

Fas Ligand Elicits a Caspase-Independent Proinflammatory Response in Human Keratinocytes: Implications for Dermatitis

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Fas ligand (FasL) causes apoptosis of epidermal keratinocytes and triggers the appearance of spongiosis in eczematous dermatitis. We demonstrate here that FasL also aggravates inflammation by triggering the expression of proinflammatory cytokines, chemokines, and adhesion molecules in keratinocytes. In HaCaT cells and in reconstructed human epidermis (RHE), FasL triggered a NF- κ B-dependent mRNA accumulation of inflammatory cytokines (tumor necrosis factor- α , IL-6, and IL-1 β), chemokines (CCL2/MCP-1, CXCL1/GRO α , CXCL3/GRO γ , and CXCL8/IL-8), and the adhesion molecule ICAM-1. Oligomerization of Fas was required both for apoptosis and for gene expression. Inhibition of caspase activity abolished FasL-dependent apoptosis; however, it failed to suppress the expression of FasL-induced genes. Additionally, in the presence of caspase inhibitors, but not in their absence, FasL triggered the accumulation of CCL5/RANTES (regulated on activation normal T cell expressed and secreted) mRNA. Our findings identify a novel proinflammatory role of FasL in keratinocytes that is independent of caspase activity and is separable from apoptosis. Thus, in addition to causing spongiosis, FasL may play a direct role in triggering and/or sustaining inflammation in eczemas.

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INTRODUCTION

Apoptosis (Kerr *et al.*, 1972), the principal mechanism for elimination of damaged cells in metazoan organisms (Edinger and Thompson, 2004), is initiated via two pathways, extrinsic and intrinsic (reviewed in Hengartner, 2000; Krammer, 2000; Meier *et al.*, 2000; Nicholson, 2000; Rich *et al.*, 2000; Savill and Fadok, 2000; Yuan and Yankner, 2000). In the extrinsic pathway, a specialized death ligand (such as FasL) binds to a death receptor (e.g., Fas) (reviewed in Locksley *et al.*, 2001). This event triggers the formation of a death-inducing signaling complex (Kischkel *et al.*, 1995) containing FasL, Fas, the adaptor protein FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995), and procaspase 8 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Within the death-inducing signaling complex, the

increased local concentration of procaspase 8 allows for its spontaneous autocatalytic cleavage and activation by “induced proximity” (Muzio *et al.*, 1998). “Initiator” caspases (such as caspase 8) activate proteolytically “effector” caspases (such as caspases 3, 6, and 7) (reviewed in Cohen, 1997; Shi, 2002). Once activated by the initiator caspases, effector caspases dismantle the apoptotic cell by systematically cleaving more than 100 cytoplasmic and nuclear substrates, thus interfering with virtually every cellular function.

FasL belongs to the tumor necrosis factor (TNF) family of cytokines that associate as homotrimers and less frequently as heterotrimers to bind their cognate receptors (Bodmer *et al.*, 2002). Receptors of the TNF family are activated by ligand-mediated oligomerization (Bodmer *et al.*, 2002). FasL signals cell death predominantly as a transmembrane protein by engagement of its receptor, Fas, on the surface of target cells (Suda *et al.*, 1997; Krammer, 2000). FasL plays an important role in the effector function of cytotoxic T lymphocytes and also regulates their homeostasis (Krammer, 2000). Genetic mutations that inactivate either FasL or Fas are associated with autoimmune lymphoproliferative syndrome, a hereditary condition characterized by the accumulation of atypical lymphocytes and by the development of autoimmune manifestations (Straus *et al.*, 1999). Membrane-bound FasL may be processed to a soluble form (sFasL) and shed by the action of a metalloprotease (Tanaka *et al.*, 1998). The processed sFasL does not display apoptotic activity and can even inhibit the action of membrane-bound FasL (Suda *et al.*, 1997; Schneider *et al.*, 1998; Tanaka *et al.*, 1998).

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Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; EGF(R), epidermal growth factor (receptor); FasL, Fas ligand; JNK, cjun amino terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP) ribose polymerase; RANTES, regulated on activation normal T-cell expressed and secreted; RHE, reconstructed human epidermis; RT-PCR, reverse transcriptase-PCR; sFasL, soluble form FasL; TGF, transforming growth factor; TNF, tumor necrosis factor

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Interestingly, crosslinking of sFasL restores its proapoptotic activity (Schneider *et al.*, 1998). Recently, Holler *et al.* (2003) explained the inability of trimeric sFasL to trigger apoptosis by demonstrating that a hexamer of FasL, consisting of two trimers held in close proximity, represents the minimal ligand structure required to signal apoptosis.

The FasL/Fas system has been found to exert functions different from apoptosis in certain cellular contexts. Engagement of Fas in immature dendritic cells triggered their maturation and expression of proinflammatory cytokines in the absence of apoptosis (Rescigno *et al.*, 2000). In an *in vivo* mouse model of acute lung injury triggered by intranasal instillation of Fas-activating antibody, Matute-Bello *et al.* (2001) reported the presence of both apoptosis and increased inflammatory gene expression in alveolar epithelial cells.

Over the past decade, several laboratories have begun to elucidate the importance of FasL/Fas in skin homeostasis, carcinogenesis, and inflammatory skin diseases. Epidermal keratinocytes express Fas, but not FasL (Viard *et al.*, 1998). 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 (Viard *et al.*, 1998). In addition, FasL/Fas signaling was implicated in acute cutaneous graft-versus-host disease (Langley *et al.*, 1996). Squamous cell carcinomas were found to have decreased expression of Fas, increased expression of its inhibitor cFLIP, and increased expression of FasL, all suggesting that this type of skin cancer may employ the FasL/Fas system to evade immune surveillance and tumor lysis (Bachmann *et al.*, 2001).

More recently, FasL was found to be involved in the pathogenesis of eczematous dermatitides (such as atopic dermatitis (AD) and allergic contact dermatitis (ACD)) (Schwarz, 2000; Trautmann *et al.*, 2000, 2001; Klunker *et al.*, 2003). 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 FasL, delivered to the epidermis by infiltrating T lymphocytes and acting on Fas whose expression on the surface of keratinocytes is induced by T lymphocyte-derived interferon- γ (Trautmann *et al.*, 2000).

These findings clearly demonstrated the important role of FasL in the epidermal destruction in inflammatory skin diseases. However, whether FasL is directly involved in the inflammatory process is not known. We demonstrate here that FasL elicits a proinflammatory reaction in human keratinocytes by triggering the expression of stress-responsive transcription factors, inflammatory cytokines, chemokines, and the adhesion molecule ICAM-1. Furthermore, we demonstrate that oligomerization of Fas is required both for apoptosis and for the inflammatory gene expression, and that

caspace activity is essential for apoptosis, but dispensable for the inflammatory gene expression. Our results suggest that, in specific tissue and cellular contexts, FasL may behave as a *bona fide* proinflammatory cytokine.

RESULTS

Identification of FasL-activated genes

To identify genes activated by FasL in keratinocytes, we performed Affymetrix microarray analyses on RNA extracted from HaCaT cells 2 hours after exposure to Fc:FasL. Analyses were performed on HG-U133 Plus 2.0 GeneChip arrays, which interrogate over 47,000 human transcripts. Genes whose mRNAs were consistently absent from both samples (control and Fc:FasL-treated) were excluded from the analysis, reducing the number of evaluated genes to 1146. Of these, 166 genes were identified, whose mRNA abundance was increased ≥ 2 -fold after Fc:FasL. The microarray analysis in Excel format is available upon request. The data from the microarrays were analyzed by EASE, a software application for the rapid interpretation of biological data obtained from microarray analysis (Hosack *et al.*, 2003). The EASE software automates the process of biological theme determination by analyzing the over-representation of genes that belong to categories that are functionally and structurally defined. Table S2 displays the hierarchical analysis of the genes whose mRNA abundance was increased ≥ 2 -fold after Fc:FasL. These data reveal that the most highly over-represented genes activated after Fc:FasL treatment of HaCaT cells were associated with inflammatory, stress, and wound responses. The molecular functions of these induced genes were identified as cytokines, chemokines, growth factors, and regulators of transcription (Tables S2 and S3). Among the mRNAs increased in abundance are several that encode immediate-early transcriptional regulators (cJun, cFos, ATF3, and Egr1; Table S3 and Figures 2a, 4a, and 5), whose expression has been tied to ligands that activate ERK, p38 mitogen-activated protein kinase (MAPK), and cJun amino terminal kinase (JNK) (Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996, 2001). Several of these transcriptional regulators have been shown to bind to regulatory sequences of genes encoding cytokines and chemokines. Indeed, the microarray analyses and their subsequent validation by real time RT-PCR (see below) identified the inflammatory cytokines TNF- α , IL-6, IL-1 α , and β , and the chemokines CCL2/MCP-1, CXCL1/GRO α , CXCL3/GRO γ , and CXCL8/IL-8 as genes whose mRNA abundance was increased dramatically in Fc:FasL-treated keratinocytes.

