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Iodide Excess Induces Apoptosis in Thyroid Cells through a p53-Independent Mechanism Involving Oxidative Stress*

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ABSTRACT

Thyroid toxicity of iodide excess has been demonstrated in animals fed with an iodide-rich diet; *in vitro* iodide is cytotoxic, inhibits cell growth, and induces morphological changes in thyroid cells of some species. In this study, we investigated the effect of iodide excess in an immortalized thyroid cell line (TAD-2) in primary cultures of human thyroid cells and in cells of nonthyroid origin. Iodide displayed a dose-dependent cytotoxicity in both TAD-2 and primary thyroid cells, although at different concentrations, whereas it had no effect on cells of nonthyroid origin. Thyroid cells treated with iodide excess underwent apoptosis, as evidenced by morphological changes, plasma membrane phosphatidylserine exposure, and DNA fragmentation. Apoptosis was unaffected by protein synthesis inhibition, whereas inhibition of peroxidase enzymatic activity by propylthiouracil completely blocked iodide cytotoxicity. During KI treatment, reactive oxygen species were produced, and lipid peroxide levels increased markedly. Inhibition of endogenous p53 activity did not affect the sensitivity of TAD-2 cells to iodide, and Western blot analysis demonstrated that p53, Bcl-2, Bcl-XL, and Bax protein expression did not change when cells were treated with iodide. These data indicate that excess molecular iodide, generated by oxidation of ionic iodine by endogenous peroxidases, induces apoptosis in thyroid cells through a mechanism involving generation of free radicals. This type of apoptosis is p53 independent, does not require protein synthesis, and is not induced by modulation of Bcl-2, Bcl-XL, or Bax protein expression. (*Endocrinology* **141:** 598–605, 2000)

IN ADDITION to its role as a substrate for thyroid hormone biosynthesis, iodide participates in a number of clinically important interactions with the thyroid. Acute administration of large doses of iodide determines a biphasic response of the thyroid: an increase, followed by a decrease, in the yield of organic iodide and thyroid hormones. The mechanism of the relative blockade of organic iodide yield, known as the Wolff-Chaikoff effect (1), is, in part, unknown and, in part, caused by the biochemical effects of large concentrations of the reactive form of iodide generated by oxidative mechanisms. Another important effect of iodine on the thyroid is its ability to diminish the hypervascularity and hyperplasia that characterize the diffuse goiter of Graves' disease. The molecular mechanisms of this phenomenon, widely used to facilitate surgical therapy of this disorder, are uncertain, but it has been hypothesized that iodine could bind to organic compounds and interfere with the metabolic processes necessary for the maintenance of hyperplasia and may be responsible for the involuting effect of iodine excess (2).

The toxicity of iodide excess has been demonstrated, both in animals and in cell systems. Involution of the thyroid gland has been described in rats fed with an iodide-rich diet (3, 4). *In vitro*, iodide inhibits thyroid cell growth and induces morphological changes in porcine thyroid cells (5). Some effects of iodide seem to be species specific. A cytotoxic effect of iodide has been documented in rat FRTL-5 cells but not in primary dog thyrocytes (6). The iodide-induced cytotoxic effect on rat thyrocytes included necrotic and apoptotic features, indicating the involvement of a controlled process of cell death. Apoptosis (or programmed cell death) is an active process of cell self destruction requiring the activation of a genetic program, leading to changes in morphology, DNA fragmentation, and protein cross-linking (7, 8). The apoptotic pathways are activated by physiological stimuli such as environmental signals, cytokines (9, 10), and growth factors; they can also be induced by pathological stimuli, radiation, and anticancer drugs (11–14). Excess iodide intake is never obtained in physiological conditions; however, therapeutic use of iodide-rich compounds, such as amiodarone and iodinated radiographic contrast agents, can lead to release of a large amount of iodide. Amiodarone, a potent antiarhythmic drug containing two iodine atoms per molecule, may induce either hypo- or hyperthyroidism (15–17). Amiodarone-induced hypothyroidism in rats is associated with specific ultrastructural features of necrosis and apoptosis of the thyroid gland and cytokine production (18–20). However,

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the role of iodine *vs.* the direct drug cytotoxicity in the pathogenesis of amiodarone-induced hypothyroidism is not yet fully solved. Therefore, it was of interest to investigate whether iodide itself displays cytotoxic effects on human thyroid cells and whether its cytotoxicity represents an apoptotic phenomenon. In the present study, we employed primary cultures of human thyroid cells and the immortalized thyroid cell line TAD-2, which proved to be a good model for studying apoptosis in the thyroid (9).

