Expression of Bcl-2 and Bax Proteins in Thyroid Glands of Rats in Experimental Thyroiditis

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In the recent years, iodine was associated to the development of apoptosis in thyroid diseases. The aim of the present study is to determine the expression of pro-apoptotic and anti-apoptotic proteins, Bax and Bcl-2, in a Wistar rat experimental model of thyroiditis induced by administration of different doses of potassium iodide. Immunohistochemical staining was done with chromogen diaminobenzidine on avidin-biotin peroxidase using the Animal Research Kit (ARK), stained with antibodies to Bcl-2 and Bax proteins. The intensity and distribution of positive staining were evaluated by light microscopy on a scale of 0 to 4. Bax protein was expressed in the area of regenerating follicular cells in high percent in potassium iodide treated rats, but was not expressed in thyrocytes from control rats. Bcl-2 expression was constantly observed in thyrocytes of the control group and in the mantle-zone of lymphoid follicular infiltrates. Our results show that Bax expression is significantly higher in the Wistar rat experimental model of thyroiditis than in the control group. These data suggest that the increased expression of Bax may contribute to the role of apoptosis in the pathogenesis of experimental thyroiditis.

Key words: Bcl-2, Bax, thyroid glands, experimental thyroiditis, potassium iodide.

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Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases or cathepsins), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic) (KROEMER *et al.* 2009; GALLUZZI *et al.* 2007). Apoptosis and autophagy are genetically-regulated, evolutionarily-conserved processes that regulate cell fate. Both apoptosis and autophagy are important in development and normal physiology and in a wide range of diseases. The functional relationship between apoptosis and autophagy is complex and recent studies show that despite the marked differences between these two processes, their regulation is connected and the same regulators can sometimes control both apoptosis and autophagy (SCARLATTI *et al.* 2009; THORBURN 2008; LOCKSHIN & ZAKERI 2004; LEVINE & YUAN 2005).

Also, recent evidence suggests that the initiation of apoptosis might actively suppress necrosis because activated caspases cleave and inactivate proteins required for programmed necrosis (EDINGER & THOMPSON 2004). In many cases, morphological features of autophagic and apoptotic cell death or autophagic and necrotic cell death are observed in the same cell. A possible explanation for high levels of autophagy in dying cells is that it is a clean-up or self-clearance mechanism in cells committed to die by apoptosis or necrosis (LEVINE & YUAN 2005).

There are several distinct subtypes of apoptosis that, although morphologically similar, can be triggered through different biochemical routes (KROEMER *et al.* 2009). Most drugs, including cancer chemotherapeutic agents, chemicals, irradiation as well as growth factor withdrawal, induce apoptosis by activation of the intrinsic pathway. Members of the Bcl-2 family control the integrity of the outer mitochondrial membrane (VOGLER *et al.* 2009). Anti-apoptotic proteins such as Bcl-2 and Bcl-xL appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing cytochrome C release and mitochondrial-mediated cell death initiation, whereas the pro-apoptotic proteins Bax and Bak promote the mitochondrial release of cytochrome C (SOPOTYK & ROGOWSKI 2004; TSATSOULIS 2002; KADENBACH *et al.* 2004; MITSIADES *et al.* 2003). Bax expression induces a massive increase in intracellular reactive oxygen species (ROS) concentration. ROS production is linked to cytochrome C release (PRIAULT *et al.* 2003). The relative levels of pro- and anti-apoptotic proteins determine a cell's susceptibility to apoptosis (LAWEN 2003). There is a possibility that Bcl-2 family members may function as oncogenes not only by blocking apoptosis but also blocking autophagy (LEVINE *et al.* 2008; PATTINGRE & LEVINE 2006; LEVINE & YUAN 2005). In the absence of apoptosis, autophagy can trigger a form of cell death known as autophagic cell death (CODOGNO & MEIJER 2005) and conversely, if during nutrient starvation the adaptive functions of autophagy are blocked, the result is accelerated death by apoptosis, which can be retarded by depleting Bax/Bak or inhibiting caspases (VICENCIO *et al.* 2008; MAIURI *et al.* 2007).

