Expression of Plasminogen Activator Enzymes in Psoriatic Epidermis

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The plasminogen activators, tissue type and urokinase type (tPA and uPA, respectively) have been identified in human skin under normal conditions and in various inflammatory dermatoses, including psoriasis. By Northern blot analyses, mRNA for uPA, but not for tPA, has been previously identified in epidermal extracts from normal skin, whereas in psoriasis, mRNA for tPA is readily detected. To further characterize uPA and tPA expression in psoriasis, the localization of uPA and tPA mRNAs was evaluated by *in situ* hybridization. Studies were conducted using lesional and nonlesional skin of patients with psoriasis as well as normal skin. Additionally, *in situ* zymography using casein gel overlays was utilized to assess enzymatic activity. In psoriatic lesional skin, both uPA and tPA mRNAs were demonstrated by *in situ* hybridization. Message for tPA was observed throughout the epidermis with areas of accentuation in the superficial stratum spinosum. Message for uPA was more focal and was localized primarily in the basal layer. Zymography showed tPA activity was coordinately increased in psoriatic lesions. Uninvolved skin of psoriatic patients was similar to that of normal skin with respect to expression of plasminogen activators. In normal epidermis, neither tPA nor uPA mRNA could be detected by *in situ* hybridization. Activity for uPA, but not tPA, was observed by zymography. These studies suggest that alterations in plasminogen activators expression may contribute to the pathogenesis of psoriasis. *Key words: in situ hybridization/proteases/zymography. J Invest Dermatol 102: 333–338, 1994*

The plasminogen activator (PA) enzymes, urokinase type (uPA) and tissue type (tPA) are proteases that catalyze the conversion of plasminogen to plasmin (reviewed in [1]). Plasmin is a serine protease that can hydrolyze a broad variety of proteins including many components of the extracellular matrix. Plasmin can activate the complement cascade by cleavage of c1, and it can also activate other proteases, including metalloproteases, to their active forms. Hence, the process of plasminogen activation initiates a cascade of proteolytic events that may modulate cellular activities entailing the interaction of the cell with its extracellular matrix, including migration, growth, and differentiation.

Normal human epidermis contains plasminogen-activating (PA) activity, which is primarily uPA [2]. Messenger RNA for uPA is detected by Northern analyses, whereas mRNA for tPA is not detectable [3]. A number of cutaneous disorders including psoriasis are characterized by elevated levels of epidermal PA activity [2,4]. More recently, it has been shown that the increased activity reflects induction of tPA expression [4]. The studies reported herein demonstrate, with *in situ* hybridization and zymography technologies, that the previously observed quantitative changes in PA activity and expression reflect dramatic differences in the distribution of these enzymes in psoriatic versus normal skin.

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Abbreviations: PAs, plasminogen activators; tPA, tissue type plasminogen activator; uPA, urokinase plasminogen activator.

MATERIALS AND METHODS

Tissue All specimens were collected in accordance with a protocol approved by the Committee for Protection of Human Subjects at the University of Pennsylvania. Normal skin, obtained from dermatologic surgery, was from various sites including face, breast, and abdomen. Samples for *in situ* hybridization were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4), dehydrated in graded ethanols, and paraffin embedded. Six-micron sections were applied to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Samples for zymography were immediately frozen in liquid nitrogen and then embedded in PolyFreeze (Polysciences, Warrington, PA).

