行政院國家科學委員會補助專題研究計畫成果報告

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※ 以螢光免疫法觀察自體免疫甲狀腺炎之甲狀腺細胞 ※
※ 因 FAS 造成之細胞凋零　　　　　　　　　　　　　※
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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC 89－2314－B－002－517－

執行期間：　89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：張天鈞

共同主持人：李文森

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執行單位：國立台灣大學醫學院內科

中　華　民　國　　90　年　9　月　30　日

行政院國家科學委員會專題研究計畫成果報告

以螢光免疫法觀察自體免疫甲狀腺炎之甲狀腺細胞因 FAS 造成之細胞凋零

Study of Fas-mediated apoptosis of thyroid epithelial cells of autoimmune thyroiditis with immunofluorescence method on flow cytometry

計畫編號：NSC 89－2314－B－002－517－
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中文摘要

甲狀腺細胞凋亡在自體免疫性甲狀腺炎的致病機轉扮演了重要的角色。Fas/Fas liqand (FasL)凋亡途徑在各種甲狀腺疾病的生理平衡和免疫調節特別受到重視，其中包括由於甲促素受器抗體(TRAb)與甲促素受器結合，導致甲狀腺細胞增生的葛瑞夫茲氏病（GD），其疾病活性與甲促素受器抗體強度有關。FasL 是腫瘤壞死因子家族的第二型膜蛋白，當他和 Fas 結合時會導致細胞凋亡。可溶性 FasL〈sFasL〉可以和 Fas 結合，而對可以表現 Fas 的細胞產生毒性。但可溶性 Fas〈sFas〉則是細胞凋亡之抑制劑。爲瞭解 sFasL 在調節 GD 之角色，我們檢視有各種不同程度 TRAb 之 GD 病人之甲狀腺組織的冷凍切片中的細胞凋亡現象和血中的 sFas / sFasL 值。結果發現 GD 之甲狀腺組織冷凍切片的甲狀腺細胞凋亡與 Fas 表現有密切相關，而血中 sFasL 與 TRAb 和 sFas 值有顯著相關。由於這些值與 TRAb 有相關性，因此可能可以作爲預測 GD 活性之指標。總之，GD 病人血清 sFasL 增加與甲狀腺的活性平衡有關，也和 GD 之疾病活性有關。

關鍵詞：可溶性 Fas，可溶性 Fas liqand，葛瑞夫茲氏病，甲促素受器抗體，細胞凋亡，自體免疫性甲狀腺炎

Abstract

Apoptosis of thyrocytes may play an important role in the pathogenesis of autoimmune thyroiditis. The Fas/Fas ligand (FasL) apoptosis pathway has received much attention in physiological homeostasis and immune regulation in various thyroid diseases, including Graves' disease (GD), which is characterized by hyperplasia of thyrocytes resulting from TSH receptor stimulation due to binding of anti-TSH receptor antibodies (TRAb), and disease activity is associated with the level of TRAb. FasL is a type II membrane protein of tumor necrosis factor family, and induces apoptosis when it binds to Fas. Soluble FasL (sFasL) exerts cytotoxic activity against Fas-expressing cells by producing trimerization of Fas molecule, but soluble Fas (sFas) is an apoptotic inhibitor. To determine the role of circulating sFasL in modulating disease activity of GD, we examined apoptosis in primary thyrocytes culture and in frozen section of thyroid tissue from GD patients, and the circulating levels of sFas/sFasL in GD patients with various levels of TRAb. Apoptosis of thyrocytes and Fas expression in frozen sections of Graves' thyroid tissues were found to be closely related, and circulating sFasL showed a significant correlation with TRAb and sFas levels. Because its levels change in parallel

with those of TRAb, circulating sFasL is a candidate marker for predicting disease activity in GD. In conclusion, increased serum sFasL levels in GD patients may contribute to the homeostasis in the thyroid and correlate with disease activity of GD

Keywords: soluble Fas, soluble Fas ligand, Graves' disease, anti-TSH receptor autoantibody (TRAb), apoptosis, autoimmune thyroiditis

