Constitutive and Inducible Expression of SKALP/Elafin Provides Anti-Elastase Defense in Human Epithelia

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Abstract

Skin-derived antileukoproteinase (SKALP), also known as elafin, is a serine proteinase inhibitor first discovered in keratinocytes from hyperproliferative human epidermis. In addition to the proteinase inhibiting domain which is directed against polymorphonuclear leukocyte (PMN) derived enzymes such as elastase and proteinase 3, SKALP contains multiple transglutaminase (TGase) substrate domains which enable crosslinking to extracellular and cell envelope proteins. Here we show that SKALP is constitutively expressed in several epithelia that are continuously subjected to inflammatory stimuli, such as the oral cavity and the vagina where it co-localizes with type 1 TGase. All epithelia from sterile body cavities are negative for SKALP. In general, stratified squamous epithelia are positive, whereas pseudostratified epithelia, simple/glandular epithelia and normal epidermis are negative. SKALP was found in fetal tissues of the oral cavity from 17 wk gestation onwards where it continued to be expressed up to adult life. Remarkably, in fetal epidermis SKALP was found from week 28 onwards, but was downregulated to undetectable levels in neonatal skin within three months, suggesting a role during pregnancy in feto-maternal interactions or in the early maturation phase of the epidermis. Immunoelectron microscopy revealed the presence of SKALP in secretory vesicles including the lamellar granules. In culture models for epidermal keratinocytes we found that expression of the endogenous SKALP gene provided protection against cell detachment caused by purified elastase or activated PMNs. Addition of exogenous recombinant SKALP fully protected the keratinocytes against PMN-dependent detachment whereas superoxide dismutase and catalase were only marginally effective. These findings strongly suggest that the constitutive expression of SKALP in squamous epithelia, and the inducible expression in epidermis participate in the control of epithelial integrity, by inhibiting PMN derived proteinases. (*J. Clin. Invest.* 1996. 98:1389–1399.) Key words: proteinase inhibitor • epithelium • epidermis • elastase • polymorphonuclear leukocyte

Introduction

Systemic regulation of proteinase activity is performed by numerous plasma-derived inhibitors that are involved in regulation of complement activation, clotting, and fibrinolysis. In addition to these systemic antiproteinases a number of locally acting inhibitors is known. These include inhibitors of cysteine proteinases such as the cystatins (1), and tissue inhibitors of metalloproteinases (2). Although the vast majority of the serine proteinase inhibitors is found in the circulation, a limited number of this class is locally active at the tissue level. Secretory leukocyte proteinase inhibitor (SLPI),[1] an inhibitor of the polymorphonuclear leukocyte derived proteinases elastase and cathepsin G, is produced by cells of mucosal surfaces and is found in the corresponding epithelial lining fluid (3). In previous studies we and others have described an epidermal proteinase inhibitor which was found to be partially homologous to SLPI. This inhibitor, termed skin-derived antileukoproteinase (SKALP), otherwise known as elafin or ESI, is absent in normal epidermis but is highly expressed in psoriatic epidermis and in some epithelial cell lines (4–8). SKALP inhibits PMN-derived elastase and proteinase 3 and is therefore putatively involved in regulation of cutaneous inflammation (9, 10). We have previously characterized SKALP in biochemical and cell biological studies (6, 11). The cDNA and gene of SKALP have been cloned and sequenced, and the chromosomal localization was assigned to chromosome region 20q12-q13 (6, 12, 13). Apart from the COOH-terminal domain which harbors the proteinase inhibiting properties, the SKALP molecule also contains a domain with putative transglutaminase substrate motifs which enable crosslinking to the cornified envelope proteins (6, 14). Previous studies in epidermis, cultured keratinocytes, and human epidermal tumors showed that SKALP expression patterns appear to be similar to that of cytokeratin 16, which is known to be expressed in the context of hyperproliferation (see references 15 and 16 for reviews on epidermal differentiation). We have recently obtained evidence that decreased levels of SKALP could be implicated in the pathogenesis of pustular forms of psoriasis (17). Apart from its presence in inflamed epidermis, SKALP/elafin has been reported in tracheal tissue (18) and in bronchial secretions, although the ex-

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1. *Abbreviations used in this paper:* CK, cytokeratin; KGM/FCS, keratinocyte growth medium supplemented with fetal calf serum; KGM/-GF, keratinocyte growth medium depleted of growth factors; PMN, polymorphonuclear leukocyte; SKALP, skin-derived antileukoproteinase; SLPI, secretory leukocyte proteinase inhibitor; SOD, superoxide dismutase; TGase, transglutaminase.

act cellular source of sputum-derived SKALP/elafin in vivo is unclear (7).

It is not known to what extent SKALP is expressed in normal adult human epithelia, and no functional studies have been performed so far. Here we report that SKALP is constitutively expressed in many adult epithelia that are exposed to environmental stimuli. In these tissues the presence of inflammatory cells is physiological, suggesting that SKALP provides protection against excessive proteolysis. Although normal human epidermis is negative, SKALP was transiently expressed during fetal and neonatal development of the epidermis. In functional assays using cultured cells we observed a protective effect of epithelial SKALP expression against the action of leukocytic proteinases, suggesting that SKALP is involved in the maintenance of epithelial integrity.

Materials and Methods

Materials. Goat anti–rabbit IgG biotin conjugate, avidin-alkaline phosphatase, and the chromogenic substrates 5-bromo–4-chloro–3-indolyl phosphate, nitroblue tetrazolium, Ca-ionophore 23187, and aminoethyl carbazole (AEC) were obtained from Sigma Chemical Co., (St. Louis, MO). Swine anti–rabbit Ig conjugated with horseradish peroxidase was obtained from Dakopatts (Glostrup, Denmark). The Vectastain ABC kits for monoclonal and polyclonal antibodies purchased from Vector Laboratories Inc., (Burlingame, CA). A synthetic peptide comprising amino acid 23 to 36 of SKALP was synthesized by Eurosequence, (Groningen, The Netherlands); at the COOH terminus a cysteine residue was added for coupling to a carrier protein. The sulfo-SMCC kit and Sulfolink gel were obtained from Pierce, (Rockford, IL). RNA zol™ B was obtained from Cinna/Biotex Laboratories, Inc., (Houston, TX), and α-^{32}PdCTP from Amersham, (Amersham, UK). Ficoll-paque was from Pharmacia, (Uppsala, Sweden).

Recombinant SKALP was a kind gift from Dr. Norman Russell (ICI Pharmaceuticals, Macclesfield, UK). As a probe for ribosomal 28S RNA a 2.1-kb EcoR1/BglII fragment was used (a kind gift of Dr. Jan Bauman, TNO Rijswijk, The Netherlands).

Tissues. Archival and fresh biopsy/autopsy material was used for this study. The following adult tissues were studied with respect to SKALP expression: brain; cornea; tongue; palate/lingual tonsil; gingiva; pharynx; larynx; epiglottis; vocal fold; lung; bronchus; esophagus; duodenum; colon; liver; kidney; urethra; uterine cervix; vagina; skin from various regions including head, ear, breast, axillar region, mamilla, inguinal region, pubic area, scrotum, finger, foot sole, and hair follicle. Punch biopsies from adult skin were taken from normal human volunteers and psoriatic patients. Fetal and neonatal tissues were available from: a 9-wk-old embryo which could be examined in toto, two second trimester fetuses, five third trimester fetuses, and two neonates. Specimens used for immunohistochemistry were fixed in buffered 4% formalin for at least 24 h, and embedded in paraffin. Tissues for in situ hybridization and Northern blot analysis were snap frozen in liquid nitrogen and stored at −80°C.

