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ORIGINAL ARTICLE

Radioiodide induces apoptosis in human thyroid tissue in culture

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Abstract Radioiodide (^{131}I) is routinely used for the treatment of toxic adenoma, Graves' disease, and for ablation of thyroid remnant after thyroidectomy in patients with thyroid cancer. The toxic effects of ionizing radiations on living cells can be mediated by a necrotic and/or apoptotic process. The involvement of apoptosis in radiation-induced cell death in the thyrocytes has been questioned. The knowledge of the mechanisms that underlie the thyrocyte death in response to radiations can help to achieve a successful treatment with the lowest ^{131}I dose. We developed a method to study the effects of ^{131}I in human thyroid tissue in culture, by which we demonstrated that ^{131}I induces thyroid cell apoptosis. Human thyroid tissues of about 1 mm^3 were cultured in vitro and cell viability was determined up to 3 weeks by the MTT assay. Radioiodide added to the culture medium was actively taken up by the tissues. The occurrence of apoptosis in the thyrocytes was assessed by measuring the production of a caspase-cleavage fragment of cytokeratin 18 (M30) by an enzyme-linked immunoassay. Neither variation of cell number nor spontaneous apoptosis was revealed after 1 week of culture. ^{131}I added to the culture medium induced a dose-dependent and a time-dependent generation of M30 fragment. The apoptotic process was confirmed by the generation of caspase-3 and PARP cleavage products. These results demonstrate that ^{131}I induces apoptosis in human thyrocytes. Human thyroid tissue cultures may be useful to investigate the cell death pathways induced by ^{131}I.

Keywords Radioiodide therapy · Apoptosis · Thyroid

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Introduction

Radioiodide (^{131}I) treatment is commonly used for ablation of thyroid remnant after thyroidectomy in patients with thyroid cancer, in patients with toxic adenoma and Graves' disease and may represent an alternative to surgery in nodular goiter [1–5]. The rationale for the radioiodide ablation of the thyroid remnant after thyroidectomy in patients with differentiated thyroid cancer is to enable the follow-up by serum thyroglobulin measurement. Although the results are inconsistent between studies and there is no direct evidence, some observational evidence suggest that radioiodide ablation with ^{131}I may reduce the risk of recurrence, development of metastases, and long-term mortality from differentiated thyroid cancer [6]. In patients with toxic adenoma or Graves disease, radioiodide treatment is a useful

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option alternative to surgery. Although many centers administer a standard activity of ^{131}I (100 mCi, 3.7×10^9 Bq) for ablation of thyroid remnant after thyroidectomy in patients with differentiated thyroid cancer and a tailored dose for toxic adenoma and Graves disease, there is uncertainty over the lowest effective activity of radioiodide that can achieve successful treatment. For this reason, the mechanisms by which ^{131}I exerts its cytotoxic effects must be studied more in depth. Excess of iodide has a toxic effect on thyroid cells [7, 8]. However, the amount administered is minimal and its cytotoxicity is entirely due to the thyroid cell exposure to ionizing radiations. Cell toxicity induced by ionizing irradiation is usually attributed to DNA damage to target cells, thus triggering cell death by apoptosis and/or necrosis. DNA damage from radiation occurs either by a direct effect or indirectly through the formation of free radicals [9]. Multiple signaling pathways are activated in mammalian cells by DNA-damaging agents, leading to altered gene regulation, some of them involved in DNA repair, others representing a more generalized response to injury [10]. Besides the DNA damage, ionizing radiations damage other cellular compartments through the production of reactive oxygen species and lipid peroxidation of the plasma membrane. Impairment of cellular compartments and reactive oxygen species can induce cell death also by necrosis. Following ^{131}I administration to rats, both apoptosis and necrosis findings were observed [11]. However, other experimental evidence suggested that ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells [12]. In the same study, neither exposure of human thyroid cells in vitro to ionizing radiation nor thyroid gland irradiation in the rat induced thyroid cell apoptosis. More recent data suggest that the apoptotic or necrotic nature of the cytotoxic effect of ^{131}I is dose dependent [13]. It has been supposed that high ^{131}I doses produce mainly necrotic phenomena, whereas low ^{131}I activity induces apoptosis. Hershman et al. recently developed an in vitro model in rat thyroid cells by which it is possible to study molecular aspects of the cellular damage induced by ^{131}I [14]. We developed a method to study the effects of ^{131}I in human thyroid tissue in culture, by which we demonstrated that ^{131}I induces thyroid cell apoptosis.

