



Specific Triggering of the Fas Signal Transduction Pathway in Normal Human Keratinocytes

Citation

Freiberg, Rachel A., David M. Spencer, Keith A. Choate, Peter D. Peng, Stuart L. Schreiber, Gerald R. Crabtree, and Paul A. Khavari. 1996. "Specific Triggering of the Fas Signal Transduction Pathway in Normal Human Keratinocytes." *Journal of Biological Chemistry* 271 (49): 31666–69. <https://doi.org/10.1074/jbc.271.49.31666>.

Published version

<https://doi.org/10.1074/jbc.271.49.31666>

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:41467418>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)

Specific Triggering of the Fas Signal Transduction Pathway in Normal Human Keratinocytes*

(Received for publication, June 3, 1996, and in revised form, August 26, 1996)

Rachel A. Freiberg[‡], David M. Spencer[§], Keith A. Choate[‡], Peter D. Peng[‡], Stuart L. Schreiber[¶], Gerald R. Crabtree[§], and Paul A. Khavari[‡]

From the [‡]Veterans Administration Palo Alto Health Care System, Palo Alto, California 94304 and Department of Dermatology, Stanford University, Stanford, California 94305, the [§]Howard Hughes Medical Institute, Center for Molecular and Genetic Medicine, Stanford University, Stanford, California 94305, and the [¶]Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

The epidermis is continually exposed to genotoxic injury and requires an efficient mechanism to eliminate genetically altered cells. The membrane receptor, Fas, initiates apoptosis in many cell types, including keratinocytes. Receptor cross-linking is the vital post-ligand binding step in Fas signal transduction, and we have utilized FK1012, capable of oligomerizing proteins engineered to contain the FK506 binding protein (FKBP), to trigger Fas via FKBP-linked receptor cytoplasmic domains in human keratinocytes. An FKBP chimera containing the Fas cytoplasmic domain targeted to the plasma membrane induced an up to 89% decrease in viability of keratinocytes, as reflected by the activity of constitutive promoters, in response to FK1012. Oligomerization of Fas, either with engineered Fas-FKBP by FK1012 or via antibody cross-linking of full-length Fas-induced cellular changes consistent with apoptosis. The *lpr* Fas point mutation abolished this effect. A Fas-FKBP construct unlinked to the membrane was fully active in this assay. Early developmental age or pre-treatment of cells with GM-CSF, TGF- β , EGF, KGF, IFN- γ , or phorbol ester failed to protect against Fas effects. These findings reveal that the Fas signal transduction pathway is active in keratinocytes, requires no induction, and dominantly overrides growth stimuli.

Human epidermis is composed of layers of self-renewing keratinocytes that demonstrate remarkable versatility in responses to external environmental stimuli via complex programs of gene expression. Such complex genetic programs are largely triggered by cell surface receptor signal transduction cascades in the skin and mediate adaptive tissue functions, such as cellular proliferation in wound healing, expression of cytokines in inflammation, and apoptosis in response to a range of injurious stimuli. Cell surface receptors initiating such complex programs of gene expression are activated, in many cases, by a cross-linking of their cytoplasmic domains induced by extracellular binding of cognate ligand (1). Such ligand-induced receptor oligomerization appears to be a general mechanism of activation for a variety of receptor gene families,

including TNF¹ receptor (TNFR) family proteins (2). TNF- α is among the earliest activated receptor ligands expressed in the skin following external injury, and the role of TNFR proteins in mediating complex programs of gene expression in response to cutaneous injury is becoming increasingly well appreciated (3).