Introduction

The apoptosis of thyroid follicular cells may play a pivotal role in the pathogenesis of autoimmune thyroiditis (1). The Fas/Fas ligand (FasL) apoptosis pathway has received much attention in term of a potential role in physiological homeostasis and immune regulation in various thyroid diseases, including Hashimoto's thyroiditis, Graves' disease (GD) and thyroid cancer (2-4). Giordano et al (2) even suggested that the constitutive expression of FasL on thyrocytes leads to apoptosis in normal and Hashimoto's thyrocytes, thereby resulting in clinical hypothyroidism. However, the result was questioned because they used nodular goiter thyrocytes as controls, and the specificity of the polyclonal FasL-specific antibodies was also criticized (5). Recently, Hiromatsu et al (3) and Mitsiades et al (4) found that Fas is present in normal thyroid, whereas FasL is only expressed in diseased thyroid, including GD and thyroid carcinomas. It is very important that the expression of FasL on thyrocytes seemed to be associated with the immune-privileged effect in GD. Hiromatsu's study also showed that FasL on Graves' thyrocytes had functional activity in that it induced apoptosis in target cells transfected with human Fas antigen, whereas FasL on normal thyrocytes did not (3). The increased expression of FasL in Graves' thyrocytes may maintain homeostasis in the thyroid by eliminating infiltrating lymphocytes and hyperplastic thyrocytes via Fas-mediated apoptosis (3). This result raises the important question whether Graves' thyrocytes use FasL expression to escape immune attack and whether FasL expression regulates disease activity of hyperplastic thyrocytes via apoptosis (6).

FasL is a type II membrane protein of tumor necrosis factor family, and induces apoptosis when binds to Fas antigen (7). Membrane bound FasL is converted to a soluble form (sFasL) by a metalloproteinase, and sFasL also exerts cytotoxic activity against Fas-expressing cells by causing trimerization of Fas molecules (8-10). Thus, it is likely that sFasL acts as a cytotoxic agent in peripheral autoimmune response and induces apoptosis in thyrocytes and lymphocytes.

Graves' disease, an autoimmune thyroid disorder, is characterized by hyperplasia of thyrocytes that results from binding of anti-TSH receptor antibodies (TRAb) to TSH receptors, and disease activity is clearly associated with the level of TRAb (11). However, Kawakami et al (12) reported that TRAb in Graves' patients may act in the same way as TSH to inhibit Fas/FasL-mediated apoptosis, and Hiromatsu et al (3) reported increased expression of FasL in Graves' thyrocytes and the down-regulation of Fas expression by TSH or TRAb. Since TRAb levels are higher correlated with disease activity in GD, it would be valuable to investigate the role of circulating sFas/sFasL in disease regulation in GD patients with various levels of TRAb.

In the present study, we examined apoptosis and Fas expression in primary thyrocytes culture and thyroid frozen sections from patients with GD, and the expression of circulating sFas/sFasL in GD patients with various levels of TRAb to investigate the role of circulating sFasL in modulating disease activity.

Materials and Methods

Patients

Between January 2000 and December 2000, serum samples were obtained from two groups of GD patients and one group of controls. Group I consisted of 22 untreated GD patients (6 men and 16 women, aged 20-45 years, mean± SD, 37.2±10.9 years)

with higher TRAb level (equal to, or greater than, 50 % inhibition of TSH binding, ranged 50.0-87.4%, mean± SD, 63.8±12.5 %). Group II consisted of 22 GD patients (1 men and 21 women, aged 26-65 years, mean± SD age, 42.9±14.3 years), who were euthyroid after antithyroid drugs (methimazole or propylthiouracil) treatment for less than 1 year and had low TRAb level (less than 25% inhibition of TSH binding, ranged 0.7-21.7%, mean± SD, 7.9±5.9 %; all patients in group II had TRAb levels greater than 15% before treatment). The control group (group III) consisted of 22 normal subjects (8 men and 14 women, aged 22-45 years, mean± SD age, 33.8±8.9 years). All the patients gave their informed consent before participation in this study.

