

Calmodulin-like skin protein level increases in the differentiated epidermal layers in atopic dermatitis

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Abstract: In atopic dermatitis (AD), the skin barrier is disturbed, and the expression of calcium-dependent S100 proteins and the calcium gradient is also altered in the epidermis. The calmodulin-like skin protein (CLSP), which is expressed in the differentiated epidermis, is believed to modulate the function of calcium-dependent proteins involved in barrier formation and is significantly increased in the epidermis of psoriatic patients. We, therefore, investigated the CLSP level in skin biopsies taken from patients with acute exacerbated and non-exacerbated AD as well as from healthy control subjects. Immunohistochemical, Western blot and ELISA analyses showed significant increases ($P < 0.03$) in

CLSP level in the epidermis from patients with acute exacerbated AD as compared to that from patients with non-exacerbated AD and from control subjects. Such increased expression of CLSP may help re-establish a functional epidermal barrier in acute AD.

Abbreviations: AD, atopic dermatitis; CLSP, calmodulin-like skin protein; AEAD, acute exacerbated AD; NEAD, non-exacerbated AD.

Key words: atopic dermatitis – calcium – skin barrier – stratum corneum

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Background

Atopic dermatitis is a chronic inflammatory skin disease which is characterized, in part, by dry and scaly skin. This is associated with a disturbed skin-barrier function induced by several biological changes (1). These include the decrease in the size of corneocytes (2,3) alterations in stratum corneum ceramide content, the maturation of lipid-containing Odland bodies (4–9), perforin hyper-releasability (10) and the activity of proteases (11–13). The epidermal calcium ion gradient is also disturbed in AD (14), and in certain cases, alterations in the levels of Filaggrin; a key calcium-dependent protein in skin-barrier formation which may predispose subjects to the disease (15). Recently, the levels of the S100 proteins, Hornerin and Filaggrin 2 were shown to be decreased in the epidermis of patients with AD (16).

Epidermal differentiation and barrier function are dependent on many proteins and enzymes which require calcium for their function (17–20). In skin, calcium transport and function are governed by a family of calcium-binding proteins known as calmodulins which are involved in the regulation of keratinocyte differentiation (21). CLSP is specifically expressed in the stratum granulosum and lower layers of the stratum corneum and is believed to modulate the function of calcium-dependent proteins, such as transglutaminase 3 (22,23). During ageing, the processed form of CLSP is significantly increased in the stratum corneum (24).

In the hyperproliferative skin disorder, psoriasis, both the calcium gradient and permeability barrier are disturbed (25). Furthermore, CLSP level is increased in the stratum granulosum to the mid layers of the psoriatic epidermis. This increase is not due to increased expression of CLSP but rather to an alteration of its degradation (26).

Questions addressed

To date, little is known about the role of calcium-regulating proteins in the pathogenesis of AD. We, therefore, investigated the

expression of CLSP in the epidermis of AD patients to determine whether its levels are altered compared with that in healthy skin.

Experimental design

CLSP expression was examined in biopsies taken from patients with acute exacerbated (AEAD) ($n = 9$) and non-exacerbated AD (NEAD) ($n = 7$) as well as from healthy control subjects ($n = 7$) using immunohistochemical, Western blot and ELISA analyses. See Supporting Information for details (Data S1).

Results

Immunohistochemical analysis of CLSP in biopsy sections taken from patients with AEAD, NEAD and in biopsies from normal matched controls showed that CLSP was expressed exclusively in the stratum granulosum and lower layers of the stratum corneum (Fig. 1) as previously reported (26). No expression of CLSP was observed in the dermis. A visual score indicated that the level of CLSP in the stratum granulosum of the epidermis of AEAD patients was significantly higher ($P < 0.03$) than in those from NEAD patients and in normal skin from healthy volunteers.

To determine which form of CLSP increased in AD, a Western blot analysis was performed on the second biopsy taken from each patient. The results showed that the predominant form of CLSP found in soluble protein extracts from the biopsies was full length 16 kDa isoform (Figure S1) which was significantly increased in AEAD patients compared with NEAD patients ($P < 0.05$) and normal controls ($P < 0.02$) (Fig. 2a). An ELISA analysis confirmed that CLSP levels were significantly increased in the epidermis of AEAD patients as compared to NEAD patients ($P < 0.05$) and normal controls ($P < 0.02$) (Fig. 2b).

