

# Cell necrosis and apoptosis are differentially regulated during goitre development and iodine-induced involution

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## Abstract

Necrosis and apoptosis coexist in the thyroid during goitre development and involution, but little is known about their respective causes. To test the possible role of free radicals, we analysed separately necrosis and apoptosis in male Wistar rats with depressed or normal antioxidant protection. Vitamin E-deficient and -sufficient rats were made goitrous with perchlorate in drinking water; involution was induced by repeated injection of NaI, without or with methimazole. Increase of thyroid malondialdehyde concentration and decrease of glutathione peroxidase activity confirmed the depressed antioxidant protection in vitamin E-deficient rats. Plasma thyroxine and TSH levels were not modified. Necrosis (swollen cells) and apoptosis (pyknotic cells) were quantified on histological sections. In vitamin E-sufficient rats, dead cells were very rare in control thyroids, increased 3-fold in goitre and still further during involution.

Necrotic epithelial cells predominated in the goitre and their number declined after iodide supplementation, with or without methimazole. In contrast, the number of apoptotic cells and the caspase-3 activity were increased in goitre and further increased after involution, with two-thirds of pyknotic cells being observed in the interstitium. Apoptosis was prevented by methimazole. Vitamin E deficiency significantly increased total cell death and epithelial cell necrosis and induced the occurrence of much cell debris in the follicular lumen during involution, with no modification of the apoptotic reaction. These results show that the type of cell death is differentially regulated during goitre development and involution: necrosis is related to the oxidative status of the cells, while apoptosis comes with iodine-induced involution.

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## Introduction

Cell proliferation, a hallmark of goitre development, results from increased thyrotropin (TSH) secretion associated with the release of growth factors acting as paracrine or autocrine agents (for review see Derwahl & Studer 1998). Cell death plays a central role in regulating the thyroid cell mass, especially during the goitre involution induced by iodine or by thyroid hormones (Wollman *et al.* 1968, Denef *et al.* 1981, Rognoni *et al.* 1987). In addition, a balance between cell death and proliferation has also been reported during goitre development itself (Tamura *et al.* 1998). Indeed, stimulated thyrocytes could be considered as being under oxidative stress: high TSH plasma levels activate H<sub>2</sub>O<sub>2</sub> production (for review see Dumont *et al.* 1992) which largely exceeds its consumption by thyroid hormone synthesis (Corvilain *et al.* 1991). H<sub>2</sub>O<sub>2</sub> is predominantly produced at the apical plasma membrane but can easily diffuse through the membrane

and provoke direct damage to cytoplasmic macromolecules, and even to DNA.

Two types of cell death have been described in the thyroid gland during goitre involution (Mahmoud *et al.* 1986). Necrosis, or accidental cell death, is characterized by the swelling of the cell, affecting both cytoplasm and nucleus. In contrast, apoptosis, or programmed cell death, corresponds to cytoplasmic shrinkage and nuclear densification (pyknosis). The physiopathology of either type of cell death differs completely. Apoptosis suppression may contribute to thyroid proliferative diseases including goitre, Grave's disease and cancer (Tanimoto *et al.* 1995). In contrast, necrosis may be involved in the development of autoimmune thyroiditis, favoured upon high iodine supply (Bagchi *et al.* 1995, Many *et al.* 1995), and in the development of congenital cretinism upon selenium deficiency (Contempré *et al.* 1993, 1995).

An acute toxic effect of iodide has been demonstrated *in vivo* in hypothyroid hamsters (Follis 1964), dogs

(Belshaw & Becker 1973), mice (Wollman *et al.* 1968, Mahmoud *et al.* 1986) and rats (van den Hove-Vandenbroucke *et al.* 1982, Contempré *et al.* 1995, Many *et al.* 1995), in obese chicken (Bagchi *et al.* 1995) and in human thyroid follicles *in vitro* (Many *et al.* 1992).

Intrathyroidal apoptotic cells were first detected in human chronic autoimmune thyroiditis by Kotani *et al.* (1995) and Tanimoto *et al.* (1995). More recently, apoptosis has been reported during goitre formation in rats, with a further increase during the early stage of goitre involution with normal (Patel *et al.* 2000) or high iodine doses (Tamura *et al.* 1998), but the respective roles of apoptosis and necrosis were not quantified.

*In vitro*, apoptosis can be induced in thyrocytes under various culture conditions: deprivation of TSH and serum (Dremier *et al.* 1994), addition of cytokines (Kawakami *et al.* 1996, Bretz *et al.* 1999), direct exposure to H<sub>2</sub>O<sub>2</sub> (Riou *et al.* 1999), and even iodide in excess (Feldkamp *et al.* 1999, Vitale *et al.* 2000). Apoptosis results also from the loss of normal integrin–fibronectin interactions (anoikis) (Di Matola *et al.* 2000), and is prevented by TSH (Li *et al.* 1999, Sato *et al.* 1999). In addition, when thyroid follicles are cultured in a three-dimensional system, even withdrawal of TSH or other growth factors does not induce apoptosis (Bechtner *et al.* 1999) suggesting that two-dimensional models of cultured thyrocytes do not reflect the *in vivo* regulation of apoptosis.

The cytotoxicity of excess iodine includes necrotic and apoptotic features. Whether either type of cell death is due to the formation of reactive oxidative radicals in excess, as previously suggested (Denef *et al.* 1996), remains to be established *in vivo*. As do many other organs, the thyroid contains antioxidative defences, among which are enzymes with antioxidant properties. For example, glutathione peroxidase (GPx) activity is increased during goitre development, as well as in human hyperactive pathologies (Mano *et al.* 1997) and in isolated cells stimulated by TSH (Björkman & Ekholm 1995). Recently, the expression of peroxiredoxins, very effective antioxidant enzymes (Knoops *et al.* 1999), has been reported to be modulated by TSH (Kim *et al.* 2000).

Besides enzymatic systems, cells are also protected by several intracellular vitamins. Vitamin E plays a major role in the maintenance of membrane integrity, being both a strong free radical scavenger and a structural stabilizer (Gutteridge 1978). Its depletion results in increased lipid peroxidation (Machlin *et al.* 1977, Walsh *et al.* 1993) and thus represents an appropriate *in vivo* model to analyse the role of oxidative stress radicals in the induction of cell death. In the present study, we compared the extent of intrathyroidal necrosis and apoptosis during goitre formation and iodine-induced involution in rats fed a vitamin E-deficient or -sufficient diet. In addition, we paid attention to the location of the apoptotic cells. Indeed, a high number in the interstitial tissue may be of great pathological significance.

