Open access • Journal Article • DOI:10.1002/JCB.10203

High levels of exogenous C2-ceramide promote morphological and biochemical evidences of necrotic features in thyroid follicular cells — Source link

Matilde Todaro, Mario Catalano, Diana Di Liberto, Mariella Patti ...+8 more authors

Institutions: University of Palermo

Published on: 01 Jan 2002 - Journal of Cellular Biochemistry (J Cell Biochem)

Topics: Ceramide, Bcl-2-associated X protein, Apoptosis, Necrotic Process and Bcl-2 Homologous Antagonist-Killer Protein

Journal of Cellular Biochemistry 86:162–173 (2002)

High Levels of Exogenous C_2-Ceramide Promote Morphological and Biochemical Evidences of Necrotic Features in Thyroid Follicular Cells

M. Todaro,[1] M. Catalano,[1] D. Di Liberto,[1] M. Patti,[1] M. Zerilli,[2] F. Di Gaudio,[2] G. Di Gesù,[1] G. Vetri,[1] G. Modica,[1] A. Bono,[2] M. Ciaccio,[2] and Giorgio Stassi[1]*

[1]Department of Surgical and Oncological Sciences, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy

[2]Department of Medical Biotechnologies and Forensic Medicine, Medical Biochemistry Section, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy

Abstract CD95 and ceramide are known to be involved in the apoptotic mechanism. The triggering of CD95 induces a cascade of metabolic events that progressively and dramatically modifies the cell shape by intense membrane blebbing, leading to apoptotic bodies production. Although the CD95 pathway has been abundantly described in normal thyrocytes, the effects of cell permeable synthetic ceramide at morphological and biochemical levels are not fully known. In the present study, we show that thyroid follicular cells (TFC) exposed to 20 μM of C_2-ceramide for 4 h are characterized by morphological features of necrosis, such as electron-lucent cytoplasm, mitochondrial swelling, and loss of plasma membrane integrity without drastic morphological changes in the nuclei. By contrast, TFC treated with 2 μM of C_2-ceramide for 4 h are able to accumulate GD3, activate caspases cascade, and induce apoptosis. Furthermore, we provide evidence that 20 μM of C_2-ceramide determine the destruction of mitochondria and are not able to induce PARP cleavage and internucleosomal DNA fragmentation, suggesting that the apoptotic program is not activated during the death process and nuclear DNA is randomly cleaved as the consequence of cellular degeneration. Pretreatment with 30 μM of zVAD-fmk rescued TFC from 2 μM of C_2-ceramide-induced apoptosis, whereas, 20 μM of C_2-ceramide exposure induced necrotic features. $\Delta\psi_m$ was obviously altered in cells treated with 20 μM of C_2-ceramide for 4 h (75% ± 3.5%) compared with the low percentage (12.5% ± 0.4%) of cells with altered $\Delta\psi_m$ exposed to 2 μM of C_2-ceramide. Whereas, only 20% ± 1.1% of cells treated with anti-CD95 for 1 h showed altered $\Delta\psi_m$. Additionally, Bax and Bak, two pro-apoptotic members, seem to be not oligomerized in the mitochondrial membrane following ceramide exposure. These results imply that high levels of exogenous ceramide contribute to the necrotic process in TFC, and may provide key molecular basis to the understanding of thyroid signaling pathways that might promote the apoptotic mechanism in thyroid tumoral cells. J. Cell. Biochem. 86: 162–173, 2002.

Key words: apoptosis; ceramide; CD95; thyrocytes

Apoptosis is a peculiar form of genetically-regulated cell death that plays a crucial role in the development and homeostasis of multicellular organisms [Cohen, 1993]. The morphological and biochemical changes of apoptosis are accompanied by cytoplasmic blebbing, chromatin condensation, DNA fragmentation, and membrane budding, culminating in compact packaging of the cellular debris into apoptotic bodies.

Apoptosis induced by CD95 is involved in various aspects of mammalian development and especially in the homeostasis of the immune system [Miller and J, 1998; De Maria et al., 1999; Song and Steller, 1999]. CD95 is one of the best characterized death surface receptors belonging to the tumor necrosis factor (TNF) receptor gene superfamily [Nagata, 1997]. The interactions between death receptors and their ligands play a pivotal role in controlling the

Grant sponsor: Associazione Italiana Ricerca sul Cancro (AIRC) (to G.S).

D. Di Liberto is recipient of a FIRC fellowship.

*Correspondence to: Giorgio Stassi, Department of Medical Biotechnologies and Forensic Medicine, Medical Biochemistry Section, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy. E-mail: gstassi@tiscalinet.it

Received 25 February 2002; Accepted 21 March 2002

DOI 10.1002/jcb.10203

apoptosis mechanism in physiological and pathological processes, such as autoimmune diseases [Stassi et al., 1997, 1999a,b, 2000; De Maria and Testi, 1998]. Recruitment of caspases, a family of cysteine proteases, induces proteolytic cleavage of multiple cellular targets, consequently determining specific changes in cell surface and nuclear morphology [Salvesen and Dixit, 1997; Thornberry et al., 1997]. During the apoptotic process, surface morphological changes have demonstrated discrete stages of cell rounding, surface blebbing, surface microspikes, followed by apoptotic bodies formation [Collins et al., 1997].

The triggering of CD95 receptor results in another cascade of metabolic events, thus involving the activation of a sphingomyelinase (SMase), which hydrolyses sphingomyelin (SM) to ceramide [Cifone et al., 1994; Jarvis et al., 1994; Testi, 1996; De Maria et al., 1997]. SM hydrolysis is elicited not only by molecular crosslinking of CD95 or TNF receptor, but also by other apoptotic stimuli such as UV and γ irradiation [Ballou et al., 1996]. Ceramide accumulation has been claimed as a major mediator of CD95 induced apoptosis [Cifone et al., 1994; Pushkareva et al., 1995; Tepper et al., 1997]. Moreover, recent studies showed that during this process ceramide enters the synthetic pathway and is ultimately converted to GD3 by the action of GD3 synthase, even in infiltrating T lymphocytes in proximity to CD178 producing thyroid follicules during Hashimoto's thyroiditis [De Maria et al., 1997; Stassi et al., 1999a]. According to published data, mitochondria are the immediate downstream target of GD3 [Rippo et al., 2000]. Accumulation of GD3 determines the loss of mitochondrial membrane potential ($\Delta\psi_m$) and the release of cytochrome c that binds Apaf-1 and promotes the recruitment of pro-caspase-9, forming a complex called "apoptosome," an apoptogenic structure that cleaves and activates pro-caspase-3 and other executor caspases [Kroemer et al., 1997; Green and Kroemer, 1998; Hengartner, 2000]. Subsequently, this caspase cascade generates irreversible apoptotic events, such as chromatin condensation, activation of endonucleases and DNA fragmentation. $\Delta\psi_m$ is the fundamental parameter for the evaluation of mitochondrial functionality and energy status of cells [Majno and Joris, 1995; Mancini et al., 1997; Miller and J, 1998]. The disruption of $\Delta\psi_m$ is considered one of the early events of apoptosis, but according to other points of view, the maintenance of $\Delta\psi_m$ with ATP synthesis is crucial for a variety of intracellular processes, including apoptosis [Mancini et al., 1997; Nagata, 1997; Salvesen and Dixit, 1997]. Apoptosis can be initiated by several stimulations in different cell types, and the kinetics of the metabolic events vary widely, from only a few minutes to several days depending on the cell system examined. In contrast, necrosis is a type of cell death resulting in an early lysis of plasma membrane before any significant nuclear morphological changes [Majno and Joris, 1995]. Although the hallmark features of CD95-induced apoptosis are amply characterized in thyroid follicular cells (TFC), morphological and biochemical events in cell permeable synthetic ceramide-induced cell death are largely unknown. Since downstream signals activated by exogenous ceramide may constitute part of the apoptotic pathway generated by CD95, we compared the morphological and biochemical differences between CD95- and ceramide-induced cell death.

