Hydrogen peroxide-induced production of a 40 kDa immunoreactive thyroglobulin fragment in human thyroid cells: the onset of thyroid autoimmunity?

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We recently reported that, during *in vitro* thyroid-hormone synthesis, H₂O₂ stress cleaved thyroglobulin (Tg) into C-terminal peptides. These peptides were found to contain the immunodominant region of Tg recognized by Tg autoantibodies from patients with an autoimmune thyroid disease. To test the hypothesis that Tg fragmentation is an early upstream initiating event involved in Tg autoimmune response and the consequence of oxidative injuries, we studied the effect of H₂O₂ stress on human thyroid cells. In culture conditions allowing Tg synthesis and iodine organification by the cells, we found that bolus addition of increasing millimolar doses of H₂O₂ induced a dose–response appearance of floating cells in the culture medium. These cells apparently resulted from a necrotic process, and they

bore iodinated Tg fragments. These fragments were found to be similar to those previously obtained *in vitro* from purified Tg. In both cases, Tg peptides were recognized by a well-defined monoclonal antibody directed to the immunodominant region of Tg. The smallest immunoreactive Tg peptide had a molecular mass of 40 kDa and entered human thyrocytes more efficiently than the entire Tg. These data suggest that thyrocytes exposed to locally increased H₂O₂ doses accumulate fragmented Tg for further delivery into surrounding living thyrocytes in the course of an autoimmune response.

Key words: antigen processing, endocytosis, iodine, necrosis, oxidative stress.

INTRODUCTION

In the thyroid gland, the thyroid hormones tri-iodothyronine and thyroxine are formed by iodination and intramolecular coupling of specific tyrosine residues of thyroglobulin (Tg). This process is not fully understood, but it is known to require iodine and it is limited by the production of H₂O₂, which acts as an acceptor of electrons generated during the oxidative reactions of the hormone synthesis. Iodine is trapped by the thyroid cells and transferred to their apical side, where the iodination and coupling processes take place by a specific membrane enzyme, thyroperoxidase (TPO) [1]. The H₂O₂-generating system, identified as flavoprotein Ca-dependent NADPH oxidase, also acts at the apical membrane of the thyroid cell [2]. H₂O₂ is produced in large amounts under stimulation by thyroid-stimulating hormone (TSH) [3] and can exert its toxic action through oxidative damage to the DNA, proteins and lipids of the cells. To prevent massive cellular destruction, the thyroid cells use various anti-oxidative systems, including enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as vitamins C and E, carotenoids, GSH and uric acid [4]. Depending on the H₂O₂ doses, the cells die by necrosis or apoptosis and, consequently, the method of H₂O₂ delivery for studying these various mechanisms is of major importance [5].

Like TPO, Tg is a thyroid autoantigen, and B- and T-cell responses against it were found to occur in Graves' and Hashimoto's diseases, two well-defined autoimmune thyroid diseases [6]. Evidence has been accumulating to suggest that thyrocytes would be capable of processing antigen and presenting it to T-cells [7,8]. However, as for most organ-specific auto-

immune diseases, the initial event leading to the breaking of T-cell tolerance still remains unknown. In a recent study [9] we reported that the oxidation process involved during thyroid-hormone synthesis could lead to the fragmentation of the Tg molecule into several peptides. These peptides were recognized by Tg autoantibodies from patients' sera and various Tg monoclonal antibodies (mAbs), including mAb 11 used in the present study. The smallest of the immunoreactive Tg fragments was a 40 kDa peptide (P40) and was identified as coming from the C-terminus of the Tg-bearing thyroid hormones. Interestingly, evidence has been accumulating for the involvement of this Tg region in the induction of experimental autoimmune thyroiditis [10–14]. We therefore hypothesized that Tg fragmentation would expose cryptic T-cell epitopes on the peptides and that subsequent endocytosis may direct these pretreated Tg peptides toward antigen processing by thyrocytes, leading to a restricted B-cell autoimmune response to Tg.