## Materials and Methods

### Animals and treatments

Three-week-old male Wistar rats (KUL, Leuven, Belgium), weighing approximately 40 g at the onset of the study, were fed a basal vitamin E-deficient diet (<15 mg/kg, AO3; Usine d'Alimentations Rationnelles (UAR), Epinay-sur-Orge, France) without (E<sup>-</sup>) or with (E<sup>+</sup>) 170 mg/kg vitamin E (dl- $\alpha$ -tocopherol acetate; UAR). This diet contained 480  $\mu$ g iodine and 100  $\mu$ g selenium per kg, representing a daily supply of about 6 and 1.3  $\mu$ g respectively. Rats were maintained in accordance with the principles of laboratory animal care. They were housed two per cage, in a temperature- and light-controlled room and had free access to water and their respective diets for 2 months. No specific feeding behaviour was noticed between E<sup>+</sup> and E<sup>-</sup> rats. The mean daily intake per rat was about 12–13 g dry food in both groups. However, after 2 months on these diets, the body weight gain was significantly smaller in the E<sup>-</sup> groups than in the E<sup>+</sup> groups (163  $\pm$  20 vs 235  $\pm$  27 g (s.d.),  $P < 0.01$ ,  $n = 20$ ).

Groups of rats were thereafter made goitrous with 1% sodium perchlorate in the drinking water for 16 days, while control groups received tap water. During this treatment, the body weight gain was not significantly different in E<sup>+</sup> and E<sup>-</sup> groups, at 23  $\pm$  12 and 17  $\pm$  10 g respectively ( $n = 15$ ).

To induce goitre involution, some goitrous rats were then injected daily with 200  $\mu$ g NaI i.p. for 3 days, perchlorate being withdrawn or replaced by 0.02% methimazole (MMI) (Merck, Darmstadt, Germany), an inhibitor of iodine oxidation.

Treatments led thus to eight different groups, of five rats each: control, goitre, involution without or with MMI, each with or without vitamin E.

Rats were killed under Nembutal anaesthesia 24 h after the last NaI injection. Blood was collected by cardiac puncture and plasma was stored at -20 °C until use. For biochemical determinations, one lobe of each thyroid gland was directly dissected, weighed, quick-frozen in liquid nitrogen and stored at -80 °C. Frozen individual lobes were homogenized by grinding in 100 mM KCl, 20 mM phosphate buffer (pH 7.4), for GPx and caspase-3/apopain activities, malondialdehyde (MDA), DNA, iodine and protein content determinations.

For a control, pieces of liver were also quickly frozen for MDA determination, or prepared for microscopy in order to check a possible toxic effect of either vitamin E deficiency or perchlorate on hepatocytes. No alteration was seen in any group.

### Morphological and stereological analysis

After the dissection of one thyroid lobe, rats were perfused through the heart with saline for 1 min, then with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), for

**Table 1** Plasma T<sub>3</sub>, T<sub>4</sub> and TSH (means ± s.d., n=5) in control rats, rats made goitrous with 1% sodium perchlorate in drinking water for 16 days and goitrous rats injected daily i.p. with 200 µg NaI for 3 days without (involution) or with methimazole (MMI)

|                | Vitamin E | T <sub>3</sub><br>(ng/dl) | T <sub>4</sub><br>(µg/dl) | TSH<br>(ng/ml)            |
|----------------|-----------|---------------------------|---------------------------|---------------------------|
| Control        | +         | 64.8 ± 6.8                | 1.39 ± 0.3                | 17.8 ± 4.7                |
|                | -         | 79.4 ± 9.2                | 1.68 ± 0.5                | 16.6 ± 3.3                |
| Goitre         | +         | 21.2 ± 4.4 <sup>o+</sup>  | <1.25 <sup>o+</sup>       | 71.6 ± 18.0 <sup>o+</sup> |
|                | -         | 17.6 ± 3.1 <sup>o+</sup>  | <1.25 <sup>o+</sup>       | 83.6 ± 14.0 <sup>o+</sup> |
| Involution     | +         | 48.8 ± 9.9*               | 2.40 ± 0.3 <sup>o+</sup>  | 29.7 ± 7.2*               |
|                | -         | 52.8 ± 12.0*              | 1.86 ± 0.3 <sup>o+</sup>  | 24.0 ± 2.5*               |
| Involution+MMI | +         | 32.6 ± 15.0 <sup>o</sup>  | <1.25 <sup>o+</sup>       | 83.3 ± 11.0 <sup>o+</sup> |
|                | -         | 34.6 ± 24.0 <sup>o</sup>  | <1.25 <sup>o+</sup>       | 86.0 ± 26.0 <sup>o+</sup> |

<sup>o</sup>P<0.05 vs E<sup>+</sup> control rats; \*P<0.05 vs goitre with or without vitamin E; <sup>o+</sup>P<0.05 vs involution with or without vitamin E.

5 min. The second thyroid lobe was carefully dissected, weighed and processed for light and electron microscopy (Many *et al.* 1992); after a further fixation in the same fixative, thyroid fragments were postfixed in 1% osmium tetroxide, dehydrated and embedded in plastic. Semi-thin sections (0.5 µm) were cut from the centre of each fragment and stained with toluidine blue. Ultrathin sections from adequate areas selected on semi-thin sections were stained with 3% uranyl acetate and 2.5% lead citrate and observed on a Philips CM12 electron microscope (Philips, Eindhoven, Holland), to confirm at the ultra-structural level events defined as necrosis or apoptosis at the light microscopy level.

The extent of cell death by necrosis or apoptosis was quantified from semi-thin sections at × 500 magnification by counting two whole sections per gland, based on 15–20 fields per section. Results were expressed per field (~100 cells). Cells with swollen aspect and/or pale cytoplasm and nuclei were defined as necrotic. Shrunken cells, with dense pyknotic nuclei, or with accumulation of apoptotic bodies were defined as apoptotic. Necrotic and apoptotic cells were counted separately in the follicular

epithelium or in the interstitium. Dying cells and cell debris present in the follicular lumina were also numbered but the distinction between necrotic or apoptotic features could not be made. Relative volumes of glandular compartments (epithelium, follicular lumina, blood vessels and non-vascular interstitium) were determined by the point counting method as described (Denef *et al.* 1981). All measurements and analyses were made blind of the animal code.

