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Short Communication

Discordant Expression of Bcl-x and Bcl-2 by Keratinocytes *in Vitro* and Psoriatic Keratinocytes *in Vivo*

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Apoptosis is a required event in maintaining kinetic homeostasis within continually renewing tissues such as skin. However, no systematic study of the apoptotic process in epidermal keratinocytes of the skin has been performed. In this report, we examined the expression of proteins associated with promoting (Fas) or preventing (Bcl-2, Bcl-x, CD40) apoptosis in the normal, psoriatic, and malignant keratinocyte. Immunohistochemical staining and flow cytometry analysis revealed that normal cultured keratinocytes express low levels of Fas, CD40, and Bcl-x that was enhanced by cytokines including γ-interferon (IFN-γ) and a phorbol ester tumor promoter, TPA. Only faint Bcl-2 staining was detected in cultured keratinocytes exposed to IFN-γ and TPA compared with the prominent expression of Bcl-x. Biopsies of normal skin, psoriatic plaques, and basal cell carcinomas were examined to extend the in vitro observations. Immunohistochemical staining revealed that while keratinocytes in normal epithelium express low to absent levels of Fas and Bcl-x, psoriatic keratinocytes expressed significantly higher levels of Fas and Bcl-x. In contrast, malignant keratinocytes in basal cell carcinomas expressed high levels of Bcl-2, but minimal Bcl-x, and no Fas. Immunoblot analysis revealed that the long form of Bcl-x ($Bcl\text{-}x_L$), which prevents apoptosis in lymphocytes, is expressed by cultured keratinocytes and psoriatic plaque keratinocytes. We conclude that normal cytokine-activated keratinocytes can express an apoptotic (Fas) and an anti-apoptotic protein (Bcl-x). The overexpression of Bcl-x in psoriasis, or Bcl-2 in basal cell carcinomas, may contribute to the longevity of these cells by blocking the normal apoptotic process involved in the terminal differentiation program of epidermal keratinocytes. (Am J Pathol 1995, 146:1079–1088)

In skin, as in every other organ, cell proliferation and cell death are coordinated in a fashion that promotes normal development and maintenance of homeostasis. The epidermal compartment consists primarily of proliferating keratinocytes that typically follow a highly regulated program of terminal differentiation, and ultimately cell death, thereby producing a nonviable stratum corneum that serves as the principal barrier function for skin. In noncutaneous systems such as hematopoietic and neuronal based organ systems, much has been recently learned about the role for members of the Bcl-2 family in regulating cell proliferation and apoptosis.[1] The identity of molecular mediators that regulate keratinocyte survival and cell death are largely unknown, although some preliminary evidence has suggested that keratinocytes lo-

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cated in the basal cell layer express Bcl-2, and cultured keratinocytes express the Fas antigen.[2–4] Unlike Bcl-2 and other members of this family, such as Bcl-x,[5] which promote cell survival and prevent apoptosis, Fas antigen is linked to induction of apoptosis.[6] The Fas antigen itself belongs to a family of a nerve growth factor/tumor necrosis factor (TNF) receptor superfamily,[7] which includes other cell surface molecules that either promote (p55 TNF-α receptor) or prevent (CD40) apoptosis. Keratinocytes are known to express p55 TNF-α receptor,[8] but whether they can express CD40 was not previously studied.

In this report, we examine the relative levels and patterns of expression of Bcl-2, Bcl-x, Fas antigen, p55 TNF-α receptor, and CD40. These patterns were correlated with detection of apoptosis at the cellular level in serial sections of the tissue samples with particular emphasis on psoriasis and basal cell carcinomas, using a sensitive immunohistochemical staining procedure.[9] We measured simultaneously this group of proteins involved in either the promotion or prevention of apoptosis not only to acquire as comprehensive a profile as possible, but also because there may be important interactions between various molecules within this group such as have been observed for Bcl-2-inhibiting Fas-mediated apoptosis in lymphoid cells.[10]

Materials and Methods

Cell Culture

Human adult keratinocytes were obtained from keratome samples of buttock skin after informed consent and approval of the University of Michigan Human Subjects Committee. Multipassaged (passages 3 to 5) keratinocytes were grown in a low-calcium, serum-free culture medium (KGM, Clonetics Corp., San Diego, CA) in plastic petri dishes (Corning, Inc., Corning, NY), and maintained at 37 C in a humidified atmosphere containing 5% CO_2 as previously described.[11] For some experiments, keratinocytes were grown in eight-well Lab Tek chambers (Nunc Inc., Naperville, IL). Keratinocytes were treated with either KGM alone or KGM containing various treatments for 48 hours, including recombinant γ-interferon (IFN-γ, 500 U/ml, Genentech, Inc., So. San Francisco, CA), 12-*O*-tetradecanoyl-phorbol-13 acetate (TPA, 5 nmol/L, Sigma Chemical Corp., St. Louis, MO), tumor necrosis factor-α (TNF-α, 1000 U/ml, Genentech, Inc.), or a combination of IFN-γ plus TPA.

Human Skin Samples

Skin biopsies were obtained after informed consent from the normal adult volunteers without any skin disease (N = 7), as well as from the advancing margin of patients with active, untreated psoriatic plaques (N = 10), and from patients with basal cell carcinomas (N = 7). These fresh tissue samples were immediately snap frozen by being placed into liquid nitrogen-chilled isopentane and stored at −80 C.

