Amphiregulin and Nerve Growth Factor Expression Are Regulated by Barrier Status in Murine Epidermis

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Disruption of the murine permeability barrier by solvents or tape stripping stimulates a homeostatic repair response that includes increased epidermal DNA synthesis. To identify potential mediators of the increase in DNA synthesis, we have measured epidermal levels of mRNAs encoding various growth factors after acute barrier disruption. In this study, mRNAs for amphiregulin and nerve growth factor were each shown to increase over controls at 30 min, reach peak levels of 12- to 30-fold at 1-2 h, and return to control levels by 6 h after tape stripping. A similar time course for the increase of amphiregulin and nerve growth factor mRNAs was observed after an unrelated form of barrier disruption, i.e., acetone treatment. Furthermore, artificial restoration of the barrier by Latex occlusion, immediately following barrier disruption by acetone treatment, inhibited the increase in epidermal amphiregulin and nerve growth factor mRNA levels, indicating that barrier status regulates the production of these growth factors. In contrast, mRNA levels of transforming growth factor-β1, an inhibitory growth factor, were unchanged at early times and decreased by 53% (p < 0.02) 6 h after tape stripping, whereas mRNA levels of transforming growth factor-α remained unchanged at all times after acute barrier disruption. These results suggest that barrier disruption stimulates the expression of amphiregulin and nerve growth factor. Together, these regulators of keratinocyte growth and differentiation may be responsible for the increased proliferative response that is associated with barrier disruption. Key words: transforming growth factor/cytokines/occlusion/cutaneous permeability barrier. J Invest Dermatol 108:73-77, 1997

When the integrity of the cutaneous permeability barrier is perturbed, a homeostatic repair response is stimulated that causes the rapid normalization of barrier function. This repair response includes the immediate secretion of a preformed pool of lamellar bodies, an increase in epidermal lipid synthesis (Menon et al, 1985; Grubauer et al, 1987; Holleran et al, 1991a), and the accelerated formation and secretion of nascent lamellar bodies (Feingold et al, 1990; Menon et al, 1992). Additionally, barrier disruption stimulates an increase in epidermal DNA synthesis, which may provide additional cells necessary for barrier homeostasis (Proksch et al, 1991). The mechanism(s) by which alterations to the barrier, located in the outermost anucleate stratum corneum, stimulate epidermal DNA synthesis, which occurs in the basal layer, are unknown. A number of cytokines, neuropeptides, and growth factors that are produced in the epidermis, however, are capable of increasing keratinocyte (KC) DNA synthesis.

Previous studies have shown that permeability barrier disruption leads to increased generation of cytokines (Wood et al, 1992, 1994a; Nickoloff and Naidu, 1994). In hairless mice, the mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1α and β, IL-1-receptor antagonist, and granulocyte-macrophage colony-stimulating factor increase in the epidermis (Wood et al, 1992, 1994a). Concomitant increases in TNF-α and IL-1α proteins in response to barrier disruption have also been shown (Tsai et al, 1994; Wood et al, 1996). In humans, tape stripping increased epidermal mRNAs for a number of pro-inflammatory cytokines such as TNF-α, IL-8, IL-10, interferon (IFN)-γ, intercellular adhesion molecule-1, and transforming growth factor (TGF)-α and -β (Nickoloff and Naidu, 1994). But the linkage of cytokines to barrier homeostasis remains unclear, because occlusion does not abrogate cytokine production in the context of acute barrier perturbations (Wood et al, 1994b). In the search for other signals of the epidermal metabolic response, we assess here the expression of selected KC-derived growth factors after barrier perturbations.

Epidermal growth factor (EGF) and its homologs, TGF-α and amphiregulin (AR), are known to stimulate KC growth in vitro (Coffey et al, 1987; Cook et al, 1991; Piepkorn et al, 1994). In addition, certain hyper-proliferative skin diseases such as psoriasis are associated with increased expression of TGF-α (Elder et al, 1989; Finzi et al, 1991) and AR (Cook et al, 1992). These two autocrine growth factors may also be involved in the pathogenesis of a variety of neoplastic and non-neoplastic hyper-proliferative skin disorders (Goutin et al, 1986; Finzi et al, 1991).