Discussion

We demonstrated, with three different methods, a significant increase in CLSP expression in the upper epidermis in AEAD as compared to NEAD and normal skin. This reinforces a published

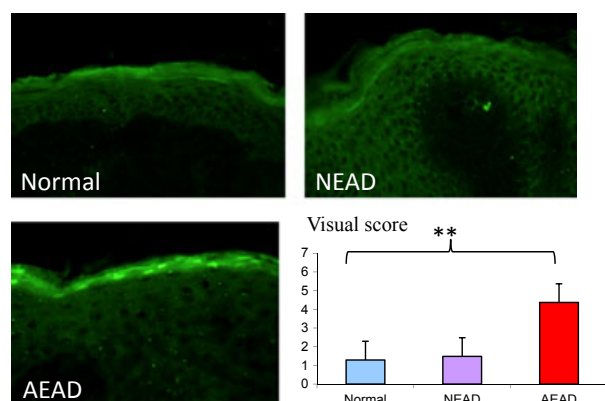


Figure 1. Immunohistochemical analysis showing CLSP expression (fluorescent green stain) in the epidermis of normal ($n = 7$), NEAD ($n = 7$) and AEAD skin ($n = 7$). The histogram shows the average relative visual score in each group. A Student's t -test showed that the visual score for CLSP increased significantly (** $P < 0.03$) in the AEAD group compared with the NEAD and normal control groups.

proteomic analysis of squame samples from severe AD revealing a significant increase in CLSP in the stratum corneum (27,28). Thus, increased levels of CLSP in the upper epidermis may be considered as a biomarker of acute exacerbated AD.

However, CLSP is not an exclusive biomarker for AD, as it was also shown to be increased in psoriatic epidermis (26). In psoriasis, in contrast to AEAD, where CLSP expression was strictly confined to the stratum corneum and granulosum, elevated levels of CLSP were also found in the spinous layers. In psoriasis, decreased degradation was reported to be responsible for the persistence of the full length form of CLSP as calcium protects CLSP from degradation by proteases (26). Our data also demonstrated the predominance of the full length 16 kDa form in AEAD. This might be explained in part by increased epidermal calcium levels occurring in the same layers of AD skin (14) where higher CLSP levels were found.

In the later stages of wound healing in human epidermis, CLSP levels were notably increased in granular and spinous keratinocytes. Once the wound had closed and barrier function was restored, CLSP returned to normal (29). Thus, CLSP may be considered as a biosensor of calcium which helps to restore the epidermal cal-

Western blot CLSP

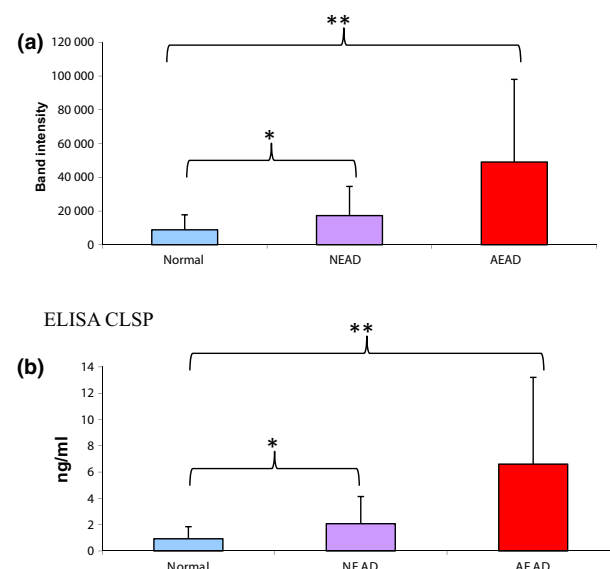


Figure 2. (a) Western blot analysis and (b) ELISA of CLSP expression in biopsies taken from subjects with normal ($n = 7$), NEAD ($n = 7$) or AEAD skin ($n = 9$). In (a) a histogram shows the average intensity of each CLSP band standardized per $1 \mu\text{g}$ of total protein. A Student's t -test showed that in both analyses, the CLSP score was significantly increased in the AEAD group compared with NEAD (* $P < 0.05$) and control groups (** $P < 0.02$).

cium gradient after barrier disruption – CLSP may also have a similar role in AD to re-establish a functional epidermal barrier which has been disrupted in this disease.

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Author contributions

MD analysed the data and wrote the manuscript; AA designed the research study, analysed the data and edited the manuscript. ATC and CP performed the research. DB analysed the data and edited the manuscript; OJ coordinated the clinical study; HG and OdeL designed the research study.

Conflict of interests

The authors declare no conflict of interest.