Materials and Methods

Cells and chemicals

Cell cultures from normal thyroids were prepared, as previously described, by collagenase digestion (21) and cultured in a 5% CO_2 atmosphere at 37 C, in F-12 medium supplemented with 10% FCS, with 1 mU/ml bovine tyreotropin (Sigma, St. Louis, MO). The TAD-2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells, was a kind gift of Dr. T. F. Davies, Mount Sinai Hospital (New York, NY). TAD-2, human dermal fibroblasts (PG1), osteosarcoma cells (SaOS), and endometrial carcinoma cells (HeLa) were cultured in a 5% CO_2 atmosphere at 37 C, in DMEM supplemented with 10% FCS. Medium was changed every 3–4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free PBS with 0.05% trypsin. TADp53cG cell mutants were generated by transfecting pLTRp53cG containing the temperature-sensitive dominant-negative p53 gene mutated at codon 135 (gift of Dr. A. Levin, Princeton University, Princeton, NJ), as described (10). KCl, KI, cycloexhimide, and o-phenylenediamine were purchased from Sigma. A 10-mM stock solution of 6-propyl-2-thiouracil (PTU) (Sigma) was prepared at basic pH, buffered at pH 7.5 by HCl.

DNA electrophoresis

Cells collected by centrifugation were washed in PBS, lysed in 300 μl 0.5% Triton-X100, 5 mM Tris-buffer pH 7.4, 20 mM EDTA for 20 min at 4 C and centrifuged at 13.000 rpm for 30 min. Centrifugation-resistant low molecular weight DNA was extracted with phenol/chloroform, precipitated with ethanol and incubated with 0.5 μg/ml deoxyribonuclease-free ribonuclease A for 30 min at 37 C. DNA with loading buffer was electrophoresed in 1% agarose, 1 μg/ml bromide at 50 V in 45 mM Tris-borate and visualized by UV.

Cell death measurements

Annexin V assay for determination of apoptosis/necrosis ratio was performed as follows: cells were washed twice with cold PBS; resuspended in 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$, and incubated for 15 min at room temperature with Annexin V-fluorescein conjugated (PharMingen, San Diego, CA) and 5 μg/ml propidium iodide. Cells were analyzed within 1 h, by flow cytometry, using a FACScan (Becton Dickinson and Co., Mountain View, CA).

Estimation of cell death, by flow cytometry, was performed as follows: floating cells and adherent cells obtained by trypsin/EDTA were collected, washed in cold PBS, and fixed in 70% cold ethanol for 30 min. Ethanol was removed by PBS wash, and cells were incubated in PBS, 50 μg/ml propidium iodide, 10 μg/ml deoxyribonuclease-free ribonuclease A overnight at 4 C. Cells were then analyzed by flow cytometry. The percent of dead cells was calculated by dividing the number of cells displaying red fluorescence lower than G0-G1 diploid peak by the total number of collected cells times 100.

Antibodies and Western-blot analysis

Mouse monoclonal antibodies to p53 were purchased from Transduction Laboratories, Inc. (Lexington, KY); mouse monoclonal antibody to Bcl-2 and rabbit polyclonal antibodies to Bcl-X and Bax were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Cells were washed in cold PBS and lysed for 10 min at 4 C with 1 ml of lysis buffer [50 mM Tris (pH 7.4), 0.5% NP40, 0.01% SDS] containing protease inhibitors. Lysates from adherent cells, collected by scraping and from floating cells, were centrifuged at 12,000 $\times$ *g* for 15 min at 4 C. The protein concentration in cell lysates was determined by Protein Assay (Bio-Rad Laboratories, Inc. Richmond, CA), and 50 μg of total protein from each sample were boiled for 5 min in Laemmli sample buffer (125 mM Tris pH 6.8, 5% glycerol, 2% SDS, 1% β-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). Acrylamide concentration was 12% for p53 and Bcl-XL, 15% for Bcl-2 and Bax. 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 μg/ml of mouse monoclonal or rabbit polyclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 C with horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies (Bio-Rad Laboratories, Inc.) diluted 1:2000 in PBS, Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Fluorescent measurement of intracellular reactive oxygen species (ROS)

TAD-2 cells were collected by mild trypsinization; washed in PBS; and resuspended in PBS, 10 μM 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Inc., Eugene, OR), 5 μg/ml propidium iodide at 37 C; and kept in DCFH-DA thereafter. DCFH-DA is a compound taken up by the cells and trapped in a nonfluorescent deacylated form (DCFH). DCFH is oxidized by ROS to a fluorescent form (22). After 1 h of incubation, cells were analyzed by FACScan with excitation at 495 nm and emission at 525 nm wavelength. Cells leaking DCFH because they were no longer intact were stained by the nonmembrane-permeable dye propidium iodide and excluded.

Lipid peroxidation measurement

The level of lipid peroxide under KI treatment was determined by measuring the production of thiobarbituric acid reactive substances (TBARS), according to the method of Esterbauer and Cheeseman (23). The cells were harvested, lysed by sonication, and incubated with 0.5% thiobarbituric acid for 30 min at 80 C. Basal levels of TBARS generated by liporeroxides were measured fluorimetrically (excitation at 530 nm, emission at 550 nm). Malondialdehyde bisdimethylacetal was used as standard. TBARS levels were normalized for cell protein content.