Antisera. A polyclonal antiserum against recombinant SKALP was obtained as described previously (10). An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide via a COOH-terminal cystein residue to chicken ovalbumin, using the sulfo-SMCC procedure according to the manufacturer's instructions. This conjugate was used for immunization of a rabbit according to previously described protocols (19). Control serum (preimmune serum) was drawn before the immunization procedure. The antiserum against the synthetic peptide was affinity purified using the synthetic peptide coupled to Sulfolink coupling gel according to the manufacturer's instructions. The two antisera against recombinant SKALP and against the synthetic peptide gave identical staining patterns in histological sections. A monoclonal antiserum against type 1 transglutaminase was obtained from Sanbio, (Uden, The Netherlands). A monoclonal antibody against human PMN elastase was obtained from DAKO, (Copenhagen, Denmark). Mon150, a monoclonal antibody against involucrin was obtained as previously described (20). Ks8.12, a monoclonal antibody recognizing CK 13 and 16 was from Sigma Chemical Co., a monoclonal antibody against CK 10 (DE-K10) was obtained from ICN.

Immunohistochemistry. Biopsies were fixed in buffered 4% formalin for at least 24 h and processed for embedding in paraffin. Sections (6 μm) were mounted on 3–aminopropyltriethoxy-silane (Sigma Chemical Co.) coated slides. Sections were deparaffinized and rehydrated and used for an indirect peroxidase technique. For antigen retrieval from paraffin sections, the slides were pretreated for 15 min in 0.1% trypsin/0.1% $CaCl_2$, pH 7.8, by 37°C for anti-cytokeratin 10, 30 min in 10 mM citrate buffer for Ks8.12, and for Mon150 a pretreatment of 10 min in 10 mM citrate buffer using a microwave oven (Miele, M720) at 450 W was performed. After preincubation with 20% normal swine or rabbit serum the slides were incubated for 60 min with the antibodies and after washing with PBS they were incubated with peroxidase-conjugated swine anti–rabbit Ig or rabbit anti–mouse Ig for 30 min. A solution of AEC in sodium-acetate buffer, pH 4.9, containing 0.01% H_2O_2 was added for 15 min after preincubation with sodium-acetate buffer pH 4.9. When desirable, the slides were counterstained with Mayer's haematoxylin (Sigma Chemical Co.) and mounted in glycerol gelatin. Appropriate control with preimmune sera or omission of the primary antibodies were performed.

Immunocytochemical staining of human keratinocytes cultured on coverslips. Human keratinocytes were cultured on tissue culture coverslips (Thermanox; Miles Laboratories Inc., Naperville, IL) using different culture conditions (see below). Cells were fixed using either paraformaldehyde (1%) or aceton/methanol (50/50). Fixed cells were stored at −20°C. Before usage the coverslips were transferred to 4°C for 30 min and then dried in air at room temperature. Paraformaldehyde fixed cells were incubated for 15 min in 50 mM NH_4Cl (in PBS, pH 7.5), and aceton/methanol fixed cells were incubated for 15 min in PBS. Cells were incubated for 10 min at 37°C with either 20% normal goat serum for the polyclonal antibodies (anti-SKALP) or 20% normal horse serum for the monoclonal anti-TGase antibody. After this incubation the coverslips were washed for 15 min in PBS and incubated with the primary antibody for 1 h at 37°C. Coverslips were washed for 15 min in PBS and incubated for 30 min at 37°C with either goat anti–rabbit-biotinylated antibody or horse anti–mouse-biotinylated antibody in a dilution of 1:200. After this incubation coverslips were washed and incubated with a complex of avidine and biotinylated horse radish peroxidase (1:50) for 30 min at room temperature. Finally, coverslips were washed for 15 min in PBS and incubated with diaminobenzidine tetrahydrochloride (DAB) substrate (metal enhanced DAB substrate Kit; Pierce; Rockford, IL), and the reaction was terminated by incubating the coverslips in demineralized water. The coverslips were lightly stained with hematoxyline solution (Harris-type; Sigma Diagnostics, St. Louis, MO) and embedded in histological mounting medium (Permount; Fisher Scientific, NJ) before making photographs.

Construction of plasmids and synthesis of RNA probes. We have previously cloned the SKALP cDNA and gene (6, 13). From the cDNA a 251-bp PCR product was obtained (bp +69 to +320, relative to the translation start site), which was subcloned into a pGEM-4 T-vector (Promega, Madison, WI) in both orientations. The plasmid was linearized with PstI to synthesize the antisense cRNA, or in the reverse orientation the control sense cRNA by T7 polymerase, using a digoxigenin RNA labeling kit (Boehringer Mannheim, Germany). The cRNA's were fragmented by limited alkaline hydrolysis and used for in situ hybridization.

In situ hybridization. Frozen sections (10 μm) were placed on superfrost slides (Menzel-Glazer, Braunscheig, Germany) and heated for 2 min at 50°C. The sections were fixed in PBS containing 4% (vol/vol) paraformaldehyde for 10 min at room temperature, air-dried, and rehydrated in 2X SSC 2X 5 min. Sections were then prehybrid-

ized in hybridization buffer for at least 1 h, consisting of 4X SSC, 10% dextrane, 50% formamide, 0.5 mg herring sperm DNA (Boehringer GmbH, Mannheim, Germany), 2 mM EDTA and 1X Denhardt's solution. Prehybridization and hybridization was performed at 37°C. Prehybridization fluid was drained off and the slides were washed in 2X SSC. Hybridization was carried out in humidity chambers using the probe in a concentration of 100 ng/ml hybridization buffer. After overnight incubation sections were washed in 2X SSC at 37°C followed by washes in 0.1X SSC/50% formamide three times for 7 min. Hybridizations signals were detected using a Nucleic Acid Detection Kit (Boehringer GmbH). Hybridized sections were processed as follows: sections were washed for 5 min in buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5) at room temperature followed by incubation in buffer 1 containing 1% blocking mix for 30 min. Anti-digoxigenin–antibody conjugate dilute 1:200 with buffer 1 containing 1% blocking mix was applied to each section at room temperature for 2 h in a humidified chamber. Slides were then washed in buffer 2 (100 mM Tris, 50 mM MgCl, 100 mM NaCl, pH 9.5). A solution of 5-bromo-4-chloro-indolyl phosphate and nitrobluetetrazolium (Sigma Chemical Co.) in buffer 2 was made immediately prior to use, filtered and placed in a light-proof glass tray. Sections were developed overnight at room temperature. The sections were washed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), counterstained with methylene green, and mounted using gelatin/glycerol.

RNA isolation and Northern blot analysis. Total RNA from cultured cells or different tissues obtained from autopsy or biopsy were extracted with RNAzol B as suggested by the supplier. The following epithelial tissues were studied: tongue, pharynx, larynx, epiglottis, vocal fold, lung, duodenum, colon, liver, kidney, urethra, and inguinal skin. For Northern blot analysis 10 μg total RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde following standard procedures (21) and blotted by capillary transfer on nylon membrane. After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). Hybridization was performed in phosphate buffer as previously described using a 0.42-kbp PvuII/EcoRI fragment of the SKALP cDNA clone pGESKA as a probe (6). Control hybridizations for equal loading were performed using a human 28S ribosomal RNA probe. All probes were labeled with ^{32}P by random priming following standard procedures. Autoradiography was done on X-Omat S film (Kodak, Odijk, The Netherlands) at −80°C with an intensifying screen.

Immunoelectron microscopy. Punch biopsies from normal and lesional psoriatic skin were fixed in 2% paraformaldehyde and divided into small pieces. Cryosubstitution and embedding in Lowicryl HM20 (Agar Scientific Ltd, Essex, England) were performed as follows: fixed tissues were stepwise infiltrated into 30% glycerol and frozen in liquid propane. Dehydration in methanol at −90°C for 48 h, and embedding in Lowicryl HM20 at -50°C were both performed in a cryosubstitution apparatus (CS-auto; Riechert). Polymerization by indirect UV-light was performed for 48 h at -50°C, followed by 24 h at room temperature. Ultrathin skin sections on carbon coated copper grids were preincubated with 0.5% BSA, 0.1% gelatin, and 0.15% glycine in PBS. The grids were subsequently incubated with a polyclonal rabbit antiserum against recombinant SKALP (1:500 in PBS/BSA) or the corresponding preimmune serum at the same dilution, at 4°C for 16 h. After washing the grids were incubated in protein A conjugated to 10 nm colloidal gold particles (1:100 in 1% BSA in PBS), for 60 min at room temperature. After washing the grids were counterstained with uranyl acetate (10 min) and lead citrate (1 min) according to standard protocols. The sections were examined in a JEOL jem1010 electron microscope at 80 kV.

