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APOPTOSIS IN DOG THYROID CELLS

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Summary : Apoptosis, a physiological cell death, has been shown to be involved in tissue homeostasis as well as in tissue regression due to hormone deprivation. The thyroid cell population slowly turns over, with cell loss compensating mitogenicity. In the absence of thyrotropin, the cell population decreases. The possible involvement of apoptosis in this loss has been studied. We show in this report that deprivation of serum, epidermal growth factor and thyrotropin triggers internucleosomal DNA fragmentation and morphological modifications characteristic of apoptotic cell death in dog thyroid cells in primary culture; cycloheximide treatment has the same effect. This indicates that thyrocytes are endowed with a constitutive apoptosis "program" and that the latter might be involved in the thyroid regression observed in vivo in the absence of full growth stimulation of the gland.

Contrary to a previously held assumption, thyroid cells divide in adult individuals and, in various species, it has been estimated that they do so 5 to 10 times in a life time (1). As the size of the organ and therefore presumably the population of cells remains roughly constant, this implies that a corresponding fraction of cells constantly disappears (2). The process by which this occurs in many cell types is apoptosis (3). This type of cell death has been occasionally observed in histological sections of thyroid, suggesting that this might also be the case in these cells (4). This work aimed at testing this assumption by checking whether dog thyroid cell in culture would develop apoptosis when treated with the protein synthesis inhibitor cycloheximide, a potent inducer of apoptosis in other cells types (5) or in response to EGF, serum and TSH deprivation (GF deprivation). Apoptosis was indeed induced in the two situations. The fact that cycloheximide did

Abbreviations:
TSH : Thyrotropin; EGF : Epidermal Growth Factors; FCS : Foetal Calf Serum; GF : Growth Factors.

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not reduce the effect of GF-startvation further indicates that the proteins necessary for this process are present in the normal thyrocytes and do not have to be induced.

Material and Methods

Material. Cycloheximide and collagenase were purchased from Sigma. The culture media and foetal calf serum (FCS) were from Gibco BRL. EGF was from Sigma and TSH from Armour Pharmaceutical Co. [^{3}H]thymidine was purchased from Amersham Belgium (Brussels). Agarose was from Bio Rad. EDTA, Tris and Triton X-100 were from Boerhinger.

Primary cultures of thyroid cells. The cells were collected from dog thyroid tissue as described by Roger *et al.* (6). Briefly, the thyroid was treated with collagenase (150 U/ml) at 37°C for 1 h to obtain mainly fragmented and intact follicles. These were seeded at a density of 10^4 cells/cm^2 in tissue culture-treated dishes and maintained in a water-saturated incubator at 37°C in an atmosphere of 5% CO_2/95% air. The culture medium was Dulbecco's modified Eagle medium (DMEM) + Ham's F12 + MCBD 104 medium (2/1/1, v/v/v) containing 100 U penicillin/ml, 100 µg streptomycin/ml and supplemented with 5 µg insulin/ml, 40 µg ascorbic acid/ml and growth factors (25 ng EGF/ml, 1mU TSH/ml and 10% FCS).

Quantification of DNA degradation. Thyrocytes seeded in 35 mm Petri dishes were maintained for 4 days in the conditions described above. They were then incubated for 24h in the same medium supplemented with 10 µCi [^{3}H]thymidine/ml, 30µM cold thymidine and 100µM desoxycytidine. They were washed twice with DMEM and incubated for 6 days in the specified medium. Every day, the cells floating in the culture medium were collected by gentle centrifugation and lysed in the lysis buffer TET (Tris HCl pH 7.4 (5mM), EDTA (20mM) and Triton X-100 (1%, v/v). At the end of the experiment, the attached cells were also lysed in the TET buffer. The lysates were submitted to a 15.000 g centrifugation to separate the fragmented from the intact DNA. The radioactivity contained in both supernatants (from floating and attached cells) reported to the total radioactivity of the cell population represents the percentage of fragmented DNA (7).

DNA electrophoresis. Thyrocytes were seeded in 100 mm Petri dishes and cultured for 5 days in the medium described above. They were then incubated for 5 days in the same medium supplemented or not with cycloheximide (2µg/ml) or in the absence of growth stimuli. The cells floating in the culture medium were collected every 24h, pooled and lysed in TET buffer. The cells yet attached to the dishes at the end of the experiment were lysed in the TET buffer. The DNA extracted from the 15.000 g supernatants was submitted to an electrophoresis on 2% agarose gel and visualized by ethidium bromide under UV.

Electron microscopic study. The culture conditions were exactly the same as for DNA electrophoresis. At the end of the culture, the cells attached to the dishes were fixed in 2.5% cacodylate buffered glutaraldehyde and embedded as previously described for microscopic examination (8). Ultrathin sections were examined in a Philips 301 electron microscope after staining with uranyl acetate and lead citrate.

