Dynamic expression of epidermal caspase-8 simulates a wound healing response

Abstract

Tissue homeostasis and regeneration are regulated by an intricate balance of seemingly competing processes - proliferation vs. differentiation and cell death vs. survival. Here we demonstrate that the loss of epidermal caspase-8, an important mediator of apoptosis, recapitulates multiple phases of a wound healing response. The epidermal hyperplasia in the caspase-8 null skin is the culmination of signals exchanged between epithelial, mesenchymal, and leukocytic cells. This reciprocal interaction is initiated by the paracrine signaling of interleukin-1α (IL-1α) which activates both skin stem cell proliferation and cutaneous inflammation. The non-canonical secretion of IL-1α is induced by a p38 MAPK mediated upregulation of NALP3 leading to inflammasome assembly and caspase-1 activation. Interestingly, the increased proliferation of basal keratinocytes is counterbalanced by the growth arrest of suprabasal keratinocyte in the stratified epidermis by IL1α-dependent NFκB signaling. Altogether our findings illustrate how the loss of caspase-8 can have an impact beyond programmed cell death to affect the local microenvironment and elicit processes common to wound repair and many neoplastic skin disorders.

The structure and function of the epidermis is maintained by an equilibrium between proliferating keratinocytes in the basal layer and differentiation of cells into the suprabasal layers that seal the body from the environment (Supplementary Figure 1a). An additional regulatory balance occurs at the epidermal surface between keratinocyte survival and death, which requires continual regeneration of this tissue. During wound healing these respective equilibria shift towards keratinocyte proliferation and cell survival to stimulate repair of the damaged tissue. Despite their importance, the mechanisms governing these regulatory nodes of tissue homeostasis remain largely undefined.

In the course of characterizing proteins that potentially affect epidermal homeostasis, we observed that epidermal caspase-8 normally fluctuates during the course of a wound healing response (Fig. 1a). In situ hybridization revealed that sites proximal (<1mm) to an excisional wound displayed a thickened epidermis coincident with a downregulation of caspase-8 RNA. However at sites distal (~5mm) to the wound in which epidermal thickness appeared normal, caspase-8 RNA was unchanged. Upon wound closure (day 14) epidermal hyperplasia subsides and caspase-8 RNA expression was restored. The development of all
epidermal layers prior to the onset of caspase-8 expression supports the notion that this protein is not involved in epidermal morphogenesis but rather monitors epidermal integrity amidst assaults from the external environment (Supplementary Figure 1b).

In order to define the contribution of reduced caspase-8 to a wound healing response, we generated an epidermal knockout of caspase-8.3, 4. By postnatal day 10 (P10) the conditional knockout mouse has flaky skin throughout its body, is slightly runted, and its epidermis is dramatically thickened (Supplementary Figure 2a-b). Interestingly, even though caspase-8 is expressed in the granular layer (Supplementary Figure 1c), it is the basal and spinous layers that are markedly expanded in the knockout epidermis (Fig. 1b). Keratinocyte proliferation contributes to this phenotype as Ki67 (Fig. 1c) and cyclin D (Supplementary Figure 3a) are elevated in the knockout skin. This observation is compatible with data showing that both epidermal and hair follicle stem cells are mobilized to help reseal a wounded epidermis5-7.

Other markers of epidermal hyperproliferation that occur during wound healing are upregulated in the caspase-8 knockout skin including keratin 6 (K6) and suprabasal expression of β4 integrin (Supplementary Figure 3 b-c). Despite this shift in balance from differentiation to proliferation, the terminal differentiation program nevertheless progressed to completion (Supplementary Figure 3d). Together these observations suggest that the loss of caspase-8 near the epidermal surface elicits a paracrine signal(s) to stimulate local stem cell pools to proliferate and fuel wound closure.