***In Situ* Hybridization** Sections were deparaffinized in xylenes, rehydrated in 50% ethanol in phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) for 10 min at 4°C. Sections were washed in 0.5 × sodium citrate/sodium chloride buffer (SSC) (1 × SSC; 0.015 M sodium citrate, 0.15 M sodium chloride), incubated with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl (pH 8) containing 0.5 M sodium chloride for 10 min at room temperature and washed in 0.5 × SSC. The sections were prehybridized for 1 h at 55°C and hybridized overnight at 58°C in a mixture of 50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 8), 5 mM ethylenediamine tetraacetic acid (EDTA), 1 × Denhardt's solution (1 × Denhardt's solution; 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidone), 10% dextran sulfate, 10 mM dithiothreitol (DTT), and 500 μg/ml transfer RNA. Hybridizations were conducted using heat-denatured, ^{35}S-labeled cRNA probes (6 × 10^6 cpm/ml). Non-specifically bound probe was removed by digestion with RNase A (20 μg/ml) in 10 mM Tris-HCl (pH 8) containing 0.5 M sodium chloride for 45 min at room temperature. Sections were washed at high stringency in 0.1 × SSC containing 1 mM EDTA and 10 mM 2-mercaptoethanol for 2 h at 68°C. The sections were dehydrated and exposed to K.2 autoradiographic emulsion (Polysciences, Warrington, PA) for 2 weeks. After development, they were stained with hematoxylin and eosin. Sections were examined and photographed using epipolarization microscopy.

cRNA Probes A 610-base pair (bp) fragment (positions 1364–1973) of the human uPA cDNA (pHUK-8) subcloned in pSP64 and pSP65 was the template for synthesis of antisense and sense uPA cRNA probes, respectively

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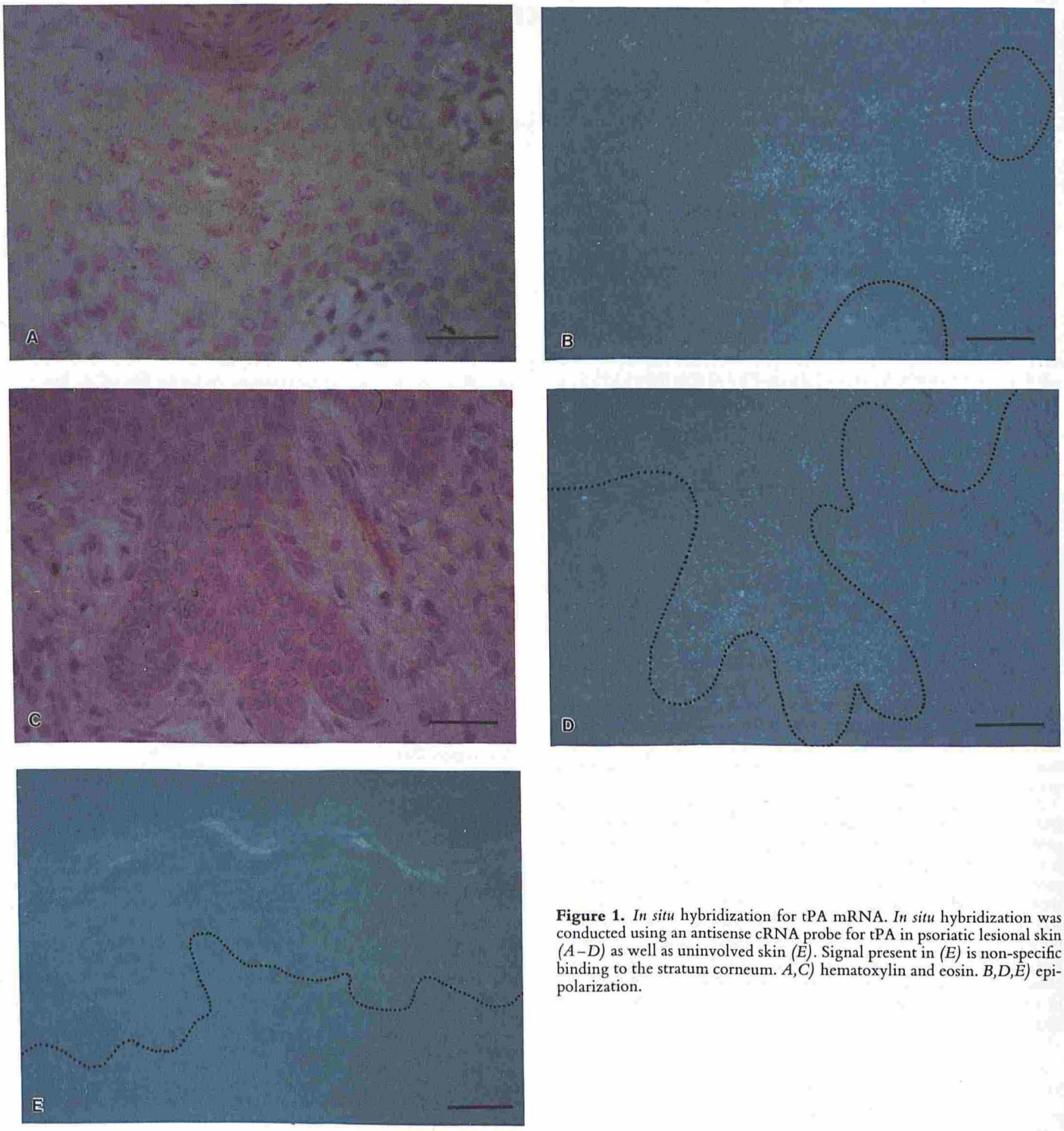


