Protooncogene Expression in Normal and Psoriatic Skin

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The expression of the c-myc, c-fos, c-jun, erbB, and c-Ha-ras protooncogenes was compared by Northern blot analysis of total RNA extracted from keratome biopsies of normal skin and psoriatic plaques. Isolation of intact RNA from frozen tissue required careful attention to technique during the early stages of extraction. Densitometric analysis revealed 1.5- to 2.5-fold elevations of c-fos transcript levels in lesional psoriatic relative to normal epidermis. Similar increases in cyclophilin and lipocortin II transcripts were also observed and may reflect characteristic differences in RNA preparations from normal and psoriatic epidermis. C-myc, c-jun, erbB, c-fos, and c-Ha-ras transcript levels were not significantly increased in lesional psoriatic epidermis when protooncogene mRNA levels were normalized to those of the cyclophilin or lipocortin genes. In contrast, transforming growth factor-α (TGF-α) transcripts were significantly increased (10- to 20-fold) with or without prior normalization. C-myc, c-fos, and c-jun transcripts were significantly induced over in vivo levels 2-4 h after organ culture of normal or psoriatic keratome biopsies, demonstrating that these genes can be highly expressed in the context of tissue injury. Our results suggest that overexpression of these protooncogenes per se is not central to the pathogenesis of psoriatic epidermal hyperplasia.

Protooncogenes are highly conserved cellular genes, initially discovered by virtue of their incorporation into oncogenic retroviruses [1]. Protooncogenes participate in the regulation of cellular proliferation and differentiation at three levels: 1) the interaction of extracellular ligands with their cell membrane receptors (e.g., c-myc identified as the PDGFrα binding protein, erbB as the EGF receptor, and c-fos as the CSF-1 receptor) [2,3]; 2) the transduction of these interactions into intracellular biochemical changes (e.g., c-Ha-ras involvement in GTP metabolism [4] and the activation of phospholipase C by receptor tyrosine kinases [5]); and 3) the induction of nuclear transcriptional events in response to cytoplasmic signals (e.g., interaction of c-fos and c-jun to function as DNA-binding transcription factors [6]). These processes result in changes in cellular differentiation and proliferation which appear to involve c-fos [7] and c-myc [8].

Psoriasis is a benign, inflammatory skin disease marked by prominent epidermal hyperplasia [9] and a disordered pattern of cellular differentiation [10]. We have recently demonstrated that transforming growth factor-α (TGF-α) is significantly overexpressed in psoriatic epidermis [11]. The cellular receptor for TGF-α, the epidermal growth factor (EGF) receptor, is encoded by the protooncogene erbB-2 [12] and has been shown to persist abnormally in the suprabasal layers of psoriatic epidermis [12]. While overexpression of the c-myc [13], N-myc [14], erbB-2 [15] protooncogenes has been shown to correlate with poor prognosis in human cancers, the role of protooncogene overexpression in the maintenance of nonmalignant neoplastic proliferation remains poorly defined. Therefore, we wished to determine whether increased protooncogene expression accompanies the epidermal hyperproliferation characteristic of psoriasis.

To accomplish this, we extracted total cellular RNA from normal and lesional psoriatic skin under denaturing conditions and analyzed these RNA by blot hybridization [16]. Taking advantage of the re-usability of the blots, we compared the expression of erbB-2, erbB-1, c-Ha-ras, c-myc, c-fos, and c-jun, representing each class of protooncogenes (cell surface receptor, signal transduction, and intranuclear) in normal skin and psoriatic lesions. Our results indicate that significant overexpression of these protooncogenes does not accompany psoriatic epidermal hyperplasia. In addition, these studies have provided useful information concerning isolation of intact RNA from human epidermis.

**MATERIALS AND METHODS**

**Skin Biopsies** After obtaining informed consent, epidermal keratome biopsies were obtained from the buttocks region of patients with chronic plaque psoriasis or from healthy nonpsoriatic controls as previously described [17].