MATERIALS AND METHODS

TFC Purification and Culture

Normal thyroid specimens were obtained at the time of thyroidectomy from the uninvolved, controlateral lobes of thyroids with tumors. Thyroid tissues were digested for 2 h with collagenase (1.5 mg/ml) (GIBCO-BRL, Grand Island, NY) and hyaluronidase (20 μg/ml) (Sigma Chemical Co., St. Louis, MO) in DMEM [Stassi et al., 2000]. TFC were maintained in culture with DMEM supplemented with 10% fetal bovine serum (Euroclone, Victoria Street, Paignton, Devon TQ4 5DN, UK). Anti-CD95 mAb (200 ng/ml; CH-11,IgM; Upstate Biotechnology, Lake Placid, NY), C_2-ceramide and C_2-dihydroceramide (1–40 μM; Biomol Research Laboratories, Inc., Plymouth Meeting, PA) were added to suspension cells cultured in polypropylene tubes at time, 0 and then cells were harvested at 0, 1, 2, 3, and 4 h for the surface, cytoplasmic, and nuclear morphological changes, GD3 localization, mitochondrial functionality, and the analysis of apoptotic factors. For caspase inhibition, cells were pretreated for 30 min with protease inhibitors (zVAD-FMK). Inhibitors were kept as stock solutions of 20 mM in DMSO and added directly to cell cultures at 30 μM final concentration.

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

For TEM cultured TFC, untreated and treated with anti-CD95 mAb, C_2-ceramide and C_2-dihydroceramide, were centrifuged, washed twice in 0.1 M phosphate-buffered saline (PBS), and immediately resuspended in 2.5% glutaraldehyde in PBS for 30 min. Then, the cells were post-fixed in 1% osmium tetraoxide (OsO_4) for 30 min and rinsed twice in PBS. Successively, the cells were dehydrated and embedded in epoxy resin (Epon 812; Fluka Chemie AG, Switzerland). Polymerization was carried out at 60°C for 48 h. Thin sections were mounted onto nickel grids, stained with uranyl acetate and lead citrate for 1–2 min. TFC were analyzed by a Jeol 1220 electron microscope.

For SEM, following post-fixation with 1% OsO_4, the cells were progressive acetone dehydrated and critical point-dried with Emscope CPD 750. After mounting on conductive carbon adhesive tabs, the specimens were gold-palladium coated by a Polaron LTD E5200. Observations were performed by a Jeol 6301 F scanning electron microscope at 4–10 kV.

Annexin-V Staining

TFC exposed to anti-CD95 and C_2-ceramide were washed with PBS and labeling of phosphatidylserine (PS) was assessed using an annexin-V staining kit (annexin-V-fluos staining kit; Boehringer Mannheim, Indianapolis, IN). Then, TFC were analyzed by flow cytometer (FACScan, Becton Dickinson, Mountain View, CA).

Immunostaining Procedure

TFC treated with anti-CD95 (200 ng/ml) and C_2-ceramide (2–20 μM) for 4 h were harvested and cytocentrifuged. Then, cells were allowed to equilibrate at room temperature, and before starting, the staining was exposed to absolute acetone for 10 min. GD3 expression (anti-GD3; S2-566, mouse IgM; Seikagaku, Tokyo, Japan) or isotype matched control at appropriate dilutions were detected following manufacturer's instructions (Dako LSAB kit; Dako Corporation, Santa Barbara, CA). The binding was revealed by a AEC colorimetric substrate. Hematoxylin aqueous formula was used as a counterstain.

$\Delta\psi_m$

TFC (3×10^5) treated with anti-CD95 mAb, C_2-ceramide and C_2-dihydroceramide were harvested, washed, and resuspended in 1 ml of complete medium. Then, the cells were stained with 2.5 μg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolylcarbocyanine iodide, T-3168; Molecular Probes, Inc. Eugene, OR) (32–34). After the dye was well dissolved, samples were kept in the dark at RT for 15 min. Successively, the cells were washed twice in PBS by centrifuging at 500*g* for 5 min, and resuspended in PBS. Analysis was performed by a flow cytometer (FACScan Becton Dickinson).

Western Blotting

Cell pellets were resuspended in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40) containing 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml). After 30 min on ice, the resulting lysates were centrifuged at 10,000*g* for 10 min to remove nuclei and cell debris. Each lysate (30 μg) was fractioned on 10% or 12% sodium dodecyl sulfate–polyacrylamide gels and blotted to nitrocellulose (Hybond, Amersham, Little Chalfont Buckinghamshire England, UK). Bound anti-Bcl-x_L (H-5, mouse IgG1, Santa Cruz Biotechnology), anti-Bcl-2 (124, mouse IgG1, Upstate Biotechnology), anti-Bax (rabbit polyclonal IgG, Upstate Biotechnology), and anti-Bak (rabbit polyclonal IgG, Upstate Biotechnology) were detected by HRP-conjugated anti-mouse or anti rabbit Abs (Amersham) and visualized by an enhanced chemiluminescence detection (ECL) system (Super Signal West Dura Extended Duration Substrate, Pierce, Rockford, IL) according to manufacturer's instructions.

Mitochondria Isolation and Cytochrome c Detection

TFC pellets were homogenized with a glass–Teflon directly into an ice-cold isolation buffer, containing 5 mM Tris-HCl, pH 7.4, 0.25 mM sucrose, 50 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF. After centrifugation, the supernatants were collected into fresh tubes and centrifuged at 25,000*g* for 10 min, at 4°C. Equivalent amounts of proteins lysated from mitochondrial pellets and cytosolic

fractions were run in a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose. Cytochrome c was detected using a monoclonal antibody (clone 7h8.2C12, PharMingen, San Diego, CA). Blot was revealed by ECL (Pierce).

