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Keratinocytes Become Terminally Differentiated in a Process Involving Programmed Cell Death

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Oral keratinocytes originate from basal cells, differentiate during migration to the surface, and finally are shed. Apoptosis occurs at the end of differentiation, but the precise relationship between terminal differentiation and apoptosis is not clear. In the present study, Bcl-xL was expressed in the basal cell and spinous cell layers, and Bax was expressed in the spinous cell and granular cell layers. In cultured keratinocytes, Bcl-xL was expressed under conditions of 0.1 m*M* calcium (low Ca^{2+}) but disappeared under conditions of 1.0 m*M* calcium (high Ca^{2+}); the latter induces keratinocyte differentiation. Bax was not expressed in keratinocytes with low Ca^{2+} but was expressed in cells with high Ca^{2+}. Finally keratinocytes with high Ca^{2+} underwent apoptosis, which was detected by the TUNEL method and by 180-bp DNA fragmentation. These results suggest that the process of terminal differentiation in gingival epithelium is a pathway to apoptosis.

The surface of oral mucosa consists of a dynamic stratified squamous epithelium that is maintained by proliferation of stem cells in the basal cell layer, by differentiation into spinous cells, then into granular cells, then into keratinocytes, ending in cell death (1). To maintain the normal architecture of adult oral epithelium, the serial events of growth, differentiation, and death are strictly modulated. This homeostatic balance undoubtedly involves integration of epithelial differentiation and apoptosis (2). Differentiation is linked to the ultrastructural and biochemical events characteristic of apoptosis, for example, pyknosis and DNA fragmentation into nucleosome-sized pieces (3–5). However, the relationship between differentiation and apoptosis is still a matter of controversy (6). Bcl-2, a 26-kDa protein encoded by bcl-2 oncogene identified in molecular analysis of the t(14;18) chromosome translocation in follicular B cell lymphomas, is one of the factors that delays or inhibits apoptosis, but that does not affect cell proliferation (7-9). In cultured human keratinocytes, overexpression of a bcl-2 transgene inhibits terminal differentiation (5, 10). Down regulating of Bcl-2 by transfecting a bcl-2-antisense expression vector promotes differentiation of a mouse keratinocyte cell line 212 (11). Therefore, Bcl-2 might have an important function in normal human tissues.

Recently some genes which share homology with bcl-2 have been identufied: bcl-xL, bax, bak, bad, etc (12-15). Bcl-xL prevents apoptotic cell death. This function is common to Bcl-2 and Bcl-xL, but Bcl-xL blocks some apoptotic pathways that Bcl-2 does not inhibit (16). Bax is a 21-kDa protein with amino acid sequence homology with Bcl-2. Bax induces apoptosis by counteracting Bcl-2 death repressor activity by forming heterodimers with Bcl-2 (13). In this study, we investigated whether the cell death that concludes terminal differentiation of gingival epithelium is a process of apoptosis, whether apoptosis occurs in the induced differentiation of cultured keratinocytes in the presence of a high concentration of calcium (1.0 mM), and the relationship between differentiation and expression of bcl-2 related proteins, using the model (17, 18) of high Ca^{2+} induced-terminal differentiation of keratinocytes.

MATERIALS AND METHODS

Culture of normal keratinocytes. Normal human gingiva samples were obtained by surgery from patients undergoing extraction of mandibular wisdom teeth at Kyushu Dental College Hospital. The samples were incubated overnight in 2% collagenase type IV (Dulbecco's C-5138, Sigma, USA) in D-MEM/F12 (GIBCO BRL, USA) at 4 °C. The epithelium was peeled off with forceps and washed with Mg^{2+}, Ca^{2+}-free phosphate-buffered saline (PBS(−)). Then it was minced and incubated in 0.1% trypsin / 0.01 % EDTA in PBS(−) at

