Sense-primer	5'-CATTGCCGACAGGATGCA-3'	
Antisense-primer	5'-CATCTGCTGGAAGGTGGACAG-3'	
Probe	5'-FAM-TGAAGATCAAGATCATTGCT-MGB-3'	

kit (Qiagen, Hilden, Germany) and quantified by A260 measurement. To control the validity of our primers, the amplified products were sequenced. Recombinant plasmid pGM-T-TRAIL served as positive control and pGM-T- β -actin served as endogenous control.

Real-Time Quantitative RT-PCR

To prepare the standard curve, the recombinant plasmids were gradient diluted with sterilized water to 1×10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , and 10^3 copies/ μ L. We synthesized cDNA from the extracted total RNA by SuperScript Platinum two-step quantitative RT-PCR kit. Real-time quantitative PCR reactions were performed in an ABI-Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) under the following cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Each reaction mixture contained 4 μ L cDNA template, 25 μ L Platinum Quantitative PCR SuperMix-UDG, 1 μ L ROX, 1 μ L sense primer, 1 μ L antisense primer, 1 μ L Taqman-MGB probe, and 17 μ L sterilized water. The results were analyzed by Sequence Detection Software (Applied Biosystems).

Determination of Erythrocyte Sedimentation Rate, TNF- α , sTRAIL, and C-Reactive Protein

Serum samples were prepared immediately by centrifugation of peripheral venous blood. All cytokine levels were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocol. TNF- α and sTRAIL kits were purchased from Bender (Grez, Austria) and Diaclone (Besançon, France), respectively. Sensitivities of various assays were as follows: TNF- α , 8 pg/mL; sTRAIL, less than 64 pg/mL. Cytokine concentrations were determined by the optical densities obtained and the standard curves. The erythrocyte sedimentation rate (ESR) was determined by AUTO e.s.r Analyzer (Electa Lab, Forli, Italy) and the serum levels of C-reactive protein (CRP) were obtained by Specific Protein Analyzer (Dade Behring, Eschborn, Germany).

Statistical Analysis

Results were expressed as mean $\pm$ SD. Student's *t*-test was used to test for differences in means between groups. The correlation of two different parameters within one group was calculated with the Spearman rank correlation coefficient. *P* values less than 0.05 were considered significant.

Fig. 1. Agarose gel electrophoresis of TRAIL gene RT-PCR products. M for DL 2000 DNA Ladder marker, 1–3 for TRAIL gene RT-PCR products (133 bp).

RESULTS

Construction of Standard Plasmids

cDNA was synthesized by reverse transcription using total RNA extracted from PBMCs as template. Amplified PCR products were visualized on 1.5% agarose gels stained with ethidium bromide and photographed under ultraviolet (UV) light (Fig. 1). The constructed plasmids and the target fragment sequence were confirmed by PCR and sequencing (Fig. 2), as an indication that the plasmids had been successfully constructed.

Creation of Standard Curve

Plasmids were purified and quantified by A260 measurement, and then converted to the number of copies. To create standard curve with the plasmid DNA template, we diluted the constructed plasmids to 1×10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 and 10^3 copies/ μ L. By such a serial dilution, the TRAIL gene was quantified in the unknown samples with known amounts of input copy numbers, by which cycle threshold (Ct) values were plotted and the standard curve was generated (Fig. 3).

Increased Expression of TRAIL mRNA in PBMCs in AS Patients

The results indicated TRAIL mRNA copy numbers ranged from 2.7×10^3 to 3.7×10^5 copies/ μ L RNA in 60 AS patients; 1.6×10^2 to 7.3×10^3 copies/ μ L RNA in 20 RA patients; and 8.3×10 to 8.1×10^3 copies/ μ L RNA in 30 healthy controls. For each sample, copy numbers of TRAIL mRNA were divided by those of β -actin mRNA to normalize for TRAIL mRNA expression and thus avoid sample-to-sample differences in RNA quantity. Normalized TRAIL expression levels in patients

Fig. 2. Sequencing for TRAIL target fragment.

