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Importance of TNF-related apoptosis-inducing ligand in pathogenesis of interstitial cystitis

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Abstract
Introduction Although interstitial cystitis is an inflammatory disease, its etiopathogenesis is not clearly understood. The objective of the present study is to investigate the distribution of TNF-related apoptosis-inducing ligand (TRAIL) and its receptors in bladder biopsy samples of patients diagnosed with interstitial cystitis and the role of TRAIL in the pathogenesis of interstitial cystitis.
Materials and methods TRAIL and its receptors were stained immunohistochemically in bladder biopsy samples of 27 patients diagnosed with interstitial cystitis, and the samples were evaluated independently by two pathologists and were scored in terms of expression intensity and distribution.
Results An evaluation of the results of the statistical analysis showed that the TRAIL-R4 receptor was immunohistochemically stained with a higher score than TRAIL-R1, TRAIL-R2, TRAIL-R3 receptors and TRAIL, with a statistically significant difference ($P < 0.05$).
Conclusion These findings indicate that TRAIL-R4 is the predominant receptor in the interstitial cystitis inflammation.

Keywords Interstitial cystitis · TRAIL · Apoptosis · Pathogenesis

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Introduction

Interstitial cystitis (IC) is an inflammatory condition of the bladder characterized by frequent and urgent urination, pain in the pelvis and/or incontinency. Because there is no established clinical definition of the disease, the criteria set by the National Institute of Arthritis Diabetes Digestive and Kidney Diseases (NIDDK) are used for diagnosis [1]. Although IC seems to be a relatively minor problem considering the number of individuals affected by the condition in the past, recent studies have reported the prevalence of IC as 67-450/100000 [2–5]. The negative effect of the condition on the quality of life results in labor loss, depression and anxiety [6].

Although IC is an inflammatory disease, its etiopathogenesis is not clearly understood. Current

theories on the pathogenesis of IC include mucosal permeability, increased mast cell activation and neuroimmune mechanisms [7–21]. Recent investigations have reported that the inflammatory and/or immunologic responses in IC may be a result of NF-κB (nuclear factor-{kappa}B) activation and that NF-κB can be reduced with treatment [22–24]. Although there are several mediators that may increase the levels of NF-κB in IC, the death ligand TRAIL (TNF-related apoptosis-inducing ligand) family, a recently discovered member of TNF (tumor necrosis factor) and evidences that TRAIL may be a further factor that stimulates NF-κB suggest that this ligand may also be involved in IC [25–27].

TRAIL is a type II transmembrane protein with a molecular weight of 40 kDa, which is known to stimulate the apoptosis pathway in transformed cells and the NF-κB signal pathway in the presence of autoimmune and inflammatory responses [25]. Currently, five TRAIL receptors have been identified, which are TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 receptors. The fifth TRAIL receptor osteoprotegerin (OPG, TRAIL-R5), as a decoy receptor, is considered to protect bone cells from the apoptosis induced by TRAIL [28].

The present study aims to investigate the distribution of TRAIL and its receptors in bladder biopsy samples of patients diagnosed with IC and the role of TRAIL in the pathogenesis of IC.

Materials and methods

A total of 27 patients diagnosed as having an IC based on the NIDDK criteria between 1997 and 2006 have been retrospectively included in the study. Following evaluations of patients through detailed medical history analysis, physical examination and voiding diary, each patient underwent urinalysis, urine culture, urine cytology and urinary tract ultrasonography analyses. None of the patients had intravesical treatment history due to urinary infection and IC. No intravesical malignant lesions were detected during cystoscopic evaluations in which hydrodistension up to a volume of 80 cm H_2O was administered, and excision biopsies were obtained from the glomerulation areas developed after the bladder is emptied.