Keratinocyte primary culture. Human epidermal keratinocytes were initially cultured according to the Rheinwald-Green system (22). Primary cultures of keratinocytes were seeded on lethally irradiated (3000 rad, 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 (3:1) (vol/vol) (Flow Laboratories, Irvine, Scotland) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research Inc., Lexington, MA), isoproterenol (10^{-6} M) (Sigma Chemical Co.), 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 6% FCS (Seralab, Nistelrode, The Netherlands), and 10 ng/ml EGF (Sigma Chemical Co.). Cells were grown at 37°C, 95% relative humidity, and 8% CO_2 in air. Liquid nitrogen stored keratinocytes from the primary culture were used in further experiments.

First passage and induction of keratinocyte differentiation. Keratinocytes were seeded at 10^5 cells in keratinocyte growth medium (KGM) as described before (23). KGM was composed of keratinocyte basal medium (KBM; Clonetics, San Diego, CA; 0.15 mM Calcium) supplemented with ethanolamine (0.1 mM) (Sigma, St. Louis, MO), phosphoethanolamine (0.1 mM) (Sigma, St. Louis, MO), bovine pituitary extract (BPE; 0.4% vol/vol) (Clonetics), EGF (10 ng/ml) (Sigma Chemical Co.), insulin (5 μg/ml) (Sigma Chemical Co.), hydrocortisone (0.5 μg/ml) (Collaborative Research Inc.), penicillin (100 U/ml) (Gibco) and streptomycin (100 μg/ml) (Gibco). For induction of differentiation, at confluence the culture medium was switched either to KGM supplemented with 5% FCS or to KGM depleted of growth factors (EGF, BPE, insulin) and hydrocortisone (11, 24). Nondifferentiated keratinocytes, present as a confluent monolayer, were obtained by keeping the cells in KGM alone. After 48 h, the cultures were harvested either from 60 mm dishes (for RNA extraction) or from 24-well plates in which the cells were cultured on coverslips for immunocytochemistry.

Purification of PMN elastase; determination of anti-elastase activity. Human PMN elastase was purified from cells of a patient with chronic myeloid leukemia and calibrated as previously described (4). Elastase activity and anti-elastase activity was measured using the fluorigenic substrate methoxy-succinyl-ala-ala-ala-pro-val-aminomethylcoumarin (Bachem, Bubendorf, Switzerland), as described previously (25). The amount of inhibitor which reduces the activity of 1 nmol elastase with 50% under the given assay conditions, is defined as 1 U of anti-elastase activity.

Cell detachment assays. Keratinocyte cultures on coverslips that were allowed to differentiate for 48 h, were extensively washed with PBS to remove all traces of the induction media. To investigate whether SKALP was secreted under these conditions, the culture medium (serum-free KGM with 1.8 mM Ca^{2+}) was subsequently collected over a 6 h time period, and the amount of SKALP was determined by a sandwich-type ELISA using a rabbit and a goat antiserum against recombinant SKALP. In addition the amount of anti-elastase activity in the medium was determined by a functional assay. PMN were isolated from peripheral blood according to standard procedures involving dextrane sedimentation, separation on a Ficoll-paque gradient and hypotonic lysis. This yielded a suspension of $>$ 95% pure PMN. Subsequently, the keratinocytes were exposed to PMN (2.10^6 per ml) activated with Ca-ionophore (1 μM), or to purified PMN elastase (10–25 μg/ml) in KGM supplemented with 1.8 mM Ca^{2+}. PMN from two different donors were used. When keratinocytes were exposed to activated PMN, the effect of excess recombinant SKALP (50 μg/ml) or the addition of superoxide dismutase (500 U/ml) plus catalase (1,000 U/ml) was tested. At regular intervals the coverslips were washed with PBS and fixed in ethanol for histological examination. The degree of cell detachment from the coverslip was quantitated by image analysis using the Vidas 2.1 system (Kontron, Germany), and expressed as a percentage of the total surface of the coverslip.

Results

SKALP is constitutively expressed in several squamous epithelia. We studied SKALP expression in 21 adult human tissues by immunohistochemistry (Fig. 1). In these tissues the stroma was always negative, whereas the epithelium in a number of tissues studied showed positive staining. The following epithelial tissues were positive for SKALP expression: tongue, palate, lingual tonsil, gingiva, pharynx, epiglottis, vocal fold,

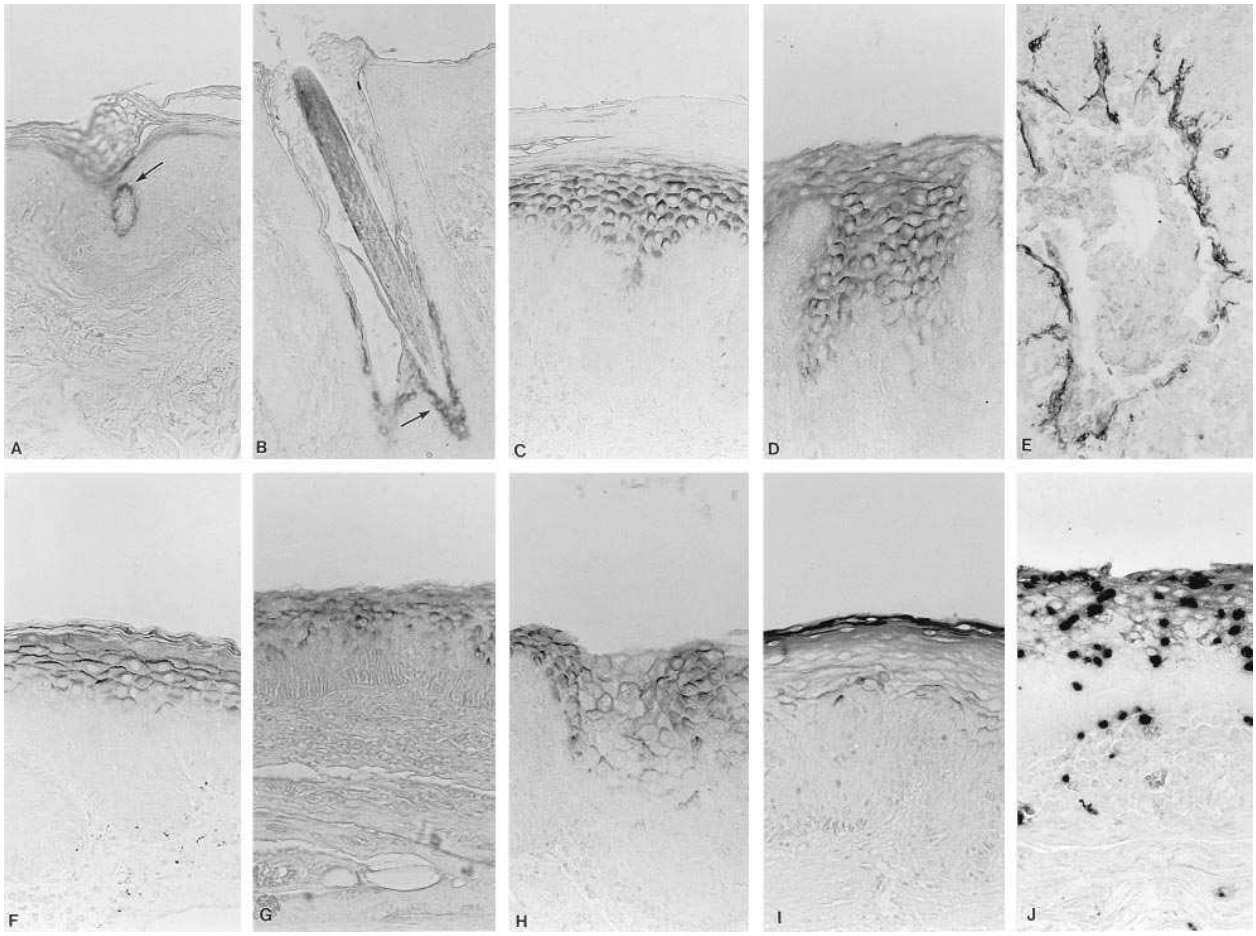

Figure 1. Immunohistochemical staining for SKALP in human epithelia. Staining was performed on formalin fixed paraffin sections, using a polyclonal rabbit antiserum. (*A*) normal epidermis is negative except for the keratinizing cells lining the sweat gland duct (*arrow*); (*B*) hair follicle, positive cells are found in the infundibulum; in the following tissues SKALP is strongly present in the suprabasal cells: (*C*) psoriatic epidermis; (*D*) tongue; (*E*) tonsil; (*F*) gingiva; (*G*) epiglottis; (*H*) esophagus; (*I*) vagina; (*J*) pharynx, double stained for SKALP, which is present in the suprabasal epithelial layers, and for PMN elastase which is present as the intensely stained dots in the epidermis and the underlying dermis. × 90 (*A* and *G*) and × 180 (*B–F*, *H–J*).

