

# Fas Ligand-induced Proinflammatory Transcriptional Responses in Reconstructed Human Epidermis

## RECRUITMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR AND ACTIVATION OF MAP KINASES<sup>\*[5]</sup>

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Fas ligand (FasL) exerts potent proapoptotic and proinflammatory actions on epidermal keratinocytes and has been implicated in the pathogenesis of eczema, toxic epidermal necrolysis, and drug-induced skin eruptions. We used reconstructed human epidermis to investigate the mechanisms of FasL-induced inflammatory responses and their relationships with FasL-triggered caspase activity. Caspase activity was a potent antagonist of the pro-inflammatory gene expression triggered by FasL prior to the onset of cell death. Furthermore, we found that FasL-stimulated autocrine production of epidermal growth factor receptor (EGFR) ligands, and the subsequent activation of EGFR and ERK1 and ERK2 mitogen-activated protein kinases, were obligatory extracellular steps for the FasL-induced expression of a subset of inflammatory mediators, including CXCL8/interleukin (IL)-8, ICAM-1, IL-1 $\alpha$ , IL-1 $\beta$ , CCL20/MIP-3 $\alpha$ , and thymic stromal lymphopoietin. These results expand the known physiological role of EGFR and its ligands from promoting keratinocyte mitogenesis and survival to mediating FasL-induced epidermal inflammation.

FasL,<sup>3</sup> a member of the tumor necrosis factor family of cytokines (reviewed in Ref. 1), signals apoptotic cell death by

engagement of its cognate receptor, Fas (2, 3). FasL/Fas triggers apoptosis by activating the caspase family of proteases (reviewed in Refs. 4 and 5). FasL plays an important role in the effector function of cytotoxic T lymphocytes and also regulates their homeostasis (3). Deficiencies in either FasL or Fas are associated with autoimmune lymphoproliferative syndrome (a hereditary condition characterized by the accumulation of atypical lymphocytes and autoimmune manifestations) and systemic lupus erythematosus (6, 7). FasL expression enables the immunoprivileged state of the cornea and the testis (8, 9), thus further underscoring the important role of this cytokine in restricting autoimmune inflammation under normal homeostatic conditions.

On the other hand, several laboratories, including ours, have revealed the importance of FasL/Fas as a positive regulator of pathological inflammation as well, particularly in inflammatory diseases of the skin. Keratinocytes in the healthy human express Fas, but not FasL (10). However, abnormal expression of lytically active FasL was found in keratinocytes of patients with toxic epidermal necrolysis, suggesting that a suicidal keratinocyte reaction contributes to the pathogenesis of toxic epidermal necrolysis (10). FasL/Fas signaling was implicated in acute cutaneous graft *versus* host disease (11). More recently, FasL was found to be involved in the pathogenesis of eczematous dermatitides or eczemas (such as atopic dermatitis and allergic contact dermatitis) (12–15). 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) (Ref. 12 and references therein), recent findings suggest that keratinocyte death plays a major role in vesicle formation (12, 13). This keratinocyte death appears to be apoptotic and to be mediated by FasL, delivered to the epidermis by infiltrating T lymphocytes and acting on Fas expressed on the surface of keratinocytes (13). Recently, expression of both FasL and Fas by epidermal keratinocytes was proposed to characterize fixed drug eruptions (16) and drug-induced maculopapular rashes (17). Finally, the Fas/FasL apoptotic pathway was proposed to contribute to the *Leishmania major*-induced cutaneous ulceration (18).

These findings clearly demonstrated the important role of FasL in epidermal destruction in inflammatory skin diseases.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1 and supplemental Tables S1–S6.

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<sup>3</sup> The abbreviations used are: FasL, Fas ligand; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; RHE, reconstructed human epidermis; MAPK, mitogen-activated protein kinase; IL, interleukin; TSLP, thymic stromal lymphopoietin; ICAM-1, intercellular adhesion molecule 1; HEKn, human epidermal keratinocytes (neonatal); siRNA, small interfering RNA; HKGS, human keratinocyte growth supplement; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; RT, reverse transcription; qRT-PCR, quantitative real time RT-PCR; CXCL14, C-X-C motif ligand 14; AREG, amphiregulin; EREG, epiregulin; HB, heparin-binding; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase.

However, whether FasL could directly trigger the inflammatory process was not known. We demonstrated recently that FasL elicits a pro-inflammatory reaction in human HaCaT keratinocytes and reconstructed human epidermis (RHE) by triggering the expression of stress-responsive transcription factors, inflammatory cytokines, chemokines, and the adhesion molecule ICAM-1 (19). We demonstrated that oligomerization of Fas was required both for apoptosis and for the inflammatory gene expression and that caspase activity was essential for apoptosis but dispensable for the inflammatory gene expression (19). These results suggested that FasL was a pro-inflammatory cytokine in eczemas. FasL stimulated the activity of the NF- $\kappa$ B transcription factor in HaCaT cells, and this activity was required for the FasL-induced activation of inflammatory genes (19). The requirement for NF- $\kappa$ B suggested that the pro-inflammatory actions of FasL were dependent on transcriptional gene activation.

In this work, we employed RHE to elucidate three novel aspects of FasL-induced inflammation in the epidermis, namely: (i) the importance of the proper organotypic stratification, (ii) the inhibitory role of caspases, and (iii) the unexpected involvement of the EGFR-ERK axis in the transcriptional inflammatory responses to FasL in the epidermis.

## EXPERIMENTAL PROCEDURES

**RHE: Primary Human Epidermal Keratinocytes (Neonatal) (HEKn) and Their Immortalized Derivatives**—HEKn-E6/E7 were established and propagated as described (20, 21). For RHE, we employed the method of Poumay *et al.* (22), with modifications as described in Ref. 19. RHEs were fixed in freshly made 4% *p*-formaldehyde, embedded in paraffin, and after sectioning were processed for immunohistochemistry following the specific protocols recommended by the respective manufacturers for each antibody.

**siRNA-mediated Knockdown of ERK1 and ERK2 in RHE**—The siRNA oligonucleotides were synthesized by the Department of Molecular Microbiology and Immunology Research Core Facility (Oregon Health & Science University, Portland, OR). Equimolar amounts of the complementary oligonucleotides were annealed in annealing buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl) by denaturing at 90 °C for 2 min followed by incubation at 37 °C for 1 h. The siRNA sequences were as follows: nonspecific control, sense 5'-CGAGUAGGCUUCGUG-ACUudTdT-3', antisense 5'-AAGUCACGAAGCCUACUC-GdTdT-3'; ERK1, sense 5'-GCCAUGAGAGAUGUCUACAdTdT-3', antisense 5'-UGUAGACAUCUCUCAUGGCdTdT-3', derived from Ref. 23; and ERK2, sense 5'-GCUAGGAACU-AUUUGCuuudTdT-3', antisense 5'-AAAGCAAUAGUCCUAGCdTdT-3'.

