H₂O₂ induces apoptosis of pig thyrocytes in culture

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Abstract

Apoptosis might be involved in the reduction of the thyroid cell population in physiopathological situations such as goitre involution and autoimmune deleterious processes. Up to now, little attention has been paid to the apoptotic phenomenon in the normal thyroid gland the specialized metabolism of which is expected to generate reactive oxygen species. Indeed, thyroid cells have the capacity to synthesize H₂O₂. In this study, we have analyzed the capacity of H₂O₂ to trigger apoptosis of pig thyrocytes in culture to try to determine whether thyrocytes exhibit a particular resistance to apoptosis induced by an oxidative stress. We show that exposure of thyrocytes cultured as monolayers to exogenous H₂O₂ induced cell death with characteristics of apoptosis. The effect of H₂O₂ was concentration-dependent; apoptotic cells were already observed after exposure to 50 µM H₂O₂. At high concentrations (millimolar range), H₂O₂ exerted toxic effects leading to rapid cell disruption. Within the first hour after the onset of exposure to 50–300 µM H₂O₂, early signs of

apoptosis, i.e. DNA fragmentation, appeared in a low (0.1–1%) but definite fraction of thyrocytes. The proportion of adherent cells exhibiting DNA fragmentation remained fairly constant after 6, 15 and 24 h. During the 24-h period, an increasing number of cells detached from the culture dish and up to 30–40% of cells in suspension displayed apoptotic features. The fraction of cells that lost contact with the culture dish amounted to up to 25% 24 h after addition of 300 µM H₂O₂.

In conclusion, as reported for other cell types, low H₂O₂ concentrations are capable of triggering apoptosis in thyrocytes cultured as monolayers. Thyrocytes that undergo apoptosis secondarily lose contact with neighbour cells and the substratum; cell detachment from the monolayer probably happens within 1–2 h after initiation of DNA fragmentation. Our data show that the apoptotic commitment can take place many hours after initiation of the oxidative stress.

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Introduction

Cells undergoing apoptosis can be distinguished from cells dying by necrosis using biochemical and morphological criteria. Necrosis leads to cell swelling, membrane lysis and release of organelles while apoptosis or programmed cell death is characterized by cell shrinkage, chromatin condensation and DNA fragmentation (Schwartzman & Cidlowski 1993). Apoptosis is expected to play, in the thyroid gland as in many different tissues and organs, a role in the regulation of cell populations. In the normal gland, thyroid cells should divide 5–10 times in a life-time, while the size of the organ is not modified (Dumont *et al.* 1992). The homeostatic control of the thyroid cell number probably brings into play a dynamic balance between cell proliferation and cell death by apoptosis. The ability of normal thyrocytes to undergo apoptosis *in vitro* has been demonstrated in dog thyroid cells in culture deprived of serum and/or growth factors (Dremier *et al.* 1994). Apoptosis might be involved in thyroid pathophysiology. The reduction of the volume of the gland that occurs during the involution of hyperplastic goitre and the loss

of thyroid epithelium or tissue destruction in chronic thyroiditis could proceed, at least in part, through programmed cell death. Many different studies have shown that the involution of hyperplastic thyroid glands in response to iodide refeeding of iodine-deficient rats or mice is largely related to cell death. Depending on the administered dose of iodide, cell deletion would take place by necrosis or apoptosis. High doses of iodide induce cell necrosis (Mahmoud *et al.* 1986, Rognoni *et al.* 1987, Wollman *et al.* 1990), whereas moderate doses of iodide cause the reduction of the cell number by apoptosis (Wollman & Breitman 1970, van den Hove-Vandenbroucke *et al.* 1982, Many *et al.* 1985, 1986). Apoptotic cells have been identified in needle-biopsied thyroid tissue samples diagnosed as Hashimoto's thyroiditis (Kotani *et al.* 1995). More recently, Kawakami *et al.* (1996) and Giordano *et al.* (1997) have provided evidence for Fas-mediated apoptosis of human thyrocytes.