In addition to its role as a substrate for thyroid hormone biosynthesis, iodine participates in a number of clinically important interactions with the thyroid. Acute administration of large doses of iodine determines a biphasic response of the thyroid. The mechanism of relative blockade of or-ganic iodide yield, known as the Wolff-Chaikoff effect (WOLFF & CHAIKOFF 1948), is in part unknown and in part caused by the biochemical effects of a large concentration of the reactive form of iodide generated by the oxidative mechanism. In recent years, iodine has been associated to the development of apoptosis in thyroid diseases. However, few studies on experimental thyroiditis induced by iodide exist.

The aim of the present study is to determine the expression of pro-apoptotic and anti-apoptotic proteins Bax and Bcl-2 in the Wistar rat experimental model of thyroiditis induced by administration of different doses of potassium iodide (KI), which can be regarded as a model of Hashimoto's thyroiditis and thus clarify the role of apoptosis in the pathogenesis of experimental thyroiditis.

Material and Methods

Wistar male rats, 6-8 weeks old, were divided into 3 groups for intraperitoneal (i.p.) administration of potassium iodide (KI) during a period of 26 days:

– group A – 22.5 μg/100g body weight (bw), n = 5;

– group B – 3 x 22.5 μg/100g (bw), n = 6;

– group C – rats treated with bovine serum albumin (BSA) – control group, n = 5.*

The rats' weights were measured at the beginning of the experiment while thyroid gland weights were measured at the end of experiment, after killing the rats. The animals were killed under ether anesthesia by bleeding on the 26th day of KI treatment.

The sera for hormone analyses had been taken after blood sampling from the heart on the 26th day of KI treatment and were kept at –18°C until hormone determination.

The determinations of T3 and T4 were carried out by radio-immune analysis (RIA) according to the method of the Institute for Application of Nuclear Energy in Agriculture (INEP, Zemun). The INEP's RIA (PEG) was intended for determination of total T4 and T3, i.e. the sum of the free and the bound hormones. The reference values of the method for T4 are from 55 to 150 nmol/l. The reference values of the method for T3 are from 1.14 to 1.40 nmol/l. TSH was determined by INEP IRMA TSH. This is the diagnostic complex intended for
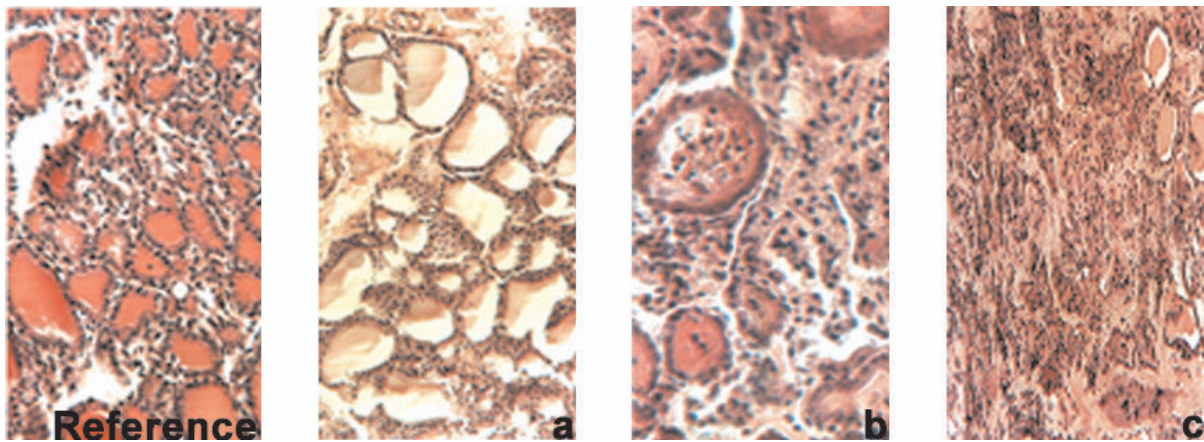

Fig. 1. The extent of lymphocytic infiltration and follicle destruction stained with H&E (x40). Reference – normal untreated thyroid gland; a – thyroid gland immunized with BSA (control group C); b – thyroid gland after administration of 22.5 μg KI/100 g body weight (A group); c – thyroid gland after administration of 3 x 22.5 μg KI/100 g body weight (B group).

