

## Expression and ultrastructural localization of plasmin(ogen) in the terminally differentiated layers of normal human epidermis

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### Abstract

**OBJECTIVE:** Plasmin, a relatively unspecific trypsin-like serine protease, is involved in many physiological and pathological conditions, particularly in dermatoses with barrier impairment. It is secreted as the inactive zymogen plasminogen and is activated to plasmin by plasminogen activators, such as urokinase. There still exists a paucity of data on the precise localization of epidermal plasmin(ogen) within the epidermis and the stratum corneum. The aim of the present study was to get information about its origin and ultrastructural localization within normal human epidermis.

**METHOD:** We performed immunoelectron transmission electron microscopy immunogold labelling in normal abdominal human skin.

**RESULT:** Plasmin was only observed in the terminally differentiated cell layers of the epidermis and was largely associated with the corneocyte envelopes and to some extent with the intercellular lipid matrix in the stratum corneum.

**CONCLUSION:** Our results indicate that in normal human skin, plasmin(ogen) is synthesized by differentiated epidermal keratinocytes of the stratum granulosum and is not serum-born.

### Résumé

**OBJECTIF:** Plasmine, une relativement peu spécifique 'trypsin-like' protéase sérine, participe aux plusieurs processus physiologiques et pathologiques et, plus particulièrement, à la physiopathologie des dermatoses caractérisées par l'altération de la barrière de perméabilité. Elle est sécrétée sous forme d'un zymogène inactif, plasminogène, et devient activée par les activateurs du plasminogène, telle urokinase. A l'heure actuelle, on manque de précision quant à la localisation de plasmine (ou son précurseur) dans l'épiderme et le stratum corneum. Le but du présent travail a été de rapporter l'information sur la provenance et la localisation ultrastructurale de plasmine/plasminogène présents dans l'épiderme humain.

**MÉTHODE:** L'étude ultrastructurale de l'épiderme humain normal (plastie abdominale) a fait appel à l'immunomarquage à l'or

colloïdal sur coupes ultrafines des tissus inclus à froid dans des résines acryliques.

**RÉSULTAT:** L'anticorps monoclonal anti-plasmine/plasminogène a détecté l'antigène situé exclusivement dans la partie la plus différenciée de l'épiderme et persistant dans la couche cornée. Il n'y a pas eu de réactivité dans les couches épineuse et basale. Le marquage a été prédominant sur les enveloppes cornifiées des kératinocytes granuleux et cornéocytes. Des foyers du marquage ont été également présents dans le cytoplasme et les espaces intercellulaires de la couche granuleuse, ainsi que dans la matrice lipidique de la couche cornée profonde.

**CONCLUSION:** Nos résultats indiquent la production *de novo* de plasmine/plasminogène dans les kératinocytes le plus différenciés et ne suggèrent pas l'origine sérique de cette enzyme dans l'épiderme.

### Introduction

The proteases of the plasminogen system in the epidermis are activated by inflammatory conditions, such as UV light exposure [1–4], or by proinflammatory cytokines, such as IL-1 $\beta$ , IL-8 and TNF $\alpha$  [5,6]. Plasmin is a relatively unspecific serine protease. In the skin, activated plasmin is thought to lead to premature desquamation, impaired maturation of corneocyte envelopes and delayed barrier recovery [7,8]. Moreover, plasmin activates pro-metalloproteinases (MMPs), which can lead to destruction of components of the extracellular matrix and the dermal–epidermal junction [9,10]. Thus, activation of the plasminogen system is detrimental for epidermal homeostasis. In inflammatory skin conditions, even in subclinical, micro- or pre-inflammatory conditions, plasmin is proposed to be a major protease involved in impaired barrier integrity [8,11–13].