Thyrocytes have a particular status with regard to the phenomenon of apoptosis. On the one hand, thyrocytes produce high amounts of H₂O₂ (Björkman & Ekholm 1984, Raspé & Dumont 1995) that acts as an acceptor of

electrons generated in the course of the oxidative reactions leading to the iodination of tyrosine residues and coupling of iodinated tyrosines within thyroglobulin (Nunez & Pommier 1982). On the other hand, oxygen reactive species including H₂O₂ are potent inducers of apoptosis. The lack of data on the susceptibility of thyrocytes to go through apoptosis in response to oxidative stresses prompted us to analyze whether pig thyrocytes in primary culture exposed to exogenous H₂O₂ exhibit signs of apoptosis.

The implication of oxidative stress in apoptosis is based on the consideration that many stimuli that do not exert a direct effect on oxidative processes induce simultaneously apoptosis and an increase in cellular oxidative metabolism (Haecker & Vaux 1994). Many inhibitors of apoptosis enhance cellular defences against oxidants (reviewed by Slater *et al.* 1995). So, H₂O₂ (and its oxidant derivatives) can directly interact with the apoptotic executive machinery.

Because the oxidative stress leads to either apoptosis or necrosis according to the intensity of the stress (Bonfoco *et al.* 1995), we have designed experimental approaches allowing the discrimination between these two processes. We report that a definite proportion of pig thyrocytes cultured as monolayers go through apoptosis after exposure to H₂O₂ concentrations as low as 50 µM.

Materials and Methods

Reagents

FITC-labelled dUTP and terminal deoxynucleotidyl transferase were purchased from Boehringer Mannheim (Mannheim, Germany). Propidium iodide and Hoechst reagent (33342) were provided by Molecular Probe (Leiden, Holland).

Isolation of pig thyroid cells and cell culture

Adult pig thyroid glands were obtained from the local slaughter-house and processed within 1 h of the animals' deaths. The isolation procedure and the culture conditions were the same as those previously reported (Munari-Silem *et al.* 1990). Briefly, after dispersion of thyroid cells by discontinuous trypsinization, the cell suspension was extensively washed in F-12 medium, then resuspended in the same medium containing 5% calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin-B (0.25 µg/ml). Thyrotropin (TSH) (1 mU/ml) was added one day after seeding. Cells were seeded in Falcon petri dishes (Falcon, Oxnard, CA, USA) at a density of 0.5×10^6 cells/cm² and cultured under a 95% air–5% CO₂ atmosphere for 2 days.

In situ terminal-dUTP-fluorescein nick-end labeling (TUNEL) method (Gavrieli *et al.* 1992)

Cells were incubated in a 4% PBS–paraformaldehyde (pH 7.2) fixative solution for 20 min and in a PBS–Triton

X-100 (0.25%, v/v) permeabilization solution for 20 min. Cells were rinsed with PBS and incubated at 37 °C for 2 h with terminal deoxynucleotidyl transferase (0.5 unit/µl) and FITC-labeled dUTP (0.5 nmol/µl). The reaction was terminated by washing with sodium citrate buffer.

Floating cells were submitted to the same protocol as that used for adherent cells. For that, cells were spread out on glass slides by centrifugation at 50 g for 5 min using a cytospin centrifuge (Shandon Elliott, Camberley, UK). Fluorescent nuclei were detected by epifluorescence using an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a PlanNeoFluar × 100 objective (N.A. 1.30).

In situ nuclei staining

Living cells or paraformaldehyde-fixed cells were incubated with the Hoechst (33342) reagent (1 µg/ml) for 20 min at room temperature. This procedure labeled the nucleus of all cells. To detect cells with altered plasma membrane permeability properties, living cells were incubated with propidium iodide (1 µg/ml) for 15 min. Contrary to the Hoechst reagent, propidium iodide does not freely cross lipid bilayers.

DNA fragmentation analysis

Cells were washed in PBS and pelleted by centrifugation at 100 g for 10 min. The pellet was resuspended and incubated in 10 mM Tris buffer containing 100 mM EDTA and 0.5% SDS for 3 h at 55 °C. DNA was extracted with buffered phenol:chloroform (5:1) mixture and then precipitated in a 3 M sodium acetate buffer (pH 8):ethanol mixture (1:2.5). Samples were submitted to electrophoresis on 2.5% agarose gels and DNA was stained with 0.1% ethidium bromide.