Fas is a TNFR member capable of triggering apoptosis and is induced in inflamed and infected human skin (4). Oligomerization of Fas by the Fas ligand (5) expressed on lymphoid cells triggers a form of cell death known as apoptosis. Apoptosis, triggered by Fas and a variety of other stimuli that include growth factor deprivation, detachment from extracellular matrix, and p53 activation, involves distinct ultrastructural and molecular alterations including chromatin condensation, DNA fragmentation, and cell shrinkage and collapse (6). Keratinocyte apoptosis has been postulated to be involved in normal differentiation, hair bulb cycling, response to physical injury such as sunburn, and prevention of neoplasia, but a role for Fas in mediating these processes is currently not firmly established (7). Expressed at low levels or not at all in normal human epidermis, Fas expression is dramatically enhanced on human keratinocytes in a number of inflammatory and infectious skin diseases (such as contact dermatitis and Herpesvirus infection) and *in vitro* in response to IFN- γ (4). The settings in which Fas triggers apoptosis in human keratinocytes and the effects of growth factors, other cytokines, and developmental state on its function are yet to be firmly established. Although TNFRs exert effects that differ by target cell and ligand dose, a potential disparity of Fas effects in differing skin cell populations, as well as biologic differences due to magnitude of receptor activation, are also not fully characterized. Among the barriers to studying the TNF superfamily is the existence of cross-reacting ligands, often making confident assignment of roles difficult (2). Another barrier is the pleiotropic effects of cytokines such as TNF- α ; they may exert opposing effects in two differing cell types (2).

We have pursued two approaches to triggering Fas oligomerization in order to determine features of Fas signal transduction in keratinocytes, cross-linking with a monoclonal antibody to full-length Fas and the FK1012 intracellular dimerizing agent for activating engineered Fas constructs. FK1012 is capable of cross-linking intracellular proteins engineered to contain the FK506 binding protein (FKBP); an FKBP-linked Fas cytoplasmic domain served as a confirmation of antibody effects as well as allowed initiation of features of Fas signal transduction in different subcellular locations. Here we present data indicating that Fas is active in normal human keratinocytes, that its downstream mediators require no induction, and

* This work was supported by the Office of Research and Development, Department of Veterans Affairs and by National Institutes of Health Grants AR/OD4337101 and AR43799 (to P. A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Chief, Dermatology Service [123], Palo Alto V. A. Medical Center, 3801 Miranda Ave., Palo Alto, CA 94304. Tel.: 415-852-3494; 415-723-8762; Fax: khavari@cmgm.stanford.edu.

¹ The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; FKBP, FK506 binding protein; IFN, interferon; CAT, choramphenicol acetyltransferase.

that it dominantly overrides growth stimuli. Features of such a cell death signal transduction pathway in the epidermis may reveal a mechanism to eliminate injured keratinocytes in avoiding potential subsequent infectious and neoplastic complications in the skin.

MATERIALS AND METHODS

Cell Harvesting and Tissue Culture—Primary human keratinocytes were isolated from neonatal foreskin and grown, as described previously (8).

Gene Transfection and Retroviral Transduction—Primary keratinocytes were cultured in 35-mm plates to approximately 70–80% confluency and then were transfected by lipofection (9). Cell extracts were prepared and analyzed for luciferase, β -galactosidase, and chloramphenicol acetyltransferase (CAT) activity 16–24 h after synthetic ligand addition. The myristylated and non-myristylated FKBP and Fas-FKBP expression constructs in pBJ5 included Fas amino acids 179–319 linked to three FKBP domains and the HA epitope tag (10). For cell morphology studies, co-transfection with RSV- β -galactosidase plasmid and triggering stimuli were followed by X-gal (Sigma) staining (11–13). An amphotropic retrovirus designed to transcribe full-length wild-type human Fas off the retroviral LTR was produced and utilized to transduce primary human keratinocytes. Briefly, keratinocytes were grown to 20% confluence in 35-mm plates and overlaid with 1.5 ml of retroviral supernatant with polybrene at 5 μ g/ml. After centrifugation at $300 \times g$ for 1 h, cells were incubated at 37 °C for 6 h and then given fresh media. 48 h later, cells were treated with CH-11 antibody or diluent alone and analyzed for morphologic changes by phase-contrast microscopy.

Keratinocyte Pretreatment—Primary human keratinocytes were incubated for 48 h with the following agents and concentrations prior to transfection: GM-CSF (10 ng/ml), EGF (20 ng/ml), KGF (20 ng/ml), TGF- β (4 ng/ml), IFN- γ (10 ng/ml), or phorbol 12-myristate 13-acetate (20 ng/ml). Cells were then rinsed with fresh media without any added agents and transfected as noted above.