**RNA Isolation and Analysis** Immediately upon removal, keratome strips were immersed in liquid nitrogen and stored at −70°C until use. Keratome strips were finely pulverized in liquid nitrogen using a mortar and pestle (see Results). The slurry of pulverized fragments in liquid nitrogen was quickly transferred to RNA extraction buffer [4M guanidium isothiocyanate, 5 mM sodium citrate, pH 7.0, 100 mM 2-mercaptoethanol, 0.5% Sarkosyl, 0.15%}

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**Abbreviations:**
- CSF-1: colony stimulating factor-1
- EGF: epidermal growth factor
- GTP: guanosine triphosphate
- PDGF: platelet-derived growth factor
- RNA: ribonucleic acid
- SSC: saline sodium citrate
- TGF-α: transforming growth factor-α

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19
Antifoam A (Sigma), 10–60 ml buffer/g wet weight tissue, resulting in the freezing of the extraction buffer. The frozen extraction buffer was immediately homogenized for 30–60 sec using a Polytron homogenizer (Brinkmann). The remainder of the RNA extraction was performed exactly as described [18]. Yields were approximately 1 μg RNA/mg wet weight tissue from both normal and psoriatic samples. RNA concentration was determined by absorbance at 260 nm. RNA concentration and intactness were verified by nondenaturing agarose gel electrophoresis and ethidium bromide staining as described [19]. Polyadenylated RNA was prepared by oligo-dT-cellulose chromatography [20].

Equal quantities of total RNA were size-fractionated by electrophoresis in 1% formaldehyde-agarose gels containing 0.5 μg/ml ethidium bromide as described [18]. After electrophoresis, gels were rinsed extensively against distilled water and photographed under long-wave UV illumination (TL-33, UV Products). Capillary transfer of RNA to positively-charged derivatized nylon membrane (Zeta-probe, Bio-Rad) was then performed in 10× saline-sodium citrate buffer (SSC; 1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Hybridizations and washings were performed under stringent conditions as described [21]. Autoradiographs of Northern blots were obtained using intensified screens (Cronex, Du Pont) and quantitated by scanning the relevant RNA bands with a laser densitometer (LKB). Blots were stripped for re-hybridization by boiling twice for 20 min in 0.1× SSC, 0.5% sodium dodecyl sulfate.

Hybridization Probes Hybridization probes were prepared by random priming [22] of the following restriction fragments, which were separated from vector fragments by electrophoresis in low-melting-temperature agarose (BRL, Bethesda, MD): a 414-base-pair (bp) Pst I fragment derived from the second exon of the c-myc gene [23]; a 372-bp Pst I fragment derived from the first exon of the c-Ha-ras gene isolated from the E) bladder carcinoma [24]; a 3,400-bp genomic Xho I-NcoI fragment spanning the first three and part of the fourth exon of the human c-fos gene [25] or a 528-bp Stal-AclI genomic fragment spanning the 3′ end of the c-fos coding sequence; a 1.4-kb HindIII-EcoRI insert containing human c-myc cDNA [26]; a 6-kb EcoR I insert containing human EGF receptor (c-erbB) cDNA [27]; a 0.7-kb Eco R I insert containing human lipocortin II cDNA [28]; and a 1.0-kb BamHI insert containing cyclophilin cDNA [29].

Organ Culture Keratome biopsy strips were cut into 1.5-cm squares immediately after removal and were either frozen immediately in liquid nitrogen or transported on ice to the laboratory within 15 min. Fragments were then placed in Keratinocyte Basal Medium (Clonetics) at 37°C in a 5% CO₂ incubator. After 2–4 h, biopsy fragments were frozen in liquid nitrogen and processed for RNA isolation as above.

Data Analysis When appropriate, integrated autoradiographic intensities obtained by densitometry were normalized to one of two control genes, lipocortin II or cyclophilin, by dividing the intensity obtained for the test gene by that of the control gene. Expression in psoriatic relative to normal epidermis was obtained by dividing the values obtained for each psoriatic patient by the mean value obtained for the normal group. Statistical analysis was by Student’s t test using a two-tailed hypothesis. Error bars represent SEM.

