

Cell Death-Induced Activation of Epidermal Growth Factor Receptor in Keratinocytes: Implications for Restricting Epidermal Damage in Dermatitis

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Recent findings have implicated Fas/Fas ligand (FasL) in mediating the death of keratinocytes in spongiotic lesions. We asked whether dying keratinocytes could potentially initiate a protective response of the skin to limit the destruction of the epidermis in the spongiotic areas. In addition to apoptosis, treatment of keratinocyte cultures *in vitro* with FasL triggers a profound phosphorylation of the epidermal growth factor receptor (EGFR) and of its downstream effectors ERK and protein kinase B (PKB/Akt). Using a variety of inhibitors and blocking antibodies, we demonstrated that: (i) apoptosis is required for the generation of the signal(s) leading to the activation of EGFR, ERK, and Akt; (ii) the activation of EGFR, ERK, and Akt by FasL is indeed mediated by its *bona fide* receptor Fas; (iii) the activation of EGFR is essential for the subsequent activation of ERK and Akt; and (iv) apoptotic keratinocytes secrete soluble EGFR ligands (including amphiregulin) that are processed from membrane-bound proligand forms by metalloproteinase(s). Our findings demonstrate a potential mechanism for the restriction and repair of spongiotic damage in eczemas.

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Atopic dermatitis (AD) and allergic contact dermatitis (ACD) are common inflammatory skin diseases that belong to the heterogeneous group of eczematous dermatitides or eczemas. A common histopathological feature of eczemas is the formation of exudative epidermal vesicles that are disruptive to the normal barrier function of the skin. Although vesicle formation in eczemas has been largely attributed to rupturing of keratinocyte attachments as a result of intercellular edema (spongiosis) (Schwarz, 2000 and references therein), recent findings suggest that keratinocyte death plays a major role in vesicle formation (Schwarz, 2000; Trautmann *et al*, 2000). This keratinocyte death appears to be apoptotic and to be mediated by Fas ligand (FasL), delivered to the epidermis by infiltrating T lymphocytes and acting on its receptor, Fas, expressed on the surface of keratinocytes (Trautmann *et al*, 2000).

The question of how vesicle formation is restricted to avoid progressive destruction of the skin is of clinical importance. The defense of the epidermis against destruction could be mediated by the eventual resolution of the under-

lying inflammatory process and the subsequent reduction in the levels of FasL in the epidermis. The epidermal keratinocytes may also respond to the wave of apoptosis by triggering pro-survival programs. Such pro-survival programs could act by rendering keratinocytes resistant to FasL-dependent apoptosis by, for instance, downregulating the expression of Fas or upregulating the expression of anti-apoptotic proteins. Alternatively, the pro-survival programs may not be directed at rescuing the keratinocytes that are targeted by FasL, but instead at increasing the proliferation of basal keratinocytes to replenish the cells lost to apoptosis.

Here, we have investigated the occurrence of both apoptosis and hyperproliferation in experimentally induced allergic contact dermatitis. Indeed, eczematous epidermis displayed the co-existence of both ectopic cell death by apoptosis and increased proliferation of keratinocytes, suggesting that the two events may be mechanistically linked. To investigate whether apoptotic keratinocytes are capable of generating and communicating mitogenic signals, we employed *in vitro* studies in a human keratinocyte monoculture system. We found that treatment of keratinocytes with FasL triggers, in addition to apoptosis, a profound phosphorylation of the epidermal growth factor receptor (EGFR) and of its downstream effectors, the extracellular signal-activated kinases (ERK) and protein kinase B (PKB/Akt). Using specific small molecule inhibitors and blocking antibodies, we found that the apoptotic caspase cascade triggered by FasL results in the increased production, processing, and shedding of soluble ligands of EGFR, one

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; AR, amphiregulin; EGF(R), epidermal growth factor (receptor); ERK, extracellular signal-activated kinase(s); FasL, Fas ligand; HB-EGF, heparin-binding EGF; HEK₁, human epidermal keratinocytes, neonatal; PARP, poly(ADP)ribose polymerase; PKB/Akt, protein kinase B; SADBE, squaric acid dibutylester; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

of which was identified as amphiregulin. Thus, we demonstrate that keratinocytes are capable of responding to pro-apoptotic stimuli by mounting an anti-apoptotic survival program. Finally, we present evidence that the pro-survival/mitogenic program(s) initiated by apoptotic keratinocytes do not rescue the keratinocytes that are directly targeted by apoptotic stimuli, but instead increase the proliferation of basal keratinocytes to replenish the loss of cells by apoptosis.

Results and Discussion

Co-existence of cell death and increased proliferation in eczematous epidermis Seven days post-application of squaric acid dibutylester (SADBE) to the skin of sensitized volunteers, biopsies from the affected area displayed abundance of epidermal vesicles and marked thickening of the epidermis, consistent with inter- and intracellular edema (Fig 1, *panels c* and *d*; Fig 2, *panels c* and *f*) and increased proliferation (see below). The appearance in the ACD lesion of epidermal cells with abnormal nuclei (either "hollow" in appearance, Fig 1, *panel c*, *filled arrowheads*, or pyknotic, Fig 3, *panel d*, *asterisk*) suggested ongoing cell death. Although programmed cell death is the end point of normal keratinocyte differentiation, such type of cell death lacks the classical features of apoptosis, e.g., caspase activation and chromatin fragmentation (Lippens *et al*, 2000; Takahashi *et al*, 2000). In contrast, the epidermal material collected either 2 or 7 d post-SADBE administration displayed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells, suggesting the possibility of ongoing apoptosis (Fig 1, *panels b–d* and Fig 2, *panels b*, *c*, *e*, and *f*) although the occurrence of other modes of cell death in our experimental model of dermatitis cannot be excluded.

To investigate the relationship between cell death and the state of differentiation of keratinocytes in ACD, we co-stained biopsied material for both TUNEL and keratin 5, a marker for *stratum basale* keratinocytes. Interestingly, the area of epidermal thickening in 2 and, especially, in 7 d ACD stained positive for keratin 5 and the majority of the TUNEL-positive cells were interspersed within this expanded area of keratin 5 expression (Fig 2, *panels b*, *c*, *e*, and *f*). Staining for keratin 1, a marker for keratinocytes in *stratum spinosum* did not display a significant expansion of this layer in ACD (not shown). Thus, the bulk of epidermal thickening in ACD was due, in addition to spongiosis, to an increase in the number of cells displaying the characteristics of undifferentiated basal keratinocytes. Furthermore, the epidermal thickening in ACD was co-existent with ongoing keratinocyte death (Figs 1 and 2).