Here we report that bolus addition of millimolar concentrations of H₂O₂ in human thyroid-cell cultures induced a dose-responsive necrotic process and that dead cells bore iodinated Tg fragments similar to those obtained by *in vitro* hormone-synthesis experiments. In addition, we found that P40 was internalized by thyrocytes more efficiently than the entire Tg.

MATERIALS AND METHODS

Thyroid-cell culture

Para-adenomatous thyroid tissues were obtained from four female adults (age range 25–50 years) with macrofollicular non-

Abbreviations used: Tg, thyroglobulin; TPO, thyroperoxidase; TSH, thyroid-stimulating hormone; mAb, monoclonal antibody; P40, 40 kDa Tg peptide; FCS, fetal-calf serum.

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toxic adenomas (diagnosed on the basis of histopathological examination at thyroidectomy). Prior to the operation the patients were not treated with any drug known to influence the thyroid gland. Human thyroid cells were prepared as previously described [15]. Briefly, the tissue was dissected free from connective tissue and minced into small fragments. The tissue fragments were washed into Coon's modification of Ham's F-12 medium (Sigma, St. Louis, MO, U.S.A.) containing 2.6 g/l Na_2CO_3 (Merck, Darmstadt, Germany), penicillin/streptomycin (100 $\mu\text{g}/\text{ml}$; Life Technologies, Grand Island, NY, U.S.A.) and kanamycin (100 $\mu\text{g}/\text{ml}$; Life Technologies). To separate the epithelial cells from the connective tissue, the fragments were digested with collagenase I (1 mg/ml) (Life Technologies) and the neutral protease dispase II (2.4 units/ml) (Boehringer-Mannheim, Meylan, France) in 10 ml of calcium- and magnesium-free PBS, pH 7.4, at 37 °C for 15 min. The enzymic digestion was then allowed to continue for 60 min with the addition of 30 ml of fresh enzyme mixture. The digested tissue was filtered through sterile gauze swabs. Medium was added and the cells were washed by successive centrifugations at 100 *g* for 5 min. Finally, the cells were counted and seeded to a density of 2×10^6 cells per 25 cm² Falcon tissue-culture flask (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) in a final volume of 6 ml of culture medium. This medium was the one described above supplemented with 5% (v/v) fetal-calf serum (FCS) (Valbiotech, Paris, France), 2 mM L-Glutamine (Life Technologies), 1% non-essential amino acids (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.) and six nutritional factors (6H medium): 1 unit/l bovine TSH (Sigma); 10 mg/l human insulin (Boehringer-Mannheim); 10 $\mu\text{g}/\text{l}$ somatostatin (Novabiochem, Lufelfingen, Switzerland); 6 mg/l human transferrin (Boehringer-Mannheim); 10^{-8} M cortisol (Calbiochem, La Jolla, CA, U.S.A.); and 10 $\mu\text{g}/\text{l}$ glycyl-histidyl-lysine acetate (Calbiochem). The cells were cultured at 37 °C in a humidified air atmosphere containing 5% CO_2 . The primary cultures of thyroid cells formed a confluent monolayer within 7–9 days. A low serum concentration in the presence of TSH and following TSH depletion for 72 h was found to significantly increase cultured thyrocyte activity [16]. Accordingly, thyrocyte culture medium was first depleted in TSH and FCS for 72 h and then it was replaced by FCS-free 6H medium containing iodide as indicated below, for 24 h.

Oxidation test

After 72 h in TSH and serum-free medium, the confluent cells were submitted to oxidative stress produced by H_2O_2 ranging from 0 to 6 mM. H_2O_2 was added at once to the flasks filled with 4 ml of FCS-free 6H medium supplemented with 5 μM KI and 1 μCi Na^{125}I with a specific radioactivity of 17.4 $\mu\text{Ci}/\text{ng}$ of iodide (Amersham Pharmacia Biotech Europe, Saclay, France) as a radioactive tracer. After 24 h, the medium was recovered and the adherent cells were washed with PBS and scraped into 1 ml of PBS. Living and dead cells from the scraped cells and culture medium supernatants were counted by the Trypan Blue-exclusion method with a Malassez cytometer, and the radioactivity linked to both the cell pellets was measured. After centrifugation, cellular pellets were freeze-dried in a Speed-Vac concentrator (Savant Instruments, Holbrook, NY, U.S.A.).