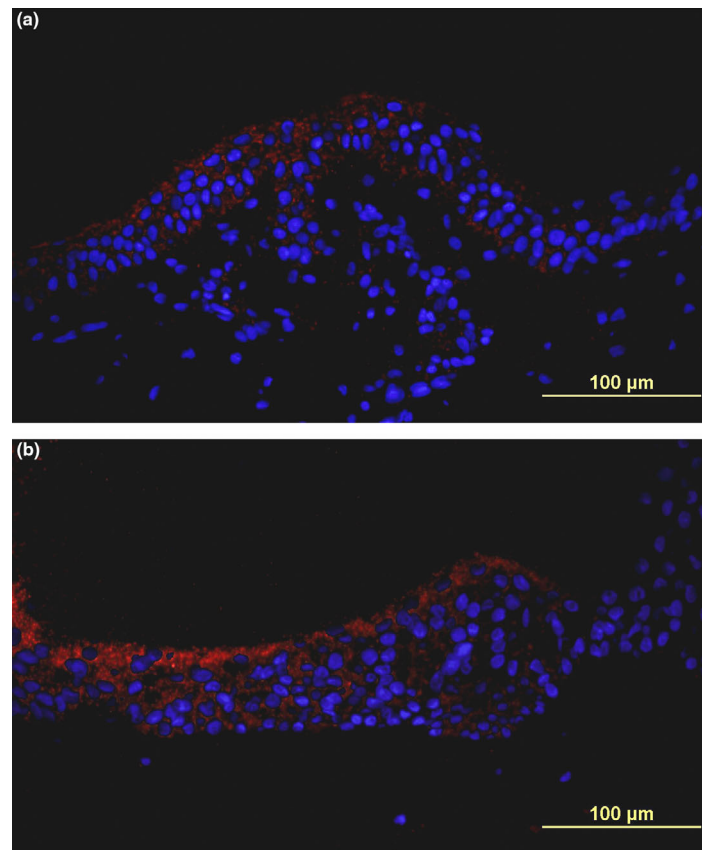
Recently, we have shown that plasmin activity in the stratum corneum (SC) was increased in photodamaged skin and tightly correlated with transepidermal water loss [14,15]. Thus, plasmin can be considered as a barrier impairing enzyme even in non-diseased skin. However, it is also increased in patients with atopic skin [16,17] and psoriasis [18].

Moreover, we have found plasminogen mRNA in the epidermis with increased levels in the upper layers after UV exposure [2] (Fig. 1), while plasmin(ogen) has been immunohistochemically localized exclusively in the SC and upper stratum granulosum of abdominal and forearm skin biopsies of healthy subjects (Fig. 2) [1]. We also identified enzymatic activity of plasmin in normal human keratinocytes cultured in plasmin-free media [19].

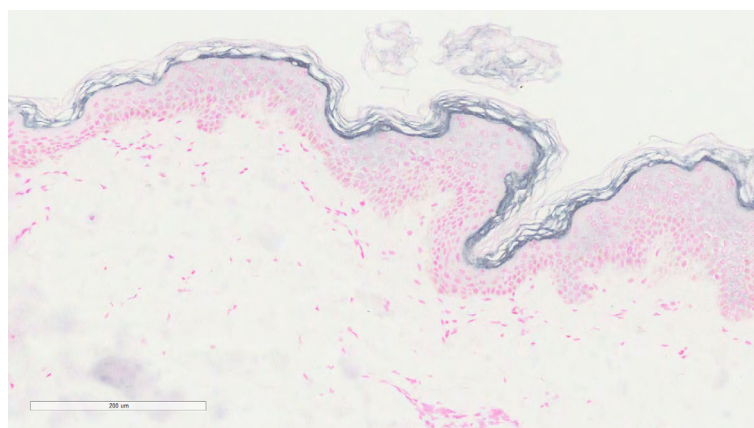
To our knowledge, no study has so far been published demonstrating the detailed subcellular localization of plasmin within

