Skin-derived antileukoproteinase (SKALP) is decreased in pustular forms of psoriasis. A clue to the pathogenesis of pustule formation?

Abstract Skin-derived antileukoproteinase (SKALP, also known as elafin) is an inducible epidermal serine proteinase inhibitor, that we have recently characterized at the protein and DNA levels. SKALP is a strong and specific inhibitor of PMN elastase, and is putatively involved in the regulation of cutaneous inflammatory processes. In order to investigate the role of SKALP in the control of elastase in psoriatic epidermis, we compared SKALP expression in normal skin, and in skin from patients with chronic plaque psoriasis and pustular forms of psoriasis. Epidermal scales and biopsies were collected and SKALP expression was studied at the mRNA level and at the protein level both functionally and immunochemically. In epidermal scales, we found that the levels of both free and total SKALP activity in pustular psoriasis were far lower than in plaque psoriasis. A significant number of pustular psoriasis patients showed latent SKALP activity, which represents the amount of SKALP putatively complexed to elastase. In addition, we found free elastase activity in 25% of the pustular psoriasis patients, indicating a total saturation of epidermal SKALP activity. In epidermal biopsies from pustular psoriasis patients, SKALP activity was significantly decreased compared with those from plaque psoriasis patients. Northern blot analysis did not reveal differences in epidermal mRNA levels between chronic plaque psoriasis and pustular psoriasis. We hypothesize that a reduced amount of epidermal SKALP contributes to an imbalance between elastase and its inhibitor, thereby promoting the formation of epidermal pustules. We suggest that these findings could provide a rationale for the treatment of pustular psoriasis with inhibitors of PMN-derived proteinases, as a new therapeutic modality.

Key words Proteinase inhibitor • Polymorphonuclear leukocyte

Introduction

Several cutaneous manifestations of psoriasis are known [1]. Erythematous lesions can vary in size from pinpoint to large plaques or even erythroderma. Psoriatic lesions can also be present as pustules rather than the more usual plaques. These lesions may be localized (persistent pustulosis palmar-plantaris) or generalized (generalized pustular psoriasis). The aetiology of psoriasis (or its subforms) is unknown. Both a defect in growth control mechanisms of keratinocytes [2], and an underlying (auto)immune process have been implicated [3]. Recently, alterations in cytokine production and responsiveness to cytokines have been described which may be causally related to the disease process [4].

One of the main features of psoriatic lesions is the presence of polymorphonuclear leukocytes (PMN) in the epidermis, either scattered or in the form of micropustules or microabcesses. In pustular forms of psoriasis an extreme local infiltration of PMN occurs. Although it is unlikely that PMN infiltration is a primary event related to the aetiology of the disease, the formation of spongiform pustules resulting from PMN infiltration is considered a disease-specific process. Activated PMN induce tissue damage, and perpetuate the inflammatory response by the secretion of proteinases (e.g. elastase), reactive oxygen metabolites, and prostanoids. We have shown in previous studies that most antipsoriatic agents interfere in vivo with leukotriene B4-induced PMN infiltration in human skin, suggesting that this might be one of the antipsoriatic mechanisms of corticosteroids, dithranol, retinoids and methotrexate [5].

Recently we have described a new, inducible, keratinoocyte-derived serine proteinase inhibitor found in psoriatic skin but absent in normal skin [6–8]. The inhibitor, termed skin-derived antileukoproteinase (SKALP), has been shown to be a potent inhibitor of human leukocyte elastase (HLE). HLE is a PMN-derived neutral proteinase implicated in
PMN migration and tissue destruction in many conditions including lung emphysema [9], arthritis [10], glomerulonephritis [11] and bullous dermatoses [12, 13]. We have characterized SKALP, which is also known as elafin [14, 15] or ESI [16], at the biochemical level [7], and have shown its presence in urine and serum of psoriatic patients [17, 18]. Furthermore, we have located its expression in differentiated epidermal keratinocytes [8]. The SKALP cDNA and gene have been cloned [19, 20] and we have been able to assign the chromosomeal localization of the SKALP gene to chromosome 20q [21]. The gene has been given the approved name of protease inhibitor, skin derived (SKALP), symbol P13, in the Genome Data base of the HUGO nomenclature committee.