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37 °C for 12 min. Trypsin was inactivated by trypsin inhibitor (Sigma, USA) in MCBD153 (Kyokuto, Japan). The cells were washed in PBS(−), and plated on 35 mm collagen-coated dishes (Iwaki, Japan). The cultures were maintained at 37 °C in 5 % CO_2, using medium MCDB 153 supplemented with 9 % Ca^{2+}-free fetal bovine serum (FBS) [FBS treated with chelating resin (Chelex 100 Resin, BIO RAD, USA) overnight at 4 °C], $CaCl_2$ 0.1mM, $NaHCO_3$ 1.2 mg/ml, HEPES 6.7 mg/ml, insulin 5 μg/ml, ethanolamine 5 mM, phosphorylethanolamine 5 mM, hydrocortisone 0.5 μg/ml, transferrin 10 μg/ml, EGF 10 ng/ml, bovine pituitary extract (Kyokuto, Japan) 40 μg/ml, fungizone 250 ng/ml, penicillin 5 U/ml, and streptomycin 5 μg/ml. When the cultured cells covered 50-70 % of the dish, they were subjected to further examinations. Control keratinocytes were incubated in the aforementioned medium including 0.1 mM calcium (low Ca^{2+}). To induce differentiation, keratinocytes were incubated for 2 days in medium with 1.0 mM calcium (high Ca^{2+}).

Immunostaining. Unfixed fresh gingival samples were cryosectioned at 6 μm for immunohistochemistry. Cultured cell samples on dishes were fixed in 4 % formaldehyde freshly prepared from paraformaldehyde in PBS for 10 min and treated with 1:1 acetone / ethanol for 10 min. Both samples were treated with 3 % H_2O_2 in PBS(−) for 10 min to quench endogenous peroxidase, then with 1:10 normal goat serum diluted in PBS(−) for 10 min to block nonspecific binding of antibodies. The primary antibodies, 1:10 Bcl-xL (B22630, Transduction Laboratories, USA), 1:100 Bax (06-499, Upstate biotechnology, USA), 1:20 cytokeratin (CK) 14 (LL002, Novocastra Laboratories Ltd., UK), 1:200 involucrin (Sy5, Novacastra Laboratories Ltd., UK), and 1:200 CK gp 56-kDa (KL-1, IMMUNOTECH, France) diluted in PBS(−) with 0.5 % goat serum albumin, were utilized. They were applied to specimens, which were incubated overnight at 4 °C. Negative control sections substituted PBS (−) for primary antibodies. Samples were washed in PBS(−), stained by the labelled streptavidin biotin method using DAKO-LSAB kit (DAKO, Denmark), and developed with diaminobenzidine (DAB) by Histofine SAB-PO (M) kit (Nichirei, Japan). These sections were lightly counterstained with hematoxylin.

TUNEL method. Apoptosis was analyzed *in situ* by the terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique using ApopTag (Oncor, Inc., Gaithersburg, MD), labeling 3′-OH DNA ends generated by DNA fragmentation (19). Cultured cells fixed in 4 % formaldehyde in PBS(−) were treated with 20 μg/ml proteinase K in PBS(−) for 10 min to digest protein, then treated with 3 % H_2O_2 in PBS(−) for 5 min to quench endogeneous peroxidase activity, and equilibrated. Terminal deoxynucleotidyl transferase (TdT) enzyme was applied to the sections, which were incubated at 37 °C for 1 hour, treated with stop/wash buffer, then exposed to anti-digoxigenin peroxidase, which was developed by DAB and counterstained with hematoxylin. After each steps, the sections were rinsed with PBS(−).