Fig. 3. Standard curve of TRAIL gene.

with HLA B27-positive (0.01 ± 0.008) and B27-negative AS (0.007 ± 0.006) and RA (0.0003 ± 0.0002) were all significantly increased compared with healthy controls (0.00002 ± 0.00001 ; $P < 0.001$ for each), and the patients with HLA B27-positive and -negative AS were higher than the RA patients, but there was no significant difference between these two AS groups. (Fig. 4).

Increased Levels of sTRAIL, TNF- α , CRP, and ESR in AS Patients

As indicated in our previous study, the serum concentrations of sTRAIL and TNF- α were signifi-

cantly elevated in AS patients compared to healthy controls ($1,514 \pm 262$ pg/mL vs. 937 ± 240 pg/mL, $P < 0.001$; 231 ± 194 pg/mL vs. 15 ± 3 pg/mL, $P < 0.001$, respectively, for sTRAIL and TNF- α), while in RA patients, only the serum concentration of TNF- α was significantly upregulated (148 ± 71 pg/mL) and no significant difference for sTRAIL ($1,067 \pm 281$ pg/mL) was observed, compared with healthy controls. In AS patients, HLA-B27-positive patients showed higher TNF- α concentration than HLA-B27-negative ones (291 ± 223 pg/mL vs. 129 ± 51 pg/mL; $P < 0.001$). However, there was no significant difference for sTRAIL between HLA-B27-positive and -negative patients

Fig. 4. Normalized TRAIL mRNA levels in PBMCs. ** and ΔΔ for $P < 0.001$, respectively, compared with Control and RA.

TABLE 2. The levels of ESR, CRP, and cytokines in populations

	Healthy controls (n = 30)	Disease controls (n = 20)	HLA-B27-negative patients (n = 22)	HLA-B27-positive patients (n = 38)
TNF- α (pg/mL)	15 $\pm$ 3	148 $\pm$ 71**	129 $\pm$ 51**	291 $\pm$ 223**
sTRAIL (pg/mL)	937 $\pm$ 240	1067 $\pm$ 281	1474 $\pm$ 329**	1538 $\pm$ 215**
ESR (mm/hr)	7 $\pm$ 4	42 $\pm$ 19**	31 $\pm$ 18**	46 $\pm$ 19**
CRP (mg/L)	5 $\pm$ 3	37 $\pm$ 26**	6 $\pm$ 3	46 $\pm$ 14**

** $P < 0.01$ vs. healthy controls.

(Table 2). Among HLA-B27-positive patients, there were significant correlations between TNF- α and sTRAIL. However, the above correlations observed in HLA-B27-positive patients were not observed in HLA-B27-negative ones. HLA-B27-positive patients showed significantly elevated ESR, compared with HLA-B27-negative patients (46 ± 19 vs. 31 ± 18 mm/hr, $P = 0.003$), and both were significantly higher than healthy controls. There was higher level of CRP in HLA-B27-positive patients than in HLA-B27-negative patients and controls. However, no significant difference was observed between the last two groups (Table 2). As disease control, RA patients showed higher levels of ESR and CRP than healthy controls.

Relationship Between TRAIL mRNA Expression and ESR or Serum sTRAIL, TNF- α , and CRP

When correlation studies were performed, close relationships between the expression of TRAIL gene in PBMCs and serum sTRAIL were found from all the populations (Fig. 5). Similar to sTRAIL, TRAIL mRNA closely correlated with serum TNF- α and CRP in HLA-B27-positive AS patients, but did not in HLA-B27-negative patients. In RA patients, no significant

association of both TRAIL mRNA and sTRAIL was observed with ESR, TNF- α , and CRP (Table 3). However, serum TNF- α showed close associations with ESR ($r = 0.536$, $P = 0.015$) and CRP ($r = 0.562$, $P = 0.01$) in RA patients. Interestingly, no close correlation was found between ESR and TRAIL mRNA or sTRAIL in all the patients, and the reason remained to be explained.