Materials and methods

Thyroid cell cultures

Thyroid tissues were obtained from controlateral lobe of papillary thyroid cancer undergoing thyroidectomy, after patients gave their written consent. The tissues were cut in small pieces of 1 mm^3, washed in PBS, and cultured in a 5 % CO_2 atmosphere at 37 °C, in F-12 medium supplemented with 10 % FCS and a mixture of thyrotropin (TSH, 10 mU/ml), insulin (10 µg/ml), hydrocortisone (5 ng/ml), transferrin (5 µg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (6H). All tissues were cultured for 1 day before any experiments. Glands with concurrent Hashimoto's thyroiditis were excluded [15]. Seven-day cultured tissues stained with hematoxylin and eosin displayed normal follicular architecture (Fig. 1).

MTT assay

Cell viability and proliferation was determined using the MTT assay [16]. For each experimental point, three tissue fragments from the same gland were put together in the same well of a 24-well plate. Following the designated treatment, the culture medium was removed and 500 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT) (Sigma Chemical Co., St. Louis, MO), 0,5 mg/ml in F-12 medium were added to each well. After 4 h at 37 °C, the medium was removed from each well and replaced with an equal volume of 0.04 m HCl/isopropanol. Following an overnight incubation in darkness, the dissolved MTT crystals were quantitated. Optical densities were obtained using a test wavelength of 550 nm (Dynatech MR5000 microelisa spectrophotometer, Chantilly, VA), and total O.D. normalized per weight of tissue.

Measurement of apoptosis of epithelial cells

To quantify the apoptosis occurring in thyroid epithelial cells, we measured the production of the neo-epitope formed upon caspase-cleavage of cytokeratin 18 at position Asp396 (M30) by an enzyme-linked immunoassay . Caspase-cleaved cytokeratin-18 was measured with M30-

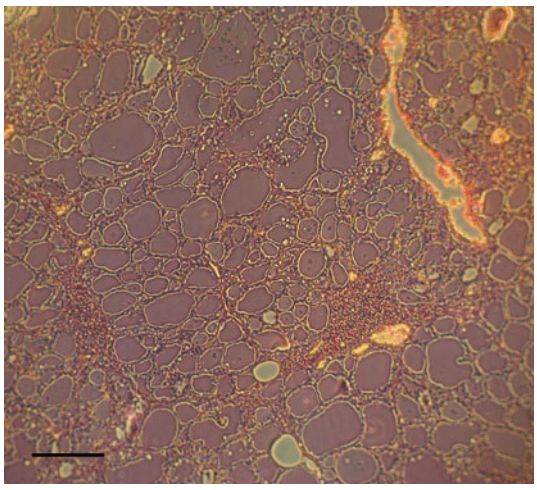

Fig. 1 Microscopic appearance of cultured thyroid tissue. Histological appearance of untreated 7-day cultured tissue, stained with H&E. The tissue shows normal follicular architecture. *Bar line* 50 µm

Apoptosence assay (Peviva, Alexis, San Diego, CA). As for the MTT assay, for each experimental point, 3 tissue fragments from the same gland were put together in the same well of a 24-well plate in 0.5 ml of medium. Following the designated treatment, 50 µl of the culture medium was removed and the assay was performed following the manufacturer protocol. Optical densities were obtained using a test wavelength of 415 nm (Dynatech MR5000), and total O.D. normalized per weight of tissue.