TGF-βs are multifunctional regulators of cell growth and differentiation and are involved in a variety of biologic processes, such as embryogenesis, carcinogenesis, wound healing, and immunomodulation (Roberts and Sporn 1990; Sporn and Roberts, 1992). Almost all cells, including KCs, (Fisher et al, 1992; Mansbridge et al, 1989) produce and respond to TGF-βs. While normal epithelial cells,
including KCs, respond to TGF-β by inhibiting DNA synthesis, transformed epithelial cell lines are typically resistant to these inhibitory effects (Krieg et al., 1991). Thus, TGF-β may play a crucial role in epidermal homeostasis by acting as a negative growth factor.

Nerve growth factor (NGF) is another protein, produced by both human and murine KCs, that is thought to regulate innervation, lymphocyte function, and melanocyte growth during normal epidermal development as well as during wound healing (Di Marco et al., 1991; Pincelli et al., 1994). Moreover, NGF is induced in KCs by ultraviolet irradiation and phorbol esters (Tzon et al., 1990). In order to begin to determine whether the epidermal metabolic response following barrier disruption could be mediated by the production of growth factors, we assessed the effect of acute barrier abrogation on TGF-α, AR, TGF-β1, and NGF mRNA levels in murine epidermis. We further determined the relationship of the observed changes to barrier homeostasis by assessing their reversibility with occlusion. In contrast to the earlier work on cytokines, these studies demonstrate a specific link between certain growth factor responses and barrier requirements.

**MATERIALS AND METHODS**

**Materials**

The rat cDNA probe for AR was kindly provided by Dr. Schubert (the Salk Institute for Biological Studies, San Diego, CA). The cDNAs for human TGF-α, human TGF-β1, and human NGF, were purchased from the American Type Culture Collection (Rockville, MD). Clone names were pHTGF-1-10,925, pHTGF-β2, and pHLN8N9, respectively. The rat cyclophilin cDNA was kindly provided by Dr. G. Strewler (Department of Veterans Affairs Medical Center, San Francisco, CA). α-32P-deoxyoxycytidine triphosphate (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Multiprimer DNA Labeling System was obtained from Amersham Corp. (Arlington Heights, IL). Sephadex G-50 spin columns were from Worthington Biochemical Corp. (Freehold, NJ). Nucleic acids (0.45 μm Nytran) were purchased from Schleicher & Schuell Inc. (Keene, NH). Oligo(dT) cellulose type 7FF was purchased from Pharmacia (Uppala, Sweden). RX Fuji films (Fischer Scientific, Santa Clara, CA) were used for autoradiography. The other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) and Fischer Scientific Co. (Fairlawn, NJ).

**Acute Barrier Disruption**

The entire torso of 6- to 8-wk-old male hairless mice (Charles Rivers Laboratory, Wilmington, MA) were treated with absolute acetone or cellophane tape as described previously (Grabauer et al., 1985; Wood et al., 1992). Both procedures were stopped when the transepidermal water loss reached 6-10 mg/cm² per h measured with an evaporimeter (Meecco Inc., Warrington, PA) (Menon et al., 1985, Wood et al., 1992). The controls consisted of untreated animals (tape-stripping experiments) or animals treated with a solution of sterile 0.9% sodium chloride (acetone experiments). Occlusion of the animal was performed as described previously by sliding mice into one finger of a latex glove (Wood et al., 1994b). Mice were killed by cervical dislocation, and the epidermis was isolated as described previously (Wood et al., 1992). Briefly, subcutaneous fat was scraped off with a scalpel, and skin was placed epidermis side up onto 10 ml of 10 mM ethylenediamine tetraacetic acid in Ca, Mg-free phosphate-buffered saline and incubated for 35 min at 37°C to separate epidermis from dermis. Tissue was stored at −70°C for subsequent RNA isolation.