Immunohistochemistry of TRAIL and its cognate receptors

First, serial sections were sliced from paraffin blocks and placed on slides. Deparaffinization procedure was employed using incubating slides at 58°C for 1.5 h. Following xylene treatment, serial ETOH washes were performed. Samples were heated in citrate buffer (pH 6) for antigen retrieval in a microwave oven at 750 W. After cooling the samples to room temperature, slides were washed in TBS. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min. Ultra V Protein Block (TA-060-UB, Lab Vision, USA) was used to inhibit nonspecific binding on slides. Then, the sections were treated with primary antibodies for 1 h at room temperature. All primary antibodies were purchased from Alexis Biochemicals (Switzerland), and the following primary antibodies (1/300) were used for the immunohistochemical analysis of prostate specimens: monoclonal antibody to TRAIL [human, (III6F) ALX-804-326-C100], monoclonal antibody to TRAIL-R1 [human (HS101) ALX-804-297A-C100], polyclonal antibody to TRAIL-R2 (ALX-210-743-C200), polyclonal antibody to TRAIL-R3 (human, ALX-210-744-C200) and monoclonal antibody to TRAIL-R4 [human (HS402) ALX-804-299A-C100]. Then, the sections were treated with a biotinylated goat antipolyvalent antibody (TP-060-BN, Lab Vision, USA) followed by streptavidin peroxidase treatment (TP-060-HR, Lab Vision, USA) for 20 min. Substrate–chromogen solution (DAB) was applied for 10 min to visualize peroxidase activity. Lastly, prostate sections were counterstained with hematoxylin for 5 min. Mainly, membranous staining was detected using these primary antibodies. Specificity of these primary antibodies was previously confirmed by Alexis Biochemicals. As a negative control, specimens were immune stained as described earlier in the absence of primary antibodies. No immune staining was detected when primary antibodies were not used.

Immunohistochemical scoring of TRAIL and TRAIL receptors

Two independent pathologists who were blinded to the names of the antibodies used for the staining performed immunohistochemical scoring. To explain

briefly, both the intensity and the marker distribution (percentage of positively stained epithelial cells) were used to obtain immune staining scores. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong staining. The marker distribution was also scored as 0, less than 10%; 1, between 10 and 40%; 2, between 40 and 70%; and 3, more than 70% of the epithelial cells stained on the specimen. Adding the scores of both the intensity and the marker distribution for a given patient attained the final immune staining score.

Statistical analysis

Statistical analyses were performed with computer software (Prism 3.0, Graphpad Software Inc.). Numeric values were reported as mean ± standard deviation. Freidman's test and Dunn's multiple comparison test were used for statistical analyses, and $P < 0.05$ values were considered as statistically significant.

Results

The present study demonstrated expression of TRAIL and its receptors (R1, R2, R3 and R4) at various levels in all patients. The distribution and intensity of TRAIL and its receptors in 27 patients with IC were evaluated with immunohistochemical staining, and the results are presented in Fig. 1.

The distribution of TRAIL receptors (R1, R2, R3 and R4) and TRAIL was determined as 33.7 ± 22.09, 37.41 ± 21.94, 40 ± 24.85, 85.19 ± 16.95 and 47.96 ± 21.97, respectively, and the statistical analysis showed that the TRAIL-R4 receptor had a statistically higher distribution compared to TRAIL and other receptors ($P < 0.05$; Fig. 2).

The expression intensity of TRAIL receptors (R1, R2, R3 and R4) and TRAIL was 1.25 ± 0.52, 1.51 ± 0.50, 1.37 ± 0.56, 2.66 ± 0.55 and 1.63 ± 0.62, respectively. Similar to the distribution analysis, the expression intensity of the R4 receptor was also statistically higher than that of other receptors or TRAIL ($P < 0.05$; Fig. 3).

