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Enhanced contact allergen and UVB induced keratinocyte apoptosis in the absence of CD95/Fas/Apo-1

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Abstract

FAS/ CD95/ Apo- 1 is an ubiquitously expressed cell surface receptor involved in the initiation of programmed cell death. Its function in epidermal keratinocytes has been incompletely defined. Available evidence from *in vitro* studies points to important roles of Fas in the pathogenesis of contact dermatitis and in keratinocyte apoptosis induced by ultraviolet light. To define functions of Fas in the epidermis *in vivo*, we have generated mice with epidermis specific deletion of the *fas* gene and have tested its requirement for DNFB induced contact dermatitis and for UVB induced keratinocyte apoptosis. We report here our unexpected finding that keratinocyte apoptosis induced by both a contact allergen and UVB irradiation was significantly enhanced in Fas negative epidermis. Expression of Fas by epidermal keratinocytes was neither necessary for the normal development of contact hypersensitivity of the skin, nor required for keratinocyte apoptosis following UVB irradiation. Our results thus demonstrate that, in the epidermis *in vivo*, Fas exerts anti apoptotic functions that outweigh its pro apoptotic role in contact hypersensitivity responses of the skin and in the tissue response of the epidermis to UVB irradiation.

Introduction

Fas (APO-1/CD 95) is a widely expressed cell surface receptor belonging to the TNF receptor superfamily. The receptor chains are expressed on the cell surface as pre associated homotrimers. Binding of its cognate ligand, FasL, is thought to result in super clustering to form oligomeric receptor complexes. As a consequence, several proteins accumulate next to the cytoplasmic domain of Fas and form the death inducing signaling complex (DISC)(1). Two important components of the DISC are the adaptor protein FADD and its binding partner caspase- 8. Caspase- 8 is a cysteine protease functioning as initiator of a cascade of proteolytic cleavage steps that finally leads to the activation of effector caspases. Effector caspases, like the cystein protease caspase- 3, cleave various intracellular proteins thereby inducing cellular changes that cause the phenomenon of apoptosis. In cells, caspase- 3 is sequestered as a zymogen (p32 caspase- 3). Upon activation of the pro apoptotic effector cascade this is converted into active caspase- 3 by proteolytic cleavage. Activation of caspase- 3 can be detected in tissues and cells by immunostaining using cleavage site specific antibodies (2).

Epidermal keratinocytes have been shown to express Fas (3) and ligation of Fas with a specific antibody can induce apoptosis of keratinocytes in culture after preincubation with gamma interferon. In addition, supernatant from activated human T- lymphocytes containing both FasL and gamma interferon induced apoptosis of cultured human keratinocytes. Apoptosis of Fas- expressing epidermal keratinocytes in association with T- lymphocytes expressing FasL has also been described in contact dermatitis and atopic dermatitis and were thought to play an important role in the pathogenesis of these conditions (4). The dissociation of epidermal keratinocytes with breakup of cell- cell contacts and formation of intra-epidermal blisters, a phenomenon called spongiosis, has been thought to be mediated by Fas/ FasL- interactions. In addition to Fas/ FasL- interactions, perforin expressed by cytotoxic T-

lymphocytes can cause killing of keratinocytes. This additional mechanism of killing was shown to be of relevance in contact dermatitis in mice (5-6).

In addition to its role in initiating apoptosis, Fas- signaling has also been proposed to activate non cell autonomous anti- apoptotic mechanisms in eczematous dermatitis. Upon induction of Fas- signaling, epidermal keratinocytes produce ligands for the epidermal growth factor receptor (EGFR) such as amphiregulin, transforming growth factor alpha (TGF- α) and others. These have been shown to activate anti apoptotic EGFR- signaling in neighboring keratinocytes to protect them from apoptosis and thus restrict tissue damage. In addition, EGFR- dependent signals induced by Fas stimulation in keratinocytes have been implicated in the production of pro inflammatory cytokines that could contribute to the inflammatory reaction in eczematous dermatitis (7-8).