Although the induction of SKALP in inflammatory dermatoses (e.g. psoriasis) and its substrate specificity suggest that SKALP is involved in the regulation of cutaneous elastase activity, no data so far exist to support this presumed role. We reasoned that under conditions of excessive elastase secretion, SKALP should be present in a complexed form with elastase. In extreme cases, when all available SKALP is consumed, one would expect free elastase activity. We therefore investigated the amounts of free and latent SKALP in scales and biopsies from patients with chronic plaque psoriasis or pustular psoriasis. To study local differences in one individual, biopsies were taken from lesional skin with pustules and without pustules. Different methods were used for studying SKALP levels including functional measurement, immunohistochemical techniques, immunohistochemical staining and Southern blot analysis. We indeed found that in the majority of the patients with pustular forms of psoriasis, SKALP was decreased at the protein level. The mechanism of pustule formation, and the therapeutic implications of these findings are discussed.

Materials and methods

Patients

Scales were collected from 16 patients with chronic plaque psoriasis and 19 patients with pustular psoriasis, either localized or generalized (acrodermatitis continua of Hallopeau n = 1; pustulosis palmoplantaris, n = 13; generalized pustular psoriasis von Zumbusch type, n = 2; nongeneralized pustular psoriasis, n = 3). All patients had active disease. Keratome biopsies (thickness 0.4 mm, area 1 cm²) were used for RNA and protein isolation. Tissue was harvested from the skin of the back and from clinically involved skin of six patients with plaque psoriasis. Two keratome biopsies were taken from six patients with pustular psoriasis (acrodermatitis continua of Hallopeau, n = 1; pustulosis palmoplantaris, n = 5; erythroderma with pustule formation, n = 2), one from a skin region with macroscopic pustules and one from a region without pustules. Punch biopsies (4 mm) for histology were taken from a patient with annular pustular psoriasis and from a patient with generalized pustular psoriasis. Before the biopsies were taken, permission of the medical ethical committee and written informed consent from the patients were obtained. Prior to analysis, scales and keratome biopsies were stored at −20°C and −80°C, respectively. Punch biopsies were fixed in buffered 4% formalin for at least 24 h and processed for routine histology. Tissue was embedded in paraffin and 5-μm sections were cut.

Preparation of the antisera

An antiserum against recombinant SKALP (a kind gift from Dr. N. Russell, ICI Pharmaceuticals, UK) was obtained as described previously [8]. An antiserum against a synthetic peptide comprising amino acid 23 to 36 of preSKALP (Eurosequence, Groningen, The Netherlands) was prepared. This peptide was coupled via a C-terminal cysteine residue to chicken ovalbumin (Sigma, St Louis, Mo., USA), using the scintillation-phosphor coupling reagent (Rochford, Ill., USA). This preparation was used for immunization of a rabbit according to previously described protocols [7]. Preimmune serum was drawn as a control.

Preparation of scale extracts

Scales were processed for determination of SKALP activity as described previously [6]. In brief, scales were weighed and homogenized (100 mg/ml) in a glass-glass grinder in a phosphate-buffered saline (PBS) and centrifuged (15 min, 12 000 g). The clear supernatant was divided into two equal portions. One portion was boiled for 2 min to inactivate elastase and to liberate the elastase-bound inhibitor and then again centrifuged (15 min, 12 000 g). Colorimetric quantification of the protein concentration was performed using the Bio-Rad DC Protein Assay according to the recommendations of the supplier (Bio-Rad Laboratories, Richmond, Calif., USA).

Protein and RNA extraction

From the keratome biopsies RNA and protein were simultaneously isolated using Tri Reagent (MRC, Cincinnati, Ohio, USA), according to the manufacturer’s guidelines. In brief, samples were homogenized in 1 ml Tri Reagent and separated with chloroform in an aqueous phase, an interphase and a phenol-chloroform phase. RNA was precipitated in the aqueous phase with 0.5 ml isopropanol, washed with 75% ethanol and subsequently after air drying dissolved in XNSE buffer (50 mM NaAc, 0.2% SDS, 2 mM EDTA). Samples were stored after adding 100% ethanol at −80°C until further study. After precipitation of DNA with 100% ethanol, proteins were precipitated from the phenol ethanol supernatant with isopropanol. The protein pellet was washed three times in 0.3 M guanidine hydrochloride in 95% ethanol and once with 100% ethanol. After vacuum drying, the protein pellet was dissolved in PBS and stored at 4°C. Colorimetric quantification of the protein concentration was performed using the Bio-Rad DC Protein Assay.