RESULTS

Engineered Fas Triggers Decreased Constitutive Promoter Activity in Response to FK1012—Antibodies to cell surface receptors have been successfully used to trigger specific signal transduction pathways. The efficiency of this approach, however, may vary with the specific antibody utilized for a given receptor and with the density of cell surface receptor expression for successful antibody-mediated threshold triggering effects (14). Therefore, we also utilized an additional approach to antibody-triggering, intracellular dimerization of engineered Fas. Keratinocytes were transfected with expression plasmids for both full-length and engineered Fas. Cells were triggered via the CH-11 Fas cross-linking antibody (15) or dimerizing agent (10), respectively, at a range of concentrations. We utilized engineered receptors as an alternative approach to antibody-triggered activation; this involves aggregation of engineered receptor cytoplasmic domains via the FK1012 intracellular oligomerizing stimulus. Because this approach may be free of problems such as receptor cross-reactivity to soluble native ligands and of varying effects of antibodies to specific extracellular epitopes, we utilized a panel of engineered cell surface receptors to allow accomplishment of this using FK1012. The cytoplasmic domains of the human receptors below were linked to trimerized FKBP domains as diagrammed, epitope tagged (16), and transfected into keratinocytes. Cells were then treated with FK1012 or diluent control for 16 h, and then reporter gene activity, driven by co-transfected constitutive reporter plasmids, was assayed (17). The latter were used as a general measure of normal physiologic gene expression, with stimuli such as Fas signal transmission expected to impact negatively on their expression. Engineered Fas, but not engineered EGFR, or KGFR (FGFR-2 spliced variant) triggered consistent decreases in constitutive reporter gene activity, as measured by gene expression from a panel of three different constitutive promoters (K5, CMV, and RSV) (Fig. 1A), suggesting a maintenance of signal transduction

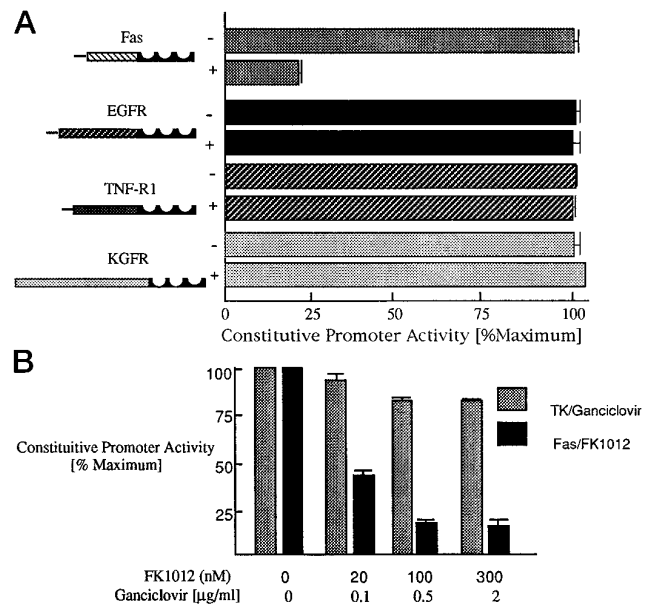


FIG. 1. Decreased constitutive promoter activity triggered by engineered Fas in response to FK1012. A, non-transformed normal human keratinocytes were transfected with the panel of engineered receptor expression vectors diagrammed above along with a constitutive reporter plasmid (keratin 5-luciferase, RSV- β -galactosidase, CMV-CAT). Cell extracts were made 16 h after addition of 300 nM FK1012 (+) or diluent alone (-), and reporter gene activity was analyzed, demonstrating a significant decrease only in cells transfected with engineered Fas and triggered with FK1012. B, normal keratinocytes were transfected with either engineered Fas or the herpes simplex thymidine kinase gene (TK) along with constitutive reporter plasmids. Ganciclovir or FK1012 was added in the concentrations noted, and 16 h later, cell extracts were made and analyzed, demonstrating an FK1012 dose-dependent decrease in constitutive reporter gene activity and a less dramatic effect for TK.

pathway specificity in these engineered hybrid receptors. This decreased constitutive reporter gene activity was dose-dependent (Fig. 1B). While longer assay times of up to 72 h post-oligomerization demonstrated no alteration in this pattern for this panel of engineered receptors, the TNFR1 showed a 51% decrease in reporter gene activity at this time point, consistent with the longer killing kinetics identified for this receptor.

