Summary. The human epidermis is characterized by a constant renewal of keratinocytes embedded in a matrix enriched with lipids. Numerous proteins involved in lipid metabolism are found in human epidermis, especially in keratinocytes. Long-chain acyl-CoA derivatives, which are catalyzed by human ACSL5, are important metabolites in several biochemical pathways, including ceramide de novo synthesis. The aim of the present study was to investigate expression of acyl-CoA synthetase isoform 5 (ACSL5) in human epidermis by an in situ, as well as a molecular approach. We show that ACSL5 mRNA and protein are found in human epidermis, as well as in non-differentiated and differentiated HaCaT cells. Keratinocytes of stratum spinosum are the main source for ACSL5 expression in both meshed facial or abdominal skin and ridged skin of upper or lower extremities including TUNEL-positive cells in upper cellular layers. Single keratinocytes of chronic solar-exposed meshed facial epidermis occasionally display a stronger ACSL5 immunostaining. In conclusion, our study indicates that epidermal ACSL5 expression might be involved in differentiation and the stress response of keratinocytes.

Key words: Acyl-CoA synthetase 5, Epidermis, Human, Keratinocyte

Introduction

The human epidermis, an epithelium with constant cellular turnover, consists of at least four different cell types, including keratinocytes, melanocytes, Langerhans cells, and Merkel cells, which are distinguished by specific marker profiles. S100 protein is expressed by melanocytes and Langerhans cells, whereas CD1a is exclusively found in Langerhans cells. Merkel cells are characterized by Cytokeratin 20 expression. Discrimination of different epidermal cell types is of relevance in order to understand complex intraepidermal pathways. Stress response and protective properties of human epidermis are due to a well-balanced homeostasis of different cell types and a permanent renewal of keratinocytes, embedded in a matrix enriched with lipids. It is suggested that intraepidermal lipid metabolism is essential for extracellular matrix composition and a key factor in stress response (de Jager et al., 2004; Jennemann et al., 2007).

Long-chain free fatty acids and their derivatives, like ceramide, are the main components of intercellular lipid matrix, as well as stratum corneum (Pyne and Pyne, 2000; Ponec et al., 2003). This structural and biochemical feature is reflected by the expression of several enzymes and molecules which are involved in lipid metabolism (Harris et al., 1998; Schmuth et al., 2005). In detail, expression of fatty acid binding proteins (FABP), fatty acid transport proteins (FATP), and acyl-CoA synthetase isoforms (ACSL) has been described in human epidermis, as well as in cultured keratinocytes and melanocytes (Harris et al., 1998; Kucharekova et al., 2003; Cario-Andre et al., 2005; Schmuth et al., 2005; Yen et al., 2005; Scott et al., 2006). ACSL proteins and other molecules, like FATP4, are able to catalyze acyl-CoA derivatives from long chain fatty acids and coenzym A (Herrmann et al., 2001; Lewin et al., 2001; Mashek et al., 2004). Acyl-CoA derivatives represent the metabolic active form of fatty acids which are involved in several biochemical pathways of lipid metabolism. In mammalians, synthesis of long-chain acyl-CoA-thioesters is the most important step in cellular long-chain fatty acid metabolism (Mashek et al., 2004).

Up to now, five different isoforms of human acyl-CoA synthetase have been characterized by molecular and biochemical techniques (Mashek et al., 2004).
**ACSL5 expression in keratinocytes**

Biochemical parameters of ACSLs, as well as their tissue distribution, reflected by normalized abundance of ACSL transcripts, vary (Lewin et al., 2001; http://harvester.fzk.de). ACSL1 through ACSL4 transcripts, are found in epithelia of trachea, mouth, liver, and skin, whereas ACSL6 is found in neural tissues and leukocytes. Isoform 5 is abundantly expressed in intestinal surface epithelia (Gassler et al., 2006). The ACSL5 gene is located on chromosome 10q25.1-q25.2, spans approximately 46 kb, and comprises 21 exons coding for the ACSL5 protein (fatty acid CoA ligases; AMP forming; E.C. 6.2.1.3.) (Yamashita et al., 2000). However, data concerning the synthesis of ACSL proteins and their functional relevance in human epidermis are very rare up to now. An in-depth analysis of ACSL5 expression in human skin is not available.

