Abnormal Bcl-2 and "Tissue" Transglutaminase Expression in Psoriatic Skin

Luca Bianchi, Maria Grazia Farrace,* Gabriele Nini, and Mauro Piacentini*
Departments of Internal Medicine and *Biology, University of Rome "Tor Vergata," Rome, Italy

Cell death by apoptosis plays a key role in skin development and homeostasis. Previous studies have shown that increased apoptosis characterizes several pathologic conditions affecting human skin. Thus, the pathogenesis of cutaneous diseases may involve an imbalance in the homeostatic mechanisms determining whether the death of keratinocytes will occur by terminal differentiation or apoptosis. We investigated the involvement of apoptosis in psoriasis. For this purpose, we assessed, in addition to morphology and DNA fragmentation, the expression of two putative apoptotic genes, bcl-2 and "tissue" transglutaminase, in normal and psoriatic skin. A large number of keratinocytes showing biochemical and morphologic features of cells undergoing apoptosis was observed in all the suprabasal layers of the psoriatic epidermis. The plaques from all patients analyzed showed a dramatic reduction in the number of bcl-2-positive cells localized in the basal cell compartment. In contrast, the psoriatic lesions presented a marked induction in "tissue" transglutaminase, which was localized specifically to the cytoplasm of apoptotic keratinocytes. "Tissue" transglutaminase protein staining was undetectable in normal epidermis. The bcl-2 and "tissue" transglutaminase staining pattern observed in psoriasis also was found in the skin of patients affected by lichen planus. These findings indicate that these two genes are regulated in an opposite fashion in psoriatic keratinocytes undergoing apoptosis, thus confirming their antithetic role in the cascade of events leading to the establishment of the mature apoptotic phenotype. Key words: lichen planus/psoriasis/programmed cell death/melanocytes/DNA fragmentation. J Invest Dermatol 103:829–833, 1994

Despite the increasing number of studies, the pathogenesis of psoriasis remains unclear [1]. Cell death plays a fundamental role in skin development and in the regulation of cell numbers in mature epidermis [2,3]. Cell death by apoptosis, which shares several structural and regulatory events with keratinocyte death by terminal differentiation, is active in removing excessive and abnormal cells from the epidermis [2–5]. A current important topic in skin biology is the understanding of how autonomous and/or environmental signals determine whether the death of post-mitotic keratinocytes occurs by terminal differentiation or by apoptosis [4,5]. A large number of genetic, cellular, and biochemical studies have indicated that several genes must be expressed in a strictly regulated manner to allow a cell to die in a way compatible with physiologic conditions [3]. According to their distinct roles within the cell death program, the putative apoptotic genes can be classified into three different functional groups: permissive, regulatory, and effector elements [6]. A key role in the initiation of apoptosis is played by proto-oncogenes such as c-fos and c-myc, as well as by the tumor suppressor gene p53 [7–9]. The product of the bcl-2 gene [10,11], in association with a family of recently characterized related genes [12], has emerged as a suppressive element of the cell death program; i.e., a block of bcl-2 expression is required to initiate apoptosis [11]. Among the genes identified recently as effector elements of the apoptotic cell death program are those coding for the Ca++-dependent enzymes "tissue" transglutaminase (tTG) and endonuclease [3,12,13]. Transglutaminases are a family of intracellular and extracellular enzymes that catalyze Ca++-dependent reactions establishing e(-glutamyl)lysine bonds and/or di- and polyamines derived cross-linkages into proteins [3,12]. The tTG gene is expressed in cells undergoing apoptosis in various in vivo and in vitro contexts [3,14,15]. Overexpression of tTG in Balb/c 3T3 fibroblasts produces in the transfected cells the cytoplasmic changes characteristic of apoptosis [16]. tTG activation in the dying cells leads to the formation of intracellular protein polymers insoluble in detergents and chaotropic agents [3,12]. Another biochemical event occurring during apoptosis is cleavage of the DNA at the internucleosomal linker regions [3,13].