To identify similarities between the pattern of Fc:FasL-induced mRNA accumulation and the patterns triggered by other stimuli, we employed the L2L database maintained by the University of Washington (Seattle, WA; <http://depts.washington.edu/l2l/>). L2L is a database of published microarray gene expression data, and a software tool for comparing that published data to a user's own microarray results. Our Affymetrix data displayed the highest degree of similarity with the program of gene expression stimulated by TNF- α in HeLa cells (Zhou *et al.*, 2003). Specifically, 15 actual matches between the two lists were identified, with 0.13 matches

expected by random coincidence (118-fold enrichment, $4.64e-28$ binomial P -value). Among the overlapping genes were those encoding TNF- α -induced proteins 2 and 3, Egr1, IL-6, cyclooxygenase 2, JunB, and CCL2/MCP-1. These results strongly suggest that, in HaCaT cells, FasL behaves as a *bona fide* inflammatory mediator similar to TNF- α .

Validation of microarray data by real time RT-PCR

To validate the Affymetrix microarray data and gain a better understanding of the temporal behavior of Fc:FasL-induced accumulation of specific gene transcripts, we treated HaCaT cells with Fc:FasL and then investigated, using real time RT-PCR, the levels of specific mRNAs at 0.5, 1, 2, and 4 hours after the treatment. The results shown in Table S4 and Figures 1a and 2 are representative of at least six highly reproducible repetitions of the same experiment. Typically, treatment of the cells with Fc:FasL (250 ng/ml) resulted in progressive apoptosis that, assessed morphologically (not shown) and by the cleavage of poly(ADP)ribose polymerase (PARP), a well-characterized substrate of effector caspases, affected $\geq 50\%$ of the cells by the fourth hour after treatment (Figures 1c, d, 6 and 7). We therefore restricted our analysis of mRNA detection to time points not later than 4 hours after Fc:FasL, as at later times massive death and detachment of cells occurred. Another rationale to study the mRNA accumulation within the described time frame was that between 0.5 and 4 hours after the treatment the majority of the MAP kinase (ERK, JNK, and p38 MAPK) activation occurred (Figure 1c). The group of transcripts increasing in abundance in response to Fc:FasL and encoding immediate-early transcription factors (cJun, cFos, ATF3, and Egr1) is presented in Table S4 and Figure 2a. Table S4 and Figure 2b demonstrate the analysis of Fc:FasL-induced accumulation of mRNAs encoding inflammatory mediators such as the cytokines TNF- α , IL-6, IL-1 β , and the chemokines CCL2/MCP-1, CXCL1/GRO α , CXCL3/GRO γ , and CXCL8/IL-8. All these mRNAs were induced to accumulate manifold in response to Fc:FasL. In addition to these inflammatory and chemotactic ligands, we detected a more than 10-fold (at 2 hours) increase in the levels of the mRNA for ICAM-1 (Table S4 and Figure 2b). We were intrigued to find that the mRNA for CCL5/RANTES (regulated on activation normal T cell expressed and secreted) was consistently unchanged in response to Fc:FasL treatment (Table S4 and Figure 9). As addressed later experimentally, it appeared that Fc:FasL-triggered apoptosis created conditions nonpermissive for the expression of this chemokine (Figure 9). Finally, we identified an important novel group of Fc:FasL targeted mRNAs, namely those encoding four epidermal growth factor receptor (EGFR) ligands: amphiregulin, epirigulin, heparin-binding EGF, and transforming growth factor- α (TGF- α) (Table S4 and Figure 2c). Interestingly, the levels of mRNA encoding EGF were not increased in response to Fc:FasL (Table S4), despite the ability of HaCaT cells to upregulate EGF mRNA expression in response to other stimuli (such as EGF itself; not shown).

Since both the EASE and the L2L database analyses suggested strongly that Fc:FasL triggered a proinflammatory gene expression program in HaCaT cells, we interrogated, by

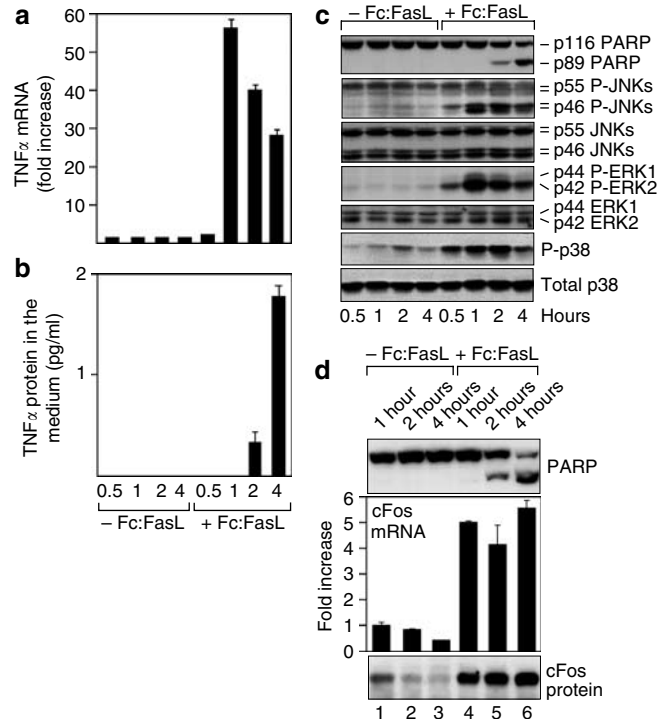


Figure 1. Time course of Fc:FasL-dependent apoptosis and signaling. HaCaT cells were plated and grown in BKM+exoGF. The cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 2 hours before treatments. The cells were then treated, for the indicated times and in duplicated plates, with Fc:FasL (250 ng/ml). (a) One plate of each duplicate was harvested for RNA analysis and (c) the other was harvested for immunoblot analyses. (b) The medium from both plates was combined and used for TNF- α protein detection by ELISA. Apoptosis was assessed by determining the cleavage of PARP. The phosphorylation states of JNK, ERK, and p38 MAPK were assessed using phosphoepitope-specific antibodies and the levels of the respective total proteins were determined using antibodies that are independent of the phosphorylation status. TNF- α mRNA levels were assessed by real-time RT-PCR. Error bars represent standard deviation from (a) triplicated RT-PCR reactions or (b) ELISA wells. (d) Apoptosis and cFos expression in Fc:FasL-treated HaCaT cells. Cells were treated exactly as in (a-c), except that the 0.5 hours time-point was omitted. One plate of each duplicate was harvested for RNA analysis and the other was harvested for immunoblot analyses. Apoptosis was assessed by the cleavage of PARP. cFos expression was determined at the levels of both mRNA and protein abundance.

real-time RT-PCR, the gene expression profiles of HaCaT cells to TNF- α and IL-1 β , two *bona fide* proinflammatory cytokines, and compared these profiles to the response of HaCaT cells to Fc:FasL. The results of these analyses are summarized in Figure 2. We observed that all the genes upregulated by Fc:FasL treatment were also strongly responsive to TNF- α and IL-1 β . As a rule, however, both TNF- α and IL-1 β displayed a more rapid attenuation of the induced mRNA accumulation over time than the attenuation of the signal observed after Fc:FasL. Typically, most TNF- α - or IL-1 β -induced mRNAs were downregulated at 4 hours after treatment, whereas several Fc:FasL-induced mRNAs were at their maximum values at 4 hours post-treatment (Figure 2, see, for instance, the examples of cJun, IL-1 β , and CXCL8/IL-8 mRNAs).

