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Increased Expression of TRAIL and Its Receptors on Peripheral T-Cells in Type 1 Diabetic Patients

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Increased Expression of TRAIL and its Receptors on Peripheral T-cells in Type 1 Diabetic Patients

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ABSTRACT

Background: Type-I diabetes is an autoimmune inflammatory disease in which pancreatic ß-cells are selectively destroyed by infiltrating cells. TNF-related apoptosis-inducing ligand (TRAIL) is a type-II membrane protein of the TNF superfamily which is expressed in different tissues, including pancreas and lymphocytes. In humans, TRAIL interacts with four membrane receptors. TRAIL-R1 and TRAIL-R2 have cytoplasmic death domains, and can activate both caspases and NFκB pathways. The other two receptors, TRAIL-R3 and TRAIL-R4, are decoy receptors not capable of activating caspase cascade but may activate NF-κB and block apoptosis. As human beta cells are sensitive to TRAIL induced apoptosis, signaling via these molecules is considered to be a probable way of beta cell destruction. These molecules also are important in suppression of autorective T cells and immunoregulation. **Objective:** To explore the importance of TRAIL and its receptors at pathogenesis of type-I diabetes, we compared expression of these molecules on T-cells of diabetic patients and healthy controls. **Methods:** In this study, expression of TRAIL and its receptors at protein and mRNA levels were studied in freshly isolated peripheral T cells of 55 type I diabetic patients and 50 healthy individuals by flowcytometry, western blot and RT-PCR. **Results:** We found that expression of TRAIL and its receptors in peripheral T-cells at both protein and mRNA levels are significantly increased in patients (except for TRAIL-R2 mRNA which was slightly higher in controls) but increase in TRAIL, TRAIL-R3 (2.7% vs. >0.5%) and TRAIL-R4 (2.6% vs. >0.5%) is more considerable. sTRAIL in sera of patients was significantly lower than in controls (p=0.01). **Conclusion:** Our results explain resistance of autoreactive T-cells to immunoregulatory mechanisms. Besides, increased expression of TRAIL in autoreactive T-cells may play an important role in beta-cell destruction. Lower level of sTRAIL in diabetic patients may be a reason for hyperactivation of autoreactive T-cells.

Keywords: Type I diabetes; TRAIL; TRAIL receptors; T-cell

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INTRODUCTION

Type 1 diabetes (T1D) is generally considered to be an autoimmune disease resulting from damage to insulin- producing pancreatic islet cells initiated by activated CD4+ and CD8+ T cells and macrophages infiltrating the islets and is mediated through the production of soluble proinflamtory cytokines such as IFN-g, IL-1β and/or cell surface death inducing ligands such as Fas ligand, TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), lymphotoxin (LT)α1β2, LTα2β1 and LIGHT(1-3). The disease accounts for about 10% of all cases of diabetes (2).

TRAIL or APO-2 ligand, is a type 2 membrane protein of the TNF superfamily(4). Although TRAIL induces apoptosis of tumor cells, but most normal cells , with the exception of hepatocytes, neural cells, and thymocytes, are resistant to TRAIL-induced apoptosis. In humans, TRAIL interacts with at least four membrane receptors that all belong to the TNF receptor superfamily. TRAIL receptor 1 (TRAILR1 or death receptor 4) and TRAIL receptor 2 (TRAILR2, death receptor 5) have cytoplasmic death domains, and can activate both caspases and nuclear factor (NF)-κB pathways. The other two receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), are not capable of activating caspases and induce apoptosis but may activate NF-κB and act as decoy receptors (5). In addition, osteoprotegerin is a soluble low affinity receptor for TRAIL, which besides its known physiologic role in bone density regulation, sequesters the ligand from the signaling death receptors. In contrast to these receptors in humans, only one membrane TRAIL receptor has been identified in mice, which shares the highest homology with human death receptor (DR5). The mouse decoys receptors share low homology with those of humans and do not have intracellular domains. Although expression of TRAIL and its receptors have been studied and seen in a variety of mouse and human tissues, their roles in health and disease are largely unknown.