Immunoperoxidase and Immunofluorescence Staining

Five-micron thick cryostat tissue sections and cultured keratinocyte monolayers were acetone fixed and immunostained to detect a variety of antigens using a highly sensitive avidin-biotin immunoperoxidase technique (Vectastain Kit, Vector Labs, Burlingame CA), as previously described.[11] The panel of reagents used is summarized in Table 1. The rabbit polyclonal Bcl-x antisera have been extensively characterized using a series of stable cell transfectants as previously described.[5] This Bcl-x antiserum is specific for Bcl-x, as it strongly reacts with Bcl-x transfectants, but not Bcl-2 or Bax transfectants (data not shown), and specifically immunoprecipitates Bcl-x (see Figure 4). Single-cell suspensions of keratinocytes were prepared for fluorescence-activated cell sorter analysis using a brief (5 minutes; 37 C) exposure to 0.01% trypsin, 0.03% ethylenediaminetetraacetic acid as previously described.[12] After this mild trypsinization procedure, cells were washed with PBS/5% heat-inactivated FCS/0.02% sodium azide, and exposed to the unconjugated primary antibodies

Table 1. *Antibodies Used in Immunoperoxidase and Immunofluorescence Staining*

Antigen	Antibodies (Isotype)	Source*
Bcl-2	124 (mouse IgG1)	DAKO
Bcl-x	Rabbit polyclonal	Gottschalk, et al[16]
CD3	Leu-4 (mouse IgG1)	Becton-Dickinson
CD40	5C3 (mouse IgG1)	Pharmingen
CD54/ICAM-1	RR 1/1 (mouse IgG1)	Genzyme
TNF-α receptor	p55 subunit (mouse IgG1)	Genzyme
CD95/Fas	DX2 (mouse IgG1)	Pharmingen

*Becton-Dickinson, San Jose, CA; DAKO, Carpinteria, CA; Genzyme Corp., Cambridge, MA; Pharmingen, San Diego, CA.

(Bcl-2, Bcl-x, CD3, ICAM-1, TNF-α receptor) or fluorescein isothiocyanate (FITC)-conjugated antibodies (CD40, Fas) on ice for 30 minutes. After two washes, cells were exposed to an FITC-conjugated goat anti-mouse IgG or phycoerythrin PE-conjugated goat anti-rabbit IgG secondary antibody (Tago Inc., Burlingame, CA) followed by additional wash cycles. To detect cytoplasmic expression of Bcl-2 or Bcl-x, cells were permeabilized with 0.3% saponin during antibody incubations.[5] Saponin-treated cells stained only with goat anti-mouse IgG-FITC, or normal rabbit serum plus goat anti-rabbit IgG-PE were used as negative controls for Bcl-2 and Bcl-x staining, respectively. Cells stained with anti-CD3 were used as negative controls for Fas, CD40, and ICAM-1 staining. To detect DNA strand breaks, which are associated with the apoptotic response, an *in situ* apoptosis peroxidase detection kit,[9] based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay, was used (Apoptag, Oncor, Gaithersburg, MD). To detect apoptosis in keratinocytes within cryostat section of normal and psoriatic epidermis a fluorescein Apoptag kit (S7110, Oncor) was used that included propidium iodide to highlight nuclear morphology. Using this double-staining protocol with epi-illumination microscopy and appropriate filters, we established that cells undergoing apoptosis have nuclei that contain DNA strand breaks, as well as nuclear chromatin that is condensed, clumped, or fragmented (data not shown).

Immunoblot Analysis of Bcl-x and Bcl-2 Proteins

Triton-soluble fractions of cultured keratinocytes or keratomes were prepared as described.[13] Lysates of murine FL5.12 cells individually transfected with plasmids containing *bcl-x*$_S$ or *bcl-x*$_L$ were used as positive controls for Bcl-x expression.[14] 100 µg of each protein sample was electrophoresed under reducing conditions through a 15% (see Figure 4, top) or 12% (see Figure 4, bottom) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE),[15] and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Staining for Bcl-x was performed using a rabbit polyclonal Bcl-x antibody[16] followed by goat anti-rabbit IgG-peroxidase (Jackson Immunoresearch, West Grove, PA). Protein expression was detected by a chemiluminescent detection system (ECL Kit, Amersham, Arlington Heights, IL).

Results

Immunohistochemical staining of confluent monolayers of cultured keratinocytes revealed no detectable constitutive expression of Bcl-2 (Figure 1A), but moderate levels of Bcl-x (Figure 1B). This Bcl-x expression was predominately cytoplasmic and perinuclear, and was present in >95% of keratinocytes. There was weak cytoplasmic Fas expression, but no plasma membrane positivity in the unstimulated keratinocytes (Figure 1C). Less than 1% of the cultured keratinocytes constitutively expressed ICAM-1 (Figure 1D). After exposure to IFN-γ plus TPA, only faint and focal cytoplasmic staining for Bcl-2 was observed (Figure 1E). This combination was selected based on our past experience in which levels of various cytokine-inducible proteins were synergistically upregulated by IFN-γ plus TPA in keratinocytes including ICAM-1 and BB1/B7–3.[12] In contrast to the Bcl-2 results, there was marked enhancement after IFN-γ-plus-TPA treatment of keratinocytes for cytoplasmic expression of Bcl-x (Figure 1F) and plasma membrane-associated expression of Fas (Figure 1G) and ICAM-1 (Figure 1H). There were strong constitutive levels of expression of the p55 subunit of the TNF-α receptor, which was not significantly enhanced by exposure to IFN-γ plus TPA (data not shown).