Detection of DNA-fragmentation pattern. For analysis of the 180-bp nucleosomal units characteristic DNA cleavage, DNA laddering was detected as follows (20). The cells were scraped from dishes and collected by centrifugation. The pellets were washed with PBS(−), and used for DNA extraction. The cells were incubated for 1 hour at 37 °C in 300 μl lysis buffer containing 0.5 mg/ml proteinase K, 0.5 mg/ml RNase, 1.0 % SDS, pH 7.2. Then DNA was purified using 25:24:1 phenol/chloroform/isoamyl alcohol and 24:1 chloroform/isoamyl alcohol, and dissolved in 10 μl TE buffer. DNA was electrophoresed on 2 % agarose gel in 0.5xTris-borate EDTA (TBE) at 100 V for approximately 40 min. The gel was stained by ethidium bromide or SYBR Green I (TaKaRa, Japan) to visualize DNA laddering and photographed with a Polaroid camera.

RESULTS

Expression of bcl-2 related proteins in gingival epithelium. Bcl-xL was expressed in almost all cells of the basal cell layer and the lower region of the spinous cell layer (Fig. 1a). Bax was expressed in many cells of the upper region of the spinous cell layer and of the granular cell layer (Fig. 1b).

Differential expression of Bcl-xL, Bax, cytokeratin, and involucrin under conditions of low Ca^{2+} and high Ca^{2+}. When cultured in the medium containing low Ca^{2+}, oral keratinocytes grew as a monolayer; they were small and polygonal (Fig. 2). Almost all cells ex-

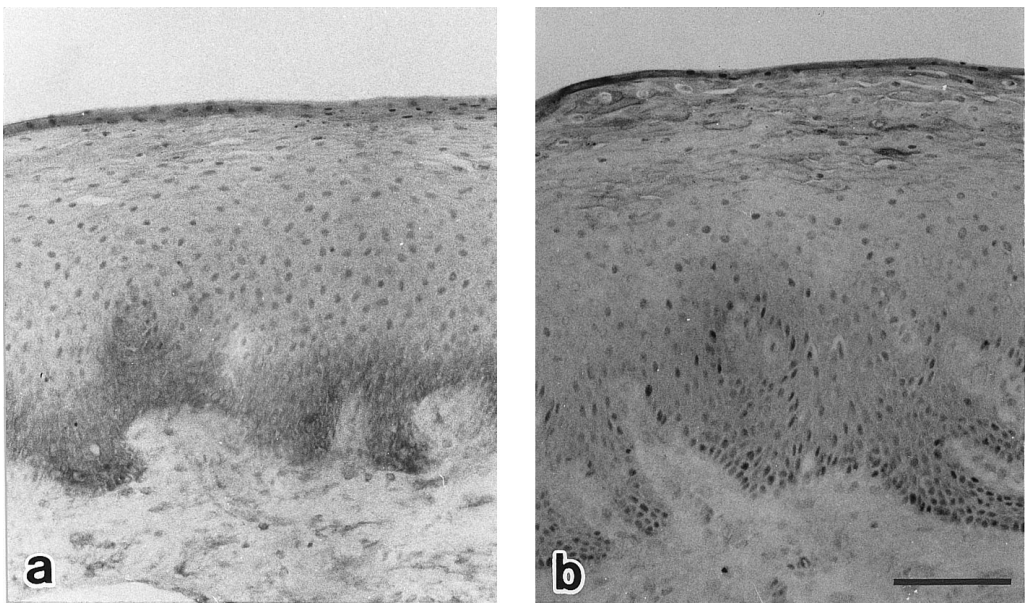

FIG. 1. Expression of Bcl-xL (a) and Bax (b) in gingival epithelium. Bar, 100 μm.