Among the early phases of a wound healing response is the infiltration of immune cells at the wound site.8 The increased dermal cellularity in the knockout skin (Supplementary Figure 2b) suggests that this is likewise occurring in response to the loss of caspase-8. Analysis with different leukocyte markers demonstrates that the caspase-8 knockout skin contains a dramatic increase in the population of granulocytes and macrophages (Fig. 2a). Probing with the pan T-cell marker, CD3, revealed elevated levels of T-cells in both the epidermis and dermis (Fig. 2b). Both CD4 helper T-cells and CD8 cytotoxic T-cells were elevated in both the epidermal and dermal compartments (Fig. 2b). Due to the pivotal role they play in wound healing, we investigated for the status of γδT-cells. In wild type skin, these cells are restricted to the basal layer and hair follicle and have a dendritic morphology (Fig. 2b). In the caspase-8 knockout skin the number of γδT-cells is increased in the suprabasal layers and dermis with a spherical morphology indicative of their activation. The γδT-cells in the dermis stain positive for Vγ3 (Fig. 2b) which suggests that they originated from cells that proliferated and migrated from the epidermis.9 These immune cells were mediating an inflammatory reaction similar to a wound healing response as evidenced by the induction of NFκB-stimulated inflammatory genes (Fig. 2c). Unlike other mutant mice with hyperplasia and inflammation phenotypes10 11, suppression of the immune cell infiltration did not completely abrogate cell proliferation (Supplementary Figure 4 a-d). The diminished hyperplasia in the presence of immunosuppressants implies that epidermal and immune cell signaling work in concert to maximally stimulate keratinocyte proliferation.

How, then, does the loss of caspase-8 near the epidermal surface stimulate proliferation only in the cells contiguous with the basal layer? A clue was provided by the observation that phosphorylated ERK (p-ERK; Fig. 3a and Supplementary Figure 5a) and β4 integrin

*Nature. Author manuscript; available in PMC 2009 September 26.*
(Supplementary Figure 3c) were elevated in the knockout tissue and expressed in the suprabasal layers. This is noteworthy as transgenic mice designed to suprabasally express active MEK1 (the upstream activator of ERK) or β4 integrin phenocopy the caspase-8 knockout mouse12, 13. Both mouse models have an increased level of active interleukin-1 (IL-1) which has an established role in regulating keratinocyte proliferation and triggering cutaneous inflammation14, 15. Since IL-1β is primarily secreted from leukocytes we focused our investigation on the activity of IL-1α which is highly expressed in keratinocytes16 and is secreted from the knockout epidermis (Fig. 3b). It has been proposed that epidermally derived IL-1α mediates keratinocyte proliferation by first stimulating fibroblasts of the underlying dermis to secrete cytokines which then incite keratinocyte division15. Consistent with this scenario, using normal serum levels we found that conditioned media (CM) from the dermis of knockout animals stimulated keratinocytes to proliferate at a faster rate than those exposed to wild type CM or normal culture media (Fig. 3c). Since the dermal explants are a heterogeneous population of cells we investigated whether dermal fibroblasts are singularly competent to stimulate keratinocyte proliferation. Given our hypothesis that IL-1α is provided by the epidermis we first primed the dermal fibroblasts with media exposed to epidermal sheets and then treated keratinocytes with this CM. Priming the dermal fibroblast with KO epidermal media caused a 40% increase in keratinocyte growth compared to wild-type-epidermal-primed dermal fibroblast or regular keratinocyte culture media (Fig. 3d). Adding recombinant IL-1α directly to the dermal fibroblast was sufficient to induce them to secrete proliferation stimulating cytokines while an IL-1α neutralizing antibody abolished the ability of the knockout epidermis to prime the dermal fibroblasts (Fig. 3d-e). This data suggests that IL-1α is a required component secreted by the epidermal sheet to render the dermal fibroblast capable of stimulating keratinocyte proliferation.