Keratinocyte apoptosis in skin exposed to ultraviolet light (UV light) is characterized by the formation of sunburn cells (SBC). This is thought to be the main mechanism by which potentially transforming mutations are eliminated from the skin. UV light induces DNA damage which leads, in case of irreparability, to p53- mediated apoptosis of the damaged cell (9). The activity of DNA repair mechanisms, which determines whether keratinocytes undergo apoptosis or not, can be regulated by factors of the extracellular milieu (10). In addition to this DNA damage response, apoptosis of epidermal keratinocytes induced by UV light is regulated by Fas dependent mechanisms (11). Irradiation of human epidermal keratinocytes with UVB induces clustering of Fas on the cell surface in a ligand independent manner and recruitment of FADD to the cytoplasmic domain of Fas. This is thought to trigger the same caspase- 8 dependent pro apoptotic signaling cascade that is also induced after binding of FasL to Fas and leads to the activation of Caspase- 3 (11-12). Similar results have been described for murine epidermal keratinocytes (13-14). In line with this proposed

mechanism, studies in mice with mutated FasL suggested that up regulation of Fas and FasL-expression after irradiation with high doses of UVB contribute to apoptosis of epidermal keratinocytes (15). However, UVB induced keratinocyte apoptosis has also been described to proceed via the internal, mitochondrial pathway. UV light is thought to damage the mitochondrial membrane thus leading to the activation of caspase- 9, a critical step in the initiation of apoptosis via this pathway. Blocking caspase- 9 as well as targeted deletion of JNK can inhibit UV- induced apoptosis (16-17), while blocking of caspase- 8 did not prevent UV- dependent apoptosis in murine embryonic fibroblasts (MEFs) (18-19). This suggested that caspase- 8 mediated Fas signaling may not be essential for the proapoptotic response upon UVB- irradiation in MEFs.

Given partially contradictory results regarding functions of Fas in keratinocytes we decided to use conditional gene targeting technology to further elucidate the role of Fas in the epidermis *in vivo*.

Results

Generation of epidermis specific Fas knockout mice

Mice with conditional ablation of Fas alleles have been described previously (20). Female mice homozygous or heterozygous for the floxed Fas allele were bred to male mice homozygous or heterozygous for the floxed Fas allele and expressing Cre- recombinase under the control of the keratin 14- promoter (21). Samples of epidermis from ear and back skin as well as total tissue lysates from mouse tails for comparison were analysed for the presence of wild type and mutant Fas- DNA (supplem. Fig. 1a) and of Fas- protein using PCR and Western blotting, respectively. Whereas epidermal samples from ear and back skin showed complete deletion of floxed Fas- alleles, Fas was readily detected in total tissue lysates from tails (Fig. 1a, supplem. Fig. 1b). This demonstrated epidermis specific deletion of Fas in mice with the genotype $K14Cre^+ Fas^{fl/fl}$ which will be referred to as Fas^{E-KO} mice. Mice with the genotypes $K14Cre^- Fas^{fl/fl}$, $K14Cre^- Fas^{fl/+}$, $K14Cre^+ Fas^{+/+}$ and $K14Cre^- Fas^{+/+}$ are referred to as control mice. Mice with the genotype $K14Cre^+ Fas^{fl/+}$ were excluded from the analysis.

We also analysed primary keratinocytes isolated from control and Fas^{E-KO} mice. Western blot and FACS- analysis showed expression of Fas by keratinocytes from control mice. This was not detectable in keratinocytes isolated from Fas^{E-KO} mice (Fig. 1 b, c). In addition, we tested the response of interferon gamma treated control and Fas KO keratinocytes to stimulation of Fas using the agonistic antibody Jo2 (22). Whereas control keratinocytes showed an increase in the percentage of apoptotic cells upon Jo2 stimulation, the number of apoptotic cells was not increased in the Fas KO keratinocyte population (supplem. Fig. 2a).

Fas^{E-KO} mice did not show an overt phenotype (supplem. Fig. 2b). Their skin was macroscopically and histologically indistinguishable from that of control mice. We also did not observe differences in the hair coat. Staining for markers of proliferation and

differentiation in the skin of Fas^{E-KO} mice and control mice did not show any differences (suppl. Fig. 3).