Results

Potassium iodide displays cytotoxicity restricted to thyroid cells

TAD-2 cells, primary human thyroid cells, and cells of nonthyroid origin were treated with varying concentrations of KI and KCl for 48 h. Whereas KCl at concentrations as high as 50 mM did not produce any detectable effects, treatment with KI induced a dramatic change in the morphology of thyroid TAD-2 cells, from flat-adherent to round-detached (Fig. 1). The number of dead cells with hypodiploid DNA content was determined by flow cytometric analysis of floating and adherent cells fixed and permeabilized by ethanol and stained with propidium iodide as previously shown (12). Because both apoptosis and late necrosis determine a decrease of cellular content of DNA, cytotoxicity was determined by measuring the percent of hypodiploid cells as shown in Fig. 2A. The cytotoxic effect of KI was time dependent (Fig. 2B), affecting 50% of the cells at 20 mM concentration by 48 h, and dose-dependent, whereas no effect was observed in the cells treated with KCl (Fig. 3A). Potassium iodide also displayed a dose-dependent cytotoxicity, although at higher concentrations, in primary cultures of normal thyroid cells; whereas no effect was observed in cells of nonthyroid origin such as osteosarcoma (SaOS) and car-

cinoma (HeLa) cells and fibroblasts (PG-1) at the same molarity (Fig. 3B).

Cytotoxicity by iodide excess is an apoptotic process.

Although apoptosis and necrosis are two distinguishable modes of cell death, they can be induced by the same toxin at different concentrations, and secondary necrosis can follow late stages of a slow apoptotic process (24–26).

To determine whether cytotoxicity by iodide excess involves apoptosis, TAD-2 cells were treated with KI, and the morphologic changes were observed under light and fluorescence microscope, plasma membrane phosphatidylserine exposure was analyzed by annexin binding, and DNA fragmentation was analyzed by agarose gel electrophoresis. Under light microscope and fluorescence microscope after acridine staining, all the characteristic morphological features of programmed cell death, picknosis, karyorrhexis, cell blebbing, and cell shrinkage were observed (not shown). DNA analysis by agarose gel electrophoresis also demonstrated a massive apoptotic process after 48 h of treatment, showing the characteristic DNA fragmentation pattern (Fig. 4A). Loss of plasma membrane asymmetry before loss of membrane integrity was demonstrated by simultaneous staining of the cells with annexin V and propidium iodide (Fig. 4B). After 24 h of treatment with 20 mM KI, 41.8% of the cells bound annexin V, and 62.3% of annexin V stained cells still retained plasma membrane integrity, remaining impermeable to propidium iodide, thus demonstrating the involvement of an apoptotic process followed by secondary necrosis. DNA laddering and annexin V staining demonstrated that also in thyroid primary cultures, as in TAD-2 cells, KI induced apoptosis (not shown).

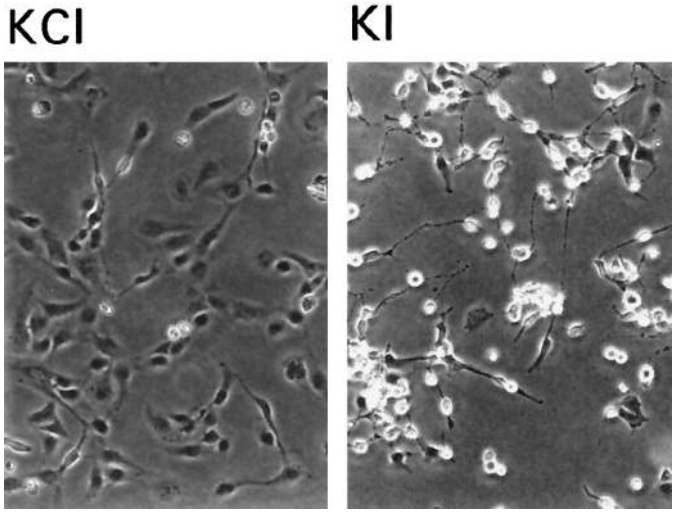

FIG. 1. Phase contrast photomicrographs of iodide-induced change of thyroid cell morphology. TAD-2 cells were cultured for 24 h in FCS containing medium, and then 50 mM KCl (*left*) or KI (*right*) was added to the medium. After 48 h of culture, only the cells treated with KI were rounded and detached from the plate. Magnification: 100×.

Effect of inhibition of protein synthesis and thyreoperoxidases on iodide-induced apoptosis

Apoptosis has been described to be differentially affected by protein synthesis inhibitors. The role of protein synthesis in iodide excess-induced apoptosis was determined by treating TAD-2 cells for 48 h with 20 mM KI in the presence of increasing concentrations of cycloheximide. This protein synthesis inhibitor, used at nontoxic concentrations (<1 μM), was ineffective on the apoptosis induced by iodide, as determined by flow cytometry (Fig. 5A).