Interestingly, we found that either epidermal CM alone or recombinant IL-1α can stunt keratinocyte growth (Fig. 3f). We investigated the possibility that IL-1α may induce NFκB signaling which promotes growth inhibition and cell survival in keratinocytes 17, 18 and found elevated nuclear phospho-NFκB in the knockout and wounded epidermis (Fig. 3g). Bcl-xL (Fig. 3g) as well as other NFκB transcriptional target genes (Supplementary Figure 6) were likewise increased. To determine whether cell growth inhibition was due to IL-1α signaling, we stained for nuclear translocation of NFκB in response to cells treated with CM from wild type and knockout epidermal sheets (Fig. 3h). Keratinocytes treated with wild type CM had NFκB predominantly localized in the cytoplasm whereas cells treated with knockout media showed nuclear localization. The nuclear translocation could be inhibited when knockout CM was added together with an IL-1α neutralizing antibody and rescued with recombinant human IL-1α (which is not recognized by the inhibitory antibody; Fig 3h). Moreover, addition of knockout dermis CM can overcome the growth inhibitory effects of the knockout epidermal CM and block the nuclear translocation of NFκB, which is consistent with the former’s ability to stimulate keratinocyte proliferation. Thus the induction of NFκB in the suprabasal layers is a contributing factor towards limiting proliferation to cells along the basement membrane in the knockout epidermis.

Given the complex effects of IL-1α signaling in the mutant mouse, it became imperative to determine how the loss of caspase-8 regulates the secretion (and therefore activity) of this
cytokine. Two proteases have been implicated in the conversion of the interleukin-1 precursor into its active form - caspase-1 and calpain 18. In both the caspase-8 null epidermis and wounded skin there is an upregulation of caspase-1 RNA and suprabasal localization of the protein (Fig. 4a-b). Furthermore, the amount of IL-1α secreted from epidermal sheets is significantly higher in the knockout mouse than its wild type littermate and this increase is lost when treated with a caspase-1 (but not the calpain) inhibitor (Fig. 4c). These results are supported by reports that the overexpression of caspase-119 or IL-1α20 in the epidermis phenocopies the caspase-8 conditional knockout.

Caspase-1 itself is synthesized as a zymogen and must be processed into its active form. We found that phosphorylated p38 MAPK is upregulated in both the caspase-8 knockout and wounded skin (Fig. 4d and Supplementary Figure 5b) and inhibition of its activity abolishes the secretion of IL-1α from the knockout epidermis (Fig. 4c). The p38 MAPK inhibitor did not abrogate the increase in caspase-1 protein expression in the knockout epidermis, but did efficiently restrain caspase-1 processing into its active form and thus its catalytic activity (Fig. 4e and Supplementary Figure 8a). A consequence of blocking p38 MAPK-mediated activation of caspase-1 is the reduction in epidermally derived IL-1α signaling as measured by nuclear translocation of NFκB (Supplementary Figure 8b).

In order to probe the mechanistic link between p38 MAPK and caspase-1, we analyzed the status of the NALP3 inflammasome, a multiprotein complex which is required for activation of inflammatory caspases such as caspase-1 22. This complex is composed of the adaptor protein Asc and pro-caspase-1, both of which have a basal expression level in keratinocytes, and NALP3 whose expression must be induced22. Interestingly, we found that NALP3 RNA is markedly upregulated in the suprabasal layers of both the knockout and wounded epidermis (Fig. 4f). This expression pattern coincides with active p38 MAPK expression in the knockout epidermis and we found that NALP3 induction is dependent upon the activity of this kinase (Fig. 4g). Furthermore, treatment of epidermal sheets with the p38MAPK chemical activator anisomycin is sufficient to induce NALP3 expression in wild type epidermal sheets (Fig. 4h). The human NALP1 protein can also form an inflammasome complex with caspase-1 to mediate its proteolytic activation, but its closest murine relative, NALP1b, is unaffected in the caspase-8 knockout epidermis and is unaltered in response to modulating p38 MAPK activity (Fig. 4g-h). Consistent with its role in stimulating inflammasome-dependent activation of caspase-1, the p38 MAPK activator anisomycin can increase the amount of IL-1α secreted from epidermal sheets (Figure 4i). The ability of both UV-irradiated 23 and anisomycin treated 24 keratinocytes to expel their inflammasome components prompted us to determine whether p38 MAPK can also impact the secretion of caspase-1. Conditioned media from caspase-8 null epidermal sheets contained elevated levels of released pro-caspase-1 which was reduced to wild type levels when p38 MAPK activity was compromised (Supplementary Figure 8c). The p20 activated form of caspase-1 was also higher in the extracellular media from the knockout epidermis and both p38 MAPK and caspase-1 inhibition abolished the release of this protein from the cell.