Enhanced contact dermatitis induced keratinocyte apoptosis in Fas^{E-KO} mice

Fas has been proposed to exert an important function in the pathogenesis of contact dermatitis by mediating apoptosis of epidermal keratinocytes (4). We induced a contact hypersensitivity (CH) reaction in the ears of 16 Fas^{E-KO} mice (9 females and 7 males) and 14 control mice (6 females and 8 males) by challenging with 2, 4-di-nitrofluorobenzene (DNFB) in acetone/olive oil 4:1, 6 days after sensitization. Ear swelling was determined at different time points after challenge as a marker for the strength of the inflammatory reaction. Ear thickness was determined on the day of sensitization and immediately prior to challenge. The average of these measurements formed the baseline value for the determination of ear swelling. Measurements of ear thickness were repeated 24 h, 48 h, 72 h and 96 h after challenge. No significant differences were detected between Fas^{E-KO} mice and control mice at any time point (Fig. 2 a). Females showed stronger ear swelling than males through all time points. Both Fas^{E-KO} mice and control mice showed edema and an inflammatory infiltrate in the dermis with similar density and comparable numbers of inflammatory cells staining positive for CD3 (T- lymphocytes) or CD11b (myeloid inflammatory cells) (Fig. 2 b). Similar numbers of F4/80 positive inflammatory cells (macrophages) were detected in the epidermis at sites of spongiosis both in control and Fas^{E-KO} mice.

Spongiosis was present in all skin samples of challenged Fas^{E-KO} mice, to a similar extent as in control mice (Fig. 2 b). We used immunostaining of activated caspase- 3 and TUNEL-staining (Fig. 3 a) to visualize intra epidermal apoptotic cells in challenged ears of Fas^{E-KO} mice and control mice 96 hours after challenge. Unexpectedly, the number of apoptotic cells in the epidermis was higher in both female and male Fas^{E-KO} mice as compared to controls.

The differences between Fas^{E-KO} mice and control mice were significant (Fig. 3 b). We conclude that, in the DNFB- induced CH reaction in mice, 1) Fas expressed by epidermal keratinocytes is dispensable for the ear swelling reaction, the accumulation of an inflammatory infiltrate and spongiosis, and 2) the presence of Fas in the epidermis partially protects epidermal keratinocytes from apoptosis.

Enhanced UVB induced keratinocyte apoptosis in Fas^{E-KO} mice

Fas has also been implicated in the apoptosis of keratinocytes following UVB- irradiation. We therefore irradiated 15 Fas^{E-KO} mice and 13 age matched control mice with a dose of 1 J/cm² UVB on their back after shaving. After 18 and 24 hours, back skin from 8 male Fas^{E-KO} mice and 9 male control mice or 7 female Fas^{E-KO} mice and 4 female controls, respectively, was taken for analysis. Histological sections of paraffin embedded skin were used for counting sun burn cells and for TUNEL- staining. While there was no difference in the number of TUNEL- positive cells in the epidermis of control and Fas^{E-KO} mice, there were significantly more sun burn cells present in the skin of Fas^{E-KO} mice (Fig. 4 b). Staining with an antibody against the macrophage marker F4/80 did not show positive cells within the epidermis. We conclude that expression of Fas by epidermal keratinocytes is not required for UVB- induced keratinocyte apoptosis in vivo and that the presence of Fas reduces the number of sunburn cells upon UVB- irradiation.

No requirement for Fas in UVB induced keratinocyte apoptosis in vitro

In vivo, there is constant exchange of information between the epidermis and other skin compartments. To investigate whether deletion of Fas had an influence on cell autonomous mechanisms of keratinocyte apoptosis we isolated keratinocytes from Fas- negative epidermis and control epidermis. Western blot and FACS- analysis showed that keratinocytes isolated

from Fas^{E-KO} mice (Fas^{KO} keratinocytes) did not express Fas protein (Fig. 1b, c). Control and Fas^{KO} keratinocytes, 3 different lines each, were then irradiated in vitro with 100 and 300 mJ/cm² UVB and analyzed by FACS using an antibody against annexin V and by TUNEL-staining. Both Annexin V and TUNEL-staining showed similar numbers of apoptotic control and Fas^{KO} keratinocytes (Fig. 5). We conclude that apoptosis of keratinocytes upon UVB-irradiation does not depend on the presence of Fas.