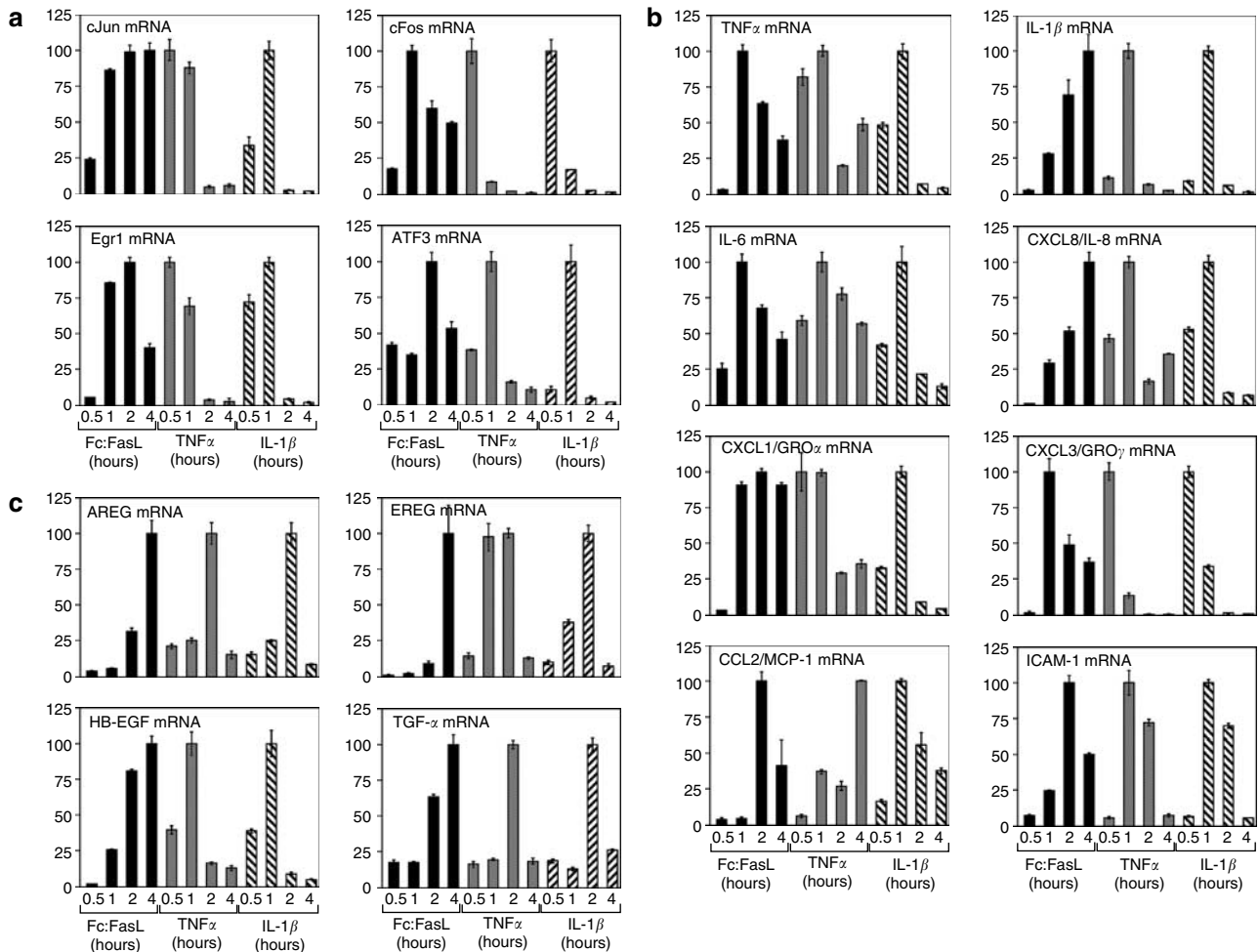


Figure 2. Time courses of Fc:FasL-, TNF α -, and IL-1 β -dependent selective mRNA accumulation. Total RNA from the same experiment shown in Figure 1 (Fc:FasL) or from TNF α - or IL-1 β -treated (20 or 5 ng/ml, respectively) HaCaT cells was interrogated in real-time RT-PCR analyses for the steady-state levels of indicated mRNAs. The relative increase in mRNA accumulation is represented as *per cent* of maximum achieved accumulation within each treatment group (Fc:FasL, TNF α , or IL-1 β). This allows us to compare kinetics of activation for each treatment irrespective of the differences in maximum fold activation observed within each treatment group. The levels of each Fc:FasL-induced mRNA expressed as *fold increase* are shown in Table S4. Error bars represent standard deviation from triplicated RT-PCR reactions. (a) mRNAs for transcription factors. (b) mRNAs for cytokines, chemokines, and ICAM-1. (c) mRNAs for EGFR ligands.

To investigate whether the increased accumulation of Fc:FasL-induced mRNAs was mediated by Fas, the *bona fide* receptor for FasL, we employed ZB4, an Fas-neutralizing antibody. Indeed, ZB4 abrogated not only the apoptotic response of HaCaT cells to Fc:FasL but also the accumulation of all Fc:FasL-regulated transcripts described in Figures 1a and 2 (not shown, but see Figure 5 below). In summary, based on the Affymetrix microarray analysis, the EASE and L2L comparisons, and the real-time RT-PCR data, we concluded that Fc:FasL triggers a specific program of proinflammatory and stress-responsive mRNA accumulation in HaCaT cells.

Dependence of the FasL-activated inflammatory genes on NF- κ B

To investigate whether the inflammatory gene expression triggered by Fc:FasL was dependent on the activity of NF- κ B, we first examined whether Fc:FasL activated NF- κ B in HaCaT

cells. To this end, we investigated, by immunocytochemistry, the translocation of NF- κ B from the cytoplasm to the nucleus in response to either TNF α , a paradigmatic NF- κ B activator, or Fc:FasL. As expected, TNF α triggered a strong nuclear translocation of NF- κ B at 0.5 hours after addition and this translocation was largely reversed at 1 hour after stimulation (Figure 3a). Fc:FasL triggered a somewhat less-intense, but clearly detectable, nuclear translocation of NF- κ B at 0.5 hours after addition; however, unlike TNF α , the nuclear localization of NF- κ B persisted and was even stronger at 1 hour after stimulation (Figure 3a). These results strongly suggest that Fc:FasL is capable of activating NF- κ B in HaCaT cells.

To address directly the question of whether the inflammatory gene expression triggered by Fc:FasL was dependent on NF- κ B activation, we undertook to “knockdown”, by means of RNA interference (RNAi), the p65/RelA subunit of NF- κ B in HaCaT cells. Figure 3b shows that p65/RelA-directed

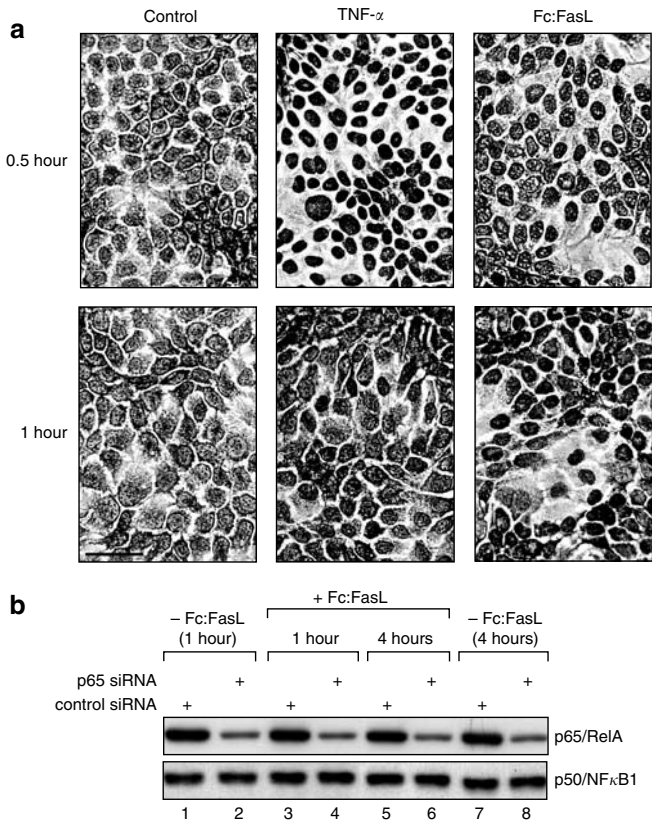


Figure 3. Activation of NF-κB by Fc:FasL and p65/RelA knockdown. (a) HaCaT cells were plated and prepared for treatments as in Figure 1. The cells were treated for the indicated times with either TNF-α (20 ng/ml) or Fc:FasL (250 ng/ml). Immunocytochemical detection of p65/RelA. Bar = 50 μm. (b) HaCaT cells were transfected in duplicates with either p65/RelA-directed siRNA or control (scrambled) siRNA as described in Materials and Methods. Cells were then treated as in Figure 1. One plate of each duplicate was harvested for RNA analysis (Figure 4, below) and the other was harvested for immunoblot analyses presented here.

small-interfering (double-stranded) RNA (siRNA), but not a control (scrambled) siRNA, specifically reduced the steady-state levels of p65/RelA protein, while not affecting the levels of its dimerization partner, p50/NF-κB1. By real-time RT-PCR, we observed, in the same experiment, highly specific effects of the p65/RelA “knockdown” on Fc:FasL-induced mRNA accumulation (Figure 4). Some of the interrogated mRNAs (e.g., the ones encoding cFos, Egr1, amphiregulin, and TGF-α) displayed independence of NF-κB (Figure 4a and c). In contrast, all interrogated mRNAs for inflammatory mediators displayed varied, but substantial, degrees of NF-κB dependence, the most strongly affected being the mRNA for ICAM-1; the accumulation of ICAM-1 mRNA in response to Fc:FasL was practically abolished in the presence of p65/RelA siRNA (Figure 4b).

Direct and secondary effects of Fc:FasL on the levels of selected mRNAs

It was conceivable that some of the mRNAs that were elevated in response to Fc:FasL treatment responded indirectly to Fc:FasL through the production and/or release of

products of earlier Fc:FasL-responding genes (e.g. cytokines). To address this possibility, we employed an approach represented schematically in Figure S1. We reasoned that conditioned medium from Fc:FasL-treated “donor” cells would contain both Fc:FasL as well as, potentially, a mixture of other cytokines secreted into the medium by the “donor” cells in response to Fc:FasL. When given to naïve (“acceptor”) cells, this conditioned medium would elicit responses in these cells that would be attributable to the actions of both Fc:FasL and other secreted cytokines. If we could eliminate the activity of Fc:FasL from the conditioned medium, we would be able to observe the “secondary” effects of the cytokines released to the medium in response to Fc:FasL by the “donor” cells. ZB4, the Fas-blocking monoclonal antibody, offers such an opportunity. The outcomes of these experiments are presented in Figure 5a and b. First, we investigated the group of four genes encoding transcription factors (cJun, cFos, ATF3, and Egr1). Pretreatment of “acceptor” cells with ZB4 abolished the accumulation of the mRNAs of these transcription factors in response to conditioned medium from Fc:FasL-treated “donor” cells, suggesting that Fc:FasL was the primary inducer to which these mRNAs responded (Figure 5a). We next analyzed the effects of ZB4 on the behavior of selected cytokines and chemokines. ZB4 abolished the accumulation of the mRNA for IL-6, suggesting that, like the transcription factors, this mRNA responded exclusively to Fc:FasL (Figure 5b). However, several other mRNAs (the one for CXCL8/IL-8 being the most pronounced example) were substantially, but not completely, suppressed in the presence of ZB4 (Figure 5b). We concluded, therefore, that the mRNAs for some inflammatory mediators (e.g. IL-6) responded exclusively to Fc:FasL, whereas others (e.g. CXCL8/IL-8) displayed some ability to be activated by “secondary” cytokines, presumably released into the medium in response to Fc:FasL.