Quantitative measurements from fluorescent labelings

Images obtained with an Axiovert 35 M inverted microscope (Zeiss) equipped with an AchroStigma × 32 objective (N.A. 0.40) were monitored using a SIT camera (Lhesa LH4036) and converted to digital images using Crystal Sapphire equipment (Quantel, Montigny le Bretonneux, France). FITC-labeled nuclei (TUNEL-positive cells) and propidium iodide-labeled nuclei in a given microscope field were counted on the screen. The number of Hoechst-labeled nuclei (total number of cells) in each microscope field was determined automatically by the image analyzer as the value of the fluorescent area divided by the average fluorescent area of a nucleus. The proportion of apoptotic cells in a given culture condition was assessed from serial determination of the number of nuclei labeled by FITC, propidium iodide and Hoechst reagent on at least ten microscope fields.

The total number of cells in a dish was obtained by multiplying the average number of nuclei per field by the

Figure 1 Identification of apoptotic cells in monolayers of pig thyrocytes exposed to 100 μM H_2O_2 for 24 h. Cells were incubated with propidium iodide for 15 min then fixed and submitted to the TUNEL labeling method and finally incubated with the Hoechst reagent to label all nuclei as described in Materials and Methods. (a), (b), (c) and (d) correspond to control cells and (a'), (b'), (c') and (d') correspond to H_2O_2 -treated cells. Each series of four images was taken from the same microscope field. (a) and (a'), Hoechst-fluorescence; (b) and (b'), phase contrast images; (c) and (c'), FITC-fluorescence identifying TUNEL-positive cells; (d) and (d'), propidium iodide-fluorescence. Bar=100 μm .

total number of fields in the dish. The average number of nuclei per field was determined from measurements performed on 10–20 microscope fields taken at random.

Results

Exposure of thyrocyte monolayers to 100 μM H_2O_2 for 24 h did not modify the overall cell morphology (compare panels b and b', Fig. 1). However, careful examinations of both phase contrast (Fig. 1b and b') and Hoechst-fluorescence (Fig. 1a and a') images revealed that H_2O_2 treatment caused a reduction of the cell density. A decrease in the number of Hoechst-labeled nuclei per unit area was reproducibly observed. Additional fluorescent labeling of nuclear DNA was used to determine whether the disappearance of a fraction of the thyrocyte population was related to a toxic effect of H_2O_2 or resulted from apoptosis induced by H_2O_2 . As a first step, living cells were incubated with propidium iodide which is known to enter cells and to label DNA only if cells have altered plasma membrane permeability. The second step carried out on fixed cells aimed at the detection of DNA fragments by the TUNEL method. As shown in Fig. 1c', a low but definite fraction of thyrocytes (that had been exposed to H_2O_2 for 24 h) exhibited an FITC-labeled nucleus i.e. were TUNEL-positive. None of the TUNEL-positive thyrocytes had a propidium iodide-labeled nucleus (Fig. 1d'). Thus, DNA fragmentation occurred in cells with intact plasma membrane permeability properties. These data led us to consider that the reduction of the thyrocyte population after H_2O_2 treatment was, at least in part, related to apoptosis. Microscopic examination at higher magnification (Fig. 2) showed that TUNEL-positive nuclei exhibited different morphological features. The TUNEL-positive nucleus shown in Fig. 2b had a regular shape and was homogeneously labeled. In contrast, the TUNEL-positive nucleus of Fig. 2a presented fluorescent patches probably corresponding to chromatin fragments; these fragments were also identifiable with Hoechst labeling (Fig. 2a'). The former case was far more frequent than the latter. These observations clearly indicate that the TUNEL method allows the detection of an early apoptotic event i.e. DNA cleavage that occurs before any detectable change in chromatin organization and nuclear morphology. From Figs 1 and 2, it can be seen that thyrocytes exhibiting apoptotic features were scattered throughout the cell monolayer. Similar results were obtained when cycloheximide, which has been reported to be an inducer of apoptosis (Dremier *et al.* 1994), was used instead of H_2O_2 (results not shown).