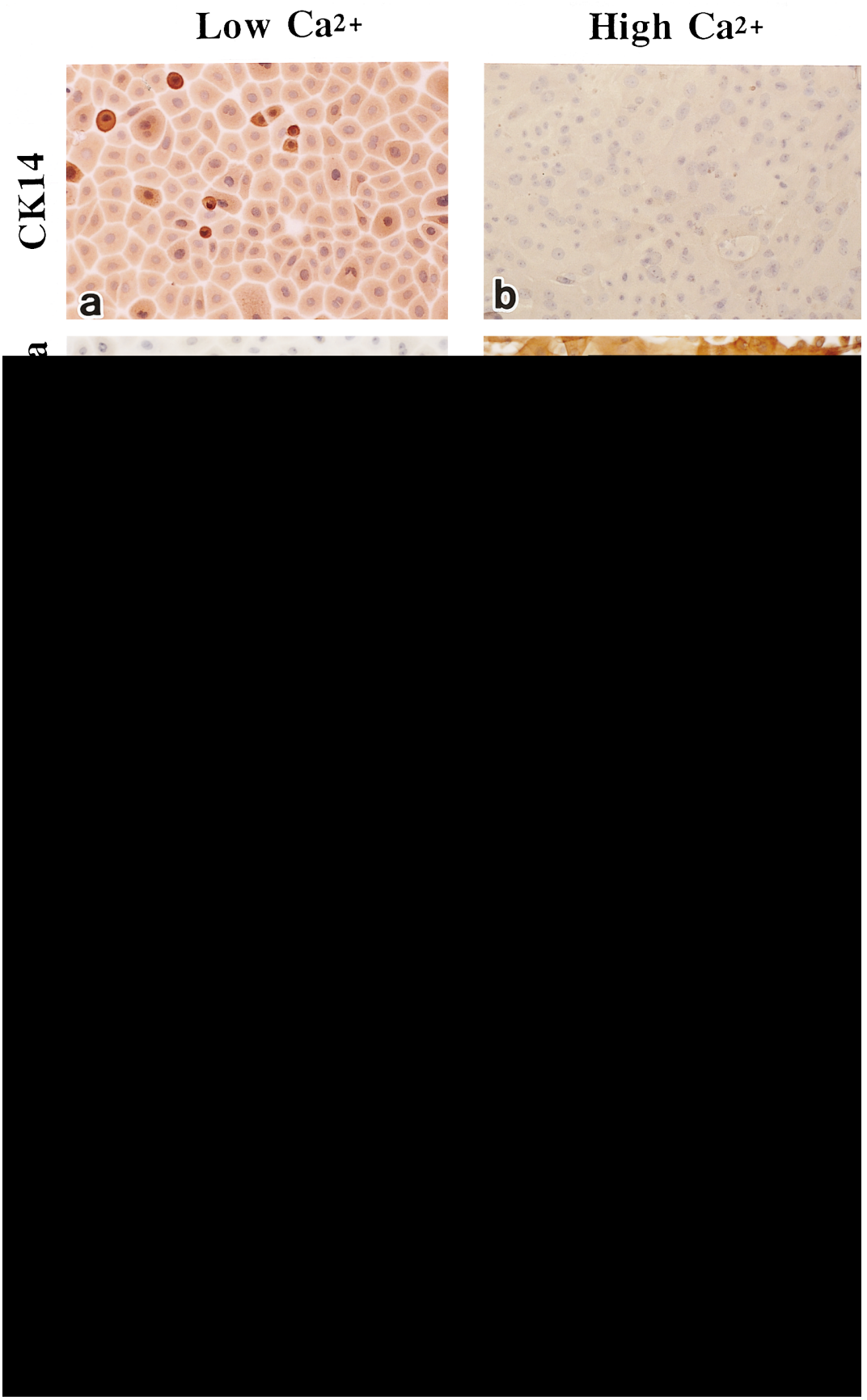

FIG. 2. Expression of CK14 (a, b), CK gp 56-kDa (c, d), involucrin (e, f), Bcl-xL (g, h), Bax (i, j) *in vitro* (original magnification, ×100). Left column is keratinocytes incubated with low Ca^{2+}, right column is keratinocytes incubated with high Ca^{2+} for 2 days.