SDS/PAGE and Western blotting

The cell content of each flask was analysed by SDS/PAGE under non-reducing conditions. After freeze-drying, the cell pellets were dissolved with 10 μl of 65 mM Tris/HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol and 0.025% Bromophenol Blue. The cell samples were vortex-mixed and submitted to

sonication for 30 min. This step was repeated once. The samples were then loaded on to a minigel (10% acrylamide; 80 mm $\times$ 100 mm; 0.5 mm thick). After electrophoresis, peptides were directly electrotransferred on to a 0.45 μm -pore-size Immobilon-P PVDF membrane (Millipore, Bedford, MA, U.S.A.). Western-blotting experiments were carried out by incubating the blotted membrane with 100 μg of Tg mAb 11 [9] in PBS, pH 7.3, containing 3% BSA for 3 h at room temperature with constant shaking, after saturation of the membrane with PBS containing 3% BSA. The membrane was then washed three times for 15 min in PBS. The second, anti-mouse antibody labelled with horseradish peroxidase (Sigma) was incubated for 2 h at room temperature with shaking. After additional washes, the blots were developed with 4-chloro-1-naphtol as substrate before being scanned for radioactivity with a phosphorimager apparatus (FujixBass1000; Fuji, Tokyo, Japan) equipped with a Tina 2.09 computer program (Raytest, Courbevoie, France).

Inside-out follicles

Inside-out follicles were obtained from cultured human thyroid cells by adaptation of a method previously described for primary culture of porcine thyroid cell [17]. Briefly, freshly isolated human thyroid cells were grown in 6H medium as described above. At confluence, the cells were treated with trypsin and suspended in TSH-free 6H medium. The cells were cultured as an unstirred suspension in polystyrene dishes (10 cm diameter) coated with 1% agarose. The medium was changed every 5–6 days by centrifuging the cells and suspending them in an equivalent volume of fresh medium. After 5–8 days, inside-out follicles began to appear in the cultures. After 10–12 days, the cultures were considered ready for use.

Radioactive labelling

Human Tg and its corresponding P40 were prepared as described previously [9] and iodinated with Na^{125}I (Amersham) by the chloramine-T method. Labelled fractions of the whole Tg molecule and Tg peptide were separated from free iodine on a PD-10 Sephadex G-25M column (Pharmacia Biotech), pooled and stored at –20 °C until use. Tg and P40 were similarly labelled to a specific radioactivity of 35 $\mu\text{Ci}/\mu\text{g}$.

Binding and endocytosis experiments

The follicles were treated as described in [17]. Briefly, the suspended follicles were centrifuged at 100 *g* for 10 min and suspended in Dulbecco's modified Eagle's medium (Life Technologies), pH 7.2, without bicarbonate, but with 20 mM Hepes, containing 3% BSA and 5 mM Ca^{2+} and 3 mM Mg^{2+} (binding medium). Binding and endocytosis experiments were performed at 36 °C under gentle agitation in 25 ml flasks containing 2.5 ml of cell suspension. Incubations were started by the addition of labelled protein (0.25 nM). After 2 h incubation, samples (1 ml each) were collected in 5 ml test tubes, centrifuged at 100 *g* for 5 min and washed twice in 2 ml of ice-cold binding medium. They were then suspended in 1 ml of the same ice-cold medium, transferred to clean tubes, and centrifuged again. The supernatant was discarded and Pronase treatment (5 $\mu\text{g}/\text{ml}$ in 1 ml of PBS, pH 7.4) of the pellet allowed separation of internalized and cell surface-associated ligand. After 5 min at 36 °C, cells were collected by centrifugation and washed once in PBS. The pooled supernatants of centrifugation contained the surface-associated labelled ligand, whereas the final pellet contained the internalized ligand. The radioactivity of the various supernatants and pellets was measured in a γ -radiation counter.