The purpose of the present study was to analyze ACSL5 expression in human epidermis by in situ techniques and a molecular approach.

**Materials and methods**

**Tissue and cell culture**

Formaline-fixed paraffin-embedded (n=20), as well as snap frozen (n=10) specimens, derived from surgical resections of meshed or ridged skin from lower extremities, abdominal as well as facial regions, and the back were used. The use of human tissues was approved by the local ethics committee at the RWTH Aachen University. For molecular analysis unfixed epidermal cells, a spontaneously immortalized keratinocyte cell line displaying a non-tumorigenic phenotype (Fusenig and Boukamp, 1998), was used for all cell culture experiments. Cells were cultured under standard conditions with DMEM high glucose supplemented with 10 % FCS (both substances from Sigma, Deisenhofen, Germany) and harvested in TRI reagent (Sigma).

**RNA and protein isolation**

Tissue specimens or cultured cells were homogenized in TRI reagent (Sigma) followed by simultaneous preparation of RNA and protein according to Chomczynski (1993). Briefly, phase separation was induced by application of 200 µl chloroform per ml suspension and subsequent centrifugation. Precipitated RNA was dissolved in distilled water/RNasin (Promega, Mannheim, Germany). Spectrophotometric measurements were performed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Protein concentrations were estimated using BioRad assay reagent (BioRad, München, Germany). Concentrated Laemmli’s buffer was added to preparation volumes in order to obtain one fold final concentrations.

**Reverse transcription and polymerase chain reaction (RT-PCR)**

SuperScript amplification system (Invitrogen, Karlsruhe, Germany) was used for oligo (dT) primed first-strand cDNA synthesis from DNase-treated total RNA (5 µg). Reverse transcription was terminated with an incubation step at 70°C for 15 min, followed by digestion with RNase H (20 min at 37°C). In control experiments, transcription of a commercially provided RNA or substitution of the enzyme reverse transcriptase by distilled water was performed. A segment of human ACSL5 sequence (accession no. NM_016234) was amplified by PCR using LightCycler equipment (Roche Diagnostics, Mannheim, Germany) and a pair of corresponding primers: 5'-TTT TTG TAC ACG GGG AGA GC-3', 5'-ACA GGC TGT CAA TTT GGG TC-3'. Amplifications were performed in a total volume of 20 µl (3 mM MgCl₂; 0.5 µM each primer; 2 µl SYBR green mix) under the following conditions [35 x (10 s, 95°C; 15 s, 68-58°C; 10 s, 72°C)]. Amplicon integrity was further evaluated by agarose gels (2%) stained with ethidium bromide (Sigma).

**SDS-PAGE and Western blot analysis**

Proteins diluted in Laemmli’s buffer were resolved by one-dimensional SDS-PAGE (7.5% gels) and transferred to PVDF Immobilon-P membranes (Millipore Corp., Bedford, USA) by semidry blotting. Transfer of proteins was controlled by Coomassie staining of gels and membranes. For detection of ACSL5 protein the monoclonal rat anti-human ACSL5 antibody, clone KD7, the secondary anti-rat horseradish peroxidase-conjugated antibody (dilution 1:10.000; Santa Cruz, Santa Cruz, USA), and ECL substrate (Amersham Pharmacia Biotech, Little Chalfont, UK) were applied sequentially. Normal small intestinal mucosa was used as a positive control, in negative controls the primary antibody KD7 was omitted.