#### Thyroid hormone assays

Concentrations of plasma thyroid hormones were determined in duplicate by RIA using commercially available kits for tri-iodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) (Corning, Meldfield, MA, USA). Plasma TSH concentrations were determined using a specific kit for rat TSH (Amersham International, Amersham, Bucks, UK).

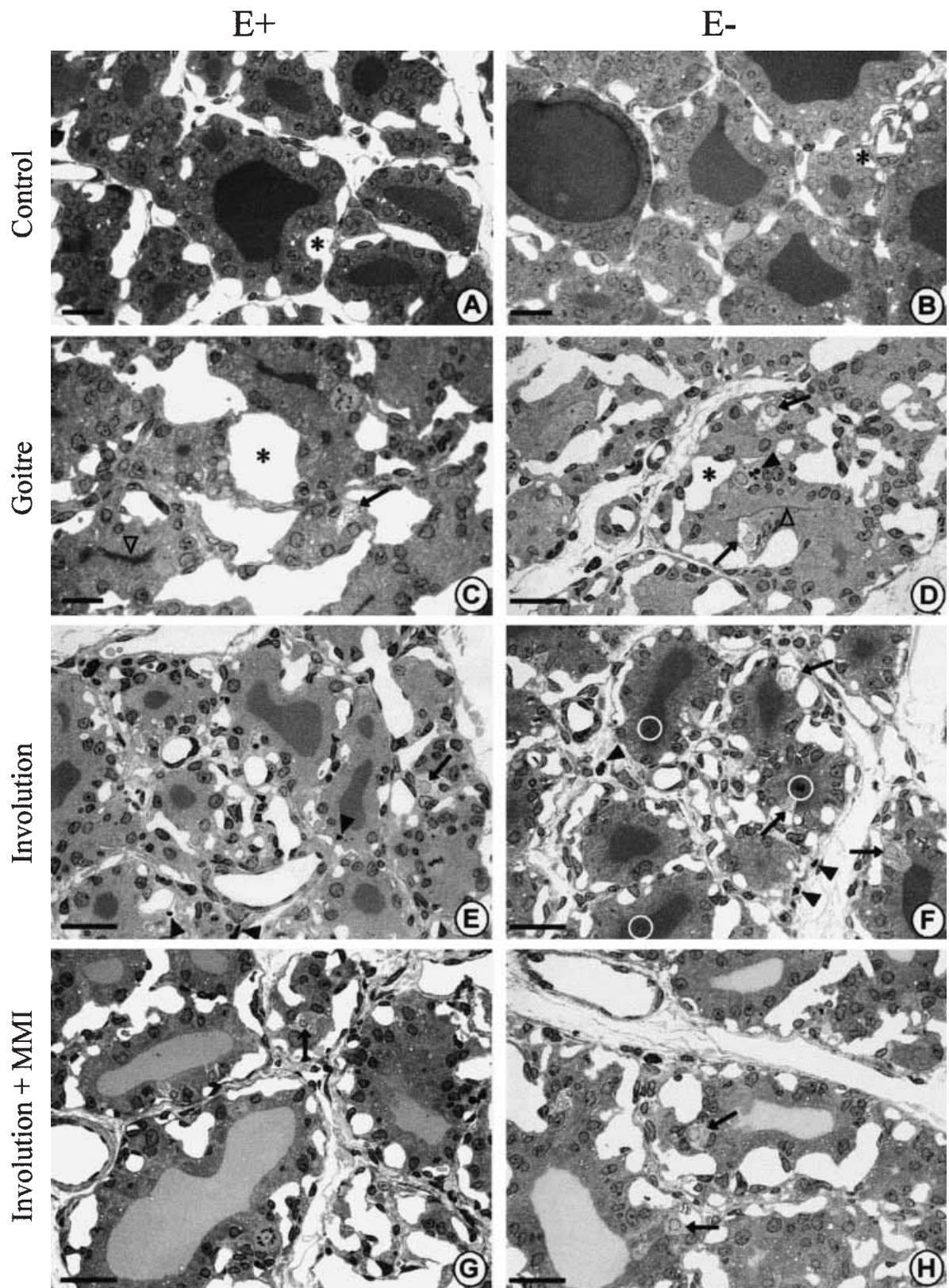
#### DNA, iodine and protein determination

DNA and stable iodine were determined on the individual thyroid homogenates as described (van den Hove *et al.*

**Table 2** Thyroid glandular parameters (means ± s.d., n=5). Same protocol as in Table 1

| Group          | Vitamin E | Relative weight<br>(µg/g) | DNA<br>(µg/mg) | Iodine<br>(ng/mg) | Relative volume of compartments (%) |                         |                        |
|----------------|-----------|---------------------------|----------------|-------------------|-------------------------------------|-------------------------|------------------------|
|                |           |                           |                |                   | Epithelium                          | Follicular lumina       | Vessels                |
| Control        | +         | 53 ± 1                    | 3.3 ± 0.7      | 719 ± 224         | 45 ± 1.9                            | 25 ± 1.1                | 10 ± 1.2               |
|                | -         | 66 ± 1 <sup>o</sup>       | 3.1 ± 0.8      | 871 ± 84          | 44 ± 1.1                            | 25 ± 1.1                | 11 ± 1.6               |
| Goitre         | +         | 155 ± 2 <sup>o</sup>      | 2.9 ± 0.7      | 31 ± 19           | 47 ± 1.8                            | 0.9 ± 0.3 <sup>o+</sup> | 35 ± 2.2 <sup>o</sup>  |
|                | -         | 137 ± 1 <sup>o</sup>      | 3.1 ± 0.4      | 22 ± 17           | 49 ± 1.7                            | 1.1 ± 0.5 <sup>o+</sup> | 34 ± 2.7 <sup>o</sup>  |
| Involution     | +         | 103 ± 0 <sup>o*</sup>     | 2.7 ± 0.4      | 347 ± 217         | 48 ± 3.7                            | 11 ± 2.1 <sup>o*</sup>  | 22 ± 2.0 <sup>o*</sup> |
|                | -         | 112 ± 2 <sup>o*</sup>     | 3.5 ± 0.2      | 396 ± 135         | 47 ± 3.4                            | 11 ± 2.1 <sup>o*</sup>  | 23 ± 3.0 <sup>o*</sup> |
| Involution+MMI | +         | 133 ± 2 <sup>o+</sup>     | 3.2 ± 0.7      | 17 ± 10           | 45 ± 1.9                            | 3.6 ± 0.5 <sup>o+</sup> | 33 ± 2.3 <sup>o+</sup> |
|                | -         | 152 ± 4 <sup>o+</sup>     | 3.2 ± 0.3      | 48 ± 30           | 44 ± 2.0                            | 6.0 ± 3.3 <sup>o+</sup> | 32 ± 4.1 <sup>o+</sup> |

<sup>o</sup>P<0.05 vs E<sup>+</sup> control rats; \*P<0.05 vs goitre with or without vitamin E; <sup>o+</sup>P<0.05 vs involution with or without vitamin E.



