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► **To cite this version:**

Eleni Zingkou, Georgios Pampalakis, Dimitra Kiritsi, Manthoula Valari, Nathalie Jonca, et al.. Activography reveals aberrant proteolysis in desquamating diseases of differing backgrounds. *Experimental Dermatology*, 2019, 28 (1), pp.86-89. 10.1111/exd.13832 . hal-03464170

HAL Id: hal-03464170


<https://ut3-toulouseinp.hal.science/hal-03464170>

Submitted on 10 Feb 2022

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Activography reveals aberrant proteolysis in desquamating diseases of differing backgrounds

Eleni Zingkou¹ | Georgios Pampalakis¹ | Dimitra Kiritsi² | Manthoula Valari³ |
Nathalie Jonca⁴ | Georgia Sotiropoulou¹ 

¹Department of Pharmacy, School of Health Sciences, University of Patras, Rion-Patras, Greece

²Department of Dermatology, Faculty of Medicine, Medical Center-University of Freiburg, Freiburg, Germany

³Aghia Sofia Children's Hospital, Athens, Greece

⁴Epithelial Differentiation and Rheumatoid Autoimmunity Unit (UDEAR), Hôpital Purpan, UMR 1056 Inserm - Université de Toulouse, Toulouse, Cedex 9, France

Correspondence

Georgia Sotiropoulou, Department of Pharmacy, School of Health Sciences, University of Patras, Rion-Patras, Greece.
Email: gdsotiro@upatras.gr

Abstract

The role of epidermal proteolysis in overdesquamation was revealed in Netherton syndrome, a rare ichthyosis due to genetic deficiency of the LEKTI inhibitor of serine proteases. Recently, we developed activography, a new histochemical method, to spatially localize and semiquantitatively assess proteolytic activities using activity-based probes. Activography provides specificity and versatility compared to in situ zymography, the only available method to determine enzymatic activities in tissue biopsies. Here, activography was validated in skin biopsies obtained from an array of distinct disorders and compared with in situ zymography. Activography provides a methodological advancement due to its simplicity and specificity and can be readily adapted as a routine diagnostic assay. Interestingly, the levels of epidermal proteolysis correlated with the degree of desquamation independent of skin pathology. Thus, deregulated epidermal proteolysis likely represents a universal mechanism underlying aberrant desquamation.

KEYWORDS

activity-based probes, activography, histochemistry, rare skin diseases, skin desquamation

1 | BACKGROUND

Epidermal desquamation is a physiological process regulated by proteolytic pathways involving serine proteases, especially kallikrein-related peptidases (KLKs), and other enzymes, *for example*, cathepsins. Various skin disorders display altered epidermal desquamation.^[1-4] Recently, we developed a new histochemical method termed activography that allows the fine mapping of enzymatic activities on tissue sections. It is based on the reaction of an activity-based probe (ABP) with the active form of the corresponding enzyme in clinical/biological samples and detection of the ABP adduct with typical histochemical reagents.^[5] Here, activography was validated side-by-side with in situ zymography to investigate the levels and spatial localization of proteolytic activities in skin biopsies obtained from patients with distinct diseases manifested by abnormal epidermal desquamation. Aberrant proteolysis due to

KLK5 hyperactivity has been causally implicated in epidermal inflammation and overdesquamation in Netherton syndrome (NS).^[6,7] Recently, we reported that in acral peeling skin syndrome (APSS), epidermal proteolytic activities are aberrantly elevated likely due to the lack or diminished expression of endogenous protease inhibitors.^[8] Interestingly, KLK5 has been implicated in the development of atopic dermatitis (AD) skin architecture^[9] and altered expression of KLKs has been observed in AD patients.^[10] In X-linked ichthyosis (XLI), there is accumulation of cholesterol sulphate, a putative inhibitor of serine proteases,^[11] but whether proteolytic activities in the epidermis are altered has not been investigated as also in seborrheic dermatitis (SD), ichthyosis vulgaris (IV) and autosomal recessive congenital ichthyosis due to *TGM1* mutations (ARCI-TGM1). Overall, the findings presented here suggest that abnormal epidermal proteolytic activities underlie the aberrant desquamating phenotype in many more inflammatory, desquamating skin disorders.