Poly (ADP-Ribose) Polymerase Cleavage Assay

Untreated cells and those treated for 4 h with 200 ng/ml anti-CD95 and 2–20 μM of both C_2-ceramide and C_2-dihydroceramide were rinsed in PBS, collected by centrifugation, suspended, and sonicated on ice in a buffer containing 50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 mM sodium fluoride, 10 mM sodium ortovanadate, and 1 mM PMSF at a concentration of 10^6 cells/20 μl. Following the addition of a solution containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 6% β-mercaptoethanol, 3% SDS, and 0.003% bromophenol blue, cell lysates were heated for 15 min at 65°C. Equivalent amounts of protein (30 μg) were resolved on 10% SDS–PAGE, transferred to nitrocellulose (Hybond, Amersham), and probed with 1:1,000 anti-PARP mAb (C2-10, IgG_1; PharMingen). Blot visualization was performed using ECL (Pierce).

Analysis of DNA Fragmentation

Untreated cells and those treated for 4 h with anti-CD95, C_2-ceramide, and C_2-dihydroceramide were lysed in a buffer containing 0.5% SDS, 5 mM EDTA, 0.2% Triton X-100. Following 5 min incubation, lysates were extracted with phenol/chloroform and DNA was precipitated with 3 M sodium acetate, pH 5.2 in cold absolute ethanol for 20 min in dry ice. The dry DNA pellet was dissolved in TE (50 mM Tris, 1 mM EDTA, pH 8) and incubated with RNase for 45 min at 37°C before re-extraction and precipitation, as above. Then, DNA was dissolved in TE, quantified by spectrophotometry at 260 nm and run in 1.8% agarose gel with 1 kb DNA standard (GeneRuler™, MBI Fermentas, Vilnius, Lithuania).

Statistical Analysis

Results are shown as mean ± SEM. We compared data on the basis of morphological and functional parameters using an imaging analyzer (Image Pro-Plus). All samples were analyzed in blind fashion.

RESULTS

Dose-Dependent Effect of Cell-Permeable Synthetic C_2-Ceramide

Cell-permeable synthetic C_2-ceramide treatment and extracellular addition of SMase led to the induction of cell death with DNA fragmentation and apoptotic morphology [Cifone et al., 1994; Jarvis et al., 1994].

These results prompted us to test a dose-dependent response of TFC exposed to C_2-ceramide (1–40 μM) to evaluate whether exogenous ceramide can induce a morphologically different kind of cell death (apoptosis or necrosis). Annexin-V assay of TFC treated with 2 μM C_2-ceramide for 4 h showed about 60% of apoptotic cell death, whereas TFC treated with 20 and 40 μM C_2-ceramide, showed about 70% and 90% of necrotic cell death, respectively (Fig. 1A).

To compare the induction of cell death by anti-CD95 and C_2-ceramide, TFC were treated in kinetics experiments (0–4 h) with different doses of C_2-ceramide (2–20 μM) and with 200 ng/ml of anti CD95 (CH-11) and successively stained with Annexin-V fluos and analyzed by cytofluorimeter and fluorescence microscopy. At 1 h, approximately 12% of TFC, treated with anti-CD95 and C_2-ceramide (2 μM), presented typically apoptotic features. This population grew to approximately 85% and 70%, respectively at 4 h (Fig. 1B), whereas TFC treated with C_2-ceramide (20 μM) did not present typically apoptotic features achieving a peak of 20% at 4 h.

When we evaluated the typically necrotic features, the predominant population was TFC treated with C_2-ceramide (20 μM). In fact, at different time points, this population grew up to 85% at 4 h, while cells treated with anti-CD95 and C_2-ceramide (2 μM) were little represented (Fig. 1B).

Furthermore, fluorescence microscopy detection of cell-surface PS with annexin-V showed a strong positivity on the surface of TFC treated with anti-CD95 at all time points analyzed. Arrowheads indicate apoptotic cells with bright green staining and characteristic condensed and fragmented nuclei (Fig. 1C, left panel). In contrast, cells cultured in the presence of 20 μM C_2-ceramide for 4 h were mostly stained with propidium iodide (PI) (red color), showing cellular destruction with typically necrotic features, and a negligible number of them