esophagus, uterine cervix, vagina, and hair follicle. The expression varied considerably from one weakly stained cell layer (as in vocal folds and hair follicles) to many intensely stained suprabasal layers (as in esophagus and vagina). Staining was never seen in the basal cells. When multiple layers were stained, the density of the cytoplasmatic staining increased with increasing differentiation, often in a distinct polarized (apical) localization in the cytoplasm, while in other cases staining appeared to be localized close to the cell membrane.

Normal epidermis and skin adnexae are negative, except for the keratinocytes lining the epidermal part of the sweat gland ducts (Fig. 1 *A*) and the infundibular part of the hair follicle (Fig. 1 *B*). The suprabasal cells of psoriatic epidermis are strongly positive (Fig. 1 *C*) as described previously (10). The tongue dorsum was strongly positive: up to 10 cell layers were intensively stained (Fig. 1 *D*). In contrast the ventral part of the tongue was negative (not shown). The furrows surrounding the vallate papillae of the tongue stained intensely, but the taste buds were negative. In palate/lingual tonsil a distinct SKALP expression was observed in destroyed epithelial lining (Fig. 1 *E*), while staining was absent in the intact cover of the tonsil. Keratinizing gingiva was moderately positive for three or four most differentiated cell layers (Fig. 1 *F*). The lining of the epiglottis was positively stained in multiple layers (Fig. 1 *G*). In between, areas of pseudostratified columnar cells, with ciliated cells common to respiratory epithelia, were negative with respect to expression of SKALP. True vocal fold was slightly positive (not shown). Esophageal lining, showed many positive cell layers, especially the superficial layers which were intensively stained in a polarized pattern (Fig. 1 *H*). The uterine cervix and vagina were positive for multiple cell layers, with varying intensity. The staining appeared to be more cell membrane associated than cytoplasmatic (Fig. 1 *I*). In most of these epithelia, infiltrating mononuclear cells and few PMNs are found in the connective tissue or in the epithelium itself, and their presence appears to be physiological. In pharynx we found a considerable number of PMN present. Fig. 1 *J* shows a double staining for SKALP and PMN elastase. In addition to the tissues shown in fig. 1 *A–J* we examined a number of epithelial and non-epithelial tissues that were negative for SKALP expression, such as brain, cornea, larynx, lung, bronchus, duodenum, colon, liver, kidney, urethra, and skin from various body regions (not shown).

Because SKALP contains a number of repeats in the NH_2-terminal domain which can be used as TGase substrate a lim-

ited number of tissues was examined for type 1 TGase. The known tissue distribution (26) and our own observations indicate that in psoriatic epidermis, esophagus and oral epithelia SKALP and TGase colocalize in the upper half of the suprabasal compartment (not shown).

SKALP is developmentally regulated in fetal epidermis. Fetal tissues (9, 17, 22, and 28/29 wk gestation) and neonatal tissues (1 and 3 mo post partum) were stained for SKALP. We studied the expression in oral epithelia, that were shown to be positive in the adult, and in epidermis which was negative in the adult (see above). For comparison we stained the tissues for other known differentiation-related markers such as CK 10, CK 13/16, and involucrin (data not shown). In a 9-wk-old embryo, which could be examined in toto, no SKALP could be detected in any tissue. The periderm which is at this timepoint starting to show the first signs of stratification was also negative for CK 10, CK 13/16, and involucrin. At 22 wk gestation, CK 10, CK 13/16, and involucrin positive cells are found in the upper suprabasal layer of the epidermis, whereas SKALP is absent at this timepoint (Fig. 2 *A*). At 28 wk, SKALP expression is found in the granular layer and upper spinous layer of the interfollicular epidermis (Fig. 2 *B*). At this timepoint the morphology of the epidermis is similar to adult human epidermis, including full stratification, keratinization, suprabasal expression of CK 10 and involucrin, and the absence of CK 13/16 in the suprabasal cells, as previously described by others (27, 28). In neonatal epidermis 1-mo–post partum the expression of SKALP is still faintly detectable in the upper layer of the stratum granulosum and in the stratum corneum (Fig. 2 *C*). In neonatal epidermis 3 mo post partum SKALP expression is completely downregulated as in normal adult skin. The expression pattern of CK 10 remains unchanged during the postnatal period. Involucrin expression which was found in the fetal stratum granulosum and stratum spinosum, is only found in the stratum granulosum of adult epidermis. In adult interfollicular epidermis SKALP is completely absent, but is re-expressed during the hyperproliferative differentation program as seen in psoriasis (Fig. 1 *C*) and injury, where it is always co-expressed with CK 16. Remarkably, in the fetal and neonatal epidermis that was positive for SKALP no positive staining was seen with the Ks8.12 (anti-CK13/16) antibody, indicating that SKALP and CK 16 (at least in fetal development) are subjected to distinct regulatory mechanisms.

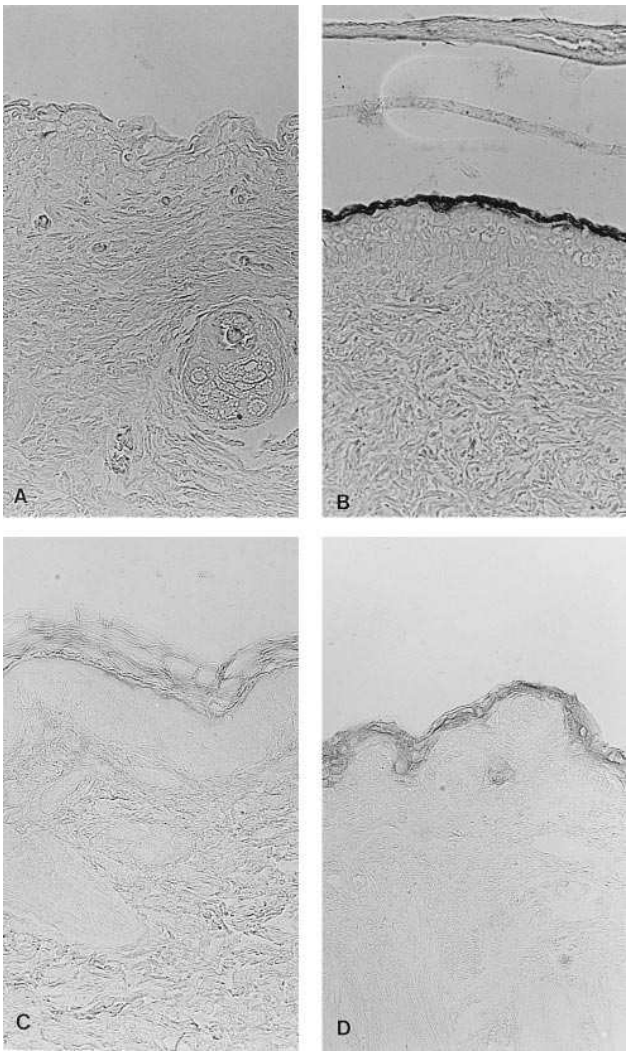

Figure 2. SKALP expression in fetal epithelia. Staining was performed on formalin fixed sections. Fetal epidermis was negative up to week 20 (*A*). From week 27–28 onwards strong epidermal staining was seen in the suprabasal cells (*B*). SKALP expression was rapidly downregulated after birth. In neonatal epidermis after 1 mo a faint staining was visible in the upper layer of the stratum granulosum (*C*) and SKALP was undetectable at 3-mo (as in adult skin). In oral epithelia SKALP was expressed as early as wk 17 (*D*) and continued to be expressed during adult life (not shown). × 90.