A model summarising how a wound-induced downregulation of epidermal caspase-8 is translated into epithelial-mesenchymal-leukocyte interactions is shown in Supplementary Figure 9. Consistent with this proposed role, caspase-8 is elevated in diabetic mice with...
impaired wound healing and humans deficient in caspase-8 have clinical symptoms including eczema, which has features of a chronic wound response. In addition to its effect on wound healing, the intercellular communication stimulated by the loss of epidermal caspase-8 and the activation of the inflammatory apparatus may be a paradigm for tumor-stroma interactions and other hyperplastic skin diseases.

METHODS SUMMARY

Generation of caspase-8 conditional knockout mice

Epidermis specific knockouts were obtained by crossing mice carrying the floxed caspase-8 allele (Casp8 fl/fl) to K14-Cre mice.

Histology, in situ hybridization and immunohistochemistry

Please refer to supplementary methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Drs. Steve Hedrick, Wendy Havran, Benjamin Yu, Deborah Witherden, Alexander Hoffmann, David Stachura and members of the Jamora lab for providing reagents and helpful discussions. This work was supported by grants from the NIH and the American Skin Association, and a Career Award from the Dermatology Foundation.

Appendix

ONLINE METHODS

Histology, in situ hybridization and immunohistochemistry

Mouse skin isolated from P10 wild type and knockout animals were either frozen in OCT (Tissue-Tek) or fixed overnight in Bouin’s fixative and embedded in paraffin depending on the application. Paraffin sections were prepared for in situ hybridization and histology, and counterstained with hematoxylin and eosin (H&E). In situ hybridization was performed using a digoxigenin-UTP (Roche) labeled probe against the 3′-UTR of mouse caspase-8 and NALP3 probe was generated as described previously. Staining for anti-cyclin D1 (Zymed), phospho-MAPK (Cell Signaling), phospho-NFκB (Ser276, Cell Signaling), phospho-p38 (Cell Signaling), Caspase-1, p-20 (M-19, Santa Cruz) and Ki67 (Vector Labs) was performed on 8 μm paraffin sections after antigen-retrieval. Epidermal differentiation markers K5, K1, Loricrin, Filaggrin and the wound healing marker K6 (Covance) were stained in 8 μm frozen sections after the tissues were fixed for 10 min in 4% paraformaldehyde. Immune cell infiltrates were stained using FITC-conjugated anti MAC-1, Gr-1, CD3, CD4, CD8, γδ TCR and Vγ3 (BD Biosciences). For nuclear staining Hoechst 33342 (Calbiochem) was added in a final concentration of 1 mg/ml to the secondary antibody dilution. Immunofluorescence was detected using rhodamine-X or FITC.
conjugated secondary antibodies (Jackson Immunoresearch) or expression was developed using the Vectastain ABC kit (Vector Labs) according to the manufacturer’s instructions.

**Cornified envelope assay**

Isolation of corneocytes in the squamous layer was performed as previously described 28.

**Quantitative real-time PCR**

Total RNA was extracted from whole skin of WT (n=3), KO (n=3) mice at postnatal day 10 (P10) or 7 days post-wounded skin (n=3) using Trizol reagent (Invitrogen) according to manufacturer’s instructions. Similarly, epidermis from P3 mice WT (n=3) and KO (n=3) was separated with dispase treatment for 1 hour at 37°C and total RNA isolated using the Trizol protocol. cDNA was synthesized by reverse transcription using oligo-dT as primers (Superscript III kit, Invitrogen). Real time-PCR analysis was performed with previously described primers 29 30 using a Stratagene Mx-3000 system. Reactions were performed using the Brilliant SYBR Green QPCR reagent mix (Stratagene) and experiments were carried out in triplicate from cDNA isolated from six different animals. Data shown is a representative example. Specificity of reactions was determined by dissociation curve analysis and quantification analysis was performed using the Mx-3000 software.