References

- Cork M J, Danby S G, Vasilopoulos Y *et al.* *J Invest Dermatol* 2009; **129**: 1892–1908.
- Hölzle E, Plewig G. *J Invest Dermatol* 1977; **68**: 350–356.
- Kashibuchi N, Hirai Y, O'Goshi K *et al.* *Skin Res Technol* 2002; **8**: 203–211.
- Melnik B, Hollman J, Erler E *et al.* *J Invest Dermatol* 1989; **92**: 231–234.
- Fartasch M, Diepgen T L. *Acta Derm Venereol* 1992; **176**: 26–31.
- Hara J, Higuchi K, Okamoto R *et al.* *J Invest Dermatol* 2000; **115**: 406–413.
- Mecheleideit O, Kaiser H W, Sanhoff K. *J Invest Dermatol* 2002; **119**: 166–173.
- Janssens M, van Smeden J, Gooris G S *et al.* *J Invest Dermatol* 2011; **131**: 2136–2138.
- Janssens M, van Smeden J, Gooris G S *et al.* *J Lipid Res* 2012; **53**: 2755–2766.
- Ambach A, Bonnekeh B, Gollnick H. *J Allergy Clin Immunol* 2001; **107**: 878–886.
- Tarroux R, Assalit M F, Licu D *et al.* *Skin Pharmacol Appl Skin Physiol* 2002; **15**: 55–62.
- Komatsu N, Saijoh K, Kuk C *et al.* *Exp Dermatol* 2007; **16**: 513–519.
- Seguchi T, Cui C Y, Kusuda S *et al.* *Arch Dermatol Res* 1996; **288**: 442–446.
- Forslind B, Werner-Linde Y, Lindberg M *et al.* *Acta Derm Venereol* 1999; **79**: 12–17.
- Palmer C N, Irvine A D, Terron-Kwiatkowski A *et al.* *Nat Genet* 2006; **38**: 441–446.
- Pellerin L, Henry J, Hsu C Y *et al.* *J Allergy Clin Immunol* 2013; **131**: 1094–1102.
- Elias P M, Ahn S K, Denda M *et al.* *J Invest Dermatol* 2002; **119**: 1128–1136.
- Bikle D D, Oda Y, Xie Z. *J Steroid Biochem Mol Biol* 2004; **90**: 355–360.
- Candi E, Schmidt R, Melino G. *Nat Mol Cell Biol* 2005; **6**: 328–340.
- Kypriotou M, Huber M, Hohl D. *Exp Dermatol* 2012; **21**: 643–649.
- Fairley J A, Marcelo C L, Hogan V A *et al.* *J Invest Dermatol* 1985; **84**: 195–198.
- Méhuil B, Bernard D, Simonetti L *et al.* *J Biol Chem* 2000; **275**: 12841–12847.
- Méhuil B, Bernard D, Schmidt R. *J Invest Dermatol* 2001; **116**: 905–909.
- Delattre C, Winstall E, Lessard C *et al.* *Exp Dermatol* 2012; **21**: 205–210.
- Menon G K, Elias P M. *Arch Dermatol* 1991; **127**: 57–63.
- Méhuil B, Bernard D, Brouard M *et al.* *Exp Dermatol* 2006; **15**: 469–477.
- Broccardo C, Mahaffey S B, Strand M *et al.* *J Allergy Clin Immunol* 2009; **124**: 1113–1115.
- Broccardo C J, Mahaffey S, Schwarz J. *J Allergy Clin Immunol* 2011; **127**: 186–193.
- Hwang J, Kalinin A, Hwang M *et al.* *J Biol Chem* 2007; **282**: 18645–18653.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Figure S1. Western blot analysis of CLSP expression in biopsies taken from patients with acute exacerbated ($n = 9$) and non-exacerbated AD ($n = 7$) as well as from healthy control subjects ($n = 7$).

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Data S1. Materials and Methods

Tissue samples

Two 4 mm punch biopsies were taken from patients with acute exacerbated AD (AEAD) (n= 7) and non exacerbated AD (NEAD) (n= 7) who were recruited from the Clinic for Dermatology, Otto-von-Guericke University Magdeburg, Germany. 2 additional 4 mm punch biopsies taken from AEAD subjects were included in Western and ELISA analyses. AEAD and NEAD were clinically characterized by their SCORAD index an objective measuring of erythema, oedema/papulation, excoriations, lichenification, oozing/crusts and dryness of patient's skin as determined by a European AD task force (Dermatology 1993 186 23-31) and atopy score (Table 1). All AEAD-patients were characterized by a medium to high SCORAD (mean: 38.25 ± 11.12), all NEAD-patients had no or only residual areas of chronic AD (SCORAD 18.2 ± 12.55). Healthy, sex and age matched volunteers with no history of inflammatory skin, allergic rhinoconjunctivitis or asthma served as controls (n = 7). All individuals gave their informed consent and the study was approved by the local ethic committee of the Otto-von-Guericke University.

Skin type	SCORAD (mean +/- SD)	Atopy score (mean +/- SD)
Normal	0	1.71 +/- 1.33
NEAD	18.2 +/- 12.55	13.85 +/- 3.18
AEAD	38.25 +/- 11.12	17.21 +/- 5.94

Table 1. Mean SCORAD index and Atopy scores of each patient group.