Morphologic studies have shown that an increased number of apoptotic cells characterizes several pathologic conditions affecting mature human skin, such as lichen planus, Darier's disease, "sunburn cells," graft-versus-host reactions, and tumors [2,4,5,15–19]. In this report, we demonstrate that in psoriatic patients an abnormal bcl-2 and tTG expression, indicative of an increased apoptotic rate, parallels the typical hyperplastic syndrome.

MATERIALS AND METHODS

Chemicals Streptavidin-biotin immunoperoxidase staining systems were obtained from Biogenex (San Ramon, CA). Other chemicals were of reagent grade and used without further purification.

Antibodies The human red blood cell-soluble tTG antibody was kindly provided by Prof. Laszlo Fesus (Department of Biochemistry, University Medical School of Debrecen, Hungary). The anti-human keratinocyte transglutaminase antibody was a gift of Prof. Renata Polakowska (Depart-
Figure 1. Characterization of the specificity of bcl-2 expression in normal human skin. Western blotting analysis was carried out on total protein extracts (approximately 100 µg, lane 1, and 50 µg, lane 2) prepared from normal human skin and subjected to SDS-PAGE as described previously [16]. Arrows, relative positions of the molecular weight standards.

ment of Dermatology, University of Rochester). The monoclonal anti-human oncoprotein bcl-2 was purchased from Dako. The OKT6 anti-human CD1 and the anti-human leukocyte antigen (HLA)-DR antibodies were obtained from Ortho Diagnostic (Milano, Italy).

Skin Biopsies Human skin biopsy specimens were obtained from five chronic plaque-type untreated psoriatic patients and quickly placed into the fixative fluid (Bouin’s). After 10 min, pieces of the skin were cut and further fixed in Bouin’s solution for an additional 4–6 h. Additional samples from the same psoriatic plaques were frozen rapidly in liquid nitrogen and subsequently stored at −80°C until used. Identical procedures were used for healthy human skin and lichen planus specimens as normal and pathologic controls.

Western Blotting For Western blotting analysis, aliquots of total protein extracts from normal human skin were electrophoresed (sodium dodecylsulfate-polyacrylamide gel electrophoresis [SDS-PAGE], 12.5% acrylamide; Fig 1), blotted on Hybond-ECL nitrocellulose filters (Amersham, Bucks, UK), and stained, using as primary antibody the mouse monoclonal anti-bcl-2 antibody (Dako, Denmark) as described previously [16].

Immunohistochemistry and DNA Nick End Labeling Immunohistochemical staining of human skin was performed using the following as primary antibodies: affinity-purified monospecific IgG raised in rabbits to chemical staining of human skin was performed using the following as primary antibodies: affinity-purified monospecific IgG raised in rabbits to c hcmical staining of human skin was performed using the following as primary antibodies: affinity-purified monospecific IgG raised in rabbits to keratinocyte (Fig 2A). Similar to other transglutaminase [3,14]; monoclonal mouse anti-human oncoprotein bcl-2 (1:40) (Dako); specific anti-human “keratinocyte” transglutaminase (TgT) antibody (1:100); and anti-human OKT6 and anti–HLA-DR antibodies (1:50) (Ortho). Incubations with the primary antibody were carried out in a wet chamber overnight at 4°C. A biotinylated goat anti-rabbit and mouse were used as second antibody IgG, followed by a preformed streptavidin–horseradish peroxidase complex (Biogenex). The reaction was developed using aminochrome as chromogen substrate and 0.01% H2O2. Cells were counterstained in Mayer’s hematoxylin. Endogenous peroxidase activity was blocked by methanol-H2O2. Biopsy specimens were fixed in 4% paraformaldehyde for 15 min at room temperature and then were washed extensively in phosphate-buffered saline. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) was performed as described by Gavriel et al [20] with few modifications. Endogenous peroxidase was inactivated using 3% H2O2 for 15 min at room temperature; slides then were incubated in terminal deoxynucleotidyl transferase buffer containing: terminal deoxynucleotidyl transferase (0.5 U/µl), biotinylated-dUTP (0.025 nmol/µl), and CoCl2 (2.5 mM) for 60 min at 37°C in a humid atmosphere. The reaction was blocked by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The cells were rinsed in distilled water, covered with 2% aqueous solution of bovine serum albumin for 10 min, and then immersed in phosphate-buffered saline for 5 min. The slides were covered with peroxidase-conjugated streptavidin (BioGenex), incubated for 20 min, and stained with aminoethylcarbazole for 10 min.