**Immunostainings**

For immunohistochemistry, sections of formalin-fixed paraffin-embedded tissues were used. Briefly, sections were dewaxed and then incubated with
monoclonal anti-ACSL5 antibody KD7 for 1 h in a moist chamber at room temperature. The ABC detection kit with DAB as chromogen was applied according to manufacturer’s protocols (DakoCytomation, Golstrup, Denmark). For co-localization studies, double immunofluorescence on 4 µm cryosections was performed. Sections were fixed in acetone, air-dried, and then incubated with KD7 antibody followed by appropriate Cy2-labeled anti-rat antibodies. In the next step, incubation with one of the antibodies against S100 protein (DakoCytomation), CD1a (Immunotech, Marseille, France), or cytokeratin 20 (DakoCytomation) was performed followed by appropriate Cy-3-labeled secondary antibodies. Sections in which the primary antibody was replaced by buffer or the appropriate normal serum were used as negative controls. TUNEL-staining was performed following manufacturer’s recommendations (Roche Diagnostics). Evaluation of immunofluorescence stainings was performed with a Nikon 80i fluorescence microscope (Nikon, Düsseldorf, Germany).

mRNA in situ hybridization

In order to transcribe riboprobes for mRNA in situ hybridization experiments a 970 bp ACSL5 sequence spanning exon 21 and an untranslated region was amplified by PCR and cloned into the PCR-bluntII-topo vector using Topo TA cloning system (Invitrogen). The cloned sequence and respective orientation were controlled by sequencing with BigDye Terminator kit (ABI PRISM, Weiterstadt, Germany) using an ABI 3700 capillary sequencer according to manufacturer’s recommendations. Riboprobes in antisense or sense orientation were transcribed with T7 RNA polymerase (Roche Diagnostics) after a restriction step with BamHI (NEB, Frankfurt, Germany). Alkaline hydrolysis was used for shortening of riboprobes to a calculated average length of 250 bases. Hybridization was carried out on deparaffinized tissue sections of human skin and small intestine (positive control). Briefly, deparaffinized tissue sections were postfixed with 4% paraformaldehyde in PBS for 10 minutes and afterwards treated with 8 µg/ml proteinase K (Roche Diagnostics) at 37°C for 30 minutes. After an acetylation step with triethanolamine and acetic anhydride, slides were dehydrated and air-dried. Prehybridization step and hybridization procedure were carried out in a moist chamber at 47 °C. Hydrolysed riboprobes were used at a final concentration of about 6-10 ng per µl hybridization mixture.

Results

ACSL5 expression in human epidermis

ACSL5 expression in keratinocytes was detectable with both mRNA in situ hybridization and immunohistochemistry (Fig. 1a-d). These findings were corroborated by molecular analyses of mechanically dissected epidermal layers derived from meshed or ridged skin. In such preparations, ACSL5 mRNA was detected with RT-PCR, ACSL5 protein with immunoblotting (Fig. 1e). These in vivo findings were consolidated by cell culture experiments using non-tumorigenic keratinocytic HaCaT cells (Fusenig and Boukamp, 1998). In undifferentiated HaCaT cells, ACSL5 expression was detectable with RT-PCR, and small amounts of ACSL5 protein were found with Western blot analysis (Fig. 1e). Differentiation of HaCaT cells to epidermal equivalents was induced by organotypic co-cultures with postmitotic fibroblasts in collagen type-I gels. Strong ACSL5 immunostaining was also found in such differentiated and multilayered HaCaT cells (Fig. 1f).