2 | QUESTIONS ADDRESSED

We validated activography as a new assay to map the epidermal proteolytic activities in skin biopsies obtained from patients suffering from various skin diseases that affect epidermal desquamation.

3 | EXPERIMENTAL DESIGN

Biopsies were obtained from patients with NS, APSS, AD, XLI, ARCI with *TGM1* mutations, SD and IV. All patients have provided written consents, and the studies were approved by the Ethics Committees of the respective institutions. A detailed description of tissues is provided

TABLE 1 Results of activography and in situ zymography analysis of clinical specimens

Sample	Activity		Spatial localization of protease activities by activography	Disease severity/severity of desquamation ^a
	In situ zymography	Activography		
Normal (healthy)				
N1	+/-	+/-	SC	Normal
N2	+/-	+/-	SC	Normal
N3	+	+	SC	Normal
N4	+	+	SC	Normal
N5	+	+	SC	Normal
Netherton syndrome (rare 1:200 000, loss of function of <i>SPINK5</i> encoding LEKTI)-Severe overdesquamation and atopic diathesis ^[15]				
NS1	+++	+++	All epidermal layers	Severe ^b
NS2	+	+++	Low epidermal activity-absence of SC	Severe
Seborrheic dermatitis (common, relation with <i>Malassezia</i> yeasts and undetermined genetic factors)-flaking and scaling skin, pruritus at the scalp and face ^[16]				
SD1	++	+++	All epidermal layers	Absence of desquamation
Acral Peeling Skin syndrome (very rare >1:1000 000, loss of function of <i>TGM5</i> encoding transglutaminase 5)-overdesquamation in hands and feet ^[17] APSS2 and 3 are shown in Supporting data				
APSS1	+++	+++	All epidermal layers	Severe ^{b,c}
APSS2	++	++	All epidermal layers	Moderate
APSS3	+++	+++	All epidermal layers	Severe
Atopic dermatitis (common, multifactorial)-pruritus and recurrent eczematous lesions ^[18]				
AD1	+++	++	All epidermal layers	60% eczema, atopy ^d , high IgE levels (25 111 kU/L, normal <100 kU/L)
AD2	++	++	All epidermal layers	50% eczema, atopy ^d , high IgE levels (2071 kU/L)
AD3	+/-	+	Very slight epidermal staining	50% eczema, no signs of atopy, normal IgE levels
X-linked ichthyosis (relatively common 1:1800, loss of function of <i>STS</i> encoding steroid sulphatase)-no desquamation, no inflammation ^[19]				
XLI1	-	+/-	Almost no epidermal staining	No shedding ^{e,f}
XLI2	-	+/-	Almost no epidermal staining	No shedding ^f
ARCI (rare 1:90 000-1:300 000, loss of function of <i>TGM1</i> encoding transglutaminase 1)-epidermal scaling may have collodion membrane at birth ^[20]				
ARCI1	+/-	+	SC/SG interface	
ARCI2	+/-	++	Low staining in all epidermal layers	Dermis with activography
Ichthyosis vulgaris (relatively common 1:250, mutations in filaggrin)-no desquamation similar to XLI ^[21]				
IV1	+/-	+/-	No epidermal staining	No shedding ^f
IV2	+/-	+/-	No epidermal staining	No shedding ^f

SC, stratum corneum; SG, stratum granulosum.

^aDisease severity was based on clinical/macrosopic assessment.

^bSevere indicates extensive macroscopic desquamation and easy of peeling.

^cIn situ zymography and clinical assessment have been published previously.^[7]

^d60% or 50% body surface coverage.

^eActivity was found in the dermis.

^fAbsence of shedding was assessed based on the clinical image of the patient.

in Table 1. Activography was conducted with the B24P ABP that reacts with active serine proteases and esterases.^[2] In situ zymography^[2,8] and immunohistochemistry (IHC) were conducted as described.^[6] Detailed experimental conditions are found in Supporting Information.