In addition to epidermis we investigated the expression of SKALP, CK 10, 13 /16, and involucrin in fetal and neonatal tongue and tonsils. SKALP was found in the suprabasal keratinocytes of tongue epithelium as early as 17 wk gestation (Fig. 2 *D*). In the lingual tonsils SKALP expression was found from 28 wk onwards (no material was available from earlier time points). In contrast to epidermis, SKALP expression in these epithelia was not downregulated in neonatal skin, but continued to be present in adult life, as shown in Fig. 1.

Expression of SKALP at the mRNA level. Fresh autopsy material was obtained, stored at −80°C, and used for Northern blot analysis. In tongue, pharynx, and psoriatic skin a strong expression of SKALP could be demonstrated whereas in epiglottis and vocal fold moderate levels were present (Fig. 3). In larynx, lung, kidney, urethra, and normal skin no SKALP mRNA was found. These findings correlated with the expression of SKALP at the protein level, as found with immunohistochemistry.

Because our studies suggested a discrepancy with data from Nonamura et al. (38) who reported the focal expression of SKALP/elafin in normal adult epidermis as shown with in situ hybridization, we have examined epidermis from many different locations. We were unable to demonstrate SKALP expression at the protein level in adult epidermis from ear, breast, axilla, scrotum, mamilla, palms, and soles (not shown). Using mRNA in situ hybridization on frozen sections of normal and psoriatic skin from the trunk, our results paralelled the findings with immunohistochemistry (Fig. 4). With the antisense cRNA probe a strong expression was found in the suprabasal compartment of psoriatic epidermis, whereas normal epidermis was negative. All tissues were negative with the sense cRNA probe. Our findings at the mRNA level are in agreement with the findings at the protein level as shown in Fig. 1.

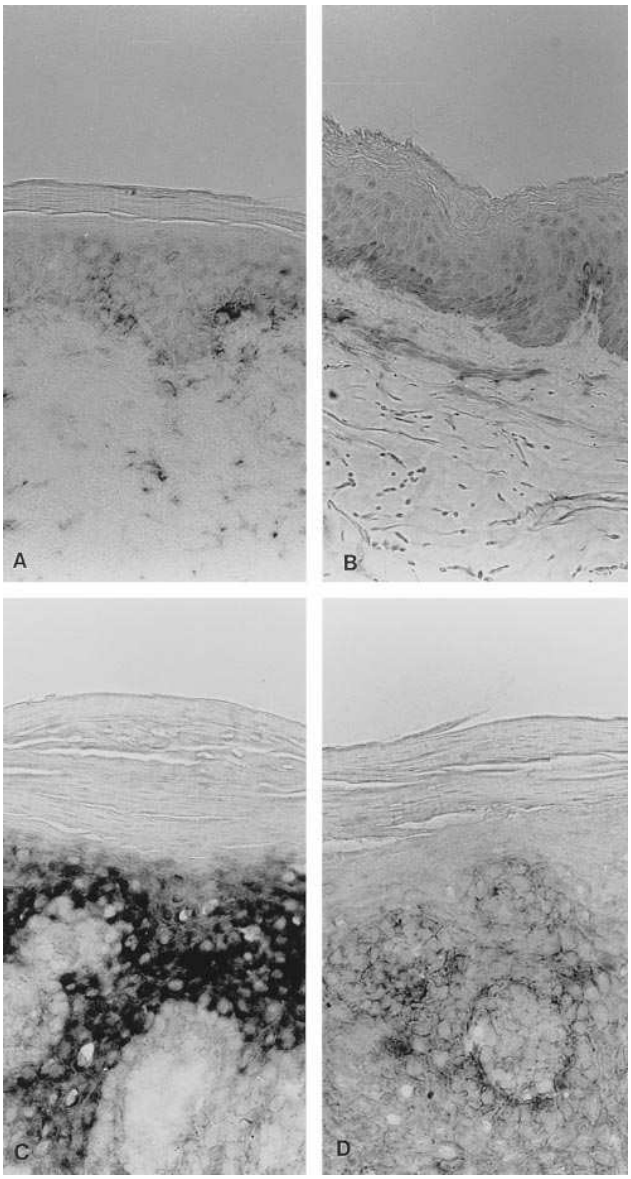

Figure 3. In situ hybridization of SKALP mRNA in human epidermis. Cryosections of normal human skin and lesional psoriatic skin were used for in situ hybridization with a digoxigenin-labeled SKALP cRNA probe. Normal skin was negative both with the antisense and sense probe (*A* and *B*). Lesional psoriatic skin was strongly positive in the suprabasal cells, using the antisense probe (*C*). No signal above background was detected with the sense probe (*D*). The occasional alkaline phosphatase precipitate adjacent to the basal membrane is non-specific, and was seen both with the sense and antisense probe. × 90.

SKALP is present in secretory vesicles of epidermal keratinocytes. Electron microscopic examination of normal and lesional psoriatic epidermis revealed a positive staining of suprabasal keratinocytes and the stratum corneum of psoriatic epidermis. No significant signal was present in keratinocytes from normal epidermis or in melanocytes, which is in accordance with the findings at the light microscopical level. Staining with the preimmune serum was negative (Fig. 5 *A*). As shown in Fig. 5 *D* staining was mainly observed in the intercellular spaces of the stratum corneum and over the lamellar

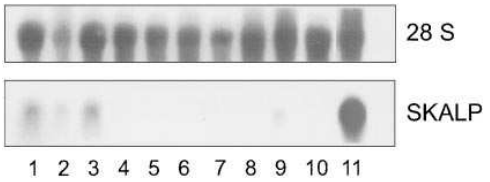

Figure 4. Northern blot of SKALP mRNA in various adult tissues. Lower panel. A 0.8-kb message was found in tongue (lane *1*), epiglottis (lane *2*), pharynx (lane *3*), vocal fold (lane *9*), and in lesional psoriatic skin which was used as the positive control (lane *11*). Larynx, lung, kidney, urethra, skin of the inguinal region, and liver (lanes *4–8* and *10*, respectively) were negative. In the upper panel a probe for 28S ribosomal RNA was used for control hybridizations to check for equal RNA loading.

granules of the keratinocytes in the upper spinous and granular layer (Fig. 5 *B*). In addition, intracellular labeling could also be observed over small vesicular structures and in the Golgi apparatus of these cells (not shown). Since these structures are implicated in secretion, these findings indicate synthesis and secretion of SKALP from suprabasal keratinocytes.