Western blot

Tissues were washed in cold PBS and homogenized with a mortar and pestle in RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate in PBS) supplemented with protease inhibitor phenylmethylsulfonyl fluoride (Sigma). Lysates were centrifuged at $12{,}000 \times g$ for 15 min at 4 °C and supernatant was recovered. The protein concentration in tissue lysates was determined by protein assay (Bio-Rad Laboratories, Inc. Richmond, CA), and 50 mg of total protein from each sample was boiled for 5 min in Laemmli sample buffer (125 mm Tris pH 6.8, 5 % glycerol, 2 % SDS, 1 % b-mercaptoethanol, and 0.006 % bromophenol blue). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Hybond-ECL Nitrocellulose, Amersham Pharmacia Biotech, Rainham, UK). Membranes were blocked by 5 % nonfat dry milk, 1 % ovalbumin, 5 % FCS, and 7.5 % glycine; and after three washes, the membranes were incubated for 1 h at 4 °C with 0.5 mg/ml of rabbit polyclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 °C with horseradish peroxidase-conjugated antirabbit secondary antibodies (Bio-Rad Laboratories, Inc.) diluted 1:2,000 in PBS, Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Rabbit polyclonal antibodies to Caspase-3 and PARP were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using the *t* test. The level of significance was set at $p < 0.05$. Bonferroni multiplicity correction was applied to adjusts the threshold for statistical significance.

Results

Thyroid cell viability in tissue cultures

We applied the MTT assay to evaluate the viability of thyroid cells in tissue cultures. Because the thyroid gland is largely perfused by blood vessels, the erythrocytes are abundant in tissue cultures. The mitochondria present in the erythrocytes affects the MTT assay and must be removed. We tested the efficacy of ammonium chloride to lyse the erythrocytes, treating the tissues with a lysis buffer (NH_4Cl–NaH_2CO_3–EDTA) up to 7 h and then performing the MTT assay. The test showed a rapid reduction of the O.D. that achieved a plateau by 2 h of treatment (Fig. 2). On the basis of this result, the subsequent MTT assays were preceded by 2 h treatment with ammonium chloride lysis buffer. To test the cell viability in thyroid tissue cultures, MTT assays were performed up to 3 weeks of culture (Fig. 3). After the first week of culture, no significant change in MTT assay was observed. By 2 weeks, the O.D. increased and only after 3 weeks of culture a significant O.D. increase was demonstrated. These results indicate that small thyroid tissues can be cultured in vitro up to 3 weeks without loss of cell viability.

^{131}I uptake in thyroid tissues cultures

To determine whether the thyroid cells in tissue cultures were still able to trap and concentrate iodide, tissue fragments were cultured for 1 day and then incubated with fresh medium containing 1×10^5 cpm ^{131}I, in the presence of serum and 6H, with or without 1 mM $KClO_4$. After 3–6 h, the tissues were washed and the incorporated radioactivity was counted in a gamma counter (Fig. 4). After 3 h of incubation, $KClO_4$ reduced the incorporated radioactivity although the difference was not significant. By 6 and 12 h, $KClO_4$ significantly affected the incorporation of radioactivity, being respectively 48 and 38 %

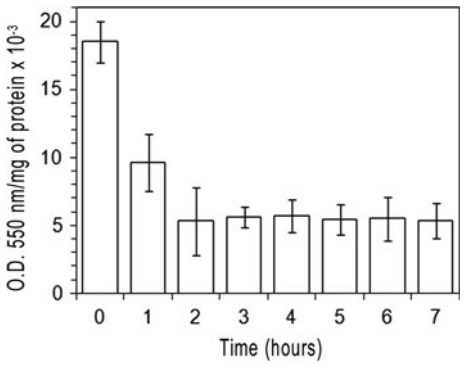

Fig. 2 Interfering effect of erythrocytes on MTT assay in thyroid tissue cultures. One mm^3 thyroid tissue samples were carefully weighted and treated at 37 °C for the indicated time with 1 ml ammonium chloride erythrocyte lysis buffer. Then, the tissues were washed and MTT assay was performed. Data are presented as mean and s.d. of triplicates O.D./mg of thyroid issue. All experimental points versus time 0 were significant after Bonferroni correction, $p < 0.007$ by Student's *t* test