Discussion

Epidermal keratinocytes constitutively express CD95 (3) and Fas induced keratinocyte death is currently thought to play a relevant role in the pathogenesis of a number of severe skin diseases such as eczema and toxic epidermal necrolysis (4, 23). Although apoptosis of epidermal keratinocytes is considered as a hallmark of eczematous dermatitis (24) the biological meaning of this is not well understood. The results from experimental models of contact dermatitis are inconclusive: The absence of Fas or FasL in mice has been reported to have no influence on the contact hypersensitivity response to DNFB (5). In another study, however, oxazolone- induced contact dermatitis was found to be attenuated in Fas- mutant *lpr* mice (25). Since both studies were performed in animals with constitutive functional Fas or FasL deficiency, they were not able to specifically address contributions of epidermal keratinocytes to the tissue reaction. Our approach of an epidermis specific deletion of Fas clearly shows that Fas expression by epidermal keratinocytes is dispensable for a normal contact hypersensitivity response, as ear swelling and inflammatory features were identical in control and Fas deficient skin. The formation of spongiotic vesicles, which is seen as a characteristic sign of acute eczematous dermatitis and has been proposed to be induced by Fas- mediated keratinocyte killing (4), occurred normally in the absence of epidermal Fas expression, showing that it is not Fas dependent. It is likely that, in the absence of Fas, keratinocyte killing is executed by perforin/ granzyme dependent mechanisms (5).

In addition to its role in the induction of apoptosis, Fas has been suggested to elicit anti-apoptotic and pro- inflammatory signals in the epidermis through the production of EGF- receptor ligands such as amphiregulin, TGF- α and numerous pro inflammatory cytokines (7- 8, 26). Recent work shows that the intracellular domain of Fas can be tyrosine phosphorylated, which can lead to the recruitment and activation of phosphatidylinositol-3- kinase (PI- 3K) (27). These findings are based on in vitro studies of immortalized and primary

human keratinocytes and other cell types; their relevance for the in vivo situation within the epidermis remained therefore unclear. Our unexpected result showing that keratinocyte apoptosis upon DNFB- challenge is enhanced in Fas negative epidermis demonstrates an anti apoptotic function of Fas and it is possible that EGF- receptor ligands or PI- 3K signaling mediate this protective signal. This mechanism could indeed restrict extensive tissue damage in response to pro apoptotic stimuli thus contributing to the maintenance of an intact skin barrier (7). Probably, this function of Fas is mediated in a non cell autonomous juxtacrine way, since we did not detect the protective effect against apoptosis in cultures of Fas^{KO} keratinocytes. Although this is, in our view, the most likely mechanism explaining the accumulation of apoptotic cells in the epidermis of Fas^{E-KO} mice, we cannot completely exclude the possibility that the deficiency of Fas in epidermal keratinocytes leads to an inhibition of their autophagocytic activity. This would result in a decreased elimination of apoptotic cells and thereby in their accumulation. Such a function for Fas has, however, so far not been reported. In contrast, the death effector domain containing cellular FLICE- like inhibitor protein c- FLIP which can be recruited to the death inducing signaling complex (DISC) via the adapter protein FADD upon Fas- ligation, has been shown to suppress autophagy (28).

Stimulation of Fas in epidermal keratinocytes has also been reported to result in the production of pro inflammatory cytokines that could facilitate the inflammatory response in eczematous dermatitis (8, 26). On the basis of these findings, a possible pathogenic role of Fas- induced cytokine production in eczema has been assumed (29). We show here that epidermis specific deficiency of Fas does not alter the contact hypersensitivity response to a DNFB- challenge in mice. While our results do not exclude production of pro inflammatory cytokines in the epidermis upon Fas ligation they show that the presence of Fas is not required for the pathogenesis of contact dermatitis in vivo.

Keratinocyte apoptosis upon UV irradiation and subsequent elimination of apoptotic bodies from the skin is believed to be the mechanism that protects the epidermis from cumulating DNA- damage in sun exposed skin. UV- light is absorbed by nuclear, cytoplasmic or cell membrane attached molecules that serve as chromophores. Excitation of these chromophores can activate a number of signaling pathways leading to apoptosis. Fas has been shown to cluster on the surface of human epidermal keratinocytes upon UVB- irradiation in a FasL- independent manner. This is followed by the recruitment of the adapter molecule FADD to the receptor complex which then enables the recruitment of caspase- 8. Both a dominant negative FADD construct and inhibition of caspase- 8 could reduce UVB induced apoptosis suggesting a causal role of the Fas- FADD- caspase 8 interaction in the induction of apoptosis (11-12, 30). This role of Fas was further supported by the finding that 500 mJ/cm² UVB irradiation of C3H/HeJ mice with mutated FasL (*gld/gld* mice) resulted in less apoptosis as compared to wild type controls (15). In contrast to these findings, we show here that in C57/Bl6 mice the Fas/ FasL- signaling system is not essentially involved in UVB- induced keratinocyte apoptosis. Both counting of sun burn cells and TUNEL- staining revealed that there is no decrease in the numbers of apoptotic keratinocytes in Fas negative epidermis as compared to control epidermis. This is further supported by our in vitro analysis of UVB- irradiated Fas KO and control keratinocytes which did not show differences in Annexin V- and TUNEL- staining. These results are consistent with data showing that deletion or blockade of caspase- 8 does not prevent UVB- induced apoptosis of MEFs (18-19). Furthermore, the increased number of sunburn cells in the epidermis of Fas^{E-KO} mice suggests that, in vivo, Fas exerts anti apoptotic functions. TUNEL staining did not show a difference in the numbers of apoptotic keratinocytes between Fas^{E-KO} mice and control mice. This is most