RESULTS AND DISCUSSION

The morphologic analysis of psoriatic skin revealed many keratinocytes showing chromatin and cytoplasmic condensation typical of cells undergoing apoptosis [4,5]. To verify whether these morphologic changes were associated with the expression of putative apoptotic genes such as bcl-2 and tTG, we conducted a systematic investigation of their expression in five patients.

Proliferation in the epidermis is limited to the basal cell compartment, where a subset of cells, the stem elements, is responsible for maintaining its own number as well as balancing proliferation with cell loss by terminal differentiation [19]. Modification in the rate of terminal differentiation is not involved in the control of skin cell numbers, which instead seem to be regulated by apoptosis [5,19]. As reported recently [21], bcl-2 protein was expressed in all cells localized in the basal layer of normal human epidermis (Fig 2). In contrast, in the psoriatic plaques we noted a drastic reduction in bcl-2 in the proliferative cell compartment, and the protein was sequestered only in a subset of cells showing a spindle-like morphology (compare Fig 2A and 3A). These bcl-2–positive cells were localized regularly along the basal cell layer and were not stained by the OKT6 and HLA-DR antibodies (Table I). The absence of HLA-DR and CD1a expression and their exclusive location in the basal cell compartment and not in the suprabasal layers led us to identify them as melanocytes; by contrast, Langerhans cells normally are spread through the suprabasal layers and usually are positive for HLA-DR and CD1a (Fig 3A; Table I).

To verify whether bcl-2 down-regulation is typical of psoriasis or is present in other cutaneous disorders involving apoptosis, we investigated its expression in the skin of patients affected by lichen planus (Fig 2). Immunohistochemical analysis indicated that reduced expression of bcl-2 also was present in the basal cell compartment of these patients (Fig 2C). However, in unaffected skin from the same patients, the bcl-2 staining pattern was identical to that found in normal epidermis (Fig 2A,E). Bcl-2 is considered to be a survival but not a mitogenic factor that is expressed mainly in the stem cell compartments [10,11,21,22]. In keeping with this, the drastic reduction in the number of bcl-2–positive cells observed in the basal layer of psoriatic epidermis suggests the involvement of undifferentiated keratinocytes in the disease.

To substantiate this hypothesis further, we investigated the localization of tTG protein in psoriatic patients (Fig 3B,D). In normal epidermis, the reaction with the tTG antibody was limited to a few scattered cells showing apoptotic morphology and to the endothelial cells in the dermis (Table I). In contrast, in all psoriatic patients studied, intense staining was detected in the upper epidermal cell layers (Fig 3B,D). The reaction with the tTG antibody was particularly intense in cells showing the morphologic features of apoptosis (Fig 3D). Similar results were obtained in the lesional skin of lichen planus (Fig 2D), whereas the bcl-2 and tTG staining pattern observed in unaffected skin from psoriatic patients was identical to that found in normal skin (data not shown).

To confirm the specificity of the tTG staining pattern, we studied the localization of the membrane-bound TgT, which is important in the assembly of cornified envelopes in differentiating keratinocytes [23,24]. In psoriasis, in contrast with the results for tTG, TgT was localized specifically on the plasma membrane of all suprabasal keratinocytes (Fig 3E), thus confirming previous observations showing abnormal expression of TgT in psoriasis [25]. By using the TUNEL staining of DNA breaks, we detected a large number of cells showing chromatin fragmentation throughout all cell layers in psoriatic epidermis (Fig 3F). Taken together, these findings strongly indicate that in psoriatic lesions, many keratinocytes undergo apoptosis.