RNA interference was performed as outlined schematically in Fig. 8A and as described below. On Day (−5), HEKn-E6/E7 were cultured in 10 ml of EpiLife® medium supplemented with human keratinocyte growth supplement (HKGS) as per the manufacturer's instructions (Cascade Biologics, Portland, OR) in 100-mm tissue culture dishes (Sarstedt, Newton, NC) to a final confluency of ~40% after ~18 h of incubation at 37 °C/5%CO<sub>2</sub>. On Day (−4), the medium was replaced with 8 ml of EpiLife®-HKGS. Lipofectamine®2000 reagent (Invitrogen) was

diluted to 20  $\mu$ g/ml in 1 ml of OPTIMEM®-reduced serum medium (Invitrogen) and incubated for 5 min at room temperature. Annealed siRNA was diluted to 500 nM in 1 ml OPTIMEM® medium and then mixed with 1 ml of dilute Lipofectamine™2000 for 20 min at room temperature. Two ml of siRNA/reagent mixture was added to the 8 ml of EpiLife®-HKGS medium/plate. The cells were transfected with siRNA (50 nM final concentration of each annealed siRNA) for 48 h. On Day (−2), transfected cells were trypsinized and counted. The cells were diluted in EpiLife®-HKGS medium to a concentration of  $1 \times 10^6$  cells/ml, and 0.5 ml was plated per Millicell-PCF 0.4- $\mu$ m culture plate insert (Millipore, Billerica, MA). Inserts were placed in 6-well plates containing 2.5 ml of EpiLife®-HKGS medium supplemented with 1.5 mM CaCl<sub>2</sub>, 10 ng/ml human recombinant keratinocyte growth factor (PeproTech, Inc., Rocky Hill, NJ), and 50  $\mu$ g/ml ascorbic acid. On Day 0, the RHEs were switched to differentiation as described previously in Ref. 19 and used typically at Day 4 as depicted in Fig. 8.

**Chemicals, Fc:FasL, Blocking Reagents, Growth Factors, and Cytokines**—All of the commonly used chemicals and EGF were from Sigma. Caspase inhibitors (z-VAD-fmk and z-IETD-fmk), UO126, SB203580, SP600125, and AG1478 were from Calbiochem. Fc:FasL has been described previously (24). The LA-1 antibody was from R & D Systems (Minneapolis, MN).

**RNA Isolation, Affymetrix Microarray, and Quantitative Real Time RT-PCR (qRT-PCR)**—These techniques were performed as previously described in Ref. 19. The following additional primers were employed here: CCL20/MIP-3 $\alpha$  mature mRNA forward, 5'-CGAATCAGAAGCAGCAAGCA-3'; CCL20/MIP-3 $\alpha$  mature mRNA reverse, 5'-AGCATTGATGTCACAGCCTTCA-3'; CCL20/MIP-3 $\alpha$  primary transcript forward, 5'-CCACCTCTGCGGCGAATCAGAAG-3'; CCL20/MIP-3 $\alpha$  primary transcript reverse, 5'-CTTAGGGACCCCCAGTTG-AGCTC-3'; ICAM-1 primary transcript forward, 5'-CATCT-ACAGTAAGAAGGGGCAGGG-3'; ICAM-1 primary transcript reverse, 5'-TCGTGAGAATCACGTTGGGC-3'; TSLP mature mRNA forward, 5'-CCGTCTCTGTAGCAATCGGC-3'; and TSLP mature mRNA reverse, 5'-GGCAGCCTTAGTTTTCATGGC-3'. For the detection of primary transcripts by qRT-PCR, 3  $\mu$ g of total RNA were reverse-transcribed in the presence of SuperScript III and random hexamer primers (Invitrogen).

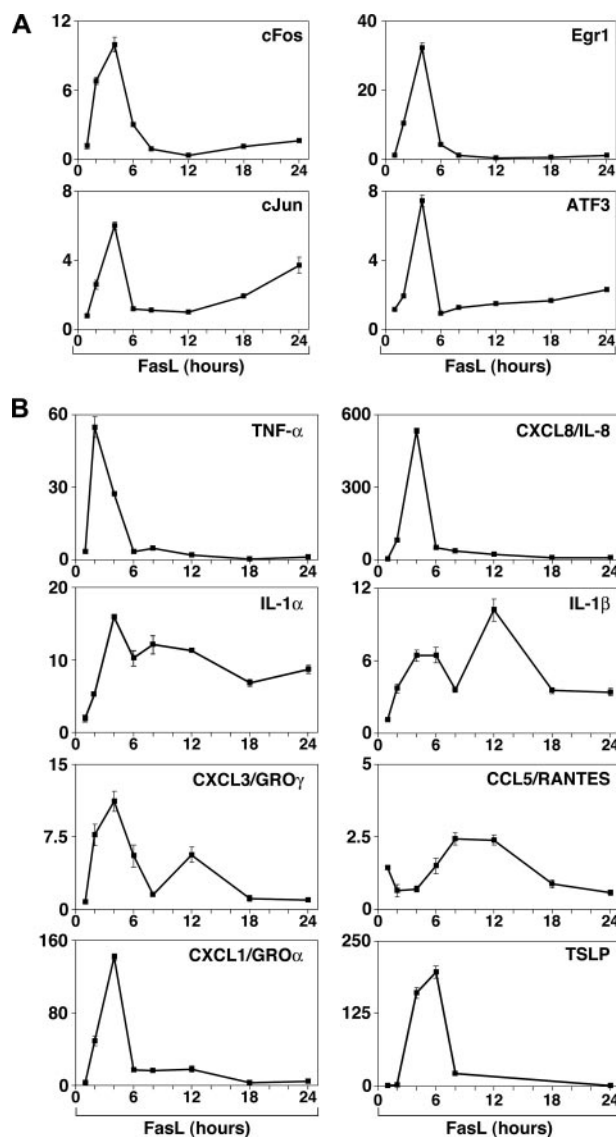
## RESULTS

**Identification of FasL-regulated Genes in RHE**—To identify genes activated by FasL in an epidermotypic context, we performed Affymetrix microarray analyses on RNA extracted from RHE 4 h after exposure to FasL. The analyses were performed on HG-U133 Plus 2.0 GeneChip arrays, which interrogate over 47,000 human transcripts. Subsequent analysis identified 571 genes that were up-regulated at least 2-fold and 186 genes that were up-regulated at least 3-fold after FasL (available online at: [www.ohsu.edu/cellbio/faculty/Iordanov\\_data/Farley\\_et\\_al\\_\(2007\).xls](http://www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_(2007).xls)). The data were analyzed with both EASE (25) and L2L (26) software, which automate the process of biological theme determination by analyzing the overrepresentation of genes that belong to categories that are functionally and structurally defined. Supplemental Tables S1 and S2 display the L2L-generated hierarchical analyses of the genes whose mRNA

abundance was increased at least 3-fold after FasL. Overrepresented genes in the Biological Process categories were associated with wound, chemotaxis, inflammatory, immune, and stress responses (supplemental Table S1). The Molecular Function categories associated with these genes were identified as cytokine, chemokine, and growth factor activities (supplemental Table S2). EASE analysis produced similar results (not shown). A selection of 37 FasL-induced genes in the organotypic model is shown in supplemental Table S3.

We also analyzed the genes that were down-regulated in response to FasL (supplemental Tables S4–S6). For this purpose, we first excluded from the Affymetrix data all genes that were categorized as “absent” or “marginally present” in the control (untreated) RHE group. We excluded, additionally, all genes from the control group that displayed basal levels of expression below 100 arbitrary Affymetrix units. Of the remaining 985 genes, we selected those that were down-regulated at least 2-fold by the FasL treatment (a total of 442 Affymetrix probe IDs; *i.e.* 45%). Of these, 287 were annotated in the public databases Entrez Gene ([www.ncbi.nlm.nih.gov/sites/entrez?db=gene](http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)) or Gene Cards ([www.genecards.org](http://www.genecards.org)). The L2L overrepresentation analysis of these genes by biological process and molecular function is shown in supplemental Tables S4 and S5, respectively. In the biological process categories, FasL-repressed genes appear to be overrepresented in the categories related to metabolic processes (including DNA, RNA, and protein metabolism). By molecular function, genes belonging to the categories peptidase, helicase, and kinase activities were down-regulated by FasL. Supplemental Table S6 contains the 68 genes that were down-regulated at least 2-fold by FasL and whose basal expression in the control RHE was the highest (*i.e.* at least 1000 arbitrary Affymetrix units). The top ranking FasL-repressed gene was the chemokine C-X-C motif ligand 14 (CXCL14), whose expression was down-regulated ~26-fold in the presence of FasL (see “Discussion”). Interestingly, the ability of FasL to trigger the repression of CXCL14 was significantly attenuated in the presence of the pan-caspase inhibitor z-VAD-fmk.