RESULTS

Isolation of Intact RNA Figure 1 depicts the effect of different extraction techniques on intactness of RNA extracted from frozen or freshly-harvested keratome biopsies of normal human epidermis. In Fig 1A, lanes a and b, snap-frozen tissue samples that were stored at −70°C were placed directly in RNA extraction buffer and homogenization began within 15 sec. Note the extensive RNA degradation, especially in lane b. Lanes c–f contain RNA extracted from frozen biopsies pulverized in liquid nitrogen prior to addition of RNA extraction buffer. The samples shown in lanes c and d were allowed to stand for 30 sec after thawing of RNA extraction buffer (See Materials and Methods) before beginning homogenization, while those in lanes e and f were homogenized immediately after freezing the extraction buffer by addition of pulverized tissue fragments in liquid nitrogen. In contrast, extensive RNA degradation was not encountered when freshly-removed, non-frozen tissue was directly homogenized. (Figure 1B, lanes a–f represent independent isolations.) Moreover, tissue fragments placed in organ culture for up to 4 h showed no appreciable RNA degradation, as judged by RNA yields and intactness of ribosomal RNA bands (Fig 1C,D).

Comparison of Transcript Levels in Normal and Psoriatic Epidermis We chose to analyze total rather than polyadenylated RNA in order to more easily compare large numbers of samples. Therefore, it was necessary to demonstrate that normal and psoriatic epidermis do not differ in terms of the percentage of total polyadenylated RNA. Polyadenylated RNA was prepared from normal (n = 4) and lesional psoriatic (n = 4) epidermis by oligo-(dT) cellular lose chromatography and quantitated by absorbance at 260 nm. The normal group yielded 2.35% ± 0.48%, and the psoriatic group yielded 2.70% ± 0.58% polyadenylated RNA (mean ± SD), and no significant difference was found between the groups (p > 0.10).

Figure 2 displays the results of RNA blot hybridization experiments comparing the levels of c-myc and c-Ha-ras protooncogene transcripts in normal and lesional psoriatic epidermis. The pattern of ethidium bromide staining is shown in Fig 2A. Some degradation of two of the normal samples (solid arrows, Fig 2A) is evidenced by increased migration of the 285 RNA band.

Figure 1. Effects of extraction conditions on RNA intactness. RNA samples (1 μg) isolated from keratome biopsies of normal epidermis under various conditions were subjected to electrophoresis through a 1% agarose gel as described [19]. Mobilities of 28 and 18S ribosomal RNA are indicated to the left. A) frozen biopsies: lanes a and b, pulverization of frozen keratome strips was omitted, and homogenization began within 15 sec of addition of tissue to extraction buffer. Lanes c and d, frozen keratome strips were pulverized with a mortar and pestle in liquid nitrogen, then added to extraction buffer. Homogenization began 30 sec after thawing of homogenization buffer. Lanes e and f, same conditions as lanes c and d except that homogenization began immediately after the extraction buffer was frozen by addition of pulverized tissue fragments in liquid nitrogen. B) freshly-frozen biopsies: lanes a–f. RNA isolated from six independent keratome biopsies immediately after tissue removal without freezing or liquid nitrogen pulverization. C) and D) organ culture: biopsy fragments were either frozen immediately after removal in liquid nitrogen (lane a), after transport of a fresh biopsy to the laboratory on ice (lane b), or after incubation at 37°C for 2 h (lanes c, d) or 4 h (lanes e, f), as described in the text. Samples shown in lane c, 1C, and lane b, 1D were presumably degraded during processing.
Rehybridizations of the blot prepared from this gel against c-myc, c-Ha-ras, and lipocortin II probes are shown in Fig 2B–D, respectively. Lipocortin II was chosen as a potential reference gene (or internal standard) because it is highly expressed in cultured human keratinocytes and epidermis [30]. In each case, a transcript of the predicted size (c-myc, 2.4 kb; c-Ha-ras, 1.4–1.6 kb; lipocortin II, 1.6 kb) was obtained (solid arrows). The faint upper band seen in Fig 2B (open arrow) probably represents cross-hybridization to the 28 S ribosomal RNA, as it is absent in blots prepared from psoriatic polyadenylated RNA (data not shown). In addition to the band at 1.4 kb, the c-Ha-ras probe also detected two bands migrating at approximately 2.6 and 5.0 kb in Fig 2C (open arrows, see Discussion).