In recent studies on human skin development, we demonstrated...
Figure 2. Bcl-2 and tTG: abnormal localization in lichen planus versus normal and unaffected human epidermis. Normal or lichen planus (both unaffected and lesional) skin was biopsied and stained with bcl-2 or anti-tTG antibodies as described in Materials and Methods. A) Bcl-2 localization in the basal layer of normal epidermis. B) Staining of normal epidermis with the anti-tTG antibody. C) Higher magnification of bcl-2-positive cells in lesional skin from lichen planus. D) Cells stained by the tTG antibody were present in all suprabasal layers of the affected skin from lichen planus. No changes in the bcl-2 (E) and tTG (F) staining pattern with respect to the normal epidermis were detected in unaffected skin from the patient with lichen planus (compare with C and D, respectively). Bars, A,B,F, 20 μm; C,E, 7 μm.
Figure 3. Bcl-2 down-regulation in the basal layer of psoriatic epidermis is paralleled by DNA fragmentation; tTG and TGI increase in the suprabasal epidermal layers. Psoriatic lesional skin was biopsied and stained with bcl-2, anti-tTG, and anti-TGI antibodies and examined by TUNEL for DNA fragmentation as described in Materials and Methods. A) Note the marked reduction in the number of cells stained by the bcl-2 antibody in the basal layer of psoriatic epidermis when compared with normal skin (compare with Fig 2A). By contrast, a large number of cells stained by the tTG antibody was present in the suprabasal layers of the affected skin from the same patient (B; compare with Fig 2B). C) Higher magnification of bcl-2-positive cells in the basal cell compartment of a psoriatic patient. D) Higher magnification of tTG-positive cells in the suprabasal cell layers of epidermis from a psoriatic patient. Note that the staining by the tTG antibody was localized in phenotypically typical apoptotic cells showing condensed cytoplasm and pyknotic chromatin. E) TGI was localized on the plasma membrane of all the suprabasal cells of psoriatic epidermis. F) TUNEL staining of psoriatic plaques showed several nuclei with fragmented chromatin in all suprabasal epidermal layers, but no staining in the dermis. Bars, A,B, 40 μm; C,D, 7 μm; E,F, 20 μm.
that the different transglutaminases are expressed in a stage-specific manner during epidermal morphogenesis, confirming their specific functions [23]. tTG is active in apoptosis of relatively undifferentiated keratinocytes, whereas TGI is expressed in the terminally differentiated cells of mature epidermis [23,24]. The intense tTG staining we observed in the keratinocytes localized in the upper layers of the epidermis in both psoriasis and lichen planus strongly suggests that these cells are committed to the apoptotic pathway. In keeping with these findings, the amount of N\^N\^N-Bis(y-glutamyl)-spermidine cross-link was demonstrated to be abnormally high in cornified envelopes extracted from psoriatic skin as compared with normal skin [26]. It is interesting that this polyanine-derived cross-link stabilizes the detergent-insoluble polymers formed in cells undergoing apoptosis [12].

The reduced bcl-2 expression detected in the basal layer of psoriatic skin indicates that this phenomenon originates in the stem cell compartment. It is tempting to speculate that in psoriasis, a large number of apoptotic cells can originate from stem cells prematurely recruited to reenter the cell cycle but programmed to persist in a quiescent G\_0 state. In psoriatic patients, the number of proliferating versus post-mitotic quiescent cells is largely increased because of abnormal shortening of the cell cycle [19,21]. Furthermore, psoriatic keratinocytes can be primed to undergo apoptosis by various factors (growth factors, cytokines, etc.) produced by dendritic or immune cells present in the dermis. In agreement with this, psoriatic skin lesions overproduce IL-6 [27], which recently has been shown to upregulate tTG gene expression [28].

In conclusion, our results offer new insight into the pathogenesis of psoriasis, suggesting a central role for apoptosis. In psoriasis, as observed in other hyperproliferative syndromes, increased apoptosis could be a homeostatic mechanism to counteract the overproduction of cells [3,13]. This hypothesis is consistent with the emerging idea that it is not possible to induce proliferation in a cell without activating its potential for apoptosis [29].

Table I. Bcl-2 and tTG Reactivity in Normal Human Skin, Lichen Planus, and Psoriatic Plaque Samples

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REFERENCES