Figure 1. *In situ* hybridization for tPA mRNA. *In situ* hybridization was conducted using an antisense cRNA probe for tPA in psoriatic lesional skin *(A–D)* as well as uninvolved skin *(E)*. Signal present in *(E)* is non-specific binding to the stratum corneum. *A,C)* hematoxylin and eosin. *B,D,E)* epipolarization.

[5]. A 614-bp fragment (positions 188–810) of the human tPA cDNA (pW349F) subcloned into pSP65 and pSP64 provided template for antisense and sense tPA cRNA probes, respectively [6]. RNA probes were generated by *in vitro* transcription in the presence of ^{35}S-UTP (1000 Ci/mmole, Amersham). Unincorporated radiolabel was removed from the probes by column purification. The specificity of the antisense uPA and tPA cRNA probes was verified by Northern analysis of total RNA prepared from human epidermis (normal and psoriatic) and keratinocyte cultures [4].

Zymography Zymography on cryostat tissue sections were conducted as described previously [7]; the overlay mixture consisted of 200 μl of PBS, 133 μl of a 2.5% agar solution in water, and 20 μl of a 0.9 mg/ml solution of purified human plasminogen, 67 μl of an 8% Carnation instant nonfat dry milk solution in PBS and 1.2 μl of 10% sodium azide. This overlay mixture was prepared at 50°C and 100 μl were applied to prewarmed 10-mm–thick cryostat sections mounted on Superfrost slides and spread evenly under 24 × 50–mm glass coverslips. Slides were incubated at 37°C in humid chambers, and the development of zymograms was assessed after 6, 24, and 48 h. Identical experiments were carried out with overlay mixtures from which plasminogen was omitted to determine whether the proteolysis was plasmin dependent. Inhibition studies were conducted to define the type of plasminogen activator present. For these studies, caseinolysis was evaluated using overlay mixtures containing plasminogen as well as 1 mM amiloride (an inhibitor of uPA, but not tPA or plasmin) [8], nonimmune immunoglobulin (Ig)G, rabbit anti-uPA IgG (final concentrations of 25, 50, or 100 μg/ml), and goat anti-tPA IgG (final concentrations of 25, 50, or 100 μg/ml). Blocking antibody to human urinary uPA was prepared as previously described [9]; antibody to tPA was purchased (product 387, American Diagnostica, Greenwich, CT). The specificity of the blocking activity of the antibodies for uPA and tPA was verified using the thiol ester substrate assay for plasmin activity described by Coleman and Green [10]. Caseinolysis was visualized as dark zones using dark field microscopy.

