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Clinical Utility of Soluble Fas and Fas Ligand in Thyroid Pathogenesis

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Abstract. **Background**: The soluble form of Fas (sFas) lacks the transmembrane domain due to alternative splicing. sFas blocks Fas-mediated apoptosis by binding to Fas ligand (FasL). This study was designed to examine whether the apoptotic-inhibitor sFas and the apoptotic inducer FasL are differentially present in two opposite phenotypes of autoimmune thyroid disorders (AITD), nodular goiter (NG), and thyroid cancer. **Methods**: sFas and FasL levels were determined using ELISA in the serum sample of a total 68 patients with Hashimoto's thyroiditis (HT) (n = 15), Graves' disease (GD) (n = 17), nodular goiter (n = 21), and thyroid carcinoma (n = 15). These patients' levels and were compared with 20 age matched, disease-free controls. In addition, for comparison, the levels of thyroglobulin and thyroid peroxidase antibodies were measured. **Results**: All studied groups had raised sFas levels. Levels in AITD patients were statistically significantly higher than in controls. FasL levels were significantly higher in all studied groups except the thyroid cancer group, as compared with controls. Compared to controls, GD patients had higher sFas level, and the HT group had a higher FasL level. Also, compared to controls and the NG group, thyroid cancer patients had higher sFas and lower FasL levels. **Conclusion**: Fas-mediated apoptosis plays an important role in the active stage of the autoimmune process of both GD and HT. Increased sFas in GD and FasL in HT may contribute to homeostasis in thyroid gland. In thyroid cancer and NG, however, sFas and FasL may provide a key protective signal that helps the cells to avoid apoptosis in a hostile environment.

Keywords Autoimmune thyroid disease • Fas ligand • Graves' disease • Hashimoto's thyroiditis • Nodular goiter • Soluble Fas • Thyroid cancer

Introduction

Thyroid problems are among the most common medical conditions, but the development of the conditions is often a slow and insidious process. Patients symptoms often appear gradually and are misunderstand so they are potentially misdiagnosed.[1]

Thyroid disorders including nodular goiter (NG), and cancer of thyroid epithelial origin as well as autoimmune thyroid diseases (AITD) such as Hashimoto's thyroiditis (HT) and Graves' diseases (GD) are well known. Studies indicated they these thyroid disorders may be associated with dysregulation of the apoptotic signaling pathway.

The processes involved in apoptosis are tightly regulated. Alterations in their functioning may result in disorders such as autoimmune diseases and cancer.[2,3] Apoptosis may play an important role in the homeostasis of thyroid follicular cells as well as destructive mechanisms in thyroid disease.

Apoptosis is a mechanism by which cytotoxic T lymphocytes can destroy thyrocytes in thyroiditis, leading in turn to hypothyroidism.[4,5] In contrast, the suppression of apoptosis may contribute to proliferative diseases of the thyroid gland, such as goiter, cancer, and GD.[5,6]

However, little is known about the mechanisms and regulation of apoptotic signaling in thyroid cells. It is important to define the signaling components of apoptosis in thyroid follicular cells. These signaling components may help in providing insights into potential pathogenic mechanism and lead to development of pharmacological interventions for the treatment of thyroid disease.[7]

The apoptotic mechanism of a cell involves several components: death receptor, adaptor molecules, the caspase cascade, mitochondria, and the Bcl-2 proteins.[8,9]

The death receptors known today belong to the tumor necrosis factor receptor (TNFR) gene superfamily which is defined by similar, cysteine-rich extra cellular domains. The death receptors, which form a subgroup of the superfamily TNFR, are composed of three domains: an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain contains a homologous domain of 80 amino acids termed the "death domain." The death domain (DD) bridges the death receptors with the apoptotic machinery or some adaptor molecules that transmit apoptotic signals from death receptors that themselves contain death domains.[10,11]