**mRNA Isolation and Northern Blotting**

Epidermis (0.2–0.4 g from entire torsos of two mice) was processed for mRNA extraction (Chomczynski and Sacchi 1987). Poly(A)+ mRNA was isolated by oligo(dT) chromatography (type 77F, Pharmacia LKB) as described previously (Wood et al., 1992). Eight to ten micrograms of poly(A)+ mRNA (extracted from the epidermis of two mice) were loaded per lane onto a formaldehyde/1% agarose gel. After electrophoresis, the gel was stained with ethidium bromide for visualization of the integrity of residual ribosomal RNA bands. RNA was transferred to Nytran membranes and subsequently fixed by baking at 80°C for 2 h. cDNA probes were [32P]-labeled by the random priming method according to the manufacturer’s instructions (Amersham Corp.). Labeled probes were purified by exclusion chromatography through G-50 mini spin columns. Hybridization to RNA was performed as described previously (Wood et al., 1992). Briefly, northern blots were pre-hybridized for 1 h at 65°C in hybridization buffer and hybridized with radiolabeled probe overnight in the same buffer at 65°C. The first wash was carried out for 30 min at room temperature, and the second wash was performed at 65°C for 1 h except for TGF-α, which was at 50°C. The blots were exposed to film at −70°C.

**Densitometry**

A model GS-670 imaging densitometer from Bio-Rad (Hercules, CA) was used for scanning films. All values are presented as ratios of relative intensity of growth factor mRNA:relative intensity of cyclophilin mRNA. Cyclophilin mRNA, a commonly used “housekeeping” mRNA, was chosen to normalize for RNA loading because it did not change after barrier disruption. The absorbance values for each mRNA examined were within the linear range of the film.

**Statistical Analysis**

Statistical significance of the results was evaluated by two-tailed Student’s t test or a paired t test.

**RESULTS**

**Tape Stripping Decreases Epidermal TGF-β1 mRNA but Has No Effect on TGF-α mRNA**

To determine the effect of acute barrier disruption on growth factor mRNA levels, we performed northern analyses on epidermal samples isolated at various times after tape-stripping or acetone treatment. We detected three transcripts for TGF-β1 (4.8, 1.9, and 1.0 kb); however, only the 1.9-kb transcript appeared consistently in every epidermal sample and was quantitated. TGF-β1 mRNA levels (1.9-kb transcript) decreased after tape stripping, reaching levels of 53% of control 6 h after barrier disruption, with partial recovery after 24 h (Fig. 1). In contrast, TGF-β1 mRNA levels remained unchanged at all comparable time points after acetone treatment, a second method of acute barrier disruption (not shown). Additionally, the levels of TGF-α mRNA remained unchanged at all times after barrier disruption by either tape stripping or acetone (not shown). Thus, tape stripping decreases epidermal TGF-β1 mRNA levels 6 h after treatment.
Acute Barrier Abrogation Increases Epidermal AR and NGF mRNAs  We next determined the effects of acute barrier disruption on epidermal AR mRNA. As early as 30 min after tape stripping, AR mRNA levels were significantly increased by 4.3-fold in the tape-stripped versus control mice (Fig 2a). The mRNA levels reached a maximum between 1 and 2 h (12.2- and 12.6-fold, respectively), returning to control levels by 6 h after barrier disruption. Similar results were found with acetone treatment, another method of acute barrier abrogation (Fig 2b). NGF mRNA levels were increased at 30 min and 1 h after tape stripping, reaching maximal levels (35-fold higher than controls) at 2 h after treatment and returning to control levels by 6 h (Fig 3a). Similar results were observed with the acetone model (Fig 3b). These results show, first, that acute disruption of the barrier causes a rapid increase in epidermal AR mRNA levels, which return to normal by 6 h. Second, that NGF mRNA levels are elevated 2 h after acute barrier abrogation.