To quantitate these results, we performed flow cytometry with and without permeabilization to measure constitutive and inducible levels of these proteins. Figure 2 represents a summary of composite profiles for untreated and IFN-γ-treated, as well as IFN-γ plus TPA-treated keratinocytes. These flow cytometry profiles confirmed and extended the immunostaining results of intact monolayers. A summary of the fluorometric results is provided in Table 2. The treatment regimens included KGM alone or KGM plus either IFN-γ, TPA, IFN-γ plus TPA, or TNF-α. As can be seen in Figure 2 and Table 2, there was little to no detectable Bcl-2 expression by keratinocytes under any of the culture conditions. However, untreated (KGM alone) keratinocytes had consistently detectable levels of Bcl-x, which were marginally increased after exposure of the keratinocytes to either IFN-γ or TPA alone, and markedly increased by IFN-γ plus TPA (approximately ninefold) but not by TNF-α (Table 2). Fas antigen was constitutively expressed at low levels on the plasma membrane and was only marginally increased by IFN-γ, compared with no effect by TPA or TNF-α. A twofold increase in mean channel fluorescence for Fas was produced by the IFN-γ-plus-TPA treatment. ICAM-1 was clearly inducible and expressed at low levels after either TPA or TNF-α

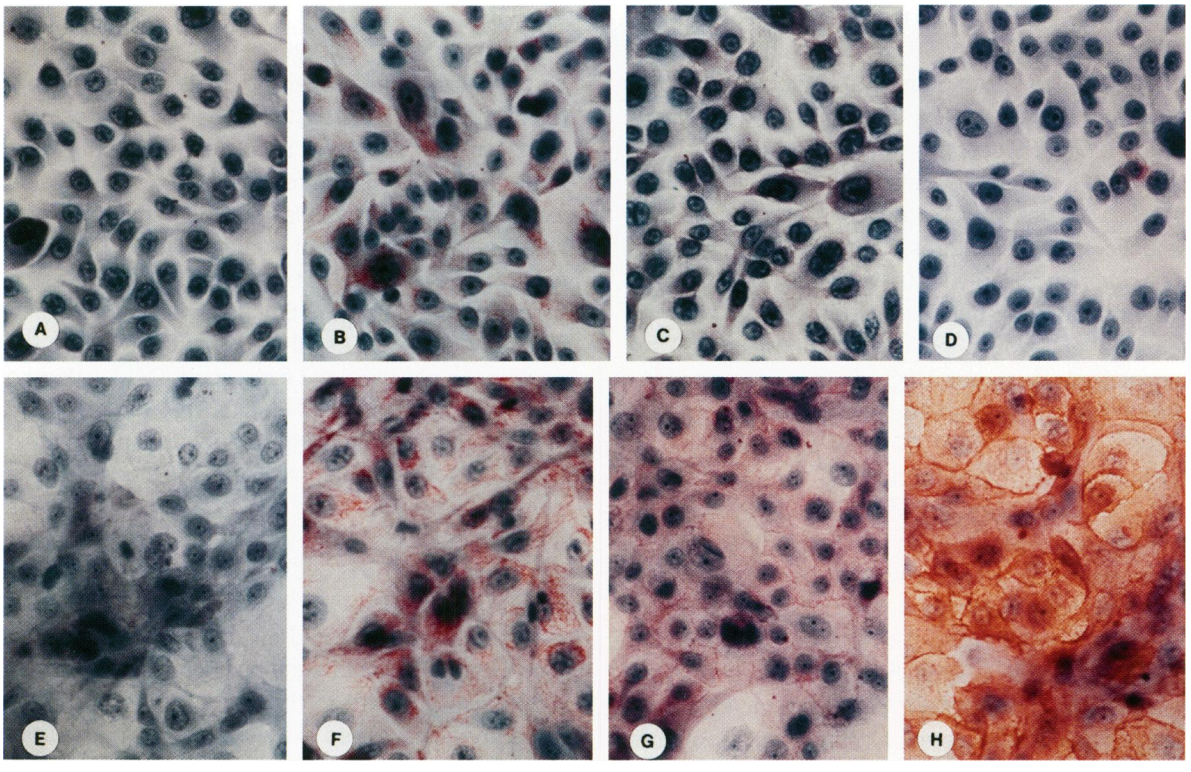

Figure 1. *Intact cultured keratinocyte monolayers constitutively express Bcl-x but not Bcl-2 immunoreactivity. The upper panels* (A *to* D) *are keratinocytes grown in KGM alone, whereas the lower panels* (E *to* H) *are keratinocytes grown in KGM containing IFN-γ plus TPA* (*48 hours*). *The following antigens were examined in unstimulated and stimulated cultures: Bcl-2* (A, E); *Bcl-x* (B, F); *Fas* (C, G); *and ICAM-1* (D, H). *After exposure to IFN-γ plus TPA treatment, there is enhanced expression of Bcl-x and induction of Fas and ICAM-1, but not Bcl-2. Immunoperoxidase stain,* ×*150.*

exposure, and strongly expressed after IFN-γ treatment. Note the synergy of ICAM-1 induction by the combination of IFN-γ plus TPA. There were no significant CD40 levels constitutively expressed by keratinocytes or after stimulation with TNF-α (and only slight increase after TPA), but after IFN-γ or IFN-γ-plus-TPA, treatment there was detection of CD40 expression.

Biopsies of normal skin, basal cell carcinomas, and psoriatic plaques were chosen to extend our *in vitro* studies. These *in vivo* samples were selected to provide some clinical relevance to the *in vitro* studies (particularly related to IFN-γ and TPA) because phorbol esters are known to produce skin cancer after epicutaneous exposure, and psoriasis is associated with elevated IFN-γ levels.[17] Because we noted expression of Bcl-x in cultured keratinocytes, the tissue samples were first examined for this protein. Figure 3A demonstrates only relatively weak and focal expression of Bcl-x by superficially located keratinocytes near the stratum corneum. No other epidermal or dermal cell type was positive for Bcl-x. In basal cell carcinomas, the budding basaloid tumor cells projecting from the lower epidermis had low to moderate expression, but focal areas were positive for Bcl-x, as were the overlying epidermis (Figure 3B). Within the abrupt budding of tumor cells from the lower epidermis, heterogeneity among tumor cells for Bcl-x can be seen, with less staining of the tumor cells on the left compared with the infiltrating tumor cells on the right of Figure 3B. In contrast, psoriatic plaques had strong and diffuse keratinocyte expression of Bcl-x with marked perinuclear accentuation (Figure 3C).