Immunohistochemistry

5 µm cryosections from the first biopsy from each patient were stained for CLSP using a mouse monoclonal antibody, 15C9, and AlexaFluor 488-conjugated rabbit anti-mouse secondary antibody (Invitrogen San Diego CA USA) as described previously (22). Representative images of the staining on each section were captured and analysed using a DMR microscope (Leica Microsystems Nanterre France). Identical exposure times were used for the capture of each image. The staining intensity of each image was scored blind by three independent investigators using a five-point scale where grade 1 denotes low CLSP expression in the SG and grade 5 denotes very strong CLSP expression.

Protein extraction

The second biopsy from each patient was homogenised in a buffer of TBS/1% Triton-X 100/1 M NaCl plus protease inhibitors – complete EDTA free (Roche Diagnostics Meylan France) . Resulting supernatants were clarified by centrifugation and filtration using Ultrafree –MC centrifugal filter units according to the manufacturer's instructions (Millipore Molsheim France). Protein determination of soluble extracts was performed by BCA assay (Thermo Scientific_Courtaboeuf France).

Western blot analysis

10 μ l aliquots of soluble protein extract from each second biopsy were electrophoresed on Criterion® 10-20% gradient gels (Biorad Marnes-la coquette France). Proteins were then transferred to PVDF membranes (Immobilon P, Millipore Molsheim France) under standard conditions. Membranes were incubated with the primary antibody (CLSP monoclonal 15C9) followed by a Horseradish Peroxidase conjugated anti-mouse secondary antibody (Dako Courtaboeuf France). Protein bands were revealed by ECL Plus® (GE Healthcare Orsay France). Signals were imaged using a FluroS_{max} scanner (Biorad Marnes-la coquette France) and quantified using Quantity One® software (Biorad Marnes-la coquette France). Results were expressed as intensity of the signal per 1 μ g total protein.

ELISA Assay

Custom CLSP ELISA assays, developed with MesoScale Discovery (Gaithersburg, MD USA), were used to measure the quantity of CLSP in soluble protein extracts from each biopsy. The capture antibody CLSP (monoclonal clone 15C9) was coated at a concentration of 200 μ g/ml on standard small spot 96 well plates (MesoScale Discovery Gaithersburg, MD USA). A standard curve (0.4 to 25 ng/ml) was established using a full-length recombinant CLSP protein (22). Each well was blocked with TBS/0.2%Tween-20/3% BSA for 1 hour at room temperature (RT). 25 μ l of each sample and standard was added in triplicate to each well and incubated with shaking for 1 hour at RT. The plate was washed four times with TBS/0.2%Tween-20. Plates were incubated with shaking for 1 hour at RT with 25 μ l of detection antibody at a concentration of 1 μ g/ml (CLSP clone 7G12 labeled with sulfo tag from Mesoscale Discovery Gaithersburg, MD USA). Plates were washed four times with TBS/0.2%Tween-20 before adding 150 μ l of 1 X READ buffer T (MesoScale Discovery Gaithersburg, MD USA). Plates were read with a Sector Imager 6000. Data was standardized as ng CLSP per 1 μ g of total protein.

Figure S1: Western blot analysis of CLSP expression in biopsies taken from patients with acute exacerbated (n = 9) and non-exacerbated AD (n = 7) as well as from healthy control subjects (n = 7).

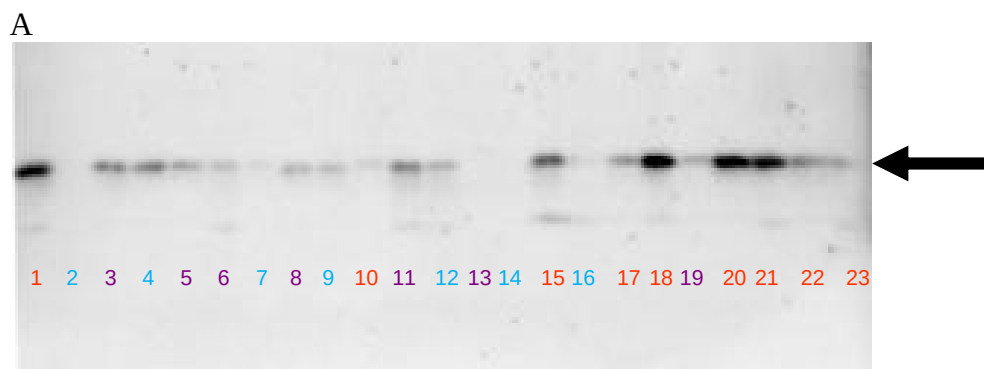


Figure S1: A) Western blot analysis using the 15C9 monoclonal antibody showing CLSP expression in biopsies taken from subjects with normal (lanes 2, 4, 7, 9, 12, 14, 16), NEAD (lanes 3, 5, 6, 8, 11, 13 & 19) or AEAD skin (lanes 1, 10, 15, 17, 18, 21, 22, 23 & 24). A 16 kDa band representing the full length form of the protein is indicated by an arrow.