In vitro hormone synthesis

We used an enhanced oxidative protocol named Ox4 for 4-fold doses of KI, TPO and H₂O₂ as compared with the classic protocol [9]. Briefly, 1 μ M human Tg dissolved in 50 mM Tris/HCl buffer, pH 7.2, was incubated for 30 min at 37 °C with 80 μ M KI in the presence of 200 μ g/ml human TPO and the H₂O₂-generating system (4 mg/ml glucose and 10 μ g/ml glucose oxidase).

RESULTS

H₂O₂ induces thyroid-cell death

Without H₂O₂ in the culture medium, human thyrocytes were able to synthesize Tg, trap iodine and transfer it to Tg. The production of Tg was evidenced by Western blotting experiments performed on the freeze-dried pellets of the adherent cells with

Figure 1 Cultured human thyrocytes produced Tg and organified iodine

Western-blotting experiment performed on the freeze-dried pellets of living cells with Tg mAb 11 shows a 330 kDa band corresponding to the Tg (lane 1); the same band was visualized by gel scanning for radioactivity using a phosphorimager apparatus (lane 2). The molecular masses (kDa) of the standards are indicated on the left.

Figure 2 Optical micrograph of human thyrocytes in monolayers

(A) Control cells without H₂O₂. (B) Cells exposed to 2 mM H₂O₂ for 24 h. The scale bar represents 80 μ m.

Figure 3 Effect of millimolar H₂O₂ concentrations on cell death and radioactive content of floating cells

The number, expressed as a percentage of total cells, (continuous line) and radioactivity, expressed as c.p.m./flask, (broken line) of floating dead cells were evaluated after 24 h incubation with various H₂O₂ concentrations. Results are the arithmetic mean $\pm$ S.D. for triplicates.

Figure 4 Effect of millimolar H₂O₂ concentrations on Tg fragmentation

Dead cells obtained with various H₂O₂ doses were analysed by Western-blotting experiments using mAb 11 (A) and the blot was scanned for radioactive Tg fragments (B). The smallest radioactive peptide was identified as P40 because it was also revealed by mAb 11 on the Western blot. For comparison, purified Tg was submitted to the *in vitro* Ox4 protocol for hormone synthesis and analysed by Western-blotting experiment using Tg mAb 11 (C). The molecular masses (kDa) of the standards are indicated on the left.

Tg mAb 11 (Figure 1). Iodine organification was ascertained by the visualization of radiolabelled Tg using a phosphorimager apparatus (Figure 1). Culture medium was then supplemented with increasing H₂O₂ concentrations from 0 to 6 mM for 24 h. H₂O₂ at concentration of 2 mM induced a dramatic change in the morphology of thyrocytes from flat-adherent to round-floating cells (Figure 2). However, when analysed by agarose-gel electrophoresis, the DNA of the floating cells failed to demonstrate the characteristic ladder pattern of fragmentation for an apoptotic process (results not shown). The number of dead cells was determined by Trypan Blue staining. All the floating cells were dead and consequently appeared blue-stained under the optical microscope. The cytotoxic effect of H₂O₂ was dose-dependent, affecting nearly 100 % of the cells at 6 mM by 24 h (Figure 3). The increase in radioactivity associated with the floating cells paralleled the cell number until two-thirds of the cells were dead, and then reached a plateau value at 2500 c.p.m. for concentrations of H₂O₂ higher than 2 mM (Figure 3).