Functional measurement of SKALP activity in scales and biopsies

Because of the complex situation of both free elastase and elastase complexed with inhibitor, we used a protocol previously described for the measurement of anti-elastase activity in urine [17] based on the differential heat stability of elastase and SKALP. Elastase is denatured by boiling the scale extract for 2 min, whereas SKALP is heat stable. The bound elastase inhibitor (latent SKALP) is released from the enzyme-inhibitor complex. In all scale extracts we measured the free SKALP activity in untreated samples and the total (latent plus free) SKALP activity in heat-inactivated samples. In protein solutions of biopsies only total SKALP activity could be measured because of the denaturing isolation procedure.

Serial dilutions were made from the samples in assay buffer (1 M NaCl, 0.4% CTAB, 0.1 M Tris, pH 8.5). Elastase activity was measured as the release of the fluorogenic substrate methoxy-succinyl-Ala-Ala-Pro-Val-Ala-NH₂ (MAAPV-AMC, Sigma, St Louis, Mo., USA) as described previously [11]. Fluorescence was measured at 375 nm (ex) and 440 nm (em) in a Perkin-Elmer LS-5 fluorimeter.

SKALP activity was measured as the percentage inhibition of a standard amount of elastase using the same assay. An extract of 500 PMN in assay buffer (equivalent to approximately 1 ng HLE) to a total volume of 10 μl was mixed with 10 μl serial dilutions of
scale extract and then incubated for 15 min at 37°C. Elastase substrate solution (20 μl, 250 μM) was then added and the reaction was allowed to proceed for 15 min at 37°C. The incubation was terminated by the addition of 1 ml stop buffer (100 mM Na2CO3; pH 10.5). Inhibition of elastase activity in this assay was calculated as the percentage inhibition of the activity of 500 PMN, within the linear range of the assay. Recombinant SKALP (a kind gift from Dr. N. Russell, ICI Pharmaceuticals, UK) was used to obtain a calibration curve from which the SKALP concentration in the scale extracts was read. After correction for protein concentration, SKALP activity was expressed as nanograms SKALP per milligram protein.

Enzyme-linked immunosorbent assay for measurement of SKALP levels

SKALP concentrations were measured in scale and biopsy extracts, using a competitive-type ELISA, as described previously [18]. Scale extracts were boiled and supernatants were taken for quantification of SKALP. Biopsy isolates were measured directly. In brief, the following mixture was prepared to contain 80% sample: 0.1 M Tris, 0.1% Tween-20, 1% BSA, and rabbit antiserum against recombinant SKALP (1:9500 diluted, and incubated overnight. Microtitre plates (96 flat bottom wells) were coated overnight with 50 ng/ml recombinant SKALP in PBS. After washing of the plate with PBS/0.05% Tween-20, the microtitre plates were blocked, probed with the test samples, and developed for 60 min with peroxidase-conjugated swine-antirabbit Ig using α-phenylenediamine dihydrochloride (OPD) as chromogenic substrate for 15 min. Human recombinant SKALP in PBS with 0.1% BSA was used as a standard; a calibration curve was prepared using recombinant SKALP in the range of 2.9–36 ng/ml. The SKALP concentrations in scales or biopsy samples were read from this curve. All ELISA steps were performed at 4°C, except development with OPD which was done at room temperature. The results were read with a Biorad ELISA reader, and evaluated using the Excel spreadsheet program. After correction for protein concentration, SKALP activity was expressed in nanograms SKALP per milligram protein.