Fig. 2. Bcl-2 gene expression at normal untreated thyroid gland, after administration of different doses of KI and control group immunized with BSA (x40). Reference – normal untreated thyroid gland; a – thyroid gland after administration of 22.5 μg KI/100g body weight (A group); b – thyroid gland after administration of 3 x 22.5 μg KI/100g body weight (B group); c – thyroid gland immunized with BSA (C group).
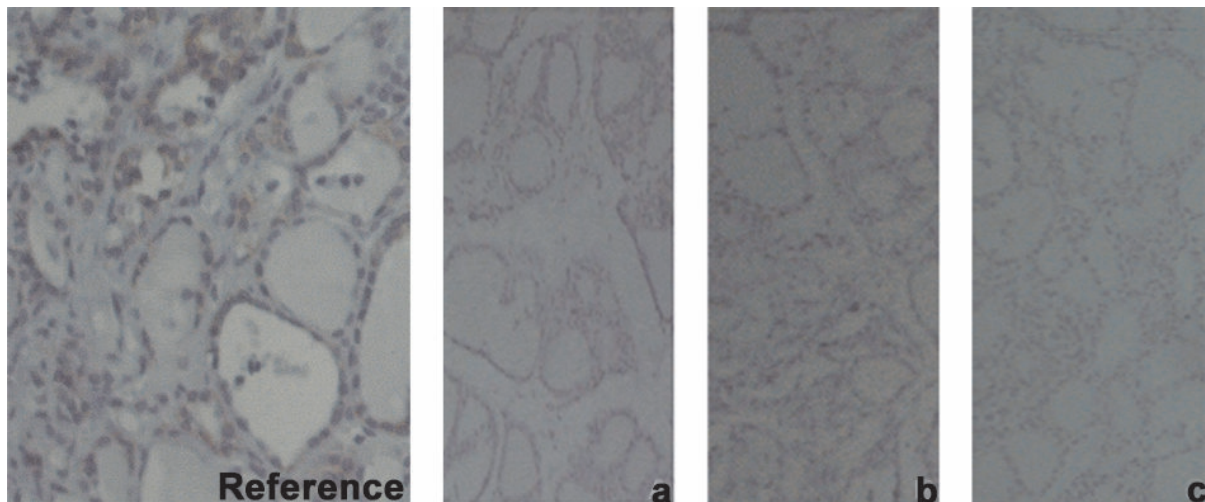

Fig. 3. Bax gene expression at normal untreated thyroid gland, after administration of different doses of KI and control group immunized with BSA (x40). Reference – normal untreated thyroid gland; a – thyroid gland after administration of 22.5 μg KI/100g body weight (A group); b – thyroid gland after administration of 3 x 22.5 μg KI/100g body weight (B group); c – thyroid gland immunized with BSA (C group).
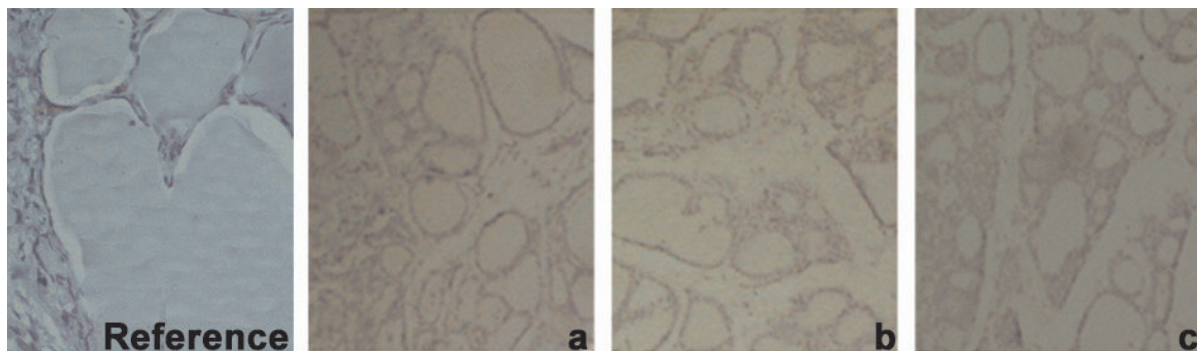
the quantitative immune radiometric determination (IRMA) of TSH in serum. The normal values for TSH by this method are up to 6 mIU/l and the sensitivity of the test is 0.035 mIU/l.