Patients who had undergone radioiodine therapy or surgery were excluded from the study. The diagnosis of GD was made on the basis of clinical and laboratory criteria. GD patients had elevated concentrations of free thyroid hormones and undetectable or clearly reduced TSH levels in the serum, had TRAb (thyrotropin-binding inhibitory immunoglobulin [TBII]) in the serum, and showed diffuse increased uptake of radionuclide on the scintiscan.

Antibodies to the TSH receptor

Anti-TSH receptor antibodies in patients' sera were assayed using a thyrotropin receptor autoantibody kit (RSR Limited, Cardiff, United Kingdom) according to the manufacturer's instructions.

Thyroid tissue

Thyroid tissue was obtained from two patients with GD, one man aged 45 years and one woman aged 38 years. They were euthyroid at the time of operation, and treated with propylthiouracil before surgery. TRAb was present in the serum at binding inhibition titers of 38.3% and 46%, respectively.

Preparation of thyrocytes

Thyroid tissues were minced and digested by collagenase (Sigma) over a period of two hours at 37°C. After washing, the dispersed cells were cultured in monolayers in RPMI 1640 (Gibco) supplemented with 10% FBS containing 2.5 μg/mL penicillin/streptomycin and 200nM/L L-glutamine. Thyrocytes were used within 48 hours after establishing primary culture.

Detection of apoptosis

Apoptosis was determined via FACS analysis (Becton Dickinson analyzer) using FITC (fluorescein isothiocyanate)-labeled annexin V. One of the membrane alterations in the early stages of apoptosis is the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer, by which phosphatidylserine becomes exposed at the external surface of the cell. Annexin V is a phopholipid-binding protein with high affinity to phosphatidylserine. As translocation of phosphatidylserine to the external cell surface occurs also in cell necrosis, the measurement of annexin V binding to the cell surface was performed simultaneously with a dye exclusion test with propidium iodide.

Immunocytochemistry

Apoptosis in thyroid tissue was detected by the terminal deoxynucleotidal transferase -mediated deoxyuridine 5' – triphosphate nick end-labeling (TUNEL) method (MEBSTAIN Apoptosis Kit II, Medical & Biological Laboratories Co., Nagoya, Japan). Immuno- histochemical staining was performed using mouse anti-human Fas monoclonal antibody on primary thyrocytes culture and frozen thyroid tissue sections (UB2, IGg1, Medical & Biological Laboratories Co., Nagoya, Japan), with mouse IgG1 (DAKO Corp.) as negative control. Cryostat sections of thyroid tissue were prepared and stained with antihuman Fas monoclonal antibody and positive reactivity detected using an avidin-biotin-peroxidase detection system (DAKO Corp. LSAB kit).

sFas/sFasL ELISA assay

Detection of sFas/sFasL was performed using sandwich enzyme-linked immunosorbent assay (Medical & Biological Laboratories, Nagoya, Japan) on serum

samples kept frozen at - 20℃ (13, 14). The sFas assay uses Fas antibodies against two different epitopes. One of the antibodies was a polyclonal antibody and recognized the intracellular domain (No. 305-319 a.a.), while the other one was a monoclonal antibody and recognized the extracellular domain (No. 110-120 a.a.). All reactions were at room temperature. In wells coated with anti-Fas polyclonal antibody, 1:4 diluted serum samples or standards were incubated for 1 hour. After washing, a peroxidase conjugated anti-Fas monoclonal antibody was added to the microwell and incubated for 1 hour. After another washing, a chromogenic substrate was added and allowed to incubate for 30 minutes. The reaction was stopped and absorbance at 450 nm was measured. A standard curve was prepared from sFas calibrators, and the concentration of sFas in serum samples was determined by interpolation. All samples were assayed in duplicate. The intra-assay coefficient of variation was less than 8% and the inter-assay coefficient of variation was less than 9%.