Engineered and Wild-type Fas Trigger Morphologic Changes of Apoptosis in Keratinocytes—Fas cross-linking does not trigger apoptosis in all cells under all conditions (18, 19), and we examined the ability of this engineered receptor to recapitulate features of this process in keratinocytes. Signal transduction by TNFR members is activated by receptor cross-linking, and while specific antibodies have been utilized effectively in receptor activation, the success of this may depend on the individual antibodies as well as on the density of cell surface receptor expression (14). Thus, as an additional control for the CH-11 Fas cross-linking monoclonal antibody, we employed the FK1012 dimerizing agent (10) and FK1012-oligomerized engineered Fas. FK1012 is a dimeric macrolide FK506 that is lacking in unwanted immunosuppressant effects and may cross-link any 2 proteins engineered to contain the 105-amino acid FK506 binding protein (FKBP) (10). Co-expression of the *Escherichia coli lacZ* gene is a marker of cell transfection and useful as an aid to identifying the morphologic changes seen in apoptosis (11–13). Decreased constitutive reporter gene activity triggered by engineered Fas was associated with morphologic changes of cell shrinkage and rounding seen in apoptosis and was comparable to that seen with full-length Fas triggered with the anti-Fas monoclonal antibody CH-11 (Fig. 2A). These findings were confirmed with an amphotropic retroviral ex-

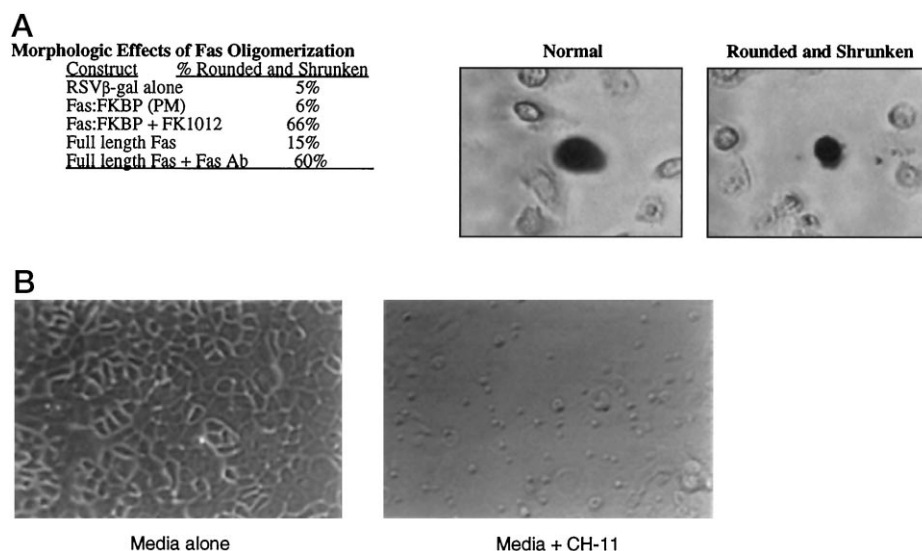


FIG. 2. Oligomerization of engineered and wild-type Fas triggers apoptotic changes in keratinocytes. A, normal human keratinocytes were co-transfected with engineered or wild-type Fas constructs along with an RSV-β-galactosidase reporter plasmid. Cells were treated with either FK1012, anti-Fas antibody CH-11, or diluent control. 16 h after addition of FK1012 or antibody, cells were fixed and stained for β-galactosidase expression to highlight transfected cell morphology, and a marked increase in the number of cells displaying the rounded and shrunken changes of apoptosis were noted with engineered Fas and wild-type Fas triggered with FK1012 and CH-11, respectively. Representative normal and rounded and shrunken keratinocytes are shown. B, normal human keratinocytes were transduced with a retroviral vector for expression of the full-length wild-type human Fas protein in the absence of normal inductive stimuli for Fas up-regulation in keratinocytes. Cells were then either incubated in media alone (*left*) or media plus the CH-11 Fas cross-linking antibody at 1 μg/ml (*right*). Cellular morphologic changes of apoptosis were analyzed by phase-contrast microscopy.