Keratinocytes are the main source of intraepidermal ACSL5 expression

Using KD7 antibody, ACSL5 protein was preferentially found in stratum spinosum keratinocytes of ridged and meshed facial/ abdominal skin (Fig. 1a-d). Occasionally, ACSL5 immunostaining was slightly stronger in the upper part and adjacent to the granular layer (Fig. 1c). Keratinocytes regularly displayed a cytoplasmic immunostaining, sometimes with a fine granular aspect. ACSL5 immunostaining of cellular membranes or in extracellular space was not detectable. In order to evaluate ACSL5 expression in different epidermal cell types, double immunofluorescence of ACSL5 protein and alternatively one of the different cell marker molecules, like S100 protein (melanocytes; Langerhans cells), CD1a (Langerhans cells), or cytokeratin 20 (Merkel cells) was performed. Immunofluorescence studies revealed co-localization of cytoplasmic immunostaining, sometimes with a fine granular aspect. ACSL5 immunostaining was detectable with RT-PCR, and, occasionally, co-localization of ACSL5 and CD1a (Langerhans cells) or S100 protein (melanocytes and Langerhans cells). Strong ACSL5 immunostaining of cellular dendrites of CD1a or S100 protein positive cells was not found (Fig. 2a-i). This in situ approach provided further evidence that keratinocytes are the main source of ACSL5 expression in human skin, as shown by mRNA in situ hybridization and ACSL5 immunohistochemistry (Fig. 1a,d). Additionally, in double fluorescence stainings ACSL5 expression was also found in TUNEL-positive keratinocytes (Fig. 2j-l).

Expression patterns of ACSL5 protein in chronically solar-exposed epidermis

Solar radiation is assumed to interact with several molecular pathways, including lipid metabolism of different epidermal cell types (Patton and Davies, 2006). In solar-exposed meshed facial epidermis, ACSL5 expression profile was occasionally changed from a homogeneous immunohistochemical pattern to a
Fig. 1. ACSL5 expression in human epidermis and cultured HaCaT cells. Sections of formalin-fixed paraffin-embedded ridged dermis of the thumb after hybridization with ACSL5 antisense riboprobes (a), ACSL5 sense riboprobes (b), and diaminobenzidine immunostaining with monoclonal KD7 antibody against ACSL5 (c). d. ACSL5 immunostaining of keratinocytes in higher magnification. e. RT-PCR analyses of ACSL5 transcripts (left) and Western blot analyses (WB) of ACSL5 protein (right) from mechanically dissected human epidermis (upper half) or HaCaT cells (lower half); s, experimental sample; n, negative control. f. ACSL5 immunostaining of HaCaT cells after 19 days or 7 days (insert) in organotypic co-cultures. Scale bars: a-c, 150 µm; d, 20 µm; f, 50 µm.
heterogeneous one. ACSL5-expressing epidermal cells intermingled with a variable number of strong ACSL5-expressing keratinocytes were found (Fig. 3).

Discussion

The human epidermis is well characterized as a tissue with a strong requirement for fatty acids in order to synthesize cellular membranes and a lipid-enriched extracellular matrix. Expression of several enzymes involved in epidermal metabolism of lipids has been shown (Harris et al., 1998; Wanner et al., 2004). Fatty acid uptake by human keratinocytes is mediated by a complex transport system with high specificity for long chain fatty acids (Schürer et al., 1994; Harris et al., 1998). Here we provide for the first time evidence that enzyme acyl-CoA synthetase isoform 5 (ACSL5), which is preferentially found in human small intestinal mucosa,
is additionally expressed in human meshed and ridged skin. In order to validate ACSL5 expression, a two-step setting was used, including molecular and \textit{in situ} techniques for detection of mRNA expression as well as protein synthesis. With these techniques, stratum spinosum keratinocytes were shown as the main cellular source of ACSL5 expression, which reflects their important role in intraepidermal lipid metabolism, as suggested by other groups (Harris et al., 1998; Schmuth et al., 2005). Data were additionally confirmed by the observation that ACSL5 expression was also found in undifferentiated, as well as differentiated HaCaT cells (Fusenig and Boukamp, 1998; Mass-Szabowski et al., 2003).