1995). Total protein was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany) using BSA as standard.

#### *Thyroid and liver MDA levels determination*

The level of lipid peroxides in thyroid and liver tissues was determined by measuring the reaction of MDA with 2–2-thiobarbituric acid (Janssen Chemica, Geel, Belgium) according to Bidder & Sipka (1989). MDA levels were normalized for DNA content.

#### *GPx activity determination*

GPx activity was determined according to Ekholm & Björkman (1997) and expressed in nanomoles of NADPH oxidized, i.e. H<sub>2</sub>O<sub>2</sub> degraded, per min and per mg of DNA.

#### *Caspase-3/apopain activity determination*

Caspase-3/apopain activity was determined with the FluoroAce Apopain assay kit, according to the manufacturer's instructions (Bio-Rad).

#### *Statistical analysis*

Results are expressed as means ± s.d. ( $n=5$ ). Comparisons between groups were made by one- or two-way ANOVA followed by an *F*-test. Significant differences between means were performed after ANOVA by the least significant differences test. The overall variation coefficient of the point counting method was about 6% (Many *et al.* 1992). For morphometric analysis, differences were considered as significant when  $P<0.05$  (not significant=ns). A logarithmic transformation was applied to data from the analysis of follicular necrotic and apoptotic cells before the ANOVA.

## Results

### *Vitamin E deficiency does not affect thyroid hormone concentrations in plasma during goitre development and involution*

Plasma T<sub>3</sub> and T<sub>4</sub> levels were not different in control E<sup>+</sup> and E<sup>-</sup> rats, but markedly depressed after 16 days of goitre

development (Table 1). They were significantly increased after 3 days of high iodide supply, suggesting the absence of an inhibitory Wolff–Chaikoff effect. Administration of MMI together with iodide to goitrous animals blocked the iodine-induced rise in hormone levels. Vitamin E deficiency did not significantly modify T<sub>3</sub> and T<sub>4</sub> levels whatever the experimental group.

Plasma TSH level was increased significantly at day 16 of goitre development and returned close to control values after iodide supplementation for 3 days. MMI given to goitrous rats abolished this decrease. Vitamin E deficiency had no effect.

### *Size and DNA content of the goitre induced in vitamin E-deficient rats are proportional to animal growth*

In controls, the absolute thyroid weight was significantly smaller in E<sup>-</sup> rats, while the thyroid/body weight ratio was slightly increased ( $P<0.05$ ) (Table 2). After 16 days of goitre development, thyroid weights increased 3- and 2-fold in E<sup>+</sup> and E<sup>-</sup> rats respectively. After 3 days of iodide administration, glandular weight decreased significantly in all treated rats, although remaining higher than in controls. This decrease was blocked by MMI.

In control groups, thyroid contained 23 ± 5 µg DNA/lobe in E<sup>+</sup> animals and 20 ± 5 µg DNA/lobe in E<sup>-</sup> animals. In E<sup>+</sup> rats, the thyroid DNA content rose to 63 ± 11 µg/lobe ( $P<0.001$ ) during goitre and decreased to 41 ± 8 µg/lobe ( $P<0.01$ ) during involution, while remaining high with MMI (65 ± 10 µg/lobe). In E<sup>-</sup> animals, the DNA content increased to 45 ± 4 µg/lobe ( $P<0.01$ ) during goitre development and was not reduced after iodine treatment without or with MMI. The DNA content varied largely in parallel with the thyroid weight.

After 16 days of perchlorate treatment, the iodine concentration decreased from 719 ± 224 ng/mg of tissue in control rats to 31 ± 18 ng/mg of tissue in goitrous rats. After iodide administration to goitrous rats, the thyroid iodine concentration remained about half the value observed in controls. MMI administration maintained the iodine concentration close to goitrous values. Changes observed in E<sup>-</sup> animals did not differ from those observed in E<sup>+</sup> animals.

**Figure 1** (Opposite). General thyroid histology. (A, B) Control rats received a vitamin E (E<sup>+</sup>) (A) or E<sup>-</sup> (B) diet. Glands have a normal morphology and the blood vessels are not dilated (asterisks). (C, D) E<sup>+</sup> (C) or E<sup>-</sup> (D) rats were made goitrous by drinking perchlorate. Blood vessels are markedly enlarged (asterisks), epithelial cells are hypertrophic and follicular lumina are very narrow (empty arrowheads). Some follicular necrotic cells are present in the epithelial wall (arrows). No effect of vitamin E deficiency is noticed on the thyroid morphology, but the proportion of follicular necrotic cells is increased in the E<sup>-</sup> goitrous thyroid. Note also the presence of apoptotic bodies in this condition (arrowhead). (E, F) Goitres in E<sup>+</sup> (E) or E<sup>-</sup> (F) rats were caused to involute by repeated Nal injections. Blood vessels are constricted as compared with (C) and (D). Follicular lumina are widened and contained a dense colloid. Some follicular necrotic cells are present in the epithelial wall (arrows). Vitamin E deficiency increases the number of necrotic cells (arrows) without altering the thyroid morphology. Note the presence of cell fragments in the follicular lumina (open circles) and of several apoptotic cells in the epithelium as well as in the interstitium (arrowheads). (G, H) E<sup>+</sup> (G) or E<sup>-</sup> (H) rats received Nal plus methimazole (MMI). Some blood vessels are constricted. Follicular lumina are widened or narrow. Some follicular necrotic cells are observed in the epithelial wall (arrows). Bars=20 µm.

Protein content showed the same evolution as DNA and was not affected by vitamin E deficiency.