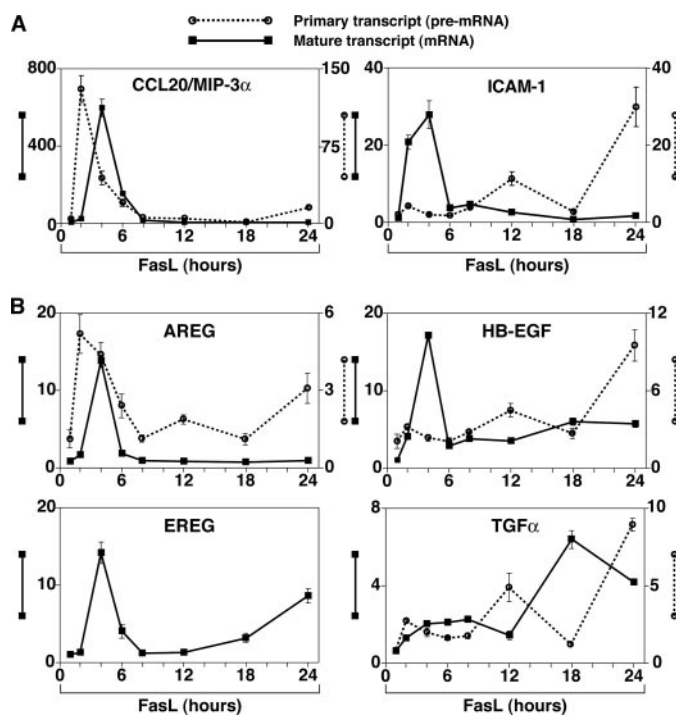
qRT-PCR was employed to investigate the temporal appearance of a variety of FasL-up-regulated gene transcripts that belonged to the molecular function categories identified as overrepresented in our Affymetrix array analyses. The mRNAs encoding transcription factors c-Fos, c-Jun, Egr1, and ATF3 displayed maximum increases in abundance 4 h after FasL treatment; their levels declined thereafter (Fig. 1A). The mRNAs encoding proinflammatory cytokines and chemokines and the adhesion molecule ICAM-1 were activated maximally between 2 and 6 h post FasL (specifically, 2 h for tumor necrosis factor- $\alpha$ , 4 h for ICAM-1, IL-1 $\alpha$ , CCL20/MIP-3 $\alpha$ , CXCL3/GRO $\gamma$ , CXCL1/GRO $\alpha$ , and CXCL8/IL-8, and 6 h for TSLP), with the exception of IL-1 $\beta$  (maximum at 12 h) (Figs. 1B and 2A). Previous studies from our laboratory have demonstrated that exposure of monolayers of keratinocytes to FasL resulted in the activation of the EGFR via the secretion of EGFR-binding ligands (21). Whereas the mRNA encoding amphiregulin (AREG) demonstrated a single transient peak of accumulation (at 4 h), mRNAs encoding epiregulin (EREG) and heparin-binding EGF (HB-EGF) displayed a prominent transient peak at 4 h



**FIGURE 1. Time course of FasL-induced accumulation of mRNAs encoding transcription factors and inflammatory mediators in RHE.** RHEs (14 days after the differentiation switch) were treated with FasL (250 ng/ml) for the indicated times as previously described (19) and then interrogated, using qRT-PCR, for the levels of specific transcripts at 1, 2, 4, 6, 12, 18, and 24 h of exposure to FasL. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicate qRT-PCRs. A, mRNAs encoding transcription factors. B, mRNAs encoding inflammatory mediators.

and a delayed second wave of activation that occurred between 18 and 24 h post FasL (Fig. 2B). In contrast to mRNA encoding the former EGFR-binding ligands, the mRNA for transforming growth factor- $\alpha$  was not induced more than 2-fold within the first 12 h after FasL stimulation but became elevated at 18 and 24 h post FasL.

*Epidermal Organotypic Context Is Essential for the Inflammatory Responses of Human Keratinocytes to FasL*—Surprisingly, treatment with FasL of either primary HEKn or immortalized (HEKn-E6/E7) keratinocytes in two-dimensional tissue culture conditions resulted in the strong activation of the immediate-early genes encoding transcription factors (c-Jun, c-Fos, ATF3, and Egr1), as in Fig. 1A, but failed completely to trigger the activation of genes encoding inflammatory media-



**FIGURE 2. Time course of FasL-induced accumulation of primary transcripts versus their corresponding mature mRNAs.** Treatment and analyses performed as for Fig. 1. Primary transcripts (dotted lines, y axes on the right-hand side of the graphs) and mature mRNAs (filled lines, y axes on the left-hand side of the graphs) encoding CCL20/MIP-3 $\alpha$  and ICAM-1 (A) or EGFR-binding ligands (B). EREG appeared not to have introns and therefore was not suitable for primary transcript analyses.

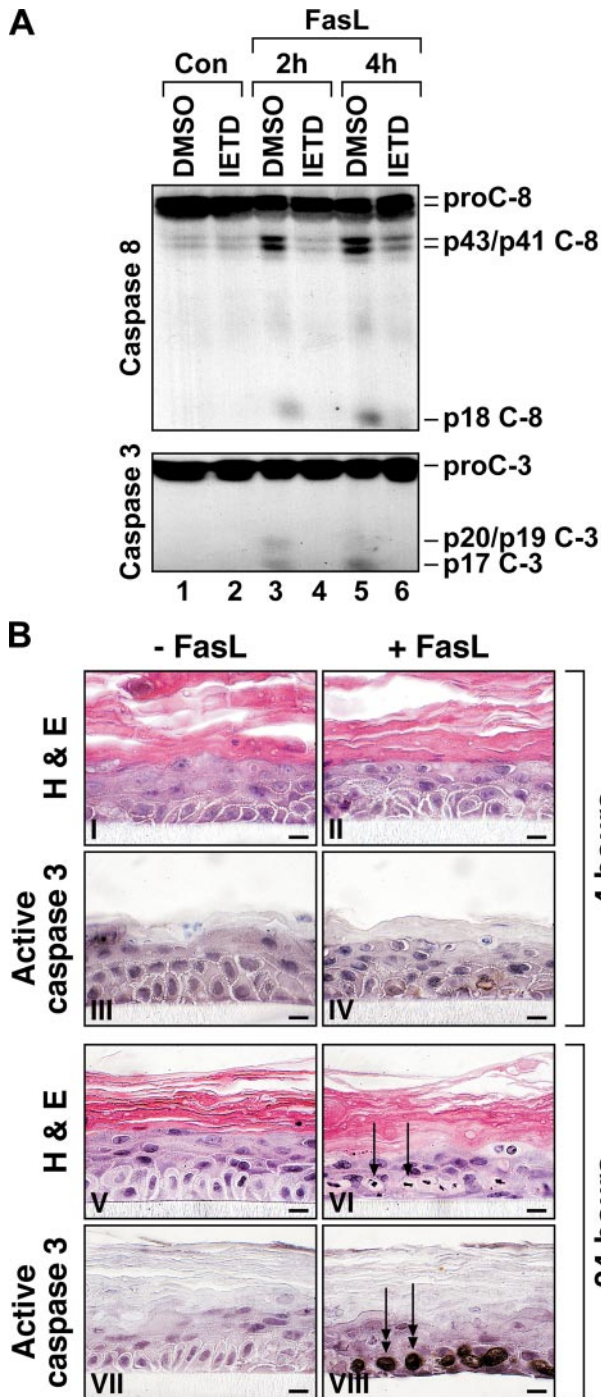
tors (such as cytokines and chemokines, as well as ICAM-1) or EGFR-binding ligands (not shown). These experiments have been repeated a number of times with identical outcomes. This lack of proinflammatory gene response to FasL in cultured keratinocytes was not affected by variations of  $\text{Ca}^{2+}$  levels or choice of growth medium (e.g. Dulbecco's modified Eagle's medium, with or without fetal calf serum) (not shown), suggesting that the proper epidermotypic context (and not growth conditions) was absolutely essential for the proinflammatory response to FasL.