Zymography performed in the presence of plasminogen showed a well-demarcated zone of lysis throughout the epidermis of normal and psoriatic skin. Caseinolysis occurred in a time-dependent manner. The optimal incubation period was 24 h, at which time the results were interpreted and photographed. Total clearing was evidenced by 48 h for both psoriatic and normal skins. No caseinolysis was observed when the overlay gels were prepared without plasminogen. Because of irregularities and artifacts of sectioning (clefts between collagen bundles, folds in the tissue, knifemarks, etc.), the localization and types of PA activity within the dermis could not be interpreted by *in situ* zymography.

RESULTS

***In Situ* Hybridization** Expression of uPA and tPA message in normal skin and in biopsies of involved and uninvolved skin from patients with psoriasis was evaluated by *in situ* hybridization. In the psoriatic epidermis, message for both uPA and tPA was evident. The induction of tPA expression was present throughout the epidermis. Areas of accentuation in the mid to upper stratum spinosum were identified (Fig 1). Lower levels of tPA mRNA were present throughout the rete ridges. Message for uPA was more restricted in distribution, being present primarily in the lower epidermis, particularly in the basal layer (Fig 2). Neither uPA nor tPA mRNA could be detected in uninvolved epidermis from psoriatic patients (Figs 1 and 2) or in normal epidermis (data not shown).

The specificity of the hybridization conditions was verified by the absence of signal above background levels in all specimens when radiolabeled sense probes replaced the antisense probes.

Data for psoriatic skin are representative of six involved plaques from three patients, two of whom also donated uninvolved skin for analyses. Each sample was hybridized in three independent experiments. All lesional skin samples showed similar results. Normal skin was obtained from 10 donors and was analyzed in three independent experiments and in no case could specific signal for uPA or tPA be identified.