The thyroid cell membrane contains a tissue-specific peroxidase (thyroid peroxidase, TPO) that oxidizes ionic iodide into its molecular form (I_2). To investigate whether I_2 mediates the apoptotic effect of KI excess, the enzymatic activity of TPO was inhibited by increasing concentrations of PTU. Inhibition of endogenous peroxidases and percent of cell death were determined after 48 h (not shown). At concentrations higher than 600 μM, PTU displayed cytotoxic effects; whereas at lower concentrations, it had no effect on cell viability. To measure inhibition of TPO activity by PTU, cells were incubated with 1 mg/ml o-phenylenediamine, and absorbance at 450 nm was measured after 48 h. PTU inhibited, in a dose-dependent manner, the oxidation of o-phenylenediamine. A total of 10^4 TAD-2 cells/well were plated in microtiter wells and treated with 20 mM KI in the presence of increasing concentrations of PTU. At 300 μM, PTU completely blocked any morphological change induced by KI, and the cells remained flat, polygonal, and adherent. Flow cytometric analysis

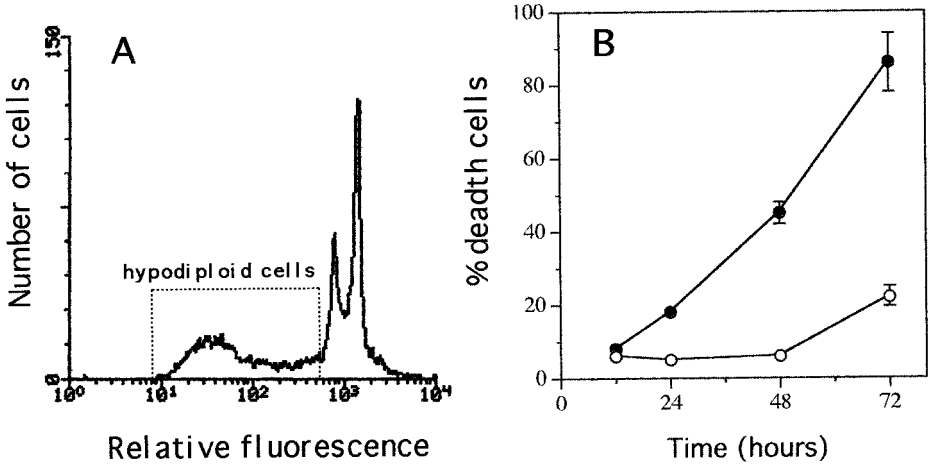

FIG. 2. Time course of iodide-induced cytotoxicity measured by flow cytometry. TAD-2 cells were cultured for 24 h, then treated with 20 mM KI or KCl for different times. Floating and adherent cells were collected, stained with propidium iodide, and analyzed by flow cytometry. The percent of death cells was determined by gating the cells with hypodiploid DNA content as shown in A (cells treated with 20 mM KI for 48 h). B reports the mean percentages of death cells induced by KI (*solid circles*) or KCl (*open circles*) determined in three experiments.

showed that the cytotoxic effects induced by KI were inhibited by PTU in a dose-dependent fashion, demonstrating that iodide excess requires peroxidase enzymatic activity to induce apoptosis (Fig. 5B).

Production of ROS and lipid peroxidation during KI-induced apoptosis

To assess whether ROS were generated during apoptosis, we used the oxidation-sensitive fluorescent probe