Results and Discussion

It is generally accepted that cell death can be classified into passive cell death (also called necrosis) or active cell death, the latter including apoptosis (9,10). As apoptosis has been implicated in tissue regression or after hormone deprivation (11-13), we hypothesized that it could be involved in growth factor deprived thyrocytes.

Our first aim was to confirm that thyrocytes are able to undergo apoptosis. We tested first the effect of cycloheximide, a protein synthesis inhibitor, on the viability of these cells as this drug has been shown to trigger apoptosis in some cell types (5,7). As shown in Fig 1-3, thyroid cells

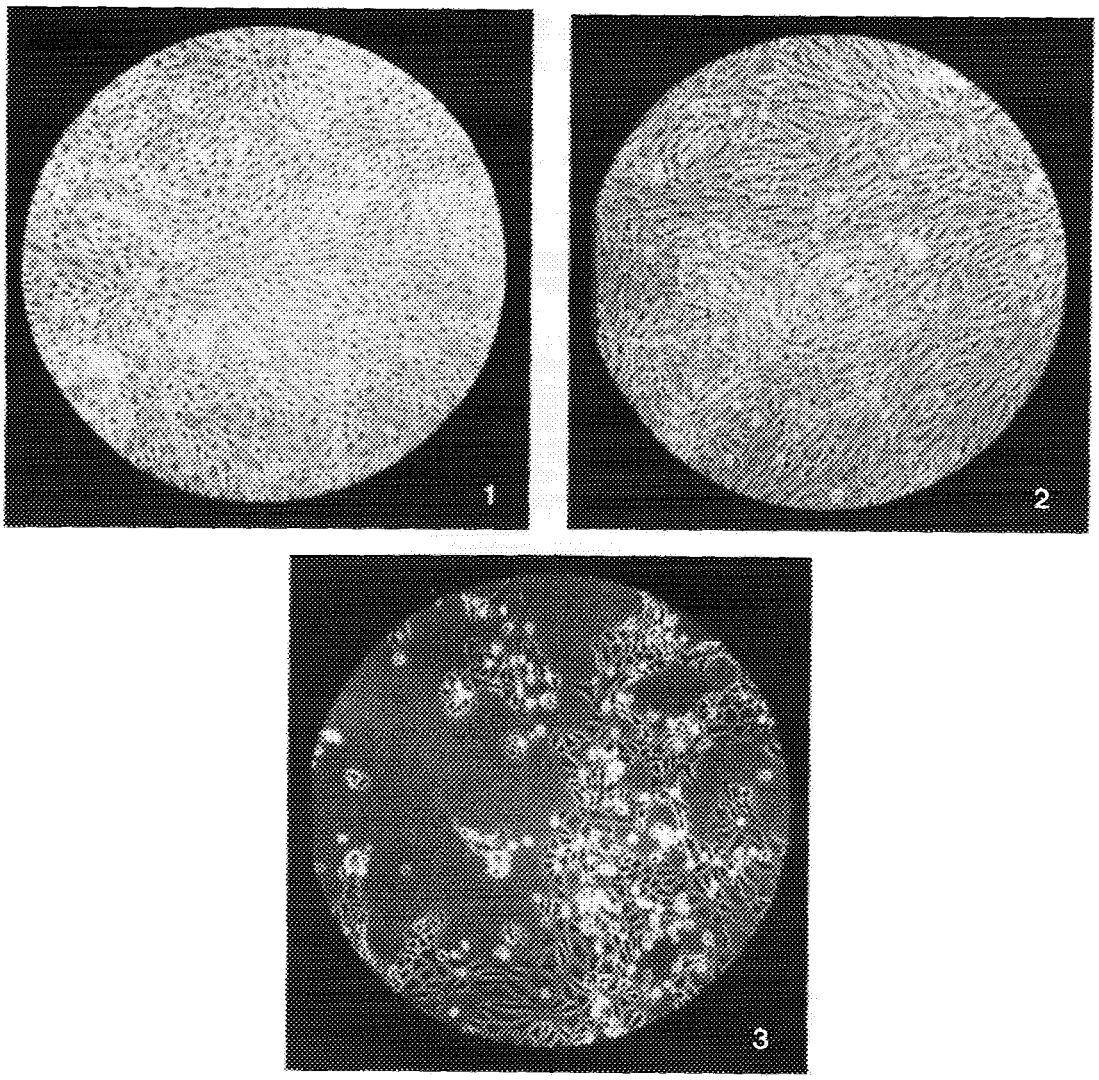

Figure 1. Thyrocytes were seeded and incubated for 5 days as described above. When the cells were deprived of growth factor for 6 additive days (2), some of them rounded and were floating in the culture media; such a phenomenon was not observed in thyrocytes incubated in complete medium (1). When they were incubated with cycloheximide (2μg/ml, 3) for 6 days, a greater number of cells rounded up and was floating in the culture medium. (X100).