pressed CK 14, a differentiation marker peculiar to the lowest spinous cell layer of normal gingiva, and Bcl-xL (Figs. 2a and 2g). In this culture, expression of CK gp 56-kDa, involucrin, and Bax was not observed (Figs. 2c, 2e, and 2i). Upon being switched to high Ca^{2+} solution and being incubated for 2 days, the culture showed large, flat, and squamous cells similar to differentiated keratinocytes *in vivo* (Fig. 2). When cultured for two days in the medium containing high Ca^{2+}, the cells expressed CK gp 56-kDa and involucrin, which are differentiation markers peculiar to the spinous and the granular cell layers (Figs. 2d and 2f). Simultaneously, Bax was expressed (Fig. 2j). In contrast, CK 14 and Bcl-xL disappeared (Figs. 2b and 2h).

Detection of apoptotic cells in cultured keratinocyte-induced differentiation. Beyond 5 days exposure to high Ca^{2+}, TUNEL positive cells appeared. (Fig. 3a). We analyzed DNA laddering of the positive cells by agarose gel electrophoresis to unambiguously identify apoptotic cells. Agarose gel electrophoresis of DNA extracted from these cells showed fragmentation into 180-bp nucleosomes (Fig. 3b, "High" lane). But in the condition of low Ca^{2+}, an apoptotic DNA-fragmentation pattern was not observed (Fig. 3b, "Low" lane).

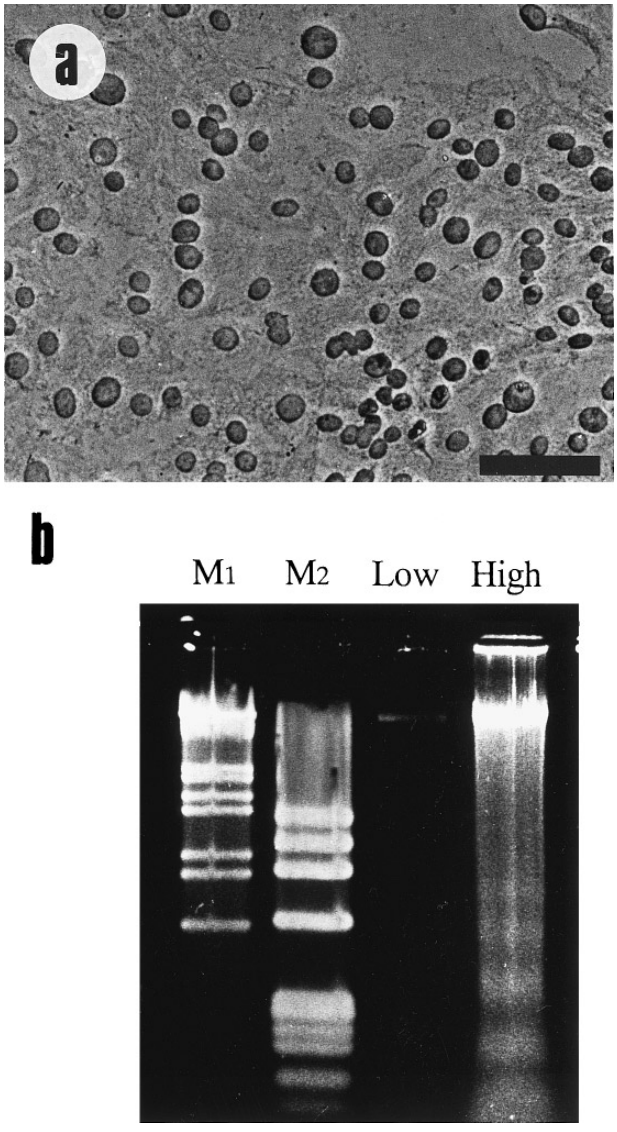

FIG. 3. Cultured keratinocytes induced to apoptosis by high Ca^{2+}. (a) Apoptotic cells detected by the TUNEL method (bar, 100 μm). These cells were incubated with high Ca^{2+} for 5 days. (b) Electrophoresis of DNA. Low = DNA from keratinocytes in low Ca^{2+}. High = DNA from keratinocytes in high Ca^{2+}. M1=λ/*Hin*d III•*Eco*R I double digest. M2=ϕX174/*Hae* III digest.