DISCUSSION

The death ligand TRAIL primarily aroused tremendous interests due to its ostensible selectivity in killing tumor cells, but recently, there has been a growing understanding of the physiological functions of endogenous TRAIL in immunity, and several studies have demonstrated the role of TRAIL in autoimmune diseases appears varied and controversial. Some studies showed that TRAIL inhibited autoimmune arthritis (19), diabetes induced by cyclophosphamide and streptozotocin (20), and experimental autoimmune encephalomyelitis (EAE) (21) in animal models. In contrast, some emerging findings showed that TRAIL might promote autoimmune diseases. Aktas et al. (22) provided evidence for a detrimental role of TRAIL in

Fig. 5. Associations between TRAIL mRNA and sTRAIL in AS patients, RA patients, and healthy controls. (a) HLA-B27-positive AS patients; (b) HLA-B27-negative AS patients; (c) RA patients; and (d) healthy controls.

TABLE 3. The correlations of TRAIL mRNA and sTRAIL with ESR, TNF- α , and CRP

	HLA-B27-positive patients				HLA-B27-negative patients				RA patients			
	sTRAIL		TRAIL mRNA		sTRAIL		TRAIL mRNA		sTRAIL		TRAIL mRNA	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
TNF- α	0.497	0.002	0.355	0.029	-0.136	0.548	-0.188	0.402	0.551	0.012	0.288	0.218
CRP	0.609	0.000	0.389	0.01	0.287	0.195	0.197	0.38	-0.03	0.9	0.295	0.207
ESR	0.299	0.068	0.08	0.631	0.093	0.68	0.164	0.466	0.362	0.116	0.358	0.121

neural death in T-cell-induced EAE. The dual role of TRAIL has also been proposed in RA, where TRAIL was shown to play a role in stimulating proliferation or apoptosis of fibroblast-like synoviocytes (23). Although numerous studies have discussed the role of TRAIL in autoimmune diseases in experimental animal models, less is known about its role in humans, especially in the AS population. In the present study, the levels of TRAIL mRNA in PBMCs and sTRAIL in serum were determined by real-time PCR and ELISA, respectively, and significant elevation was found in both HLA-B27-positive and -negative patients with AS, compared with

healthy individuals and RA patients. Although normalized TRAIL mRNA levels showed statistical correlations with serum sTRAIL levels, surprisingly, magnitude differences in normalized TRAIL mRNA gave only rise to minimal changes in sTRAIL levels in present study. We presumed one reason might be that TRAIL protein includes membrane-bound TRAIL and sTRAIL, both of which are regulated by TRAIL gene. Additionally, many factors may alter the expression of sTRAIL levels. Although it was reported that TRAIL levels that exceed 10 ng/mL can exert an apoptotic response in vitro (24), it might be more complex and

quite different *in vivo*. The detailed biological function of elevated sTRAIL levels *in vivo* remains uncertain. AS shows a strong association with HLA-B27, but the advantages and disadvantages of HLA-B27 as a diagnostic test for early AS has been a subject of longstanding debate that needs to be clarified (25). The current study indicates that increased expression of TRAIL may be associated with pathogenesis of AS, independent of HLA-B27. However, the close correlations between the mRNA level of TRAIL or sTRAIL and CRP are clearly influenced by HLA-B27. HLA-B27-positive AS patients showed close correlation, but HLA-B27-negative patients did not. It can also be explained by a lower disease activity in the group of HLA-B27-negative patients reported by another study (26).

Unlike serum TNF- α , which was significantly elevated in both AS patients and RA patients, sTRAIL showed significantly higher level only in AS patients, compared with RA patients and healthy controls. The mRNA levels of TRAIL were increased in both AS patients and RA patients, but far higher in AS patients than in RA patients. Furthermore, TNF- α closely correlated with ESR and CRP in RA patients, while TRAIL did not. These results demonstrated that as far as TRAIL was concerned, there might be a difference in the mechanisms between AS and RA, and TRAIL might be more specific for AS than TNF- α . In addition, if the study is confirmed in a larger sample size, TRAIL expression can be further exploited clinically, for instance to predict a clinical response of AS to TNF- α blocking agents, just like TRAIL, as a candidate for pretreatment assessment and prognostic marker of treatment response to interferon beta in MS (27).

To summarize, it has been indicated, in our study, that TRAIL might be associated with the pathogenesis of AS. Furthermore, the relationship between TRAIL and the progression of AS might depend on HLA-B27 status. However, the molecular mechanism driving TRAIL overexpression and the detailed roles of TRAIL and HLA-B27 in the pathogenesis of AS remain to be clarified.

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