Expression of SKALP protects against PMN-elastase mediated cell detachment. The specificity of SKALP for the PMN-derived proteinases elastase and proteinase 3, and its constitutive expression in epithelia that are subjected to inflammatory stimuli suggests a role in the control of inflammation. We therefore investigated whether SKALP expression could protect against elastase-dependent cell damage in an in vitro system. Human epidermal keratinocytes were cultured in KGM to form a confluent monolayer. At this stage, no differentiation is induced and SKALP is not expressed. Differentiation was induced either by growth factor depletion (24) or by the addition of fetal calf serum (11). KGM depleted of growth factors (KGM/-GF) induces a differentiated phenotype, as assessed by type 1 TGase expression, and resembles normal skin as witnessed by the expression of CK 1/10 and the absence of SKALP (29). KGM with fetal calf serum (KGM/FCS) induces differentiation, as assessed by the expression of type 1 TGase, with a psoriasiform phenotype such as sustained hyperproliferation and expression of SKALP (29). Fig. 6 shows the expression of SKALP in these culture systems at the mRNA level. After induction of differentiation, these cultures were washed extensively to remove the induction media and were placed in the culture medium that was used in subsequent experiments for coculture with PMN (KGM with 1.8 mM Ca^{2+}, without serum). The secretion of SKALP in the medium was measured by ELISA and in a functional assay as anti-elastase activity. Fig. 7 shows that during a 6 h timecourse SKALP is secreted in the medium by cells that were allowed to differentiate in KGM/FCS, whereas the cells differentiated in KGM/-GF were negative. Having established that the culture models differed in SKALP expression, the keratinocytes were exposed to purified human PMN elastase. During a 4-h time course, cell detachment from the coverslips was visualized by H&E staining and quantitated by image analysis (Figs. 8 and 9). Keratinocytes cultured in KGM/FCS (positive for SKALP synthesis and secretion) were found to be protected against elastase-dependent detachment, whereas cells cultured in KGM/-GF (negative for SKALP synthesis and secretion) were fully detached from the tissue culture dish. Elastase-dependent loss of adhesion was concentration dependent (not shown). Next we

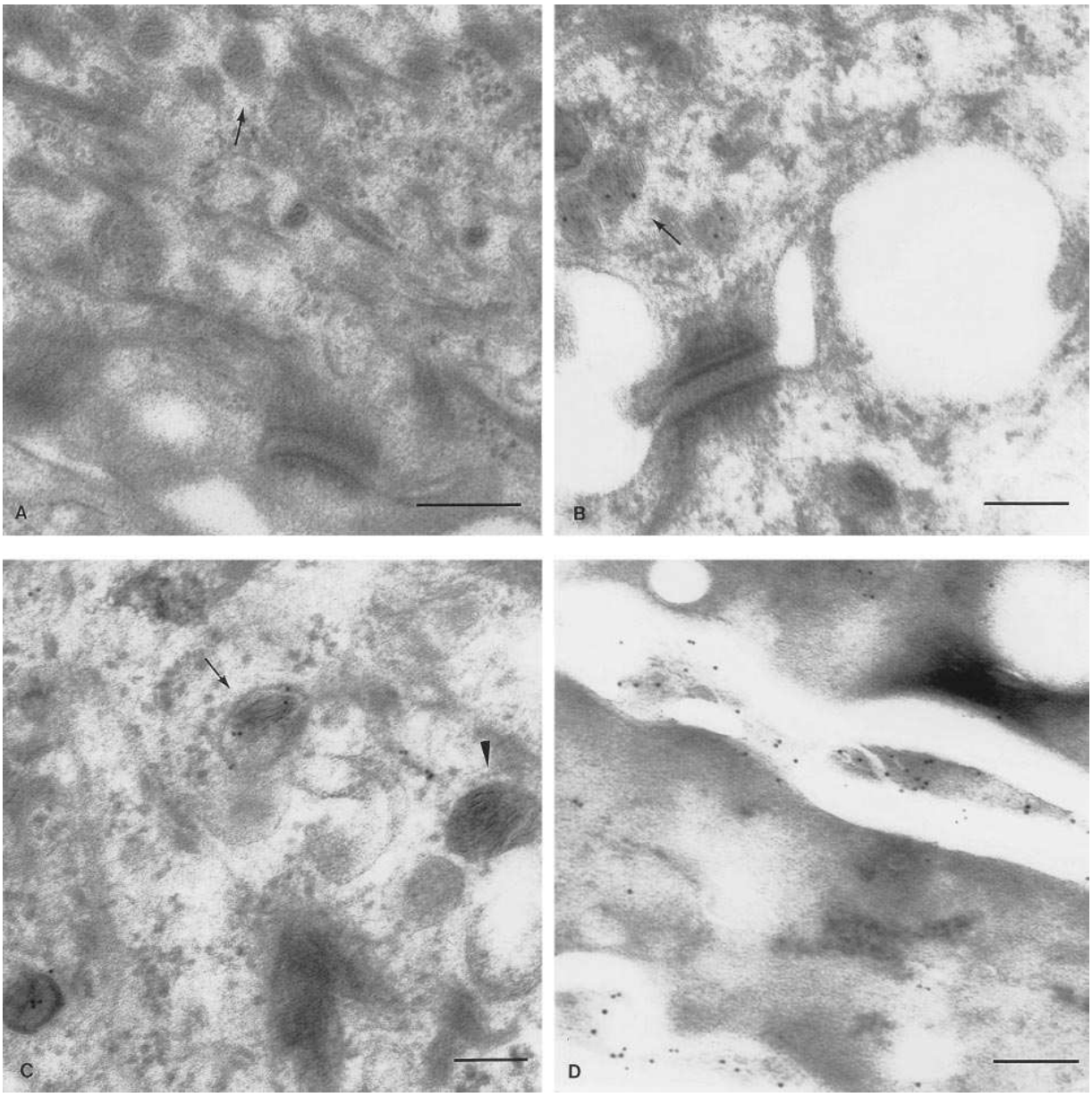

Figure 5. Immunoelectron microscopy of SKALP in human epidermis. (*A*) Psoriatic epidermis stained with pre-immune serum. An area of a keratinocyte of the spinous layer is shown, near a desmosome. No immunogold labeling was seen in any of the structures including the lamellar granules (*arrow*). (*B*) psoriatic epidermis stained with anti-SKALP serum. A group of labeled lamellar granules is shown (*arrow*) near a desmosome, at the cell membrane. (*C*) High power magnification of the same preparation as in *B*, with a labeled lamellar granule (*arrow*) and an empty lamellar granule (*arrowhead*). (*D*) Stratum corneum of psoriatic epidermis stained with anti-SKALP serum. Clear labeling of the outer layers of the corneocytes is visible. Bar, 100 nm.

investigated whether activated PMN could also induce the effects seen with the purified enzyme. Differentiated cultures of keratinocytes were extensively washed and exposed to calcium-ionophore activated PMN. Experiments were performed in duplicate with PMN from two different donors. Within 12 h, activated PMN caused cell detachment in keratinocyte cultures grown in KGM/-GF, although to a lesser extent than observed in the concentration range of purified elastase ($\sim$ 10–15% detachment after 12 h). The SKALP-expressing keratinocytes (grown in KGM/FCS) were fully protected against cell detachment (Figs. 10 and 11). Addition of recombinant SKALP (50 μg/ml) to keratinocytes cultured in KGM/-GF gave complete protection against PMN-mediated damage (Fig. 11 *E*), whereas addition of SOD (500 U/ml) plus catalase (1,000 U/ml) was only marginally effective (Fig. 11 *D*).

Discussion

The proteinase inhibitor SKALP/elafin was first discovered at the biochemical level in psoriatic skin, which is reflected in the acronym SKALP (4), as a specific inhibitor of leukocyte elastase

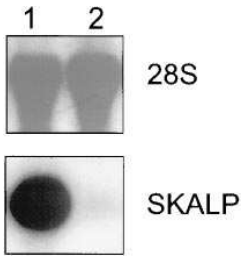

Figure 6. Northern blot analysis of SKALP expression in cultured keratinocytes. Human epidermal keratinocytes were induced to differentiate either in KGM depleted of growth factors (KGM/-GF) or in KGM with KGM/FCS. In KGM/-GF (lane *2*) SKALP is not expressed, whereas a strong signal is seen in KGM/FCS (lane *1*). In the upper panel control hybridization with 28S ribosomal RNA is shown to check for equal loading.

which is reflected in the name elafin (5). Retrospectively, both these names are unfortunate, since we now know, firstly, that SKALP is not normally present in epidermis but is constitutively expressed in other epithelia, and secondly that the inhibitory profile of SKALP also includes leukocyte proteinase-3 (9). In view of its specificity for leukocytic proteinases, it was hypothesized that SKALP is involved in the regulation of cutaneous inflammation either by interfering with PMN migration or by providing protection against excessive proteolysis of extracellular matrix components. In previous studies we have documented the expression of SKALP in psoriatic epidermis, in injured skin and in cell culture (11). A striking parallel was found with the expression pattern of CK16 which is not normally expressed in the interfollicular epidermis but is induced in psoriasis, after injury and in cell culture (30, 31). We hypothesized that both SKALP and CK 16 were part of the hyperproliferative differentiation program of normal epidermis, and subjected to similar control mechanisms. In the present study