Apoptosis, mRNA accumulation, and protein synthesis

One potential caveat in the interpretation of the data in Figures 1a, 2, and 5 is that the Fc:FasL-induced mRNA accumulation of selected genes may not be translated productively into changes in the levels of the respective proteins encoded by these mRNAs as a result of inhibition of protein synthesis in apoptotic cells. We addressed this possibility in three ways. First, we determined the rates of protein synthesis in Fc:FasL-treated HaCaT cells by measuring the rates of [³H]-leucine incorporation at various times after the treatment and at various concentrations of Fc:FasL (125, 250, and 500 ng/ml). The degree of apoptosis was assessed by the cleavage of PARP in identically treated parallel plates. Figure 6 shows that, at any concentration of Fc:FasL, massive PARP cleavage was detected at 2 and 4 hours, but not at 1 hour. The levels of [³H]leucine incorporation also displayed a dose-dependent decrease between 2 and 4 hours, but not substantially at 1 hour (Figure 6). Importantly, however, even at 4 hours after the treatment with the two highest doses, when PARP cleavage was estimated to be between 70 and 80% (Figure 6, see immunoblot and graph) and the majority of the cells displayed blebbing and other signs of apoptotic

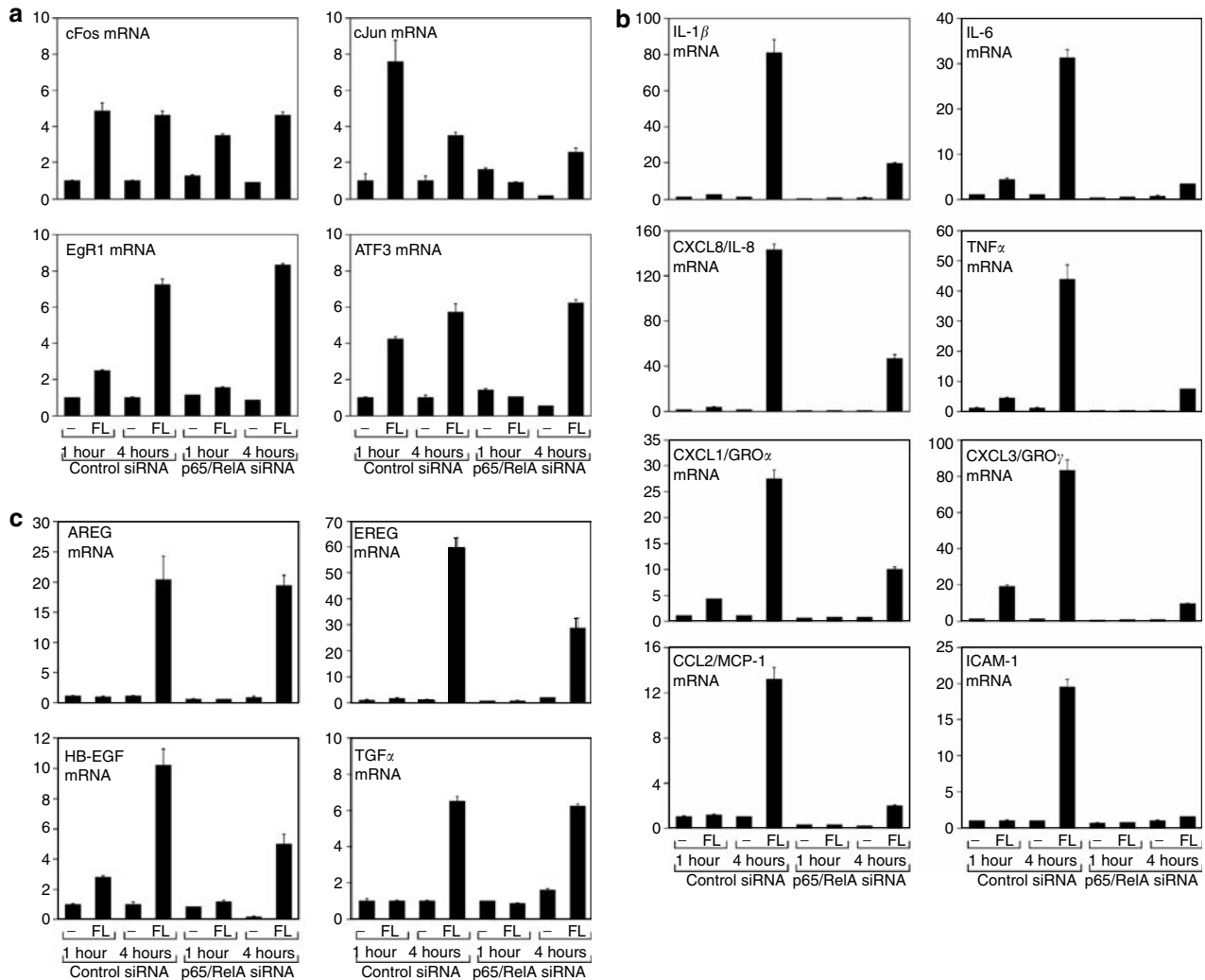


Figure 4. Effects of p65/RelA knock-down on Fc:FasL-induced mRNA accumulation. HaCaT cells were transfected in duplicates with either p65/RelA-directed siRNA or control (scrambled) siRNA as described in Materials and Methods. Cells were then treated as in Figure 1. One plate of each duplicate was harvested for immunoblot analyses (Figure 3, above) and the other was harvested for real time RT-PCR RNA analyses presented here. (a) mRNAs for transcription factors. (b) mRNAs for cytokines, chemokines, and ICAM-1. (c) mRNAs for EGFR ligands. FL, Fc:FasL.

morphology (not shown), the levels of [³H]leucine incorporation were maintained within 55–65% of the control levels (Figure 6). This suggested that substantial levels of protein synthesis were available to translate mRNAs in Fc:FasL-treated cells. Second, we directly assessed the levels of cFos protein in Fc:FasL-treated HaCaT cells. Figure 1d demonstrates that both cFos mRNA and protein levels were increased 1 hour after the treatment. At this time, PARP cleavage was not yet detectable (Figure 1d). Importantly, the levels of both c-Fos mRNA and protein remained high at 2 and 4 hours after the treatment, despite the progressively increasing cleavage of PARP (Figure 1d). Finally, we determined the levels of TNF- α protein secretion into the medium of Fc:FasL-treated cells. Figure 1b demonstrates that detectable levels of secreted TNF- α protein were found 2 and 4 hours after Fc:FasL administration. Taken together, these results strongly argue that apoptosis does not prevent at least some of the increased mRNAs from being efficiently translated.

Limited oligomerization of FasL is a prerequisite for FasL-dependent inflammatory mRNA accumulation

Recent findings suggest that Fas-dependent apoptosis requires the close proximity of two trimeric Fas ligands (Holler *et al.*, 2003). We asked whether such limited oligomerization of FasL was also required to trigger the increased accumulation of the mRNAs for inflammatory mediators. To address this question, we inserted a PreScission™ protease cleavage site in the linker region of Fc:FasL, generating a cleavable version of Fc:FasL designated Fc:PS:FasL (Figure S2a). Cleavage of the Fc:PS:FasL protein with PreScission™ protease disrupts the hexameric structure of the Fc:PS:FasL and results in the release of a trimeric FasL protein very similar to the naturally occurring sFasL (Figure S2a–c). When a Fc:PS:FasL preparation that was not subjected to cleavage with PreScission™ protease was given to HaCaT cells, the cells displayed time-dependent apoptosis (see PARP panel, Figure 7) and activation of MAP kinases (Figure 7), although the activity

