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The caspase-1 inhibitor CARD18 is specifically expressed during late differentiation of keratinocytes and its expression is lost in lichen planus

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ABSTRACT

Background: CARD18 contains a caspase recruitment domain (CARD) *via* which it binds to caspase-1 and thereby inhibits caspase-1-mediated activation of the pro-inflammatory cytokine interleukin (IL)-1β.
Objectives: To determine the expression profile and the role of CARD18 during differentiation of keratinocytes and to compare the expression of CARD18 in normal skin and in inflammatory skin diseases.
Methods: Human keratinocytes were induced to differentiate in monolayer and in 3D skin equivalent cultures. In some experiments, CARD18-specific siRNAs were used to knock down expression of CARD18. CARD18 mRNA levels were determined by quantitative real-time PCR, and CARD18 protein was detected by Western blot and immunofluorescence analyses. *In situ* expression was analyzed in skin biopsies obtained from healthy donors and patients with psoriasis and lichen planus.
Results: CARD18 mRNA was expressed in the epidermis at more than 100-fold higher levels than in any other human tissue. Within the epidermis, CARD18 was specifically expressed in the granular layer. *In vitro* CARD18 was strongly upregulated at both mRNA and protein levels in keratinocytes undergoing terminal differentiation. In skin equivalent cultures the expression of CARD18 was efficiently suppressed by siRNAs without impairing *stratum corneum* formation. Epidermal expression of CARD18 was increased after ultraviolet (UV)B irradiation of skin explants. In skin biopsies of patients with psoriasis no consistent regulation of CARD18 expression was observed, however, in lesional epidermis of patients with lichen planus, CARD18 expression was either greatly diminished or entirely absent whereas in non-lesional areas expression was comparable to normal skin.
Conclusions: Our results identify CARD18 as a differentiation-associated keratinocyte protein that is altered in abundance by UV stress. Its downregulation in lichen planus indicates a potential role in inflammatory reactions of the epidermis in this disease.

1. Introduction

The epidermis is the outermost layer of the skin and the first line of defense against external physico-chemical injuries and invasion of microbial pathogens [1–3]. After detachment from the basal layer, epidermal keratinocytes undergo a differentiation program and ultimately convert into anuclear corneocytes which form the *stratum corneum*. Besides establishing the body's barrier to the environment, keratinocytes participate also actively in tissue inflammation by secreting a wide variety of pro- and anti-inflammatory mediators [4,5].

Abbreviations: CARD, caspase recruitment domain; IL, interleukin; siRNA, small interfering RNA; UV, ultraviolet.

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It is well documented that human keratinocytes are a major source of pro-inflammatory cytokines such as IL-1β and IL-18. Both IL-1β and IL-18 are generated as inactive precursors (pro-IL-1β and pro-IL-18) in healthy skin [6,7]. Upon exposure to specific stimuli such as UV irradiation, the pro-forms of these cytokines undergo proteolytic maturation and are released [8]. This process is catalyzed by caspase-1, an aspartate-specific protease that is activated within inflammasome complexes [9,10]. The catalytically inactive procaspase-1 interacts with other components of the inflammasome *via* its amino-terminal CARD domain [11].

CARD18, also known as ICEBERG [12], is a human CARD-only protein that shares 53% sequence similarity with the CARD domain of pro-caspase-1 [13]. The *CARD18* gene is located on chromosome 11q22, adjacent to the caspase-1 (*CASP1*) locus and has probably arisen by duplication of the 5′-terminal exons of the *CASP1* gene [14]. It was reported that CARD18 inhibits caspase-1 by binding to the CARD present in procaspase-1. This interaction blocks the activation of procaspase-1 and indirectly suppresses the secretion of IL-1β [11,15]. Expression of CARD18 was detected by RT-PCR in various cell lines and tissues [11,15] as well as in the epidermis of normal skin and non-lesional skin of patients with psoriasis and atopic dermatitis [16].

During the preparation of this manuscript, Göblös and coworkers reported that CARD18 is expressed in all layers of normal human epidermis and that its expression levels are increased in psoriasis [17]. Suppression of CARD18 expression enhanced the secretion of IL-1β from cultured keratinocytes in response to poly (dA:dT) treatment [17], indicating that CARD18 might reduce the activation of caspase-1 by the cytosolic DNA-responsive AIM2 inflammasome. Here, we utilized a new antibody against CARD18 and re-defined the *in situ* expression profile of CARD18 by demonstrating specific localization to the granular epidermal layer and the *stratum corneum* in normal human skin. In line with this immunochemical pattern, we show that keratinocyte differentiation is associated with upregulation of CARD18 *in vitro*, although CARD18 is dispensable for cornification. In addition we report here that CARD18 expression is induced by UVB irradiation and that it is vastly reduced in lichen planus lesions.