Similar to FasL, TRAIL participates in the direct tissue destruction observed in most autoimmune diseases, but in addition, it regulates proliferation and activation of autoreactive T-cells (6), and negative selection of thymocytes (7), Thus, dependent on time, dose and location of TRAIL expression and engagement of special receptors, it may exert a beneficial as well as a destructive potential (8). It has been shown that most human primary Beta cells express TRAIL and its receptors. While TRAIL induces strong cytotoxicity and apoptosis to beta-cell lines, normal pancreatic beta-cells escape from apoptosis by unidentified inhibitors (9). It has been shown that systemic TRAIL blockade exacerbates autoimmune diabetes in mice models and TRAIL deficient mice are more prone to induction of autoimmune diabetes (10, 11). In this study, to clarify the role of TRAIL – TRAIL-R system in pathogenesis of type 1 diabetes, we have measured protein and mRNA expression of TRAIL and its 4 receptors on T lymphocytes derived from type I diabetic patients and healthy controls.

MATERIALS AND METHODS

Subjects. 55 patients with diabetes type 1 from the Iranian Society of Diabetes whose diagnosis had been made by an endocrinologist and had been under insulin therapy were collected. 50 Control subjects had been chosen from the healthy volunteers who had been matched for sex and age with the case group. 5 ml venal blood sample was taken

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from each subject mixed with EDTA, and 2 ml of whole blood was also collected in separate tube for serum preparation.

Antibodies. and reagents for their detection were as follows: mouse anti-human TRAIL (MAB687 R&D systems USA), mouse anti-human TRAIL-R1 (MAB347 R&D systems USA), mouse anti-human TRAIL-R2 (MAB6311 R&D systems USA), mouse anti-human TRAIL-R3 (MAB6302 R&D systems USA), Mouse anti-human TRAIL-R4 (MAB633 R&D systems USA), biotinylated anti-mouse IgG(R&D BAF007), streptavidin-phycoerythrin(R&DF0040), streptavidin-HRP detection solution (R&D4800-30-06).

Isolation of T-cells. PBMC were isolated from anticoagulated blood of patients and healthy donors by Ficoll density gradient (Lympholyte-H Cederlane, Canada, Catalogue# CL5020). PBMCs were washed and CD3+ T cells purified using enhanced human T cell recovery column kit following manufacturer's instructions (Cederlane, Canada, Catalogue# CL100). The purity of the isolated subsets was >95% as determined by flow cytometry.

RNA Extraction and RT-PCR. Isolation of RNA was performed using a total RNA purification kit (RNX-PLUS cinagen RN7713C). DNAse-digestion (Boehringer Mannheim, Mannheim, Germany) was carried out to remove possibly contaminating genomic DNA. From 1 μg of RNA, cDNA was synthesized using a RevertAid^{TM} first strand cDNA synthesis kit (Fermentas #K1622). The PCR conditions were as follows:

	Forward primer	Reverse primer	PCR condition
TRAIL	5'-AGACCTGCGTGCTGATCGTG-3'	5'-TTATTTTGCGGCCCAGAGCC-3'	29 cycles, 45 s/95°C, 45 s/60°C, 60 s/72°C,
TRAIL-R1	5'-CGATGTGGTCAGAGCTGGTACAGC-3'	5'-GGACACGGCAGAGCCTGTGCCATC-3'	29 cycles, 45 s/95°C, 45 s/60°C, 60 s/72°C
TRAIL-R2	5'-GGGAGCCGCTCATGAGGAAGTTGG-3'	5'-GGCAAGTCTCTCTCCCAGCGTCTC-3'	28 cycles, 45 s/95°C, 45 s/608C, 60 s/728C
TRAIL-R3	5'-GTTTGTTTGAAAGACTTCACTGTG-3'	5'-GCAGGCGTTTCTGTCTGTGGGAAC-3'	33 cycles, 45 s/95°C, 45 s/72°C, 45 s/72°C
TRAIL-R4	5'-CTTCAGGAAACCAGAGCTTCCCTC-3'	5'-TTCTCCCGTTTGCTTATCACACGC-3'	32 cycles, 45 s/95°C, 45 s/64°C, 60 s/72°C
β-Actin	5'-GTGGGGCGCCCCAGGCACCA-3'	5'-CTCCTTAATGTCACGCACGATTTC-3'	26 cycles, 45 s/95°C, 45 s/54°C, 60 s/72°C