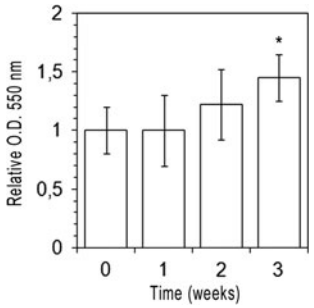

Fig. 3 MTT assay in thyroid tissue cultures. Thyroid tissues of 1 mm^3 were carefully weighted and cultured up to 3 weeks in the presence of serum and 6H. At the indicated time, the tissues were washed, erythrocytes were lysed, and MTT assay was performed. Total O.D./mg of tissue was calculated. Results are reported as mean and s.d. relative O.D. 550 nm of triplicate experiments. *$p = 0.012$, Student's t test

lower. These results indicate that the ability to uptake iodide was retained by thyroid tissues in culture.

Measurement of apoptosis in thyroid tissues

To assess the apoptosis of thyroid cells in cultured tissues, we used an immunometric assay which determines the amount of caspase-3-induced degradation of cytokeratin-18. Thyroid tissues were treated for 24 h with the apoptotic inducer staurosporine, or the necrosis inducer NaN_3 (Fig. 5). A large amount of M30 cytokeratin fragment was generated by the staurosporine treatment, while as expected NaN_3 did not.

Apoptosis induction by ^{131}I in thyroid tissue cultures

Thyroid tissues were cultured for 1 day and then placed in 24-well plates with medium containing ^{131}I. After 1 h, 1, 3,

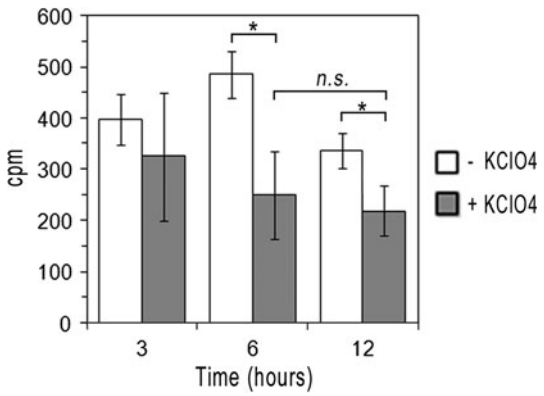

Fig. 4 ^{131}Iodide uptake in thyroid tissues in culture. One-week-old thyroid tissues were cultured up to 12 h in the presence of serum, 6H and 1×10^5 cpm ^{131}I, with or without 1 mM $KClO_4$. At the indicated time, the tissues were washed and incorporated cpm was counted by a gamma counter. *$p < 0.01$, Student's t test; *n.s.* not significant

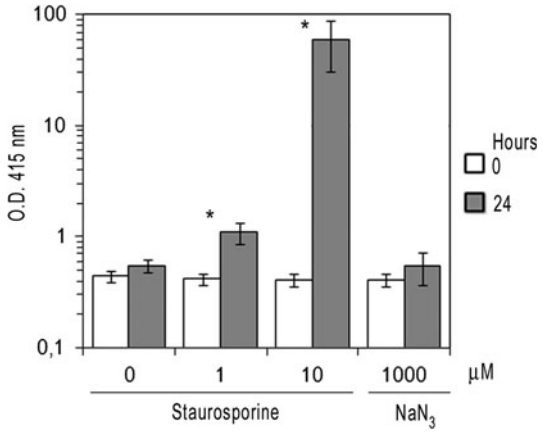

Fig. 5 Assessment of apoptosis in cultured thyroid tissues. Thyroid tissues were treated for 24 h with 0.1–1 µM staurosporine or 1 mM NaN_3. Then, the culture mediums were recovered and the presence of the M30 cytokeratin fragment was assessed by the M30 Apoptosense assay. Results are reported as total O.D. at 415 nM. *$p < 0.01$, Student's t test