Occlusion Significantly Reduces the Increase of AR and NGF mRNAs in Response to Acute Barrier Abrogation  Previous studies have shown that artificial restoration of barrier function by occlusion with a water vapor-impermeable membrane inhibits multiple metabolic responses, including DNA synthesis, after barrier disruption (Proksch et al., 1991). Thus, we next examined the effect of occlusion on epidermal AR and NGF mRNA levels at 1 h and 2 h after barrier abrogation by acetone treatment. Occlusion with a Latex membrane, immediately after barrier disruption by acetone, did not block either the severalfold increase in AR mRNA or the modest increase in NGF mRNA levels that occur 1 h after acetone treatment (not shown). In contrast, as shown in Figs 4 and 5, occlusion significantly blocks the increase in both AR and NGF mRNA levels that occurs 2 h after acetone treatment. Both AR and NGF mRNAs still increase significantly in comparison to controls, even under the occlusive membrane. The increase in AR and NGF mRNAs induced by tape stripping was also reduced by occlusion for 2 h after treatment (data not shown). These results indicate that, under these experimental conditions, artificial restoration of the barrier partially blocks the increase of AR and NGF mRNA levels in the epidermis.

DISCUSSION

Following acute perturbations of the murine permeability barrier, epidermal lipid and DNA synthesis, as well as the lamellar body secretory system, is stimulated (Menon et al., 1985; Gruber et al., 1987; Holleran et al., 1991a; Proksch et al., 1991). All of these alterations are blocked by occlusion with water vapor-impermeable membranes, suggesting that these responses are linked to barrier function (Feingold, 1991). The signals that mediate these metabolic responses, however, remain unknown.

In this study, we demonstrate that acute barrier abrogation with both acetone treatment and tape stripping leads to an increase of epidermal AR and NGF mRNAs. Moreover, these increases were partially blocked by occlusion, suggesting that barrier abrogation and occlusion play a role in the regulation of these responses.
largely blocked by occlusion, indicating that the increases were related to barrier dysfunction rather than to injury. Both AR and NGF are potent stimulators of KC proliferation, and the overexpression of AR in hyper-proliferative skin diseases such as psoriasis has been demonstrated (Coffey et al., 1987; Elder et al., 1989; Cook et al., 1992). Thus, it is tempting to speculate that one or both of these growth factors stimulate the DNA synthetic response to barrier disruption. Although TGF-α and AR belong to the same EGF family and bind to the same receptor, barrier disruption in mice induced a selective increase of epidermal AR mRNA, without producing comparable changes in TGF-α. One explanation for this result is that AR may supplant the requirement for TGF-α or EGFr
murine KC growth (Coffey et al., 1987). To understand redundancy within the EGF family, others have shown that extensive auto-induction and cross-induction occurs within the EGF-related peptide family in several EGF-responsive epithelial cell types (Barnard et al., 1994). In these studies, AR mRNA was rapidly induced by EGF-related peptides (within 30 min) and peaked 1–2 h after stimulation. The induction occurred by a transcriptional mechanism, and AR is considered to be an immediate-early gene (Barnard et al., 1994). Its transient expression may be an important component of cell cycle activation and progression to DNA synthesis. In contrast, TGF-α auto-induction in nontransformed human KCs occurs primarily by prolongation of mRNA half-life (Coffey et al., 1992). Therefore, the disparate responses by these two EGF relatives in murine epidermis may reflect heterogeneity in the molecular mechanisms for induction between AR and other members of the EGF-related family.

NGF has a well established role in the development and survival of the central and peripheral nervous system (Levi-Montalcini, 1987). Yet there also is increasing evidence that NGF, in addition to its actions within the nervous system, mediates other biologic processes such as the modulation of inflammatory responses and the stimulation of wound healing (Pearce and Thompson, 1986; Otten et al., 1989; Nonogaki et al., 1996). In response to barrier abrogation, a rapid increase of NGF mRNA levels was observed in murine epidermis. In vitro studies have shown that human KCs both secrete and respond to NGF by increasing proliferation (DiMarco et al., 1991, Pincelli et al., 1994). Taken together, these observations suggest that AR and NGF may play important roles in barrier homeostasis in murine epidermis.