**Transcriptional Regulation of FasL-induced mRNAs**—To investigate the FasL-induced accumulation of mRNAs encoding inflammatory mediators at a level mechanistically and temporarily closer to true transcriptional activation of the corresponding genes, we determined the accumulation of selected primary transcripts following FasL administration. To this end we employed a random hexamer-primed method of cDNA synthesis and exon-intron border-specific primers for qRT-PCR. As shown in Fig. 2, some of the primary transcripts tested displayed kinetics of increase that preceded those of the corresponding mature mRNAs (best exemplified by CCL20/MIP-3 $\alpha$ , Fig. 2A; and AREG, Fig. 2B, RNAs). These examples suggest that FasL-induced elevation of the mRNAs encoding inflammatory mediators and EGFR-binding ligands in RHEs involves true transcriptional activation of the corresponding genes. These analyses further permitted us to identify increased levels of primary FasL-induced transcripts at later times (24 h) after the addition of FasL (e.g. ICAM-1, Fig. 2A; and AREG and HB-EGF, Fig. 2B). Interestingly, this delayed accumulation of

primary transcripts was not mirrored by a corresponding increase in the levels of their mature mRNAs (Fig. 2), suggesting a level of regulation of these genes that escapes detection when RT-PCR is employed to detect levels of mature mRNA transcripts.

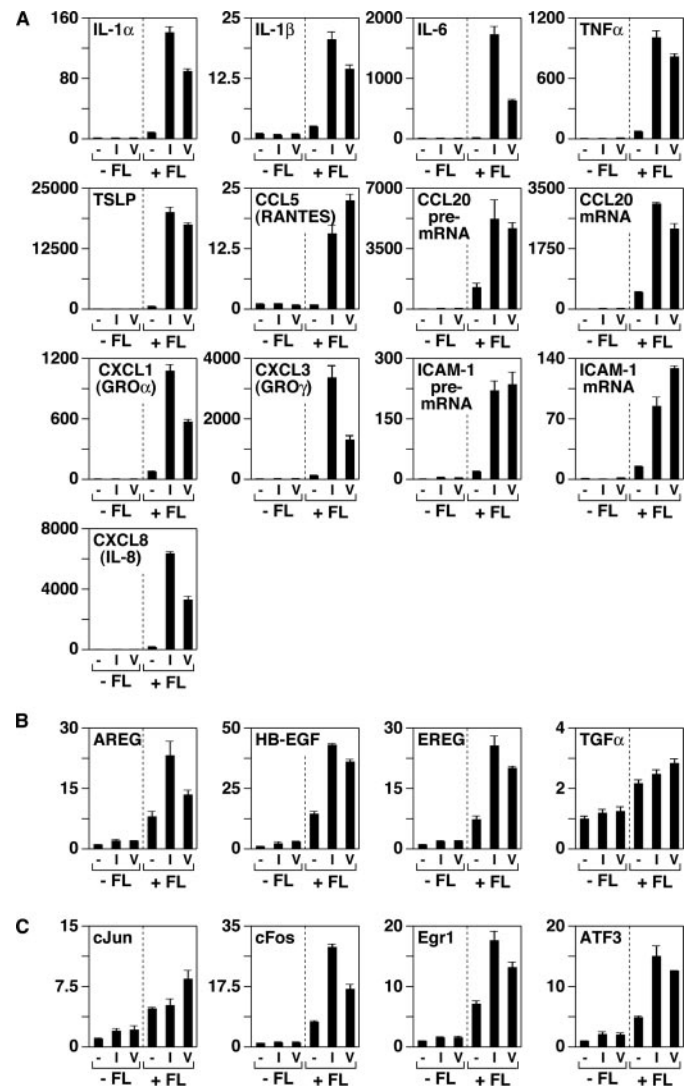
**Caspase Activity Is a Potent Antagonist of the Pro-inflammatory Action of FasL in RHE**—Because FasL is known to activate proapoptotic pathways in keratinocytes and other cell types, we set out to investigate the potential role of FasL-activated caspases in regulating the expression of proinflammatory mRNAs following exposure of RHEs to FasL. First, we asked whether caspases were activated within the time frame of onset and optimal expression of FasL-induced genes. RHEs treated with FasL displayed processing of procaspase 8 into the p43/p41 intermediate fragments and the p18 mature large subunit as early as 2 h (Fig. 3A). The processing of caspase 8 was inhibited by the caspase 8-specific inhibitor z-IETD-fmk (27, 28) (Fig. 3A). The activation of caspase 8 was accompanied by a small, but detectable, processing of the effector procaspase 3, first to the p20/p19 intermediates and subsequently to the p17 mature large subunit of the active caspase 3 (Fig. 3A). Apoptotic nuclei were not detectable at 4 h after FasL but became evident by 24 h in the basal layers of FasL-treated RHEs (as shown by hematoxylin and eosin staining; Fig. 3B). Basal layers of RHEs at 24 h (but not 4 h) post FasL also revealed positive immunoreactivity for active caspase 3 (Fig. 3B). These data demonstrate that FasL engaged the apoptotic cascade as early as 2 h post addition but that the morphological manifestation of apoptosis was not yet evident within the time frame of onset and optimal expression of FasL-induced genes (2–4 h).

We then employed either z-IETD-fmk or the pan-caspase blocker z-VAD-fmk to investigate whether the engagement of apoptotic cascades by FasL would modify the accumulation of proinflammatory transcripts in response to FasL. Fig. 4 demonstrates that the application of either z-IETD-fmk or z-VAD-fmk resulted in a dramatic increase in expression of a variety of transcripts that encode cytokines and chemokines. For example, in the absence of caspase inhibitors, FasL triggered a 475-fold ( $\pm 58$ ) increase in the expression of TSLP mRNA at 4 h post addition. However, in the presence of either z-IETD-fmk or z-VAD-fmk, the FasL increased the abundance of TSLP mRNA to 20,000- and 17,000-fold, respectively (Fig. 4A). Similarly, in the absence of caspase inhibitors, FasL triggered a 11-fold ( $\pm 2.6$ ) increase in the expression of IL-6 mRNA at 4 h post addition. In the presence of either z-IETD-fmk or z-VAD-fmk, the IL-6 mRNA was increased by FasL to 1,721-fold ( $\pm 130$ ) and 622-fold ( $\pm 21$ ), respectively (Fig. 4A). In addition to these quantitative differences, inhibition of caspases affected the responsiveness to FasL qualitatively as well. For instance, FasL alone failed to induce the expression of CCL5/RANTES mRNA, but FasL in the presence of either z-IETD-fmk or z-VAD-fmk caused a 15–25-fold increase in expression of the CCL5/RANTES mRNA (Fig. 4A and Ref. 19). The potentiating effect of caspase inhibitors on FasL-induced gene expression patterns appeared biased in favor of inflammatory mediators. For example, in the presence of z-IETD-fmk, FasL effected a >40-fold increase in TSLP mRNA and >160-fold increase in IL-6 mRNA (Fig. 4A). By comparison, the addition of FasL



**FIGURE 3. Activation of caspases by FasL in RHE.** A, RHEs were treated with FasL as in Fig. 1 in the presence of either Me<sub>2</sub>SO (vehicle) or z-IETD-fmk (IETD, 50  $\mu$ M), both administered 30 min prior to FasL. Activation of caspases 8 and 3 was determined in immunoblot analyses. B, hematoxylin and eosin (H & E) staining or immunohistochemical detection of active caspase 3 at either 4 or 24 h after treatment with FasL. The experiments were performed as described in Ref. 19. Examples of apoptotic cells, determined by morphology (hematoxylin and eosin staining) or the presence of active caspase 3, are identified by single- or double-headed arrows, respectively. DMSO, dimethyl sulfoxide.