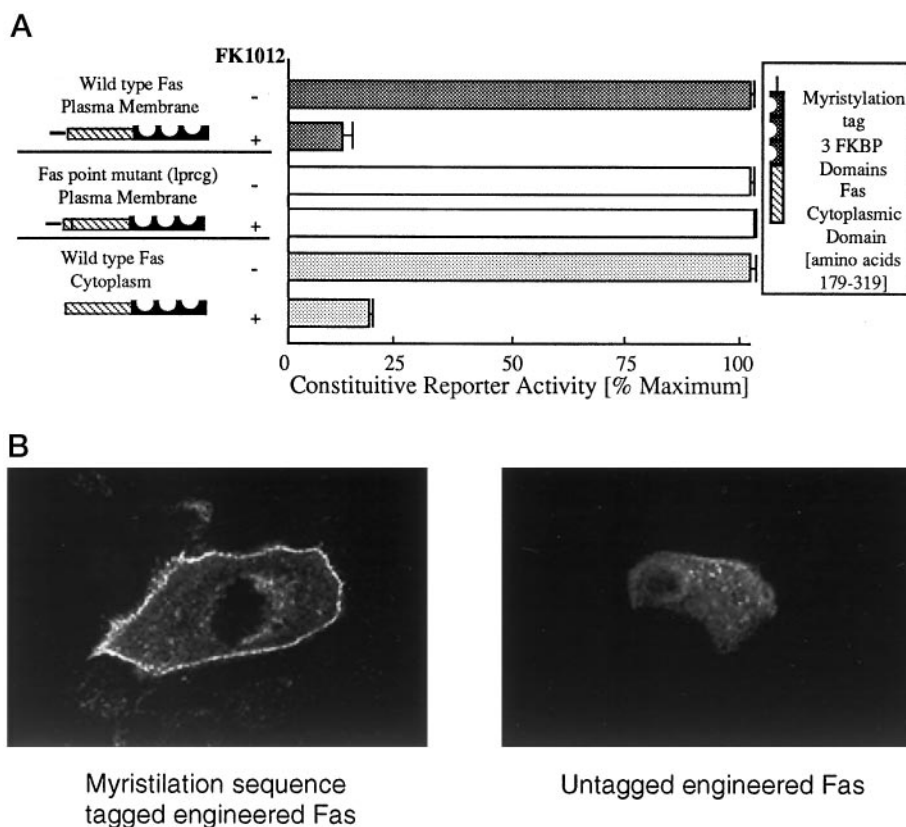


FIG. 3. Effects of subcellular localization on Fas signal transduction in keratinocytes. A, normal keratinocytes were transfected with engineered Fas proteins containing either the wild-type or *lprcg* point mutant Fas cytoplasmic domain with the myristylation tag from the v-Src protein for membrane localization or with the wild-type Fas cytoplasmic domain with no membrane localization signal. Cell extracts were made 16 h after addition of 300 nM FK1012, and reporter gene activity was analyzed, demonstrating a significant decrease in cells transfected with tagged or untagged engineered Fas but not with the *lprcg* point mutant. B, laser confocal immunofluorescence microscopy was used to examine the subcellular localization of either myristylation tagged or untagged engineered Fas in transfected keratinocytes using the 12CA5 antibody. Engineered Fas lacking the myristylation sequence appeared diffusely within the cell (*left*) while the myristylation tagged engineered Fas localized primarily to the cell perimeter.

pression vector for full-length wild-type human Fas. Keratinocytes transduced with this vector and treated with CH-11 displayed widespread apoptotic changes while Fas-overexpressing keratinocytes not treated with CH-11 did not (Fig. 2B). These findings provide further support that activation of Fas signal transduction triggers apoptosis in keratinocytes.