Under light microscopy (40x) the intensity of histological lesions, stained with hematoxylin and eosin (H&E), was graded from 0 to 4 (SUGIHARA *et al.* 1990) in the following manner: 0 – normal structure of the gland; 1 – one or two focuses of infiltration; 2 – three or more focuses of infiltration; 3 – diffuse infiltration with scattered destruction of the follicles; 4 – diffuse infiltration with massive destruction of the follicles and infiltration by the connective tissue.

Immunohistochemical staining was performed using chromogen diaminobenzidine (DAB) on avidin-biotin and peroxidase methodologies using the Animal Research KIT, Peroxidase (The DAKO ARK) on formalin-fixed, deparaffinized and rehydrated specimens by rabbit policlonal IgG Ab sc-526 (Santa Cruis Biotechnology Inc.) raised against a peptide mapping at the amino terminus of mouse origin and stained with antibodies to: Bcl 2 oncoprotein (Monoclonal Mouse Antihuman Bcl 2 Oncoprotein, Clone 124 ND) and Bax (p-19) SC-S26, rabbit polyclonal IgG. The negative control was the DakoCytomation Antibody Diluent. We evaluated, by light microscopy, the intensity and distribution of positive DAB staining of thyrocytes and lymphocytes on a scale of 0 to 4 (0 = 0%; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; and 4 = 76-100% of thyrocytes/lymphocytes) by two independent observers, whose agreement was almost complete (MITSIADES *et al.* 1998).

Statistical Analysis

Values of hormones and weights of animals and thyroids are expressed as mean ± SEM and given in Tables 1, 2 and 3. Tests of significant differences in individual parameters between all groups were conducted by the variance analysis method (ANOVA). Inter-group testing was carried out using the Student *t*-test. The results are given in the same Tables (1, 2 and 3). P values of less than 0.05 were considered significant.

Results

After potassium iodide administration, the thyroid shows the extent of lymphocytic infiltration and follicle destruction, stained with H&E (Fig. 1). According to SUGIHARA *et al.* (1990), Figure 1Reference is a normal, untreated thyroid gland (grade 0); Figure 1a is group C – control group of rats treated with bovine serum albumin

(grade 2); Figure 1b is group A – experimental group of rats treated with 22.5 μg KI/100g body weight (grade 3) and Figure 1c is group B – experimental group of rats treated with 3 x 22.5 μg KI/100g body weight (grade 4). These figures clearly show that higher doses of potassium iodide cause more extensive destruction of the thyroid.

Due to follicle destruction, values of thyroid hormones are lower in groups A and B than in control group C (P<0.05). Due to the destruction of follicles, diffuse lymphocytic infiltration and infiltration by the connective tissue, thyroid glands in group A and B (especially in group B) are significantly heavier than in group C (Tables 1 & 2).

There is no significant difference among values of thyroid hormones from group A and B, although a significant difference exists among the weights of thyroid glands from these groups (Table 3).