In order to determine the proliferation potential of ACD epidermis, we immunostained for Ki-67, a marker for cycling cells (Gerdes *et al*, 1991). A ~ 2 -fold (2.07 ± 0.39 , $p < 0.05$) increase in the appearance of Ki-67-positive cells was detected in the ACD skin in comparison to the healthy skin of the same volunteer (exemplified in Fig 3). Both cell death (Fig 3, *panels b* and *d*, see pyknotic nuclei labeled with an *asterisk*) and increased renewal of the epidermal cells (Ki-67 staining) were concurrent within the eczematous

lesion. To confirm that the increased epidermal thickness and Ki-67-positivity in ACD is indeed associated with increased keratinocyte number, we also counted nucleated cells per millimeter of basal lamina in healthy (control) and 48 h ACD biopsies from the volunteer presented in Figs 1 and 2. Although the control epidermis contained 382 ± 29 nucleated cell per millimeter of basal lamina, the ACD epidermis displayed 529 ± 18 nucleated cell per millimeter of basal lamina, indicative of $\sim 39\%$ ($p < 0.05$) increase in cell number in ACD epidermis. Due to the massive epidermal disruption in 7 d ACD (Figs 1 and 2), an exact count of nucleated cells per millimeter of basal lamina could not be obtained.

The origin and nature of the mitogenic signals in the eczematous epidermis: a hypothesis What is the source of mitogenic signals that govern the increased proliferation of keratinocytes detected in ACD? Although the diffusion of keratinocyte-stimulating mitogens from the dermis is well established (Szabowski *et al*, 2000; Angel *et al*, 2001; Maas-Szabowski *et al*, 2001; Angel and Szabowski, 2002), we and others have reported that keratinocytes sustain active proliferative and pro-survival signaling cascades by means of autocrine production of secreted ligands for the epidermal growth factor receptor (Cook *et al*, 1991; Pittelkow *et al*, 1993; Piepkorn *et al*, 1998; Jost *et al*, 1999; Iordanov *et al*, 2002). Therefore, it is possible that at least some of the mitogenic signals driving the increased proliferation in ACD epidermis are keratinocyte-derived. We further speculated that the dying keratinocytes themselves might generate some of these mitogenic signals. To test this hypothesis, we took advantage of the recent progress made in understanding keratinocyte cell death in eczemas. Trautmann *et al* (2000) have discovered that epidermal apoptosis in eczemas (both AD and ACD) is mediated by activation of the death receptor Fas on the surface of keratinocytes. Therefore, we employed FasL-mediated apoptosis in keratinocyte monoculture to investigate the ability of apoptotic epidermal cells to produce mitogenic signals.

Hexameric soluble Fc:FasL triggers apoptosis and phosphorylation of EGFR, ERK, and Akt in human keratinocytes To determine the susceptibility of human keratinocytes to FasL-triggered apoptosis, we treated either immortalized human keratinocytes (HEKn (human epidermal keratinocytes, neonatal)-E6/E7) or primary human keratinocyte explants (HEKn, passages 3–6) with Fc:FasL, a recombinant hexameric form of human FasL (Holler *et al*, 2003). Both HEKn and HEKn-E6/E7 displayed time-dependent apoptosis in response to Fc:FasL as measured by the cleavage of caspase substrate poly(ADP)ribose polymerase (PARP; Fig 4A, *lanes 4–6*; Fig 4B, *lanes 4–6* and *10–12*). Surprisingly, the treatment with Fc:FasL resulted in a pronounced phosphorylation of EGFR, as determined by immunoblot analyses using two phosphoepitope-specific antibodies (Fig 4A, *lanes 4–6*; Fig 4B, *lanes 4–6* and *10–12*; directed against phosphotyrosine residues 1173 and 1068). Whereas the effect of Fc:FasL on the phosphorylation of EGFR in HEKn-E6/E7 was straightforward to interpret, the effect of Fc:FasL on the phosphorylation of EGFR in HEKn was superimposed on the more pronounced decrease in

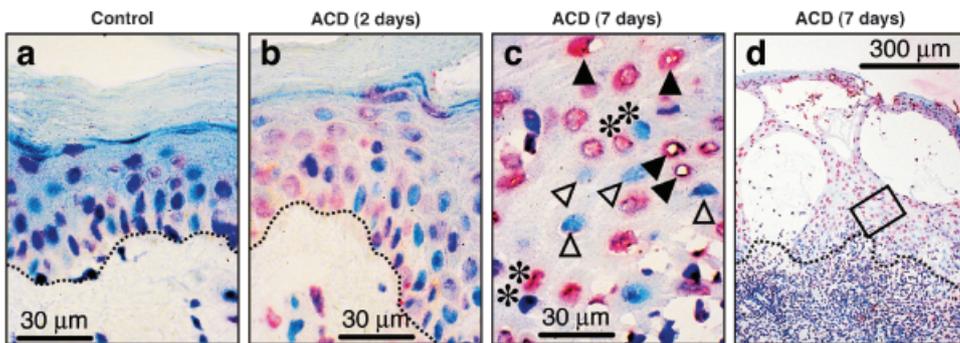


Figure 1
Spongiosis and apoptotic cell death in allergic contact dermatitis (ACD). Representative biopsies from squaric acid dibutylester (SADBE)-induced ACD and adjacent normal skin taken either 2 or 7 d after the challenge with SADBE. The subject developed massive vesiculation. The sections were stained first for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (crimson; examples indicated with filled arrowheads) and then counterstained with hematoxylin (blue; examples indicated with empty arrowheads). Note that the

TUNEL-positive nuclei exclude hematoxylin, thus allowing a clear distinction between apoptotic and non-apoptotic cells. Paired asterisks indicate examples of neighboring apoptotic (crimson) and healthy (blue) nuclei. The rectangle in panel d indicates the location within the thickened epidermis of the area represented in panel c. Dashed lines indicate the location of the basement membrane.

the total EGFR levels that is coincidental with apoptosis (Fig 4B, compare *PARP* and *EGFR* panels, lanes 11 and 12) in these cells. Taking into consideration the levels of both total EGFR and phosphorylated EGFR (Fig 4B, compare lanes 8 and 9 to lanes 11 and 12), it is apparent that there is an net increase in the phosphorylation of EGFR in the Fc:FasL-treated HEK_n at 2 and 4 h after the addition of Fc:FasL.