We have also shown that the epidermal mRNA levels for the anti-proliferative growth factor, TGF-β1, are decreased by 53% at 6 h after tape stripping, but levels of TGF-β1 mRNA did not change in response to barrier disruption induced by acetone treatment. Thus, this modest reduction in TGF-β1 mRNA levels following tape stripping may not be related to barrier status but, rather, may reflect an injury response. Furthermore, we consistently detected (and quantified) a transcript of 1.9 kb and not the 2.4-kb transcript that predominates in most tissues (Thompson et al., 1989). The 1.9-kb mRNA species of TGF-β1 was structurally characterized in infarcted rat heart (Qian et al., 1991) and was observed in a variety of other mouse and rat tissues, including skin (Akhurst et al., 1988; Thompson et al., 1989; Manthey et al., 1990). It differs from the 2.4-kb transcript in the 5’- and 3’-untranslated regions. The highly increased level of expression of the 1.9-kb mRNA in infarcted heart tissue has been interpreted as an “emergency response” to injury, and it strongly suggests that the 1.9-kb TGF-β1 mRNA may play an important role in the response of a variety of tissues to injury and subsequent repair processes (Qian et al., 1991). In the skin, 60% of barrier recovery has already occurred by 6 h after barrier disruption (Feigold, 1991). Therefore, the decreased levels of the 1.9-kb transcript at this time point may reflect a downregulation of this injury/repair response. Furthermore, a decrease in TGF-β1, an inhibitory type growth factor, may be a prerequisite for the increase in DNA synthesis that occurs 18 h after barrier disruption.

Nickoloff and co-workers have shown an increase in TGF-α and TGF-β mRNA levels in human epidermis after tape stripping (Nickoloff and Naidu, 1994). The disparity with our results may relate to differences in the detection techniques used and also to the difference between human and murine skin. These authors used polymerase chain reaction amplification of reverse-transcribed RNAs as opposed to northern blotting. Moreover, the authors pointed out that variations in cytokine mRNA signals were observed in punch biopsy samples taken from the same test area as well as those taken from different experimental subjects. In our study we used the entire torsos of mice, which provided a larger sample area, thereby minimizing regional or “site to site” variations that may have contributed to the non-uniform levels of cytokine mRNAs observed in the human studies.

Previous studies have demonstrated that increased mRNA levels of TNF-α and IL-1 are observed in murine epidermis in response to barrier abrogation (Wood et al., 1992). TNF-α and IL-1 have been shown to stimulate KC proliferation either directly or by initiating a cascade of cytokine production (Hancock et al., 1988; Pillai et al., 1989; Barker et al., 1991). Given the complexity and redundancy of cytokine- and growth factor-signaling mechanisms, it is likely that a large number of cytokines and growth factors could influence epidermal metabolism, and it will therefore be very difficult to determine directly which molecules play a role in stimulating the increase in DNA synthesis that occurs 18 h after barrier disruption.

In summary, this study shows that acute barrier abrogation by both acetone and tape-stripping treatment increase epidermal AR, and NGF mRNA levels in murine skin, demonstrating that murine epidermis can respond rapidly to barrier perturbations by upregulating mRNAs encoding growth factors that could contribute to metabolic changes necessary to restore cutaneous homeostasis.

REFERENCES


Figure 5. Occlusion, immediately after barrier disruption by acetone, partially blocks the increase of epidermal NGF mRNA. Mice were treated with acetone, and one group was occluded as described in Fig 4. Northern blotting was performed as described in Fig 1, except that the blot was probed with 32P-labeled NGF and cyclophilin cDNAs. (a) Northern blot. Lanes 1–3, acetone; lanes 6–10, control; lanes 11–15, acetone/occluded. (b) Quantification by densitometry of epidermal NGF mRNA levels. The northern blot shown in Panel A was scanned as described in Materials and Methods. The data were analyzed as described in Fig 1b. Error bars, mean ± SEM; n = 5 for each group. *p < 0.0001 versus control.


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