A cDNA fragment of the housekeeping gene β-Actin was amplified as reference for the relative expression of the other genes. The PCR fragments were separated in 2% agarose gels and visualized by ethidium bromide (12). To quantify the expression of the TRAIL receptors, optical density (OD) was determined using ImageQuant™ TL software (Amersham, Biosciences). Relative Expressions are presented as an arbitrary value of OD index, calculated as ratio of the integrated OD of the TRAIL and its receptors in patients to to the appropriate OD of healthy controls.

Flowcytometry Analysis. To detect expression of TRAIL and TRAIL receptors on T-cells, 100000 cells were incubated with specific antibodies or isotype control in separate tubes for 30 minutes. Cells were washed with PBS, 3 times, and then incubated with biotinylated anti-mouse IgG for 30 minutes. Washing step was repeated and cells were subsequently incubated with streptavidin-phycoerythrin for 30 minutes at 4°C. Cell analysis was performed with a FACScalibur- flow cytometer (Becton Dickinson and Co., Mountain View, CA, USA) equipped with CELLQuest software.

ELISA for Detection of sTRAIL. After centrifugation of whole blood at 2000g, sera were taken and stored at -70°C. Levels of sTRAIL were determined in duplicate by a commercially available sandwich ELISA with a detection limit of 2.86 pg/mL, according to the manufacturer's instructions (Quantikine_Human TRAIL/TNFSF10 Immunoassay Catalog Number DTRL00, R & D Systems, Minneapolis, Minn., USA). Briefly, samples were incubated in plates precoated with a murine monoclonal anti-TRAIL antibody. After washing step, HRP-conjugated polyclonal anti-TRAIL was added to bind sTRAIL captured by the first antibody. Substrate solutions reactive with HRP were added to the wells. The color reaction was terminated by adding 2 N sulfuric acid, and absorbance was measured at 450 nm. A standard curve was prepared using dilutions of an sTRAIL standard to determine sTRAIL concentrations.

Western Blotting Analysis. To determine the amount of TRAIL and its receptors in T-cells of type 1 diabetic patients and healthy volunteers, 5×10^6 T-cells were lysed on ice for 20 minutes in 1 milliliter of lysis buffer containing 50 mM HEPES (N-2-hydroxyethylpiperazine- N-2-ethanesulfonic acid), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a cocktail of protease inhibitors (Roche). The lysates were spun, and the 20μL supernatants were collected and the same volume (20μL) of double-strength sample buffer (20% glycerol, 6% sodium dodecyl sulfate (SDS), 10% 2-Mercaptoethanol) was added. The samples were boiled for 10 minutes. Proteins were analyzed on 12% polyacrylamide gels by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred electrophoretically to Immobilon-FL Membrane (IPFL 000 10) after methanol activation of membranes at 15V for 30 minutes by using a semidry system(BioRad). The membranes were incubated in 5% defatted dry milk in cold room overnight and then for 60 minutes with specific antibody or mouse anti–human actin monoclonal antibody, followe by washing with PBS-Tween20, 3×5mins and by a biotinylated anti-mouse IgG (R&D BAF007) for 60 minutes. Membranes were incubated in streptavidin-HRP detection solution (R&D 4800-30-06). Peroxidase activity was visualized by the enhanced chemiluminescence detection system (Amersham RPN2124). To quantify the level of proteins, bands were quantified based on the optical density of bands using ImageQuant™ TL software (Amersham, Biosciences).

This work was approved in ethical committee of Tehran University of Medical Sciences and informed consents were taken from all patients and healthy donors. All data were collected and analyzed by SPSS 11.5 software. A P-value of <0.05 was regarded as significant.