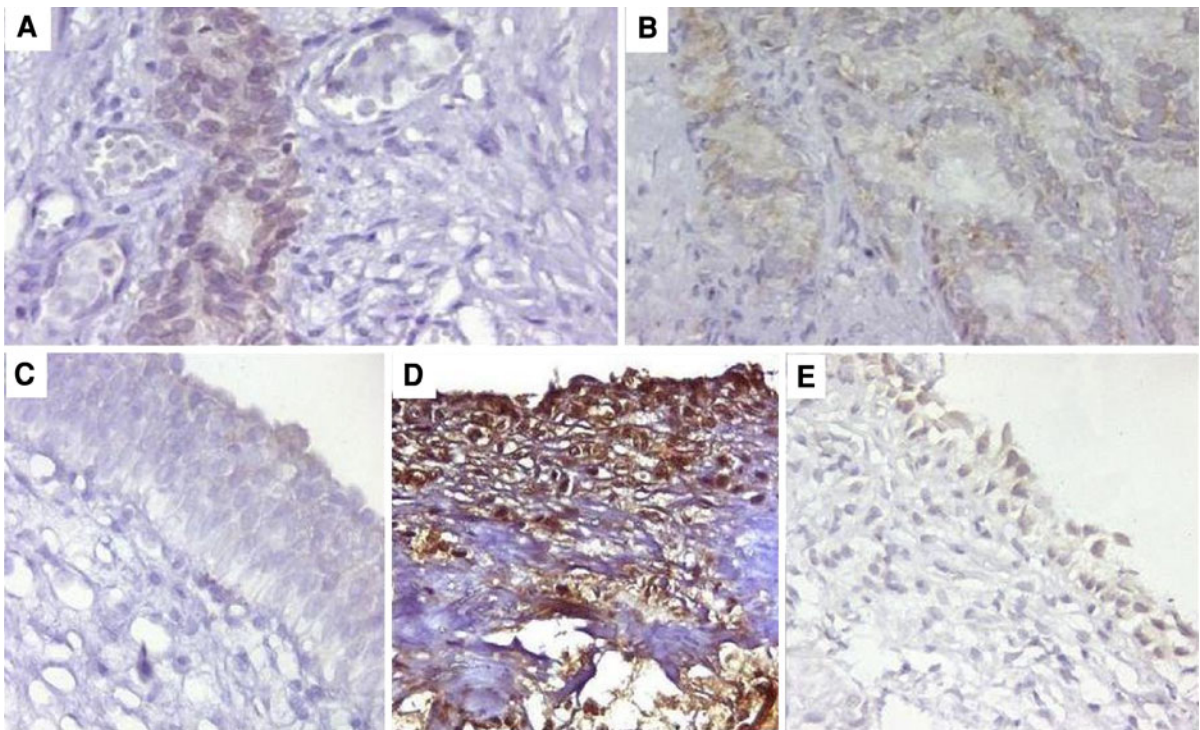


Fig. 1 Appearances of bladder sections following immunohistochemical staining. *Brown-colored zones* indicate areas stained with TRAIL antibodies. **a** TRAIL-R1 receptor expressions. **b** TRAIL-R2 receptor expressions. **c** TRAIL-R3 receptor expressions. **d** TRAIL-R4 receptor expressions. **e** TRAIL expressions

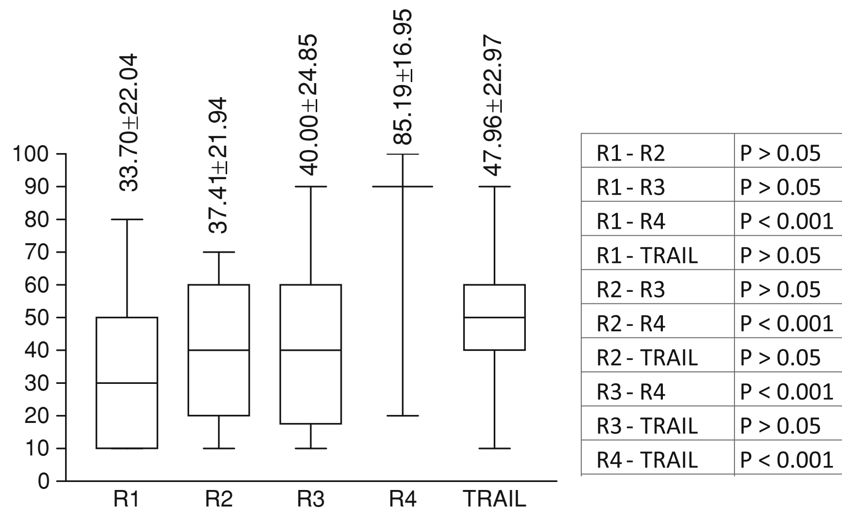


Fig. 2 Distribution of TRAIL and TRAIL receptors (R1, R2, R3 and R4), mean values