Figure 2E depicts the result of a different experiment comparing hybridization of total RNA from normal or lesional psoriatic epidermis to the c-fos probe. Using the 3.4-kb XhoI-NcoI probe, hybridization to the 2.2 kb c-fos mRNA (closed arrow) was faint, and cross-hybridization to the region of the blot containing 28 S ribosomal RNA was evident (open arrow). Rehybridization of the blot used in Fig 2A against the 3.4 kb c-fos probe is not shown because of an unacceptably high background hybridization.

The band intensities of the c-myc and c-Ha-ras transcripts appeared to be moderately increased in psoriatic lesions; however, hybridization to the lipocortin II reference probe also appeared to be similarly increased. Moreover, the variation in band intensities from sample to sample was similar for all three probes, suggesting that increased hybridization to protooncogene probes in the psoriatic samples might reflect differences in the quality and/or nature of RNA preparations obtained from normal and psoriatic skin. Therefore, we repeated the experiment with RNA samples from different patients and selected a different reference probe, cyclopamin. This probe has been shown to be expressed at high levels in a variety of tissues [29], and levels of cyclophilin protein are not significantly different in normal and psoriatic epidermis [31]. The results of these studies are shown in Fig 3.

The blot shown in Fig 3A was hybridized with a mixture of c-myc and cyclophilin probes and again demonstrates an increase in the 2.4-kb c-myc transcript in psoriatic lesions. However, a similar increase in the 1.0-kb cyclophilin mRNA was also noted and is more apparent on a lighter exposure of the autoradiogram (Fig 3A, lower panel). A duplicate blot was prepared and hybridized to a mixture of cyclophilin probe and the 528-bp StuI-AccI c-fos 3′ end probe, as shown in Fig 3B. Note that 2 of the normal samples display high levels of c-fos transcripts, and that the signal-to-noise ratio using the 3′ end probe is improved relative to that obtained with the 3.4-kb Xba-NcoI probe used in the experiments shown in Fig 2.

These blots were stripped and rehybridized to the c-jun, c-Ha-ras, and c-erbB protooncogene probes, as shown in Figs 3C–E. Each probe detected transcripts of the expected sizes (c-jun, 2.7 kb [26]; c-erbB, 12, 6, and 2.5 kb [27]). Other bands were also observed (see Discussion). For comparison of one of these blots to the TGF-α probe [11] is shown in Fig 3F.

The signal intensities of mRNA bands detected by the c-myc (2.4 kb), c-Ha-ras (1.6 kb), c-fos (2.2 kb), c-jun (2.7 kb), c-erbB (2.5 kb), cyclophilin (1.0 kb), lipocortin II (1.6 kb), and TGF-α (4.8 kb) probes shown in Figs 2 and 3 were quantitated by laser densitometry, and the results are summarized in Fig 4. Only the increase or decrease of intensity in psoriatic lesions relative to the mean value obtained for normal epidermis is shown. In addition, the data are compared either as raw integrated optical densities (black bars) or after normalization to either lipocortin II or cyclophilin (hatched bars). c-myc mRNA levels were significantly (p < 0.05) increased (approximately twofold) in psoriatic lesions when the raw intensities were compared. However, similar significant increases were observed for lipocortin II and cyclophilin transcripts, and all significant differences in protooncogene expression were lost after normalizing the data to either lipocortin II or cyclophilin (hatched bars). In contrast, expression of TGF-α transcripts remains significantly and highly significantly elevated in lesional epidermis with or without prior normalization.

Protooncogene Expression in Organ Culture In order to test the hypothesis that protein kinase C is down-regulated in psoriatic
lesions [32], we wished to compare the responses of normal and psoriatic keratome biopsies to phorbol ester treatment in organ culture. However, in pilot experiments, we found that large inductions of several protooncogene transcripts occurred in the absence of phorbol ester treatment. The results of these experiments are shown in Fig 5 and quantitated in Table I. C-mye, c-fos, and c-jun was induced from twofold to greater than 150-fold after 2–4 h of organ culture, while c-Ha-ras, TGF-α, and cyclophilin were not induced (<1.5-fold).