Northern blot analysis

For Northern blot analysis, 10 μg of total RNA was electrophoresed on a 1.4% agarose gel following standard procedures [22]. In brief, 10 μg total RNA was dissolved in 3 μl RNase-free water (prepared using a Milli-Q ultrafiltration apparatus, Millipore), and then 2 μl 0.1 M sodium phosphate (pH 7.0), 10 μl dimethyl sulphoxide (DMSO) and 4.5 μl 6 M glyoxal was added. The mixture was incubated for 60 min at 50°C. After cooling the RNA samples to 0°C, 3.5 μl glyoxal /DMSO gel-loading buffer was added and the mixture immediately loaded into the wells of the gel. The gel was poured and run in 10 mM sodium phosphate. The buffer was recirculated to maintain the pH within acceptable limits. The gel was then blotted by capillary transfer onto a nylon membrane (Boehringer, Mannheim, Germany). After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm²). Hybridization was performed as previously described, using a 0.42 kbp PvuI/EcORI fragment of the SKALP cDNA clone pGESKA as a probe [19]. Control hybridizations for equal loading were performed using a human β2S submaxillary RNA probe. The probe was labelled with [3P] by random priming, following standard procedures. Autoradiography was done using an X-Omat S film (Kodak, France) at -80°C with an intensifying screen.

Histology and immunohistochemistry

Paraffin sections were deparaffinized, rehydrated and immunostaining was performed according to a previously described method [8]. First, the sections were preincubated with normal swine serum and incubated with antiserum against recombinant SKALP at a dilution of 1:100. Detection of SKALP with an antiserum raised against a synthetic peptide gave an essentially similar localization pattern (not shown). After incubation with peroxidase-conjugated swine antirabbit Ig (Dako, Glostrup, Denmark), the sections were developed with aminoethylcarbazole as the chromogenic substrate. Preimmune serum from the immunized rabbit was used as a control.

Results

SKALP in scale extracts

In all samples we measured the free SKALP activity in untreated samples and the total (latent plus free) SKALP activity in heat-inactivated samples using a functional assay. The values in the scales of patients with pustular psoriasis (n = 19) and patients with chronic plaque psoriasis (n = 16) are presented in Fig. 1. A remarkable finding was that SKALP activity was significantly lower in the pustular psoriasis group than in the plaque psoriasis group (P < 0.01, Mann-Whitney test). This was found both for total SKALP and for free SKALP. Heat inactivation of the scale extracts revealed latent SKALP activity in a considerable number of patients in the pustular psoriasis group, while in the plaque psoriasis group no latent SKALP was found. In contrast, SKALP activity was higher after direct measurement of the scale extracts than after heat inactivation.

In some patients SKALP activity was undetectable; in these patients free elastase activity was measurable. In the pustular psoriasis group this was found in five patients out of 19 (elastase activity equivalent to 41 ± 29 × 10³ PMN per mg scales), and in the plaque psoriasis group only one patient was found with a small amount of free elastase (6000 PMN per mg scales). Remarkably, in both patients with generalized pustular psoriasis of the von Zumbusch type, free elastase activity was found. In all six patients with free elastase activity, latent SKALP activity could be recovered after heat inactivation of elastase (not shown).

SKALP levels as measured using the ELISA in the supernatant boiled scale extracts were the same as those obtained using the functional assay. The SKALP concentration was significantly lower in patients with pustular psoriasis than in those with plaque psoriasis (P < 0.01, Mann-Whitney test). The SKALP levels measured using the ELISA were within the same range as those found with the functional assay, indicating that the anti-elastase activity found was indeed largely due to SKALP.

SKALP in skin biopsies

Biopsies were taken from six healthy volunteers and from the involved skin of six patients with chronic plaque pso-
Fig. 1 SKALP concentration in scale extracts from patients with chronic plaque psoriasis (n = 16) and pustular psoriasis (n = 19). SKALP was determined using a functional assay after heat inactivation, i.e. boiling for 2 min to inactivate elastase and to liberate the elastase-bound inhibitor, (filled bars) and directly (hatched bars). The open bars represent SKALP concentration as measured using an ELISA. SKALP levels were significantly lower in the pustular psoriasis group than in the plaque psoriasis group. This was found with functional measurements as well as with ELISA (P < 0.01, Mann-Whitney test). Heat inactivation resulted in a significant increase in SKALP concentration in the pustular psoriasis patients (P < 0.03, Wilcoxon Signed Ranks’ test for paired samples). Bars means ± SE