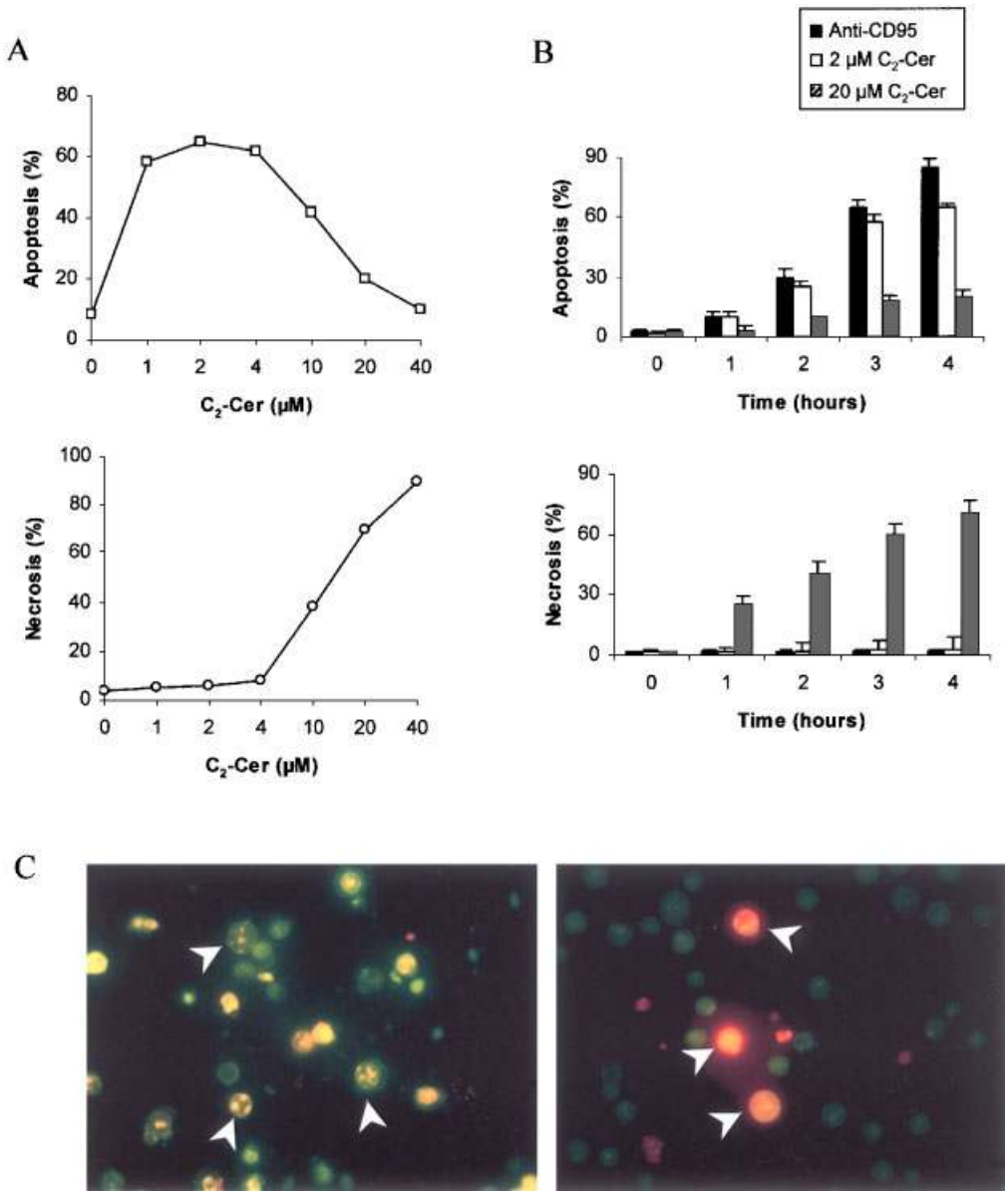

Fig. 1. Response evaluation by annexin-V staining of TFC to ceramide exposure. **A**: Apoptosis and necrosis evaluation on TFC exposed to various doses of C_2-ceramide. **B**: Kinetic evaluation of apoptosis and necrosis on TFC exposed to anti-CD95 (200 ng/ml) or C_2-ceramide (2 and 20 μM). **C**: Left panel: Cells exposed to anti-CD95 for 2 h. Arrowheads indicate apoptotic cells with bright green staining and characteristic condensed nuclei. Right panel: Cells treated with 20 μM C_2-ceramide for 2 h. Arrowheads show necrotic cells stained with PI (red color) and advanced cellular destruction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

stained in green, can be regarded as apoptotic cells (Fig. 1C, right panel).

CD95 Stimulation and Ceramide Exposure at Ultrastructural Levels

To determine whether CD95 is functional in TFC, and therefore able to induce a death signal, we incubated the cells with an agonist anti-CD95 mAb and examined them at different time points for evidence of apoptosis. At 2 h of incubation, we found 30% of cells at an early apoptosis morphological stage, as evidenced by the margination of chromatin, rounding of cells together with the onset of cell surface blebbing (Figs. 2 and 3). Several cells demonstrated chromatin capping as well as its segregation into dark and lucent areas (not shown). When we analyzed 4 h cultures treated with anti-CD95,

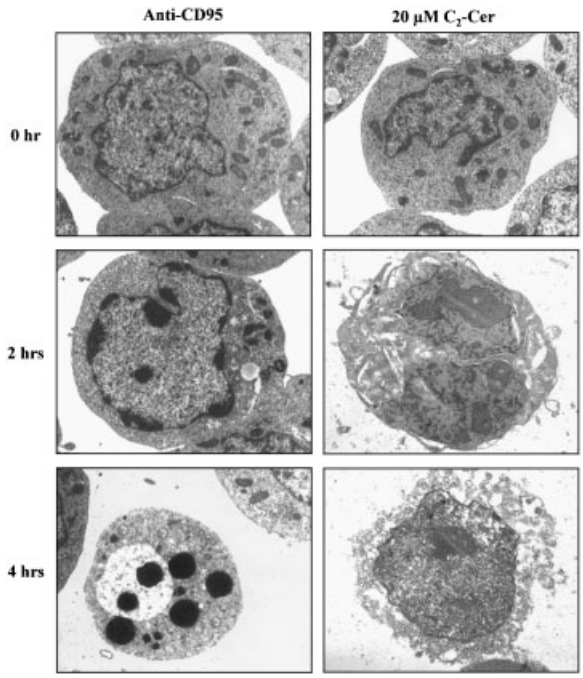

Fig. 2. TEM analysis following anti-CD95 and C_2-ceramide exposure. Upper Panels: Untreated TFC (6,000 × and 5,000 × respectively). Middle panels: TFC treated with anti-CD95 for 2 h, characterized by peripheral chromatin margination (5,000 ×) and TFC exposed to 20 μM C_2-ceramide for 2 h showing the initial cell swelling with membrane lysis, surface blisters, and disruption of cytoplasmic organelles (8,000 ×). Lower panels: TFC treated with anti-CD95 for 4 h demonstrating the apoptotic bodies formation (5,000 ×), and TFC exposed to 20 μM C_2-ceramide for 4 h characterized by cytoplasmic disintegration (8,000 ×).

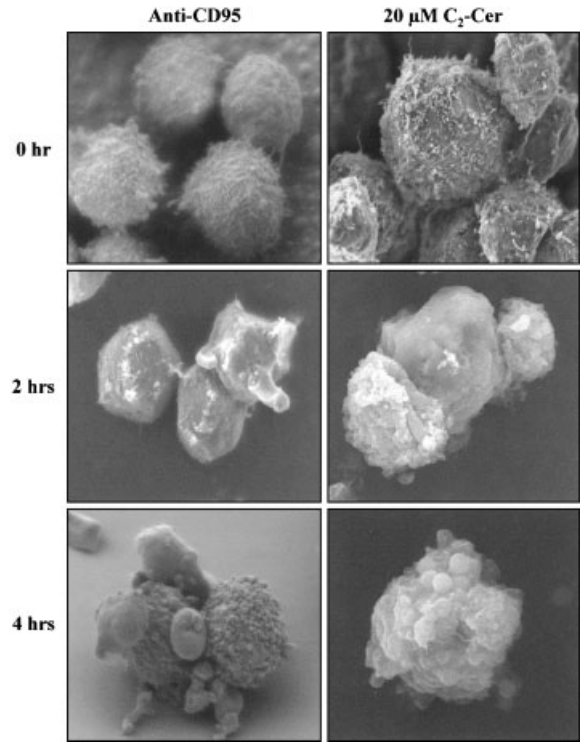

Fig. 3. SEM of TFC treated with anti-CD95 and C_2-ceramide. Upper panels: Untreated cells (3,500 × and 4,500 ×, respectively). Middle panels: Cells treated with anti-CD95 for 2 h, characterized by intense membrane blebbing formation (3,500 ×) and cells exposed to 20 μM C_2-ceramide for 2 h with the presence of surface blisters (5,000 ×). Lower panels: Apoptotic bodies formation and echinoid spikes in CD95 treated cells for 4 h (3,500 ×), and membrane disruption in C_2-ceramide treated cells for 4 h (5,000 ×).

85% of cells were at late stages of apoptosis, as shown by the formation of multiple apoptotic bodies and echinoid spikes (Figs. 2 and 3). Chromatin in several apoptotic bodies was enclosed in morphologically intact nuclear membranes and cytoplasm was electron-dense (Fig. 2).

In contrast, TFC exposed to C_2-ceramide (20 μM) for 2 h showed cell swelling, formation of surface blisters separated by deep and irregular furrows, and initial membrane lysis (Figs. 2 and 3). We also found a low percentage of apoptotic events ranging from 4 to 10% after exposure for various times to ceramide (data not shown). In addition, about 80% of TFC treated for 4 h with C_2-ceramide (20 μM), were typified by membrane lysis, mitochondrial swelling, and dispersal of cytoplasmic organelles (Fig. 2). In particular, Figure 3 at 4 h revealed the presence of several ruffles and membrane breaking. The main feature of this process, however, was the apparent integrity of nuclear membrane (Fig. 2).