It is well-known that human epidermis consists of cell types other than keratinocytes, such as melanocytes, Langerhans cells, and Merkel cells. In order to evaluate ACSL5 expression in different epidermal cell types, double immunofluorescence for ACSL5 and various respective marker molecules were performed. Using this approach we found that ACSL5 was abundantly expressed by human keratinocytes, and, to a lesser extent, in Merkel cells, Langerhans cells and melanocytes. However, cellular dendrites of Langerhans cells and melanocytes did not clearly show ACSL5 immunostaining, whereas cell bodies were occasionally stained. This spatial distribution of ACSL5 immunostainings could reflect different roles of such cells in epidermal lipid metabolism. Actually, the importance of free long chain fatty acids in the regulation of pigmentation and melanogenesis has been shown recently (Ando et al., 2004). Linoleic acid or palmitic acid, both substrates of ACSL5 (Oikawa et al., 1998), regulate tyrosinase activity via post-transcriptional events, including the modulation of tyrosinase ubiquitination. In addition, Langerhans cells have been identified as components in lipid metabolism, which are involved in antigen presentation via CD1 family molecules, especially CD1a (Pena-Cruz et al., 2003).

**Fig. 3.** Expression patterns of ACSL5 protein in chronic solar-exposed facial epidermis. \textbf{a-d.} ACSL5-immunostaining of dewaxed tissue sections of formalin fixed paraffin embedded meshed skin derived from face after different DAB-incubation periods. \textbf{b.} Image (a) at higher magnification. \textbf{d.} Image (c) at higher magnification. Scale bars: \textbf{a, c}, 100 µm; \textbf{b, d}, 50 µm.
In normal epidermis, immunostaining of ACSL5 and TUNEL-staining were found in some keratinocytes of upper epidermal layers. The coincidence indicates a possible association of ACSL5 expression and apoptosis in keratinocytes. Apoptotic cellular death of keratinocytes in the upper stratum spinosum has already been well defined by several other groups (Eckert et al., 1997, 2002; Bachmann et al., 2001; Bowen et al., 2004). Interestingly, in chronic solar-exposed skin, a variable number of strong ACSL5-expressing keratinocytes was found in the middle and upper epidermal layers. These cells were characterized by cellular shrinking and nuclear condensation, indicating apoptotic status. However, our data do not clarify the role of ACSL5 as an enhancer or silencer of apoptotic cell death. Acyl-CoA, the important product of ACSL5 activity, is able to interact with several mitochondrial proteins, including adenine nucleotide translocase, which is possibly involved in cytochrome C release from mitochondria during apoptosis (Woldegiorgis et al., 1995; Marzo et al., 1998; Kokoszka et al., 2004). A potential pathway to mediate ACSL5 antiapoptotic activities involves the diminishing of free fatty acids, which have been shown to promote the opening of the mitochondrial permeability transition pore (Wieckowski et al., 2000) or to cause cytochrome C release (Scorrano et al., 2001). The reduction of free fatty acids could be induced by ACSL5 activity. Moreover, a functional link between ACSL5 activity and intrinsic antiapoptotic pathways has recently been shown (Mashima et al., 2005). In contrast to these putative antiapoptotic pathways, experimental evidence for a proapoptotic ACSL5 activity via ceramide is given (Modrak et al., 2006). C16-ceramide is assumed to be an important second messenger of apoptosis, because ceramide levels rapidly increase before the onset of apoptosis (Tepper et al., 2000; Osawa et al., 2005; Siskind et al., 2006). Pamitoyl-CoA, which is provided by enzymes like ACSL5, is an important metabolite for de novo synthesis of proapoptotic N-palmitoyl-sphingosine (C16-ceramide), which is known to form channels in mitochondrial outer membranes during the induction phase of apoptosis (Pyne and Pyne, 2000; Osawa et al., 2005; Modrak et al., 2006; Siskind et al., 2006).

In conclusion, our study indicates that keratinocytes are the main source of intraepidermal ACSL5 expression, which might be involved in cellular differentiation and apoptosis. However, the precise function of ACSL5-associated acyl-CoA synthesis in lipid metabolism of human keratinocytes has still to be shown.

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