*Vitamin E deficiency aggravates epithelial cell necrosis without influencing cell apoptosis during goitre development and involution*

In both E<sup>+</sup> and E<sup>-</sup> control animals, thyroid contained normal large follicular lumina lined by a cuboidal epithelial layer. The follicles were surrounded by capillaries with very thin lumina (Fig. 1A and B).

A typical hyperplastic goitre was formed after 16 days of perchlorate treatment (Fig. 1C). The follicular lumina were narrow, and lined by a thickened epithelium with hypertrophied cylindrical cells and numerous mitotic figures. Blood vessels were markedly enlarged as confirmed by the morphometric analysis (Table 2). In contrast, the relative volumes of epithelium (Table 2) and of non-vascular interstitium (data not shown) were not modified. Again, vitamin E deficiency had little influence on the morphology and on the morphometric parameters of goitrous glands, except that more numerous necrotic cells were seen in the epithelial layer (Fig. 1D).

After 3 days of iodine-induced goitre involution, the follicular lumina were slightly but significantly widened (Table 2) and contained a dense colloid. Blood capillaries were constricted and typical dense pyknotic nuclei were observed either in the epithelial wall or in the interstitium (Fig. 1E). In E<sup>-</sup> animals, follicular lumina size and blood capillary constriction were similar to those observed in E<sup>+</sup> ones (Fig. 1F). Meanwhile, cellular debris was more numerous in some follicular lumina. Apoptotic cells were also observed, either in the epithelial wall, or in the interstitium. A very modest inflammatory cell infiltration was observed in the interstitium or in the follicles in either group.

After administration of iodide with MMI to the iodine-deficient rats, the follicular lumina were slightly dilated, containing a pale colloid, and the blood vessels were still enlarged. The gland aspect was similar in both E<sup>-</sup> (Fig. 1H) and E<sup>+</sup> animals (Fig. 1G). Cell death was not as frequent as after iodine administration alone. The iodine-induced expansion of the follicular lumina and the reduction of blood vessels observed during involution were partly impaired (Table 2). Again, vitamin E deficiency did not alter any of these parameters.

Apoptosis and necrosis were confirmed at the ultra-structural level. The dense inclusions, often rounded, sometimes elongated, seen in the interstitium or in the basal pole of the epithelial follicular cells on semi-thin sections (Fig. 2A) corresponded to condensed nuclei with very dense chromatin (Fig. 2B). On the contrary, faintly coloured cells, often with a round shape (Fig. 2A), consisted of necrotic cells with diluted or vacuolated cytoplasm and numerous damaged organelles (Fig. 2C).

Debris present in the follicular lumina was often dense (Fig. 2D) but some was pale in aspect.

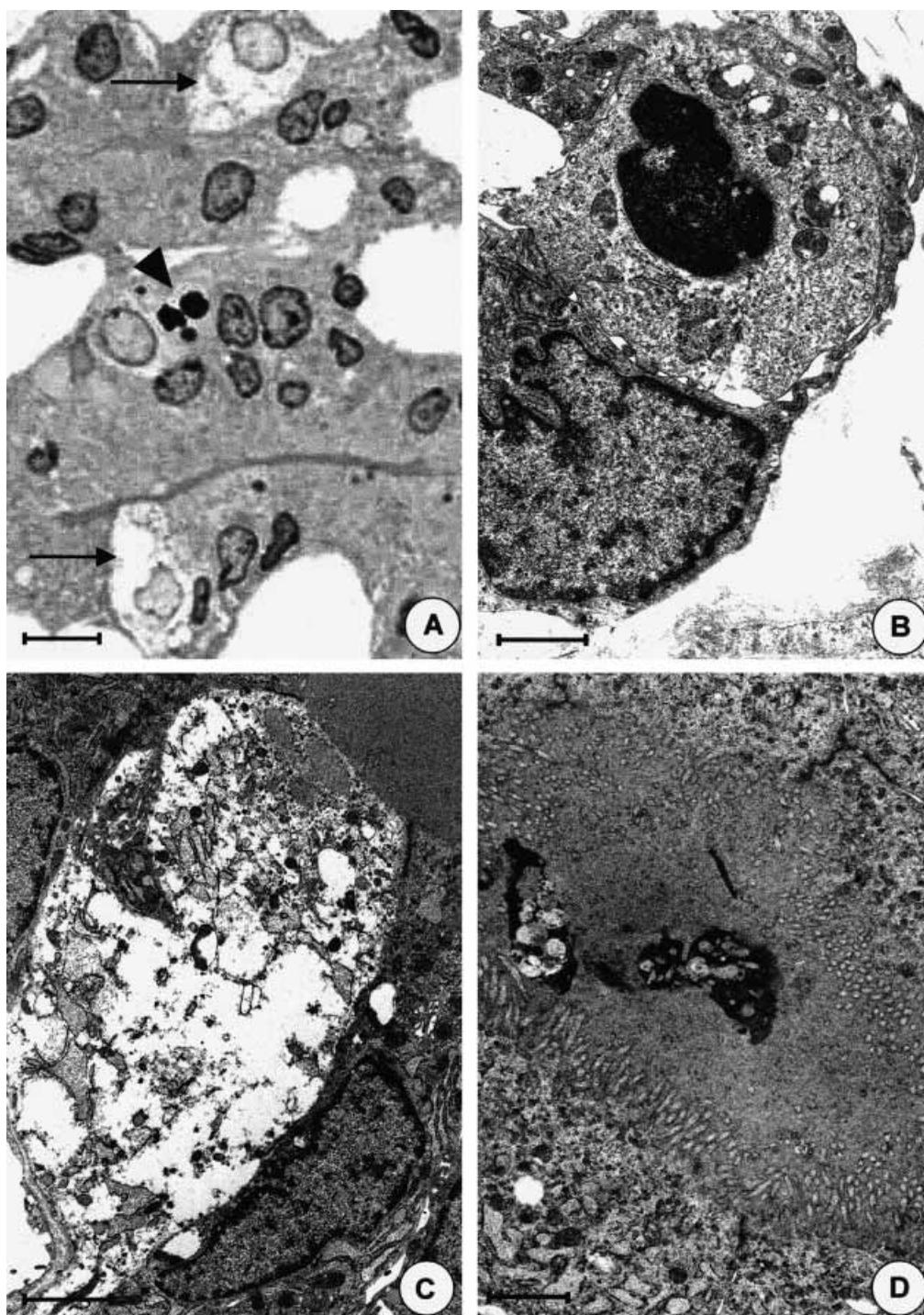
Results of counting for necrotic or apoptotic cells are reported in Fig. 3. The total number of dead cells per field was low in control thyroids and significantly increased in goitrous glands of E<sup>+</sup> or E<sup>-</sup> animals (Fig. 3A). It further increased during involution, mainly in the E<sup>-</sup> group, while it was not changed in the presence of MMI. A significant proportion of the total dead cells observed during involution corresponded to interstitial cells, whatever the vitamin E supply. These cells were almost all in apoptosis. In addition, intracoloidal cell debris was mainly observed during involution in the E<sup>-</sup> group.