H₂O₂ induces Tg fragmentation

Thyrocytes committed to cell death were analysed for their immunoreactive Tg content by Western-blotting experiments

Figure 5 Integrity of the radiolabelled probes used for the binding and internalization experiments

Tg (lane 1) and P40 (lane 2) were analysed by performing SDS/PAGE and scanning the radioactivity. The molecular masses (kDa) of the standards are indicated on the left.

using Tg mAb 11. Without H_2O_2 , only entire Tg was revealed. Then, as the H_2O_2 concentration increased, P40 gradually appeared on the blot on addition of Tg (Figure 4A). In contrast, whatever the concentration of H_2O_2 used, but without iodide in the culture medium, we only found entire Tg on the blot from the dead cells (results not shown). Figure 4(B) further shows on the blot that the organized radioactive iodine was distributed into entire Tg and several Tg fragments, the smallest of which is the 40 kDa peptide, as revealed by mAb 11. The intensity of the bands gradually increased using H_2O_2 concentrations from 0.5 to 2 mM, but they were qualitatively similar whatever the H_2O_2 concentration used. H_2O_2 concentrations below 0.5 mM did not permit us to discern any radioactive material, and H_2O_2 concentrations above 2 mM did not result in an increased Tg fragmentation (results not shown). Interestingly, the Tg fragmentation pattern was similar to the one previously obtained using the *in vitro* Ox4 protocol for thyroid-hormone synthesis described in the Materials and methods section, the smallest Tg fragment identified by the mAb 11 being P40 (Figure 4C). Analysed under similar conditions, the living cells were consistently found to be deprived of Tg fragments whatever the H_2O_2 dose used, suggesting that Tg fragments, notably the P40, only accumulated in dead cells (results not shown).

P40 enters thyrocytes

Checking for Tg fragment binding and internalizing into thyrocytes, we performed experiments with inside-out follicles of human thyrocytes as described in the Materials and methods section. These follicles appeared as hollow spheres with the apical surface facing the culture medium. In the experiments, we used radiolabelled P40 as the smallest representative Tg fragment, and native, non-fragmented radiolabelled Tg as a control. The integrity of the *in vitro* iodinated materials was checked by subjecting the probes (1000 c.p.m./lane) to SDS/PAGE under non-reducing conditions prior to scanning the gel for radioactivity. Figure 5 shows that most of the radioactivity localized in one band for both the probes. To discriminate between cell-surface binding and internalized radiolabelled probes, cells were treated with Pronase to remove surface-associated ligand before counting radioactivity. Comparative measurement of cell-surface-associated and internalized radiolabelled Tg and P40 showed that there was more P40 than entire Tg bound to the cells and

Figure 6 Binding and internalization of Tg and P40 by thyroid cells

Experiments were performed on inside-out follicles of human thyroid cells. Results are expressed as a percentage of total radioactivity (mean $\pm$ S.D. for triplicates).

that thyrocytes internalized P40 far more efficiently than entire Tg (Figure 6).

DISCUSSION

Hypothesizing that autoantigen fragmentation through oxidative stress would be the initial event leading to a Tg autoimmune response [9], we examined the effect of H_2O_2 during Tg iodine organization in human cultured thyrocytes. For this purpose, we set up thyrocytes culture conditions allowing Tg synthesis and iodide incorporation. To mimic the hypothetical rise in H_2O_2 production that would occur during *in vivo* thyroid-hormone synthesis, cultured human thyrocytes were exposed to bolus addition of millimolar doses of H_2O_2 . Such high levels of H_2O_2 represent abrupt and acute non-physiological stresses, but would be representative of local thyroid oxidative status in some pathological situations.

We first looked at the impact of these oxidative stresses on the human thyrocytes. In our test, millimolar concentrations of H_2O_2 were found to kill thyrocytes quickly in a dose-response way through a necrotic pathway apparently unrelated to apoptotic cell morphology and DNA fragmentation [18–20]. Cell necrosis was commonly reported to arise at high concentrations of H_2O_2 , while lower concentrations induced apoptosis [21] in various cell types, including pig thyrocytes [22]. However, evaluation of the H_2O_2 threshold dose for moving from apoptosis to necrosis was largely related to the cell type and experimental conditions. Here we suggest that, under special circumstances, high doses of H_2O_2 can be achieved locally in human thyroid. However, owing to their short lifetime [23], the doses of H_2O_2 we added at once in culture medium could reflect those of H_2O_2 continuously produced *in vivo* by a stimulated gland. It is therefore difficult to come to conclusions about the physiopathological relevance of the H_2O_2 concentrations we used in our tests. As a matter of comparison, it was recently reported that the extracellular H_2O_2 concentration was 7-fold higher than that of cytosol [24]. However, even taking into account that the physiological H_2O_2 concentration ranges between 10^{-8} and 10^{-7} M [25], we do not truly know how much would reach a local intra-thyroidal H_2O_2 concentration in a stimulated gland.