The sFasL assay was identical to the above assay. In wells coated with anti-FasL monoclonal antibody, 4H9, 1:2 diluted serum samples or standards were incubated for 1 hour. After washing, a peroxidase conjugated anti-FasL monoclonal antibody, 4A5, was added to the microwell and incubated for 1 hour. After another washing, a chromogenic substrate was added and allowed to incubate for 30 minutes. The reaction was stopped and absorbance at 450 nm was measured. A standard curve was prepared from sFasL calibrators, and the concentration of sFas in serum samples was determined by interpolation. All samples were assayed in duplicate. The intra-assay coefficient of variation was less than 4.2% and the inter-assay coefficient of variation was less than 7.3%.

Statistics

Results were analyzed by paired Student's t test. $P<0.05$ was considered statistically significant. Linear regression test was used to examine the correlation between sFasL levels and other parameters.

Results

sFas and sFasL serum level

The demographic characteristics of patients with GD are shown in Table 1.

As shown in Fig. 1, serum levels of sFas were significantly higher in the untreated GD patients with higher TRAb level (Group I, mean ± SD, 1.56±0.26 ng/ml) than in the GD patients with low levels of TRAb (Group II, 0.76±0.26 ng/ml, $p < 0.01$), and to control subjects (Group III, 0.79±0.24 ng/ml, $p < 0.01$).

As shown in Fig. 2, serum levels of sFasL were also significantly higher in group I patients (mean ± SD, 0.153±0.018 ng/ml) than in group II patients (0.126±0.012 ng/ml, $p < 0.01$). Serum concentrations of sFasL in control subjects (group III) were all below the level of 0.1 ng/ml (mean ± SD, 0.076±0.010 ng/ml).

Correlation between sFas, sFasL and TRAb

A significant correlation was seen between serum levels of sFas, sFasL and TRAb.

A significant correlation was found between sFasL levels and TRAb activity in group I patients (n=22, r=0.43, $p < 0.05$). When group II patients were included in this analysis, a more significant correlation was found (n=44, r=0.69, $p < 0.01$, Fig. 3).

Fig. 4 shows that there was also a significant correlation between sFas levels and TRAb activity in GD patients (n=44, r=0.91, $p < 0.01$).

Since sFas is an apoptosis inhibitor while sFasL induces apoptosis, it is interesting that a significant correlation was observed between serum levels of sFas and sFasL in GD patients (n=44, r=0.71, $p < 0.01$, Fig. 5).

Detection of apoptosis on primary thyrocytes culture of GD

Thyrocytes were harvested and prepared for FACS analysis within 48 hours after establishing primary culture. Apoptosis was found in 4.8±1.8% in primary thyrocytes culture (Fig. 6).

Immunocytochemical staining of Fas expression and apoptosis on primary thyrocytes culture and frozen sections of GD

Apoptosis was detected in thyroid tissues from GD patients by the TUNEL method. Positive nuclear staining of the thyrocytes was found. We performed immunocytochemistry using anti-human Fas monoclonal antibodies to detect Fas expression on primary thyrocytes culture and frozen section in thyroid tissues from GD patients. Prominent Fas expression was detected on the primary culture and the basal surface of thyroid tissue from patients with GD (Fig. 7 and 8). The results from FACS analysis and thyroid sections showed that apoptosis which could be detected, and Fas expression were closely related.

Discussion

FasL, a member of the tumor necrosis factor family which can induce apoptosis of Fas-bearing cells, was firstly reported to be expressed in activated T cells and NK cells (15). Although membrane-associated FasL is more efficient than sFasL in aggregating Fas, sFasL also has cytotoxic activity against Fas-expressing cells, as it causes trimerization of Fas molecules, thus inducing apoptosis (9,16).

Recently, sFasL was reported to induce epithelial cell apoptosis in acute lung injury (17), and other reports indicated that serum sFasL levels increase with the severity of heart failure in patients with myocarditis (18) and in patients with advanced congestive heart failure (19). In our study, using FACS analysis and frozen sections of Graves' thyroid tissue, we found that apoptosis and Fas expression were closely related. These findings suggest that sFasL and Fas-induced apoptosis play a role in modulating disease activity of certain diseases. GD is an autoimmune disease in which sFas is reported to be a pathological factor (14). However, although FasL expression in GD thyrocytes has been shown to have functional activity in inducing apoptosis of Fas-bearing target cells (3), the cytotoxic effect of circulating sFasL requires further investigation. In the present study, we analyzed circulating sFasL levels in GD patients with different disease activity and found that serum sFasL may be a marker for predicting disease activity in Graves' hyperthyroidism.