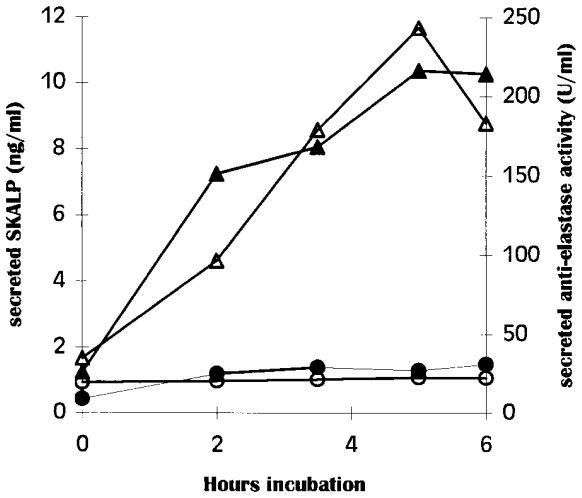

Figure 7. Secretion of SKALP in the medium of cultured keratinocytes. After induction of differentiation in KGM/FCS or in KGM/-GF, the cells were extensively washed, and incubated in KGM supplemented with 1.8 mM Ca^{2+}, which is the medium used for detachment assays (see below). During a 6-h-time course, secretion of SKALP in the medium was measured at the protein level by ELISA (left-hand Y-axis, *open circles* and *triangles*) or measured functionally and expressed as units anti-elastase activity per ml (right-hand Y-axis, *closed circles* and *triangles*). Under these conditions the keratinocytes cultured in KGM/FCS (*triangles*) continued to secrete SKALP whereas the keratinocytes cultured in KGM/-GF (*circles*) remained negative.

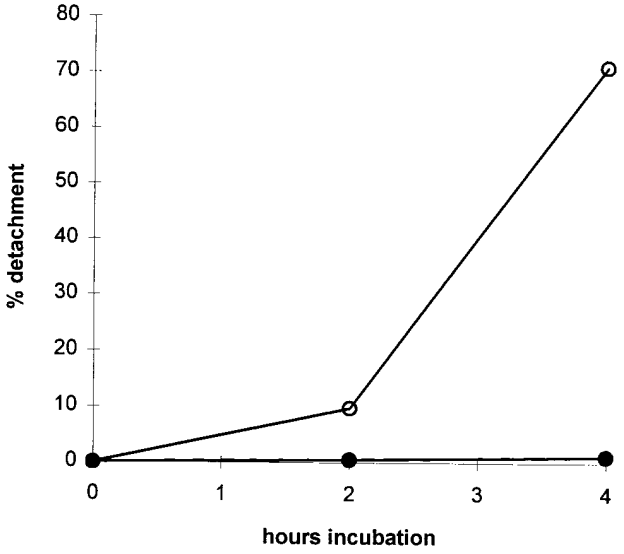

Figure 8. Time course of cell detachment by purified PMN elastase. Human epidermal keratinocytes cultured on coverslips in KGM/-GF (*open circles*) or in KGM/FCS (*closed circles*), were extensively washed, and subsequently exposed to PMN elastase (10 μg/ml) in KGM with 1.8 mM Ca^{2+}. After 2 and 4 h cells were washed, fixed, and stained with H&E. Cell detachment was quantitated by image analysis.

we show that SKALP and CK 16 are not co-expressed during fetal and neonatal development. CK 16 is not expressed in any stage of development of fetal epidermis as previously described by others (28), whereas SKALP is expressed in the third trimester and downregulated in neonatal skin. In contrast to previous reports that dealt with SKALP in epidermal keratinocytes, either in pathology or in cell culture, here we demonstrate SKALP to be expressed in normal squamous epithelia. The adjective "normal" in this respect should be used with caution. Several normal tissues are subjected to continuous mechanical stress or inflammatory stimuli, which may influence the structure and organization of the lining epithelium. In normal epiglottis, squamous metaplasia can be observed, that is partly functional or due to mechanical stress. Pathogens may be present in the pharynx of healthy individuals, and in normal pharynx and esophagus PMN infiltration is not considered to be pathological. Also in the infundibular part of the hair follicle the presence of bacteria that may induce an inflammatory response is common. SKALP expression was demonstrated in the tonsillar crypts where destruction of the epithelial lining by lymphocytes was observed. Lymphocytes transmigrate the tonsillar epithelium into the crypts that also contain the desquamated cells of the stratified squamous epithelium. This passing through is accompanied by desolution of the epithelium, which may be the trigger for SKALP induction as part of the ongoing inflammatory response.

SKALP is specific for elastase and proteinase 3 which are proteinases present in PMN. In addition, elastase is found in monocytes, albeit at lower levels than in PMN. Continuous trafficking of PMNs and mononuclear cells to epithelia could expose these tissues to breakdown of extracellular matrix proteins as is known from various pathological conditions (25, 32). Studies by several authors have shown that PMN can in vitro degrade extracellular matrices and cause cell detachment by

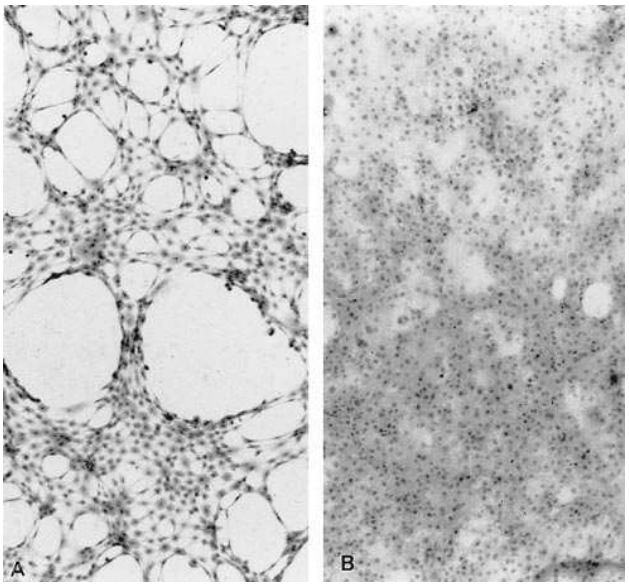

Figure 9. H&E staining of keratinocyte cultures exposed to PMN elastase. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN elastase (10 μg/ml) in KGM with 1.8 mM Ca^{2+}. Cells were washed, fixed and stained with H&E. After 4 h, cell detachment and retraction was seen in keratinocytes cultured in KGM/-GF (*A*) whereas no signs of cell detachment were visible in cells cultured in KGM/FCS (*B*). × 45.

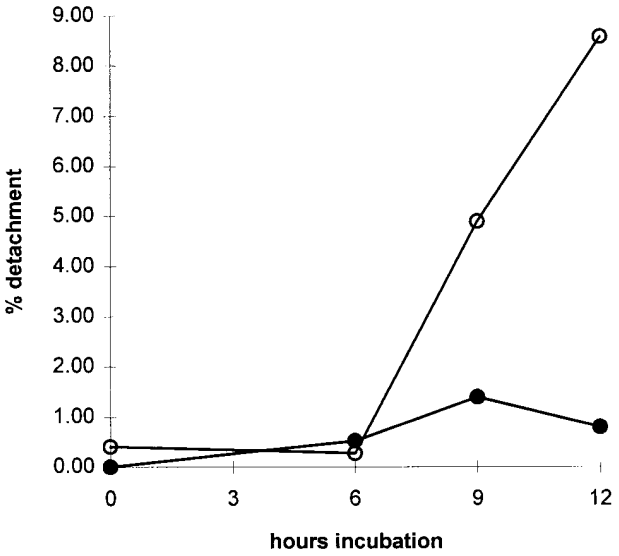

Figure 10. Time course of cell detachment by activated PMN. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN (2.10^6/ml) activated by Ca-ionophore (1 μM). Experiments were performed in duplicate with PMN from two different donors. The coverslips were washed, fixed, and stained with H&E. Cell detachment was quantitated by image analysis. Between 6 and 12 h incubation, cells detached from the cultures that did not express SKALP (keratinocytes cultured in KGM/-GF, *open circles*), whereas the SKALP expressing culture (keratinocytes cultured in KGM/FCS, *closed circles*) was fully protected.

