

Immunohistochemical Evidence of Apoptosis in Different Cells of Hashimoto's Thyroiditis and Graves' Disease Thyroids

Hashimoto Tiroiditi ve Graves Hastalığında Farklı Hücrelerin Apoptosisi üzerine İmmunohistokimyasal Kanıtlar

Michèle Véronique El May, Soumeya Arafa, Zeineb Oueslati, Aida Mihiri, Faouzi Ben Slimane, Abderrahmen Ladghan, Ahmed El May

Faculty of Medicine, Histology Embryology, Tunis, Tunisia

*Institut Salah Azaiez, ImmunoHistoCytology, Tunis, Tunisia

**Institut Salah Azaiez, ORL, Tunis, Tunisia

****Institut Salah Azaiez, Nuclear Medicine, Tunis, Tunisia

Abstract

Objective: Hashimoto's (HT) and Graves' (GD) diseases are thyroid autoimmune diseases that share similar pathogenic mechanisms. Apoptosis is often related to an autoimmune process and is present in HT thyroid follicular cells. We compared the apoptosis characteristics in these two thyroid diseases considering three thyroid cell types, namely lymphocytes, follicular cells and macrophages.

Material and Methods: Nineteen surgically removed thyroids coming from fourteen patients suffering from HT and five patients from GD, have been processed for immunohistochemistry. Apoptosis has been revealed using a terminal deoxynucleotidyl transferase - mediated dUTP nick end-labeling (TUNEL) and an anti active caspase-3 antibody respectively showing DNA fragmentation and activation of the last apoptosis effector. Bcl2, bax and p53 proteins were also immunohistochemically investigated.

Results: Lymphocytic infiltration, TUNEL positivity, caspase-3 activation, p53 and bax immunoreactivities were much more present in HT than in GD. Lymphoid cells reacted with anti bcl2 antibody only, with different patterns in HT and in GD. In GD, almost all the follicular cells were immunoreactive with anti bcl2 antibody. In HT, some follicular cells in the vicinity of lymphoid follicles were TUNEL, active caspase-3 and bax positive; on the contrary, far from lymphoid follicles, the follicular cells were bcl2 positive. Our main result showed the presence of numerous macrophages in HT thyroid follicular lumina, expressing strong bax and p53 immunoreactivities along with bcl2, TUNEL and active caspase-3 negativities.

Conclusion: Apoptosis is rare in GD thyroids and characteristic of HT thyroid follicular cells in the vicinity of lymphoid follicles. In HT, macrophages initiated apoptosis in follicular lumina and seemed either to stop apoptosis before its end or to commit suicide by a caspase-3 independent pathway without DNA fragmentation. *Turk Jem 2007; 11: 73-8*

Key words: Apoptosis, autoimmune thyroid diseases, TUNEL, caspase-3, bax, bcl2, p53

Özet

Amaç: Hashimoto (HT) ve Graves (GH) hastalıkları tirodin otoimmün hastalıkları olup aynı patojenik mekanizmaları paylaşmaktadır. Apoptosis sıklıkla otoimmün olaylara bağlı olarak HT tiroid follikül hücrelerinde bulunmaktadır. Biz bu iki tiroid hastalığında üç tip tiroid hücrelerini lenfositler, follikül hücreleri ve makrofajları karşılaştırdık.

Gereç ve Yöntem: On-dokuz cerrahi olarak alınmış tiroid (14'ü HT, 5 hasta GH) immunohistokimyasal incelemeye alındı. Apoptosis "terminal deoxynucleotidyl transferase - mediated dUTP nick end-labeling" (TUNEL) ve anti aktif kaspaz 3 antikor kullanılarak sırasıyla DNA fragmentasyonunu ve son apoptoz etkeni aktivasyonu bakılarak apoptosis değerlendirildi. Bcl2, bax ve p53 proteinleri de immunohistokimyasal olarak araştırıldı.