or 7 days, an aliquot of medium was removed and the M30 concentration was determined by the M30 Apoptosense assay (Fig. 6). In the absence of ^{131}I, no spontaneous M30 production occurred after 3 days of culture, while it was detectable after 7 days. ^{131}I induced a significant M30 production already by 3 days of culture, demonstrating that ^{131}I induced apoptosis of thyroid epithelial cells. In a parallel experiment, thyroid tissues were cultured for 3 days as above. Then, the tissues were homogenized, proteins were extracted and analyzed by Western blot (Fig. 7). Following the treatment with 3.7×10^6 and 3.7×10^7 Bq ^{131}I, cleaved caspase-3 and PARP fragments were evident, confirming that apoptosis was occurring.

Discussion

Thyroid follicles are three-dimensional structures embedded in extracellular matrix which cannot be retained for a long term in conventional monolayer, floating, or organ cultures, [17, 18]. Thyrocyte interactions with the extracellular matrix components are mediated by integrins and generate intracellular signals that regulates cell physiology [19–21]. Furthermore, the absence of blood flow in tissue culture hampers gas and nutrients diffusion. Thus, we cultured human thyroid tissue in fragments of about 1 mm^3, sufficiently small to allow adequate O_2/CO_2 exchange and metabolites diffusion with culture medium. Cell viability was investigated by the MTT assay, based upon the tetrazole reduction to formazan in the mitochondria of living cells. MTT assay demonstrated that no significant variation of cell viability occurred in the first 2 weeks of culture. By that time, a moderate increase of absorbance was noted,

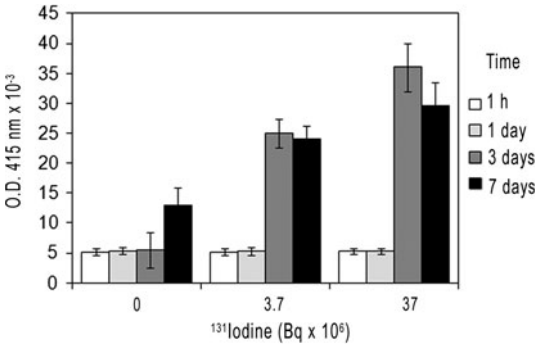

Fig. 6 Induction of apoptosis by ^{131}Iodide in cultured thyroid tissues. Thyroid tissues were cultured up to 7 days in the presence of ^{131}I. At the indicated time, 10 % of culture medium was recovered and the presence of M30 was assessed by the M30 Apoptosense assay. Results are reported as mean O.D. 415 nM of triplicate experiments. 3.7×10^6 and 3.7×10^7 Bq for 3 and 7 days, and no ^{131}I for 7 days versus 1 h, $p < 0.01$; 3.7×10^6 and 3.7×10^7 Bq for 3 and 7 days versus corresponding time without ^{131}I, $p < 0.016$ by Student's t test

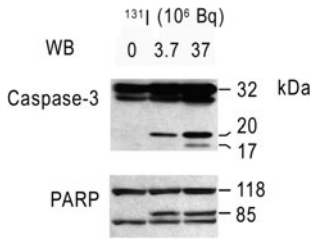

Fig. 7 Western blot analysis for caspase-3 and PARP thyroid tissues were cultured for 3 days in the presence of ^{131}I. Then, the tissues were washed, homogenized, lysed in RIPA buffer and protein extracts were separated by SDS-PAGE with 12 % acrylamide concentration. The proteins were blotted onto nitrocellulose membrane, incubated with polyclonal antibodies to intact and fragmented caspase-3 and PARP proteins and evidenced by ECL system