Localization of Fas Oligomerization Away from the Plasma Membrane Fails to Abolish Fas Signal Transduction in Kera-

tinocytes—A number of cell surface receptors appear to interact closely with plasma membrane-linked signal transduction machinery (1, 20); however, some of the effectors of Fas signal transduction have recently been identified and lack obvious features suggesting plasma membrane localization (11–13). To examine as yet unidentified plasma membrane localization requirements for the initiation of Fas signal transduction in keratinocytes, we utilized a Fas:FKBP construct targeted to the

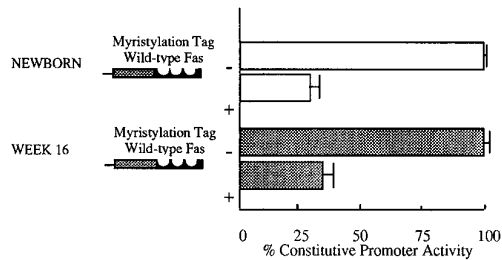


FIG. 4. **Fas signal transduction is active in keratinocytes from developing skin.** Keratinocytes were prepared from normal human skin at 16 weeks gestation or during the neonatal period and then co-transfected with the engineered Fas construct above and constitutive reporter plasmids and analyzed as in Fig. 1, demonstrating a comparable decrease in constitutive promoter activity in response to FK1012.

cytoplasm and demonstrated it retained activity (Fig. 3A). The *lprcg* point mutation at Fas amino acid 238, a residue known to abrogate apoptosis (21), in this construct totally abolished this effect (Fig. 3A). Laser confocal immunofluorescence microscopy was used to confirm subcellular distribution of myristylated Fas-FKBP at the keratinocyte cell perimeter while the untagged construct appeared diffusely within the cell (Fig. 3B), suggesting that certain features of the initiation of Fas signal transduction may not require strict plasma membrane localization.

Fas Signal Transduction Is Active in Keratinocytes from Developing Skin—Because Fas function appears to vary with developmental age in certain lymphoid subpopulations (7, 19), we wished to determine the existence of any differential Fas signal transduction in keratinocyte development. 16-week-old human fetal keratinocytes responded to Fas oligomerization in a comparable fashion to mature cells (Fig. 4), suggesting that aspects of Fas signal transmission are active in earlier stages of keratinocyte development.

Fas Effects Override Pre-Treatment of Normal Keratinocytes with Selected Cytokines and Growth Factors—Based on the hypothesis that Fas may trigger a dominant override death mechanism in keratinocytes, we determined the effect of keratinocyte pre-treatment with selected growth factors and cytokines, as well as phorbol ester. Transfection studies bypass the need for IFN- γ -induced up-regulation of Fas expression by expressing Fas via a constitutive promoter in keratinocytes, thereby allowing direct testing of whether the signal transduction machinery downstream of Fas also requires induction by IFN- γ or another agent or is ready at all times in a “standby” mode. Stimuli for growth factor receptor tyrosine kinase triggering failed to impact on Fas signal transduction as did TNF- α and IFN- γ or PMA (Table I), suggesting the inability of strong stimuli for proliferation, expression of a pro-inflammatory program of gene expression, or cell cycle arrest to impact Fas effects in these cells.

DISCUSSION

While apoptosis has been implicated in a variety of processes in the skin, the role of Fas in triggering this process is as yet not fully characterized. The prominent up-regulation of Fas expression in skin in infectious skin disease such as herpes simplex viral infection as well as in non-infectious inflammatory disease (4) suggests that, in situations of potential genotoxic injury or microbial infection, keratinocytes present infiltrating T lymphocytes with a rapid mechanism to eliminate suspect cells in the epidermis. Our studies suggest that the signal transduction machinery downstream of Fas may require no induction. This may indicate that the key control point of Fas-triggered apoptosis resides in receptor expression and oligomerization, implying a dramatic lack of the redundant checkpoints that prevent other biologically dangerous processes such

TABLE I
Fas effects override pre-treatment of normal keratinocytes with selected cytokines and growth factors

Normal human keratinocytes were co-transfected with engineered Fas and constitutively active reporter plasmid and then treated for 48 h with cytokine, growth factor, or phorbol ester as noted prior to addition of 300 nM FK1012. 16 h after FK1012 addition, cell extracts were prepared and constitutive promoter activity was determined, demonstrating a consistent effect of Fas triggering regardless of pre-treatment.