**Proliferation assays**

Epidermis of newborn caspase-8 WT and KO mice were separated in dispase overnight at 4°C. Isolated epidermis or dermis was incubated in 3 ml of keratinocyte media 30 for three days. For Figure 4 these conditioned media were diluted 1:5 with keratinocytes media and applied to a well containing 10,000 keratinocytes. Cells were collected by trypsinization every 24 hours for four days and counted on a hemocytometer and results are representative of three independent experiments done in triplicate. To prime dermal fibroblasts, 400 μl of epidermal-conditioned media was diluted in 1.6mL keratinocytes media and added to a confluent 6 cm dish of fibroblasts and incubated for three hours at 37°C. This epidermal primed, dermal fibroblast conditioned media was then diluted 1:2 in keratinocytes media and added to primary keratinocytes with 2 ng/mL IL-1α neutralizing antibody/control antibody, or recombinant human IL-1α.

**NFκB Translocation Assay**

Diluted (1:5) wild type and knockout epidermal and dermal conditioned media described above was applied to coverslips seeded with 30,000 primary keratinocytes 16 hours earlier. Cells were incubated with this conditioned media and control goat antibody, 2 ng/mL IL-1α neutralizing antibody, or 10 pg/mL recombinant IL-1α for 3 hours at 37°C. Cells were stained with an antibody recognizing NFκB (Santa Cruz)30.

**IL-1α ELISA**

Isolated epidermis from three day old (P3) caspase-8 WT and KO mice were incubated for 24 hours in 2 mL of keratinocyte media containing DMSO or inhibitors of AKT (20 μM), p38 MAPK (SB 203580) (2.4 μM), caspase-1 (80 μM) or calpain (200 μM) (all from...
Calbiochem). Secreted mouse IL-1α levels were detected using the Quantikine kit from Stratagene according to manufacturer’s instructions.

**Caspase-1 Activity Assay**

Explants were treated as described above and following 24 hours of incubation with vehicle control (DMSO) or inhibitors they were trypsinized for 20 minutes at room temperature. Protein lysates were extracted from isolated epidermal cells and 100 μg of protein was analyzed for caspase-1 activity as previously described. Data shown is representative of three independent experiments done in triplicate.

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Figure 1. Effect of caspase-8 downregulation in the epidermis
A. In situ hybridization of Caspase-8 RNA on 0, 1, 7 and 14 days after excisional wounds. Arrows denote wound sites. Distal (>5mm) and proximal (<1mm) refer to the distance from the wound site.
B. Differentiation in the wild type (WT) and knockout (KO) epidermis. K5, K1, and loricrin stain the basal, spinous, and granular layers respectively. Dotted line denotes the basement membrane.
C. Hyperproliferation of the epidermis in the KO skin is revealed by increased expression of Ki67.
Figure 2. Characterization of the inflammatory response in the KO skin

A. Increased number of granulocytes (Gr-1) and macrophages (MAC-1) are detected in KO skin.

B. Pan (CD3), helper (CD4), cytotoxic (CD8), and \(\gamma\delta\) (\(\gamma\delta\) TCR) T-cell markers are increased in the KO skin and infiltrate the epidermis (arrowheads). Insets are magnified views of \(\gamma\delta\) T-cell morphology. Epidermal derived pool of \(\gamma\delta\) T cells is stained with \(V\gamma3\).

C. Quantitative RT-PCR of pro-inflammatory NFkB target genes in WT (orange) and KO (blue) skin of 10-day old mice and wounded skin (purple) samples three days after wounding.
Figure 3. Control of keratinocyte proliferation through epithelial-mesenchymal interactions

A. Expression of phospho-ERK (p-ERK), loricrin and K5 in serial sections from P10 WT and KO skin and skin three days post wounding.