Bax gene expression was significantly higher in rats with potassium iodide induced lymphocytic experimental thyroiditis and correlated with the extent of lymphocytic infiltration. The Bax protein was expressed in the area of regenerating follicular cells in high percent in potassium iodide treated rats, but was not expressed in thyrocytes from control rats. Bcl-2 expression was constantly seen in thyrocytes of the control group and in the mantle-zone of lymphoid follicular infiltrates (Table 4, Figs 2 & 3).

Discussion

Normal thyroid glands show a low level of apoptosis, suggesting a role of this process in basal thyroid cell turnover (ARSCOTT & BAKER 1998). In contrast, thyroid cells undergoing apoptosis (as determined by immunohistochemical and morphological analyses) occur with increased frequency in thyroids from patients with destructive thyroiditis (BRETZ & BAKER 2001; STASSI & DE MARIA 2002). Immunohistochemical studies of thyroid tissue sections revealed an increased number of apoptotic follicular cells in Hashimoto's thyroiditis, most of them on the periphery of infiltrating lymphocytes (ANDRIOKULA & TSATSOULIS 2001).

The increased expression of Bax in thyrocytes and lymphocytes in our study agrees with findings of KOGA and colleagues (1999). Their study confirmed that normal thyroid follicular cells expressed Bcl-2 and Bak, but not Bax, suggesting that Bcl-2 and Bak expression in differentiated normal thyroid follicular cells might regulate apoptosis in the physiological state. In contrast, in the area of granulomatous changes in subacute thyroiditis, Bax expression was detected in follicu-

Table 1

Values of hormones and weights of animals and thyroids between A and C group (all data presented as mean ± SEM); bw – body weight

	Group A	Group C	P value
weight of animal	272.21 ± 15.35	268.32 ± 16.51	P>0.05
weight of thyroid (mg/100 g bw)	6.04 ± 0.09	4.25 ± 0.05	P<0.05
T3 (nmol/l)	0.76 ± 0.02	1.24 ± 0.06	P<0.05
T4 (nmol/l)	62.53 ± 4.21	123.06 ± 7.08	P<0.05
TSH (mIU/l)	0.17 ± 0.01	0.21 ± 0.013	P>0.05

Table 2

Values of hormones and weights of animals and thyroids between B and C group (all data presented as mean ± SEM); bw – body weight

	Group B	Group C	P value
weight of animal	241.84 ± 12.82	268.32 ± 16.51	P>0.05
weight of thyroid (mg/100 g bw)	7.86 ± 1.02	4.25 ± 0.05	P<0.05
T3 (nmol/l)	0.80 ± 0.03	1.24 ± 0.06	P<0.05
T4 (nmol/l)	60.07 ± 4.07	123.06 ± 7.08	P<0.05
TSH (mIU/l)	0.16 ± 0.002	0.21 ± 0.013	P>0.05

Table 3

Values of hormones and weights of animals and thyroids between A and B group (all data presented as mean ± SEM); bw – body weight

	Group A	Group B	P value
weight of animal	272.21 ± 15.35	241.84 ± 12.82	P>0.05
weight of thyroid (mg/100 g bw)	6.04 ± 0.09	7.86 ± 1.02	P<0.05
T3 (nmol/l)	0.76 ± 0.02	0.80 ± 0.03	P>0.05
T4 (nmol/l)	62.53 ± 4.21	60.07 ± 4.07	P>0.05
TSH (mIU/l)	0.17 ± 0.01	0.16 ± 0.002	P>0.05