DISCUSSION

Differentiation of keratinocytes is regulated by many factors, i.e., paracrine or autocrine growth factors (21-27), cell adhesion molecules (28-30), and the microenvironment (31-33). The mode of cell death at the end of terminal differentiation of gingival epithelium was apoptosis, which was shown by the TUNEL method and analysis of DNA fragmentation (5). But the precise relationship between apoptosis and the process of differentiation has not been well defined. In present study, bcl-2 related proteins, namely, Bcl-xL and Bax, were analyzed in gingival epithelium and in cultured keratinocytes. Bcl-2 was expressed in the basal layer (5) and Bcl-xL were expressed in the basal and lower spinous layer in gingival epithelium, both suppressing factors of apoptosis. In contrast, Bax, an inducing factor of apoptosis, was expressed in the upper layer of epithelium. These data suggest that bcl-2 related proteins are expressed in a manner correlated with the progress of differentiation in gingival epithelium. To examine the relationship between differentiation and bcl-2 related proteins, we used an *in vitro* differentiation model. In this model, keratinocytes adopt a basal or differentiated cell phenotype depending on the concentration of calcium in the medium (17, 27). Keratinocytes in low Ca^{2+} solution morphologically resemble basal cells of gingiva, proliferate rapidly, and express differentiation markers peculiar to the innermost cells of gingival epithelium, for example CK 14, but do not express differentiation markers peculiar to the suprabasal cell layer. On the other hand, keratinocytes incubated in high Ca^{2+} solution are differentiated morphologically and express only differentiation markers peculiar to the suprabasal cell layer, for example CK 10, involucrin, and cornified envelope precursor (17). Therefore, the state of cells in this model were considered similar to that of differentiation of gingival epithelium *in vivo*. In addition, we showed that differentiated keratinocytes in this model ended in apoptosis as *in vivo*.

Bcl-2 was not detectable in cultured keratinocytes under both low and high Ca^{2+} conditions (data not

shown). It is unknown why Bcl-2 was expressed only *in vivo*, but not *in vitro*. In epithelial tissues, Bcl-2 is predominantly expressed in undifferentiated and long-lived cells (34), and is considered as a stem cell marker (35). Stem cells are defined as having the capacity both to self-renew and to generate differentiated progeny, and as having a long lifetime. In contrast, almost all normal keratinocytes *in vitro* are committed to terminal differentiation under the proper conditions, and their lifetime is limited. The keratinocytes *in vitro* might not have the character of stem cells. Bcl-xL was expressed in keratinocytes in low Ca^{2+} simultaneously with a differentiation marker peculiar to less differentiated keratinocytes. Keratinocytes from which Bcl-xL has disappeared because of their exposure to high Ca^{2+} are still capable of differentiation. Bax was not detectable in keratinocytes under low Ca^{2+} conditions, simultaneous with the absence of differentiated keratinocytes markers. But Bax was expressed with differentiated keratinocytes markers in keratinocytes under high Ca^{2+}. These data suggest that expression of bcl-2-related proteins is correlated with differentiation of keratinocytes; terminal differentiation of keratinocytes was accompanied by a decrease of Bcl-xL and an increase of Bax, as well as by the progress of apoptosis. The expression pattern of Bax both *in vivo* and *in vitro* echoes the differentiated condition. The same holds true for the expression of Bcl-xL. Indeed, keratinocytes in which differentiation was suppressed by low Ca^{2+} expressed a suppressing factor for apoptosis, Bcl-xL; in contrast, keratinocytes induced to differentiate by high Ca^{2+} expressed an inducing factor for apoptosis, Bax. These findings support the notion that some apoptosis regulating factors relate not only to apoptosis but also to differentiation, and are expressed according to the progress of differentiation.

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