An overall significant increase in epithelial dead cells was noticed in the E<sup>-</sup> groups (Fig. 3). The proportion of dead thyrocytes was 3-fold increased during goitre development, including mostly cells with a necrotic aspect (Fig. 3B), but not further increased when iodine was administered alone or in association with MMI. The proportion of epithelial necrotic cells slightly decreased in the E<sup>+</sup> animals as compared with the goitre condition when iodine was administered, alone or with MMI. This decrease was not observed in the E<sup>-</sup> group. Epithelial apoptosis was mainly increased during involution, with no significant difference upon vitamin treatment (Fig. 3B). Caspase-3/apopain activity, an indicator of apoptosis biochemistry, was not detected in control glands, increased in goitres and further increased during involution (Table 3). The effect of vitamin E supply taken as a whole was not significant, differences between E<sup>+</sup> and E<sup>-</sup> values varying in an opposite way in goitre and involution.

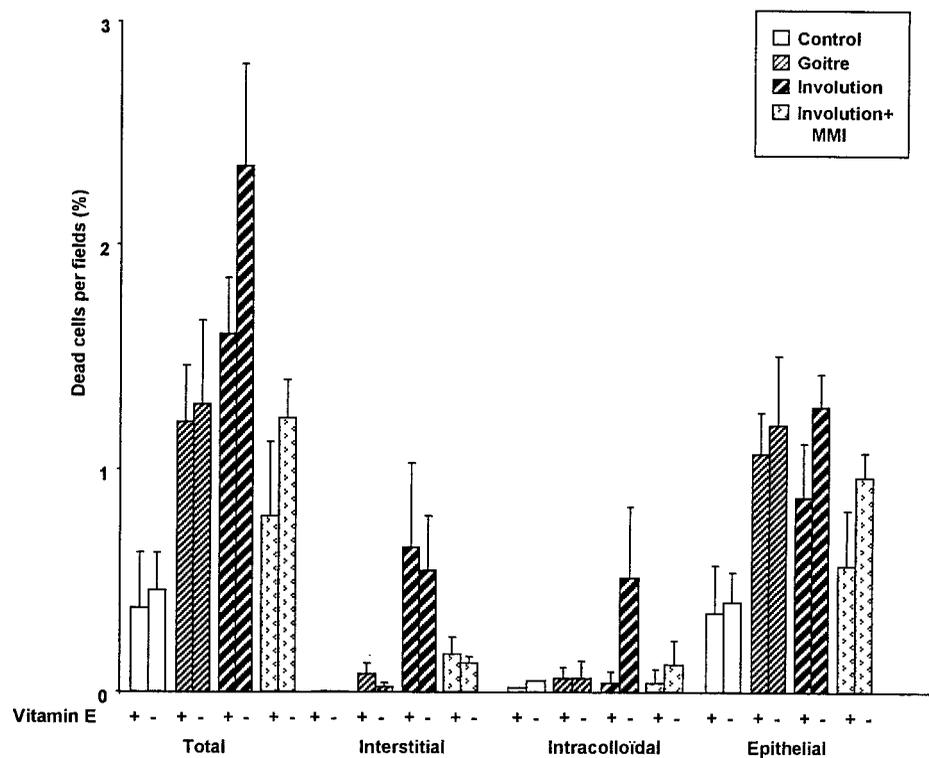
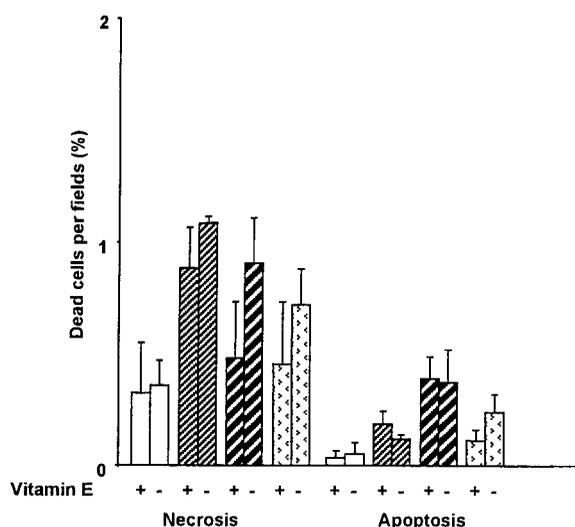
*Vitamin E deficiency increases MDA concentration and decreases GPx activity in the thyroid*

In E<sup>+</sup> control rats, the MDA concentration in the thyroid tissue ( $82.9 \pm 19.0$  nmol/mg DNA) was one-third of the values observed in the liver of the same animals ( $276.0 \pm 46.7$  nmol/mg DNA). In E<sup>-</sup> animals, the increase was proportionally higher in the thyroid ( $144.5 \pm 32.6$  nmol/mg DNA) than in the liver ( $352.7 \pm 58.3$  nmol/mg DNA,  $P < 0.001$ ). The thyroid MDA concentration was not changed during the goitrogenic treatment whatever the vitamin E supply (Fig. 4A). The thyroid MDA concentration rose slightly but significantly in E<sup>+</sup> goitrous rats exposed to iodine in excess. This effect was absent in the presence of MMI. ANOVA analysis clearly demonstrated a significant increase of the MDA concentration, as a whole, in E<sup>-</sup> animals as compared with E<sup>+</sup> ones.

GPx activity increased significantly during goitre development and was not changed after iodine treatment (Fig. 4B). ANOVA analysis showed a significant general decrease of GPx activity in E<sup>-</sup> groups, which was aggravated after 3 days of iodine involution without MMI.