B. Extracellular IL-1α is detected by staining in the absence of detergent (denoted as unpermeabilized [unperm.] samples). Detergent was incorporated in the staining protocol to visualize total IL-1α levels in the skin (denoted as permeabilized [perm.] samples).

C. Primary keratinocyte growth rate was measured over four days after incubation in caspase-8 WT dermal conditioned media (der CM) in blue, KO der CM (red) or normal growth media (green).

D. Keratinocyte growth was measured after incubation in CM from dermal fibroblasts (df) that were primed with either wild type epidermis exposed media (WT epi + df CM; light blue), media incubated with caspase-8 null epidermis (KO epi + df CM; red [***]), normal media (media; green), normal media supplemented with recombinant human IL-1α (media + rhIL-1α; orange), df CM primed with WT epidermal media treated with rhIL-1α (WT epi + rhIL-1α + df CM; dark blue [*]). *-p<0.05; ***-p<0.001 compared to the media which was used as a control.

E. Keratinocyte growth rate after incubation in normal media (green), df CM primed with KO epi CM and either control antibody (KO epi + df + con Ab; red), IL-1α inhibitory antibody (KO epi + df + inh. Ab; purple) or recombinant human IL-1α (which is not inhibited by the antibody) added (KO epi + df + inh. Ab + rhIL-1α; pink).

F. WT epi CM (light blue), KO epi CM (dark blue) or normal media (green) was added to primary keratinocytes and growth rate measured. Addition of recombinant human IL-1α to normal media (media + rhIL-1α; red) is sufficient to inhibit growth.
G. Skin sections from 10 day old caspase-8 WT and KO mice and adult mouse skin three days after excisional wounding were stained for pNFkB, its target gene Bcl-XL (green) and K5 (red). Arrowhead marks wound site.

H. WT epi CM and KO epi CM were added to primary keratinocytes and stained for NFκB nuclear translocation. Inhibitory IL-1α antibody (Inh. Ab) which specifically neutralizes the mouse cytokine and/or rhIL-1α was added to the KO epi CM. Keratinocytes were also treated with KO epi CM, WT der CM, KO der CM or a neutralizing antibody specific for mouse IL-1β. Results are representative of three independent experiments.
Figure 4. Mechanism regulating IL-1α secretion
A. Quantitative RT-PCR for caspase-1 in P10 WT and KO skin, and wounded skin (wound) three days after excision. Immunofluorescence of caspase-1 in P10 wild type (WT) and knockout (KO) skin, and wounded skin (wound) three days after excision.
B. Expression of caspase-1 in wild type, knockout, and wounded skin.
C. IL-1α ELISA from media conditioned by wild type (orange) or knockout (blue) epidermal sheets treated with either DMSO, or inhibitors for caspase-1, p38MAPK, or calpain. Note: the efficacy of the calpain inhibitor was verified by its ability to inhibit processing of filaggrin in differentiating keratinocytes (Supp. Fig. 7).
D. Expression of phosphorylated p38 MAPK [(P)-p38] in P10 WT and KO skin and three days post wounding.
E. Western blot of caspase-1 and β-actin from cell lysates generated from the epidermal sheets in (C).
F. In situ hybridization of NALP3 RNA on P10 WT, KO, and 3 day post-wounded skin sections.
G. Quantitative RT-PCR of inflammasome components using RNA isolated from epidermal sheets of WT and KO skin treated with DMSO vehicle control or p38 MAPK inhibitor (p38 inh) for 16 hours.
H. Quantitative RT-PCR on RNA from WT epidermal sheets treated with DMSO vehicle control, anisomycin (aniso), or anisomycin + p38 MAPK inhibitor (aniso + p38 inh) for two hours.
I. Measurement of IL-1α secretion into conditioned media from WT epidermal sheets treated with DMSO, anisomycin (aniso), p38 inhibitor (p38 inh), and/or caspase-1 inhibitor (casp1 inh). KO epidermal sheets treated with DMSO (KO DMSO) is used as comparison.