likely due to the relatively late time point of 18 hours post irradiation, which was chosen primarily for the detection of sunburn cells.

The difference between our results and results from other models of UVB- induced keratinocyte apoptosis may be explained by variations in the UV response between mouse strains. Another explanation could be a gradual involvement of different pro apoptotic mechanisms depending on the UVB- dose, since Hill et al. (15) used 500 mJ/cm² whereas we used 300 mJ/cm². A third hypothetical explanation is offered by the fact that *gld/gld* mice are not deficient for FasL but carry a point mutation within the protein that disables pro apoptotic Fas- signaling (31). As a consequence, mutant FasL- mRNA is constitutively and strongly expressed in lymphoid cells of *gld/gld* mice (32). It is therefore conceivable that mutant FasL in these mice, although not able to stimulate pro apoptotic Fas signaling, can still bind to CD 95 expressed on the surface of epidermal keratinocytes and activate its anti apoptotic signaling mechanisms. This would then lead to the stimulation of survival signals in keratinocytes and thus result in decreased apoptosis. The ability of Fas to stimulate pathways involved in cell survival has been demonstrated recently (33). In addition, signaling via c-FLIP, which is known to exert strong anti- apoptotic effects by binding to the adapter protein FADD and competing for the recruitment of caspase 8, could be a mechanism mediating anti apoptotic activities of Fas (34).

In summary, we here provide genetic evidence showing conclusively that, in murine skin, Fas is an essential element of pathways that restrict keratinocyte apoptosis in eczematous dermatitis and upon UVB- irradiation. Neither contact allergen nor UVB- induced keratinocyte apoptosis depend on the expression of Fas by epidermal keratinocytes in vivo. Moreover, both the inflammatory tissue reaction and spongiosis in acute eczematous dermatitis can proceed in the absence of epidermal Fas- expression. The exact mechanisms of

anti apoptotic Fas- signaling remain unclear here; both Fas- mediated autocrine stimulation of EGF- receptor dependent signals (7) and/ or activation of phosphatidylinositol- 3- kinase (33) could be relevant in this context. Both in contact dermatitis and upon UVB- irradiation the protective effects of Fas apparently outweigh Fas- induced keratinocyte death. It therefore seems possible that, for the maintenance of tissue integrity within the epidermis, anti apoptotic functions of Fas are at least as important as its pro apoptotic functions.

***Materials and Methods**

Generation of mice with epidermis specific Fas deficiency and UV irradiation

Mice with conditionally targeted Fas- alleles have been described previously (20). These mice were crossed with mice expressing Cre- recombinase under the control of the keratin- 14 promoter (21). Deletion of floxed Fas- alleles and presence of the Cre- recombinase transgene were analysed by PCR as described therein. For UVB- treatment mice were anaesthetized, shaved on their backs and irradiated with a dose of 300 mJ/cm² UVB using a Waldmann UV 801 lamp equipped with UV21 bulbs emitting UV- light in the range of 280–360 nm with an emission maximum at about 314 nm (Waldmann, Villingen-Schwenningen, BRD). The UV- dose was determined using a Variocontrol UV- radiometer (Waldmann). Mice were killed after 18 or 24 hours and skin was taken for analysis.