Fig. 2 SKALP concentration in epidermal biopsies from healthy controls (n = 6), patients with chronic plaque psoriasis (n = 6) and patients with pustular psoriasis (n = 6). From the last group, two biopsies per patient were taken: one from a region with macroscopic pustules (pustular +) and one from a region without pustules (pustular −). SKALP was measured using a functional assay (filled bars) and an ELISA (open bars). SKALP activity as measured using the functional assay was significantly lower in the pustular + biopsies than in plaque psoriasis biopsies (P < 0.01, Mann-Whitney test). Bars means ± SE

Riasis. In six patients with pustular psoriasis one biopsy was taken from a region with pustules and one from an involved region without pustules to check for local differences in the same individual. These biopsies were processed for simultaneous extraction of both the protein and RNA. The denaturing isolation procedure led to the measurement of total SKALP in all protein fractions. SKALP activity as obtained from functional measurements of anti-elastase activity is shown in Fig. 2. In normal skin no SKALP activity was found, whereas in psoriatic skin high levels were found. SKALP activity in biopsies from the pustular region of pustular psoriasis patients was significantly decreased compared with the activity in plaque psoriasis (P < 0.01, Mann-Whitney test). The ELISA showed lower SKALP levels in both the pustular region and the involved skin without pustules of pustular psoriasis patients than in plaque psoriasis patients, but the difference did not reach significance.

Northern blot analysis

Figure 3 shows the expression of SKALP mRNA in the biopsies from normal skin, lesional skin from chronic plaque psoriasis patients and lesional skin from pustular psoriasis patients. In plaque psoriatic skin a strong expression of SKALP mRNA was found (lanes 3 and 4), whereas the normal skin showed no expression (lanes 1 and 2). In paired biopsies from three patients with pustular psoriasis (lanes 5 and 6, 7 and 8, 9 and 10, respectively) similar levels of SKALP mRNA expression were found as in psoriatic skin. The expression in pustular regions (lanes 5, 6 and 9) did not differ from the areas without macroscopic pustules (lanes 6, 8 and 10).
Histology and immunohistochemistry

We have previously reported that normal human skin and nonlesional psoriatic skin are negative for SKALP. Lesional skin of chronic plaque psoriasis shows cytoplasmic staining of the upper layers of the suprabasal compartment [8]. Here we performed immunohistochemical staining for SKALP in lesions of pustular psoriasis patients and compared the expression patterns with those obtained from psoriasis lesions. The SKALP expression pattern was similar in pustular and plaque psoriasis, i.e. basal cells were negative and there was positive staining in the upper suprabasal layers. In the epidermis near pustules, SKALP expression was clearly, but variably, affected by the presence of large amounts of PMN. Figure 4 shows that several layers of SKALP-positive cells were present in the intact epidermis underlying a subcorneal pustule. No damage to the epidermis was evident. Figure 4b shows a pustule where the upper layers of the epidermis were destroyed; here, only a few SKALP-positive cells were present with low levels of expression.

Discussion

One of the putative functions of PMN elastase is to facilitate migration through connective tissue and basal membranes, towards inflammatory foci. Speculatively, SKALP could counteract this process since it is synthesized in the upper epidermis and provides a gradient of anti-elastase activity from epidermis to dermis. In support of this concept, we and others have indeed recently shown that SKALP can be found in the urine [17, 23] and serum [18] in psoriatic patients. In our view, the induction of SKALP in human epidermis is a negative feedback on cutaneous inflammation, to control PMN migration and protect against proteolysis of structural proteins.

In human skin at least three different high-affinity elastase inhibitors can be present simultaneously, either free or complexed: alpha-1-antitrypsin (alpha-1-AT), secretory leukocyte protease inhibitor (SLPI) and SKALP. From previous studies we know that SLPI activity in scales is low compared to SKALP, and disturbance by alpha-1-AT activity is eliminated in the assay by the presence of cetyltrimethylammonium bromide (CTAB). We therefore assume that most anti-elastase activity measured in our assay represents SKALP activity. Furthermore, we are dealing in the epidermis with a complex situation. In psoriatic scales both free elastase and elastase complexed with inhibitor was found, and direct quantification of the total amount of biologically active SKALP was not possible. Therefore, we used a protocol, previously described for the measurement of anti-elastase activity in urine [17], which is based on the differential heat stability of elastase and SKALP.