Bulgular: Lenfositik infiltrasyon TUNEL pozitifliği, kaspaz 3 aktivasyonu, p53 ve bax immunoreaktivitesi HT'da GH'na göre daha fazla bulundu. Lenfoid hücreler yalnızca bcl2 antikoruyla HT ve GH'da farklı paternde reaksiyon gösterdi. GH'da hemen tüm folliküller anti bcl2 antikoruna ile immunoreaktifdi. HT'da lenfoid follikül civarındaki bazı follikül hücreleri TUNEL, aktif kaspaz-3, ve bax pozitifdi. Buna karşın lenfoid folliküllerden uzak folliküler hücreler bcl2 pozitif bulundu. Ana bulgumuz olarak HT'da tiroid follikül lumeninde çok sayıda kuvvetli bax ve p53 immunoreaktivitesi ve bcl2, TUNEL ve aktif kaspaz 3 negatifliği gösteren çok sayıda makrofaj varlığı gözlemlendi.

Sonuç: Apoptosis GH tiroidinde nadir olup, HT için karakteristik olarak lenfoid folliküller çevresinde olur. HT'da makrofajlar apoptosisi follikül lumeninde başlatıyor ve apoptosisi DNA fragmentasyonu olmadan kaspaz-3'den bağımsız bir yolda durduruyor görünmektedir. *Turk Jem 2007; 11: 73-8*

Anahtar kelimeler: Apoptosis, Otoimmün tiroid hastalıkları, TUNEL, caspase-3, bax, bcl2, p53

Introduction

Human thyroid autoimmunity involves several pathological patterns. Tissue destruction leading to hypothyroidism is characteristic of Hashimoto's thyroiditis (HT) while hyperthyroidism along with tissue stimulation is described in Graves's disease (GD). T cells are probably of critical importance in the induction, development and maintenance of these diseases but the events leading to the different disease forms are unclear (1).

In HT, a variety of different models have been proposed to account for immune cell destruction of thyroid cells. Each model assumes that some sort of immune response to thyroid autoantigens occurs and stimulates an immune cell infiltration into the thyroid gland: this step is called the initiator phase. The last phase, known as the effector phase, leads to thyroid cell death. However, the existence of inflammatory cell infiltrates in a thyroid does not inevitably lead to the thyroid destruction and some thyroiditis syndromes occur without gland destruction (2). The change from non-destructive thyroiditis to destructive thyroiditis occurs by an unknown mechanism.

Apoptosis or programmed cell death is a ubiquitous process by which cells commit suicide. It is characterized by blebbing of the cell membrane, chromatin condensation, cleavage of DNA into nucleosomal size fragments by endonuclease, fragmentation of the cell with retention of cell membranes. Apoptosis is induced by initiation from internal events within the cell (mitochondrial or intrinsic pathway) or on the cell surface (death receptor or extrinsic pathway) as a response to a variety of extracellular stimuli and is controlled by a complex interplay between numerous proteins. Apoptotic pathways are usually carried out by proteolytic activation of a series of cysteine dependent aspartate directed proteases called caspases. Activation of the last one, caspase 3, leads to irreversible cellular damages.

It is assumed that immune mediated cell death in a number of autoimmune endocrine diseases is due to induction of apoptosis in target organ cells. This was demonstrated for thyroid follicular cells in HT (3), but the mechanisms underlying this cell death are not clearly understood (4). Several hypotheses were put forward suggesting the role of death-signalling molecules expressed on thyroid cells (5). P53, bcl2 and bax are among the proteins that play a part in apoptosis. P53 mediates two biological processes, cell cycle arrest and apoptosis, which seem to be separate functions. In some diseases with normal p53 expression, the later abnormality is located in the Bcl2 family proteins (6). Bcl2 and bax are homologous proteins exerting opposed effects on cell life and apoptosis. While bcl2 inhibits apoptosis, bax promotes it. It has been shown that bcl2 homodimerizes and heterodimerizes with bax (7,8) and that their relative rates may determine whether or not a cell becomes apoptotic (8,9).

Apoptosis of thyroid follicular cells has been extensively studied but very little is known about apoptosis of other thyroid cells, that is why we looked for DNA fragmentation using TUNEL, caspase-3 activation and expression of p53, bcl2 and bax expressions in thyroids of HT and GD patients with particular attention to macrophages.