The pathogenesis of GD is associated with serum TRAb, and the disease activity is closely correlated with the level of TRAb (11). In our study, we found that levels of circulating sFas and sFasL correlated closely with to TRAb levels, especially in GD patients with higher TRAb levels (>50%). Hiromatsu et al (14) have reported a significant correlation between sFas and TRAb levels, but this is the first report of a correlation between sFasL and TRAb levels. In addition, a significant correlation was seen between serum levels of sFas and sFasL, indicating that sFas/sFasL is related to disease activity of GD patients.

Conventionally, Fas, FasL, and sFasL are thought to induce apoptosis, while sFas is an apoptotic inhibitor. In Hiromatsu's study (14) and our own, circulating sFas levels and TRAb were simultaneously increased. Thus, sFas may play a role, together with TRAb in preventing the Fas/FasL-mediated apoptosis of thyrocytes, thus leading to thyroid hyperplasia and the increased disease activity. However, although Fas is expressed in both normal and diseased thyroid, FasL is only expressed in diseased thyroid. FasL is expressed in immune-privileged sites, such as retina and testis, where it affords protection against specifically activated lymphocytes (20, 21). Recent reports even indicated the greater the expression of FasL, the more down-regulation of disease activity was seen in autoimmune thyroiditis (22); and FasL expression on thyrocytes may have curative effect on ongoing experimental autoimmune thyroiditis by inducing apoptosis of autoreactive infiltrating T lymphocytes in animal model (23).

Production of FasL can be used to induce specific tolerance by apoptosis and clonal deletion of antigen-reactive T cells (24-26), but Fas L can also cause local damage. In our study, sFasL was only

detected in GD patients; in addition, sFasL levels were significantly correlated with TRAb and sFas levels. This suggests that sFasL may have a dual effect in modulating disease activity in Graves' hyperthyroidism, acting at immune-privileged sites to destroy infiltrating activated lymphocytes and also inducing apoptosis of hyperplastic thyrocytes. The explanation provided by Hiromatsu et al (3) that increased expression of FasL in GD thyrocytes may help in maintaining thyroid homeostasis, appears to be more important. Generally, higher levels of TRAb are indicative of more aggressive disease activity in GD. In our study, since higher sFasL levels were noted in those GD patients with higher TRAb (>50%), it was expected that these patients would show higher expression of apoptosis. However, the immune-privileged effect on activated infiltrating lymphocytes may be more important, as the conventional Fas/FasL-mediated apoptosis of thyrocytes is usually prevented by TRAb (12), or blocked by a protein inhibitor in thyrocytes (27).

On the other hand, in our study and in other reports (13,14,28), although circulating sFas can be readily detected in both GD patients and normal subjects, serum levels of sFasL in normal subjects are lower than 0.1 ng/ml. These results are compatible with the finding that FasL is only expressed in diseased thyroid, as in GD and thyroid carcinoma. However, circulating level of sFasL may be a good marker for predicting disease activity in GD, since changes in sFasL levels and TRAb levels occur in parallel. In conclusion, increased serum sFasL in patients with GD may contribute to homeostasis in the thyroid. Serum sFasL may be useful as a marker for predicting disease aggression or regression in Graves' hyperthyroidism.

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Legends for figures

Fig. 1. Serum levels of sFas in untreated GD patients (group I) with higher TRAb level, treated GD patients with low levels of TRAb (group II), and control subjects (group III).

Fig. 2. Serum levels of sFasL in untreated GD patients (group I) with higher TRAb level, treated GD patients with low levels of TRAb (group II), and control subjects (group III).

Fig. 3. Correlation between serum sFasL levels and serum TRAb activity in all patients with Graves' disease.

Fig. 4. Correlation between serum sFas and serum TRAb activity in all patients with Graves' disease.