Western blot analysis of epidermis and cultured keratinocytes

Epidermis was separated from dermis with forceps after 30 minutes of incubation in 3.8% ammonium thiocyanate dissolved in borate buffer pH 7.6 at 4 °C. Epidermis samples were minced and homogenized for 2 minutes on ice using an MM300 ultrasound tissue homogenizer (Retsch GmbH, Haan, Germany), extracted for 1 h in modified RIPA- buffer containing 5 mM EDTA, 1% Triton X 100, 1% NP40, 0,1% SDS, 0,5% deoxycholate, 20 µM leupeptin, 1 mM PMSF, 0.5 mg/ml soybean trypsin inhibitor, 0.5 mM NaVO₃ and 10 mg/ml p-nitrophenylphosphate, and centrifuged at 14,000 x g for 10 minutes at 4 °C. The supernatant was used for protein analysis. Keratinocytes were lysed in situ using the same buffer, scraped from the dishes and sonicated for 30 seconds at full power. Lysates were centrifuged at 14,000 x g for 10 minutes and the supernatant was used for Western blot analysis. 30 µg of protein were separated by SDS- PAGE and blotted onto nitrocellulose LC 2000 membranes (Invitrogen, Carlsbad, USA). After blocking with 5% milk powder solution the membrane was probed with the following antibodies: rabbit polyclonal anti Fas M- 20

(Santa Cruz Biotechnology, USA) and mouse monoclonal anti actin antibody (MP Biomedicals, Aurora, USA). Immunoreactive proteins were visualised with HRP coupled secondary antibodies on Hyperfilm by chemiluminescence (Western Lightning, Perkin Elmer, Rodgau, USA). Protein quantification was carried out using the Pierce protein quantification kit (Pierce).

Induction of contact dermatitis

Mice were anaesthetized on day 0 and the thickness of the left ear was measured twice. Mice were shaved on the abdomen and 25 µl of a 0,5% DNFB-solution in acetone/olive oil 4:1 was applied and allowed to dry. Six days later the mice were again anaesthetized and the thickness of the left ear was determined twice. The mean of thickness measurements on day 0 and day 6 formed the zero value for the stimulation experiment. 10 µl of a 0.2% DNFB solution were applied to the front side and back side of the left ear. On days 7 – 10 after sensitization mice were anaesthetized and the thickness of the left ear was determined twice. The relative increase in ear thickness was calculated for each time point.

Histopathological analysis and immunostaining

After excision, tissue samples were fixed in 4% paraformaldehyde or embedded in OCT-compound and frozen immediately. Further processing, paraffin embedding of paraformaldehyde fixed tissue and H/E staining was according to standard histopathological procedures. Immunostaining of keratin- 14, keratin- 10 and loricrin were performed as described in (35). For immunostaining against activated caspase- 3 on paraffin embedded tissue a rabbit antibody against cleaved caspase- 3 (Cell Signaling Technology, Inc., Denver, USA) at a dilution of 1:50 was used according to the instructions of the manufacturer.

TUNEL- staining

TUNEL- staining of tissue sections and cells was carried out with the Dead End Fluorimetric TUNEL- System (Promega Corporation, Madison, USA) according to the instructions of the manufacturer.

Isolation and culture of epidermal keratinocytes, UV irradiation and Fas stimulation

Epidermal keratinocytes were isolated from transgenic and wild type newborn mice as described in (36). For UVB- irradiation, keratinocytes were grown to subconfluency without feeder cells, washed with PBS and then irradiated with 100 or 300 mJ/cm² UVB using a Bio Sun++ System at 312 nm (Vilber Lourmat, Marne-la-Vallée, France). After 16 hours of culture keratinocytes were trypsinized and analysed by FACS or TUNEL- staining. For stimulation of Fas, primary epidermal keratinocytes were incubated with 10 ng/ml IFN γ (kindly provided by Jonathan Howard, University of Cologne) to increase Fas- expression. After 48 hours cells were stimulated with 50 ng/ml Jo2 antibody (Becton Dickinson #554256) or left unstimulated. 8 hours or 20 hours later keratinocytes were fixed and numbers of TUNEL- positive cells were determined.

FACS- analysis

Keratinocytes were trypsinized and resuspended in HEPES incubation buffer. Staining of Annexin- V was performed using the Annexin- V- FLUOS Staining Kit (Roche Applied Science, Penzberg, Germany). Analysis was performed with a FACS- Calibur (Becton Dickinson, Franklin Lakes, New Jersey, USA).