Secondly, Tg fragmentation was monitored by revealing radioactive peptides which obviously came from dead cells, their having incorporated labelled iodide into Tg. We recently showed that Tg fragmentation paralleled thyroid-hormone synthesis by comparing the intensity of the bands in Western blotting with the iodoamino acid contents of the Tg molecule before and after various protocols of *in vitro* hormone synthesis. We thus

suggested that Tg was cleaved during hormone synthesis [9]. In agreement with this suggestion we observed in the present study that, without iodide, H₂O₂ had no effect on Tg fragmentation. Fragments accumulated in dead cells but not in living cells, probably reflecting the concerted action of free radicals on Tg protein and thyrocyte unity. Tg fragments were similar to those obtained using either an enhanced *in vitro* protocol for hormone synthesis or a metal-catalysed Fenton reaction [9]. The smallest radioactive peptide was identified as P40 by a Western-blotting experiment performed by using mAb 11. However, mAb 11 failed to reveal the other radiolabelled Tg peptides having a higher molecular mass on the blot. We suggest that, albeit more labelled than P40 by radioactive iodine, these peptides were only present in minute amounts in dead cells as compared with the preponderant P40. For pathological considerations, it is worth noting that efficient *in vivo* Tg fragmentation would require high H₂O₂ production, which in turn would induce cell necrosis. The rupture of plasma membranes of necrotic cells causes enzyme delivery and further tissue injuries and inflammation. Immuno-competent cells accumulate at the inflammatory site and produce chemokines [26]. High doses of P40 that accumulated in the dead cells may be simultaneously released, leading to an overload of peptide in the surrounding living thyrocytes. Being activated by chemokines, thyrocytes would act as presenting cells [7,8] and process P40 to present cryptic epitopes to T-cells.

Thirdly, we had to find out whether P40 only bound to thyrocytes or whether it was internalized into the cells. Using a well-defined test [17], we found that P40 entered thyrocytes more efficiently than the entire Tg molecule. Such a finding posed questions as to the pathological function of P40. As already reported [9], this peptide contains two hormone-forming sites, but not the major one, which is located on residue Tyr⁵ at the N-terminus of Tg. Consequently, P40 would not be involved in the physiological process of hormone release. In contrast, Tg fragmentation would be required for preferential P40 targeting to lysosomal compartments in a pathological situation leading to autoimmune processes [27]. Another view, which does not come directly within the scope of the present study, concerns the membrane receptor for P40. Megalin (a member of the low-density-lipoprotein receptor family of proteins also known as gp330) was proposed to mediate Tg endocytosis into the thyrocytes through a heparin-binding consensus sequence, and a related sequence is present in the P40 [28]. However, it was further demonstrated that this megalin-mediated process resulted in transcytosis of Tg without proteolytic cleavage [29], as observed for antigen processing in the lysosomal compartment. Moreover, a human Tg peptide which is part of the P40 was recently found to bind and enter porcine thyrocytes [30], as described in the present study for the P40 and human thyrocytes. Albeit not yet defined, it was suggested that this shorter peptide bound and internalized the thyrocytes via a Tg cell-surface component different from megalin, but which occurred widely on various cell-types [30].

In conclusion, we found that, in the presence of iodide and under defined culture conditions, human thyrocytes were able to cleave Tg into several fragments under H₂O₂ stress. Evidence of the smallest immunoreactive fragment, P40, was found in the dead cells and it was observed to enter the living thyrocytes. Considering the putative implication of P40 in the initiation of an autoimmune disease, it would be interesting to analyse its fate after internalization in thyrocytes.

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