GD3 Accumulation and $\Delta\psi_m$ Evaluation

GD3 ganglioside accumulation is one of the metabolic components involved in CD95-induced apoptosis, responsible for engendering an irreversible apoptotic signal [De Maria et al., 1997]. Since GD3 accumulation is able to directly interact with mitochondria [Rippo et al., 2000], we contemporarily studied GD3 accumulation and mitochondrial ultrastructural changes of TFC after 4 h of treatment with anti-CD95 and C_2-ceramide (2–20 μM). Immunocytochemistry demonstrated strong positivity for GD3 when TFC were exposed to anti-CD95 and C_2-ceramide (20 μM) (Fig. 4A), while a lower accumulation of GD3 was represented in cells treated with 2 μM of C_2-ceramide. Moreover, the same cells treated with anti-CD95, when analyzed by TEM, showed hyperactive mitochondria, while mitochondria of cells cultured with C_2-ceramide (2 μM) appeared

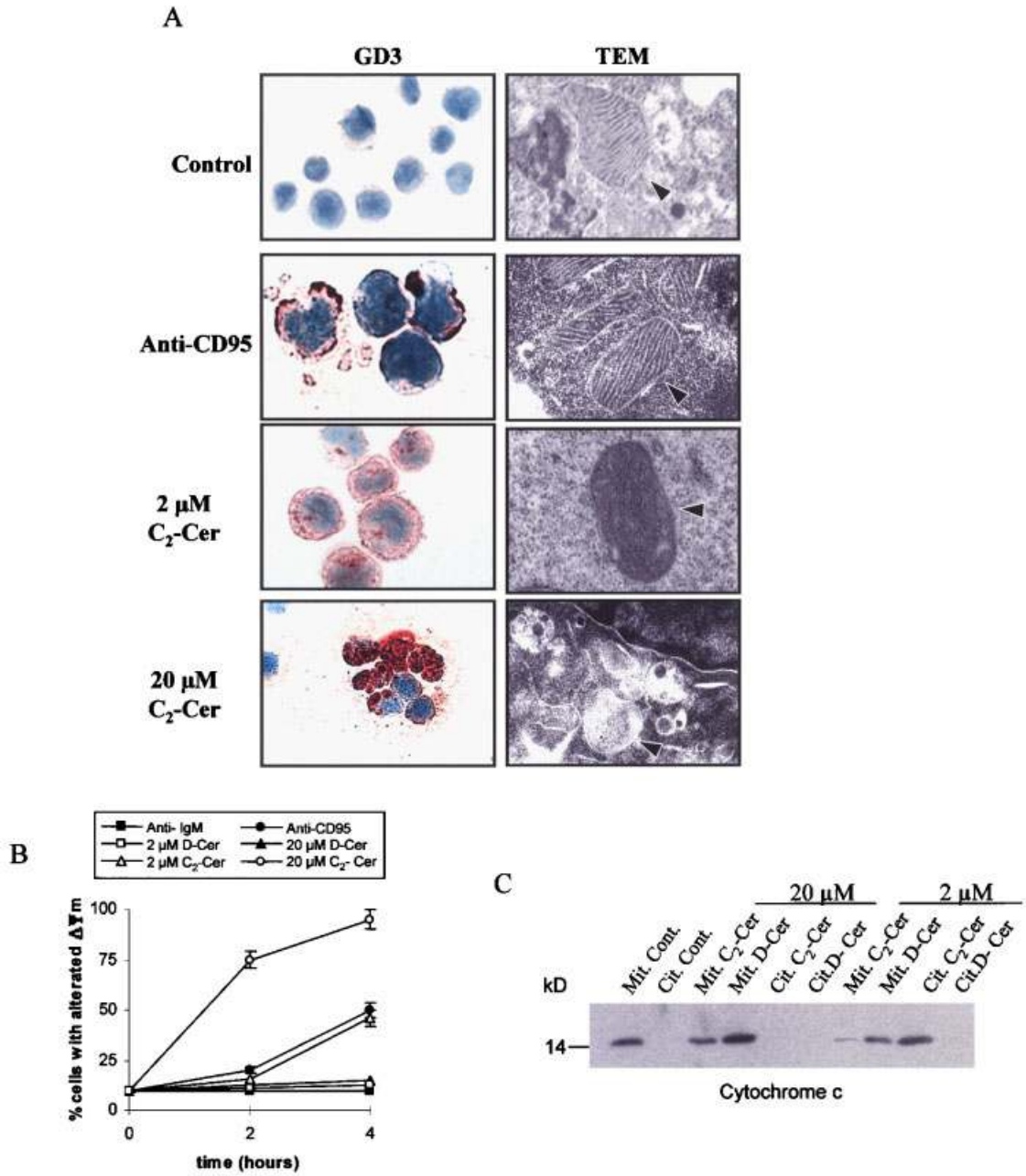

Fig. 4. GD3 accumulation and mitochondria energy status evaluation on TFC. **A**: Immunohistochemical analysis of TFC exposed to isotype-matched control (400 ×), anti-CD95 (1,000 ×), 2 (1,000 ×), and 20 μM (400 ×) C_2-cermide labeled with GD3 mAb; ultrastructural analysis of mitochondria of cells treated with isotype-matched control (30,000 ×), anti-CD95 (30,000 ×), 2 (40,000 ×), and 20 μM (23,000 ×) C_2-cermide. **B**: $\Delta\psi_m$ analysis, using JC-1 staining, in cells treated with IgM, anti-CD95, C_2-dihydroceramide (2 and 20 μM), and C_2-ceramide (2 and 20 μM), at 0, 2, and 4 h time points. **C**: Immunoblot analysis of cytochrome c on mitochondria and cytosol of TFC exposed to 2 and 20 μM C_2-ceramide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

condensed according to data that condensed mitochondria have been observed in association with apoptosis [Mancini et al., 1997; Zhuang and Cohen, 1998]. Unexpectedly, 20 μM C_2-ceramide determined mitochondrial destruction (Fig. 4A).

Analyzing the mitochondrial activity of cells with a specific probe JC-1, we found variations in $\Delta\psi_m$ from the single cell to different concentrations of ceramide and anti-CD95. Particularly, $75 \pm 3.5\%$ of TFC treated with 20 μM ceramide for 4 h showed disruption of $\Delta\psi_m$,

compared with the low percentage (12.5% ± 0.4%) of cells with altered $\Delta\psi_m$ exposed to dihydroceramide. By contrast, only 20 ± 1.1% of cells treated with anti-CD95 for 4 h showed altered $\Delta\psi_m$ (Fig. 4B).

We investigated the redistribution of cytochrome c from the mitochondrial intermembrane space to the cytoplasm. Detecting cytochrome c by immunoblotting analysis in isolated mitochondria and cytosol from TFC cultured with C_2-dihydroceramide and C_2-ceramide (2–20 μM) for 4 h, we found that it was completely absent in the cytosol of cells treated with 20 μM C_2-ceramide and abundantly present in 2 μM C_2-ceramide treated cells (Fig. 4C).