Statistics

Determination of significance between samples were made using the Student's t test (GrapPad Prism; GraphPad Software). For all statistical tests, the 0,05 level of the confidence interval was accepted as statistically significant.

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List of abbreviations

CH – contact hypersensitivity

DISC – death inducing signaling complex

DNFB – dinitrofluorobenzene

EGFR – epidermal growth factor receptor

FADD - Fas associated death domain

FasL – Fas ligand

F1 – floxed

FITC – fluoresceine isothiocyanate

H/E - hematoxylin and eosin

JNK - Jun N-terminal kinase

KO – knock out

MEFs - murine embryonic fibroblasts

PI-3K – phosphatidyloinositol-3-kinase

SBC – sunburn cells

TGF α - transforming growth factor alpha

TNF – tumor necrosis factor

TUNEL – terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling

UVB – ultraviolet light B

Figure legends

Figure 1: Deletion of Fas in epidermal keratinocytes

Western blot (**a, b**) and FACS analysis (**c**) with antibodies against Fas (upper panels) and actin as loading control (lower panels). **a**) Epidermis isolated from back skin as well as whole tissue of tails were used. **b, c**) Primary epidermal keratinocytes isolated from newborn mice were analysed for Fas expression by means of Western blot (**b**) and FACS (**c**). ct = control mice/ keratinocytes; ; Thy = thymus extract as positive control, unst = unstained, sec AB = secondary antibody.

Figure 2: DNFB induced contact dermatitis in the absence of epidermal Fas expression

a) 16 Fas^{E-KO} mice (9 females and 7 males) and 14 control mice (6 females and 8 males) were challenged with DNFB 6 days after sensitization. Ear swelling was determined before (0h) and 24, 48, 72 and 96 hours after challenge. Individual data points show the mean increase of ear swelling for each time point calculated from three consecutive measurements of ear thickness. **b**) Micrographs and immunofluorescent images of ear skin of control and Fas^{E-KO} mice 96 hours after challenge with DNFB. The lower panel of pictures of H/E- stained sections shows spongiotic blister formation. The immunostainings show green fluorescence for CD3 and CD11b, respectively. Red staining shows nuclei. Scale bars in H/E- staining: Upper: 100 µm; lower: 50 µm. Scale bar in immunofluorescent staining: 100 µm.

Figure 3: Contact hypersensitivity induced keratinocyte apoptosis in Fas- negative epidermis

Apoptotic cells were detected 96 hours after challenge by staining with an antibody against cleaved caspase- 3 and by TUNEL- staining. **a**) microscopic images of skin sections of Fas^{E-}

^{KO} mice and control mice stained for active caspase- 3 (light microscopy) or with the TUNEL method (fluorescence microscopy), respectively. Arrows indicate apoptotic cells within the epidermis. **b)** Bar graphs show mean numbers \pm SD of caspase- 3 positive or TUNEL- positive epidermal cells per power field in skin sections of male and female control and Fas^{E-} ^{KO} mice. 160 power fields of caspase- 3 and TUNEL stained sections were counted, respectively.

* indicates statistical significance of the difference, p values are given below.