Material and Methods

Patients

Thyroid specimens were obtained from fourteen patients undergoing thyroidectomy for HT and five for GD at Salah Azaiz Institute of Cancer in Tunis. Female predominance was evident in both diseases: among HT patients 86 % were females ranging in age from 20 to 59 years (median age: 39 years) while in GD patients 80 % were females ranging in age from 31 to 66 years (median age: 48 years). All these patients presented with a suspicious nodular thyroid and had not been treated before. Disease duration extended from some months to several years, but was difficult to determine precisely. In HT patients, diagnosis was always determined by pathologists; serum hormone determinations revealed 4 cases of hypothyroidism, 2 of hyperthyroidism and 8 of euthyroidism. GD was diagnosed on the basis of histology and clinical and biological evidence of hyperthyroidism. Thyroid autoantibody status was rarely determined because nodules were suspected to be cancerous. Informed consent was obtained from all subjects and protocol was approved by the institutional board.

Immunohistochemical Staining

Formalin-fixed, paraffin-embedded tissues from HT and GD thyroids were used for CD68, CD20, CD3, DNA fragmentation, active caspase-3, bcl2, bax and p53 detection. Consecutive sections (3µm) were mounted on silane-coated slides. After deparaffinization with toluene and rehydration through ethanol series, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide. Antigen retrieval was carried out using microwave treatment (750W, 15 min) in a 0.01M sodium-citrate buffer (pH 6.0). Incubation time with the specific antibody depended on the antibody used: it lasted 30 min with anti CD68 (clone KD1, anti human macrophages; Dako, Denmark), anti CD20 (clone 26; Dako; for B-cells) and anti CD3 (polyclonal; Dako; for T-cells) antibodies. Sections were incubated overnight with anti active caspase-3 (pAb; Promega), anti bcl2 (clone 100; Immunotech), anti bax (clone 4F11; Immunotech) and anti p53 (clone MO-1; Immunotech, France) antibodies. Immunostaining was completed with avidin-biotin-peroxidase enzyme system and 0.03% 3-3' diaminobenzidine (DAB) - 0.1% hydrogen peroxide in Tris-HCl-NaCl buffer. Sections were counterstained with Mayer's hematoxylin. Double immunohistochemical staining was performed in order to detect p53 and CD68 in the same sections: p53 was revealed using peroxidase-DAB and CD68 using phosphatase-AEC.

TUNEL staining

A terminal deoxynucleotidyl transferase (TdT) - mediated dUTP nick end-labeling (TUNEL) was performed for in situ detection of apoptotic cells, using biotin-streptavidin-alkaline phosphatase system and BCIP/INT substrate (Enzo, USA) in order to label the 3'-OH DNA ends generated by DNA fragmentation in apoptotic cells. Quantification was performed independently by two persons on ten contiguous x400 fields.

A slide from each sample was stained with hematoxylin-eosin (HE) and negative and positive controls were routinely performed.

Results

Results are presented in Table 1 and in figures n°1, 2 and 3.

HT and GD morphological aspects

HT was characterized by lymphocyte and plasmocyte infiltrations in the majority of thyroid glands. This infiltration appeared diffuse, scattered or usually organized as lymphoid follicles with a germinal centre in place of thyroid follicles (figure 1a). Numerous normal thyroid follicles still existed in HT thyroids but sometimes their lumina contained cell clusters. In contrast, GD showed a milder lymphocytic infiltration and lymphocytes were scattered among the thyroid follicles. B and T lymphocyte distribution was not the same in HT and in GD. Seventy per cent of HT cases presented numerous lymphoid follicles with B cells restricted to the germinal centre and T cells to the periphery. T cells were also scattered throughout the thyroid follicles. In GD thyroids, the infiltration was scanty, diffuse and essentially formed by T lymphocytes. In these two auto-immune diseases, numerous CD 68

positive cells were detected (fig 1d and 2a). In HT they were frequently located within lumina of the thyroid follicles but they were also found in the thyroid stroma and in lymphoid follicles, both in the germinal centre and in the peripheral corona. Sometimes, they assembled under and inside the follicular epithelium.