Normal skin stained for Bcl-2 revealed only melanocyte reactivity along the basement membrane zone, in agreement with a previous report[2] (data not shown). In basal cell carcinomas, the overlying benign epidermis also contained Bcl-2-positive melanocytes, but the malignant basaloid tumor cell islands were strongly and diffusely positive for Bcl-2 (Figure 3, D and E). Virtually every tumor cell was Bcl-2-positive, as were scattered dermal lymphocytes between tumor cell islands. In psoriatic plaques, epidermal hyperplastic keratinocytes were Bcl-2-negative, but melanocytes and infiltrating lymphocytes were occasionally Bcl-2-positive (Figure 3F).

In normal skin, there was no significant Fas expression (data not shown), but in the epidermis overlying basal cell carcinomas, there was focal and intense

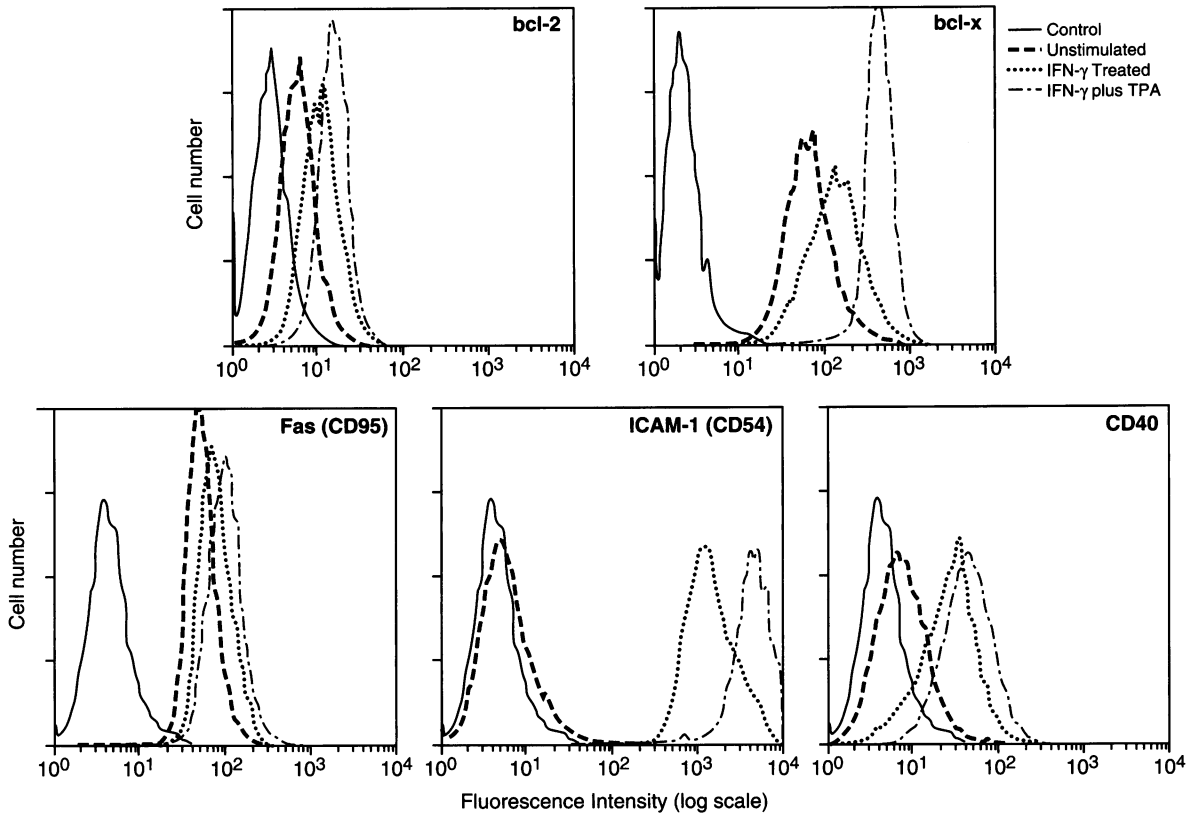

Figure 2. *Composite flow cytometry profiles for untreated (ie, unstimulated), IFN-γ treated, and IFN-γ plus TPA-activated keratinocytes demonstrating Bcl-x but no Bcl-2 expression, and induction of Fas, CD40, and ICAM-1 on the cell surface of keratinocytes* in vitro.

Table 2. *Summary of Flow Cytometry Results Using Normal Adult Cultured Keratinocytes**

Antigens	Untreated	IFN-γ	TPA	IFN-γ + TPA	TNF-α
Bcl-2	0.614 (0.284)†	1.08 (0.391)	0.855 (0.473)	1.59 (0.544)	0.689 (0.343)
Bcl-x	6.07 (0.230)	13.1 (0.287)	17.2 (0.377)	45.0 (0.644)	7.15 (0.237)
Fas	5.34 (0.468)	7.65 (0.563)	5.71 (0.830)	10.0 (1.05)	5.92 (0.494)
ICAM-1	0.616 (0.468)	137.1 (0.563)	9.71 (0.830)	369.7 (1.05)	5.09 (0.494)
CD40	0.736 (0.468)	2.43 (0.563)	1.76 (0.830)	4.3 (1.05)	0.957 (0.494)

*Mean channel fluorescence values (log scale).
†Values in parentheses, mean channel fluorescence values of negative control reactions.

keratinocyte expression on the plasma membranes producing a "chicken-wire" appearance (Figures 3G and 3H). There was absolutely no Fas expressed by any of the basaloid tumor cells, which was exactly the same pattern (ie, positive overlying benign keratinocytes and negative tumor cells), seen by ICAM-1 in this study, as well as for human leukocyte antigen-DR as previously reported.[18] Scattered peritumoral lymphocytes were also Fas-positive (Figure 3H). In psoriatic plaques, there was diffuse and strong keratinocyte Fas expression on the plasma membranes (Figure 3I). No keratinocyte CD40 expression was identified in normal skin, and only weak to moderate predominately cytoplasmic immunoreactivity was present on keratinocytes within psoriatic plaques or in tumor cell islands of basal cell carcinomas. Adjacent epidermal and dermal dendritic cells were strongly CD40-positive (data not shown).