treated 6 days with cycloheximide (2μg/ml) were mostly rounded and floated in the medium. The cytosolic DNA extracted from these floating cells or from the cells attached to the dishes after this treatment showed (Fig 2) the characteristic ladder-like electrophoretic pattern, indicating apoptotic cell death (9). Moreover, electron microscopy examination of cycloheximide treated cells revealed a majority of cells exhibiting typical morphological features of apoptosis, namely apoptotic bodies enclosing intact cytoplasmic organelles and/or condensed chromatin, (Fig 3-2). Secondary necrosis was present in some of those apoptotic bodies. The fact that morphological characteristics of

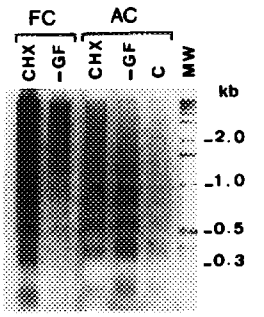

Figure 2. Agarose gel electrophoresis of DNA extracted from the 15000g supernatant of floating (FC) or attached (AC) thyrocytes incubated for 6 days with (c) or without serum,EGF,TSH (-GF) supplemented with cycloheximide (2µg/ml) (CHX). MW: restriction fragments of Hind III and EcoRI of phage lambda.

apoptosis and internucleosomal DNA degradation were observed in attached cells demonstrates that the apoptotic process started before their detachment and did not result from it.

These results thus clearly show that apoptosis can be triggered in primary cultures of dog thyroid cells. The cycloheximide (2µg/ml) concentration we used inhibits within two hours by more than 95% the incorporation of [^{35}S]-methionine into total proteins (14). This suggests therefore that thyrocytes in culture constitutively express the proteins necessary for the apoptotic process.

Our second aim then was to investigate whether apoptosis could be triggered in our cells by growth factor deprivation. Therefore, after a 5 days incubation in the presence of growth factors (EGF 25 ng/ml, TSH 1 mU/ml and FCS 10%), thyrocytes were incubated for 6 additional days in the absence of these growth factors. At the end of this treatment an abundance of cells were seen rounded up and floating in the culture medium (Fig 1-2). This phenomenon was exceptionally observed in continuously stimulated cells (Fig 1-1). As apoptosis is mainly characterized in light microscopy by condensation and rounding of the cells (9), this suggested that a fraction of the cell population underwent apoptosis when deprived of growth factors. At the electronic microscopic level, we observed that some cells, in the bottom of the dishes, display classical features of apoptosis as described above (Fig 3-1). This was confirmed by the electrophoretic analysis of the DNA extracted from the floating as well as from attached cells after 5 days of GF deprivation revealing in both cases the characteristic DNA laddering pattern (Fig 2).

We determined the percentage of DNA degradation, which has been shown in other models to be correlated to the percentage of apoptotic cells (7,15). A cycloheximide treatment was used as positive control. As shown in Fig. 4, after 24 h of incubation in the presence of cycloheximide (2µg/ml), almost 10 % of total incorporated radioactivity was recovered in the cytosol of floating cells. This effect increased rapidly to reach more than 30 % after 6 days. When thyrocytes were deprived of growth factors, a slight but significant DNA fragmentation was already