Pre-treatment	Decrease in constitutive gene expression by engineered Fas + FK1012
	%
None	71 $\pm$ 3
IFN- γ	71 $\pm$ 4
EGF	75 $\pm$ 6
KGF	72 $\pm$ 2
TNF- α	67 $\pm$ 3
PMA	69 $\pm$ 2

as uncontrolled cellular proliferation. Such a lack of checkpoints would support the contention that it is more adaptive for multicellular organisms to readily eliminate infected or genetically injured epithelial cells than to risk microbial invasion or subsequent neoplastic transformation. We did not observe differences in Fas effectiveness to indicate that keratinocyte susceptibility to Fas-triggered cell death varies, as seen in hematopoietic and lymphoid cells (7, 19). The latter may be due to altered levels of molecules downstream of Fas oligomerization with impact on signal transduction outcomes, such as Bcl-2 family proteins and ICE.

Recently, a number of downstream effectors of the TNFR family member signal transduction have been identified (11–13). The fact that none, unlike the case with growth factor receptor tyrosine kinases, appear to require strict plasma membrane localization is consistent with our finding that engineered Fas, triggered in the cytoplasm, appears to trigger changes of apoptosis in keratinocytes. In summary, our data indicate that Fas oligomerization in normal human keratinocytes activates a signal transduction pathway leading to apoptosis and suggests that this pathway functions dominantly in these cells. Such a powerful cell death stimulus in the epidermis may underlay a strategic preference of multicellular organisms to eliminate infected or injured epithelial cells rather than risk microbial invasion or subsequent neoplastic transformation.

REFERENCES

- Heldin, C.-H. (1995) *Cell* **80**, 213–223
- Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) *Cell* **76**, 959–962
- Nickoloff, B. J., and Naidu, Y. (1994) *J. Am. Acad. Dermatol.* **30**, 535–546
- Sayama, K., Yonehara, S., Watanabe, Y., and Miki, Y. (1994) *J. Invest. Dermatol.* **103**, 330–334
- Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) *Cell* **75**, 1169–1178
- Cleveland, J. L., and Ihle, J. N. (1995) *Cell* **81**, 479–482
- Haake, A. R., and Polakowska, R. R. (1993) *J. Invest. Dermatol.* **101**, 107–112
- Rheinwald, J. G., and Green, H. (1975) *Cell* **6**, 331–344
- Jiang, C. K., Connolly, D., and Blumenberg, M. (1991) *J. Invest. Dermatol.* **96**, 969–973
- Spencer, D. M., Belshaw, P., Chen, L., Ho, S. N., Randazzo, F., Crabtree, G. R., and Schreiber, S. L. (1996) *Curr. Biol.* **6**, 7–11
- Hsu, H., Xiong, J., and Goeddel, D. V. (1995) *Cell* **81**, 495–504
- Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995) *Cell* **81**, 513–523
- Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) *Cell* **81**, 505–512
- Merino, R., Grillot, D. A., Simonian, P. L., Muthukkumar, S., Fanslow, W. C., Bondada, S., and Nunez, G. (1995) *J. Immunol.* **155**, 3830–3838
- Yonehara, S., Ishii, A., and Yonehara, M. (1989) *J. Exp. Med.* **169**, 1747–1756
- Kolodziej, P. A., and Young, R. A. (1991) *Methods Enzymol.* **194**, 508–519
- Khavari, P. A., Peterson, C. L., Tamkun, J. W., and Crabtree, G. R. (1993) *Nature* **366**, 170–174
- Aggarwal, B. B., Singh, S., LaPushin, R., and Totpal, K. (1995) *FEBS Lett.* **364**, 5–8
- Williams, G. T., Smith, C. A., Spooner, E., Dexter, T. M., and Taylor, D. R. (1990) *Nature* **343**, 76–79
- Kazlauskas, A. (1994) *Curr. Opin. Genet. & Dev.* **4**, 5–14
- Nagata, S. (1994) *Semin. Immunol.* **6**, 3–8

Specific Triggering of the Fas Signal Transduction Pathway in Normal Human Keratinocytes

Rachel A. Freiberg, David M. Spencer, Keith A. Choate, Peter D. Peng, Stuart L. Schreiber, Gerald R. Crabtree and Paul A. Khavari

J. Biol. Chem. 1996, 271:31666-31669.
doi: 10.1074/jbc.271.49.31666

Access the most updated version of this article at <http://www.jbc.org/content/271/49/31666>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 21 references, 2 of which can be accessed free at <http://www.jbc.org/content/271/49/31666.full.html#ref-list-1>