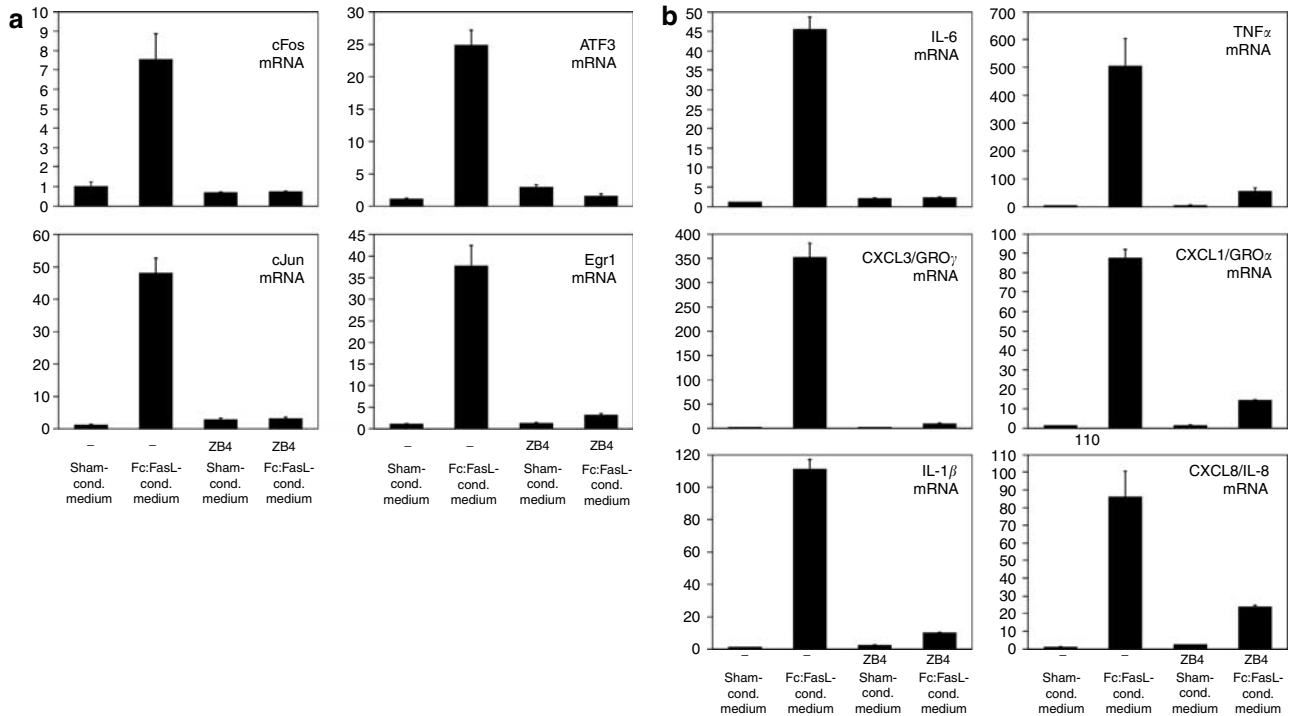


Figure 5. Direct and secondary effects of Fc:FasL on the levels of selected mRNAs. “Donor” HaCaT cells were plated and grown in BKM + exoGF. The cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 2 hours before treatments. The cells were then left untreated (control cells) or were treated with Fc:FasL (250 ng/ml) for 4 hours. Conditioned medium from the “donor” cells was collected and cleared by centrifugation to remove dead cells. Conditioned medium from the untreated cells was designated “sham-conditioned medium”. Conditioned medium from the Fc:FasL-treated cells was re-supplemented with fresh Fc:FasL (250 ng/ml), so that the concentration of Fc:FasL to which the “acceptor” cells (see below) would be exposed was at least as high as the one experienced by the “donor” cells. The “donor” cells, which have produced the conditioned media, were discarded. Naïve (“acceptor”) HaCaT cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 1.5 hours. Then, where indicated, the ZB4 Fas-blocking antibody (2 μ g/ml) was given to the cells for 0.5 hours. Finally, the medium of the “acceptor” cells was exchanged, as indicated, with either one or the other conditioned media for 2 hours. 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. Total “acceptor” cell RNA was interrogated in real-time RT-PCR analyses for the steady-state levels of indicated mRNAs. The levels of each mRNA are expressed as fold increase relative to the basal levels of expression in the sham-conditioned medium-treated “acceptor” cells. Error bars represent standard deviation from triplicated RT-PCR reactions. (a) mRNAs for selected transcription factors. (b) mRNAs for selected cytokines and chemokines.

of Fc:PS:FasL was reduced compared to the parental Fc:FasL. Cleavage of Fc:PS:FasL with PreScission™ protease abolished both apoptosis and MAP kinase activation (Figure 7). However, identical treatment of Fc:FasL with PreScission™ protease did not affect the ability of Fc:FasL to trigger apoptosis or to induce the activation of MAP kinases (Figure 7). Similarly, cleavage of Fc:PS:FasL with PreScission™ protease abolished completely the ability of Fc:PS:FasL to trigger the accumulation of all Fc:FasL-regulated mRNAs (exemplified here by three mRNAs, TNF- α , CXCL1/GRO α , and IL-6; Figure 8). In contrast, PreScission™ protease-treated Fc:FasL protein retained its ability to trigger the accumulation of Fc:FasL-regulated mRNAs (Figure 8). We concluded, therefore, that limited oligomerization of FasL is a prerequisite for FasL-dependent inflammatory mRNA accumulation.

Caspase activity is not required for FasL-dependent gene expression

We next examined whether caspase activity is essential for the accumulation of Fc:FasL-regulated mRNAs. As expected, pretreatment of HaCaT cells with either caspase 8 inhibitor

zIETDfmk or pancaspase inhibitor zVADfmk, blocked Fc:FasL-dependent cleavage of PARP (Figure 9a, a representative result using zIETDfmk), indicating efficient inhibition of caspase activity by these agents. Under these conditions of caspase inhibition, we observed that all proinflammatory mRNAs (cytokines, chemokines, ICAM-1, and transcription factors) presented in Figures 1a, d, and 2 were induced to accumulate by Fc:FasL to levels very similar to those observed in the absence of caspase inhibitors (Figure 9b, top panel; TNF- α , a representative mRNA is shown). Surprisingly, the mRNA for CCL5/RANTES (which was not induced to accumulate by Fc:FasL in the absence of caspase inhibitors; see above and Figure 9b, bottom panel) was dramatically increased in abundance in Fc:FasL-treated cells in the presence of either zVADfmk or zIETDfmk (Figure 9b, bottom panel). These results not only demonstrated that caspase activity is dispensable for the proinflammatory gene expression program triggered by Fc:FasL, but also strongly suggested that caspase activity may be suppressive for some aspects of the pro-inflammatory action of Fc:FasL in keratinocytes.

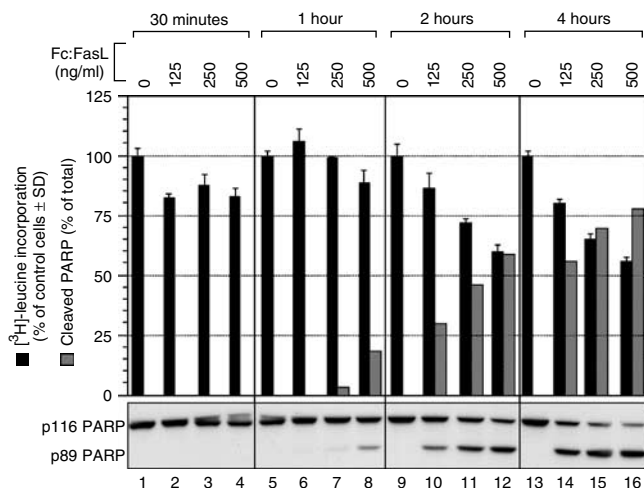


Figure 6. Apoptosis and protein synthesis in Fc:FasL-treated HaCaT cells. Cells were plated in 12-well tissue culture dishes 24 hours before treatments in BKM + exoGF. The cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 2 hours before treatments. The cells were then treated, in triplicated wells, with Fc:FasL (250 ng/ml). The cells were pulse-labeled with 5 μ Ci of [3 H]leucine in 0.3 ml of BKM-exoGF for the last 15 minutes before harvesting. At the desired times (0.5, 1, 2, and 4 hours after Fc:FasL), the incorporation of [3 H]leucine was stopped by the addition of equal volume of 10% TCA. Cells (both adherent and detached) were washed 3 \times with 5% TCA, followed by solubilization of the TCA-insoluble proteins in 88% formic acid. The combined samples (containing both adherent and detached cells, solubilized in formic acid) were counted in a scintillation counter. Error bars represent standard deviation from experimental point in triplicates. Cells from identically treated wells (without [3 H]leucine labeling) were harvested for immunoblot detection of PARP cleavage. Quantification of PARP cleavage was performed as described in Materials and Methods.