We have previously demonstrated that the basal activities of the ERK and Akt protein kinases in primary HEK_n and in HEK_n-E6/E7 are exclusively regulated by autocrine stimulation of the EGFR (Iordanov *et al*, 2002). Consistent with the activation of EGFR by Fc:FasL, we observed that both ERK and Akt became phosphorylated following administration of Fc:FasL (Fig 4A, lanes 4–6). The phosphor-

ylation of EGFR, ERK, and Akt in response to EGF was, as expected, not accompanied by apoptosis (Fig 4A, compare lanes 4–6 to lanes 7–8). To investigate whether the phosphorylation of EGFR in response to Fc:FasL was mediated by Fas, the *bona fide* receptor for FasL, we employed ZB4, a Fas-neutralizing antibody. Indeed, ZB4 abrogated not only the apoptotic response of HEK_n-E6/E7 to Fc:FasL, but also the phosphorylation of EGFR (Fig 5A, compare lanes 3 to lanes 4).

Secretion of soluble EGFR ligand(s) by Fc:FasL-treated keratinocytes We next tested whether Fc:FasL-treated keratinocytes produce soluble ligands capable of activating EGFR. To this end, we prepared conditioned media from either untreated or Fc:FasL-treated HEK_n-E6/E7 (referred

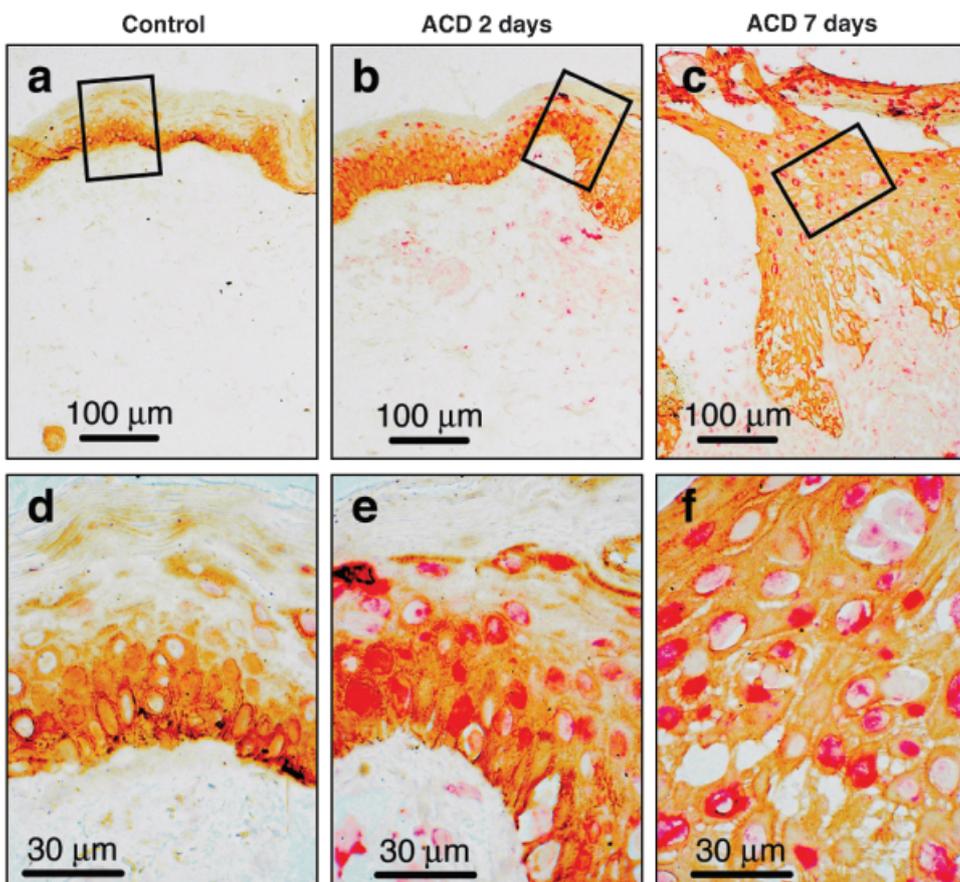


Figure 2
Spongiosis, apoptosis, and keratin 5 expression in allergic contact dermatitis (ACD). Representative biopsies from the same subject as in Fig 1. The sections were stained first for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (crimson) and followed by immunostaining for keratin 5 (yellow-brown). The rectangles in panels a–c indicate the respective locations in the epidermis of the areas represented in panels d–f.

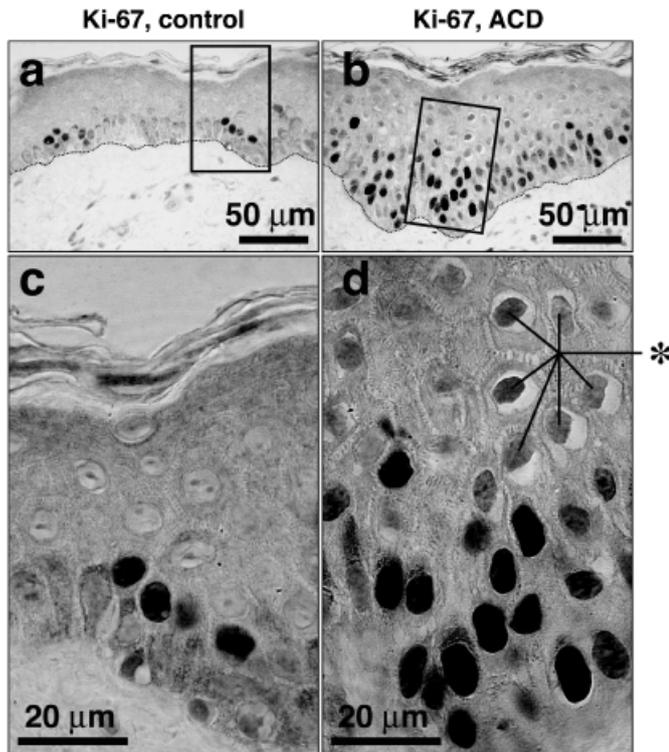


Figure 3
Increased proliferation in allergic contact dermatitis (ACD). Representative biopsies from a subject (different from the one presented in Figs 1 and 2) taken 7 d post-SADBE and stained immunohistochemically for Ki-67. Note the concurrent existence within the eczematous lesion of both cell death (pyknotic nuclei labeled with an asterisk) and increased Ki-67 staining. The rectangles in panels a and b indicate the respective locations in the epidermis of the areas represented in panels c and d.