To quantitate the levels of Bcl-2 and Bcl-x as well as to determine whether the long or short form of Bcl-x was being expressed by keratinocytes *in vitro* and *in vivo*, immunoblot analysis was performed. Figure 4A (top) revealed that cultured keratinocytes produce only Bcl-x_L, but not Bcl-x_S. In agreement with the flow cytometry results, cytokine activation of the keratinocytes enhanced Bcl-x_L production over untreated keratinocyte cultures. The activation treatments that increased Bcl-x_L production included IFN-γ, IFN-γ plus TPA, TPA, and TNF-α. Bcl-x_L was barely detectable in normal skin, but in psoriatic keratome samples there

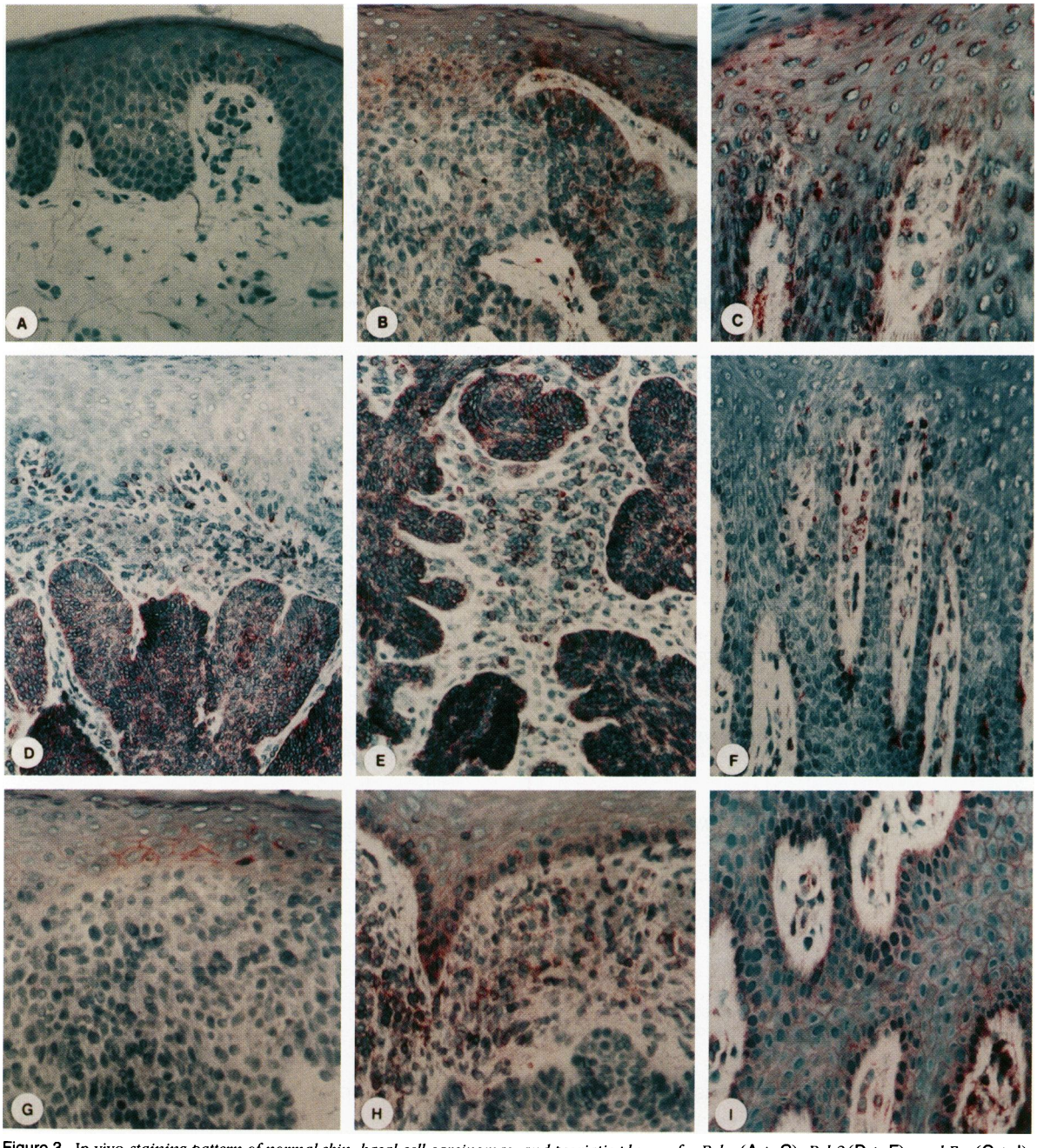

Figure 3. In vivo *staining pattern of normal skin, basal cell carcinomas, and psoriatic plaques for Bcl-x* (**A** *to* **C**), *Bcl-2* (**D** *to* **F**), *and Fas* (**G** *to* **I**). *Compared with normal skin* (**A**), *the epidermis immediately overlying the infiltrating basal cell carcinoma tumor cells* (*which are connected to the lower epidermis*), *as well as the invasive tumor cells, express more readily detectable levels of Bcl-x* (**B**). *Note that there is some heterogeneity in expression of Bcl-x by the invasive tumor cells projecting downward from the epidermis* (**B**). *However, strong and diffuse Bcl-x expression in the perinuclear zone of keratinocytes is present throughout all layers of the epidermis in psoriatic plaques* (**C**). *In basal cell carcinomas, the invasive tumor cells seen in* (**D**) *below the epidermis are strongly and diffusely positive for Bcl-2. Only melanocytes in the lower level of the overlying epidermis express Bcl-2, as do the infiltrating lymphocytes in the dermis seen at higher magnification in* (**E**). *Within psoriatic plaques, the keratinocytes do not express Bcl-2, but the melanocytes and dermal lymphocytes are Bcl-2-positive* (**F**). *Basal cell carcinoma tumor cells budding from the lower epidermis are not expressing Fas, but the adjacent* (**G**) *and overlying normal epidermis* (**H**) *have Fas-positive keratinocytes. The dermal lymphocytes also include a subset of cells positive for Fas* (**H**). *In psoriatic plaques, there is strong and diffuse keratinocyte Fas expression highlighting the plasma membrane zones* (**I**).

was considerably more Bcl-x_L present in the hyperplastic keratinocytes (Figure 4, bottom). Using the same immunoblot assays, no Bcl-2 was consistently detected in any of the keratinocyte cultures (data not shown). Similarly, in agreement with the immunohistochemistry results, only low levels of Bcl-2 were detected in normal and psoriatic keratome samples by Western blot analysis (data not shown).