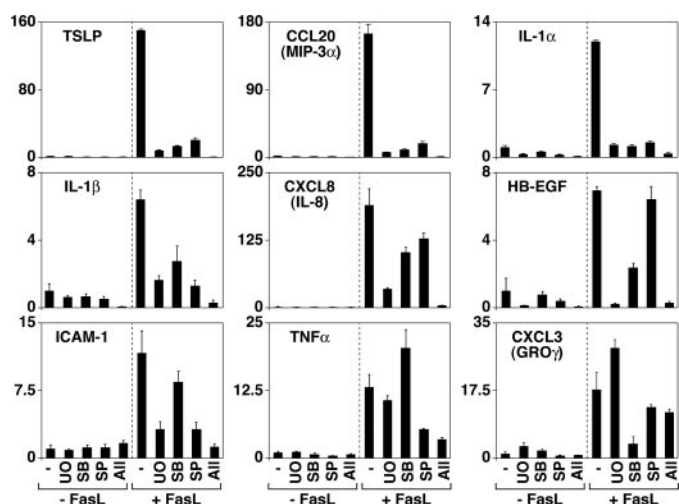
increased the expression of mRNA encoding EGFR-binding ligands (AREG, HB-EGF, and EREG) only ~3-fold in the presence of z-IETD-fmk and even less in the presence of z-VAD-fmk (Fig. 4B). Furthermore, FasL-induced transforming growth factor- $\alpha$  mRNA expression was not substantially increased by



**FIGURE 4. Effects of caspase inhibitors on the FasL-induced expression of inflammatory mediators (A), EGFR-binding ligands (B), or transcription factors (C).** RHEs were treated with FasL as in Fig. 1 in the presence of a 30-min pretreatment with Me<sub>2</sub>SO (vehicle, lanes -) or 50  $\mu$ M of either z-IETD-fmk (lanes I) or z-VAD-fmk (lanes V). qRT-PCR analyses were performed 4 h after FasL. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. Note that the qRT-PCR analyses of CCL20/MIP-3 $\alpha$  and ICAM-1 RNA expression were performed both for the primary transcripts and the mature mRNAs.

either caspase inhibitor (Fig. 4B). In a similar fashion, the potentiation of FasL-induced expression of the mRNAs encoding the transcription factors c-Fos, c-Jun, Egr1, and ATF3 was within the range of ~3-fold (Fig. 4C). The measurement of primary transcripts encoding CCL20/MIP-3 $\alpha$  and ICAM-1 suggested that the potentiating effect of caspase inhibitors on the FasL-induced proinflammatory gene expression occurred at the level of transcription (Fig. 4A).

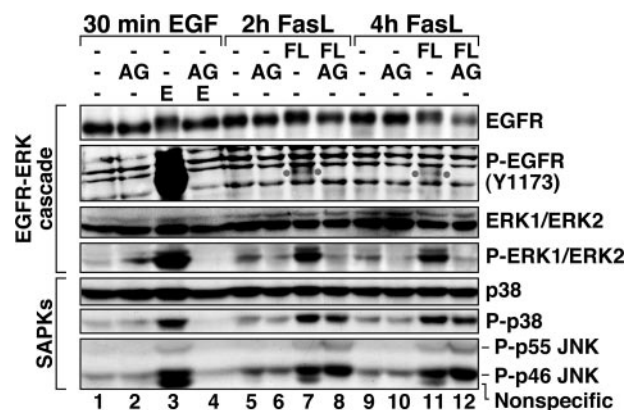
**ERK, JNK, and p38 MAP Kinases Contribute Nonredundantly to the Inflammatory Responses of Human Keratinocytes to FasL**—We have previously found that FasL activated JNK, p38, and ERK MAP kinases in monolayer cultures of human keratinocytes (21). To investigate the contribution of MAPKs in the inflammatory response triggered by FasL in RHE, we applied specific inhibitors, singly and in combination, that are



**FIGURE 5. Effects of MAP kinase inhibitors on the expression of FasL-induced genes.** RHEs were treated with FasL as in Fig. 1 in the presence of 30-min pretreatments with either  $\text{Me}_2\text{SO}$  (vehicle, —) or various antagonists as labeled. UO, UO126, 10  $\mu\text{M}$ ; SB, SB203580, 10  $\mu\text{M}$ ; SP, SP600125, 40  $\mu\text{M}$ ; All, all three inhibitors together. qRT-PCR analyses were performed 4 h after FasL. The data are presented as fold change relative to the corresponding vehicle-treated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs.

known to interfere with effective transduction of the ERK, JNK, and p38 MAPK cascades: UO126, an inhibitor of MEK, the upstream activator kinase for ERK; SP600125, a direct inhibitor of JNK; and SB203580, a direct inhibitor of p38 MAPK. The effectiveness and specificity of these inhibitors was confirmed in immunoblot analyses using phospho-specific antibodies against these kinases and/or their downstream phosphorylated targets (data not shown). We investigated the effects of inhibiting MAPKs on the FasL-stimulated expression of various mRNAs encoding proinflammatory mediators and EGFR-binding ligands. The FasL-induced expression of all genes tested was abolished in the presence of all three MAPK inhibitors, suggesting that MAPK activity in general is essential for FasL-induced mRNA expression (Fig. 5). Several FasL-induced mRNAs (e.g. IL-1 $\alpha$ , IL-1 $\beta$ , CCL20/MIP-3 $\alpha$ , and TSLP) were strongly inhibited by each of the three MAPK inhibitors when applied singly (Fig. 5), whereas other FasL-induced mRNAs were dependent on one or two of the MAPK members for their activation (Fig. 5). Taken together, these results suggest that the ERK, JNK, and p38 MAPKs are instrumental in a nonredundant fashion in orchestrating the proinflammatory responses to FasL in RHE. Furthermore, HB-EGF (Fig. 5), AREG, EREG (not shown here), IL-1 $\alpha$ , IL-1 $\beta$ , CCL20/MIP-3 $\alpha$ , TSLP, CXCL8/IL-8, and ICAM-1 (Fig. 5) were identified as FasL-induced mRNAs that displayed a strong dependence on the ERK family of MAPKs.

**Recruitment of EGFR to Mediate the ERK-dependent Inflammatory Responses to FasL**—We further investigated the subset of FasL-induced genes encoding growth factors and inflammatory mediators whose induction by FasL displayed strong dependence on the ERK family of MAPKs. We previously reported that FasL triggers the activation of ERK MAPKs in cultured human keratinocytes through stimulation of the auto-crine secretion of soluble EGFR-binding ligands, one of which we identified as AREG (21). To determine whether EGFR and



**FIGURE 6. Activation of EGFR and MAP kinases in RHE.** RHEs were treated with FasL (FL) as in Fig. 1 in the presence of 30-min pretreatments with either vehicle ( $\text{Me}_2\text{SO}$ , lanes —) or AG1478 (10  $\mu\text{M}$ , lanes AG). The phosphorylated states of EGFR and MAP kinases were assessed using phospho-specific antibodies at the indicated times. Treatment with EGF (lanes E, 100 ng/ml) served as positive control. Immunoblot detections of total ERK1/2 and p38 MAPK were used to monitor for expression levels and equal loading.

its ligands mediate FasL-induced pro-inflammatory responses in RHEs, we first investigated whether FasL treatment would trigger EGFR activation in the reconstructed epidermis in a manner similar to the one observed in cultured keratinocytes. Indeed, RHEs treated with FasL for 2 or 4 h displayed increased phosphorylation of EGFR (Fig. 6, lanes 7 and 11). FasL-induced EGFR phosphorylation was abolished in the presence of AG1478, a small molecule inhibitor of the kinase activity of EGFR (29) (Fig. 6, lanes 8 and 12). FasL also triggered the phosphorylation of p44 ERK1 and p42 ERK2 (Fig. 6, lanes 7 and 11), and this phosphorylation was abrogated by AG1478 (Fig. 6, lanes 8 and 12). FasL stimulated the phosphorylation of JNK and p38 $\alpha$  MAP kinases (Fig. 6, lanes 7 and 11), but in contrast to the phosphorylation of ERK, the phosphorylation of JNK and p38 $\alpha$  was not dependent on EGFR activity (Fig. 6, lanes 8 and 12).