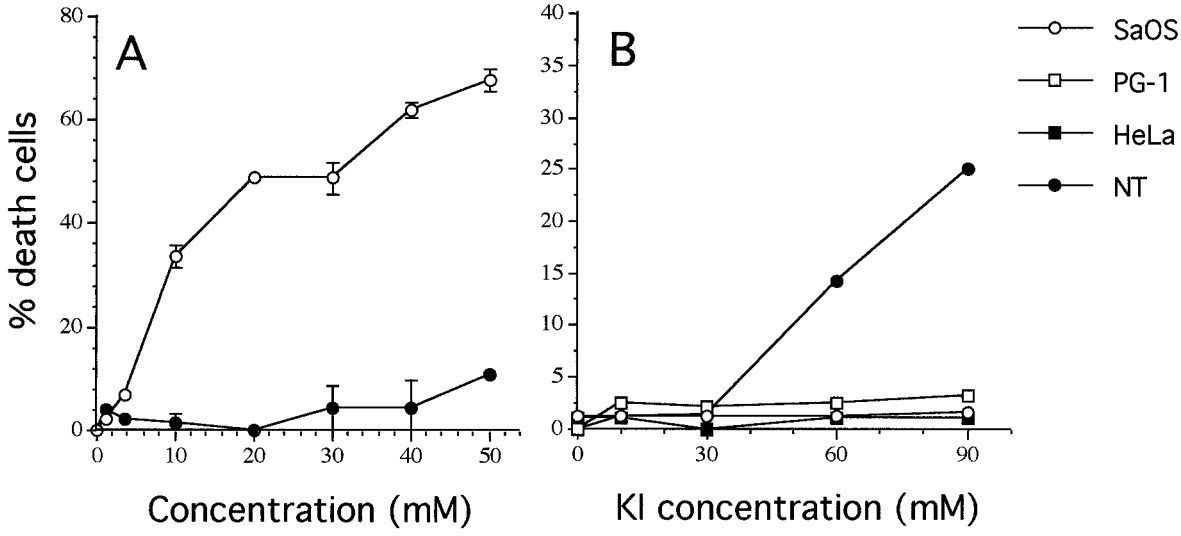

FIG. 3. Dose response of KI-induced cytotoxicity. A, TAD-2 cells were cultured for 24 h, then treated with KI (*open circles*) or KCl (*solid circles*) at different concentrations for 48 h, and dead hypodiploid cells were quantitated by flow cytometry as described; B, primary thyrocytes of normal thyroids (NT, *solid circles*), SaOS (*open circles*), PG-1 (*open squares*), and HeLa (*solid squares*) cells were cultured for 24 h and then treated for 48 h with varying concentrations of KI. Percentages of dead hypodiploid cells were determined by flow cytometry as described.

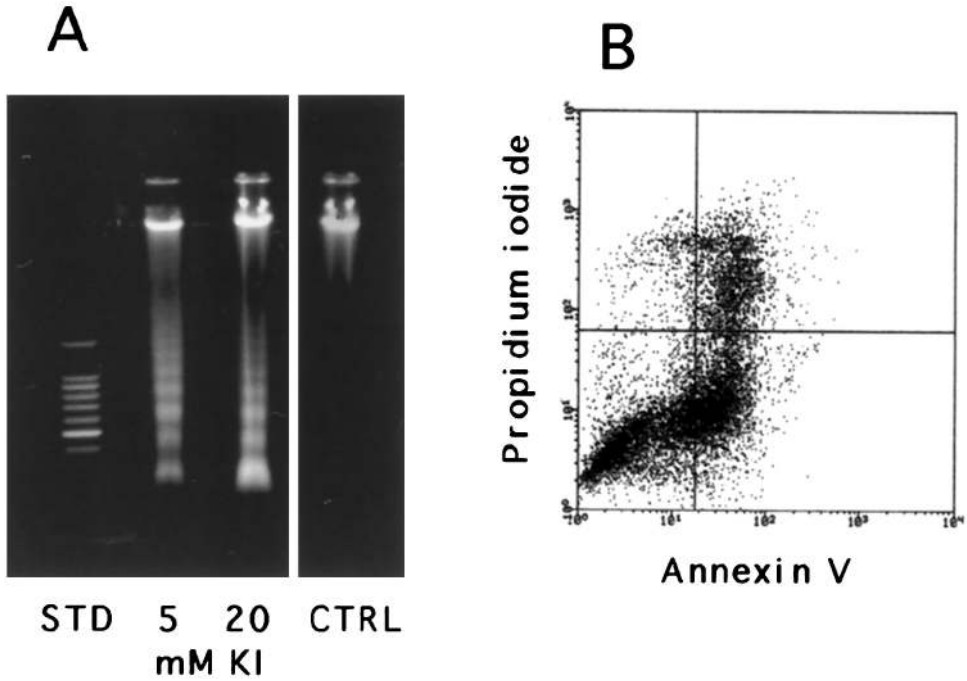

FIG. 4. A, Gel electrophoresis analysis of low-molecular-weight DNA from TAD-2 cells cultured for 48 h in the presence of 5 and 20 mM KI. Centrifugation-resistant low-molecular-weight DNA was extracted from the cells and electrophoresed in 1% agarose, 1 μg/ml propidium bromide in Tris-borate buffer and visualized by UV. Low-molecular-weight DNA with characteristic apoptotic internucleosomal fragmentation was evident in the presence of KI. STD, Marker of DNA molecular weight; CTRL, DNA of untreated cells. B, Annexin V assay for determination of apoptosis/necrosis ratio was performed by incubating the cells treated with 20 mM KI for 24 h, with annexin V-fluorescein conjugated (*abscissa*) and propidium iodide (*ordinate*), and analyzing the cells by flow cytometry. Intact cells are located in the *lower left quadrant*, necrotic cells permeable to propidium iodide are in the *upper right* and *left quadrants*, and the apoptotic cells stained by annexin V and unstained by propidium iodide are in the *lower right quadrant*.