RESULTS

Demographic information of study groups is shown in table 1. Hb A1c level in patients showed that they were all well managed and the disease was under control with insulin therapy. Patients with diabetic complications were excluded. Control group was matched for age and sex ratios with patients. The flowcytometry results (Figure 1) showed that TRAIL and the level of its receptors were higher in membranes of T lymphocytes in type 1 diabetic patients compared to healthy controls p<0.05), Albeit this increase was more considerable for TRAIL-R3 and TRAIL-R4 but it was slight for membrane-bound TRAIL expression. We could not observe a significant correlation between cell surface expression of studied markers with the HbA1c level or the age of onset of the disease. The related results are shown in Table 2. Serum level of sTRAIL was decreased significantly (p=0.01) in patients in comparison with healthy controls

as shown in figure 2. Western blot analysis shows that expression of TRAIL and its 4 receptors are elevated in T–cell lysates of patients when compared to control group (Figure 3). Data were quantified and normalized according to beta-actin level. We could demonstrate a good positive correlation between westernblot and flowcytometry results (r=0.85, p>000). RT-PCR experiments showing an increased expression of mRNA confirmed our flowcytometry and western blot results, with exception for TRAIL-R2. As shown in Figure 4 mRNA expression of TRAIL-R2 was slightly higher in healthy controls in contrast to the amount of protein checked by flowcytometry and western blot.

Table 1. Demographic data and clinical information of study population

	Type 1 Diabetic Patients	Normal Control
Number	55	50
Male/ Female	25/30	24/26
Median age (range)	17(3-52)	18(5-54)
Disease onset age	8.8±7.3	-
Disease duration	9.0±7.6	-
Hb A1c	7.0±1.6	-

Table 2. Differences in membrane TRAIL and its receptors determined by flowcytometry on peripheral T-cells. Values denote percentage of related surface protein expressing T-cells

	Type 1 Diabetic Patients	Healthy Controls	P-value
TRAIL	2.5±1.4	1.62±1.7	0.035
TRAIL-R1	2.8±1.6	1.2±0.8	0.000
TRAIL-R2	3.2±1.6	1.3±0.6	0.000
TRAIL-R3	2.7±2.0	<0.5	0.000
TRAIL-R4	2.6±1.9	<0.5	0.000

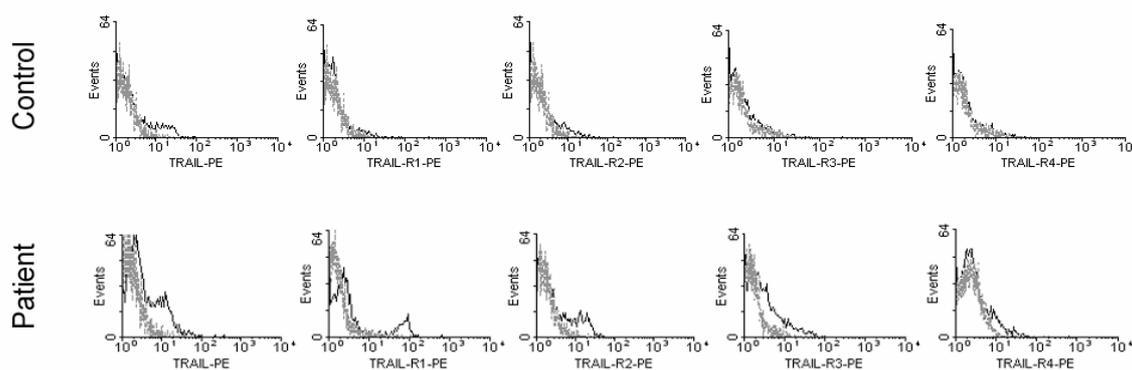*P-value calculated from independent-sample t test