Bax and Bak Are Not Oligomerized in TFC Exposed to High Concentrations of C_2-Ceramide

We analyzed the expression levels of Bcl-xl, Bcl-2, Bak, and Bax by immunoblot in the mitochondrial and cytoplasmic compartment of untreated and treated cells with 20 μM C_2-ceramide for 2 h and 4 h. Mitochondria are the principal site of the action of some of the Bcl-2 family members [Kluck et al., 1997] that are composed of both anti-apoptotic (Bcl-2, Bcl-xl) and pro-apoptotic (Bax, Bak) molecules.

In line with $\Delta\psi_m$ results, we found low Bcl-xl and Bcl-2 expression levels in the mitochondrial and cytoplasmic compartment at both time points of culture (Fig. 5A). In contrast, Bak and Bax were scarcely present in mitochondria, while being abundantly expressed in the cytoplasmic compartment (Fig. 5A). These findings suggest that Bax and Bak are not oligomerized and inserted into the outer mitochondrial membrane where they classically elicit their pro-apoptotic role.

High Levels of C_2-Ceramide Are Not Able to Induce PARP Cleavage and DNA Laddering

A well-characterized substrate of caspase-3 is PARP, which maintains the integrity of chromosomal DNA and plays a pivotal role in the endonucleolytic degradation of DNA. We examined untreated and treated cell lysates for PARP cleavage products and DNA cleavage into nucleosome-sized fragments to evaluate ceramide effects. As demonstrated in Figure 5B,C (lane 4), cleavage of PARP and DNA laddering were not observed in 20 μM C_2-ceramide. In contrast, PARP cleavage and DNA fragmentation were seen in cells treated with 200 ng/ml of CH-11 and 2 μM C_2-ceramide (Fig. 5B,C, lane 2). These results support a ceramide-induced death mechanism that occurs without leading to cleavage of nuclear substrates for manifestation of ceramide effects.

zVAD Failed in the Rescue of TFC From C_2-Ceramide Effects

To determine whether apoptosis induced by C_2-ceramide in TFC required activation of caspases, we pre-treated cells with the broad spectrum caspases inhibitor, zVAD-fmk. Accordingly, when we analyzed 2 h and 4 h C_2-ceramide (2 μM) TFC pretreated with zVAD (30 μM) for 30 min, no apoptotic feature was observed (Fig. 5D), while zVAD was not able to rescue TFC from necrosis when they were cultured with 20 μM C_2-ceramide (Fig. 5D). These data confirm that TFC necrosis induced by high levels of C_2-ceramide does not require caspases activation.

DISCUSSION

As shown by several studies, ceramide, generated by SMase activation, seems to be involved in cell growth, differentiation, and apoptosis, essential for the development and maintenance of tissue homeostasis [Cifone et al., 1994; Testi, 1996; De Maria et al., 1997].

Our findings focused on the signaling pathway study following ceramide exposure in human normal TFC.

The involvement of CD95/CD178 system in the regulation of thyroid cell apoptosis has been extensively studied [Borgerson et al., 1999]. Moreover, deregulation of CD95-mediated apoptosis has been proposed as a possible common effector of tissue destruction in organ-specific autoimmune diseases, such as Hashimoto's thyroiditis [Giordano et al., 1997; Hammond et al., 1997; Stassi et al., 2000, 2001].

Several studies have demonstrated the association of interleukin-1β (IL-1β) with thyroidal autoimmune diseases [Bendtzen et al., 1989]. IL-1β induces ceramide formation and SM degradation in porcine thyroid cells [Schneider et al., 2001]. An atypical protein kinase C (PKC), PKC-ξ, seems to be the direct target of IL-1β and ceramides action, in response to TNF-α stimulation. The regulation of PKC-ξ by ceramide could contribute to NF-κB induction, leading to cell survival [Muller et al., 1995; Wang et al., 1999; Schneider et al., 2001]. Therefore, these data established a new role for ceramide