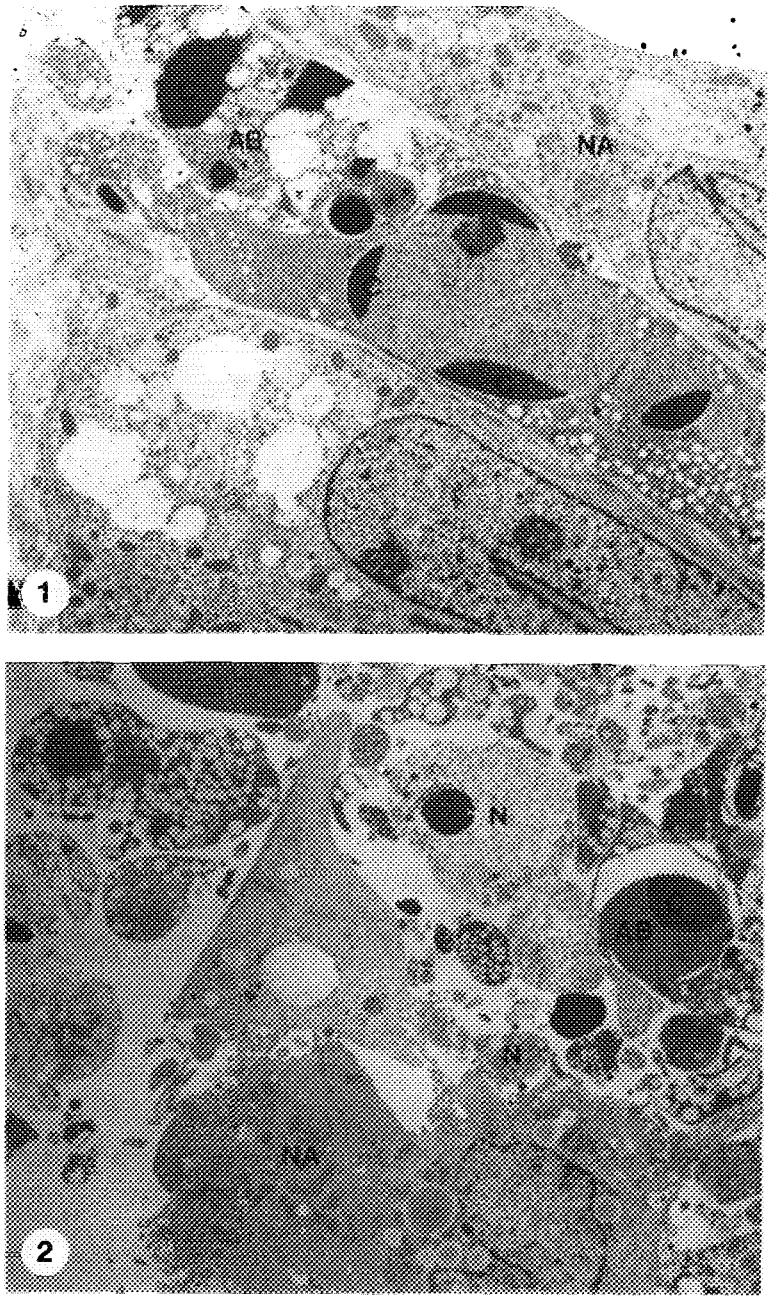

Figure 3 -1. Electron micrograph of cells attached in the dish after GF deprivation. Condensed chromatin (C) was observed in apoptotic cells and apoptotic bodies (AB) but not in nonaffected cell (NA) (X 4,400).
Figure 3 -2. Electron micrograph from attached cells after a 6-day treatment with cycloheximide (2μg/ml). Apoptotic bodies containing nuclear fragments with condensed chromatin (C) and secondary necrosis (N) of apoptotic bodies were observed, NA: nonaffected cells (X 3,000).

observed after 24h. After 6 days of growth factor withdrawal almost 10% of the total DNA was fragmented contrasting whith the 2% of spontaneous DNA degradation measured at this time in the control cultures.

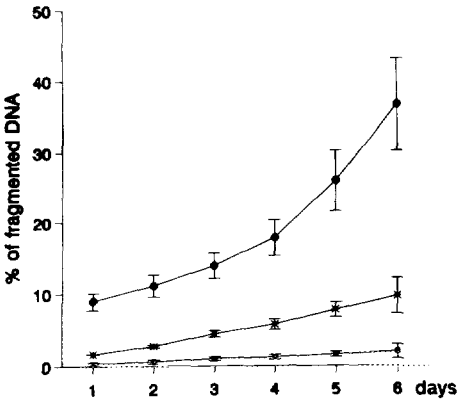

Figure 4. Kinetics of the induction of DNA fragmentation in thyroid cells by cycloheximide (2μg/ml) treatment (-●-) and growth factor deprivation (-✳-). Control medium (-■-). Average value (± SD) of triplicate measurements is taken from one representative out of three experiments.

We then investigated the requirement of protein synthesis to the GF deprivation induced apoptosis of the thyrocytes. After GF stimulation of the cells for 5 days, the cells were cultured 30h with or without EGF, FCS and TSH in the presence or absence of cycloheximide (2μg/ml). The percentage of fragmented DNA triggered by the drug alone and by GF-starvation were 7.4 ± 0.2% and 3.8 ± 0.3% respectively; spontaneous DNA fragmentation was of 2.1 ± 0.6%. GF deprivation and cycloheximide had additive effect, 16.9 ± 2.1% of the DNA being fragmented when the two treatments were combined. To our knowledge, our experimental system is the first in which induction of apoptosis by hormone deprivation is shown to be independent from protein synthesis. It is tempting to postulate that GF could act as survival factors in these cells by inducing the synthesis of (a) protein(s) that inhibit the constitutively expressed apoptotic machinery.

The effect observed in cells after acute withdrawal of a growth stimulation could correspond to the classical in vivo experiment in wich adrenocortical cells also enter into apoptosis when ACTH treatment is stopped (3). It is known that a similar abrupt cessation of stimulation in rat thyroid in vivo also induces cell death but the nature of this death has not been established (16,17). The present results show that the apoptotic program exist in thyroid cells and suggest that this process may be involved in vivo with homeostatic control of the size of the thyrocyte population.

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