to as “donor” cells; see *Experimental design* in the legend to Fig 5B) and tested the ability of these conditioned media to trigger phosphorylation of EGFR in naïve (“acceptor”) cells. Indeed, the conditioned medium from the Fc:FasL-treated donor cells, but not the conditioned medium from the untreated donor cells, contained activity that triggered an early (within 0.5 h) phosphorylation of EGFR and ERK (Fig 5B, compare lanes 1 and 5). For two reasons, this activity was clearly different from the Fc:FasL itself present in the conditioned medium. First, the phosphorylation of EGFR and ERK in the acceptor cells occurred before the onset of apoptosis, triggered by the residual Fc:FasL in the conditioned medium (Fig 5B, lane 5, compare PARP to the phosphorylation of EGFR and ERK). Second, the phosphorylation of EGFR and ERK in response to the Fc:FasL-conditioned medium proceeded unaffected when the acceptor cells were pretreated with ZB4 to neutralize Fas (Fig 5B, compare lanes 5 and 7).

Caspase activity is required for the production of EGFR ligands by Fc:FasL-treated keratinocytes To determine whether the production of soluble EGFR ligands in response to Fas ligation could be mediated by caspase activity, we employed zVADfmk, a non-specific pan-caspase inhibitor, and zIETDfmk, a more specific caspase 8 inhibitor (Thornberry *et al*, 1997; Garcia-Calvo *et al*, 1998). As shown in Fig 6, both inhibitors abrogated completely the apoptosis

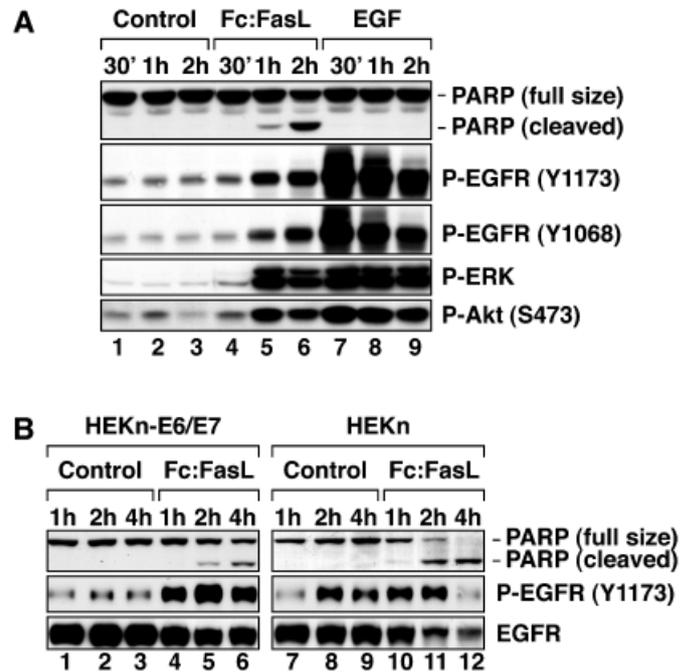


Figure 4
Induction of apoptosis and phosphorylation of epidermal growth factor receptor (EGFR), extracellular signal-activated kinase (ERK), and protein kinase B (Akt)1 in response to Fc:FasL (Fas ligand) treatment of keratinocytes. (A) HEK293 (human epidermal keratinocytes, neonatal)-E6/E7 were plated and grown in basal keratinocyte medium (BKM) + exoGF. The cells were extensively washed with BKM + exoGF and incubated in BKM + exoGF for 2 h before treatments. Therefore, at the time of treatments, the basal levels of phosphorylation of EGFR and its downstream effectors are determined by the secretion of autocrine factors by the cells, as we have shown previously (Iordanov *et al*, 2002). The cells were then treated, for the indicated times, with either Fc-FasL (see Materials and Methods for concentrations used) or EGF (50 ng per mL). The cells were harvested as described in Materials and Methods and cell lysates were processed for immunoblot analyses. Apoptosis was assessed by determining the cleavage of poly(ADP)ribose polymerase. The phosphorylation state of EGFR, ERK, and Akt1 was assessed using phosphoepitope-specific antibodies. (B) HEK293-E6/E7 and HEK293 were deprived of exogenous growth factors and treated with Fc-FasL for the indicated times with as in (A) and processed for immunoblot analyses with the indicated antibodies.

triggered by Fc:FasL (measured by determining the cleavage of either caspase 8 or PARP; Fig 6, compare lanes 10–12 to lanes 13–18). Under these conditions, both inhibitors strongly diminished the Fc:FasL-induced phosphorylation of EGFR, ERK, and Akt (Fig 6, compare lanes 10–12 to lanes 13–18). We concluded, therefore, that caspase activity is required for the activation of production and/or shedding of EGFR ligands by apoptotic keratinocytes.

Metalloprotease activity is required for the secretion of EGFR ligands by Fc:FasL-treated keratinocytes Members of the EGF family of growth factors, including transforming growth factor- α (TGF- α), amphiregulin (AR), and heparin-binding EGF (HB-EGF), are expressed as transmembrane precursors that are cleaved at one or more sites in the extracellular domain to release soluble growth factor (Lee *et al*, 2003). The available experimental evidence implicates the tumor necrosis factor- α converting enzyme (TACE/ADAM17) as one relevant protease responsible for the maturation of TGF- α , AR, and HB-EGF *in vivo* (Sunn-