We then studied whether thyrocytes exhibiting early signs of apoptosis (i.e. DNA cleavage within an apparently intact nucleus) remained within the cell monolayer or detached from the culture dish to go through the executive phase of apoptosis. When the culture medium of

Figure 2 Morphological characteristics of the nucleus of adherent thyrocytes undergoing apoptosis. Twenty-four hours after exposure to 100 μM H_2O_2 , monolayer cells were submitted to the TUNEL labeling method and the nuclei were labeled with the Hoechst reagent. (a) and (b), FITC-fluorescence images identifying TUNEL-positive cells; (a') and (b'), Hoechst-fluorescence labeling of all nuclei in the corresponding fields. The arrows identify apoptotic cells. Bar=10 μm .

H_2O_2 -treated cells was centrifuged at 50–100 g , a small cell pellet was obtained; this cell population was submitted to the triple fluorescent labeling procedures. A large proportion (up to 30–40%) of thyrocytes in suspension appeared as typical apoptotic cells; they were shrunk and rounded and displayed membrane blebs (Fig. 3). These floating cells exhibited a TUNEL-positive but propidium iodide-negative nucleus. In most cases, FITC fluorescence divided amongst several pieces, indicating a fragmentation of the nucleus. Disruption of the nuclei either led to a small number of big fragments (Fig. 3a) or to numerous smaller fragments (Fig. 3b). Labeled nuclear fragments were frequently observed within cell buds, probably indicative of the formation of apoptotic bodies. Thyrocytes in suspension exhibited features of advanced stages of apoptosis. The electrophoretic analysis of DNA extracted from floating cells is reported in Fig. 4. Cells collected from the culture medium of thyrocytes treated with either H_2O_2 or cycloheximide contained DNA fragments deriving from internucleosomal cleavages. The DNA cleavage products formed the typical ladder, the size of the bands being a multiple of about 186 bp. The bands obtained from DNA extracts of H_2O_2 -treated cells were more diffuse. This might reflect a further processing of the DNA fragments as reported in other cell types (Bonfoco *et al.* 1995). These observations indicated that thyrocytes undergoing apoptosis secondarily detached from the cell monolayer.

Figure 3 Apoptotic features of thyrocytes in suspension. Twenty-four hours after exposure to 100 μ M H₂O₂, floating cells were collected, spread on glass slides using a cytospin centrifuge and submitted to the TUNEL labeling method. (a) and (b), FITC-fluorescence identifying TUNEL-positive cells; (a') and (b'), phase contrast images of the corresponding cells. The arrows identify a cell bud containing DNA, probably indicative of the formation of an apoptotic body. Bar=10 μ m.

Figure 4 Analysis of DNA fragmentation by agarose gel electrophoresis. DNA was extracted from control thyrocytes (lane 1) and from thyrocytes collected from the culture medium after 24 h treatment with 10 μ g/ml cycloheximide (lane 2) or 100 μ M H₂O₂ (lane 3). DNA size markers are shown (Std). DNA was stained with 0.1% ethidium bromide. The tracing presented on the right of the Figure gives the intensity and distribution of the fluorescent bands of lane 3. The average distance between bands numbered 1 to 4 corresponds to about 186 bp.

This sequence of events was further documented by analyzing the H₂O₂ effects at different times after the onset of treatment (Table 1). Three parameters were studied:

(i) the number of cells exhibiting a TUNEL-positive nucleus in the adherent cell population, (ii) the number of cells that detached from the culture dish and (iii) the

Table 1 Effects of H₂O₂ on the triggering of apoptosis and cell detachment during a 24-h period. Thyrocytes were cultured for two days, exposed to 100 µM H₂O₂ for 6 h and further incubated in normal medium for up to 18 h. The number of adherent cells and the number and proportion of cells with a TUNEL-positive nucleus were determined 6, 15 and 24 h after the onset of treatment. To assess the number of cells that detached from the support and the proportion of TUNEL-positive nuclei in this cell population, the culture medium was collected during two periods of 9 h: between 6 and 15 h and between 15 and 24 h. Cells in suspension were spread on glass slides using a cytocentrifuge for fluorescence labeling. In control conditions (no treatment), the number of adherent cells remained constant during the 24-h period; the fraction of cells in suspension was negligible. The proportion of control cells with a TUNEL-positive nucleus was lower than 0.08%. Results are expressed as the means ± S.E.M. of three determinations. Numbers in parentheses give the fraction of floating cells as a percentage of the total number of adherent cells