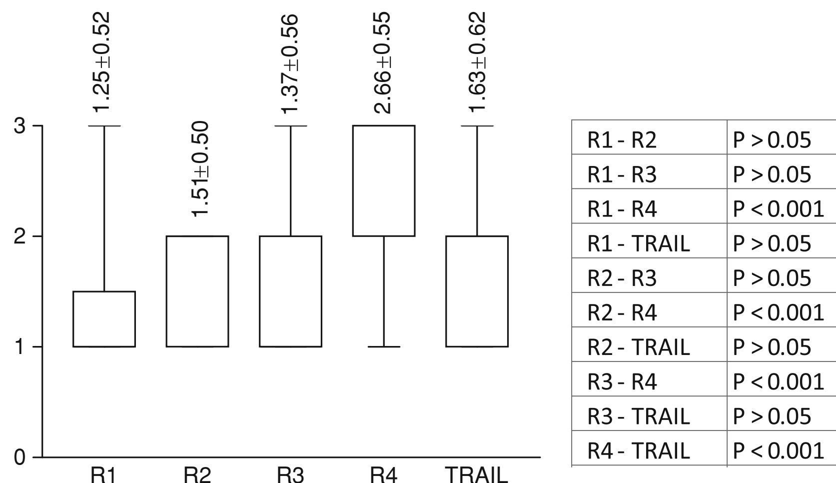
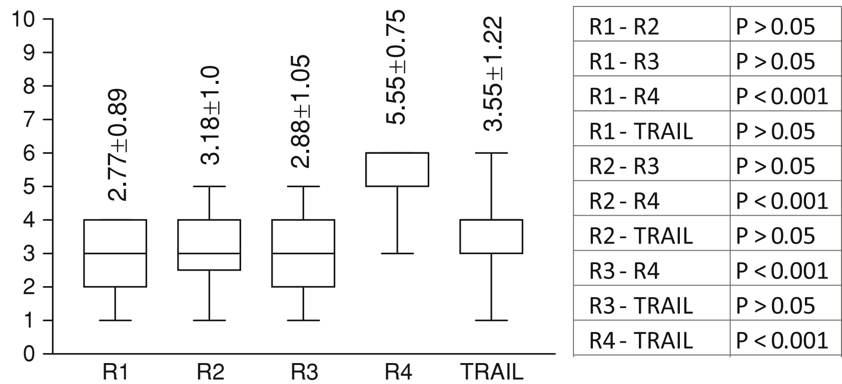


Fig. 3 Expression intensity of TRAIL and TRAIL receptors (R1, R2, R3 and R4), mean values

Immune staining scores, which is the total of intensity and distribution of expression scores of TRAIL receptors (R1, R2, R3 and R4) and TRAIL, were 2.77 ± 0.89, 3.18 ± 1.0, 2.88 ± 1.05, 5.55 ± 0.75 and 3.55 ± 1.22, respectively. The statistical analysis demonstrated that the TRAIL-R4 receptor was immunohistochemically stained with a higher score than TRAIL-R1, TRAIL-R2, TRAIL-R3 receptors and TRAIL, with a statistically significant difference ($P < 0.05$; Fig. 4).

Discussion

Interstitial cystitis is a condition characterized by frequent and urgent urination, pain in the pelvis and/or incontinency. The diagnosis and treatment of IC have not been established despite the many studies on the etiopathogenesis of the disease carried out on human and animal. Although there is no consensus on the pathophysiology of IC, three mechanisms are extensively studied: mucosal dysfunction, mast cell activation and neuroimmune mechanism. Recent studies have reported that the inflammatory and/or immunologic responses in IC may be a result of NF-κB activation and that NF-κB can be reduced with treatment [22–24, 29]. Although there are several mediators that may increase the levels of NF-κB in IC, the death ligand TRAIL, a recently discovered member of TNF (tumor necrosis factor) family, and evidences that TRAIL may be a further factor that stimulates NF-κB suggest that this ligand may also be



R1 - R2	P > 0.05
R1 - R3	P > 0.05
R1 - R4	P < 0.001
R1 - TRAIL	P > 0.05
R2 - R3	P > 0.05
R2 - R4	P < 0.001
R2 - TRAIL	P > 0.05
R3 - R4	P < 0.001
R3 - TRAIL	P > 0.05
R4 - TRAIL	P < 0.001

Fig. 4 Immune staining scores of TRAIL-L and TRAIL receptors (R1, R2, R3 and R4), mean values

involved in IC [25–27]. In addition, presence of TRAIL and receptors was identified in mast cells, which are known to be involved in the pathogenesis of IC, and mast cells have been reported to have a role in the apoptosis stimulated by TRAIL [30, 31]. Present in cells involved in inflammation (mast cells, neutrophiles, CD4+ T cell, natural killers, macrophages, etc.), TRAIL is also believed to have a regulatory role in physiologic and pathologic immune responses [32–35].