In this study we examined the amounts of SKALP in scales and biopsies of psoriatic patients, either of the plaque type of pustular type of psoriasis. Most striking was the finding that in scale extracts the levels of free and...
total SKALP were considerably lower in pustular psoriasis than in plaque psoriasis; this was highly significant in all subtypes of pustular psoriasis, despite the large variation in levels of SKALP in each group. Furthermore, an unexpected finding was that SKALP activity after boiling of the scale extracts was lower than after direct measurement. We have previously studied [17] the effect of boiling on elastase activity. Boiling for 1–2 min gives an optimal recovery of anti-elastase activity. Prolonged boiling causes denaturation of SKALP. This could be a fraction of SKALP that is already in a partly denatured state. Therefore, a possible explanation for this observation is that a certain amount of SKALP is denatured by boiling the scale extracts. Alternatively, it could be due to the presence of small amounts of other heat-labile proteinase inhibitors.

In biopsies from pustular psoriasis patients from a region with pustules, SKALP levels as measured using the functional assay were significantly decreased compared with plaque psoriasis. This discrepancy is not related to differences in the body region, since in both scales and biopsies from patients with generalized pustular psoriasis (obtained from trunk and extremities) and in samples from pustulosis palmoplantaris patients (all obtained from the palms) low amounts of free and total SKALP were found. In scales from five patients with pustular psoriasis no free SKALP activity could be measured. In these cases free elastase activity was found, indicating that all available SKALP was saturated with elastase. Latent SKALP in these scales was liberated by heat inactivation of the scale extracts. This phenomenon has been found previously in urine from patients with severe erythrodermic psoriasis [17]. Apart from these five patients in whom all SKALP activity was in a latent form, we found that a considerable number of the pustular psoriasis patients had SKALP partially in a latent form. These findings support the notion that SKALP has a biological function in vivo to control elastase activity. Interestingly, it has recently been reported by others [24] that free elastase activity can be extracted from lesional psoriatic skin by a noninvasive method, suggesting that in the stratum corneum of plaque type psoriasis a local imbalance of elastase and inhibitor might also exist.

From our results we clearly can not conclude whether the appearance of pustules is a consequence of saturation of epidermal SKALP with elastase, or vice versa. We would hypothesize that in psoriatic skin (or under inflammatory conditions in general) SKALP activity in the epidermis attenuates accumulation of PMN in the epidermis. In the case of a large, continuous PMN influx with subsequent secretion of elastase, SKALP is consumed by complex formation with elastase. Whether SKALP-elastase complexes are cleared by endocytosis as described for other proteinase inhibitors [25], is not known. However, this could explain the low amounts of SKALP in pustular psoriasis patients. Gene expression of SKALP as visualized by Northern blot analysis demonstrated similar amounts of mRNA in pustular and plaque psoriasis, and in both a transcript length of approximately 0.8 kb was found. These findings make it less likely that the observed low SKALP levels have a genetic basis, although this possibility cannot be totally dismissed.

The role of SKALP in the mechanism of pustule formation is unclear. Speculatively, SKALP consumption could lead to local SKALP deficiencies which allow the formation of pustules of Kogoj or microabscesses of Munro as seen in psoriasis vulgaris. In extreme cases, macroscopic pustules arise as seen in pustular forms of psoriasis. Three basic findings in patients with pustular psoriasis support this concept: (1) the presence of latent (complexed) SKALP in scale extracts, (2) the presence of free elastase in 25% of the patients, and (3) low levels of SKALP in pustular psoriasis.

The fact that in the pustular psoriasis group not all patients showed significant amounts of latent SKALP activity in the scale extracts could be explained by the methodology. We used scales obtained from the entire surface of the trunk or the hand. Since pustule formation is a localized phenomenon, the information we obtain from our measurements is an average in which the information concerning the local situation of a pustule is ‘diluted’. Furthermore, we showed with immunohistology that SKALP expression can vary between individual patients.

Irrespective of the mechanism for SKALP deficiency, which could have a genetic basis or be an extracellular mechanism, it is clear that it would lead to excessive proteolysis of structural epidermal proteins. The data from the present study provide evidence that control of elastase activity is a pathogenic factor in pustular forms of psoriasis. We would hypothesize that the use of (preferably topical) low molecular weight drugs with anti-elastase activity could be a new therapeutic modality in pustular forms of psoriasis.

References