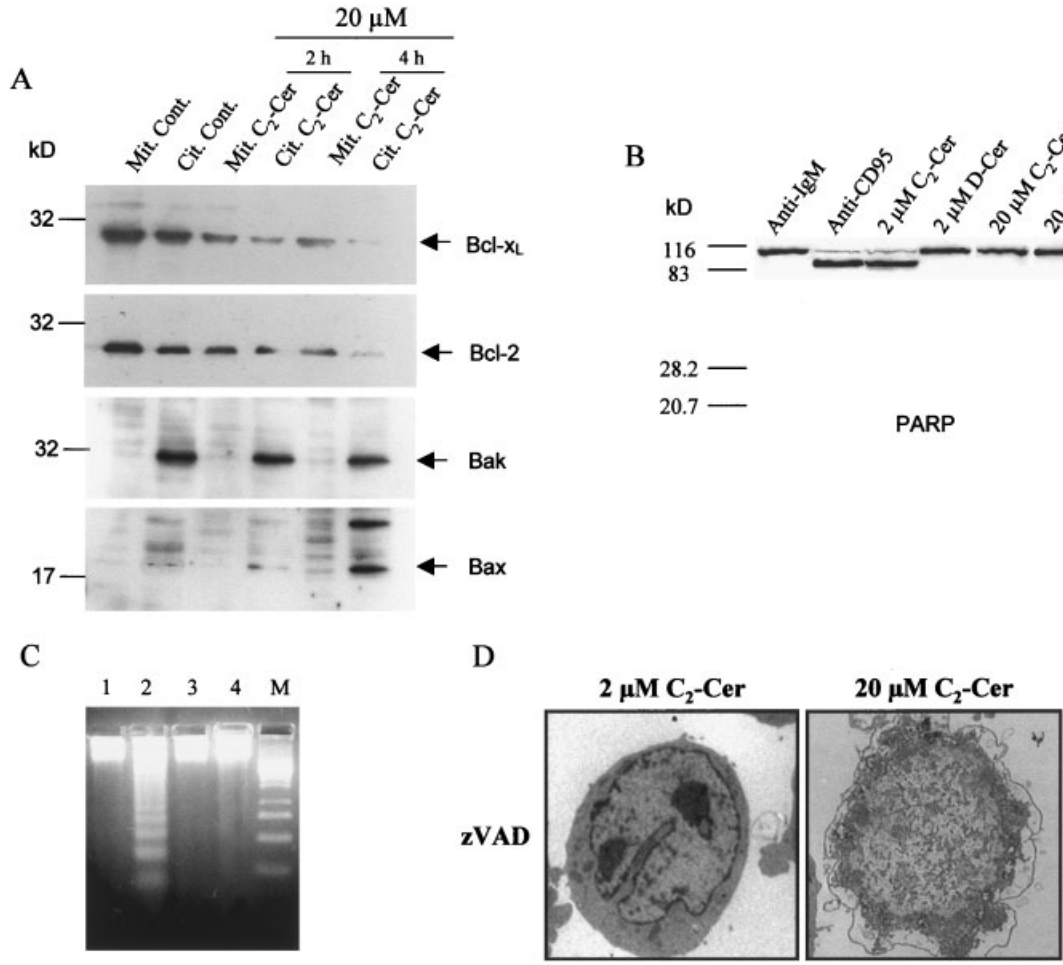

Fig. 5. Apoptotic molecule expression and nuclear substrate evaluation on TFC. **A**: Immunoblot analysis of anti-apoptotic molecules (Bcl-xL and Bcl-2) and pro-apoptotic molecules (Bak and Bax) in the purified mitochondrial and cytoplasmic compartment from cells untreated and exposed to 20 μM C2-ceramide for 2 and 4 h. **B**: Immunoblot detection of cleaved fragments of PARP on cells treated with isotype-matched control, anti-CD95, C_2-dihydroceramide, and C_2-ceramide (2–20 μM) for 4 h. **C**: DNA laddering of TFC exposed for 4 h to 2 μM C_2-dihydroceramide (**lane 1**), 2 μM C_2-ceramide (**lane 2**) 20 μM C_2-dihydroceramide (**lane 3**), and 20 μM C_2-ceramide (**lane 4**). **Lane M** represents standard DNA fragments (1 kb). **D**: TEM analysis of TFC pre-cultured with 30 μM of zVAD and subsequently exposed to 2 and 20 μM C_2-ceramide.

in modulating thyroid cell dedifferentiation, characterized by a SMase-induced thyrocyte proliferation that was accompanied by the loss of their ability to iodinate proteins and decrease the adenylate cyclase system response. Moreover, ceramide is known to be recruited in CD95, TNF-α, ionizing radiations, and anticancer drugs apoptotic-signaling cascade [Ballou et al., 1996].

Several studies have shown that C_2- and C_8-ceramide treatment mimicked the TNF-α-mediated induction of apoptosis, prevented by exposure to DAG, PLC, or PMA, thereby suggesting that PKC activation antagonizes the SM pathway [Jarvis et al., 1994; Santana et al., 1996]. In addition, using histochemical criteria and DNA fragmentation, Jarvis et al. [1994] have demonstrated that apoptosis is induced by C_2- and C_8-ceramide. The effects of ceramide on DNA fragmentation were inhibited by zinc, suggesting the involvement of a Ca^{2+}-dependent endonuclease [Obeid et al., 1993].

CD95 triggering determines SMase activation, responsible for SM hydrolysis and resulting in ceramide accumulation [Cifone et al., 1994; Santana et al., 1996]. Then, ceramide is gradually converted into the apoptotic GD3 ganglioside through the final contribution of a GD3 synthase (ST8) [De Maria et al., 1997]. We recently demonstrated that the majority of lymphocytes located in closed proximity to $CD178^+$

thyrocytes, during Hashimoto's thyroiditis, are committed to apoptosis, as shown by high levels of expression of GD3 ganglioside [Stassi et al., 1999b]. GD3 accumulation seems to be involved in the ongoing apoptotic process generated by ceramide [De Maria et al., 1997; Rippo et al., 2000]. Although ceramide-signaling pathways have been implicated in cell death, little is known about the morphological aspects and nuclear target cleavage in TFC. Toward this end, we report the biological effects of exogenous ceramide at high concentrations as defined by morphological criteria, gene apoptotic factors, PARP cleavage, and DNA laddering.

Our findings show that high levels of ceramide (20 μM) exposure determine early mitochondrial damage and necrotic features. These results suggest that the energy status of cells is altered and unable to support the metabolically active apoptotic process.

Several studies have demonstrated that ceramide induces cell necrosis through disruption of mitochondrial function and ATP depletion in rat hepatocytes [Arora et al., 1997; Ha and Snyder, 1999].

Moreover, studies on MCF-7 breast cancer cells showed that the apoptotic process can occur only when sufficient oxygen and energy supply are available [Formigli et al., 2002]. During ATP depletion, energy levels rapidly decline [Doctor et al., 1994], and aponecrosis, a novel type of cell demise sharing the features of both apoptosis and necrosis, can ensue as the result of an incomplete execution of the apoptotic program [Formigli et al., 2000].

The data presented here suggest that ceramide is an additional component of apoptosis induction in TFC. Moreover, although endogenous ceramide accumulation may promote the apoptotic process in TFC, exposure to high levels of synthetic ceramide is likely to result in necrotic cell death. Therefore, for evaluating the effects of exogenous ceramide on gene apoptotic factors, we assessed whether Bax and Bak, two proapoptotic proteins necessary for apoptosis in many cell types, are required for apoptosis induction by triggering cytochrome c release from TFC mitochondria [Liu et al., 1996].

In fact, activation and oligomerization of Bax or Bak have been proposed to result in the formation of a homomultimeric pore [Saito et al., 2000; Wei et al., 2001], formation of a voltage-dependent anion channel-containing pore [Shimizu et al., 1999] or the permeabilization of mitochondrial membranes [Kluck et al., 1999] to initiate cytochrome c release. Release of cytochrome c activates the Apaf-1-caspase-9 apoptosome and downstream effector caspases [Li et al., 1997], with a progressive mitochondrial dysfunction and consequent cell death [Mootha et al., 2001]. Loss-of-function studies have revealed that the absence of proapoptotic Bax and Bak molecules creates a profound block, preserving mitochondria and inhibiting apoptosis [Wei et al., 2001], thereby suggesting that the mitochondrial apoptotic pathway requires both Bax and Bak oligomerization into the mitochondrial membranes.

In our model, our findings show that Bax and Bak are not oligomerized or inserted into the outer mitochondrial membrane where they classically elicit their pro-apoptotic role, proving that high concentrations of exogenous ceramide are not able to induce apoptotic cell death in TFC.

Recently, PARP cleavage has been proposed to prevent depletion of NAD (a PARP substrate) and ATP, which are thought to be required for later events in apoptosis [Herceg and Wang, 1999]. Futhermore, PARP activation has been shown to prevent induction of necrosis during apoptosis and ensure appropriate execution of caspase-mediated programmed cell death, influencing intracellular ATP levels [Kolesnick and Fuks, 1995]. Our data show that since ceramide treatment is not able to determine the proteolytic cleavage of PARP and the internucleosomal DNA fragmentation, resulting in depletion of ATP, treated cells undergo necrosis. This is consistent with the evidence that the depletion of ATP can transform an ongoing apoptotic process into necrosis, thereby suggesting that intracellular ATP levels control the quality of cell death.

REFERENCES

Arora AS, Jones BJ, Patel TC, Bronk SF, Gores GJ. 1997. Ceramide induces hepatocyte cell death through disruption of mitochondrial function in the rat. Hepatology 25:958–963.

Ballou LR, Laulederkind SJ, Rosloniec EF, Raghow R. 1996. Ceramide signalling and the immune response. Biochim Biophys Acta 1301:273–287.