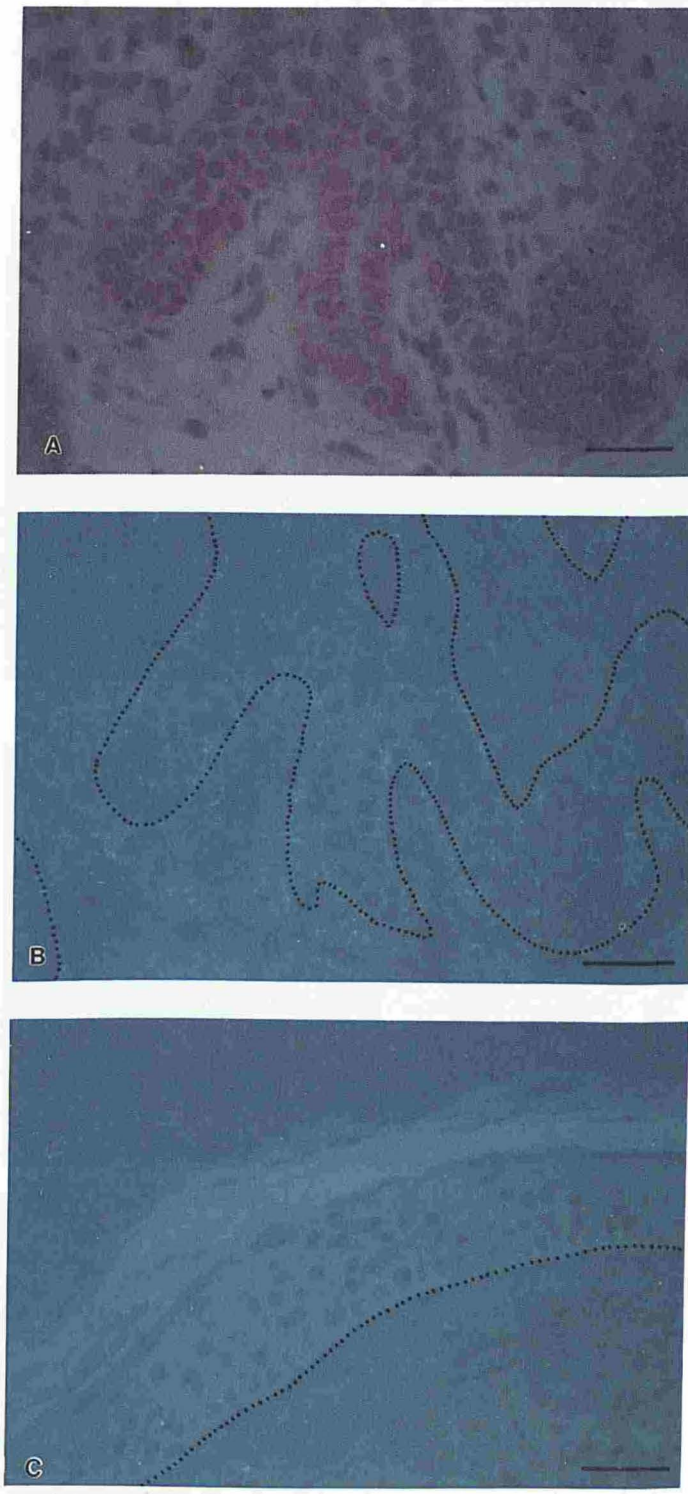
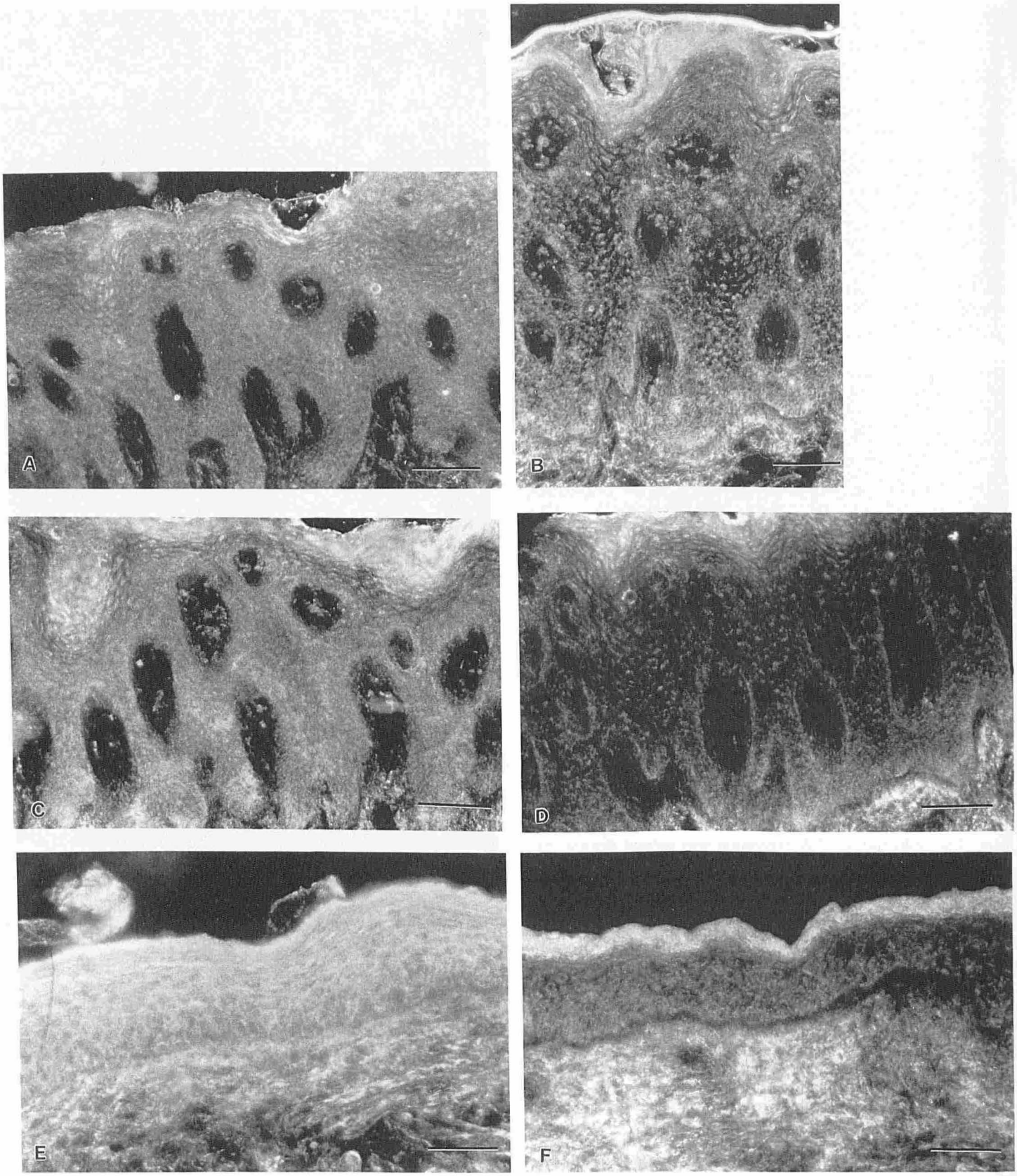