Fig. 5. Correlation between serum levels of sFas and sFasL in all patients with Graves' disease

Fig. 6. Apoptosis of harvested primary thyrocytes culture (left upper quadrant) was determined via FACS analysis.

Fig. 7. Immunocytochemical staining of Fas expression (brownish) on the primary thyrocytes culture from patients with GD. Final magnification x400.

Fig. 8. Immunocytochemical staining of Fas expression on the basal surface of thyrocytes (arrowhead, brownish) in thyroid tissue from patients with GD. Final

magnification x400.

Fig. 4

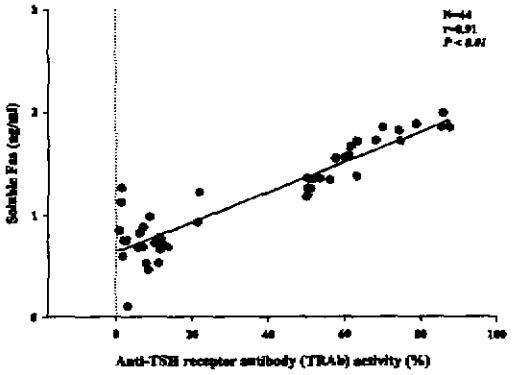


Fig. 5

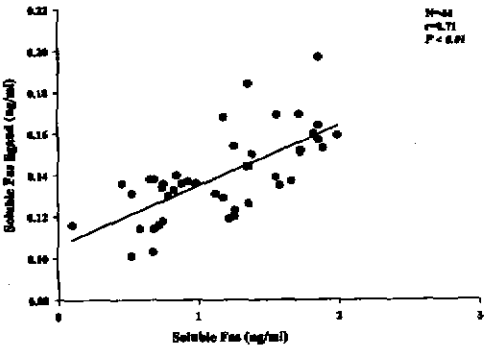


Fig. 6