Bendtzen K, Buschard K, Diamant M, Horn T, Suensen M. 1989. The thyroid cell group. Possible role of IL-1, TNF-α and IL-6 in insulin-dependent diabetes mellitus and autoimmune thyroid disease. Lymphokine Res 8(3):335–340.

Borgerson KL, Bretz JD, Baker JR, Jr. 1999. The role of Fas-mediated apoptosis in thyroid autoimmune disease. Autoimmunity 30:251–264.

Cifone MG, De Maria R, Roncaioli P, Rippo MR, Azuma M, Lanier LL, Santoni A, Testi R. 1994. Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 180:1547–1552.

Cohen JJ. 1993. Apoptosis. Immunol Today 14:126–130.

Collins JA, Schandi CA, Young KK, Vesely J, Willingham MC. 1997. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem 45:923–934.

De Maria R, Testi R. 1998. Fas–FasL interactions: a common pathogenetic mechanism in organ-specific autoimmunity. Immunol Today 19:121–125.

De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R. 1997. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis [published erratum appears in Science 1998 Apr 17;280 (5362):363]. Science 277:1652–1655.

De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, Riccioni R, Felli N, Samoggia P, Peschle C. 1999. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 93:796–803.

Doctor RB, Bacallao R, Mandel LJ. 1994. Method for recovering ATP content and mitochondrial function after chemical anoxia in renal cell cultures. Am J Physiol 266: C1803–C1811.

Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S, Orlandini SZ. 2000. Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol 182:41–49.

Formigli L, Zecchi Orlandini S, Capaccioli S, Poupon MF, Bani D. 2002. Energy-dependent types of cell death in mcf-7 breast cancer cell tumors implanted into nude mice. Cells Tissues Organs 170:99–110.

Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R, Galluzzo A. 1997. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 275: 960–963.

Green D, Kroemer G. 1998. The central executioners of apoptosis: caspases or mitochondria? Trends Cell Biol 8:267–271.

Ha HC, Snyder SH. 1999. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 96:13978–13982.

Hammond LJ, Lowdell MW, Cerrano PG, Goode AW, Bottazzo GF, Mirakian R. 1997. Analysis of apoptosis in relation to tissue destruction associated with Hashimoto's autoimmune thyroiditis. J Pathol 182:138–144.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Herceg Z, Wang ZQ. 1999. Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. Mol Cell Biol 19:5124–5133.

Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S. 1994. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc Natl Acad Sci USA 91:73–77.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136.

Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR, Newmeyer DD. 1999. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J Cell Biol 147:809–822.

Kolesnick R, Fuks Z. 1995. Ceramide: a signal for apoptosis or mitogenesis? [comment]. J Exp Med 181:1949–1952.

Kroemer G, Zamzami N, Susin SA. 1997. Mitochondrial control of apoptosis. Immunol Today 18:44–51.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147–157.

Majno G, Joris I. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death [see comments]. Am J Pathol 146:3–15.

Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB, Hockenbery DM. 1997. Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. J Cell Biol 138:449–469.

Miller LJ, J M. 1998. Apoptosis. Science 281:1301–1304.

Mootha VK, Wei MC, Buttle KF, Scorrano L, Panoutsakopoulou V, Mannella CA, Korsmeyer SJ. 2001. A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. EMBO J 20:661–671.

Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K. 1995. PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. EMBO J 14: 1961–1969.

Nagata S. 1997. Apoptosis by death factor. Cell 88:355–365.

Obeid LM, Linardic CM, Karolak LA, Hannun YA. 1993. Programmed cell death induced by ceramide. Science 259:1769–1771.

Pushkareva M, Obeid LM, Hannun YA. 1995. Ceramide: an endogenous regulator of apoptosis and growth suppression [see comments]. Immunol Today 16:294–297.

Rippo MR, Malisan F, Ravagnan L, Tomassini B, Condo I, Costantini P, Susin SA, Rufini A, Todaro M, Kroemer G, Testi R. 2000. GD3 ganglioside as an intracellular mediator of apoptosis. Eur Cytokine Netw 11:487–488.

Saito M, Korsmeyer SJ, Schlesinger PH. 2000. BAX-dependent transport of cytochrome c reconstituted in pure liposomes. Nat Cell Biol 2:553–555.

Salvesen GS, Dixit VM. 1997. Caspases: intracellular signaling by proteolysis. Cell 91:443–446.

Santana P, Pena LA, Haimovitz-Friedman A, Martin S, Green D, McLoughlin M, Cordon-Cardo C, Schuchman EH, Fuks Z, Kolesnick R. 1996. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell 86:189–199.

Schneider C, Delorme N, El Btaouri H, Hornebeck W, Haye B, Martiny L. 2001. Interleukin-1 beta (IL-1 beta) action in porcine thyroid cells involves the ceramide signalling pathway. Cytokine 13:174–178.

Shimizu S, Narita M, Tsujimoto Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483–487.

Song Z, Steller H. 1999. Death by design: mechanism and control of apoptosis. Trends Cell Biol 9:M49–M52.

Stassi G, De Maria R, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C, Trucco M. 1997. Nitric oxide primes pancreatic beta cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. J Exp Med 186: 1193–1200.

Stassi G, Di Felice V, Todaro M, Cappello F, Zummo G, Farina F, Trucco M, De Maria R. 1999a. Involvement of Fas/FasL system in the pathogenesis of autoimmune diseases and Wilson's disease. Arch Immunol Ther Exp 47:129–133.

Stassi G, Todaro M, Bucchieri F, Stoppacciaro A, Farina F, Zummo G, Testi R, De Maria R. 1999b. Fas/Fas ligand-driven T cell apoptosis as a consequence of ineffective thyroid immunoprivilege in Hashimoto's thyroiditis. J Immunol 162:263–267.

Stassi G, Di Liberto D, Todaro M, Zeuner A, Ricci-Vitiani L, Stoppacciaro A, Ruco L, Farina F, Zummo G, De Maria R. 2000. Control of target cell survival in thyroid autoimmunity by T helper cytokines via regulation of apoptotic proteins. Nat Immunol 1:483–488.

Stassi G, Zeuner A, Todaro M, Di Liberto D, Ricci-Vitiani L, De Maria R. 2001. Fas/FasL in Hashimoto's thyroiditis. J Clin Immunol 21(1):19–23.

Tepper AD, Cock JG, de Vries E, Borst J, van Blitterswijk WJ. 1997. CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3/CPP32 inhibition. J Biol Chem 272:24308–24312.

Testi R. 1996. Sphingomyelin breakdown and cell fate. Trends Biochem Sci 21:468–471.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272:17907–17911.

Wang YM, Seibenhener ML, Vandenplas ML, Wooten MW. 1999. Atypical PKC zeta is activated by ceramide, resulting in coactivation of NF-κB/JNK kinase and cell survival. J Neurosci Res 55:293–302.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292:727–730.

Zhuang J, Cohen GM. 1998. Release of mitochondrial cytochrome c is upstream of caspase activation in chemical-induced apoptosis in human monocytic tumour cells. Toxicol Lett 102–103:121–129.