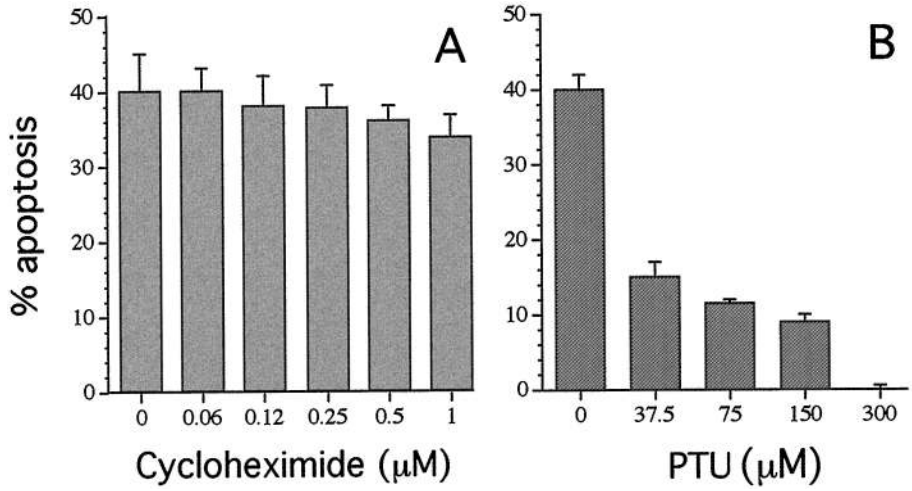

FIG. 5. Effect of cycloheximide and PTU on iodide excess-induced apoptosis. TAD-2 cells were treated for 48 h with 20 mM KI alone or with different concentrations of cycloheximide (A) or PTU (B). After 48 h, the percent of apoptotic cells was determined by flow cytometry. Cycloheximide and PTU were not toxic for the cells at the indicated concentrations. Results are reported as percent of dead cells from three separate experiments.

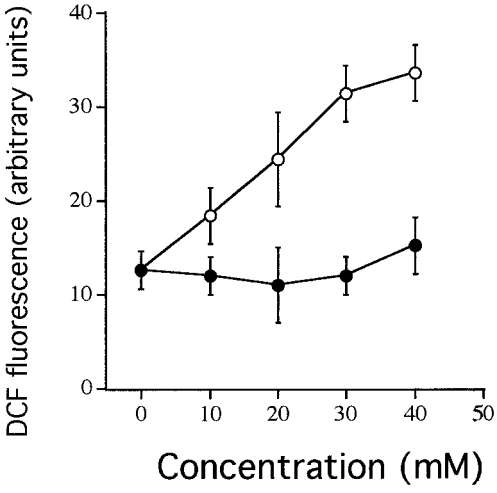

FIG. 6. Estimation of intracellular ROS level. Cells were treated for 24 h with variable concentrations of KI (*open circles*) or KCl (*solid circles*). Floating and adherent cells were collected, incubated with DCFH-DA probe for 1 h, and analyzed by FACS. Contemporary staining with propidium iodide were used to exclude cells whose membrane was no longer intact.

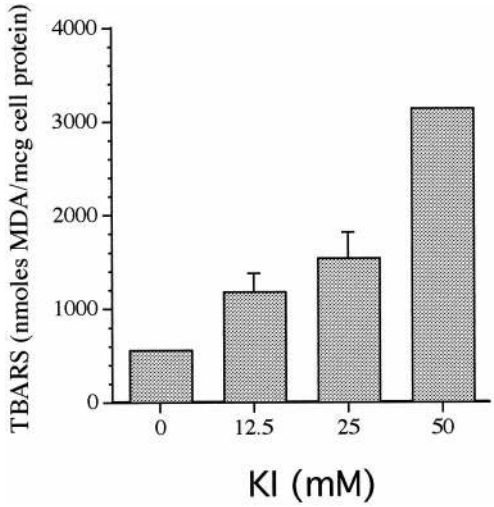

FIG. 7. Generation of lipid peroxides during KI treatment. Cells were incubated for 48 h with increasing concentrations of KI. Basal TBARS levels were measured fluorimetrically whereas KI-induced levels were measured spectrophotometrically, as described in *Materials and Methods*. Data are the means and SE of three separate experiments. MDA, Malonolialdehyde biodimethylacetal.

DCFH-DA in cells treated with variable KI or KCl concentration. DCFH-DA is a compound readily taken up by the cells and trapped in a nonfluorescent deacylated form (DCFH). DCFH is oxidized by ROS to a fluorescent form measured by FACScan. Because DCFH is leaked by late apoptotic cells whose membrane is no longer intact, cells stained by propidium iodide were excluded. Whereas KCl did not produce any detectable effects, 24-h treatment with KI induced a 2.8-fold increase of ROS cell content at 40 mM (Fig. 6). Because lipid peroxidation is considered a major mechanism of free radical-induced cell damage, we examined the generation of lipid peroxide during KI treatment (Fig. 7). Incubation of TAD-2 cells for 48 h with increasing concentrations of KI resulted in a dramatic dose-dependent generation of TBARS levels, demonstrating that cell lipids were extensively peroxidated.

KI-induced apoptosis is p53 independent

Some, but not all, forms of apoptosis require the product of the p53 tumor suppressor gene. To determine whether the apoptosis induced by KI is a p53-dependent mechanism, KI cytotoxicity was assayed in TAD-2 cells expressing a dominant-negative mutated p53 previously described (TADp53cG) (12). In these cells, stably transfected with a vector encoding a temperature-sensitive, dominant-negative murine p53 protein (p53cG), the endogenous p53 activity is inhibited at 39 C.