distinct mechanisms involving proteolytic enzymes or oxidative molecules (33–36). Recently it was shown that activated PMN can express cell-surface bound elastase and cathepsin G which provides a potent mechanism to egress from the vasculature, penetrate tissues and migrate to sites of inflammation (34). Using cultured keratinocytes exposed to purified PMN elastase or to activated PMN we show that expression of SKALP provides protection against elastase-mediated detachment in vitro. In this model system we used Ca-ionophore activation of PMN which is a powerful stimulator of lysosomal enzyme secretion (up to 30% of the total elastase content) and gives a moderate superoxide production. After 9 h of exposure

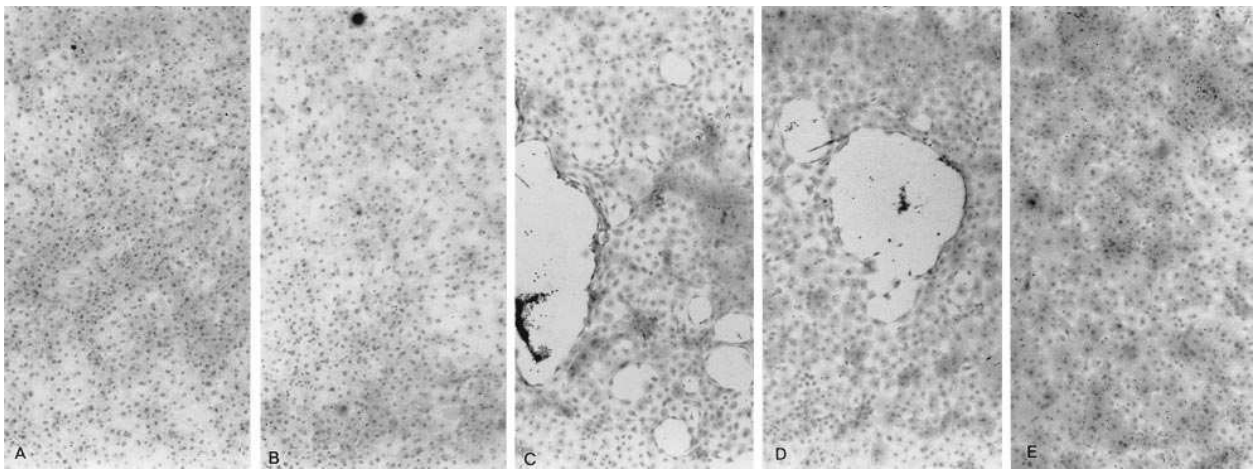

Figure 11. H&E staining of keratinocyte cultures exposed to activated PMN. Human epidermal keratinocytes cultured on coverslips in KGM/-GF or in KGM/FCS, were extensively washed, and subsequently exposed to PMN (2.10^6/ml) activated by Ca-ionophore (1 μM). After 12 h incubation, the cells were washed, fixed, and stained with H&E. *A* shows a confluent culture (KGM/FCS) after 12 h without PMN, which was taken as the control value. No detachment was seen in these cultures; this was also found for the KGM/-GF cultures (not shown). SKALP-expressing cultures (KGM/FCS) exposed to activated PMNs did not show significant detachment of cells (*B*), whereas in the cultures that lacked SKALP-expression (KGM/-GF) considerable cell detachment was noted (*C*). Recombinant SKALP (50 μg/ml) could completely prevent cell detachment in the KGM/-GF cultures (*E*) whereas addition of SOD (500 U/ml) plus catalase (1,000 U/ml) had only a marginal protective effect (*D*).

to activated PMN a cytopathic effect was seen on keratinocytes that lacked SKALP expression. The cell damage during this period was clearly elastase/proteinase 3 dependent since the addition of recombinant SKALP provided protection. In contrast, a high concentration of SOD and catalase did not protect against cell detachment and retraction. SKALP could protect against elastase mediated damage in two ways. The presence of a signal peptide and its presence in secretory vesicles and lamellar bodies suggests that SKALP is secreted in the extracellular space, where it can neutralize its target proteinases. On the other hand, the TGase substrate motifs of SKALP enable crosslinking to the pericellular matrix and the cornified envelope. SKALP/elafin was recently found to be crosslinked to loricrin, a component of the cornified envelope (14), suggesting that SKALP is crosslinked to intracellular proteins, and can also act as an anchored proteinase inhibitor. We have recently obtained indirect evidence that SKALP is involved in protection of epidermal integrity in pustular forms of psoriasis (17). It was shown that in forms of pustular psoriasis, which are characterized by a massive influx of PMN, the amount of epidermal SKALP is much lower than in plaque type psoriasis. It remains to be established whether this phenomenon is caused by an intrinsic (genetic) defect in these patients, or by inactivation/clearance of SKALP.

Assuming that induction of SKALP controls excessive proteolysis in adult skin, the function of fetal SKALP expression is not clear at the moment, since no obvious signs of inflammation are present in the fetal skin itself. It was, however, recently reported that at the end of gestation increased levels of inflammatory cytokines are found in the amion fluid, and evidence for an inflammatory response has been found in the forebag compartment of the uterus presumably as a result of leakage of microbial stimuli from the vagina and cervix into the uterus (37). Speculatively, SKALP expression could be induced via this route to ensure protection of the fetal skin against proteolytic activity in the amnion fluid at the end of gestation. However, no evidence for the presence of inflammatory cells or proteinases derived thereof in the amnion fluid, has been published to our knowledge. Alternatively, SKALP induction could be a mechanism to provide temporary protection to the epidermis of the newborn, when switching from the fetal submerged, sterile environment to a dry environment which allows colonization of the skin with microorganisms. Although a fully keratinized epidermis is present at term, the barrier function of neonatal skin is incomplete (27). The observed downregulation of SKALP in neonatal epidermis could therefore be related to the functional maturation of barrier function. It has to be noted that under conditions that compromise barrier function such as tape stripping and psoriasis, SKALP is again induced. Recently Nonamura et al. (38) have described the focal presence of SKALP mRNA in normal epidermis, using in situ hybridization. Here, we have studied a large number of samples from various locations of normal skin, but we failed to demonstrate significant expression of SKALP at the protein level. Also with in situ hybridization we were unable to demonstrate the presence of SKALP in normal skin. We do occasionally observe SKALP expression in the epidermis near hair follicles and acrosyringia, which may explain the focal expression found by Nonomura et al. (38).

Remarkably, SKALP in tongue and tonsil epithelium is induced much earlier than in epidermis and continues to be expressed in the neonatal phase and throughout adult life. In many of the moist oral epithelia (and of course in the tonsils) the presence of inflammatory cells is physiological, as a result of continuous exposure to environmental and microbial stimuli. In adult epidermis which is a dry, keratinizing epithelium, inflammatory cells are not normally present, which may explain the difference in SKALP expression.

On the basis of the pattern of SKALP expression, one would surmise that SKALP expression is induced by inflammatory cells such as PMN. Interestingly, Sallenave et al. demonstrated induction of SKALP expression in tumor cell lines by the cytokines interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α), but also by the PMN-derived proteinases human leukocyte elastase and cathepsin G (39). This finding is in line with that of Perlmutter et al. who reported that neutrophil elastase regulates the synthesis of its inhibitor α1-proteinase inhibitor in human monocytes and bronchoalveolar macrophages (40). The cytokines IL-1 and TNF-α are considered to be initiating cytokines in inflammatory processes in cutaneous tissues (32). In normal tissues where continuous monitoring of the tissue by inflammatory cells is desired for reasons of host defense, this control mechanism might explain SKALP presence in epithelia such as esophagus, pharynx, tongue and vagina. The difference in SKALP expression in the various epithelia could be related to differences in inflammatory stimuli to which the tissues are subjected. However, ultimate proof for its supposed relevance in control of epithelial inflammation has to come from studies using transgenic mice lacking a functional SKALP gene, which will be the direction for future research.

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