We next employed either AG1478 (at a low dose, 100 nM, to minimize possible “off target” effects; Fig. 7A) or LA1, an EGFR-neutralizing antibody (30–32) (Fig. 7B) to investigate the potential contribution of EGFR to FasL-induced gene expression. Either mode of EGFR inhibition substantially suppressed the FasL-induced activation of HB-EGF, IL-1 $\alpha$ , IL-1 $\beta$ , CCL20/MIP-3 $\alpha$ , CXCL8/IL-8, TSLP, ICAM-1 (Fig. 7), and AREG (not shown) but not that of tumor necrosis factor- $\alpha$  mRNA (Fig. 7). We concluded, therefore, that part of the inflammatory action of FasL was mediated through activation of the EGFR.

**Contribution of Both ERK1 and ERK2 to the FasL-induced Gene Expression**—To assess the relative contributions of ERK1 and ERK2 to the FasL-induced pro-inflammatory gene expression, we developed a technique for siRNA-mediated gene knockdown in RHE preparations (outlined schematically in Fig. 8A). In the optimization phase, it appeared that a sustained siRNA-mediated knockdown of various gene products could be maintained for ~4 days after the switch to organotypic differentiation (Fig. 8A). To assess how early organotypic reconstructions develop epidermal characteristics, we measured barrier function and the expression of differentiation markers. RHEs established proper barrier function by day 3 after the switch to

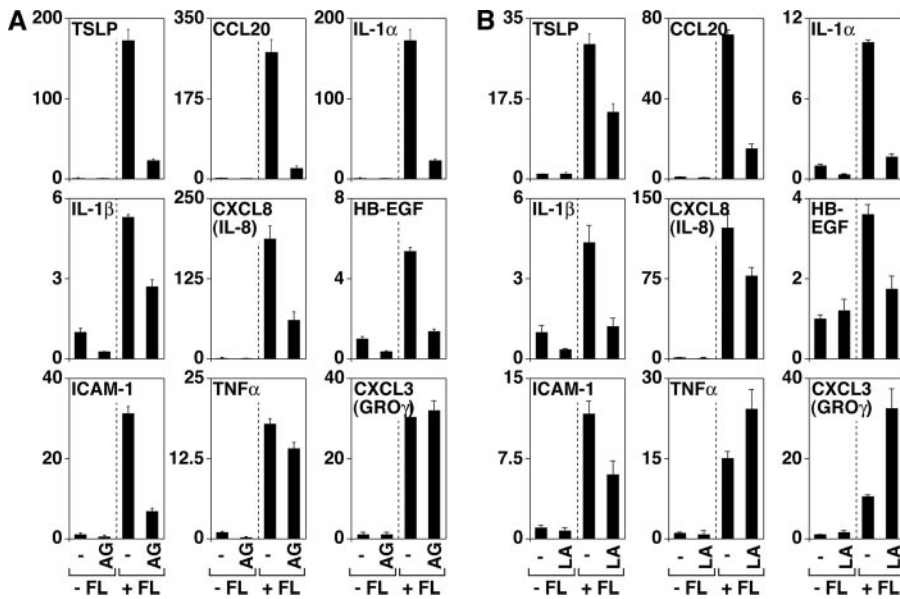


FIGURE 7. Effects of EGFR antagonists on the expression of FasL-induced genes. RHEs were treated with FasL as in Fig. 5 in the presence of 30 min pretreatments with either AG1478 (A, lanes AG, 100 nM) or LA-1 antibody (B, lanes LA, 10  $\mu$ g/ml). qRT-PCR analyses were performed 4 h post FasL. The data are presented as fold change relative to the corresponding vehicle-treated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. FL, FasL.

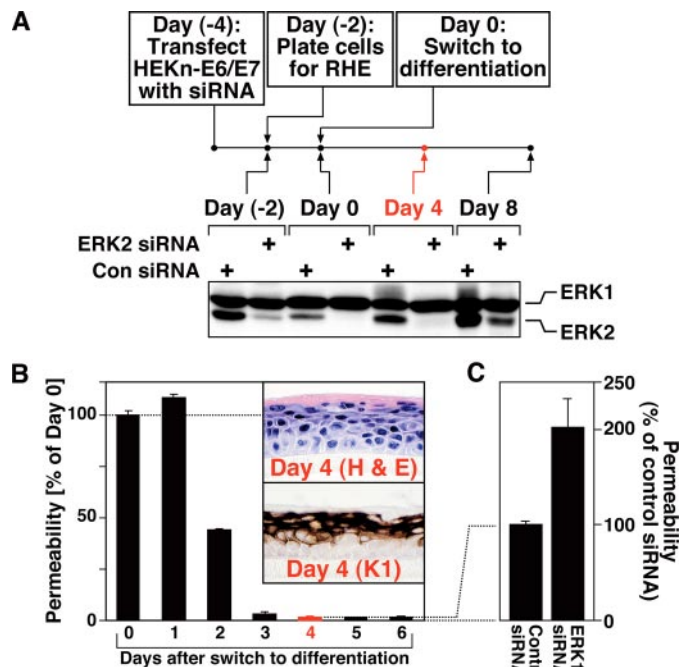


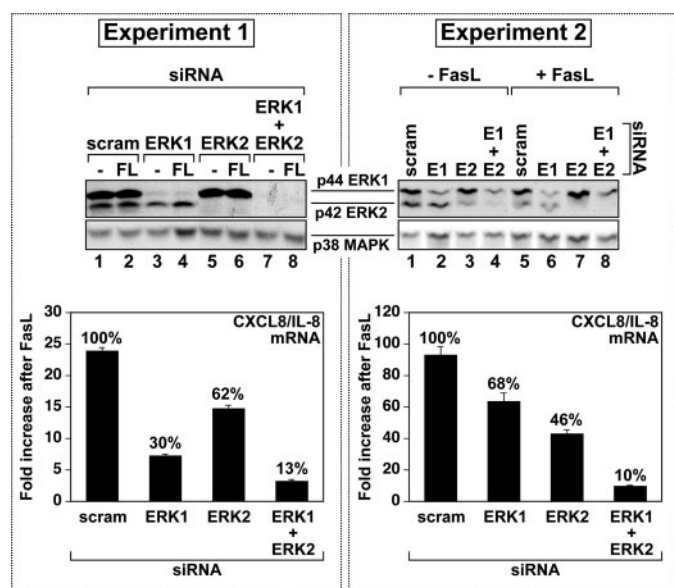
FIGURE 8. A, efficient siRNA-mediated knockdown of ERK2 at day 4 after the switch to differentiation. HEK293-E6/E7 were transfected with the indicated siRNAs, plated, and differentiated into RHEs as depicted in the experimental scheme. Immunoblot analysis with an antibody against ERK1 and ERK2 (pan-ERK antibody). ERK1 serves as a loading and specificity control. B, proper barrier function, morphology, and keratin 1 expression of RHEs at day 4 after the switch to differentiation. "Outside-In" barrier function was assessed by Toluene Blue O dye penetration assay, and it is presented as a percentage of permeability relative to the permeability of the reconstructs immediately after the initiation of differentiation (Day 0). Insets, epidermal morphology (hematoxylin and eosin staining, H & E) and detection of keratin 1 (K1) expression. C, effect of double knockdown of ERK1 and ERK2 on the barrier function. Note that the decrease of barrier function (i.e. increase in permeability) after the knockdown of ERK is small compared with the range of permeability decrease occurring during normal epidermal differentiation (dotted lines connecting B and C).

differentiation (Fig. 8B), and when examined at day 4, they displayed mature epidermal stratification, including the presence of *stratum corneum* and proper expression of keratin 1 (Fig. 8B, inset). For these reasons, the experiments described below were performed using RHEs at day 4 after the initiation of differentiation. RHEs that were subjected to a double knockdown of ERK1 and ERK2 (Fig. 9) showed a pattern of epidermal differentiation that was similar to control RHEs (supplemental Fig. S1). However, the double knockdown cultures exhibited a slightly delayed onset of differentiation, manifested by the decreased thickness of the *stratum corneum* (supplemental Fig. S1), a weaker (but properly stratified) keratin 1 immunoreactivity (supplemental Fig. S1), and a 2-fold decrease in barrier function (Fig. 8C).