DISCUSSION

Using the Northern blotting technique, we have demonstrated expression of the c-mye, c-Ha-ras, c-fos, c-jun, and c-erbB protooncogenes in normal and psoriatic skin. We found that careful attention to technique was necessary during the early stages of the extraction procedure in order to ensure the isolation of intact RNA (Fig 1A). This is not surprising, because ribonucleases are abundant in human epidermis [33]. We also found that the process of freezing and thawing increases the degradation encountered during isolation of epidermal RNA. This could be due to disruption of cellular organelles and membranes, causing increased access of enzyme to substrate during the early stages of isolation. This interpretation is consistent with the observed stability of epidermal RNA in fresh biopsies (Fig 1B) and in epidermal organ cultures for up to 4 h (Fig 1C,D).

These observations suggest that modest differences in mRNA transcript levels in normal and psoriatic skin analyzed by these methods should be interpreted cautiously. We have consistently found an approximately twofold increase in c-mye mRNA in psoriatic lesions; however, these differences have been paralleled by those of two putative reference genes, lipocortin II and cyclophilin. In contrast, TGF-α mRNA is consistently and significantly increased in psoriatic lesions [11] to a much greater extent than either reference gene. The other protooncogenes tested were not consistently increased in psoriatic lesions, and c-fos transcript levels appear to be slightly decreased in psoriatic lesions (Figs 3B, 4).

The presence of additional hybridizing bands in our studies can usually be explained by non-specific hybridization to the large amounts of 28S ribosomal RNA present in our preparations. However, in the case of c-Ha-ras, a 2.6-kb transcript has been observed by ourselves and by Ogiso et al, who have suggested that it is due to hybridization to Ki-ras transcripts [34].

Figure 3. RNA blot hybridization of protooncogene transcripts (experiment 2). Shown to the left are 18 and 28S ribosomal RNA markers. -cy denotes location of cyclophilin transcripts. Solid arrows indicate transcripts of previously-reported sizes. Open arrows indicate cross-hybridization to 28S RNA.
In contrast, TGF-α mRNA and protein are significantly increased in psoriatic lesions [11]. Because TGF-α appears to be the endogenous epidermal ligand for the EGF receptor, these results suggest that regulation of TGF-α synthesis is more central to the molecular pathogenesis of psoriasis than is regulation of the synthesis of its receptor.

We have observed significant increases in mRNA levels of three proto-oncogenes, c-myc, c-fos, and c-jun, after placing normal and psoriatic keratome biopsies into organ culture. These observations increase our confidence that these genes are expressed at low but detectable levels in normal and psoriatic skin in vivo because our probes detect greatly increased amounts of the same-sized transcripts under our in vitro conditions. Increased expression of these genes occurs upon entry into the cell cycle [37], and antisense inhibition experiments have demonstrated a requirement for c-myc [8] and c-fos [38] in cell cycle progression. Increased expression of these protooncogenes is induced in vitro by phorbol esters, certain growth factors, and ultraviolet light, and in vivo by injurious stimuli such as tape stripping of the epidermis [39–41]. In the latter setting, these protooncogenes may participate in the initiation of reparative epidermal hyperplasia. However, unlike TGF-α, these protooncogenes are not overexpressed significantly, if at all, in the steady-state hyperplasia that characterizes psoriatic epidermis. It is possible that the molecular mechanisms that maintain epidermal hyperplasia in psoriasis are different from those that initiate it after epidermal injury.

Our results document the expression of several protooncogenes at the level of steady-state mRNA in normal and psoriatic human skin. The presence of the same transcripts, at similar levels, in normal epidermis suggests that these genes are important regulators of epidermal cell proliferation and differentiation in both normal and psoriatic skin.