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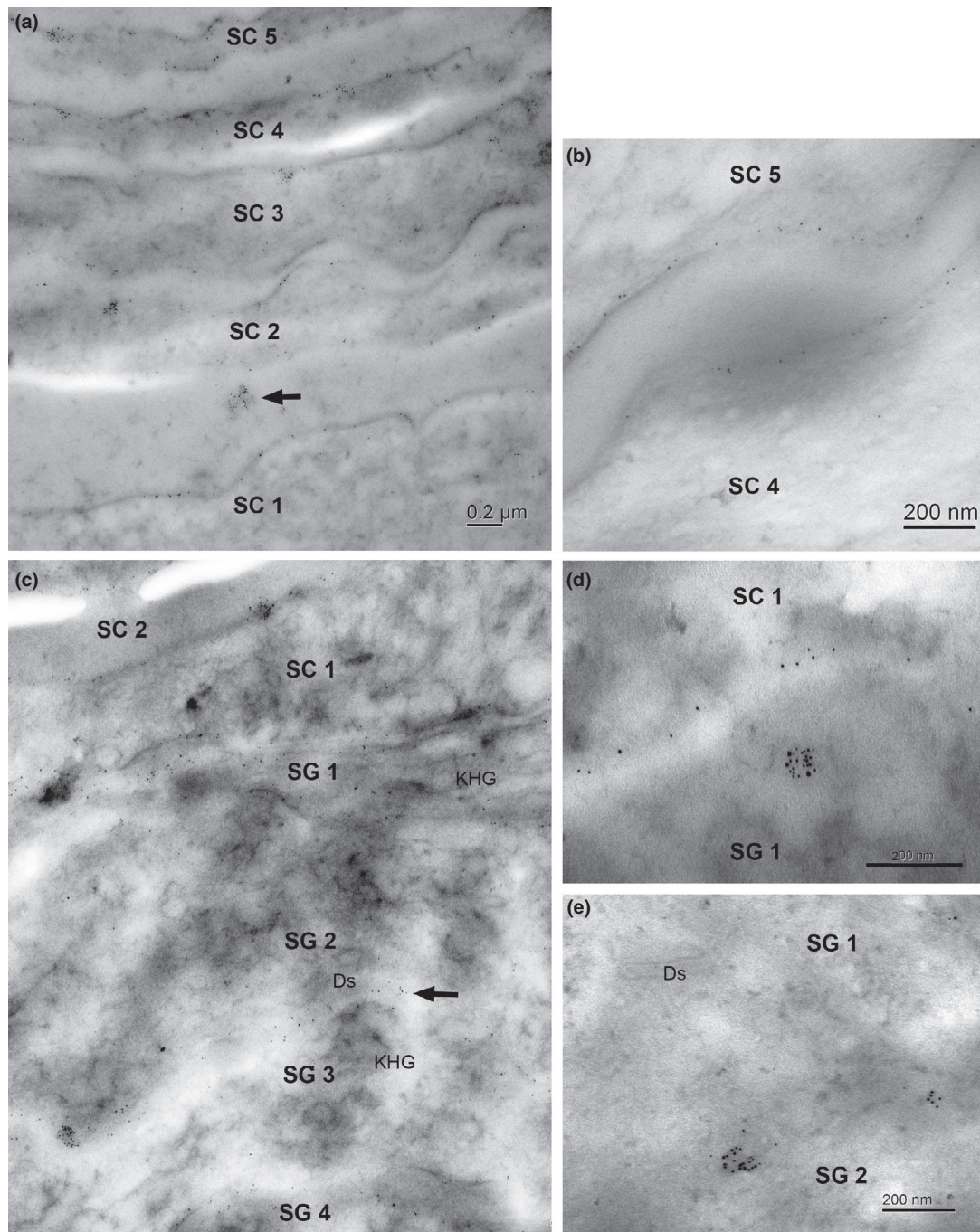
Presentation at Scientific Meetings: This manuscript has not been published and is not under consideration for publication elsewhere, but was presented as a poster at the International Investigative Dermatology Meeting in May 2018 in Orlando FL, US.



**Figure 1** Plasminogen mRNA in situ hybridization. Biopsies of excised abdominal skin were formalin-fixed, paraffin-embedded and -sectioned (5 µm). Plasminogen mRNA was detected using Affimetrix' ViewRNA ISH Tissue 1-Plex Assay Kit (#BMSQVT0050), ViewRNA Chromogenic Signal Amplification Kit (#BMSQVT0200) and ViewRNA TYPE 1 Probe Set (#BMSVX-01). The sections were counterstained with DAPI (4', 6-diamidino-2-phenylindole). (a) In the untreated sample, plasminogen mRNA was shown to be in the epidermis (red fluorescence). (b) The level was increased in UVA/B-exposed skin, particularly in the upper epidermis. Cell nuclei are displayed in blue. Scale bar = 100 µm. Courtesy Marco Massironi, Cotech Srl, Padova, IT.



**Figure 2** Immunohistochemical staining of plasmin with nuclear fast red counterstaining. Forearm skin biopsies were cryo-sectioned (7 µm) and acetone-fixed. The sections were incubated with a biotinylated rabbit polyclonal antibody to plasmin (Abcam Cat# ab48350; Cambridge, UK, RRID:AB\_882071). The primary binding was visualized using ImmPACT™ SG HRP substrate (Vector Laboratories, Peterborough, UK). Plasmin is exclusively localized in the stratum corneum and upper stratum granulosum (black–blue staining). Cell nuclei are in pink/red. Scale bar = 200 µm. Courtesy Victoria Newton, University of Manchester, UK.



**Figure 3** Anti-plasmin immunogold labelling. Peripheral labelling of corneocyte envelopes and, focally, of the intercellular space in the terminally differentiated epidermis. (a, b) Anti-plasmin labelling was most prominent in the stratum corneum (SC). Immunogold granules decorated the periphery of corneocytes, essentially within the corneocyte envelope structures. Rarely, clusters of anti-plasmin immunogold labelling could be found within the intercorneocyte spaces of lowermost SC (a; arrow). (c) In the zone of transition from the stratum granulosum (SG) to the SC, the immunolabelled vesicles were observed occasionally to open into the extracellular space and the plasmin labelling was present both at the periphery of the cells and within the intercellular spaces. (d) Peripheral labelling of the corneocyte (SC1) and focal expression in the apical portion of the SG keratinocyte (SG1). In the SG, plasmin was detected in the cytoplasm of uppermost keratinocytes, localized within the ovoidal structures of size and shape of the lamellar bodies (d and e). In this Figure, cells in the SC and SG were numbered starting from the interface between the two layers. Ds: desmosome; KHG: keratohyalin granule. Scale bar = 200 nm in (a) is also valid for (c).

normal human epidermis and the SC. This should improve the knowledge about the biological functions of this enzyme in the skin.