Figure 2. *In situ* hybridization for uPA mRNA. *In situ* hybridization was conducted using an antisense cRNA probe for uPA in psoriatic lesional skin *(A,B)* as well as uninvolved skin *(C)*. *A)* hematoxylin and eosin. *B,C)* epipolarization.

***In Situ* Zymography** *In situ* zymography studies were conducted to assess whether the focal increases in PA mRNA expression within the psoriatic epidermis were reflected in a coordinate enhancement of PA enzymatic activity. In zymography of psoriatic skin, caseinolysis was present throughout the full thickness of the epidermis, with the exception of the basal epithelial cells in sections incubated for 24 h (Fig 3). This proteolysis was completely inhibited in gels prepared with anti-tPA IgG (25, 50, 100 μg/ml), but was not inhibited by inclusion of anti-uPA IgG or normal goat IgG (25, 50, 100 μg/ml). Identical results were obtained from lesional skin of three patients with psoriasis in three independent experiments. These data demonstrate that the PA activity detected in psoriatic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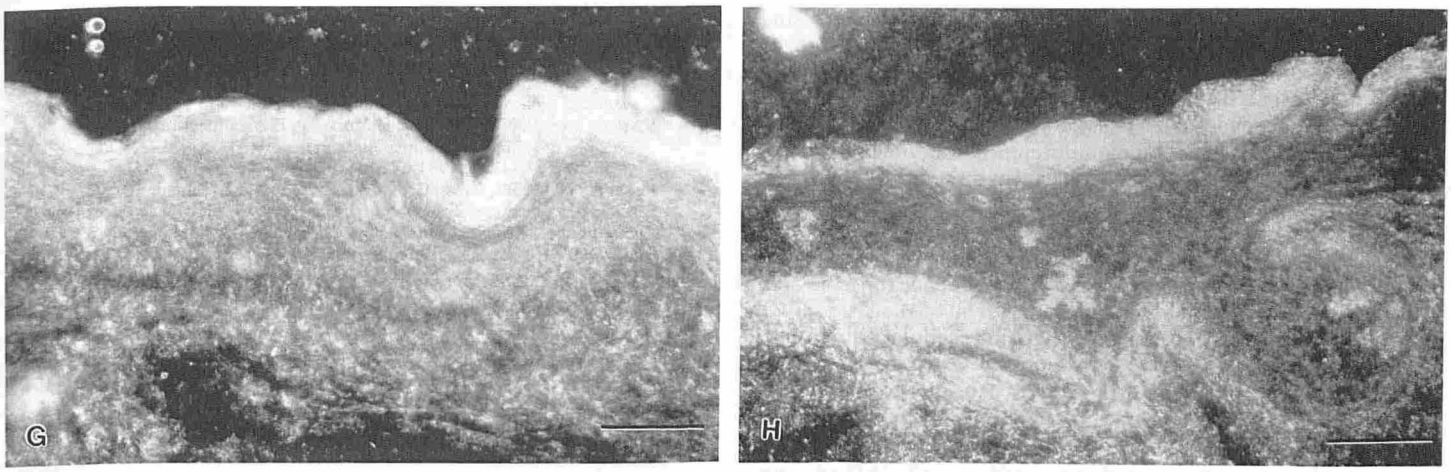