**Figure 4.** Quantitation of protooncogene transcripts in normal and lesional psoriatic epidermis. The bands indicated by the solid arrows in Figs 2 and 3 were quantitated by laser densitometry and expressed as fold-change relative to normal epidermis as described in Materials and Methods. Solid bars: raw integrated autoradiographic intensities; hatched bars: integrated autoradiographic intensities normalized to lipocortin II (experiment 1) or cyclophilin (experiment 2) as described in Materials and Methods; dashed line: mean levels obtained in normal epidermis.

Mordovtsev and co-workers have compared the expression of several protooncogenes in normal psoriatic skin by RNA dot blotting [35]. While this technique is insensitive to RNA degradation, it does not discriminate between signal and background hybridization. For example, our hybridizations with c-fos (Fig 2E) and Ki-ras (not shown) probes have revealed high levels of diffuse background hybridization. Nevertheless, our results agree that c-myc and c-fos genes are expressed in psoriatic skin, and c-myc mRNA levels are increased in psoriatic epidermal RNA. However, we did not find increased levels of c-fos mRNA in the psoriatic RNA samples. Also, we did not observe the significant variation in epidermal c-myc expression nor the correlation between epidermal c-myc and c-fos expression reported by Ogiso et al [34]. Variability in tissue sampling and in quality of RNA preparations could account for the covariation of c-myc and c-fos transcripts observed by these workers.

Thompson et al [19] have demonstrated that c-myc expression is independent of position in the cell cycle in actively cycling cells, while its expression is greatly reduced or absent in quiescent ("G₀") cells. It is generally agreed that the growth fraction of the germinative layers of psoriatic epidermis approaches 100%, whereas estimates for normal epidermis range from 10% to 50% [9]. On this basis, a greater increase in c-myc expression than we have observed in psoriatic epidermis might have been expected. Our results suggest that c-myc expression may display a more complex relationship to cellular growth state in epidermal keratinocytes in vivo than in growth-arrested vs actively cycling cells in vitro [36].

King and co-workers described an altered distribution of EGF receptors in psoriatic epidermis [12]. However, they did not find a significantly increased amount of EGF receptors in psoriatic epidermis on a protein or DNA basis [12]. This is consistent with our finding that c-erbB transcripts are not increased in psoriatic lesions.

**Figure 5.** Induction of protooncogene transcripts in organ culture. Only the relevant hybridizing bands are shown. See text for details.
psoriatic skin. Future insights into the roles of these protooncogenes in epidermal growth regulation will be gained through the use of in situ detection methods for protooncogene mRNA and protein and through further exploration of the organ culture model system.

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REFERENCES


Table 1. Fold-Change in mRNA Levels in Organ Culture of Normal and Psoriatic Epidermis

<table>
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<th>Gene</th>
<th>Normal* mRNA Level</th>
<th>Psoriatic* mRNA Level</th>
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<td></td>
<td>2 hr(n)</td>
<td>4 hr(n)</td>
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<tr>
<td></td>
<td>2 hr(n)</td>
<td>4 hr(n)</td>
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<td>Cyclophilin</td>
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* Means of the indicated number of samples are shown. Values are expressed as fold-change over mean integrated optical density at time zero.

MEETING ANNOUNCEMENT AND CALL FOR PAPERS

The Eighth International Symposium on Bioengineering and the Skin is to be held at Stresa, Lake Maggiore, Italy on the 13th to the 16th of June 1990. Local Organizers: G. Rabbiosi, S. Sacchi, G. Borroni, E. Berardesca, Department of Dermatology, University of Pavia, 27100 Pavia, Italy. Dermatologists, pharmacologists, plastic surgeons, cosmetic chemists, and other scientists in the field will gather to discuss technologies and methods for the non-invasive evaluation of skin physiology, pathology, therapy, and skin product assessment. Invited speakers will present updates on current bioengineering issues. Deadline for abstract submission is January 31, 1990. Abstracts should be typed on A4 paper with 1-inch left margin and should include title, author’s name and affiliation, and a summary not exceeding 300 words. Abstracts should be sent to: Dr. Jorgen Serup, Department of Dermatology, Rigshospitalet H 5132, 9 Blegdamsvej, 2100 Copenhagen, Denmark or to: Dr. Randy Wickett, S.C. Johnson & Son, 1520 Howe Street, Racine WI 53403-5011.