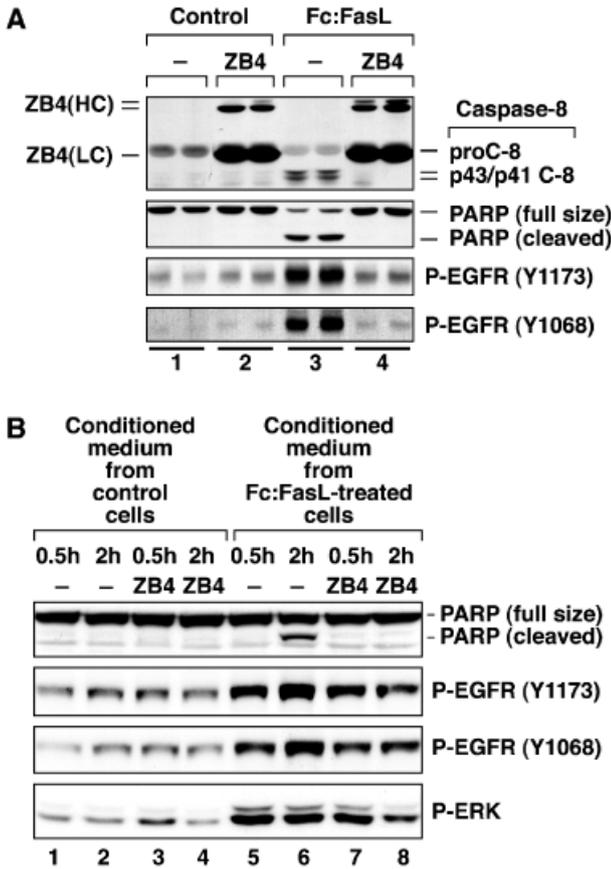


Figure 5
The phosphorylation of epidermal growth factor receptor (EGFR) in response to Fc:FasL (Fas ligand) depends on Fas ligation and on secreted soluble EGFR ligands. (A) HEK₁n (human epidermal keratinocytes, neonatal)-E6/E7 were treated with Fc:FasL for 2 h as in Fig 4, except that, where indicated, zVADfmk (50 μM) or ZB4 (2 μg per mL) were given to the cells for 30 min before the treatment. Each experimental point was performed in duplicate. Immunoblot analyses using phosphoepitope-specific antibodies to EGFR as indicated. Apoptosis was assessed by determining the cleavage of caspase 8 or poly(ADP)ribose polymerase (PARP). The light and heavy chains of the ZB4 antibody appear in the gel because of internalization and/or adherence of the antibody to the cell surface. (B) *Experimental Design:* HEK₁n-E6/E7 were left untreated ("control cells") or were treated with Fc:FasL. Both control and treated cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 2 h before treatment with Fc:FasL. Two hours after the treatment, the media from the control and treated cells were collected and cleared from cell debris by centrifugation. These media were designated "conditioned medium from control cells" and "conditioned medium from Fc:FasL-treated cells", respectively. The "donor" cells, which have produced the conditioned media, were discarded. Naïve ("acceptor") HEK₁n-E6/E7 were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 1.5 h. Then, where indicated, the ZB4 Fas-blocking antibody was given to the cells for 0.5 h. Finally, the medium of the "acceptor" cells was exchanged, as indicated, with either one or the other conditioned media for the indicated times (0.5 or 2 h). During the time of incubation with conditioned media, the ZB4 antibody was continuously present where indicated, in order to prevent the residual Fc:FasL from the conditioned medium from acting on the "acceptor" cells. That ZB4 indeed effectively prevented residual carry-over Fc:FasL from acting on the "acceptor" cells is evident from the lack of PARP cleavage in lane 8 (compared to lane 6). The phosphorylation states of EGFR and ERK were assessed in immunoblot analyses using phosphoepitope-specific antibodies.

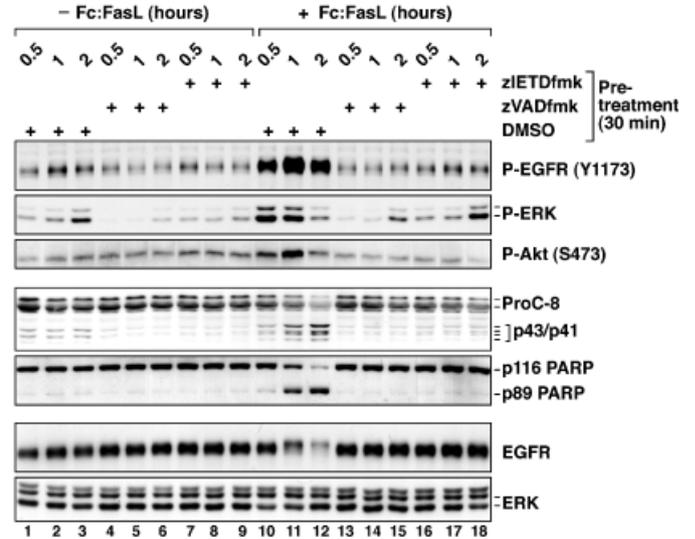


Figure 6
Caspase activity is required for the activation of epidermal growth factor receptor (EGFR), extracellular signal-activated kinase (ERK), and protein kinase B (Akt)1 by Fc:FasL (Fas ligand). HEK₁n (human epidermal keratinocytes, neonatal)-E6/E7 were treated with Fc:FasL. Where indicated, zVADfmk or zIETDfmk (all at 50 μM) or solvent vehicle (DMSO) were given to the cells for 30 min before the treatment. At the indicated times, the phosphorylation states of EGFR, ERK, and Akt1 were assessed in immunoblot analyses using phosphoepitope-specific antibodies. Apoptosis was assessed by determining the cleavage of poly(ADP)ribose polymerase.

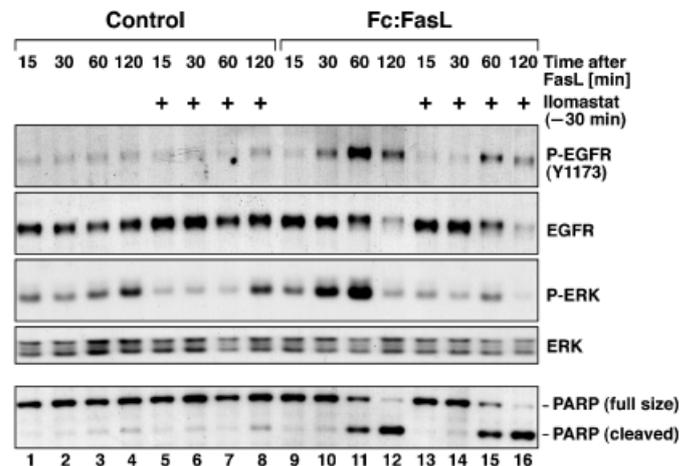


Figure 7
The Fc:FasL (Fas ligand)-induced soluble epidermal growth factor receptor (EGFR) ligands are secreted following proteolytic cleavages of their membrane-bound proforms. The cells were treated with Fc:FasL. Where indicated, ilomastat (25 μM) or solvent vehicle (DMSO) were given to the cells for 30 min before the treatment. At the indicated times, the phosphorylation states of EGFR and ERK were assessed in immunoblot analyses using phosphoepitope-specific antibodies. Apoptosis was assessed by determining the cleavage of poly(ADP)ribose polymerase.