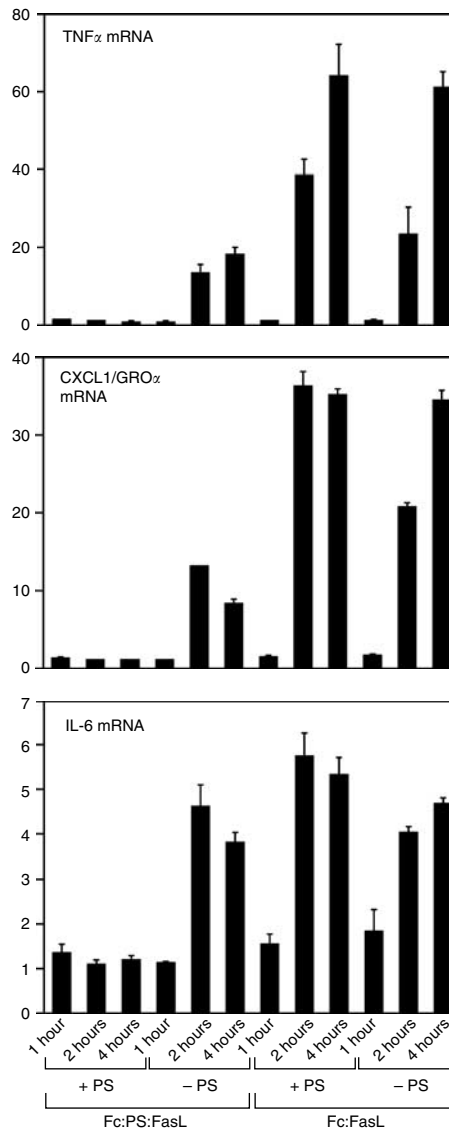


Figure 8. Limited oligomerization of FasL is a prerequisite for FasL-dependent inflammatory mRNA accumulation. HaCaT cells were treated exactly as, and in parallel to, the cells in Figure 7. Total RNA was interrogated in real-time RT-PCR analyses for the steady-state levels of indicated mRNAs. The levels of each mRNA are expressed as fold increase relative to the corresponding control (i.e. untreated) cells. Error bars represent standard deviation from triplicated RT-PCR reactions.

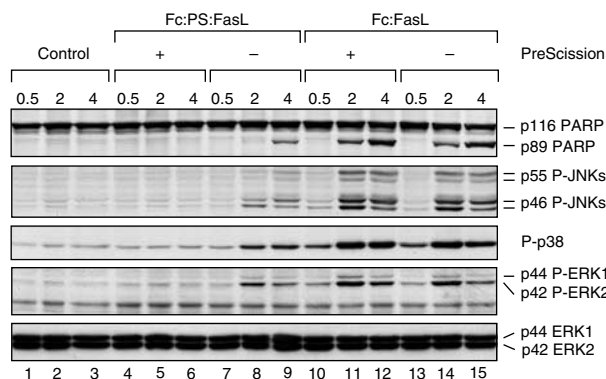


Figure 7. Limited oligomerization of FasL is a prerequisite for FasL-dependent MAP kinase activation. HaCaT cells were treated exactly as in Figures 1 and 2 (except that the treatments consisted, as indicated, of either Fc:PS:FasL or Fc:FasL, treated or not, as indicated, with PreScission protease). One plate of each duplicate was harvested for immunoblot analyses and the other was harvested for RNA analysis (see Figure 8). Apoptosis was assessed by determining the cleavage of PARP. The phosphorylation states of JNK, ERK, p38 MAPK were assessed using phosphoepitope-specific antibodies and the levels of total ERK proteins (loading control) were determined using antibodies that are independent of the phosphorylation status.

Validation of the pro-inflammatory action of Fc:FasL using reconstructed human epidermis

To gain more confidence that the results described so far are physiologically relevant and are not characteristic of a single (HaCaT) cell line, we employed reconstructed human epidermis (RHE) using the HEKn-E6/E7 keratinocytes established in our laboratory (Iordanov *et al.*, 2002, 2005). Under standard protocol conditions for RHE (Poumay *et al.*, 2004), HEKn-E6/E7 keratinocytes form a morphologically normal epidermis (including a pronounced *stratum corneum*) and proper spatial distribution of epidermal marker proteins (keratin 5, keratin 1, and filaggrin; Figure S3). Observed at various times following Fc:FasL administration, RHEs

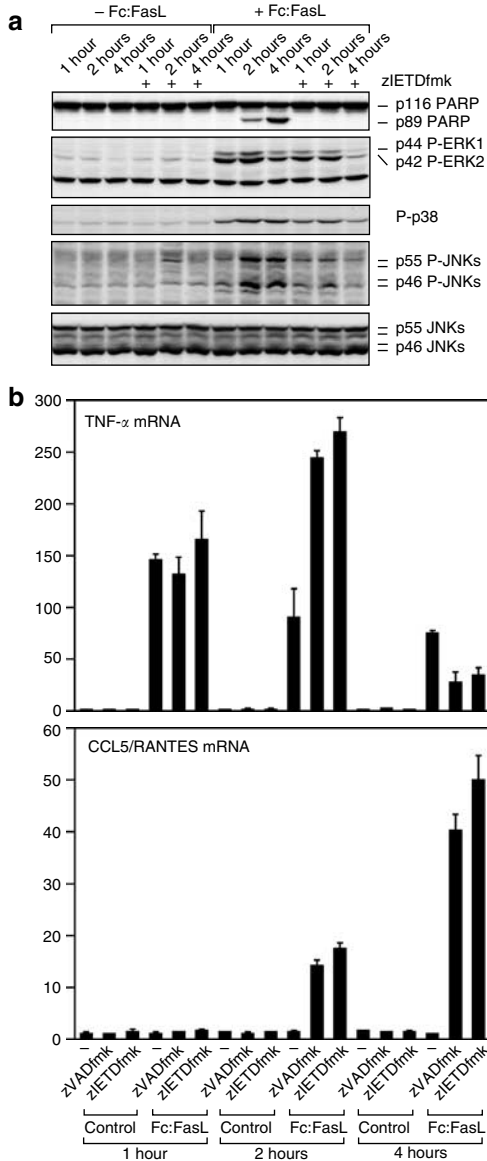


Figure 9. Caspase activity is not required for FasL-dependent gene expression. (a and b) HaCaT cells were treated exactly as in Figures 1 and 2, except that, where indicated, the cells were pretreated for 30 minutes with 25 μ M of either zVADfmk or zIETDfmk. All cells that were not pretreated with caspase inhibitors received identical amounts of the solvent vehicle (dimethyl sulfoxide). (a) Apoptosis was assessed by determining the cleavage of PARP. The phosphorylation states of JNK, ERK, p38 MAPK were assessed using phosphoepitope-specific antibodies and the levels of total JNK proteins (loading control) were determined using antibodies that are independent of the phosphorylation status. (b) Total RNA was interrogated in real-time RT-PCR analyses for the steady-state levels of indicated mRNAs. The levels of each mRNA are expressed as fold increase relative to the corresponding control (i.e. untreated) cells. Error bars represent SD from triplicated RT-PCR reactions.

displayed increasing apoptotic morphology between 1 day and 2.5 days post-treatment (Figure 10a-d, compare panels a and c; only 2.5 days shown). The presence of *bona fide* apoptosis was confirmed by positive staining for active caspase-3 (Figure 10e-h, compare panels e and g; only 2.5

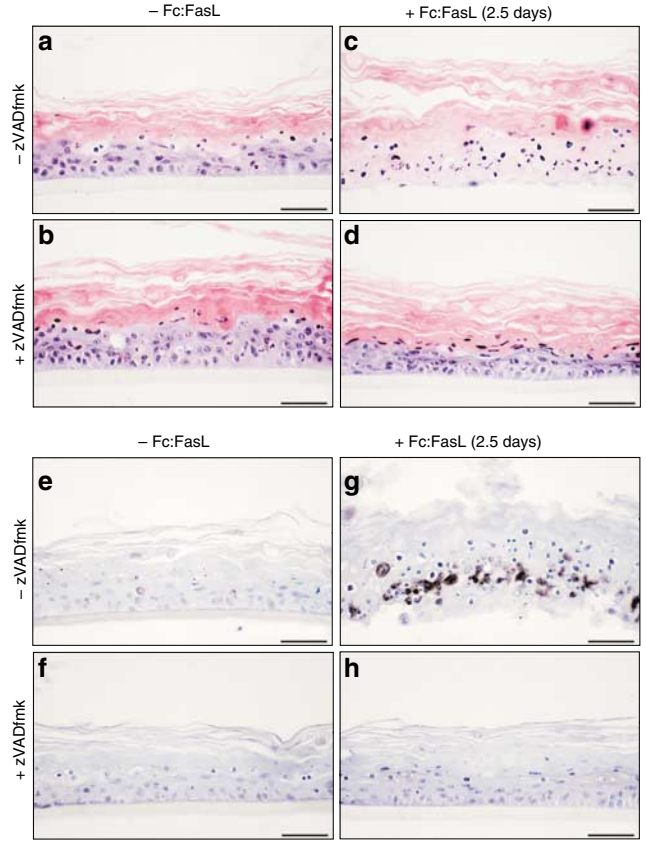


Figure 10. Caspase-dependent apoptosis in Fc:FasL-treated RHEs. RHEs using HEKn-E6/E7 cells were derived as described in Materials and Methods. Where indicated, the RHEs were pretreated for 30 minutes with 25 μ M zVADfmk (“+ zVADfmk”) or identical volume of the solvent vehicle (dimethyl sulfoxide; “- zVADfmk”). The RHEs were then left untreated (“- Fc:FasL”) or were treated with Fc:FasL (250 ng/ml; “+ Fc:FasL”). All pretreatments and treatments were given into the medium outside the inserts and, therefore, penetrated the RHE through the Millipore polycarbonate membrane. (a-d) Hematoxylin and eosin (H&E) staining. (e-h) Immunohistochemical detection of active caspase 3 (brown; with a hematoxylin counterstaining, blue). Note the detachment of the RHE from the Millipore polycarbonate membrane in the case of Fc:FasL-triggered apoptosis. Bar = 50 μ m.

days shown). The apoptotic action of Fc:FasL on the RHEs was completely abrogated by the pan-caspase inhibitor zVADfmk (Figure 10a-h, compare panels c and d and g and h). Under these experimental conditions of Fc:FasL-triggered, zVADfmk-inhibitable, apoptosis in RHEs, we observed a program of inflammatory gene expression highly similar to that found in HaCaT cells (Figure 11, exemplified by CXCL1/GRO α , CXCL8/IL-8, ICAM-1, and CCL5/RANTES mRNAs). The Fc:FasL-induced accumulation of mRNAs for inflammatory mediators was completely independent of caspase activity (Figure 11). Importantly, the behavior of CCL5/RANTES in RHEs was identical to the one in HaCaT cells: Fc:FasL was able to induce the accumulation on the mRNA for this chemokine only when caspase activity was suppressed by zVADfmk (Figure 11).