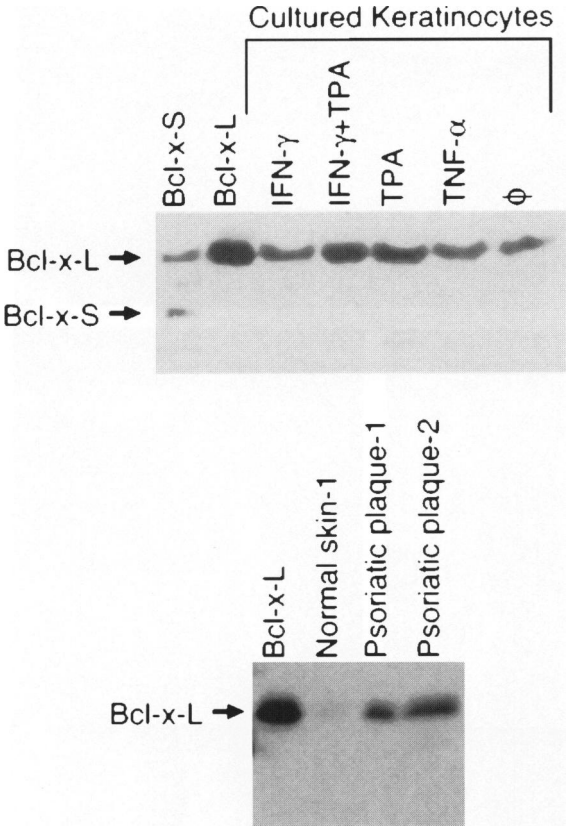

Figure 4. *Immunoblot analysis of Bcl-x and Bcl-2 proteins. The upper panel reveals that cultured human keratinocytes only express Bcl-x_L, but no Bcl-x_S, and that compared with untreated keratinocytes, exposure to various cytokines and/or TPA enhances expression of Bcl-x_L. The control lanes before the keratinocytes demonstrate the endogenous levels of Bcl-x_L produced by the cell line before and after transfection with either Bcl-x_S (first lane) or Bcl-x_L (second lane). The lower panel reveals that compared with the barely detectable levels of Bcl-x_L in normal skin, psoriatic plaques containing keratinocytes markedly overexpress this protein. The first lane in this panel reveals the same Bcl-x_L-transfected cell line as described above.*

Because the aforementioned proteins either promote or prevent apoptosis we were interested in correlating these *in vivo* staining patterns with detection of cells that were presumably undergoing apoptosis. To accomplish this goal we used the TUNEL assay, which is a highly sensitive immunostaining procedure designed to detect single strand 3′-OH DNA ends produced by DNA fragmentation and typically localized to cells undergoing programmed cell death.[9] The localization results of such single-strand DNA breaks in the nucleus are summarized in Figure 5. In normal epidermis, occasional nuclei were positive by the TUNEL assay in the most superficial levels (stratum granulosum), where terminal differentiation immediately precedes formation of the nonviable stratum corneum (Figure 5, A and B). In contrast, within psoriatic plaques keratinocytes throughout all layers were abundantly positive by the TUNEL assay (Figure 5, C and D). The control reaction, where TdT is absent, revealed no positive nuclei in psoriatic plaques (Figure 5E). When the normal skin and psoriatic plaques were examined after propidium iodide staining, there was no evidence of apoptosis in the keratinocytes, as the labeled nuclear chromatin was evenly dispersed without any condensation, clumping, or fragmentation (data not shown). In basal cell carcinomas, the invasive tumor cell islands were completely devoid of nuclear reactivity using the TUNEL assay, although scattered lymphocytes immediately adjacent to the tumor cell islands were positive (Figure 5, F, G, and H).