these EGFR ligands in keratinocytes is not known. Ilomastat (galardin, GM6001), a broad specificity metalloproteinase inhibitor (Schultz *et al*, 1992) inhibits TACE (Solorzano *et al*, 1997) and blocks bombesin- and lysophosphatidic acid-induced EGF receptor transactivation (Santiskulvong and Rozengurt, 2003). We pretreated HEK₁n-E6/E7 with ilomastat before stimulation of the cells with Fc:FasL. Both EGFR

arborg *et al*, 2002; Borrell-Pages *et al*, 2003; Gschwind *et al*, 2003; Jackson *et al*, 2003; Lee *et al*, 2003) although the identity of the protease(s) responsible for the maturation of

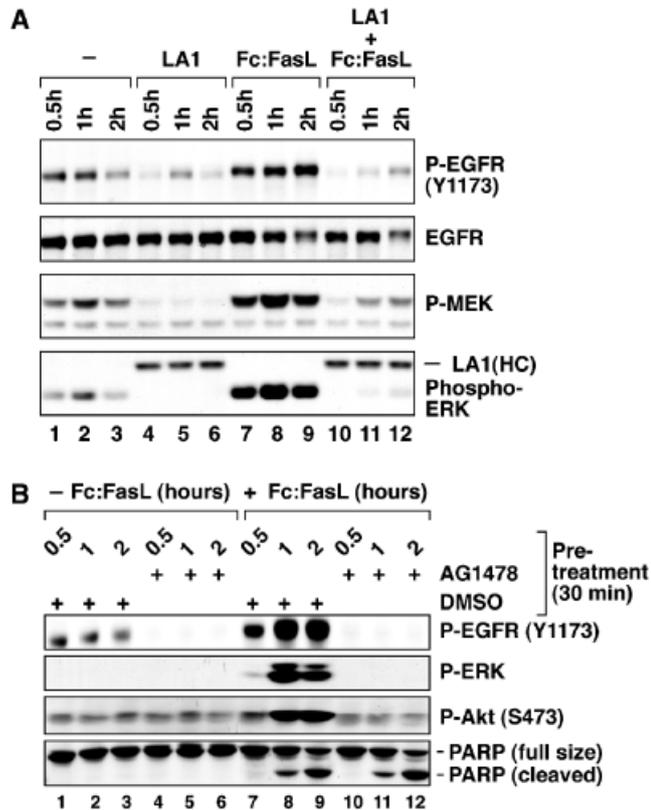


Figure 8

The activation of MEK, extracellular signal-activated kinase (ERK), and protein kinase B (Akt)1 in response to Fc:FasL (Fas ligand) is dependent on epidermal growth factor receptor (EGFR). (A) HEK_n (human epidermal keratinocytes, neonatal)-E6/E7 were treated with Fc:FasL. Where indicated, the LA1 EGFR-blocking antibody (5 μg per mL) was given to the cells for 30 min before the treatment. At the indicated times, the phosphorylation states of EGFR, MEK, and ERK were assessed in immunoblot analyses using phosphoepitope-specific antibodies. The heavy chain of the LA1 antibody appears in the gel because of internalization and/or adherence of the antibody to the cell surface. (B) HEK_n-E6/E7 were treated with Fc:FasL. Where indicated, AG1478 (10 μM) or solvent vehicle (DMSO) were given to the cells for 30 min before the treatment. At the indicated times, the phosphorylation states of EGFR, ERK, and Akt1 were assessed in immunoblot analyses using phosphoepitope-specific antibodies. Apoptosis was assessed by determining the cleavage of poly(ADP)ribose polymerase.

and ERK phosphorylations were reduced in the presence of ilomastat (Fig 7, compare lanes 9–12 to lanes 13–16). Ilomastat had no apparent effect on Fc:FasL-induced apoptosis, measured by PARP cleavage, indicating that the drug did not interfere with the binding of Fc:FasL to Fas or with caspase activity (Fig 7, compare lanes 9–12 to lanes 13–16). Furthermore, ilomastat had no effect on the ability of exogenous EGF to trigger phosphorylation of EGFR and ERK (not shown). We concluded that proteolytic maturation of membrane-bound precursors of one or more EGFR ligands is responsible for at least part of the phosphorylation of EGFR in Fc:FasL-treated keratinocytes.

The phosphorylation of EGFR in response to Fc:FasL is autocatalytic and determines the phosphorylation of ERK and Akt To investigate whether the kinase activity of EGFR itself is responsible for the phosphorylation of the receptor in Fc:FasL-treated keratinocytes, we employed LA1, an EGFR-neutralizing antibody (Kawamoto *et al*, 1992;