Various TADp53cG clones and TADneo cells, carrying only the neo resistance, were treated for 48 h at 39 C with 30 mM KI, the cells were observed, and apoptosis was determined by flow cytometry (Fig. 8). Student's *t* test paired analysis showed no statistically significant difference in sensitivity to KI between the clones, thus demon-

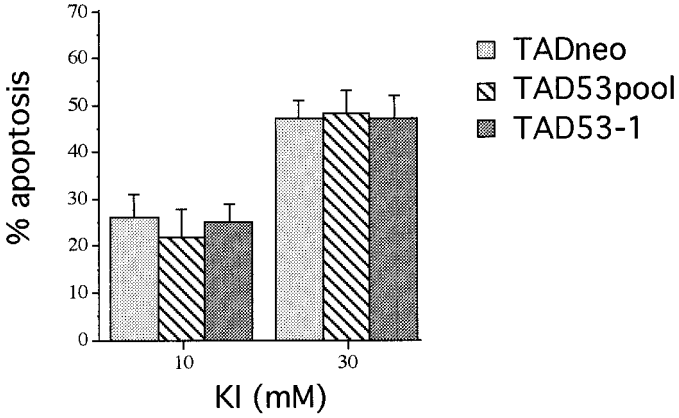

FIG. 8. Involvement of p53 in iodide-induced apoptosis. TADneo, TAD53-pool, and TAD53–1 cells were cultured in the presence of different concentrations of KI for 48 h. Results are presented as the mean ± SD from three independent experiments. Differences in sensitivity to KI of the TADp53 *vs.* TADneo cells were not statistically significant.

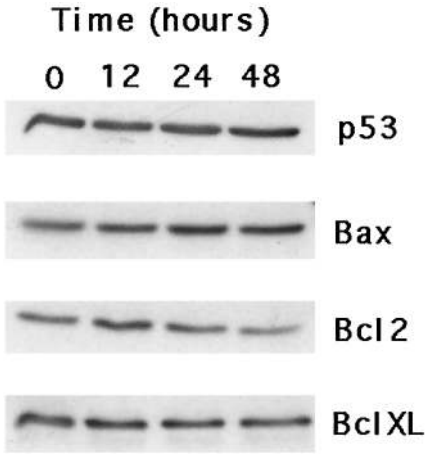

FIG. 9. Western blot analysis of p53, Bcl-2, Bcl-XL, and Bax expression in cells treated with KI. TAD-2 cells were cultured in the presence of 30 mM KI for 0, 12, 24, and 48 h; and 50 μg of each sample was loaded in the gel. Acrylamide concentration was 12% for p53 and Bcl-XL and 15% for Bcl-2 and Bax.

strating that p53 is not required in this type of drug-induced apoptosis.

Expression of p53, Bcl-2, Bax, and Bcl-XL is unchanged in KI-induced apoptosis

Western blot analysis of different pro- and antiapoptotic proteins was performed in thyroid cells cultured in the presence of 30 mM KI for 0, 12, 24, and 48 h (Fig. 9). The proapoptotic proteins p53 and Bax and the antiapoptotic Bcl-2 and Bcl-XL were clearly visible by immunoblot in untreated cells. Analysis of p53 protein expression did not show any variation induced by KI up to 48 h. Among the genes known to be under the transcriptional control of p53, the antiapoptotic Bcl-2 is down-regulated whereas the proapoptotic Bax is up-regulated (27). As predicted by the observation that p53 expression was unchanged, also Bcl-2 and Bax proteins remained constant during apoptosis; and Bcl-XL, another antiapoptotic protein belonging to the Bcl-2 family, did not show any quantitative variation during KI treatment. These results demonstrate that apoptosis induced by KI is not associated with a variation of the ratios between death agonist Bad and antagonists Bcl-2 and Bcl-XL.

Discussion

Besides the well-characterized effects on thyroid hormone metabolism, a direct toxic effect of iodide excess has been documented in thyroid gland *in vivo* and in rat and dog thyrocytes in culture. Thyroid dysfunction has also been documented in humans after the use of iodide-rich contrast agents (28, 29). Diatrozoate meglumine sodium (Hypaque) contains 370 mg iodide/ml, and 50–350 ml are injected during coronary angiography (29). This huge iodide exposure determines a serum iodide molar concentration greatly exceeding that able to induce cell death *in vitro*; however, because of iodide fast renal clearance, it is not known whether its extracellular molar concentration could reach that used in our experiments for a time sufficient to trigger apoptosis. Amiodarone can also release a large amount of iodide: a quantity of 200 mg amiodarone releases forty times the average daily requirement of iodide (18). The iodide released during amiodarone therapy is lower than the iodide load reached with use of contrast agents, but amiodarone is stored in many tissues, and iodide is subsequently released over a long period (30).