## Materials and methods

Skin biopsies from abdomen reduction surgery in three healthy adult Caucasian subjects (two women, one man) were embedded in acrylic LR White resin [20]. A mild fixation in 2% paraformaldehyde in PBS at 4°C for three hours was followed by washings in PBS, dehydration in graded ethanol (50°, 70°, 95°, 100°) and substitution with the resin (one-hour bathes in ethanol RP/LR White 2:1, 1:1, 1:2, pure resin; LR White alone overnight). Polymerization occurred at 4°C under UVA (365 nm).

Post-embedding immunogold labelling was performed on ultra-fine sections of the embedded skin. Briefly, skin sections harvested on formvar-coated nickel grids (200 mesh) were first pre-treated with saturated solution of sodium metaperiodate for antigen retrieval (6 min at 60°C), rinsed in water, blocked on drops of 2% ovalbumin/0.1% gelatin in PBS, then exposed to biotinylated rabbit polyclonal antibody to human plasmin (1:50; Abcam Cat# ab48350; Cambridge, UK, RRID:AB\_882071) overnight at 4°C, washed in PBS, and floated on drops of goat anti-rabbit IgG antibody, 5 nm gold conjugated (1:10; BBI Solutions Cat# EM GAR5; Crumlin, UK) for 1 h. Skin sections incubated without the primary antibody served as controls. After final washes in PBS and distilled water, the samples were counterstained for 7 min with Uranylless (Delta Microscopies, Toulouse, France). The samples were then observed at 80 KV with a transmission electron microscope equipped with a digital high-resolution camera.

## Results

Immunocytochemical labelling obtained on mildly fixed skin fragments embedded in LRWhite, using 5 nm immunogold goat anti-rabbit secondary antibody resulted in a high-density-specific labelling of morphologically discernable ultrastructural features.

Clusters of anti-plasmin immunogold labelling could rarely be found within the inter-corneocyte spaces of lowermost SC (Fig. 3a). Anti-plasmin labelling was most prominent in the SC. Immunogold granules decorated the periphery of corneocytes, essentially within the corneocyte envelope structures (Fig. 3b).

In the zone of transition from the SG to SC, the immunolabelled vesicles were observed to open into the extracellular space and the plasmin labelling was present both at the periphery of the cells and within the intercellular spaces (Fig. 3c). In the stratum granulosum (SG), plasmin was detected in the cytoplasm of uppermost keratinocytes, localized within the ovoidal structures of size and shape of the lamellar bodies (Figs. 3d and e). Suboptimal morphological preservation of the weakly fixed tissue did not allow for formal recognition of these vesicles as lamellar bodies, but the reactivity of the antibody on better fixed samples proved unsatisfactory. There

was no labelling in the stratum spinosum and the lower parts of the epidermis, strongly suggesting the local production of the enzyme by the terminally differentiated keratinocytes (data not shown).

According to the manufacturer, the specificity of the anti-plasmin antibody used in our experiments was verified via radioimmunoassays, Western blotting [21–23] and ELISA and does recognize plasminogen at about 20–40% (<https://www.abcam.com/plasmin-antibody-biotin-ab48350.html>). It is therefore possible that part of the observed labelling did not detect the fully active enzyme but its zymogen. However, close association of the labelling with corneocyte envelopes in the SC disjunctum (deprived of corneodesmosomes) indicates a detrimental effect of plasmin in the process of corneocyte maturation and SC desquamation [7,8,24].

## Conclusions

Plasmin is synthesized by differentiated epidermal keratinocytes of the SG and appears to be transported to the cell periphery by a vesicular system of lamellar body shape and size.

Plasmin appears to be associated with the corneocyte envelopes. Although only traces of the labelling can be found clustered in the intercellular spaces of the lower SC, there, plasmin is most probably sequestered within the hydrophobic lipid matrix. There was no plasmin labelling in the stratum spinosum and the lower parts of the epidermis, indicating that the epidermal enzyme/zymogen is not serum-born in normal human skin.

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## Conflict of interest

RV is an employee of DSM Nutritional Products Ltd., AVR is a consultant to DSM Nutritional Products, MH declares no conflict of interest.

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