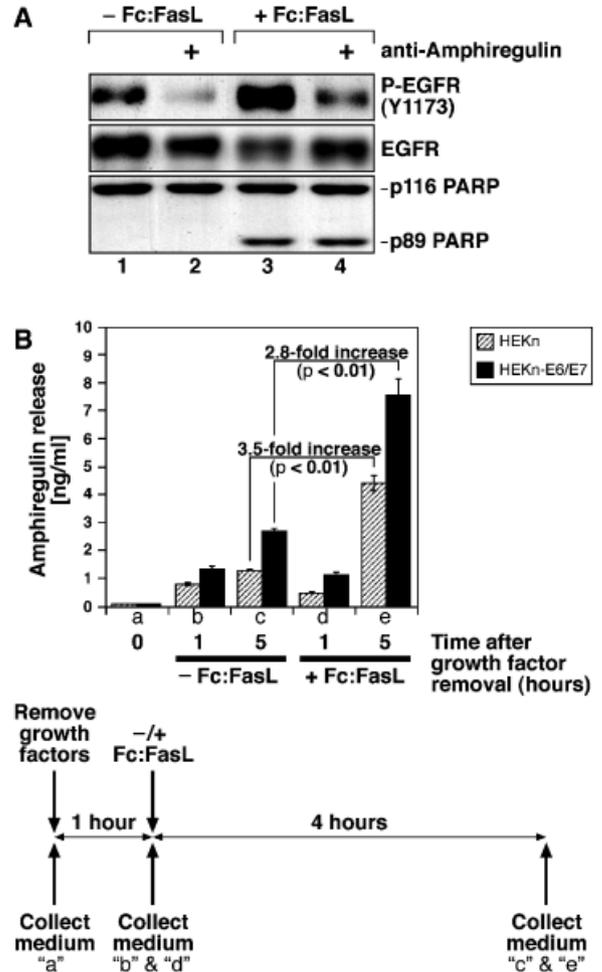


Figure 9

Role of amphiregulin (AR) in the Fc:FasL (Fas ligand)-induced phosphorylation of epidermal growth factor receptor (EGFR). (A) A neutralizing anti-AR antibody decreases both the basal and the Fc:FasL-induced phosphorylation of EGFR. The cells were treated with Fc:FasL. Where indicated, a neutralizing anti-AR antibody (50 μg per mL) was given to the cells for 30 min before the treatment. Four hours later, the phosphorylation state of EGFR was assessed in immunoblot analyses using a phosphoepitope-specific antibody. (B) ELISA detection of AR under untreated and Fc:FasL-treatment conditions. Cells (as indicated, either HEK_n-E6/E7 or HEK_n, passage 6) were washed of exogenous and endogenously produced growth factors and the cells were placed in BKM-exoGF. The medium was collected for ELISA either immediately (a) or at the indicated times after the removal of growth factors (b–e) in the absence (b, c) or in the presence (d, e) of Fc:FasL. Error bars depict standard deviation from experimental points in triplicates.

Ristow, 1996; Al Moustafa *et al*, 1999) and AG1478, a small molecule inhibitor of the kinase activity of EGFR (Osherov and Levitzki, 1994). Indeed, both LA1 and AG1478 abrogated the ability of Fc:FasL to trigger EGFR phosphorylation (Fig 8A, compare lanes 7–9 to lanes 10–12; Fig 8B, compare lanes 7–9 to lanes 10–12). As expected, also the Fc:FasL-triggered phosphorylation of ERK and Akt was strongly inhibited by blocking EGFR (Fig 8A, compare lanes 7–9 to lanes 10–12; Fig 8B, compare lanes 7–9 to lanes 10–12).

Identification of amphiregulin as an EGFR ligand produced by apoptotic keratinocytes In an attempt to identify the EGFR ligand(s) produced by apoptotic keratin-

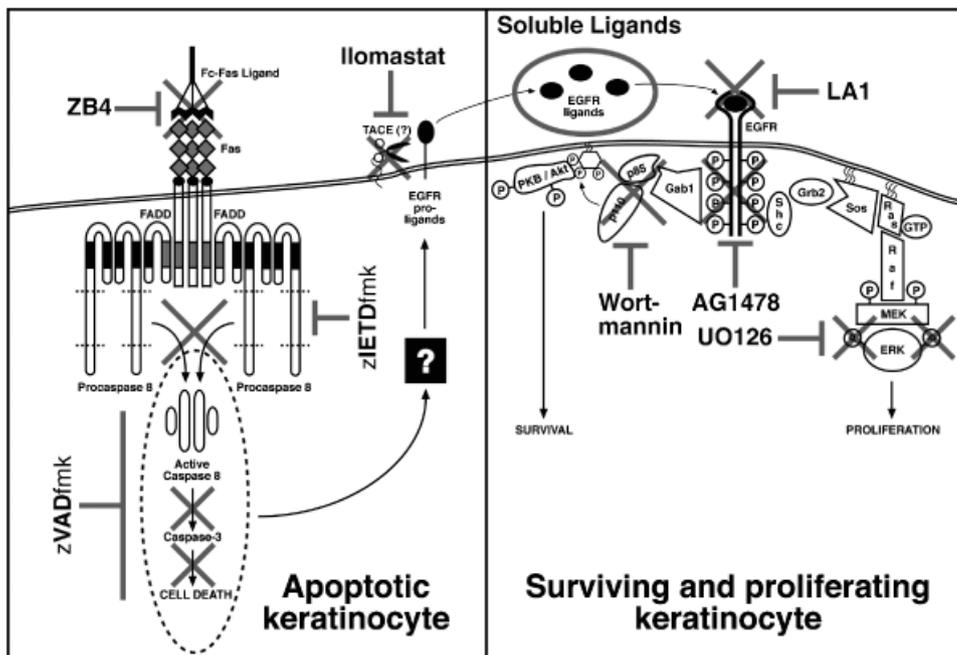


Figure 10
A model for the mechanism of Fc:FasL (Fas ligand)-induced activation of epidermal growth factor receptor, extracellular signal-activated kinase, and protein kinase B1 in keratinocytes. See explanations in the text.

ocytes, we employed blocking antibodies against three known EGFR ligands. Whereas antibodies neutralizing TGF- α and HB-EGF had consistently little or no effect on the Fc:FasL-induced EGFR phosphorylation (not shown), the AR-neutralizing antibody was able to substantially reduce both the basal and the Fc:FasL-induced phosphorylation of EGFR (Fig 9A). These results are in agreement with recent data indicating the importance of AR the autocrine activation of ERK in keratinocytes (Kansra *et al*, 2004). Furthermore, an ELISA detection of AR release by both HEK α n and HEK α n-E6/E7 demonstrated the ability of these cells to produce AR constitutively (Fig 9B and C) and to further increase the release of AR in response to Fc:FasL (Fig 9B, D and E). We concluded, therefore, that AR is an important autocrine regulator of EGFR activity in response to apoptotic stimuli.

The activation of EGFR, ERK, and Akt cannot prevent the cell death caused by Fc:FasL We considered the possibility that stimulating the potent pro-survival EGFR-Akt and EGFR-ERK signaling pathways may decrease the cytotoxicity of FasL in keratinocytes. We tested this possibility by interfering with the activation of either signaling pathway singly or in combination. Thus, abrogating EGFR activation by means of AG1478, blocked both the activation of ERK and Akt (Fig 8B, compare lanes 7–9 to lanes 10–12). Application of wortmannin, an inhibitor of phosphatidylinositol 3-kinase (Arcaro and Wymann, 1993), an upstream activator of Akt (Franke *et al*, 1995), abrogated the activation of Akt by Fc:FasL, but had a modest effect on the activation of ERK (not shown). Conversely, U0126, an inhibitor of MEK (Favata *et al*, 1998), abrogated the activation of ERK, but had no effect on the activation of Akt by Fc:FasL (not shown). Importantly, none of the employed inhibitors was capable of affecting the pro-apoptotic action of Fc:FasL, as determined by PARP cleavage (exemplified in Fig 8B for AG1478). It appears, therefore, that, for keratinocytes directly targeted by FasL, the ligation of Fas triggers a dom-

inant cytotoxic response that cannot be counteracted by the activation of EGFR, ERK, and Akt.