Figure 1. Flowcytometry analysis of membrane bound TRAIL & its receptors on peripheral T-Cells of diabetic patients and healthy controls revealing upregulation of all 5 proteins in type1 diabetic patients.
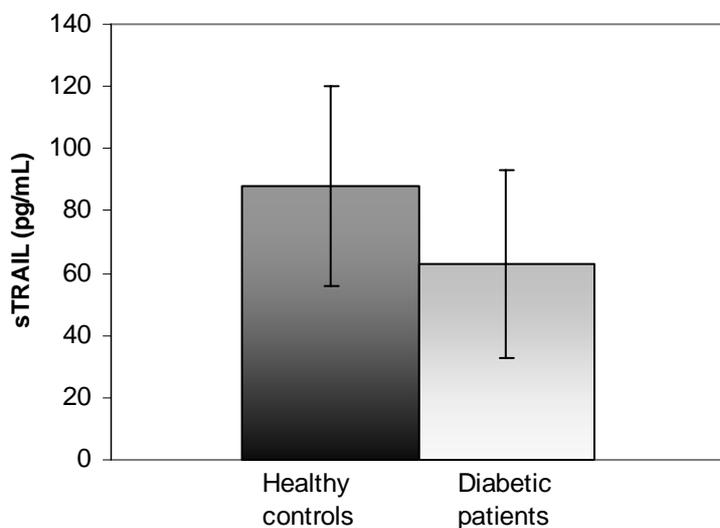
Figure 2. sTRAIL in sera measured by ELISA in diabetic patients and healthy controls.
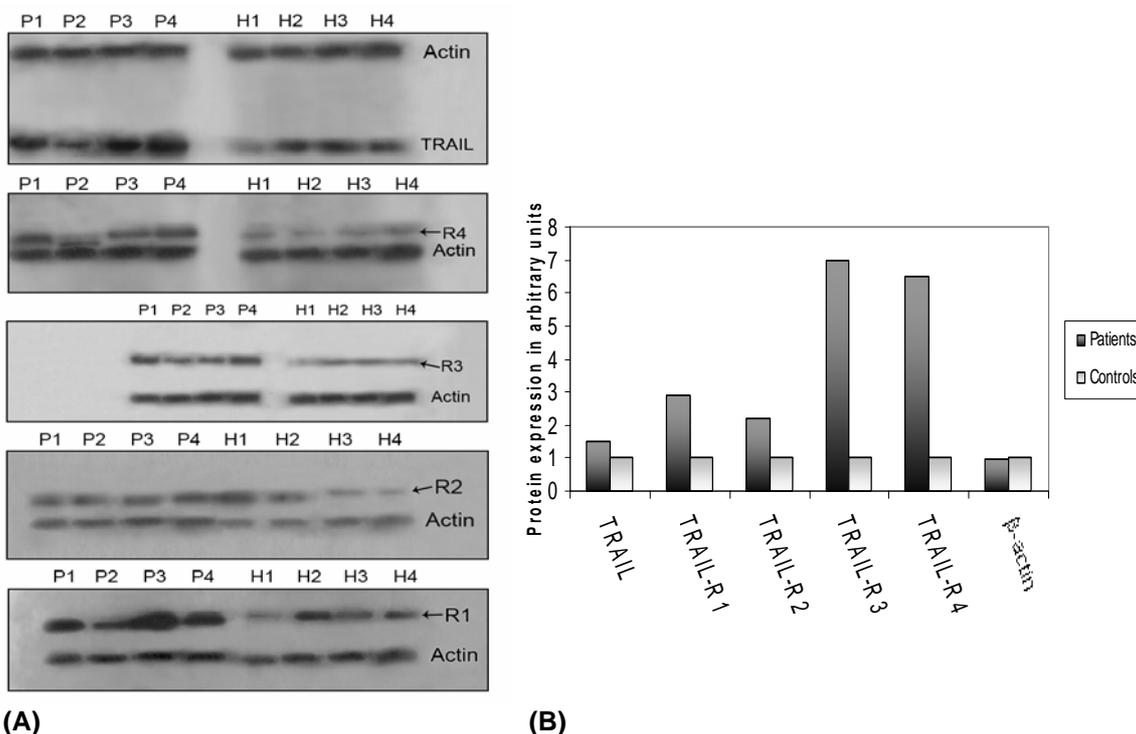

(A)

(B)

Figure 3. Protein levels of TRAIL & its receptors in T-Cell lysates of diabetic patients (P1-4 are results for four individual patients) and healthy controls (H1-4 are results for four individual controls) **(A)**. Equivalent loading was confirmed by using actin as an internal standard. Normalized protein expression ratio in type I diabetic patients and healthy controls **(B)**.
*Values in arbitrary unit are the ratios of mean density of related bands in patients to those of healthy controls. Differences for TRAIL-R1, TRAIL-R2, TRAIL-R3 & TRAIL-R4 were significant (p<0.05) but not significant for TRAIL (p=0.068).