Discussion

Using antibodies directed at Bcl-x and Bcl-2, immunohistochemical analysis revealed markedly discordant patterns of expression by keratinocytes both *in vitro* and *in vivo*. Cultured keratinocytes express almost exclusively the long form of Bcl-x, with no short form of Bcl-x detectable by immunoblotting, and only weak and barely detectable Bcl-2. Moreover, the levels of Bcl-x expressed by keratinocytes *in vitro* could be modulated by various stimuli including a synergistic enhancement by IFN-γ plus TPA. We believe these *in vitro* results are relevant *in vivo*, because in psoriatic plaques the hyperkinetic keratinocytes have been linked to IFN-γ exposure and activation of protein kinase C,[17] and there was extensive keratinocyte Bcl-x, but no Bcl-2 expression. The discordance in patterns of expression for Bcl-2/Bcl-x also apparently extends to other normal cell types in the skin besides keratinocytes, as melanocytes were prominently immunoreactive for Bcl-2, but not Bcl-x. Returning to psoriasis, the markedly overexpressed long form of Bcl-x may be of particular pathophysiological significance.

In psoriasis, research efforts have previously focused primarily on various keratinocyte mitogens such as transforming growth factor-α interacting with the epidermal growth factor receptor to try to explain the marked thickening of the epidermis.[17] However, another hypothesis emerges from our current findings that implicates overexpression of Bcl-x, which by blocking apoptosis enhances the longevity of the keratinocyte, thereby potentially promoting an alternate pathway of differentiation,[19] and concomitant overpopulation of the epidermal compartment. To further investigate this hypothesis, it was necessary to determine what role apoptosis was playing in the normal homeostatic regulation of epidermal keratinocyte growth and differentiation. The TUNEL assays revealed that in normal skin, upper-level keratinocytes

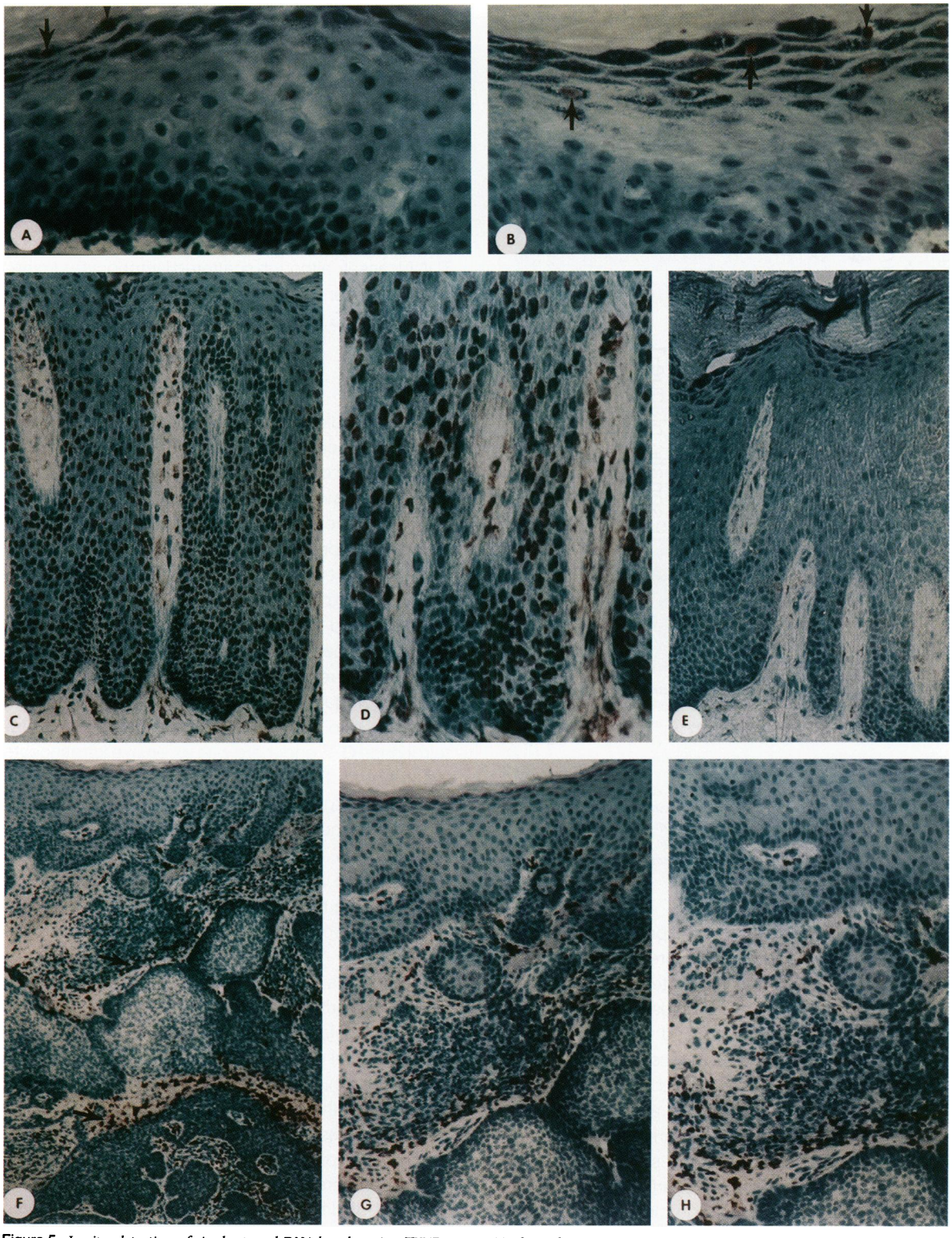

Figure 5. In situ *detection of single-strand DNA breaks using TUNEL assay. To detect keratinocytes with DNA fragmentation, the TUNEL assay was employed and the nuclei of cells examined for immunoreactivity. In normal skin superficial keratinocytes in the granular cell layer were positive* (**A** *and* **B**, *arrows*). *In psoriasis, there were innumerable positive keratinocytes at all levels of the epidermis* (**C** *and* **D**). *The negative control* (*absence of TdT*) *revealed no positive labeling of keratinocyte nuclei* (**E**). *In basal cell carcinomas, the infiltrating tumor cells were uniformly negative, although peritumoral lymphocytes were focally positive* (**F, G,** *and* **H**). *Note: TUNEL positive cells were visualized with the peroxidase-based detection method producing a dark brown reaction product.*