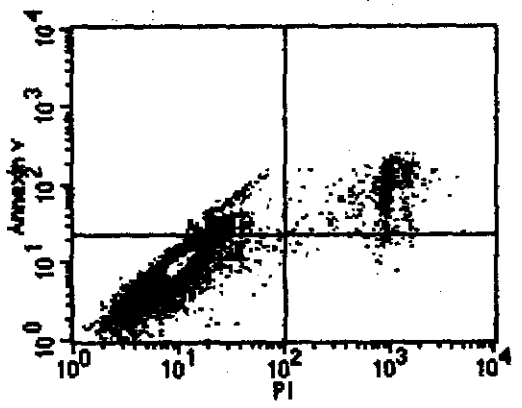


Fig. 1

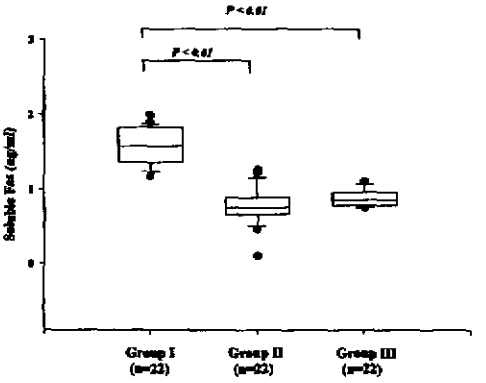


Fig. 2

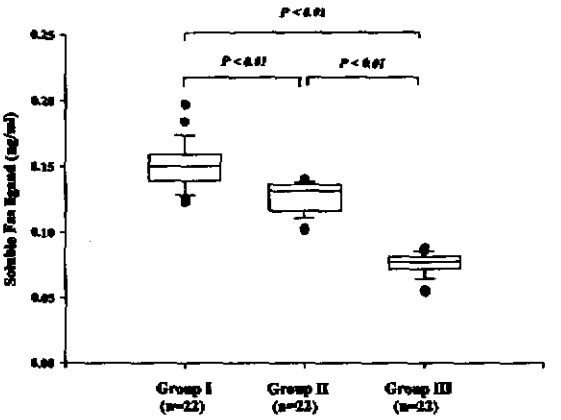


Fig. 3

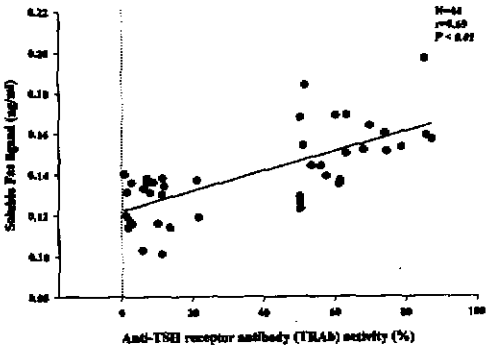


Fig. 7

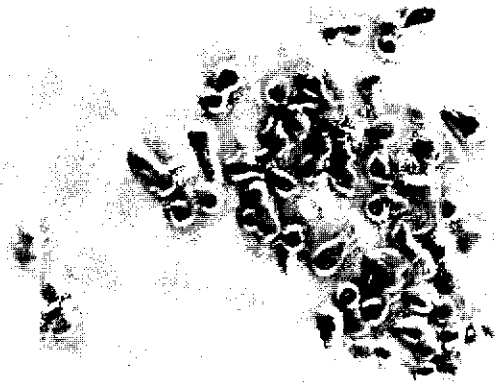
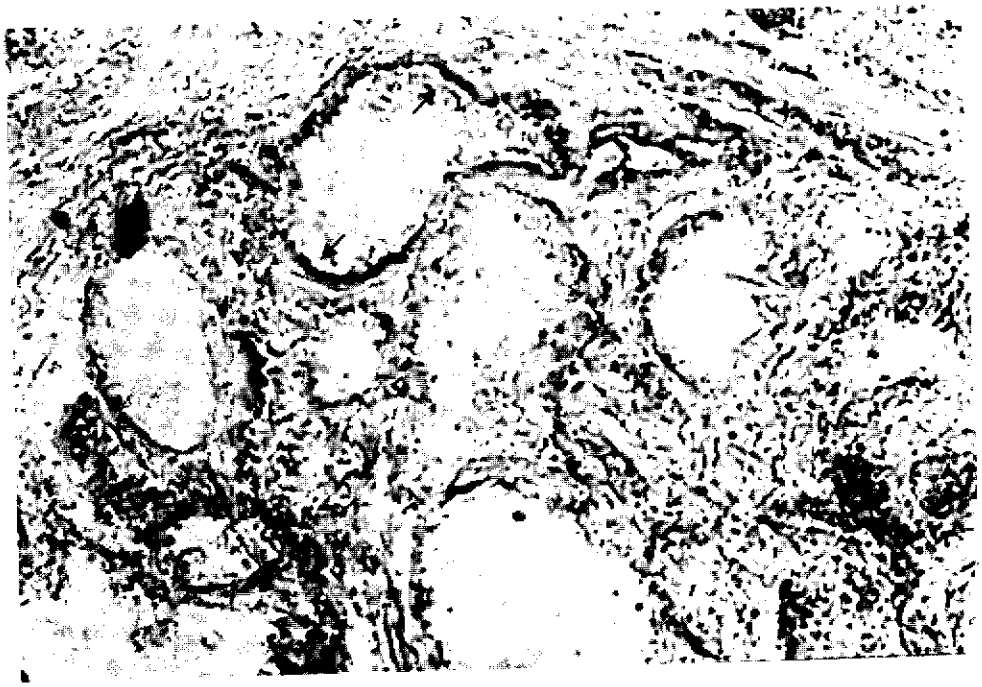


Fig. 8



Table

Table 1 Demographic characteristics of patients with Graves' disease

Characteristics	Untreated GD High TRAb (n=22) (≧ 50%)	Treated GD Low TRAb (n=22) (≦20%)
Age (years)	37.2±10.9	42.9±14.3
Men/women	6/16	1/21
sFas (ng/ml)*	1.56±0.26	0.76±0.26
sFasL (ng/ml)*	0.152±0.018	0.126±0.012
TRAb(%)*	63.8±12.5	7.9±5.8

TRAb: TSH receptor antibody; *statistically significant