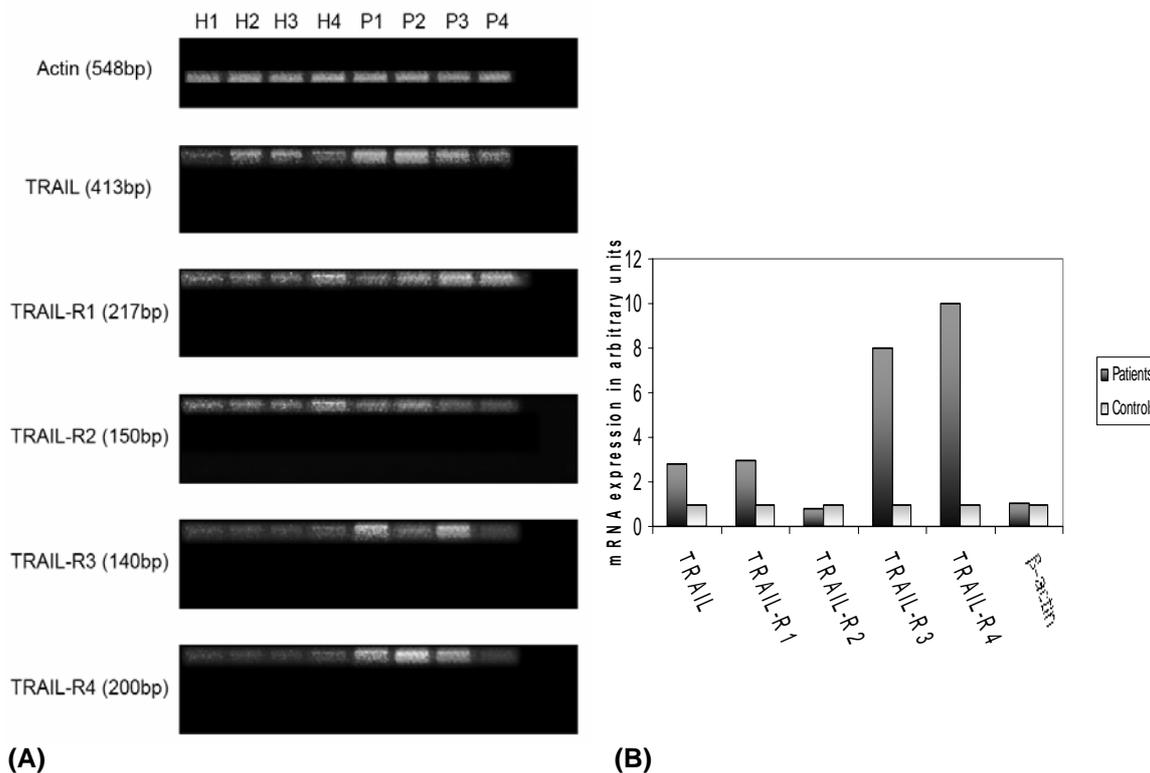Iran.J.Immunol. VOL.4 NO.4 December 2007

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Figure 4. mRNA Expression of TRAIL & its receptors in T-Cells of diabetic patients(P1-4 are results for four individual patients) and healthy controls (H1-4 are results for four individual controls) **(A)**. Normalized protein expression ratio in type I diabetic patients and healthy controls **(B)**
*Values in arbitrary unit are the ratios of mean density of related bands in patients to those of healthy controls. Differences for TRAIL, TRAIL-R1, TRAIL-R3 & TRAIL-R4 were significant (p <0.05) but not significant for TRAIL-R2 (P=0.15).