The findings described in this work can be summarized in a model presented in Fig 10. We demonstrate the existence of a novel signaling pathway triggered by ligation of Fas in keratinocytes. Downstream of the FasL/Fas-dependent activation of caspases, one (AR) or more epidermis-specific, membrane-bound forms of EGFR ligands are produced and processed by a proteolytic activity inhibitable by ilomastat. The soluble EGFR ligands, in turn, activate EGFR and trigger the activation of two downstream EGFR effector pathways: the Ras-Raf-MEK-ERK pathway and the PI3K-Akt pathway. Despite the potent pro-survival function of EGFR in the epidermis (Sibilia *et al*, 2000 and references therein), it appears that the ligation of Fas is dominant in determining the fate of an individual keratinocyte, i.e., the keratinocyte that has produced EGFR ligands in response to Fas activation is unable to “benefit” from the anti-apoptotic program it has initiated. What could be, then, the function of the activation of EGFR, ERK, and Akt in response to apoptotic stimuli in the epidermis? Work performed by Trautmann *et al* (2000) on AD and nickel-triggered ACD and by us on SADBЕ-triggered ACD (Fig 1) indicates that apoptotic keratinocytes present in the eczematous areas are surrounded by cells lacking any apoptotic morphology (as in Fig 1, panel c). We speculate that the EGFR ligands produced by apoptotic cells in the epidermis act on non-apoptotic neighboring cells to trigger their proliferation (in the case of basal keratinocytes) and/or migration into the areas of massive epidermal destruction, thus preserving the barrier function of the skin.

Materials and Methods

Cells Primary explanted HEK α n and all cell culture reagents were from Cascade Biologics (Portland, Oregon). The derivation and characterization of immortalized human epidermal keratinocytes

(HEKn-E6/E7) has been described in Iordanov *et al*, 2002. Briefly, primary human epidermal keratinocytes, neonatal (HEKn) were infected with an amphitropic recombinant retroviral vector encoding the E6 and E7 transforming proteins of human papilloma virus serotype 16. HEKn and HEKn-E6/E7 were maintained in EpiLife basal keratinocyte medium (BKM) (Cascade Biologics, Portland, Oregon) supplemented with a semi-defined human keratinocyte growth supplement (HKGS; the final concentrations of the components in the supplemented medium are: bovine pituitary extract, 0.2% v/v; bovine insulin, 5 mg per mL; hydrocortisone, 0.18 mg per mL; bovine transferrin, 5 mg per mL; and human epidermal growth factor, 0.2 ng per mL). BKM supplemented with HKGS is referred to as BKM + exoGF (BKM *plus* exogenous growth factors). Respectively, BKM lacking HKGS is referred to as BKM - exoGF (BKM *minus* exogenous growth factors).

Chemicals and Fc:FasL All commonly used chemicals were from Sigma Chemical Company (St. Louis, Missouri). Caspase inhibitors (zVADfmk and zIETDfmk), AG1478, U0126, and wortmannin were from Calbiochem (La Jolla, California). Ilomastat was from Biomol (Plymouth Meeting, Pennsylvania). For all experiments, Fc:FasL (Holler *et al*, 2003) was used at a final concentration of 250 ng per mL except for the experiment shown in Fig 4A (500 ng per mL).

Preparation of cell lysates for immunoblot analysis To avoid potential post-lysis modifications or degradation of proteins of interest, the cells were harvested by direct lysis in 2 × SDS-PAGE sample-loading buffer, followed by heat denaturation at 95°C for 5 min and ultrasonic shearing. Typically, the detached (dead) cells were sedimented from the growth medium by centrifugation, lysed in 2 × SDS-PAGE sample-loading buffer, and combined with the cell lysates from adherent cells. Cell lysates were stored at -70°C.

Antibodies and immunoblot analyses The anti-Ki-67 antibody was from Biomed (Beaufort, South Carolina). The anti-K5 and -K1 antibodies were from BabCo. The Fas-blocking antibody ZB4 and the EGFR-blocking antibody LA1 were from Upstate Cell Signaling Solutions (Waltham, Massachusetts). The amphiregulin-, heparin-binding EGF-, and TGF- α -blocking antibodies were from R&D Systems. The antibodies against PARP (H-250), ERK1 (C-16), EGFR (EGFR-1005), and the phosphorylated form of EGFR (pY1173) were from Santa Cruz Biotechnologies (Santa Cruz, California). The antibody against the phosphorylated form of EGFR (pY1068) and the antibodies against the phosphorylated forms of ERK, MEK, and PKB/Akt1 (S473), as well as the anti-caspase 8 antibody (clone 1C12), were from Cell Signaling Technology. The separation of proteins in SDS-PAGE and the electrotransfer onto polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts) were performed by standard procedures. Immunoprob- ing with specific antibodies and enhanced chemiluminescent detection (DuPont NEN Research Products, Boston, Massachusetts) were performed following the instructions of the respective manufacturers.

Immunohistochemistry, TUNEL, and ELISA The immunohistochemical detection of Ki-67, TUNEL, and detection of AR release by ELISA were performed following the protocols provided by the respective manufacturers (Biomed, Boehringer Mannheim/Roche, and R&D Systems, Indianapolis, Indiana).

Experimental ACD SADBE was used to induce allergic contact dermatitis in normal volunteers after informed consent documents were obtained, under a protocol approved by the Institutional Review Board of Oregon Health & Science University and conformed to ethical guidelines of Declaration of Helsinki. Volunteers were sensitized by applying 2% SADBE in acetone under occlusion to a 2 cm² area of normal skin on the upper arm overnight. Volunteers then washed the area thoroughly with soap and water. Volunteers were then challenged with 0.5% SADBE to the volar forearm 2 weeks later. All volunteers displayed erythema and a typical eczematous dermatitis 48 h after application. Four millimeter punch

biopsies from lesional and adjacent non-lesional skin were obtained 2 and 7 d after the application of SADBE. Biopsy specimens were cryopreserved in optimal cutting temperature (OCT) embedding medium.

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