Time or period of time after H ₂ O ₂ addition (h)	Adherent cells		Cells in suspension	
	Number per dish (× 10 ⁻⁶)	TUNEL-positive cells (% of total)	Number per dish (× 10 ⁻⁶)	TUNEL-positive cells (% of total)
6	6.55 ± 0.28	0.31 ± 0.03	—	—
15	6.30 ± 0.25	0.44 ± 0.05	—	—
24	5.90 ± 0.28	0.35 ± 0.05	—	—
6–15	—	—	0.26 ± 0.02 (3.8)	29 ± 5
15–24	—	—	0.41 ± 0.03* (6.0)	35 ± 2

*P<0.01 compared with 6–15 hours.

proportion of floating cells with a TUNEL-positive nucleus. After 6-h treatment with 100 µM H₂O₂, about 0.31% of total adherent cells were TUNEL-positive; a similar proportion of TUNEL-positive cells (0.44 and 0.35%) was found 15 and 24 h after the onset of treatment. Thus, the proportion of adherent cells exhibiting early signs of apoptosis remained nearly constant from 6 to 24 h after the onset of the treatment. It is worth noticing that TUNEL-positive, propidium iodide-negative cells have been detected as early as 1 h after H₂O₂ addition. H₂O₂ treatment caused a continuous reduction (although not statistically significant) of the adherent cell population from 6 to 24 h. The progressive release of cells from the culture dishes was demonstrated by the quantitative estimation of cells in suspension. About 0.26 and 0.41 × 10⁶ cells per dish, representing respectively 4% and 6% of the initial adherent cell population, were collected as floating cells during the two successive periods of 9 h. Among the floating cells, 30–40% corresponded to apoptotic cells. Probably, these values did not reflect the actual proportion of apoptotic cells within the floating cell population since cells in the course of apoptosis probably broke up and disappeared during the 9-h period of collection. Dead cells escaping from the analyses result in an overestimation of the actual fraction of non-apoptotic cells in the floating cell population. We do not have a definite explanation for the presence of apparently normal cells in the culture medium. Normal cells contiguous to cells committed to apoptosis could detach from the support together with the apoptotic cells. As mentioned earlier for adherent cells, there were very few propidium iodide-labeled cells in the population of cells in suspension. This indicates that alterations of plasma membrane integrity probably only occurred during a short period in the terminal phase(s) of apoptosis. In

contrast, propidium iodide-positive, TUNEL-negative cells were greatly increased after exposure of thyrocytes to 3 mM H₂O₂ (data not shown). At high concentration, H₂O₂ exhibited cell toxicity. Below 0.5 mM, H₂O₂ produced a concentration-dependent increase in the number of monolayer cells committed to apoptosis (Fig. 5a). The fraction of cells that detached from the culture substratum during a 24-h period of treatment progressively enlarged with increasing concentrations of H₂O₂. Exposure to 300 µM H₂O₂ induced the release of 27–30% of thyrocytes initially present in the cell monolayer (Fig. 5b).

Discussion

We have found that pig thyrocytes cultured as monolayers are highly responsive to H₂O₂ which causes thyrocyte cell death mostly, if not solely, through induction of apoptosis. The higher the H₂O₂ concentration, the higher the proportion of cells committed to apoptosis. However, above 0.5 mM, H₂O₂ rapidly altered cell viability through a pathway unrelated to apoptosis, but possibly akin to what is called necrosis *in vivo* and ‘accidental’ cell death *in vitro*. Apoptotic thyrocytes were readily detected after an exposure to 50 µM H₂O₂. At concentrations as low as 10–30 µM, H₂O₂ was reported to act as a potent apoptotic inducer of rat thymocytes (Sakamoto *et al.* 1996) or human HL-60 cells (Sakagami *et al.* 1996). In contrast, induction of apoptosis of human lung adenocarcinoma cells required a much higher (1 mM) H₂O₂ concentration (Kazzaz *et al.* 1996). The sensitivity of thyrocytes to undergo apoptosis following the oxidative stress produced by H₂O₂ does not appear to differ from that of other cells. This suggests that