Apoptotic cell detection

In HE stained sections from HT thyroids, some cells had characters of apoptotic cells: a shrunk dense nucleus, an eosinophilic cytoplasm and an irregular cell membrane. These characters were noticed in the follicular epithelium and inside lumina of thyroid follicles (figure 1a), especially around lymphocytic infiltrations. These cells were rare in intact follicles far from lymphoid follicles and in GD thyroid follicular lumina. In 11 TUNEL colored HT sections, more follicular thyroid cells (up to 64%) had brown-red-dish nuclei (figure 1b) than in the other HT and GD sections (only 2%), suggesting a stage of DNA fragmentation in these cells. Many of the positive cells showed shrunk nuclei but some cells were apparently preapoptotic because they showed no mor-

Table 1. TUNEL positivity and positive immunoreactivities to anti active caspase - 3, anti bcl2, anti bax and anti p53 antibodies in Hashimoto's thyroiditis and in Graves' disease thyroid

| | HASHIMOTO'S THYROIDITIS | GRAVES' DISEASE |
|--------------------|---|--|
| TUNEL | - Thyroid follicular cells | |
| Active caspase - 3 | - Thyroid follicular cells by lymphoid follicles - Rare lymphocytes | Rare thyroid follicular cells and lymphocytes |
| Bcl2 | - Lymphocytes except in the centre of lymphoid follicles - Thyroid follicular cells far from lymphocytic infiltrations | - Lymphocytes - Almost all thyroid follicular cells |
| Bax | - Thyroid follicular cells inside and by lymphocytic infiltrations - Macrophages in thyroid follicular lumina | - Rare stromal cells |
| P53 | - Numerous macrophages in follicular lumina | - Rare thyroid follicular and stromal cells |

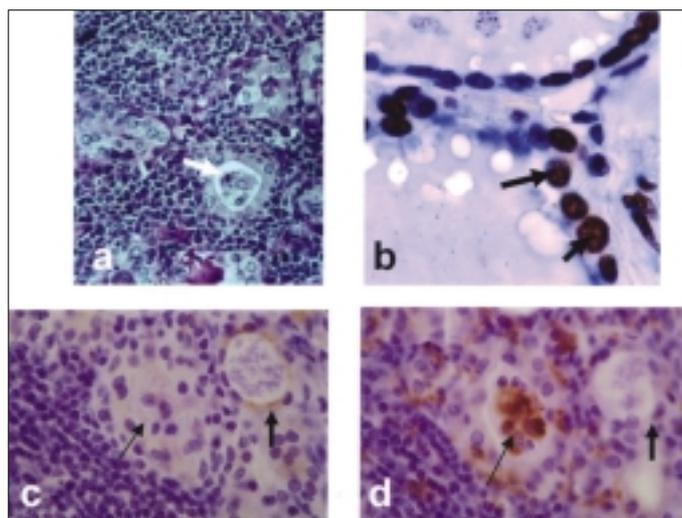


Figure n° 1. Apoptosis evidence in Hashimoto's thyroiditis thyroid
a: HE (x 400): white arrow points to thyroid follicular lumen containing four cells, one of them with a dense shrunk nucleus. b: TUNEL (x1 000): Positive thyroid follicular cells show brown reddish nucleus (arrows). c,d: homologous fields of contiguous sections coloured with anti active caspase-3 (c) and anti CD68 (d) antibody (x1 000): macrophages located in a thyroid follicular lumen (slim arrow) are caspase-3 negative and epithelium of an other thyroid follicle (thick arrow) is caspase-3 positive

phological characteristics of apoptosis. We noted a high heterogeneity in the reactivity between the cases, and in a same case, from place to place. Lymphocytes and cells located inside thyroid follicular lumina never stained with TUNEL.