**Figure 2** Characteristic aspects of necrosis and apoptosis by light (A) and electron (B–D) microscopy. (A)  $E^-$  goitrous rat; bar=5  $\mu\text{m}$ . Two necrotic cells (arrows) are visible in the epithelial wall; nuclei are pale and cytoplasm are swollen and vacuolar. Apoptotic bodies correspond to fragmented pyknotic nuclei (arrowhead). (B–D) Nal-treated  $E^-$  goitrous rats; bar=1  $\mu\text{m}$ . Necrosis corresponds to organelle swelling and disruption (C). Apoptotic bodies are endocytosed in the interstitium (B). Note that cell debris is often very dense in the follicular lumen (D).

**A : Total dead cells****B : Epithelial death type**

**Figure 3** Quantification of various aspects of total dead cells (A) and epithelial death type (B).  $E^+$  and  $E^-$  rats were either untreated (control group), made goitrous by drinking perchlorate (goitre group), further injected with Nal alone (involution group) or with MMI (involution+MMI group). There were ~100 cells/field. Means  $\pm$  s.d.,  $n=5$ . Results of statistical analysis were as follows. Total number of dead cells: vitamin effect: significant for  $P<0.001$ ; treatment effect:  $P<0.001$ ; interaction:  $P<0.05$ . Number of interstitial dead cells: vitamin effect: ns; treatment effect:  $P<0.001$ ; interaction: ns. Number of epithelial dead cells: vitamin effect:  $P<0.001$ ; treatment effect:  $P<0.001$ ; interaction: ns. Epithelial necrotic cells: vitamin effect:  $P<0.01$ ; treatment effect:  $P<0.001$ ; interaction: ns. Epithelial apoptotic cells: vitamin effect: ns; treatment effect:  $P<0.05$ ; interaction: ns.

**Table 3** Caspase-3/apopain activity (means  $\pm$  s.d.,  $n=4$ ). Same protocol as in Table 1

| Group      | Vitamin E | Caspase-3/apopain activity (fmol AFC/min per $\mu$ g DNA) |
|------------|-----------|---|
| Control    | +         | ND  |
|            | -         | ND  |
| Goitre     | +         | 135 $\pm$ 8.2   |
|            | -         | 184 $\pm$ 2.4   |
| Involution | +         | 242 $\pm$ 33  |
|            | -         | 208 $\pm$ 58  |

ANOVA analysis shows a significant difference ( $P<0.01$ ) between the goitre and the involution groups. ND=not detected. Data represent fmol of 7-amino-4-trifluoromethyl coumarin (AFC) released from the fluorogenic peptide substrate carbobenzyloxy-DEVD-AFC.

## Discussion

We present here direct evidence for a different involvement of necrosis and apoptosis in the thyroid cell death occurring during goitre development and its aggravation upon involution. During goitre development, necrosis of

thyrocytes increases to a much larger extent than apoptosis. On the contrary, apoptosis is predominantly stimulated during iodine-induced involution. In addition, necrosis but not epithelial or interstitial apoptosis is significantly increased upon vitamin E deficiency, i.e. when the antioxidative defences were reduced, as reflected by the increase in MDA concentrations and the decline in GPx activity.

### Vitamin E deficiency reduces antioxidative protection

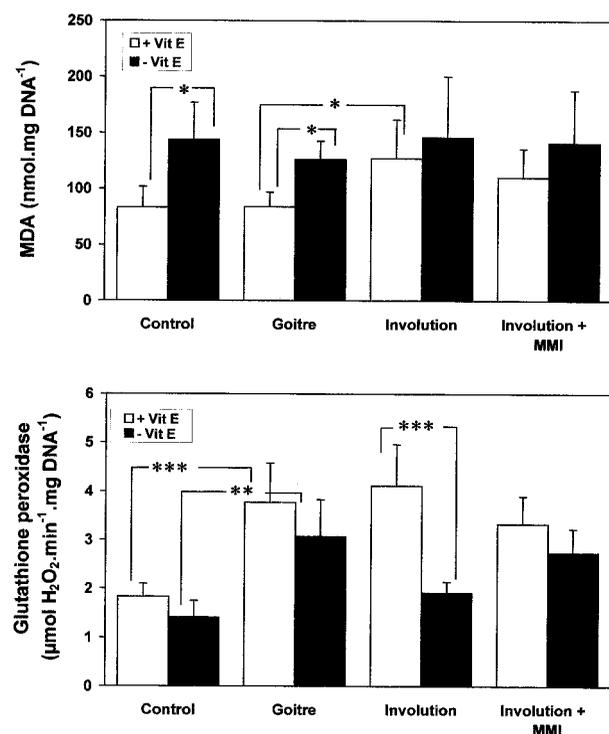
Vitamin E, which is very active in the antioxidative protection of thyroid membrane, is as concentrated in the thyroid as in the liver in control rats and 2-fold increased in goitres (Mutaku *et al.* 1998). We therefore have developed a model of experimental goitre in animals with reduced antioxidative defences to test the hypothesis of a reactive oxygen-induced cell death during goitre development and involution. Our results clearly show that vitamin E deficiency is associated with an increase in MDA concentration and a decrease in GPx activity in the thyroid and a concomitant rise of MDA in the liver. These results, in line with others (Bourre *et al.* 2000), reflect a systemic defect in antioxidative protection.

In addition to its antioxidative effect, vitamin E also has other actions; vitamin E-deficient animals have a depressed growth rate (Machlin *et al.* 1977), possibly through abnormalities of the growth hormone–insulin-like growth factor system. In the present study, vitamin E-deficient rats, despite a comparable daily food intake, gained less body weight than rats fed a control diet, with a parallel reduction of the thyroid weight.

### Vitamin E deficiency aggravates total and epithelial cell death

We show here that total cell death count, known to increase during goitre development in mice (Many *et al.* 1991) and rats (Contempré *et al.* 1993, Mutaku *et al.* 1998), is further increased after iodide administration to iodine-deficient rats. These changes are in line with previous reports (Mahmoud *et al.* 1986, Many *et al.* 1991, 1992, 1995, Contempré *et al.* 1993, 1995, Mutaku *et al.* 1998). However, it can be added that this iodine-induced increase results mostly from the appearance of dead cells in the interstitium, all with pyknotic nuclei, in E<sup>-</sup> as well as in E<sup>+</sup> animals. In contrast, the number of epithelial dead cells was not influenced by the iodine load but, as a whole, significantly increased with vitamin E deficiency.

The present work, for the first time, separates quantification of apoptotic and necrotic cells in goitre and after administration of a pharmacological dose of iodine. It clearly shows that necrosis predominates in the goitrous gland, while apoptosis is significantly induced during goitre involution.