One of the best characterized death receptors is Fas known as CD95/APO-1/TNFRSF6. It is a 36 KDa cell surface type-1 membrane glycoprotein. Fas ligand (FasL) is a type-II transmembrane protein of the same family, which has the ability to bind Fas.[12] Fas has been shown to be an important mediator of apoptotic cell death. It is also involved in inflammation. Binding of FasL induces trimerization of Fas in the target cell membrane. Activation of Fas causes the recruitment of Fas associated protein with death domain (FADD) via interactions between the death domain of Fas and FADD. Procaspase-8 binds to Fas-bound FADD via interactions between the death effecter domain (DED) of FADD. Procaspase-8 then leads to the activation of caspase-8. Activated caspase-8 cleaves (activates) other procaspases, in effect beginning a caspase cascade that ultimately leads to apoptosis.

Fas can occur both as a cell surface protein and a soluble protein. Cell surface Fas is anchored by a single membrane-spanning domain and is widely expressed in normal and malignant cells.[13] Soluble Fas (sFas), alternatively spliced Fas mRNA that results from deletion of exons 3, 4, 6 & 7 and lacks 21 amino acid residues that contain a transmembrane domain. sFas protects target cells from Fas-induced apoptosis by competitively binding with FasL and altering lymphocyte development and proliferation in response to self antigens.[14]

The Fas pathway has been demonstrated to be the important apoptotic pathway in the thyroid gland. Its role in the pathogenesis of thyroid diseases, however, is controversial and a subject of much debate.[16] Regulation or modulation of this pathway can occur at multiple levels throughout the pathway. This may include changes in the level of the expression of Fas or its ligand;[12,17,18,20] regulation of components of intracellular signaling;[9,22,23,24] and expression of proteins that promote survival, such as members of the Bcl-2 gene family.[3,9,23,26,27]

This study summarizes the role of Fas-mediated apoptosis in thyroid diseases such as goiter, thyroid cancer, and autoimmune thyroid diseases. In addition, markers of humoral immune response (thyroglobulin and thyroid peroxidase antibodies) were also evaluated to compare with sFas and FasL levels.

Materials and Method

Patients and Sample Collection. A total of 68 patients with thyroid disorder (age range: 07-to-78 years) were enrolled in the current study. All the patients were newly diagnosed and untreated for their condition at the time of blood collection. Also, due and required consent was taken from each patient to enroll in this study prior to blood collection.

The study was approved by Institutional Scientific Review Board & Ethics Committee. The diagnosis of GD and HT was based on commonly accepted clinical and laboratory criteria. In case of NG and thyroid cancer, blood samples collected prior to surgery and diagnosis was based on histopathological investigation. Of all 68 patients, thirty two (47.1%) had AITD, twenty (30.9%) had NG, and fifteen (22.1%) had thyroid carcinoma.

For comparison with patients, 20 age matched, healthy individuals were included. They had normal thyroid function test results, no goiter, and were negative for antithyriod autoantibodies.

Venous blood samples were collected in Vacutainer tubes between 9:00 to 11:00 am. The samples were allowed to clot for 30 minutes at room temperature. Sera were obtained by centrifugation, were aliquoted, and stored at -20ºC till analysis. All the samples were analyzed for thyroid hormone levels and thyroid autoantibodies.

Evaluation of Soluble Fas and Fas Ligand. Circulating sFas and FasL were estimated using sandwich ELISA (Quantikine, R&D systems, USA)

kits. Sample activation and dilution were performed according to the manufacturer's protocol. The unit of measure for the levels of sFas and FasL was ng/mL. The detection range was 0-2.0 ng/mL and 0-1.0 ng/mL for sFas and FasL, respectively. All enzymatic reaction products were determined photometrically at 450 nm by Plate reader (Thermolabsystems, Finland; Multiskan spectrum).

Evaluation of Thyroid Autoantibodies. Commercially available kits (Immunotech, France) were used for the measurement of serum anti-thyroglobulin (anti-TG), and anti-thyroid peroxidase (anti-TPO) antibodies with the automated gamma counter (Packard, cobra). The detection range was 0-to-250.0 ng /mL and 0-2100.0 IU /mL for TG and TPO, respectively. Measurements were based on radioimmunoassay and immunoradiomatric assay.