appeared to follow an apoptotic pathway as revealed by the presence of detectable DNA strand breaks in the nuclear chromatin.[9] Surprisingly, when active psoriatic plaques were examined, high levels of DNA strand breaks were detected throughout the epidermis; however, there was no cytological evidence at any level of the thickened epidermis of keratinocytes undergoing apoptosis. In psoriasis, increased keratinocyte proliferation has been associated with elevated proto-oncogene expression,[20] and in other cell systems, delivery of cell activation signals via certain oncogenes such as c-*myc* can simultaneously stimulate proliferation and apoptosis.[21] One possible explanation for the discrepancy between the TUNEL assay and the lack of cytopathic changes in the epidermis is that the overabundance of Bcl-x within the psoriatic keratinocytes suppresses the ability of cells that sustain DNA strand breaks to complete the complex, multistep process of apoptosis. An additional explanation for the striking TUNEL results in psoriasis is that there may be extensive labeling of 3′OH ends related to the high level of proliferation in which actively replicating DNA is more abundant or accessible to such detection methods. Using the propidium iodide stain, no psoriatic keratinocytes appeared to be undergoing apoptosis, because the nuclear chromatin was evenly distributed and not condensed, clumped, or fragmented. It is unclear at present whether overexpression of Bcl-x in psoriasis is a primary pathological event, or represents an aberrant physiological response secondary to locally produced cytokines or protein kinase C-agonists. To distinguish between these two possibilities, work is underway to determine whether the Bcl-x expressed by psoriatic keratinocytes contains a mutation, or whether there is linkage disequilibrium in families that have an inheritance pattern of psoriasis. Another surprising *in vivo* finding related to psoriasis was the high level of Fas expression, even though no evidence of cytotoxicity or apoptosis is observed in psoriasis.

Fas-mediated cytotoxicity requires that the target cell express Fas antigen and that the effector lymphocyte express the Fas ligand, a type II transmembrane protein that belongs to the TNF-α family.[22] The relatively lower levels of Fas antigen detected by flow cytometry of freshly trypsinized cell suspensions (Figure 2, Table 2), compared with expression of intact keratinocyte monolayers (Figure 1, C and G), may reflect the sensitivity of the Fas antigen on the keratinocyte surface to the trypsinization used to prepare the single-cell suspensions.[4] It is possible the lack of cytotoxicity seen in psoriatic lesions in which there is prominent keratinocyte expression of Fas is that the infiltrating activated T cells do not belong to an effector subset bearing Fas ligand. Unfortunately, because reagents for human Fas ligand are not currently available, we cannot directly test this possibility at the moment. Given that Bcl-2 inhibits Fas-mediated apoptosis in other experimental systems,[10] perhaps high levels of Bcl-x expression block Fas-mediated apoptosis in psoriatic keratinocytes. Moving from Fas to CD40, cytokine-activated culture keratinocytes were found to upregulate CD40 in a manner similar to that in a previous report using cultured thymic epithelial cells.[23] However, *in vivo* the strongest expression of CD40 in the diseased skin samples was observed decorating the dendritic cells, rather than the keratinocytes. In thymic tissue samples, weak to moderate epithelial cell CD40 expression was also accompanied by more intense CD40 expression on surrounding dendritic cell populations.[23] Thus, the functional significance of CD40 expressed by the proliferating keratinocytes in psoriasis and basal cell carcinomas remains to be determined. Comparing the benign hyperplasia of keratinocytes *in vivo* as seen in psoriasis with the malignant proliferation of keratinocytes in basal cell carcinomas, several other noteworthy observations were made.

First, in contrast to psoriasis in which there was no keratinocyte Bcl-2 expression,[24] the basaloid tumor cells strongly and diffusely expressed Bcl-2. This prominent Bcl-2 expression was accompanied by a total absence of Fas expression by the tumor cells. Thus, the tumor cells have apparently utilized two molecular pathways to ensure their viability and protection from T cell-mediated cytotoxicity including high levels of expression of the anti-apoptotic protein, Bcl-2, and no expression of Fas, preventing an effective cytotoxic T cell-mediated event. The assay of apoptosis for single-strand DNA breaks revealed the success of the tumor cells from evading entry into an apoptotic process, since no basal cell carcinoma islands displayed any positive reaction sites. Positive internal controls for the Fas and TUNEL groups of the basal cell carcinomas (which were entirely negative) were adjacent peritumoral infiltrating lymphocytes. It appeared that the lymphocytes nearest the tumor cell islands had higher levels of Fas and single-strand DNA breaks, whereas the lymphocytes farther away from the tumor cells had more prominent Bcl-2 expression. This suggested that the tumor cells themselves may be capable of reciprocally interacting with the adjacent lymphocytes. Obviously, more work remains to be performed to substantiate this possibility.

In conclusion, we have demonstrated that there are discordant patterns of expression of Bcl-2 and Bcl-x expression by keratinocytes *in vitro* and *in vivo*. The predominant form of Bcl-x expressed by keratino-

cytes *in vitro* and *in vivo* is the long form, with no detectable short form. There were also contrasting patterns of expression of Bcl-2 and Bcl-x when comparing benign versus malignant keratinocytes. These results indicate that the regulation of keratinocyte growth and differentiation involve the expression of several proteins linked to the apoptotic process (ie, Bcl-2 and Bcl-x), and emphasizes the need for a new perspective dealing with the immunobiology of skin in normal and diseased states.

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