In HT, active caspase-3 was detected in thyroid follicular cells (fig 1c), but with a heterogeneity in reactivity between the cases and in a same case, from place to place. These reactive cells were preferentially located nearby lymphoid follicles, and observation of contiguous serial sections showed that CD68 positive cells located inside thyroid follicular lumina never stained with anti active caspase-3 antibody. The epithelium of thyroid follicles that contained CD68 positive cells was not stained with anti active caspase-3 antibody. A few lymphoid cells were stained with this antibody in HT and GD as were some rare follicular thyroid cells in GD.

Detection of Apoptosis Related Proteins

Intensity and distribution of bcl2 positive cells depended on the type of cells and on the disease concerned. In sections from HT thyroid glands, follicular cells in the vicinity of lymphoid follicles showed significantly weak or absent staining for bcl2 (figure 2a). On the contrary, the cytoplasm of follicular cells far from lymphocytic infiltrates showed a strongly positive staining for bcl2. In addition, the lymphocytes forming the periphery of the lymphoid follicles were strongly stained with bcl2 antibody (figure 2a) as were some lymphocytes situated in the lumina and epithelium of

some thyroid follicles. In contrast, the lymphocytes present in the germinal centres weakly expressed bcl2. In sections from GD thyroid glands, follicular thyroid cells showed a strong bcl2 immunoreactivity as did lymphocytes (figure 2c). In sections from HT thyroid glands, follicular cells were usually weakly stained with bax antibody but immunostaining intensity was more important in the vicinity of lymphoid follicles (figure 2b). On the contrary, in GD sections follicular cells were not bax immunostained (figure 2c). Observation of contiguous serial sections stained with anti CD68 and with anti bax revealed that in HT, numerous macrophages (CD68 positive cells) located in follicular lumina and in the interfollicular stroma strongly expressed bax (figure 3c, d) as did some macrophages situated in the lymphoid cell infiltration. Bax staining showed a granular aspect in these CD68 positive cells.

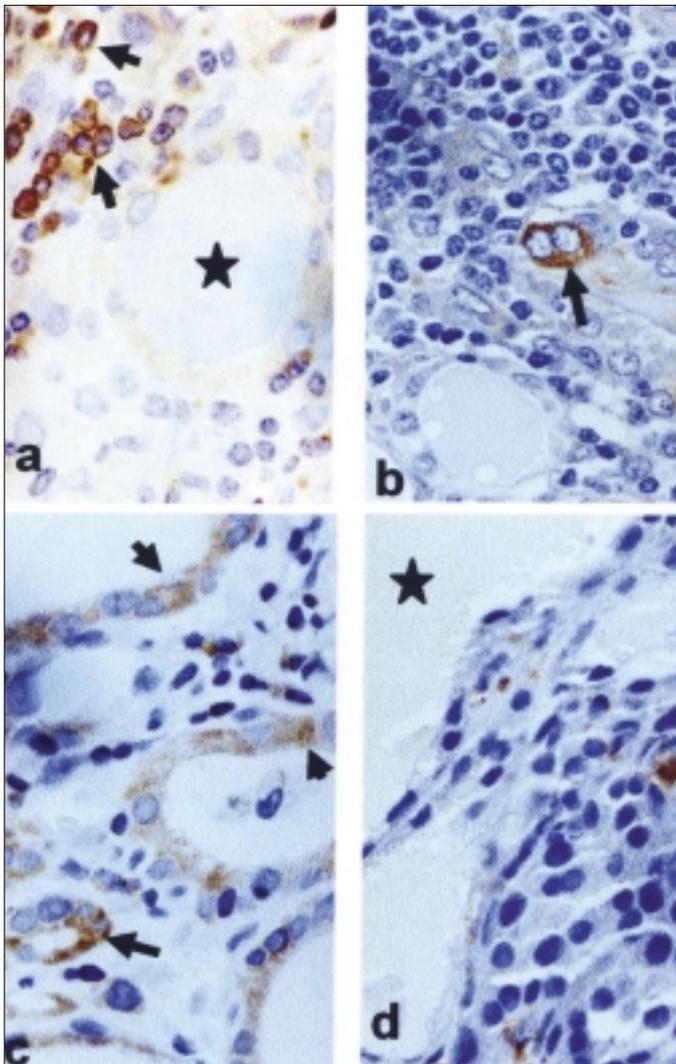


Figure n° 2. Bcl2 and bax immunoreactivities in Hashimoto's thyroiditis and Graves's disease thyroid