There are no studies in the literature reporting data on TRAIL and TRAIL receptors' expression from bladder sections of patients with IC evaluated with immunohistochemical staining assay, although there are relevant studies using other tissues. Currently, five TRAIL receptors have been identified, which are TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 receptors. The fifth TRAIL receptor osteoprotegerin (OPG, TRAIL-R5), as a decoy receptor, is considered to protect bone cells from the apoptosis induced by TRAIL [28]. Although there is no complete description on how the TRAIL mechanisms work, TRAIL-R1 and R2 are known to induce mainly apoptosis, while R3 and R4 receptors stimulate proliferation and inflammation mediated by NF-κB. On the other hand, R1 and R2 receptors were demonstrated to stimulate proliferation, contrary to what one might have expected [36]. Some studies have shown NF-κB activation with high TRAIL-R4 expression [26, 37–39].

Compared to severe systemic adverse effects observed with gene therapies using the first-discovered death ligands (TNFα, Fas Ligand), the limited systemic adverse effects of TRAIL have established its place in investigations based on gene therapy [40]. High levels of TRAIL-R4 receptors have been detected on the cell surface of synovial fibroblasts that are involved in the pathology of rheumatoid arthritis and result in abnormal inflammation at joints by secreting metalloproteinase [41]. In a study by Sanlioglu et al., no significant increase in apoptosis in synovial cells was reported following transfection of an adenovirus (AdhTRAIL) loaded with TRAIL protein-coding gene (hTRAIL), and the authors described this finding as apoptosis resistance mediated by TRAIL. When transfection was induced with plasmid coding the TRAIL-R2 receptor protein in the same study, TRAIL-mediated apoptosis resistance in the synovial cells were broken down, after which apoptosis developed and synovial cells were eliminated. The authors have shown in vitro that high levels of TRAIL-R4 produced TRAIL resistance and that this may be eliminated by gene therapy by increasing the expression of TRAIL-R2 receptor [42].

Among the research investigating TRAIL in prostate cancer, there are studies that aimed to eliminate TRAIL-mediated apoptosis resistance by using agents inhibiting the activity of NF-κB. These studies have reported that the TRAIL-mediated apoptosis sensitivity may be increased in vitro in prostate cancer by using adenoviral vectors coding the dominant negative mutants of AdhTRAIL, IKKβ and IKBα (subunits of NF-κB reducing activation) in order to prevent NF-κB activity and to increase the levels of TRAIL [43]. In a similar study, melanoma cells were made sensitive to TRAIL-mediated apoptosis through transfection of the plasmid coding the IKBα protein [44].

The present study demonstrated expression of TRAIL and TRAIL receptors at various levels in bladder biopsy samples obtained from patients with IC. It was further shown that TRAIL-R4 receptor was immunohistochemically stained with a significantly

higher score than TRAIL-R1, TRAIL-R2, TRAIL-R3 receptors and TRAIL. The death ligand TRAIL expression is much lower than that of the receptors in patients with IC. This does not come as a surprise, since the down-regulation of death ligand TRAIL expression might be important for the development of inflammation and one of the ways of escaping apoptosis. As shown recently, the absence of TRAIL expression or function increased the degree of islet inflammation and the incidence of autoimmune diabetes [45]. One of the functions of TRAIL in vivo is to inhibit autoimmune inflammation in the islets of Langerhans. Thus, unlike TNF-α, which may promote the development of type 1 diabetes, TRAIL inhibits insulitis and suppresses autoimmune diabetes [45]. And because TRAIL-transduced dendritic cells suppressed antigen-specific T cell activity and protected mice from acute graft versus host disease and leukemia relapse [46]. Dirice et al. [47] have shown that severe insulitis was detected in animals transplanted with mock infected or AdCMVLacZ-infected islets, while the severity of insulitis was reduced in animals engrafted with Ad5hTRAIL-infected islets. Thus, TRAIL overexpression in pancreatic islets extends allograft survival and function leading to a therapeutic benefit in STZ-induced diabetic rats [47]. Being expressed in the bladder epithelium in high proportions, as determined in the present study, the TRAIL-R4 receptor may contribute to the NF-κB activation resulting in inflammation in IC. These findings suggest that use of NF-κB-blocking agents in IC may decrease inflammation and that virus-mediated gene therapy may present a significant subject for research into IC.

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