Fig. 9 shows the effect of knocking down ERK MAPKs on the FasL-induced expression of CXCL8/IL-8. As expected from the previous findings using UO126 (Fig. 5), reducing the levels of both ERK1 and ERK2 in two independent experiments inhibited the responsiveness of CXCL8/IL-8 to FasL by 87 and 90%, respectively. It appeared that both ERK1 and ERK2 contributed to the activation of CXCL8/IL-8 by FasL, because single knockdowns of either kinase caused only partial inhibition of CXCL8/IL-8 expression (Fig. 9). Similar outcomes were observed for CCL20/MIP-3a, ICAM-1, and TSLP (not shown). The degree of siRNA-mediated effects on FasL-induced gene expression appeared to correlate with the achieved efficiency of knockdown of a targeted protein (compare, for instance, the knockdown of ERK1 in the two experiments presented in Fig. 9). These results strongly support the notion that both ERK1 and ERK2 contribute to the genomic responses to FasL.

## DISCUSSION

*Apoptosis and Inflammation in the Skin*—The studies presented here and recently (12–14, 21, 33) have been directed toward uncovering the possible roles of FasL and apoptosis in human eczema. Eczema is traditionally thought of as disease characterized primarily by inflammatory phenomena. However, apoptosis of keratinocytes is a key hallmark of the acute eczematous epidermis and is thought to play an important role in spongiosis and vesicle formation in dermatitis (12–14, 21, 33). FasL appears to be the most prominent candidate for the mediation of keratinocyte apoptosis in dermatitis (12–14, 21, 33). Neither apoptosis nor spongiosis characterizes the epidermal lesion in psoriasis, another skin disease that is inflammatory, but not eczematous (34). Thus, apoptosis is a defining feature specifically of the eczematous type of tissue inflammation. One important question from a clinical point of view is whether attempts to suppress keratinocyte apoptosis in the epi-



**FIGURE 9.** siRNA-mediated knockdown of ERK1, ERK2, or both, in RHE and the effect of the knockdown on the expression of CXCL8/IL-8. RHEs were transfected in two independent experiments with the indicated siRNAs (scram, “scrambled” control siRNA; for Experiment 2, E1 denotes ERK1, and E2 denotes ERK2) and analyzed at day 4 post the differentiation switch as described in the text and in Fig. 8A. The top panels depict immunoblot detections of ERK1, ERK2, and p38 MAP kinases. p38 MAPK is used as control for siRNA specificity and loading control. The bottom panels depict the expression of CXCL8/IL-8 mRNA 4 h after FasL treatment was determined by qRT-PCR at day 4 after the differentiation switch. The data are presented as fold change relative to the corresponding untreated control RHEs. Error bars, standard deviation from triplicated qRT-PCRs. The percentage of change relative to scrambled siRNA is indicated.

dermis would be beneficial to dermatitis patients. Alternatively, apoptosis could be part of the “healing” process that serves the purpose to restrict uncontrolled inflammation, even at the price of temporary tissue damage. Our findings presented here suggest that therapeutic strategies aimed at epidermal apoptosis suppression at the level of caspase activity may prove detrimental by aggravating inflammation (see below).

**Positive and Negative Regulation of Gene Expression by FasL**—The unbiased approach to gene expression analysis offered by microarray technologies allows for the identification of both positively and negatively regulated genes in experimental systems of interest. Although the main body of work presented here deals with genes that were induced by FasL in RHE, the Affymetrix microarray analysis revealed that a substantial number of RNA transcripts (as high as ~45%, see “Results”) was reduced in abundance 4 h after FasL administration. A minority (12%) of the 287 annotated repressed genes considered in our analysis was affected more than 3-fold. Of the 68 FasL-down-regulated transcripts selected for their high basal expression in untreated RHE (supplemental Table S6), also 12% (*i.e.* the eight transcripts labeled in red in supplemental Table S6) were down-regulated more than 3-fold. The chemokine CXCL14, whose expression was reduced ~26-fold after FasL, may be of particular interest for further investigation. The relatively high levels of CXCL14 mRNA expression in our RHE (1182 arbitrary Affymetrix units, see primary data available online at [www.ohsu.edu/cellbio/faculty/Iordanov\\_data/Farley\\_et\\_al\\_\(2007\).xls](http://www.ohsu.edu/cellbio/faculty/Iordanov_data/Farley_et_al_(2007).xls)) are in agreement with the findings of Moser and co-workers

(35, 36), who reported high constitutive levels of CXCL14 expression in healthy human epidermis and in RHE. Recently, the same research group reported evidence strongly supporting a critical role for cutaneous CXCL14 in targeting blood precursors of dendritic cells to the epidermis for their differentiation as Langerhans cells (36). Because CXCL14 appears to act in a constitutive manner under non-inflammatory conditions, the authors suggested that CXCL14 might play a role in the steady-state functions of dendritic cells such as presentation of self-antigens for the purpose of elimination of self-reacting T cells and/or induction of suppressor/regulatory T cells (36). It is therefore tempting to speculate that FasL affects the genomic responses of epidermal keratinocytes by both inducing pro-inflammatory chemokines (*e.g.* CCL20, CXCL1–3, and CXCL8) and repressing, simultaneously, the expression of a potentially “anti-inflammatory” chemokine, CXCL14.

**Pro-inflammatory Actions of FasL in Human Keratinocytes: Importance of the Organotypic Context**—As described here and in Ref. 19, a single trigger, FasL, reproduces key clinical and molecular hallmarks of the eczematous epidermis in RHE. However, the inflammatory genomic responses to FasL observed in RHEs are in stark contrast to the lack of the same responses under two-dimensional tissue culture conditions. *In vitro* tissue culture has been employed previously to study the behavior of the eczematous keratinocyte. For instance, cultured keratinocytes from patients with atopic dermatitis display abnormal responses to T cell-derived cytokines (37, 38). However, the inappropriate genomic responses of cultured keratinocytes to FasL observed by us underscore the limitations of the tissue culture in studying eczemas. Ironically, the pro-inflammatory potential of FasL in human keratinocytes was initially discovered in the keratinocyte cell line HaCaT (19). We were therefore surprised to observe the complete lack of inflammatory gene expression in FasL-treated primary keratinocyte explants (HEKn) or in their immortalized derivatives, HEKn-E6/E7. However, the same HEKn or HEKn-E6/E7 when grown in an epidermotypic context (RHE) “regained” the ability to respond to FasL with increased expression of inflammatory mediators. As far as we have been able to determine, the main reason for this different behavior is the inability of HEKn or HEKn-E6/E7 in tissue culture to respond to FasL with activation of NF- $\kappa$ B (not shown).<sup>4</sup> In contrast, HaCaT cells in tissue culture potently activate NF- $\kappa$ B in response to FasL (19). Indeed, siRNA-mediated knockdown of the p65/RelA subunit in HaCaT (19) or in HEKn-E6/E7 RHEs (not shown)<sup>4</sup> abrogated the FasL-triggered expression of inflammatory mediators. It should be emphasized that HEKn and HEKn-E6/E7 in tissue culture display normal NF- $\kappa$ B activation in response to cytokines other than FasL (*e.g.* tumor necrosis factor- $\alpha$ , or IL-1 $\beta$ ; not shown).<sup>4</sup> Furthermore, HEKn and HEKn-E6/E7 in tissue culture display an efficient response to FasL as determined by apoptosis (21) or expression of NF- $\kappa$ B-independent genes (such as *c-fos* or *c-jun*; not shown).<sup>4</sup> Therefore, it is reasonable to conclude that HEKn and HEKn-E6/E7 in tissue cul-