**Figure 4** Oxidation index and antioxidant potential. Thyroid malondialdehyde (MDA) concentrations (upper panel) and glutathione peroxidase (GPx) activities (lower panel) were determined. For details of the experimental groups, see legend of Fig. 3. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs the similar experimental group with different vitamin E supply or the different experimental group with similar vitamin E supply.

*Apoptosis occurs during goitre involution and is not influenced by vitamin E status*

Apoptosis, as assessed by measurement of TUNEL-positive cells, increased during goitre formation in experimental rats (Tamura *et al.* 1998). The reliability of this method is questionable when apoptosis is to be compared with necrosis. Indeed, the TUNEL method is based on the incorporation of labelled nucleotides into DNA breaks and can yield false positive results due to other mechanisms of DNA nicking such as during necrosis. In addition, methodological artefacts can be induced by aldehyde fixation (Labat-Moleur *et al.* 1998). We, therefore, decided to quantify instead apoptosis by careful histological counting of cells with pyknotic nuclei or apoptotic bodies. Using this method, we show that apoptosis of epithelial cells, which is exceptionally detected in normal thyroids, already slightly increased during goitre development. In contrast, a high iodine load rapidly induces the occurrence of pyknotic thyrocytes and apoptotic bodies at the basal pole of normal cells, indicating the participation of this type of cell death in the goitre regression. The increase of the caspase-3/apopain activity during involution confirmed these results at the molecular level.

The occurrence of apoptotic cells in the interstitium could reflect a totally different mechanism, even if apoptotic epithelial cells could extrude out of the epithelial layer. Some interstitial apoptotic cells are probably inflammatory in nature, as a consequence of the small inflammatory reaction induced by the iodine treatment; they could also be mast cells, although lymphocytes and mast cells are few. Other apoptotic cells are most probably of endothelial nature, reflecting the involution of the vascular bed. An apoptosis-specific protein was also immunolocalized predominantly in the stromal and vascular tissue (Patel *et al.* 2000).

Both the morphological counting and the direct detection of the caspase-3/apopain activity show that apoptosis evolves in the epithelium or in the interstitium independently of the vitamin E status of the animals, indicating that it is not modulated by the cellular antioxidative defences. This contrasts with experiments carried out *in vitro*, suggesting a role of free radical generation in apoptosis induction. In the first study, addition of H<sub>2</sub>O<sub>2</sub> to primary cultures of pig thyrocytes was reported to cause apoptosis with modifications of the Bcl-2/Bax regulatory pathway (Riou *et al.* 1999), and a decreased response in FRTL-5 cells overexpressing peroxiredoxins (Kim *et al.* 2000). The discrepancy with our results could be that either H<sub>2</sub>O<sub>2</sub> concentration in the goitrous glands analysed remained much below the high concentration applied to thyrocytes *in vitro*, or that H<sub>2</sub>O<sub>2</sub> takes no part in the iodine-induced apoptosis. In a second study, excess molecular iodine induced apoptosis in isolated thyrocytes independently of the modulation of Bcl-2 or Bax protein

expression or protein synthesis, but through generation of free radicals (Vitale *et al.* 2000). However, in that study, the iodine-induced cytotoxicity included necrotic and apoptotic features in equal proportions and arguments for an implication of free radicals in apoptosis were only circumstantial. As in our study, apoptosis was fully blocked by an inhibitor of thyroperoxidase, indicating a role for molecular iodine.

*Vitamin E deficiency aggravates epithelial cell necrosis*

We show here that cells with necrotic features are almost exclusively epithelial cells. They are, as a whole, more numerous in E<sup>-</sup> than in E<sup>+</sup> thyroids and no more numerous after a high iodine load. Reports on the effect of iodine on cell necrosis are discrepant, depending on the experimental model used. Indeed, necrosis is less extensive in male Wistar rats used in our study than in mice (Mahmoud *et al.* 1986, Many *et al.* 1991) or other species (Belshaw & Becker 1973, Bagchi *et al.* 1995), and several authors did not even observe necrotic thyrocytes in rats (Allen 1992, Li & Boyages 1994, Colzani *et al.* 1999). However, most of these studies were performed in rats fed a normal iodine dose (Allen 1992, Li & Boyages 1994). In a study by another group on iodine-deficient female rats, an iodide dose ten times as large as in the present study did also not result in increased cell death (Colzani *et al.* 1999). The fact that serum T<sub>4</sub> and TSH concentrations were not modified in their study, in contrast to ours, suggests that iodine has to be in its oxidized form to be toxic. Discrepancy could originate from species- or gender-linked variations either in the antioxidative defences, or in the avidity of thyroid cells for iodide. Indeed, in the mouse fed a normal iodine diet, administration of large doses of iodine does not provoke cell necrosis (Many *et al.* 1995).

Necrosis affected isolated cells and not pluricellular foci, as would be expected in the case of severe toxicity. It could therefore be interpreted as the consequence of a locally produced and rapidly degraded toxic agent, such as H<sub>2</sub>O<sub>2</sub> produced in excess at the apical membrane of goitrous glands. Indeed, necrosis of epithelial cells is increased in vitamin E<sup>-</sup> thyrocytes in which the reduced GPx activity could become limiting in some cells. These results support the free radical hypothesis previously proposed (Denef *et al.* 1996). Since the 2-fold increase in vitamin E content of goitres (Mutaku *et al.* 1998) is less than that of H<sub>2</sub>O<sub>2</sub> production (Corvilain *et al.* 1991), the administration of a vitamin E supplement to goitrous patients will improve their antioxidative defences.

*Vitamin E deficiency induces the intracoloidal accumulation of cell debris during goitre involution*

After goitre involution, cell fragments accumulate in the follicular lumen of vitamin E<sup>-</sup> thyroids while they are very rare in vitamin E<sup>+</sup> colloids. Whether those

fragments represent terminal necrotic debris or apoptotic bodies expelled apically could not be decided. However, their presence essentially in vitamin E-deficient animals suggests that the integrity of the apical membrane could have been perturbed in this situation of high membrane peroxidation.

### Conclusion

In conclusion, two types of cell death, necrosis and apoptosis, occur during goitre development and involution. They are differently modulated according to the oxidative status and/or the level of stimulation of the thyroid gland. Necrosis is related to the oxidative status of the thyroid cells while apoptosis occurs with iodine-induced involution.

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