Table 4

Intensity of positive DAB staining of thyrocytes and lymphocytes

	Group A		Group B		Group C	
	thyrocytes	lymphocytes	thyrocytes	lymphocytes	thyrocytes	lymphocytes
Bcl-2	2	0	1	1	1	0
Bax	2	1	2	4	0	1

lar cells and inflammatory cells. These data suggest that the increased expression of Bax may contribute to apoptosis in the lesion. Apoptotic nuclei were found in granulomas, especially in macrophages/histiocytes and lymphocytes, and in the regenerating follicular cells, but were rarely found in the area of fibrosis. The mean percentage of apoptotic follicular cells was significantly greater in subacute thyroiditis than in controls. Xu and colleagues (2007) also found an increased expression of Bax in thyrocytes. They demonstrated that the rates of cell apoptosis in rat thyroid cells of 10, 50 and 100 mmol/l iodine groups increased significantly compared with the normal control group. Moreover, the mRNA levels of FasL and Bax in 10 and 50 mmol/l iodine groups increased significantly while the level of Bcl-2 decreased significantly. They concluded that excessive iodine treatment increased apoptosis of thyroid cells which might be related to gene expression of Fas, FasL, Bcl-2 and Bax.

According to Mitsiades et al. (1998), follicular cells in Hashimoto's thyroiditis undergo apoptosis by concomitant up-regulation of FasL and Fas and down-regulation of the Bcl-2 protein. Lymphocytes do not seem to be directly engaged in the process with their own FasL, but they may provide the appropriate cytokine milieu that, in turn, up-regulates Fas and/or FasL leading to apoptosis. Kroemer et al. (2007) suggest that overexpression of Bcl-2 can prevent or retard necrotic cell death. On the other hand, Soeki et al. (2000) consider Bcl-2 to be essential for leukemic cell survival and its down-regulation results in autophagy. Our results (we did not detect down-regulation of Bcl-2), also support the role of apoptosis, not autophagy or necrosis, in the pathogenesis of changes in our experimental model of thyroiditis.

In our study, values of TSH in experimental groups are lower than in the control group. Our results agree with *in vitro* studies that have shown that TSH was able to inhibit Fas-mediated apoptosis in a dose-dependent manner, whereas withdrawal of TSH resulted in the induction of apoptosis (Tsatsoulis 2002).

Besides Bax, Bcl-2, Fas/FasL system and TSH (Tsatsoulis 2002), iodine may also be involved in the death of thyrocytes and the control of thyroid gland mass. High doses which patients receive during ^{131}I therapy immediately destroy thyroid tissue by simple necrosis. The neighboring tissue which survives and receives sub lethal damage by ^{131}I may undergo apoptotic changes by activating mitochondrial apoptosis. Such tissue destruction would occur later, after the activation of the apoptotic pathway (Sopotyk & Rogowski 2004). High doses of iodine induce cell toxicity both in *vivo* and *in vitro*, possibly due to an excessive production of free radicals. The role of iodine in inducing apoptosis was also investigated in thyroid cell cultures (Andrikoula & Tsatsoulis 2001). Involution of the thyroid gland has been described in rats fed with an iodine rich diet (Burikhanov & Matsuzaki 2000). The iodine-induced cytotoxic effect on rat thyrocytes included necrotic and apoptotic features, indicating the involvement of a controlled process of cell death. Studies of Vitale et al. (2000) have shown that excess molecular iodide, generated by oxidation of ionic iodine by endogenous peroxidases, induces apoptosis in thyroid cells, through a mechanism involving generation of free radicals, and possibly mitochondrial damage and cytochrome C release.

The experimental model for inducing thyroiditis in Wistar rats shows the significance of iodine effect as an initial mechanism of this disease. The mechanism of potassium iodide effect is unknown, but its effect of completely destroying the structure of thyroid gland leads to changes in expression of apoptotic proteins. The increased expression of Bax after iodine administration in our study suggests the involvement of apoptosis in the pathogenesis of experimental thyroiditis in Wistar rats. This observation agrees with the opinion of Levine et al. (2008) that Bax and Bak are not implicated in the regulation of autophagy. Lockshin and Zakeri (2004) suggest that cells preferentially die by apoptosis but will die by any alternative available route, including autophagy, if exposed to harsh enough stimuli. According to all these findings and our results, we think that the increased expression of Bax after potassium iodide administration and the excess iodide induce apoptosis in thyroid cells through a mechanism involving the generation of free radicals, and possibly mitochondrial damage and cytochrome C release.

Acknowledgements

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