<sup>4</sup> S. M. Farley, D. E. Purdy, O. P. Ryabinina, P. Schneider, B. E. Magun, and M. S. Iordanov, manuscript in preparation.



ture are specifically deficient in the proinflammatory genomic responses to FasL and that epidermotypic context (RHE) restores these responses. Because keratinocytes in tissue culture most resemble the undifferentiated transit-amplifying cells in the basal layer of the epidermis, it is tempting to speculate that the cells responding to the pro-inflammatory actions of FasL in RHEs are not the basal keratinocytes but rather keratinocytes within the *strata spinosum* or *granulosum*. Presumably, HaCaT cells differ from HEK<sub>n</sub> and HEK<sub>n</sub>-E6/E7 by having either gained a positive, or having lost a negative, FasL-triggered regulator of NF- $\kappa$ B.

**Possible Anti-inflammatory Functions of Caspases**—Our results using the caspase inhibitors z-IETD-fmk and z-VAD-fmk (Fig. 4) strongly support the notion that caspase activity may have the physiological role of limiting the extent of FasL-triggered inflammation in the skin. Initially, we considered the simplest explanation, namely that caspases limit the inflammatory response by promoting cell death. By means of rapid elimination of FasL-challenged keratinocytes, the extent of transcriptional activation and release of inflammatory mediators by the same cells would be restricted. Although this explanation cannot be ruled out, it does not fully concur with the experimental observations. As Figs. 1 and 2 demonstrate, the peak of inflammatory gene expression occurs within 4 h after the administration of FasL. At this time, the caspase activity, although clearly detectable (Fig. 3), is well below the levels observed at later time points (Fig. 3B). Importantly, at 4 h after FasL, keratinocytes in the RHEs display normal morphological characteristics (Fig. 3B, panels I and II). Nuclear pyknosis, karyorrhexis, and cell swelling were only observable at later times (e.g. 24 h; Fig. 3B, panel VI). We have also reported previously that HaCaT cells, treated with FasL for up to 4 h and displaying  $\geq 75\%$  cleaved poly(ADP-ribose) polymerase, nevertheless maintain high levels of biosynthetic activity, as determined by incorporation of [<sup>3</sup>H]leucine into newly synthesized proteins (19). Taken together, these findings argue against the conclusion that caspases limit FasL-triggered inflammation by simply eliminating inflammatory cytokine-producing cells. We propose an alternative hypothesis, *i.e.* that the initial activation of caspases triggers an active anti-inflammatory program *before* the onset of cell death. Such an early action of caspases would, therefore, define a novel function for these proteases, a function that is kinetically separable from their cytotoxic properties. The anti-inflammatory functions of caspases may be effected, for instance, through suppression of the FasL-triggered activation of NF- $\kappa$ B, a possibility being currently investigated.

One clinically relevant conclusion from these findings might be that attempts to suppress keratinocyte apoptosis by means of caspase inhibition in acute dermatitis may have the unintended consequences of aggravated inflammatory response and, ultimately, a worse outcome for the patient. An important unanswered question that remains is whether the anti-inflammatory signals associated with activated caspases are effected directly by the apical caspases 8 and 10 or further downstream by effector caspases such as caspases 3 and 7. Should the anti-inflammatory functions be specific for the apical caspases only, strategies aimed at inhibiting effector caspases may be beneficial in limiting apoptosis, yet avoiding aggravated inflammation.

**A Novel Role of the EGFR-Ras-ERK Cascade in Mediating Inflammation in the Skin**—We have previously reported that the basal levels of ERK activity in HEK<sub>n</sub> or HEK<sub>n</sub>-E6/E7 in tissue culture were determined by the autocrine secretion of ligands that bind to the EGFR (20). Interfering with either the extracellular ligand-binding portion of EGFR by means of the LA-1 antibody or the intracellular kinase domain of EGFR reduced the basal phosphorylation of ERK1 and ERK2 to undetectable levels (20). We have also reported that FasL-induced ERK activation of HEK<sub>n</sub> or HEK<sub>n</sub>-E6/E7 in culture is mediated by FasL-simulated secretion of EGFR ligands (one of which was identified as amphiregulin) and the subsequent activation of EGFR (21). FasL also activated EGFR in RHEs (Fig. 6), and interfering with the activity of EGFR prevented FasL-triggered activation of ERK (Fig. 6). A subset of FasL-induced inflammatory mediators in RHEs was found to require ERK activity (Fig. 6). Taken together, these findings had allowed us to predict that EGFR would be recruited to mediate some of the inflammatory gene expression in response to FasL in the RHE. This was indeed found to be the case (Fig. 7). However, it should be emphasized that the biological roles of EGFR in human skin are likely to be much more complex than can be appreciated with the available tools for experimentation. For instance, CXCL8/IL-8, an EGFR-dependent, FasL-induced, potent inflammatory mediator, is also a suspected keratinocyte mitogen (39). Bearing in mind that FasL triggers the production of mitogenic EGFR ligands in human keratinocytes (21), it is likely that the physiological outcomes of the presence of FasL in the human epidermis may be determined by the interplay of three seemingly disparate phenomena: apoptosis, inflammation, and mitogenesis (the last two being, at least partially, dependent on EGFR). With the exception of the findings that anti-EGFR-directed therapies of cancer produce cutaneous acne-like rashes as side effects (40), our understanding of the roles of EGFR in the human skin is still very limited.

**The Roles of ERK1 and ERK2 in the Genomic Responses of Keratinocytes to FasL**—We report here that both ERK1 and ERK2 are involved in mediating the proinflammatory gene expression in response to FasL (Fig. 9). A single siRNA-mediated knockdown of either ERK1 or ERK2 reduced FasL-induced CXCL8/IL-8 expression significantly, but a double knockdown appeared necessary to reduce CXCL8/IL-8 expression by more than 85% (Fig. 9). Recently, Vantaggiato *et al.* (41) presented evidence that ERK1 and ERK2 play opposing roles in mediating the proliferative effects of mitogenic growth factors and Ras-driven oncogenic transformation. According to this model, ERK2 mediates the proliferative and transforming effects of activated Ras, whereas ERK1 inhibits these effects by competing with ERK2 for their mutual upstream activator MEK (41, 42). Interestingly, CXCL8/IL-8 expression was recently reported to be a transcriptional target of Ras signaling (43), and based on these findings, our initial expectations were that we would observe opposing roles of ERK1 and ERK2 in mediating the expression of CXCL8/IL-8 in FasL-treated RHEs. The fact that this was not the case (Fig. 9) underscores the complexity of MAPK regulation and function and strongly emphasizes the need to investigate MAPK signaling pathways in the context of specific tissues and organs. In conclusion, the RHE-based orga-

notypic model described here could be applicable to study pharmacologic manipulations of the FasL-driven genomic responses as novel therapeutic options for the treatment of dermatitis and other inflammatory skin conditions.

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