Statistical Analysis. All the statistical analysis carried out with the SPSS 17.0 software and statistical significance was computed using students' *t*-test and anova. Receiver's operating characteristic (ROC) curve was also constructed to determine the discriminating efficacy of sFas and FasL. $p < 0.05$ was considered as a statistically significant. The correlation of TPO and TG with sFas and FasL was done by parametric analysis, i.e. Pearson's correlation and non-parametric, i.e. Spearman's correlation test.

Results

A total of 68 samples and 20 controls were analyzed for sFas and FasL levels. Higher mean levels of sFas and FasL observed in thyroid diseases were statistically significant when compared with controls. The results of circulating serum sFas and FasL levels in the examined thyroid disease patients are shown in Table-1 and 2 as mean ± standard error of the mean in ng/mL.

Levels of sFas were higher in all the studied groups as compared to the controls. However, the highly significant values were found in GD patients as compared to controls (0.791 ± 0.060 vs 0.566 ± 0.012, $p = 0.0001$) and compared to HT (0.791 ± 0.060 vs 0.645 ± 0.045, $p = 0.068$). Higher levels in HT (0.645 ± 0.045, $p = 0.065$) and thyroid cancer patients (0.734 ± 0.102, $p = 0.069$) were close to statistically significant. There was also a difference between thyroid cancer patients and NG (0.734 ± 0.102 vs 0.599 ± 0.033, p=0.164).

Table-1. Significance of Soluble Fas levels in patients with various thyroid diseases as compared to controls.

Subjects	N	Soluble Fas			
		Mean ± S.E. *(ng/mL)*	Minimum	Maximum	*p* value
Controls	20	0.566 ± 0.012	0.430	0.680	
Total patients	68	0.687 ± 0.031	0.340	2.000	0.042
Autoimmune thyroid disease	32	0.723 ± 0.040	0.400	1.500	0.004
Graves' disease	17	0.791 ± 0.060	0.530	1.500	0.0001
Hashimoto's thyroiditis	15	0.645 ± 0.045	0.400	1.050	0.065
Nodular goiter	21	0.599 ± 0.033	0.340	0.960	0.37
Thyroid cancer	15	0.734 ± 0.102	0.390	2.000	0.069

As Table-2 shows, compared to controls, the mean level of Fas ligand was significantly higher in all patient groups except thyroid cancer patients. The difference in FasL levels between GD (0.056 ± 0.003) and HT (0.062 ± 0.007) was not significant. Significantly higher levels of FasL were observed for NG patients compared to controls (0.062 ± 0.006 vs 0.035 ± 0.005, $p = 0.003$) and compared to thyroid cancer patients (0.062 ± 0.006 vs 0.042 ± 0.004, p = 0.028).

Table-2. Significance of Fas ligand levels in patients with various thyroid diseases as compared to controls.

Subjects	N	Fas Ligand			
		Mean ± S.E. *(ng/mL)*	Minimum	Maximum	*p* value
Controls	20	0.035 ± 0.005	0.005	0.064	
Total patients	68	0.056 ± 0.003	0.006	0.0154	0.001
Autoimmune thyroid disease	32	0.059 ± 0.003	0.025	0.110	0.0001
Graves' disease	17	0.056 ± 0.003	0.035	0.084	0.002
Hashimoto's thyroiditis	15	0.062 ± 0.007	0.025	0.110	0.003
Nodular goiter	21	0.062 ± 0.006	0.015	0.154	0.003
Thyroid cancer	15	0.042 ± 0.004	0.006	0.008	0.35

Levels of TPO and TG antibodies were also measured and found higher in all groups except TG in the HT group (Table-3). Parametric and nonparametric correlation tests showed significant linear correlations between sFas and TG ($r = 0.319$, $p = 0.05$), TPO ($r = 0.384$, $p = 0.019$). Significant linear correlations were also found between sFas and TPO antibodies ($r = 0.590$, $p = 0.021$) in HT patients and between sFas and TG ($r = 0.543$, $p = 0.011$) in NG patients. There was no correlation between FasL and TG or TPO antibodies.