HT(a, b) and GD (c, d) thyroid sections

a: anti bcl2 antibody (x1 000): brown lymphoid cells are bcl2 immunoreactive (arrows) – b: anti bax antibody (x1 000): two thyroid follicular cells by lymphoid follicle corona are bax immunoreactive (arrow) .

c: anti bcl2 antibody (x 1 000): almost all thyroid follicular cells are bcl2 immunoreactive (arrows) – d: anti bax antibody (x 1 000): no bax immunoreactivity. The stars localize thyroid follicular lumina

In GD, the nuclei of thyroid follicular cells and stromal cells, weakly stained with p53 antibody. Lymphocytes and HT thyroid follicular cells never stained with p53 antibody. Frequent cells exhibited a strong nuclear p53 immunoreactivity and were clustered in thyroid follicular lumina (figure 3b). Staining intensity and density of these p53 positive cells were about five times higher in HT. Using adjacent serial sections and double immunohistochemical staining (figure 3b), we found that p53 positive cells located in the follicular cavity also exhibited positive staining for CD68 in their cytoplasm suggesting that they were macrophages.

Discussion

Thyroid related autoimmune disorders comprise more than 30 % of all organ specific autoimmune diseases and exhibit a wide range of symptoms. HT and GD represent the most prevalent and well-characterized forms of thyroid autoimmunity wherein abnormal cellular and humoral immune response to thyroid follicular antigens are seen.

Like others (10), we noticed a common histopathologic feature in the thyroid glands of our patients suffering from HT, the massive lymphocyte infiltration usually organized in lymphoid follicles. On the contrary, in GD this lymphoid infiltration was diffuse. Lymphoid infiltration was phenotypically characterized by immunohistochemistry. We found that in both diseases it was formed essentially by T cells contrary to Hammond LJ et al (11) according to whom B and T lymphoid cells were equally represented. This finding was probably due to the limited number of their HT cases (only three). Lymphocyte organization changed according to the disease type. In HT, we found the usual organization for lymphoid follicles (11): a germinal centre consisting essentially of B cells and a peripheral corona made up of a mixture of B and T cells.

In this study, apoptosis was investigated in three cell types (lymphocytes, thyroid follicular cells and macrophages), in HT and GD thyroids. The histological techniques used for apoptosis evidence were: DNA fragmentation using TUNEL staining, activation

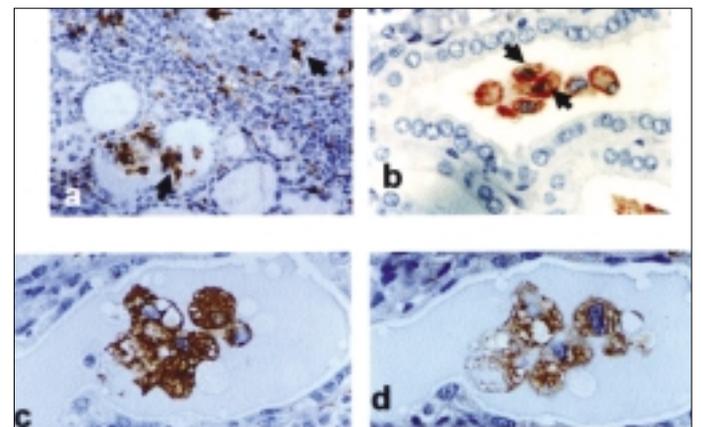


Figure n° 3. Macrophages in Hashimoto's thyroiditis thyroid

a: CD68 antibody (x 1 000) – note the presence of numerous macrophages in lymphoid follicles at the upright side and in thyroid follicular lumina (arrow), b : p53 (brown nuclei) and CD68 (red cytoplasm) immunoreactivities of macrophages in thyroid follicle lumina (x 1 000), c, d: homologous fields of contiguous sections coloured with anti CD68 (c) and anti bax (d) antibodies