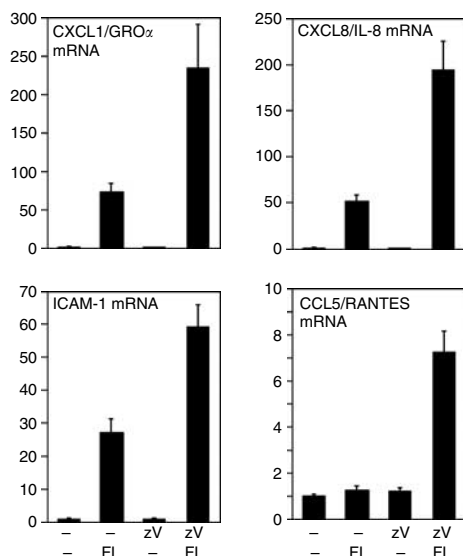


Figure 11. Caspase activity is not required for FasL-dependent gene expression in RHEs. RHEs using HEK₂₉₃-E6/E7 cells were derived as described in Materials and Methods. Where indicated, the RHEs were pretreated for 30 minutes with 25 μ M zVADfmk ("zV") or identical volume of the solvent vehicle (dimethyl sulfoxide; "--"). The RHEs were then left untreated ("--") or were treated with Fc:FasL (250 ng/ml; "FL"). All pretreatments and treatments were given into the medium outside the inserts and, therefore, penetrated the RHE through the Millipore polycarbonate membrane. Total RNA was interrogated 4 hours after the Fc:FasL treatment in real-time RT-PCR analyses for the steady-state levels of indicated mRNAs. The levels of each mRNA are expressed as fold increase relative to the corresponding control (i.e. untreated) RHEs. Error bars represent standard deviation from triplicated RT-PCR reactions.

DISCUSSION

Recruitment of T cells, monocytes, and other leukocytes to sites of cutaneous inflammation plays an important role in the pathogenesis of acute or chronic inflammatory skin diseases, such as AD, ACD, and psoriasis (Nickoloff *et al.*, 1990). Following the endothelium-regulated processes of rolling, tethering, adhesion, and extravasation, the leukocytes are attracted to the dermal and epidermal compartments by gradients of chemoattractants (chemokines) produced by resident and immigrant cells and are maintained *in situ* by adhesion molecules (Pastore *et al.*, 2004). As the major resident cell type in the epidermis, keratinocytes likely play a role in leukocyte attraction and epidermal retention. Indeed, keratinocytes from patients with AD or psoriasis were found to display distinct chemokine production profiles (Giustizieri *et al.*, 2001). In the case of AD, the second step of chemotaxis (i.e. the one after transendothelial migration) was found to be regulated by the release of chemokines by keratinocytes triggered to undergo apoptosis in response to T-cell derived IFN- γ (Klunker *et al.*, 2003).

Bearing in mind the apparent prominent role of FasL in keratinocyte apoptosis during AD and ACD (Trautmann *et al.*, 2000, 2001), we decided to investigate systematically the possibility that this cytokine may also play a direct role in inflammation. Specifically, we addressed the following three questions:

Is FasL a proinflammatory agent for keratinocytes?

The results of our Affymetrix microarray interrogation, coupled with the use of the EASE software and L2L database, and validated by real-time RT-PCR, ELISA, and immunoblotting, support strongly the conclusion that FasL is a potent inducer of a proinflammatory profile of gene expression in HaCaT cells. This proinflammatory profile of gene expression includes elevated mRNA levels of transcription factors, cytokines, chemokines, and ICAM-1. Considering the activation of MAP kinases, the increased abundance of mRNAs encoding transcription factors, and the dependence on NF- κ B, FasL-dependent increase in transcriptional activity of proinflammatory genes is a likely mechanism by which FasL triggers the increased abundance of their mRNA transcripts, although other possibilities, such as increased mRNA stability, cannot be ruled out.

Can the (trimeric) sFasL trigger apoptosis and/or inflammatory response in keratinocytes?

The answer to this important question could potentially shed light on the cellular source of FasL-mediated inflammation. Should sFasL be capable of triggering keratinocyte apoptosis and/or inflammation, this would be an indication that epidermal keratinocytes may be subject to "long-range" FasL-dependent influence by leukocytes that themselves are not resident in the epidermis. Our results using Fc:PS:FasL indicate that a minimum of two adjacent FasL trimers are required for both the apoptotic and the proinflammatory responses of keratinocytes. The potential implication of this finding is that, in inflammatory diseases of the skin, the FasL-dependent keratinocyte apoptosis and proinflammatory behavior may require transmembrane presentation of FasL and, therefore, transmigration of leukocytes into, and their retention in, the epidermal compartment. Alternatively, it is possible that sFasL is capable of signaling apoptosis and/or gene expression, but only in a specific tissue context, for instance due to its appropriate oligomerization and presentation to cells by components of the extracellular matrix.

Is FasL-induced inflammatory response dependent upon apoptosis?

The traditional view of apoptosis holds that apoptotic cell death has evolved as a mechanism of avoiding unnecessary inflammation through neat and controlled elimination of damaged cells. This is achieved by means of packaging diseased cells into apoptotic bodies without plasma membrane breakdown and their subsequent elimination by professional or bystander phagocytes (Edinger and Thompson, 2004). Apoptosis is also employed to maintain the immune-privileged state of some organs (such as the eye and the testis) and in graft-tolerance (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995). It is thought, however, that if the apoptotic cells are not efficiently eliminated through phagocytosis, the resulting process of secondary necrosis (due to the eventual breakdown of the plasma membrane) may trigger inflammation (Erjefalt, 2005). Secondary necrosis was proposed to explain the aggravation of asthma-like inflammation by apoptotic airway tissue eosinophils in a mouse model (Uller

et al., 2005). Considering the relative lack of professional phagocytes in the epidermis and the abundance of apoptotic keratinocytes observed *in vivo* in biopsies from eczematous skin (Trautmann *et al.*, 2000, 2001; Iordanov *et al.*, 2005), it is plausible to suspect that postapoptotic secondary necrosis is a major mechanism of keratinocytes' participation in the "vicious cycle" of inflammation in chronic eczemas. The results presented here, however, strongly argue that the FasL-dependent proinflammatory reaction precedes, and is independent of, FasL-dependent apoptosis. The mRNAs for most of the inflammatory mediators (e.g. TNF- α , IL-6, IL-1 β , CXCL1/GRO α , CXCL3/GRO γ , and CXCL8/IL-8) were already induced to accumulate at 1 hour post-Fc:FasL. At this time point, apoptosis in the same cells was neither detected morphologically (not shown) nor by means of caspase activity (PARP cleavage). Most importantly, caspase inhibitors not only failed to inhibit the inflammatory mRNA accumulation, but their presence appeared to be essential for the ability of Fc:FasL to induce the accumulation of the mRNA for CCL5/RANTES. Taken together with several recently published reports, our findings suggest that the relationship between FasL-induced cell death and inflammation may be different in specific tissue and cellular contexts. Engagement of Fas in immature dendritic cells triggered their maturation and expression of proinflammatory cytokines in the absence of apoptosis (Rescigno *et al.*, 2000). In an *in vivo* mouse model of acute lung injury triggered by intranasal instillation of Fas-activating antibody, Matute-Bello *et al.* (2001) reported the presence of both apoptosis and increased inflammatory gene expression in alveolar epithelial cells. Similar to our findings, the study of Matute-Bello *et al.* did not reveal an increase in CCL5/RANTES expression after Fas activation, although this work did not employ caspase inhibitors to investigate the ability of Fas ligation to activate CCL5/RANTES under conditions of suppressed apoptosis. Recently, Petrilli *et al.* reported intriguing results suggesting that caspase-cleaved PARP is an important mediator of inflammatory gene expression during experimentally induced endotoxic shock or intestinal or renal ischemia-reperfusion (Petrilli *et al.*, 2004). We demonstrate, however, the ability of FasL to induce inflammatory gene expression in keratinocytes in the absence of any detectable PARP cleavage and that the proinflammatory action of FasL in keratinocytes can be (as in the case of CCL5/RANTES) even suppressed by caspase activity. Our findings support a hypothesis that apoptosis in the eczematous epidermis (manifested clinically as spongiosis) may have the function of restricting, rather than aggravating inflammation. Such a notion may prove to be important in designing therapeutic strategies for the treatment of AD and ACD. It is important to point out that the lack of requirement for caspase activity in the FasL-dependent inflammatory response allows for the possibility that caspases may be involved in the nonapoptotic FasL-dependent signaling in a scaffolding-related function, as discovered by Kreuz *et al.* (2004).