compatible with a change in mitochondrial dehydrogenase activity or increase of cell number, due to thyrocytes or more likely fibroblasts proliferation. Significant M30 production was detected only after 7 days of culture (Fig. 5), indicating that spontaneous apoptosis was occurring by that time. In light of these results, all the experiments were performed in the first week of culture. The ^{131}I isotope emits γ-rays but it is used for thyroid ablation by virtue of its β-particles emission. Its cell toxicity is primarily the result of the β-particle radiation, which has a path length of 1–2 mm in water. This means that the thyrocytes in tissue cultures were exposed to β-particle radiations coming from the taken up ^{131}I isotope as well as from the ^{131}I isotope present in the culture medium in which tissues were embedded. Regardless the source of β-particle radiations, this model revealed to be suitable to investigate the molecular mechanisms by which ^{131}I kills thyrocytes. High doses of ionizing radiations lead to genetic damage, mutations, and finally cell death. DNA damage from radiation can occur either by a direct effect or indirectly through the formation of free radicals [9, 22]. In both cases, chromosomal damage leads to the activation of the enzymes responsible for DNA repair, cell cycle arrest, or apoptosis. A key protein in this process is p53 which, after radiation exposure, translocates to the nucleus, where it mediates the transcription of specific genes. Although this is a general mechanisms operating in all mammalian cells, the involvement of apoptosis and the role of p53 in radiation-induced cell death in the thyrocytes have been questioned. Namba H et al. demonstrated that p53-WAF1/Cip1 pathway plays a central role in induction of G1 arrest following irradiation in human thyrocytes in culture [23]. Both apoptosis and necrosis findings were observed in the thyroid of rats treated with ^{131}I [11]. At the same time, evidence have been produced in favor of a dose-dependent apoptotic or necrotic cell death, that is high ^{131}I doses can produce mainly necrotic phenomena, whereas low doses induces mainly apoptosis [13]. However, in a following study in human thyroid primary cells in culture and intact rat, exposure to ionizing radiation confirmed that DNA end-jointing activities were promoted by p53 induction in thyroid cells, but failed to demonstrate the induction of apoptosis [12]. The measurement of the M30 fragment enabled us to quantify the apoptotic phenomena involving the thyrocytes as this protein fragment is generated by the enzymatic activity of caspase-3 on cytokeratin 18, expressed in the thyroid gland only by the thyroid follicular cell. The generation of M30 fragment following 3 days of treatment with ^{131}I indicates the occurrence of apoptosis but does not exclude also the occurrence of necrotic phenomena. Apoptosis is an active mechanism involving protein–protein interactions and regulation of gene expression, with a fundamental tumor suppression role [24]. Cancer cells can reduce the pro-apoptotic machinery thus becoming resistant to apoptosis-mediated toxic factors. p53 inactivation or down regulation and NF-kB modulation are just examples of how aberrant apoptotic signaling can contribute to cancer development through resistance to apoptosis [25, 26]. Administration of ^{131}I is currently used in the treatment of differentiated thyroid carcinoma not only to ablate the thyroid remnants following surgery but also to treat the metastatic disease. Aberrant apoptotic signaling in differentiated thyroid cancer metastasis can induce resistance to radiotherapy. Cell–extracellular matrix components interactions are crucial in the normal thyroid follicle architecture as well as in tumor invasion and metastasis [27]. This is even more important when genetic alterations like mutated BRAF or soluble factors (i.e., cytokines) released by infiltrating lymphocytes alters the normal cell polarity and the expression of the sodium iodide symporter [28–30]. This experimental model using normal human thyroid tissue may be useful in investigating the cell

death pathways induced by ^{131}I and identify the mechanisms by which thyroid cancer cells become resistant to radioiodide treatment.

Conflict of interest The authors declare that they have no conflict of interest.

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