Figure 3. Characterization of PA activity in psoriatic versus normal epidermis. *In situ* zymography was performed using cryostat sections of psoriatic and normal epidermis. In psoriatic epidermis, no caseinolysis was observed in plasminogen-free gels *(A)*, whereas clearing was seen in gels containing plasminogen *(B)*. Neutralizing anti-tPA IgG (50 μg/ml) completely inhibited caseinolysis in the presence of plasminogen *(C)*. No inhibition occurred with normal goat IgG (100 μg/ml) *(D)*. In normal epidermis, no caseinolysis was observed in plasminogen-free gels *(E)*, whereas clearing was seen in gels containing plasminogen *(F)*. Neutralizing anti-uPA IgG (25 μg/ml) completely inhibited caseinolysis in the presence of plasminogen *(G)*. No inhibition occurred with normal rabbit IgG (25 μg/ml) *(H)*. *A–H)* dark field microscopy.

epidermis is predominantly tPA. This finding agrees with the results of zymographic analyses of extracts prepared from psoriatic epidermis [2,3]. Additionally, these studies localize PA activity to the suprabasal layers of the psoriatic lesion.

For normal epidermis, the caseinolysis was apparent throughout the epidermis, sparing only the stratum corneum. Clearing was most pronounced over the basal layer. Proteolysis was completely inhibited with the inclusion of anti-uPA IgG (25, 50, 100 μg/ml). No inhibition of caseinolysis was observed in the presence of anti-tPA IgG (25, 50, 100 μg/ml) or normal rabbit IgG (100 μg/ml). No caseinolysis was detected with gels containing amiloride (1 mM), a specific inhibitor of uPA but not tPA (data not shown) [8]. For normal epidermis, identical results were obtained from two patients in three experiments. These data confirm that the PA activity in normal epidermis is predominantly uPA [2]. Finally, the localization of PA activity to the basal area of the normal epidermis is notably different from that of psoriatic tissues.

DISCUSSION

Previously, the modulation of PA enzymatic activities and mRNA expression in psoriatic epidermis were quantitatively assessed using zymographic analyses of epidermal extracts and Northern hybridization, respectively [2–4]. Additionally, immunohistochemical studies have demonstrated that psoriatic lesions stained focally for uPA in the basal epidermal cells of the suprapapillary plates, whereas tPA was localized to the mid to upper stratum spinosum [11]. Neither uPA nor tPA is detected in normal epidermis by immunocytochemistry [11]. Nonetheless, biochemical analyses of epidermal extracts provide evidence for the presence of both uPA and tPA activity [2]. Thus, the enzymatic assays are tremendously more sensitive than immunocytochemical techniques for their detection.

The studies reported herein confirm and extend these observations. With *in situ* hybridization, we show the localization of mRNAs for uPA and tPA in psoriatic lesions. The transcript for tPA was broadly distributed throughout all layers of the epidermis, with more pronounced areas in the uppermost portions. The message for uPA was focal and primarily present in the basal layer. Thus, the localization of uPA and tPA mRNAs within the psoriatic epidermis is largely in agreement with the previously reported distribution of uPA and tPA antigens [11].