DISCUSSION

Although the molecular mechanism of TRAIL-induced apoptosis in tumor cells has been elaborated, the role of TRAIL in health and disease is largely unknown.(13). In this study, we demonstrated increased mRNA and protein expression of TRAIL and its receptors in peripheral T cells from type I diabetic patients. Because of complexity of TRAIL-TRAIL receptor system, our results may imply some functional consequences. First, as human primary islet cells and beta-cell lines constitutively express TRAIL receptors and TRAIL mediated apoptosis is functional in β-cell destruction(9), increased TRAIL expression on autoreactive T-cells may be responsible for apoptosis induction. This conclusion is consistent with Sze-shuen's study (1) which shows that TRAIL[+] activated T-cells are present in islet infiltrating cells. Such cytototoxic role for TRAIL expressing T-cells against different cell types has been shown by Nobuhiko Kawasaki (14) and Sato et al (15). Secondly, emerging data propose that TRAIL- TRAIL-R system is important in immune surveillance, tolerance (16) and immune regulation and suitable combination of TRAIL death and decoy receptors on T-cells keep them under tight control(8, 17). We showed that decoy/death receptor ratio is significantly increased in T-cells of diabetic patients probably leading to hyper-activation of T-cells and consequently uprising of auto-reactive T-cells which will act against susceptible self tissues.

On the other hand it has been shown that TRAIL engagement can transduce reverse signals and activate target cells (17), so increased expression of TRAIL on T-cells of diabetic patients may lead to over-activity of T-cells. Two interesting studies in mice propose critical role for TRAIL in autoimmune diabetes (10, 11). These two studies show that systemic TRAIL blockade as well as knocking out TRAIL gene will increase both incidence and severity of autoimmune diabetes in genetically prone mice. Their results support our explanation that increased expression of Dc-R1 and Dc-R2 on T-cells from diabetic patients hinders these cells from inhibitory effects of TRAIL. We also showed that sTRAIL is significantly decreased in serum samples of type I diabetic patients. As cleavage of surface TRAIL from T-cells is considered to be one of the main sources of serum TRAIL (18), this decrease may be the result of more stabilized surface TRAIL. Decrease in circulatory soluble TRAIL probably leads to autoreactive T-cells uprising. Several studies have shown that sTRAIL causes cell cycle arrest and blocks cytokine production of autoreactive T cells (19) and in this regard is a prototype of autoimmune inflammation inhibitors (20). A recent study, has demonstrated that insulin significantly down-regulated the expression of TRAIL in some cell types of both diabetic and non-diabetic rats as well as in cultured rat and human VSMC.(21) Regarding this, decrease in sTRAIL could be an effect of insulin insufficiency instead of a cause in pathogenesis of autoimmunity. The importance of TRAIL in pathogenesis of different autoimmune diseases was studied earlier(13, 22-25) and all emphasized the immunoregulatory and immunomodulatory effects of TRAIL-TRAIL-R system, but to determine the actual role of TRAIL in pathogenesis of autoimmune diabetes, we need to study expression and function of this system in the immune system and in primary beta cells simultaneously. We found that in contrast to surface and cell lysate protein level, mRNA expression of TRAIL-R2 was lower in diabetic patients. This may imply the presence of regulatory mechanisms which stabilize TRAIL-R2 mRNA in T-cells and should be clarified by more studies. In contrast to our findings, Kayagaki and colleagues could not find a detectable level of surface TRAIL on freshly isolated PBT cells and T-cells stimulated with PHA or IL-2 (26) which may be because of using insensitive antibodies, but many others detected this marker on different cell types comparable to our results(1, 14, 23, 27). In conclusion, although this research showed some immunopathologic changes in peripheral T-cells which may be the cause of autoimmune diabetes, but further studies (especially functional studies on human tissues) are needed to better understand the role of TRAIL and its receptors in the pathophysiology of type 1 diabetes.

ACKNOWLEDGMENT

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