Figure 5 Apoptotic responses of thyrocytes exposed to increasing concentrations of H₂O₂. Thyrocytes cultured for two days were exposed to different H₂O₂ concentrations for 24 h. The number of TUNEL-positive nuclei in the thyrocyte monolayers and the number of cells that remained adherent to the culture support at the end of the treatment were determined as described in Materials and Methods. (a), Number of apoptotic cells per dish; (b), number of adherent cells 24 h after the outset of H₂O₂ treatment. Results are expressed as the mean ± S.E.M. of three determinations. On day 2, culture dishes contained about 6×10^6 thyrocytes; the number of adherent thyrocytes in control conditions (no treatment) did not vary during the 24-h period.

thyrocytes are probably no more capable than most other cells of overcoming the deleterious effects of reactive oxygen species. Thus, efficient control mechanisms of cellular H₂O₂ levels are required to rescue thyrocytes from apoptosis in case of H₂O₂ overproduction during thyroid hormone synthesis processes. In thyrocytes, H₂O₂ in excess is probably eliminated by the concerted actions of several enzymes including the selenium-dependent glutathione peroxidase (Corvilain *et al.* 1991). A diminution of the glutathione peroxidase activity due to selenium deficiency alone or in conjunction with an augmented production of H₂O₂ related to iodine deficiency has been proposed as a potential cause of thyroid destructive processes (Contempré *et al.* 1995). It is not known to what

extent apoptosis could contribute to the changes of the thyroid gland following alterations of the oxidative reactions and/or antioxidant defences.

Our study provides information on the development of the apoptotic phenomenon of epithelial cells cultured as confluent monolayers. The process can be tentatively divided into three steps. At variable times after addition of H₂O₂, apoptosis is triggered in individual thyrocytes randomly distributed within the cell monolayer. The earliest sign of apoptosis that we detected corresponds to a uniform TUNEL labeling of the nucleus. Then, these thyrocytes lose contact with other cells and the culture support. Cells now in suspension in the culture medium progressively proceed towards late apoptotic phases (Fig. 3) going from nuclear to cell fragmentation. The observation that apoptosis commences with adherent cells and is completed following detachment, has been made on human endothelial cells (Zoellner *et al.* 1996), mouse fibroblasts (Kulkarni & McCulloch 1994) and rat hepatocytes (Bayly *et al.* 1994) in culture. After detachment of the apoptotic cells, remaining adherent cells modify their organization to maintain a confluent monolayer (see Fig. 1). We have used the quantitative data of Table 1 to estimate the time that elapses between the appearance of early signs of apoptosis in adherent cells and the detachment from the substratum. The fraction of TUNEL-positive adherent cells (F) was fairly constant from 6 to 24 h and equal to about 0.3%. Assuming that cell detachment occurs at a constant rate, the minimum rate of detachment of cells in the course of apoptosis (R) would vary between 0.15 and 0.3% of adherent cells per hour. This value takes into account the fact that only part of the floating cells were apoptotic cells. The turnover rate of adherent cells with early signs of apoptosis (given by the F to R ratio) would thus range from 1 to 2 h. According to this rough estimate, a cell with a TUNEL-positive nucleus would detach from the support within 1 or 2 h. A more precise evaluation would require a cell by cell analysis by videomicroscopy. As previously reported for other cell types, thyrocytes undergo apoptosis at variable times after the application of the oxidative stress. Adherent cells exhibiting early signs of apoptosis were still found 18 h after removal of H₂O₂ (Table 1). Apoptosis appears essentially as an asynchronous event. This is even true for cell cycle-synchronized cells (Earnshaw 1995). Only a limited fraction of thyrocytes is involved in apoptosis. This means that all thyrocytes did not have the same susceptibility to an oxidative stress. The origin of this diversity of cell responses should be the goal for future studies.

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