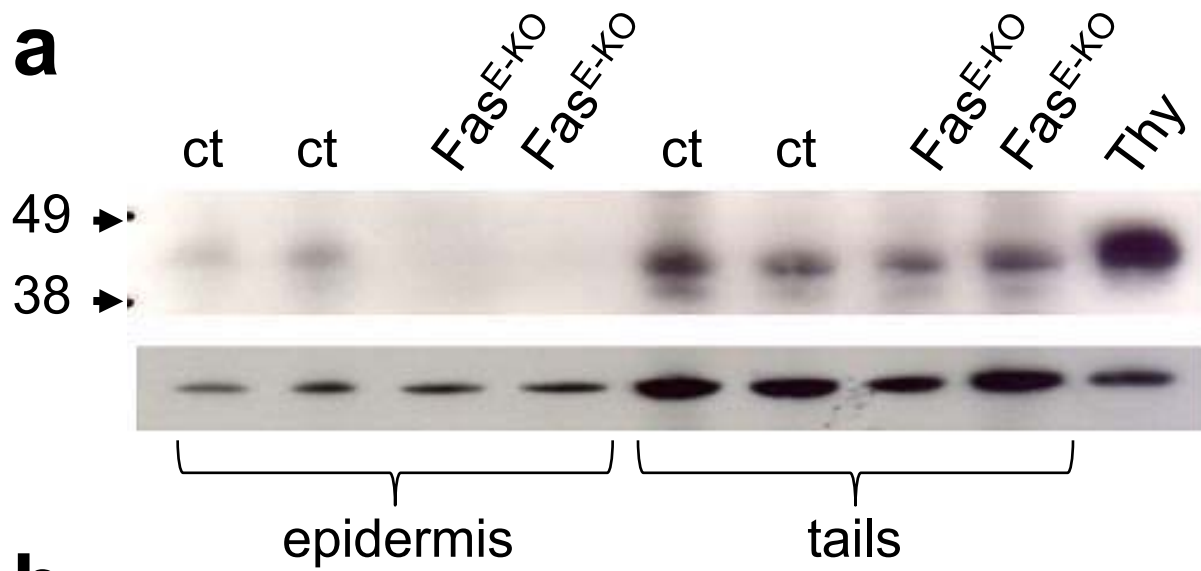
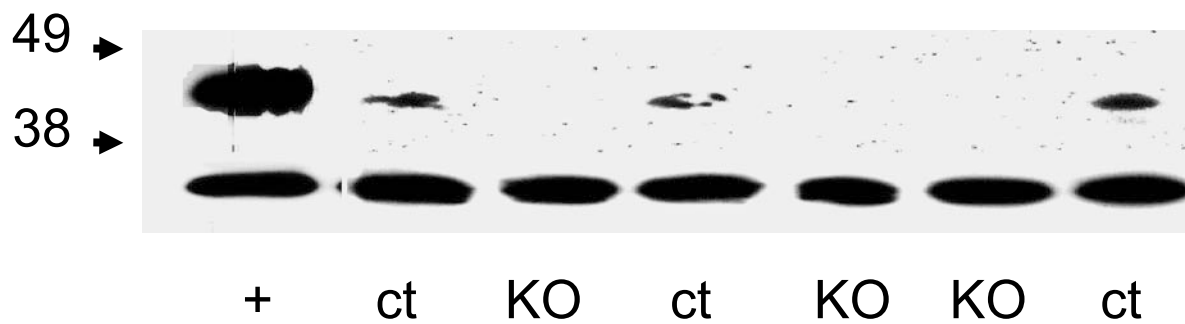
Figure 4: Apoptosis of epidermal keratinocytes upon UVB- irradiation in Fas- negative epidermis

Detection of sun burn cells and TUNEL- positive keratinocytes 18 and 24 hours after UVB- irradiation. **a)** High power images of skin sections of Fas^{E-KO} mice and control mice 18 hours after UVB- irradiation stained H/E (light microscopy) or with the TUNEL method (fluorescence microscopy), respectively. Black arrows indicate sun burn cells, white arrows indicate TUNEL- positive cells. **b)** Bar graphs show mean numbers \pm SD of sun burn cells per high power field (upper panel) and TUNEL- positive epidermal cells per power field (lower panel) 18 and 24 h after UVB- irradiation, respectively. To analyse numbers of sbc, 170 and 110 power fields were counted in the 18 hours and 24 hours experiment, respectively. For TUNEL stainings, 196 (ct) and 252 (Fas^{E-KO}) power fields were counted.

** indicates statistical significance, p- value is given above. Differences in sun burn cell number were not significant after 24 hours. Abbreviations: sbc = sun burn cells; HPF = high power field; PF = power field.

Figure 5: UVB- induced apoptosis of Fas- negative keratinocytes in vitro

Primary murine keratinocytes in passages 2- 4 isolated from control mice (ct) and Fas^{E-KO} mice (Fas KO) were irradiated with 100 or 300 mJ/cm² UVB, respectively. **Upper part:** FACS- analysis of Annexin- V exposure on the cell surface was carried out after 16 hours. Bar graphs show the percentage of Annexin- V positive keratinocytes in the propidium iodide negative population \pm SD. Results are from 3 different control and Fas KO primary isolates analysed in at least two independent experiments. **Lower part:** Primary keratinocytes were irradiated with 300 mJ/cm² UVB and labeled with the TUNEL- technique 16 hours later. Bar graphs show the percentage of TUNEL- positive keratinocytes in the total cell population \pm SD in two independent experiments using keratinocytes from three control and three Fas^{E-KO} mice.

a**b****c**