4 | RESULTS AND DISCUSSION

All healthy controls ($n = 5$) exhibited “low” staining that was localized at the stratum granulosum (SG) and stratum corneum (SC), compatible with the known protein expression pattern of KLKs. In accordance, they displayed low caseinolytic activities using in situ zymography at the SG and SC. Representative images of activography and in situ zymography are shown in Figure 1, and findings are summarized in Table 1.

NS1 skin tissue showed high activography intensity throughout the epidermis, especially at regions of SC/SG dissociation (Figure 1). Caseinolytic activities showed similar pattern. The NS2 did not show increased caseinolytic activities, most likely because SC was completely shed from the stained area. Activography found increased proteolytic activities in SG and inner layers. Thus, activography does not provide identical results with in situ zymography, as anticipated, since B24P is specific for mapping serine protease activities, while casein is recognized by other proteases as well. Strong activography signal was detected in the sample from APSS patient that exhibited severe desquamation, especially at the SC/SG interface (Figure 1, Table 1). In two other APSS patients, activography staining correlated with the severity of desquamation, (Figure S1A, Table 1).

Using activography, proteolytic activities were spatially mapped and quantified in AD, XLI, ARCI (*TGM1*) and IV skin (Figure 1, Table 1).

Elevated activities were also observed in SD, a severe form of dandruff characterized by altered corneocyte cohesion and abnormal corneodesmosome degradation. The enhanced proteolytic activities could be related to both mechanisms. The previously reported increased expression of the serine protease inhibitors LEKTI (lymphoepithelial Kazal-type-related inhibitor) and SCCA1 (squamous cell carcinoma antigen) in dandruff could reflect compensatory mechanisms.^[12] Biopsies from three AD patients exhibiting different degrees of skin inflammation and serum IgE levels were analysed, and the levels of proteolysis correlated with phenotype severity (Figure S1A, Table 1). In XLI and IV, almost no proteolysis is observed, consistent with hyperkeratosis and diminished desquamation. A slight staining in SC/SG junction in XLI may be attributed to low serine protease activities reminiscent of healthy specimens. Both ARCI-*TGM1* samples showed loss of epidermal proteolytic activities consistent with diminished desquamation. However, similarly to XLI, slight staining was found at the SC/SG junction of ARCI1 specimen. Increased dermal staining with activography in ARCI2 could suggest the presence of active serine proteases with low caseinolytic activities in the dermis.

Immunohistochemical staining for KLK5 in healthy, NS, AD, APSS and SD samples resembled activography staining (Figure S1B), consistent with the fact that KLK5 plays important roles in NS,^[6] AD^[9,10] and APSS^[8] pathology. KLK6 colocalizes with KLK5, and its staining is like activography (Figure S1C).

Using activography, we associated abnormalities in the levels of epidermal proteolysis with corresponding alterations (up or down) in the extent of desquamation in a variety of skin disorders. Except for NS, where the causative gene encodes a protease inhibitor, in the other skin diseases studied here, abnormal proteolysis was not known to be a pathological feature. We propose the concept that deregulated proteolysis could be a unifying feature in diseases characterized by abnormal desquamation. A subset of clinical specimens was also analysed with gelatin in situ zymography to determine the specificity of activography. Since matrix metalloproteinases are the main gelatinolytic enzymes, the B24P should not provide identical results to gelatin in situ zymography. Indeed, the pattern of gelatinolytic activities was completely different from that of caseinolytic activities and activography (Figure 1B). Specifically, no gelatinolytic activities were detected in most samples, except for APSS that showed high activity only in SC. To provide evidence for the specificity of B24P, skin biopsies were pretreated with aprotinin and AEBSF that effectively inhibit activity of serine proteases (Figure S2). It should be mentioned that these controls should always run in parallel with the experiments as also suggested and performed previously,^[13,14] especially in this case that the B24P does not have a proteolytic enzyme-selective “bait” and its specificity merits further characterization.