Iodide toxicity to thyroid cells at the molecular level has not yet been fully documented, although the involvement of an apoptotic process has been hypothesized. Apoptosis, or programmed cell death, is an active process of self destruction that requires the activation of a genetic program leading to changes in cell morphology, DNA fragmentation, and protein cross-linking (26). The first cellular event displayed by cells undergoing apoptosis is the loss of plasma membrane asymmetry, leading the phosphatidylserine present in the inner leaf of the cell membrane to appear in the outer leaf, with preservation of membrane integrity. In TAD-2 cells treated with KI excess, this event was clearly demonstrated when the cells were stained by fluorescent annexin V but remained unstained with propidium iodide. A later event in the apoptotic process is the activation by terminal caspases of the DNA cleavage enzymes endonucleases, documented in KI-treated cells by the electrophoretic analysis of DNA, showing the characteristic fragmentation pattern. Quantitative measurement of apoptosis was performed in this study by flow cytometry, identifying the cells with hypodiploid DNA content. This method can overestimate the apoptosis, because necrotic cells are also hypodiploid, although with a different DNA fragmentation pattern. However, this is the most accurate method to measure a slow apoptotic process, because it allows consideration of the cells undergoing secondary necrosis, occurring in the late stages of apoptosis (31). In 1986, when apoptosis was still thought to be a type of cell deletion characterized by morphological features only, Mahmoud *et al* (3). described cell shrinkage, densification of the cytosol, karyorrhexis, and karyolysis of the thyrocytes from low-iodide-fed rats injected with sodium iodide. That original observation has been confirmed by the findings in TAD-2 cells, that demonstrate that also human thyroid fol-

licular cells react to an excess of iodide activating a cell suicide program. Similar sensitivity to KI excess was also shown by thyroid primary cultures, whereas cells of nonthyroid origin were resistant, indicating that iodide cytotoxicity is tissue specific.

Some, but not all, kinds of apoptosis require protein synthesis; and programmed cell death can even be accelerated by protein synthesis inhibitors (32, 33). Whereas inhibitors of protein prenylation in TAD-2 cells induce a type of apoptosis fully inhibited by cycloheximide (12), iodide toxicity proved to be completely resistant to this drug, demonstrating it to be a process independent of protein synthesis. Thus, the entire process, from the involvement of the iodide metabolism triggering apoptosis to the executioner pathway leading to cell death, must involve proteins already present in the cell. The thyroid specificity and the ability of the TPO inhibitor PTU to fully block the toxicity of iodide strongly suggest that ionic iodide is not directly toxic for the follicular cell, whereas its molecular form I_2, produced by TPO oxidation, mediates the apoptotic effect of KI excess. I_2 is a highly reactive molecule, able to react with proteins, lipids, and nucleic acids to form iodocompounds. Different types of iodolipids are produced when iodide binds to membrane lipids, and this could determine the loss of cell and mitochondrial membrane integrity with generation of ROS and peroxidation of lipids (34). Although the molecular mechanisms behind apoptosis are only partially understood, some molecular effectors have been identified. The apoptotic pathways initiate at the cell surface, from membrane receptors such as Fas/APO1 and TNFR-1 and are executed by a class of cysteine proteases, the caspases, representing the distal effector components of the apoptotic machinery. Apoptosis has been described to be differentially affected by the transcription regulatory activity of the oncosuppressor p53, depending on the cell system and the apoptotic stimulus. Some types of apoptosis do not require macromolecular synthesis, and entry into the apoptosis pathway does not involve p53 transcriptional activity (35, 12). This type of apoptosis involves the activation of proteases of the caspase cascade already present in the cell. The toxic effect demonstrated by iodide excess in the TADp53 cell mutants at 39 C, a temperature at which endogenous p53 was inactivated by dominant-negative p53cG, indicated that this tumor suppressor gene is not involved. This, together with the observation that macromolecular synthesis inhibition did not induce resistance, were confirmed by Western blot analysis that demonstrated a constant level of protein expression of p53, Bcl-2, Bcl-Xl, and Bax throughout the apoptotic process. The overall ratio of death agonists to antagonists, which constitutes the critical intracellular checkpoint of apoptosis, is not regulated only by the synthesis of the Bcl-2 family proteins such as Bcl-2, Bcl-Xl, and Bax. The Bcl-2 family has further expanded to include antiapoptotic and proapoptotic proteins whose activity is regulated at posttranscriptional level (36). One of these proteins, Bad, does not require neosynthesis to regulate apoptosis, because it heterodimerizes with Bcl-Xl only when it is nonphosphorylated (37). Thus, factors other than those investigated can be altered by KI excess at posttranscriptional level and trigger apoptosis. Several studies have shown that exposure to ionizing radiation or anticancer drugs inducing DNA damage, evokes a variety of cellular responses, including p53 accumulation in the cell and its translocation into the nucleus (38–41). The observation that the level of expression of p53 is unchanged during KI-induced apoptosis suggests that DNA damage is not a primary event evoked by KI. A number of recent findings have contributed to the development of models for the involvement of mitochondria in apoptotic execution (26, 42). Loss in mitochondrial membrane barrier function involving opening of mitochondrial megachannels, cytocrome C release, and ROS production, is a major controlling mechanism in some apoptotic process (43–45). Whichever iodiocompounds are generated by I_2, produced by the TPO-mediated transformation of ionic iodide, mitochondria seem to be a possible target. Future studies will have to explore the nature of the toxic iodocompound(s) and whether mitochondrial damage occurs during this type of apoptosis.

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