ROC curve indicates that both sFas and FasL

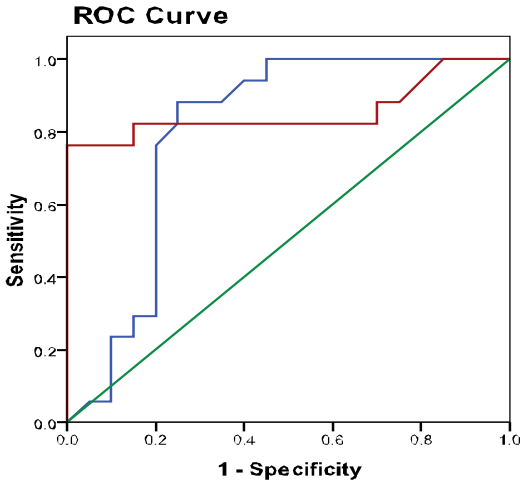
Table-3. Levels of thyroid peroxidase and thyroglobulin in patients with various thyroid diseases and controls.

Subjects	N	Thyroid peroxidase		Thyroglobulin	
		Mean ± S.E. *(IU/mL)*	*p* value	Mean ± S.E. *(ng /mL)*	*p* value
Controls	20	20.16 ± 6.63		6.16 ± 0.92	
Total patients	68	781.99 ± 172.86	0.02	56.90 ± 15.45	0.08
Autoimmune thyroid disease	32	1236.01 ± 306.12	0.003	48.56 ± 18.30	0.074
Graves' disease	17	1198.90 ± 390.55	0.002	84.47 ± 32.20	0.012
Hashimoto's thyroiditis	15	1278.07 ± 494.86	0.006	7.85 ± 4.21	0.65
Nodular goiter	21	598.50 ± 256.23	0.034	57.98 ± 33.07	0.13
Thyroid cancer	15	70.30 ± 57.59	0.32	73.19 ± 37.12	0.044

ROC curve indicates that both sFas and FasL exhibited a good discriminatory efficacy between controls and GD patients (sFas: AUC-0.856; FasL: AUC-0.801) (Figure-1).

Figure-1. ROC curve for Soluble Fas (red line) and Fas ligand (blue line) between graves's disease patients with controls.

Variable	Area under the curve	*p* value	95% CI	
			Lower	Upper
Soluble Fas	0.856	0.0001	0.712	1.000
Fas ligand	0.801	0.002	0.647	0.956



Discussion

The control of thyroid gland volume results from an equilibrium between the trophic action of TSH and thyrocyte apoptosis. Apoptosis is limited to some extent by resistance to Fas activation by producion of an inhibitor of apoptotic signal transduction.[24] In the Fas/FasL apoptotic pathway, Fas, FasL and sFasL are thought to induce apoptosis while sFas inhibits apotosis.

The current prospective study was designed to examine whether sFas, an inhibitor of apoptosis, and FasL, an inducer of apoptosis are differentially present in two opposite phenotypes of autoimmune thyroid disorders, nodular goiter, and thyroid cancer.

In the study, all patients with various thyroid diseases showed elevated levels of sFas. However, the highest concentrations were found in patients with AITD. Hiromatsu et al. demonstrated Fas expressed in cell membranes and predisposed to FasL mediated apoptosis. Fas may be present both on the thyrocytes of patients with AITD with infiltrating lymphocytes. Thus, Fas/FasL interaction considerably influences destruction of thyroid tissue on one side and plays an important part in the elimination of autoreactive lymphocytes on the other side.[25]

Moreover, we found higher levels of sFas in GD patients. This finding concurs with the study result of Shimoka et al.[26] and Hiromatsu et al.[27] They have demonstrated that increased sFas in GD suggest increased expression of an alternatively spliced mRNA variants that produces sFas proteins that decreases the cell expression of Fas and may induce thyroid cell growth and production of TSH receptor antibodies by protecting against apoptosis of thyroid cells.