The inability to detect uPA transcript in normal epidermis by *in situ* hybridization, although uPA mRNA is apparent in normal epidermis using Northern analyses, suggests that uPA mRNA is expressed at low levels and is not concentrated in a sub-population of the epidermal cells. Messages of low expression may be difficult to detect by *in situ* hybridization consequent to background signal. Likewise, the inability to detect enhanced levels of uPA mRNA by Northern analyses of mRNA from psoriatic epidermis likely reflects the discrete localization of the induction of uPA expression. Data from Northern analyses reflect changes in mRNA levels for the total epidermal cell population, whereas, *in situ* hybridization shows changes in mRNA expression at the cellular level.

With the *in situ* zymography technique, we have exploited the high sensitivity of enzymatic analyses for detection of plasminogen activators. Our studies are in concordance with biochemical studies showing that uPA is the predominant PA in epidermal extracts of normal skin. Likewise, our *in situ* zymography results for psoriatic lesions are consistent with studies showing that tPA activity is the predominant PA activity detected in extracts prepared from psoriatic plaques [2,3]. The *in situ* zymography studies reported herein additionally demonstrate that the localization of PA enzymatic activity is altered within the psoriatic epidermis.

Previous studies in which autohistographic fibrin film was used to measure PA activity *in situ* also showed tPA activity in psoriatic plaques [12]. With this technique, it was shown that a reduction in tPA-mediated fibrinolytic activity occurred with disease clearing consequent to a diversity of systemic and local treatments (betamethasone valerate, anthralin, PUVA, hydrocolloid dressing). The localization of tPA activity within the psoriatic epidermis was not apparent by this technique; however. Additionally, PA activity in normal epidermis was not detected, probably consequent to the low sensitivity of the autohistogram fibrin film technique for uPA activity [12].

The inability to detect uPA activity in the basal cell layer of the psoriatic epidermis where there is uPA message and antigen suggests that the enzyme is in an inactive form, as one might expect if the uPA were bound to a plasminogen activator inhibitor (PAI). Keratinocytes in culture express PAI-1 and PAI-2 as well as both uPA and tPA [13]. Additionally, exudation of PAI-1 from the serum may occur with the vasodilatation and inflammation characteristic of the psoriatic lesion. Thus, we hypothesize that the uPA present in the psoriatic epidermis is inactive consequent to complex formation with either of these inhibitor proteins. Studies to localize message, antigen, and activity for PAI-1 and PAI-2 are in progress to explore this possibility.

The characteristic histopathology of psoriasis includes acan-

thosis, regular elongation of the rete ridges, hypogranulosis, and parakeratosis admixed with neutrophils. These changes are believed to arise consequent to incomplete differentiation of the keratinocytes of the superficial epidermis. The induction of PA expression in the basal layer provides evidence for a phenotypic change in the keratinocytes at the initial stages of differentiation. *In vitro,* characteristic alterations in PA expression occur with keratinocyte differentiation [13,14]. Using the skin equivalent model, expression of uPA is detected in the basal layer, whereas tPA is identified in suprabasal layers [14]. Thus, the studies reported herein in conjunction with *in vitro* studies provide compelling evidence for stage-specific expression of PA enzymes.

Proteases have been postulated to affect intercellular adhesion of keratinocytes in disorders characterized by spongiosis and spongiform pustules. Psoriatic lesional skin has been shown to have elevated elastase activity, a serine protease originating from polymorphonuclear leukocytes [15]. Skin-derived antileukoproteases (SKALPs) are also present in psoriatic epidermis and may be associated with modulation of the inflammatory process [16,17]. Human leukocyte elastase activity is not specific to psoriasis, however, as it has been noted in lesional skin of atopic dermatitis and contact dermatitis [15]. Whereas aberrant PA profiles have been noted in a variety of cutaneous disorders such as Hailey-Hailey disease, pemphigus vulgaris, and psoriasis, it is possible that site-specific alterations in cellular sources may be disease related and potentially more specific.

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