Finally, the proinflammatory response elicited by FasL-treated HaCaT cells is not the first example of a nonapoptosis-related action of FasL in keratinocytes. We recently reported

that FasL-treated human keratinocytes secrete amphiregulin and activate EGFR and EGFR-dependent MAP kinase pathways (Iordanov *et al.*, 2005). We speculated that this promitogenic effect of FasL may play a role in the hyperproliferative phase of AD and ACD. Thus, the FasL/Fas system may exert a previously underappreciated variety of effects in the eczematous epidermis, including regulation of spongiosis and inflammation in the acute phase and hyperproliferation in the chronic phase.

MATERIALS AND METHODS

Cells

HaCaT cells were propagated in DMEM supplemented with 10% fetal bovine serum. For experiments, the cells were plated (24–48 hours before treatments) in EpiLife[®] basal keratinocyte medium (BKM) supplemented with a semidefined 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/ml; hydrocortisone, 0.18 mg/ml; bovine transferrin, 5 mg/ml; and human epidermal growth factor, 0.2 ng/ml). Both EpiLife[®] BKM and HKGS were from Cascade Biologics (Portland, OR). 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).

RHE and immunohistochemistry

HEKn-E6/E7 cells were established and propagated as described (Iordanov *et al.*, 2002, 2005). For RHE, we employed the method of Poumay *et al.* (2004), with minor modifications as described. HEKn-E6/E7 cells were plated at 1×10^6 cells/ml on polycarbonate membranes (Millipore: 0.4 μ m, 12-mm-diameter) and allowed to reach confluence in BKM + exoGF. Cells were then exposed to the air-liquid interface by removing the medium above the confluent monolayer. Simultaneously, the cells were exposed from underneath the monolayer to differentiation medium (BKM + exoGF supplemented with 1.5 mM CaCl₂, 50 μ g/ml ascorbic acid, and 10 ng/ml recombinant human keratinocytes growth factor (rhKGF, Peprotech, Rocky Hill, NJ)). The differentiation medium was exchanged every 48 hours for 14 days. 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. Immunocytochemical detection of p65/RelA was performed as described previously (Iordanov *et al.*, 2001).

Chemicals, Fc:FasL, and cytokines

All commonly used chemicals were from Sigma Chemical Company (St Louis, MO). Caspase inhibitors (zVADfmk and zIETDfmk) were from Calbiochem (San Diego, CA). The cloning, expression, purification, and characterization of Fc:FasL was described previously (Holler *et al.*, 2003). Fc:PS:FasL was constructed by inserting annealed oligos (5'-GATCTCTGGAGGTGCTGTCCAGGGGCCCG-3' and 5'-GATCCGGGCCCTGGAACAGCACCTCCAGA-3') in the *Bam*H1 site of the plasmid encoding Fc:FasL (Holler *et al.*, 2003), so that the encoded linker sequence between the Fc and FasL becomes RSPQPQPKPQPKPEPEGSLEVLFGQPGSLQ. Proteolytic digestion of Fc:[\pm PS]:FasL with PreScission protease, which cleaves the

sequence LEVLFQ-GP, was performed for 24 hours at 4°C in PBS 1 mM EDTA with 10 U of GST-PreScission (GE Healthcare, Piscataway, NJ), per mg of purified protein. Processing of Fc:PS:FasL was essentially complete, whereas Fc:FasL was unaffected, as assessed by Western blotting with an anti-human FasL antibody (Figure S2c). Recombinant human TNF- α was from R&D Systems and recombinant human IL-1 β was from Calbiochem.

RNA isolation, affymetrix microarray, and real time RT-PCR analyses

Cells or RHEs were lysed directly in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and RNA was extracted in accordance with the manufacturer's instructions. Integrity of RNA was determined by the appearance of distinct 28S and 18S rRNA bands when analyzed by electrophoresis on 1% agarose gels. The integrity of RNA was confirmed for all samples before Affymetrix microarray and real-time RT-PCR analyses. Gene expression profiling was performed by the Oregon Health and Science University Gene Microarray Shared Resource Affymetrix Microarray Core using HG-U133 Plus 2.0 GeneChip arrays, which interrogate over 47,000 human transcripts. Image processing and normalization were performed using Affymetrix Microarray suite 5.0 (MAS 5.0) software to obtain the estimate of the fold change for each paired group. For real-time RT-PCR analyses, 2 μ g of total RNA were reverse-transcribed in the presence of SuperScript II and oligo-dT primers (both reagents were purchased from Invitrogen Life Technologies). The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in the presence of the commercially available SYBR Green PCR Master Mix (Applied Biosystems) and 20 μ mol/L of the corresponding sense and antisense RT-PCR primers for 120-bp amplicons in a 40-cycle PCR (Table S1). Fold induction in gene expression was measured using absolute quantitation of a standard curve in arbitrary units. The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, respectively.

Preparation of cell lysates for immunoblot analysis

To avoid potential postlysis modifications or degradation of proteins of interest, the cells and RHEs were harvested by direct lysis in 2 \times SDS-PAGE sample-loading buffer, followed by heat denaturation at 95°C for 5 minutes and ultrasonic shearing. Typically, the detached (dead) cells were sedimented from the growth medium by centrifugation, lysed in 2 \times SDS-PAGE sample-loading buffer, and combined with the cell lysates from adherent cells. Cell lysates were stored at -80°C.

Antibodies and immunoblot analyses

The Fas-blocking antibody ZB4 was from Upstate Cell Signaling Solutions. The antibodies against PARP (H-250), ERK1 (C-16), JNK1 (FL), p38 MAPK (C-20), cFos (6-2H-2F), p65/RelA (C-20; rabbit for immunoblot, goat for immunocytochemistry), and p50/NF- κ B1 (E-10) were from Santa Cruz Biotechnologies. The antibodies against the phosphorylated forms of ERK, JNK, and p38 MAPK and the antibody against active caspase 3 were from Cell Signaling Technology. The anti-filaggrin antibody was from Abcam and the anti-K5 and -K1 antibodies were from BabCo. The separation of

proteins in SDS-PAGE and the electrotransfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) were performed by standard procedures. Immunoprobings with specific antibodies and enhanced chemiluminescent detection (DuPont NEN Research Products, Boston, MA) were performed following the instructions of the respective manufacturers. For immunoblot quantification, appropriately nonsaturated film exposures were selected and scanned, and the scanned images were imported into IP Lab Gel (GE Healthcare) software for quantification.

Measurement of protein synthesis

The cells were plated in 12-well tissue culture dishes 24 hours before treatments in BKM + exoGF. The cells were extensively washed with BKM-exoGF and incubated in BKM-exoGF for 2 hours before treatments. The cells were then treated, in triplicated wells, with Fc:FasL. The cells were pulse-labeled for 15 minutes with 5 μ Ci of [³H]leucine in 0.3 ml of BKM-exoGF. The incorporation of [³H]leucine was stopped by the addition of equal volume of 10% trichloroacetic acid (TCA). Cells (both adherent and detached) were washed 3 \times with 5% TCA, followed by solubilization of the TCA-insoluble proteins in 88% formic acid. The combined samples (containing both adherent and detached cells, solubilized in formic acid) were counted in a scintillation counter.

ELISA

Detection of TNF- α release by ELISA was performed following the protocol provided by the manufacturer (eBioscience, San Diego, CA).

RNAi

The p65/RelA sense (5'-GCCCUAUCUUUACGUCAdTdT-3') and antisense (5'-UGACGUAAAGGGAUAGGGCdTdT-3') oligonucleotides (Zhou *et al.*, 2003) as well as nonspecific scrambled control sense (5'-UCCUCUCCUAACCUGAUGCdTdT-3') and antisense (5'-GCAUCAGGUUAGGAGAGGAdTdT-3') oligonucleotides were synthesized by the MMI Research Core Facility (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 minutes followed by incubation at 37°C for 1 hour. Annealed siRNAs were transfected at a final concentration of 10 nM using DharmaFECTTM1 (Dharmacon, Lafayette, CO), according to the manufacturer's instructions and used for experiments 72 hours later. Measured by BLOCK-iTTM (Invitrogen) fluorescent control siRNA transfection, the transfection efficiency was \geq 90% (not shown).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. Experimental scheme to investigate direct and secondary effects of Fc:FasL.

Figure S2. Schematic representation of membrane-bound FasL, soluble FasL (sFasL), and the recombinant Fc:[±PS]:FasL proteins employed in this work.

Figure S3. Morphology and epidermal marker distribution in reconstructed human epidermis (RHE).

Table S1. Forward and reverse primer sequences used for real-time RT-PCR.

Table S2. Overrepresentation analysis of annotated fasl-induced mrna abundance.

Table S3. Selected transcripts increased in abundance in fasl-treated HaCaT cells gene.

Table S4. Increased accumulation of selected mRNAs in Fc:FasL treated HaCaT cells.

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