Fountolakis et al. have also demonstrated that elevated sFas in GD may reflect reduced full length membrane Fas and may have a role in the inhibition of Fas-mediated apoptosis of thyrocytes in this condition.[28] Circulating forms of Fas are commonly considered as a factor that inhibits membrane Fas-mediated apoptosis. These originate from both proteolytic cleavage from the transmembrane domain and direct mRNA transcription.[23] In our study, circulating sFas levels were simultaneously increased with TG and TPO antibodies in GD. Thus, sFas may play a role with these molecules in preventing the Fas/FasL-mediated apoptosis of thyrocytes in GD. The local production of sFas by thyrocytes, its regulation by cytokines, and its increased serum levels in GD have been confirmed. These studies suggest that sFas, by interfering with the Fas-FasL interaction, plays a role in the pathogenesis of GD.[26,27]

Our data have shown elevated levels of Fas ligand in HT compare to healthy individuals and GD patients. In contrast, sFas was decreased in HT compare to GD. An increase in sFas concentration especially in patients with HT compare to healthy individuals may be a consequence of the enhanced releasing of circulating forms of Fas that seem to reflect the intensity of Fas/FasL mediated apoptosis. Recent studies have revealed that FasL is also expressed in immune-privileged sites, such as the retina[29] and testis.[30]

Hiromatsu et al. has shown that in HT, a higher

degree of apoptosis is more due to thyrocytes with a more intense Fas expression on follicular cells than infiltrating lymphocytes.[25] The presence of apoptosis has also been reported in HT.[4] Recently, Giordano et al. reported the constitutive expression of FasL in normal and HT thyrocytes, indicating that they contribute to the development of clinical hypothyroidism.[31] FasL was initially reported to be expressed in activated T cells and NK cells.[16] Moreover, taking into account the high cytotoxic capacity of a TPO antibodies, a positive correlation between sFas and TPO antibodies in HT patients suggest that increased sFas may reflect the intensity of the immune-involved destruction of the thyroid follicular cells.

Andrikoula et al. concluded that increased sFas in multinodular goiter may indicate an increased expression of alternatively spliced Fas mRNA variants and decreased expression of cell surface protein. This may enhance thyroid cell proliferation by protecting thyroid cells from Fas-mediated apoptosis.[32] A similar observation was seen in our study. This indicates that sFas can play a vital role in the pathogenesis of NG. sFas simultaneously increases with TG antibodies in patients with goiter, showing unbalanced apoptosis and increased thyroid cell proliferation.

The functions of Fas have not yet been fully elucidated, but there are several investigations suggesting roles of both sFas and FasL in cancer progression. More recently, it has been reported that some tumor cells, including those of epithelial origin, express FasL.[33,34] Several studies have demonstrated high concentration of soluble Fas in large tumors in patients with ovarian cancer, cancer of the corpus uteri, colorectal cancer, thyroid cancer and adenoma, and adrenocortical cancer.[35] Using immunohistochemistry and mRNA studies, Basolo et al. demonstrated that different thyroid tissue expressions of Fas and FasL are simultaniously upregulated in adenomas and in well-differentiated papillary and follicular carcinomas. In contrast, Fas is suppressed, and FasL is strongly reduced in the most aggressive histological variants.[36] In our study, we observed that thyroid cancer patients had higher levels of sFas in lower levels FasL than patients with NG and AITD.

Summary

In summary, our study demonstrate that circulating sFas and FasL concentrations are increased in patients with AITD, especially those with GD. This indicates enhanced thyroid cell proliferation by protecting against thyroid cell from Fas-mediated apoptosis. However, in contrast to GD, decreased sFas in HT increased FasL, indicate destruction of thyrocytes. In cases of thyroid cancer and NG, sFas may provide a key protective signal that helps the cells to avoid apoptosis in a hostile environment. Further studies with larger numbers of patients that evaluate the post-therapeutic changes in the levels of these molecules may provide additional predictive value in disease differentiation and pathogenesis.

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