of caspase-3 and expression of three proteins bcl2, bax and p53 using specific antibodies. In HT, lymphocyte staining for bcl2 was in accordance with the previously reported pattern (12), i.e. negative staining of germinal centre lymphocytes and positive staining of coronal zone lymphocytes usually found in lymphoid organs (13). In HT, as well as in GD, diffusely infiltrating lymphocytes were also strongly positive for bcl2, as reported by others (14) and lymphoid cells were negative for TUNEL, active caspase-3, bax and p53, suggesting that they had not undergone apoptosis. In all cases of HT and GD the majority of thyroid follicular cells expressed bcl2 much more than bax. But in HT some follicular cells adjacent to lymphoid follicles showed a markedly decreased bcl2/bax ratio, thereby becoming more vulnerable to apoptotic stimuli, and undergoing apoptosis as suggested by TUNEL and active caspase-3 positivities. These data agree very well with results from other studies (5,11,14-17). But we must emphasize that we found heterogeneous results in follicular cell staining with TUNEL and caspase-3 probably because, though apoptosis is a rapid process, it takes a long time to spread through the whole thyroid. This result suggests that regional stimuli are necessary to induce localized apoptosis. Moreover, as positive staining was frequently found in the vicinity of lymphoid follicles, these regional stimuli probably had an immunological origin. Immunostaining of thyroid follicular cells with p53 was low or absent in the two diseases, because thyrocytes do not usually undergo apoptosis by this pathway but by other pathways such as Fas-Fas ligand (16,18, 19) and tumor necrosis factor-related ligand (e.g.: TRAIL and DR3) (20).

The outstanding result of our study was the presence of numerous macrophages in HT cases, particularly in the lumina of thyroid follicles probably as a consequence of immune events (4). The majority of these macrophages exhibited a strong staining for p53 and bax and were negative for bcl2, suggesting an apoptotic process, but were also negative for TUNEL and active caspase-3. Two different hypotheses may explain these contradictory results: either macrophage apoptosis was inhibited after expression of p53 and bax, or the macrophages used a caspase-3 independent pathway without DNA fragmentation to commit suicide. P53 and bax expressions commonly occur in macrophage apoptosis. It has been demonstrated that p53 may inhibit innate immune responses and so plays a crucial role in the pathogenesis of autoimmune diabetes and in the regulation of macrophage innate functions (21). Causative factors include reactive oxygen and nitrogen species such as nitric oxide (NO) and oxygen peroxide (H₂O₂). Nitric oxide is known to play an important role in the pathogenesis of organ specific autoimmunity (22) and elevated NO synthesis follows activation of macrophages (23). Short-lived NO induces rapid apoptosis in macrophages by increasing the levels of p53 and bax expressions (24) before cytochrome c (25) and apoptosis-inducing factor release (26), activation of caspases and DNA fragmentation. On the contrary, continuously supplied NO (27) or H₂O₂ (28) contribute to inhibition of caspases-3 and -8 and to up-regulation of the inhibitor of apoptosis proteins (27). In HT, the thyroid macrophages entering the follicular lumina may be continuously exposed to H₂O₂ released by thyroid follicular cells as a consequence of thyroglobulin synthesis. Thus, macrophages may stop their apoptotic process after its initiation. Considering the

second hypothesis, macrophages may commit suicide using a caspase-3 independent pathway without DNA fragmentation. This hypothesis has to be explored, as this type of apoptosis has been described (29,30) in other cell types. This may explain our apparent contradictory results for macrophages. The question is why so many macrophages move to the thyroid follicular lumina. Is it to initiate thyroid follicular cell apoptosis or to commit their own suicide? Our findings show the complexity of the physiological mechanisms involved in apoptosis regulation, depending on cell type and pathological state, and they also confirm that HT and GD are two different autoimmune thyroid diseases with very different pathophysiology.

Acknowledgments

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