2. Materials and methods

2.1. Patients

For the analysis of inflammatory skin diseases, paraffin-embedded tissue obtained for diagnostic purposes were used. Samples were from patients with lichen planus ($n = 7$) and from patients with psoriasis ($n = 6$) at the outpatient department of the Huashan Hospital, Fudan University, Shanghai, China. With the approval of the Committee for the Use of Human Subjects in Research, informed consent was obtained from all subjects.

For studying normal skin and for *in vitro* culture of skin cells, we used specimens derived from abdominal skin reduction surgery, obtained from the Department of Plastic Surgery of the Medical University of Vienna, Austria. The donors provided written informed consent. This study was carried out under the approval of the Ethics Committee at the Medical University of Vienna (EK2011/1149).

2.2. Cell culture and siRNA transfection

Normal human dermal fibroblasts and normal human epidermal keratinocytes were isolated from skin specimens derived from abdominal skin reduction surgery, obtained from the Department of Plastic Surgery of the Medical University of Vienna, Austria. The epidermis and dermis were separated by dispase II. Then keratinocytes were obtained by incubation of the epidermis in 0.25% trypsin/EDTA (Lonza, Basel, Switzerland), and fibroblasts were prepared by incubation of the dermis collagenase I (Gibco, Vienna, Austria). All keratinocytes and fibroblasts were cultured in serum-free keratinocyte growth medium (KGM, Lonza) and Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA), respectively, as described previously [18]. Three Stealth siRNAs specific for CARD18 (RNA duplex sense sequences: 5′-GCAAAUUUAUCAAGCAUCUCUGUGA-3′, 5′-GGCACAAUAAAUGCCUUGCUGGAUU-3′, and 5′-GCUCGAGUCUUGAUUGACCUUGUUA-3′) and a negative control (scrambled) were obtained from Invitrogen (Carlsbad, CA, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to a published protocol [19]. Briefly, third-passage keratinocytes were grown to 50–60% confluence in a T75 culture flask. 50 μl Lipofectamine 2000 was mixed with 5 ml Opti-MEM medium (Gibco) and 65 μl of a 20 μM siRNA solution (Invitrogen) or the scrambled control RNA solution (Invitrogen). After incubation at room temperature for 30 min, the solution was added to 20 ml KGM (Lonza) and transferred to the cells. The keratinocytes were then incubated for 24 h and seeded onto a fibroblast collagen gel as described below. As further control, cells were treated with Lipofectamine 2000 alone.

2.3. Preparation of 3D skin equivalent cultures

In vitro 3D skin equivalent cultures were generated as previously described [18]. Briefly, a suspension of collagen type I (Vitrogen 100, Collagen, Palo Alto, CA) containing 1×10^5 fibroblasts per ml, balanced by Hank's buffered salt solution (HBSS, GIBCO), was added into a special cell-insert (3 μm pore size, BD Bioscience, Bedford, MA, USA) and incubated at 37 °C for 2 h in a humidified atmosphere, and then equilibrated with KGM for 2 h. 1.5×10^6 transfected or control keratinocytes were suspended in 2 ml KGM and seeded onto the collagen gel. After overnight incubation at 37 °C, the KGM medium was removed from both inserts and external wells, and replaced in the external wells by serum-free keratinocyte defined medium (SKDM), consisting of KGM without bovine pituitary extract and supplemented with 1.3 mM calcium (Sigma, Vienna, Austria), 10 μg/ml transferrin (Sigma), 50 μg/ml ascorbic acid (Sigma) and 0.1% bovine serum albumin (Sigma). Keratinocytes were allowed to form a multilayered epidermis for 7 days and medium was changed in every other day for 7 days.

2.4. UVB irradiation of skin ex vivo

Irradiation of human skin was performed with a Waldmann F15 T8 tube (Waldmann Medizintechnik, Villingen-Schwenningen, Germany). The energy output of the UVB source, monitored with a Waldmann UV meter, was 1.1 mW/cm^2 at a tube-to-target distance of 30 cm. Human skin obtained from abdominal skin reduction surgery was freed of subcutaneous fat and cut into pieces of 1 cm × 2 cm, placed in 6-well culture plates and irradiated with 150 mJ/cm^2 of UVB. After irradiation, the skin was cultured in 1 ml pre-warmed keratinocyte basic medium (KBM) in 6-well plates for 48 h. Then the epidermis was harvested for RNA and protein analysis. As control, skin was treated identically except for the exposure to UV radiation.

2.5. RNA isolation, cDNA synthesis and quantitative real-time PCR

Keratinocytes and 3D skin equivalent cultures were lysed by TriFast reagent (VWR) and RNA was extracted according to the manufacturer's instructions. For cDNA synthesis, RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and real-time PCR was carried out with LightCycler 480 SYBR