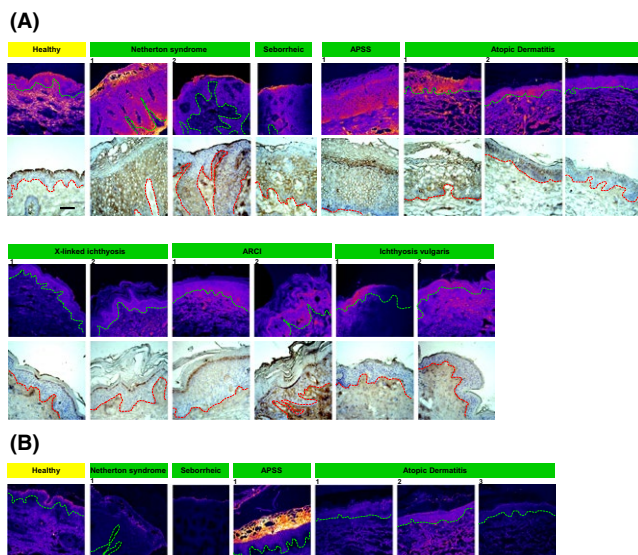


FIGURE 1 Analysis of clinical specimens with activography and in situ zymography. A, Comparison of activography and casein in situ zymography. For clarity, only one control sample of healthy skin is shown. B, Gelatin in situ zymography of clinical specimens. The dashed lines (green or red) indicate the dermo-epidermal junction zone. Scale bar: 20 μ m

5 | CONCLUSIONS

Activity-based probes have a well-recognized ability to visualize proteases in vivo.^[13,14] Here, we showed that activography provides similar spatial and semiquantitative information as the in situ zymography

method but is advantageous in that activography allows the mapping of enzyme specificities thus opening possibilities for new applications in clinical diagnostic laboratories. In the present study, the B24P-ABP used does not have proteolytic enzyme-selective "bait," and therefore, it is expected to react with both proteases and esterases. However in skin, using the appropriate control inhibitors, it was demonstrated that B24P mainly reacts with proteases. The development of highly specific ABPs that could react with only one enzyme will enable spatial localization of the active forms of unique enzymes. Finally, we highlight for the first time that deregulated skin proteolysis is a common feature in a wide range of distinct skin diseases, all characterized though, by aberrant desquamation and barrier defect. This could open a new window targeting proteolytic pathways.

CONFLICT OF INTERESTS

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

EZ performed experiments and analysed data; GP participated in experiment design and data analysis; DK, MV and NJ collected and characterized the clinical samples; GS designed and supervised research; EZ, GP and GS wrote the manuscript.

ORCID

Georgia Sotiropoulou  <https://orcid.org/0000-0003-0747-4914>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Comparison of activography (A) with KLK5 (B) and KLK6 (C) IHC staining. In activography, two more APSS biopsies were included (marked with asterisk). APSS1 (Figure 1) and APSS3 show severe desquamation compared to APSS2 that exhibits moderate desquamation. In situ casein zymography has been published previously^[7]. Negative controls include absence of B24P in (A) and absence of incubation with primary anti-KLK5 (B) and anti-KLK6 (C) antibodies. Scale bar: 20 µm.

Figure S2. Representative activography controls. To demonstrate that the B24P-ABP reacts preferentially with serine proteases than other serine hydrolases present in skin we have pre-incubated samples (2 h at room temperature) for NS, and SD, with either 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), or aprotinin serine protease inhibitors. Since the ABP used here does not have proteolytic selective "bait" it is necessary to include the above mentioned controls to confirm the specificity of the assay. Indeed as shown, both inhibitor pretreatments resulted in absence of B24P-staining confirming the specificity of the assay. Negative controls that do not include reaction with B24P to test the specificity of streptavidin-peroxidase polymer are also shown. Scale bar: 20 µm.

How to cite this article: Zingkou E, Pampalakis G, Kiritsi D, Valari M, Jonca N, Sotiropoulou G. Activography reveals aberrant proteolysis in desquamating diseases of differing backgrounds. *Exp Dermatol.* 2019;28:86–89. <https://doi.org/10.1111/exd.13832>