Inhibition of erbB Receptor Family Members Protects HaCaT Keratinocytes from Ultraviolet-B-Induced Apoptosis

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In the human epidermis, the cells most at risk for the development of cancer due to sunlight exposure are the keratinocytes. In animal models, ultraviolet-B is a complete carcinogen, capable of inducing and promoting the development of malignant cells. A key element of ultraviolet-B-induced carcinogenesis is the ability of ultraviolet-B to induce the expression of a number of cellular proteins and activate growth factor receptor tyrosine kinases, including the erbB receptor family. Keratinocytes express the erbB1 (also called EGF-R, HER1), the erbB2 (also known as neu or HER2), and the erbB3 (HER3) subtypes. In general, activation of the erbB receptor family leads to a cellular proliferative response. In certain instances, however, activation of an erbB receptor can induce differentiation, cell cycle arrest, and even apoptosis. The inhibition of tyrosine kinase activity in rodent models and human skin has been shown to inhibit some ultraviolet-B response pathways. We have shown that the inhibition of erbB receptors, by both pharmaceutical and immunologic means, will inhibit ultraviolet-B-induced apoptosis in the HaCaT human keratinocyte cell line. This inhibition was specific for the erbB receptor family and specific for ultraviolet-B-induced apoptosis. These results suggest that, in certain instances, ultraviolet-B-induced apoptotic signaling requires erbB family receptor activity. Key words: UVB/keratinocytes/apoptosis/erbB.

inhibition of the erbB receptor family of tyrosine kinases. Our findings demonstrate that the inhibition of erbB receptors in HaCaT cells leads to a protection from UVB-induced apoptosis.

MATERIALS AND METHODS

Reagents  Pharmacologic inhibitors AG 825 (4-hydroxy-3-methoxy-5-(benzothiazolylmethyl)-benzylidenecanoacetamide), AG 1295 (6,7-dimethyl-2-phenylquinoxaline), AG 1478 (4-(3-chloromethyl)-6,7-dimethoxyquinazoline), and neutralizing antibodies to erbB1 (clone 528) and erbB2 (clone 9G6) were all obtained from Calbiochem, La Jolla, CA. 4,5-Diaminophthalimidine (DAPH) was obtained from Sigma, St. Louis, MO. All other chemicals were obtained from Sigma or Roche Molecular Biochemicals, Indianapolis, IN.

HaCaT cell culture  HaCaT keratinocyte cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Santa Ana, CA) supplemented with 10% fetal bovine serum (Nova-Tech, Grand Island, NY) and 1000 U penicillin, streptomycin (Roche). Cells were grown to approximately 80% confluence before experiments were carried out.

UVB irradiation  UVB irradiation of HaCaT cells was accomplished using a Philips F20T12/UV-B source (270–390 nm; containing 2.6% UVB, 43.6% UVB, 53.8% UVA). The intensity of the UVB source was measured using a Philips F2000 spectrophotometer (excitation, 380 nm; emission, 460 nm). The fluorescent intensity was converted to picomoles of AMC by comparison with the fluorescent intensity of standards of AMC (Molecular Probes, Eugene, OR). The activity of caspase 3 was determined by immunoblotting with antiphosphotyrosine horseradish peroxidase (HRP) conjugated antibody Ab-4 (Oncogene, Boston, MA). Relative activities were determined by comparing the specific activities derived from UVB-treated cell lysates with those derived from untreated cell lysates.

erbB tyrosine phosphorylation  Activation of erbB receptor family was assayed from HaCaT cell lysates. Briefly, HaCaT cells were washed twice with ice-cold phosphate-buffered saline and lysed in RIPA buffer [50 mM NaCl, 0.1% Triton–HCl, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P-40] containing complete protease inhibitor cocktail (Roche), 10 mM sodium orthovanadate, and 5 mM sodium fluoride for 20 min on ice. The cell lysates were harvested from the culture dishes and cellular debris was removed by centrifugation. Tyrosine phosphorylation of the erbB receptors was determined by immunoblotting with antiphosphotyrosine horseradish peroxidase (HRP) conjugated antibody Ab-4 (Oncogene, Boston, MA). Proteins were detected using the ECL-Plus chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). To identify total erbB1 receptors, the same immunoblot was stripped of antiphosphotyrosine and re-probed with anti-erbB1 antibodies (Cell Signaling Tech, Beverly, MA).

RESULTS

Time and dose dependence of UVB-induced apoptosis in HaCaT cells  The response of HaCaT cells to UVB irradiation has been extensively studied. Various investigators have shown that HaCaT cells undergo apoptosis following UVB irradiation in a time- and dose-dependent manner. Unfortunately, the actual dose of UVB required to induce apoptosis varies with each report depending on the methodology of irradiation used and the methods used to detect apoptosis. For that reason, it is important to define the response of HaCaT cells to UVB in these experiments. We have assessed the induction of apoptosis using an assay that measures the activity of the caspase 3 enzyme. Previous data from our laboratory have shown that the induction of caspase 3 perfectly correlates with the induction of other measures of apoptosis (Barber et al, 1998; Peus et al, 2001a). Because the erbB1 receptor is not the only erbB family member that becomes activated in increased sensitivity of UVB-induced apoptosis (Stoll et al, 1998; Peus et al, 2001a). Because the erbB1 receptor provides a protective function from UVB-induced apoptosis. Inhibition of the erbB1 receptor using high-affinity, specific inhibitors resulted in increased sensitivity of UVB-induced apoptosis (Stoll et al, 1998; Peus et al, 2001a). Because the erbB1 receptor is not the only erbB family member that becomes activated in increased sensitivity of UVB-induced apoptosis.

Pharmaceutical inhibition of erbB family members suppresses the induction of apoptosis by UVB  Previous studies have demonstrated that the erbB1 receptor provides a protective function from UVB-induced apoptosis. Inhibition of the erbB1 receptor using high-affinity, specific inhibitors resulted in increased sensitivity of UVB-induced apoptosis (Stoll et al, 1998; Peus et al, 2001a). Because the erbB1 receptor is not the only erbB family member that becomes activated in increased sensitivity of UVB-induced apoptosis.

Figure 1. Dose- and time-dependent induction of HaCaT cell apoptosis by UVB. HaCaT cells were irradiated with UVB as described in Materials and Methods using the doses indicated in the figure (A) or at 400 J per m² for the various times listed (B). For (A), the cells were harvested and assayed for caspase 3 activity at 9 h following irradiation.
activated in response to UVB irradiation (Jost et al., 2000; Olayioye et al., 2000; Bogdan and Klambt, 2001; Yarden and Sliwkowski, 2001), we used an inhibitor of both erbB1 and erbB2 receptors (DAPH) and assayed for the influence of both receptors on the sensitivity of HaCaT cells to UVB (Noonberg and Benz, 2000). HaCaT cells were irradiated with a dose of 0 or 400 J per m² of UVB. Immediately following irradiation, the cells were treated with 0, 1, 5, 10, or 30 µM DAPH. Cells were photographed 9 h following UVB irradiation.

Figure 2. The erbB family inhibitor DAPH reduces the induction of apoptosis by UVB. HaCaT cells were irradiated with a dose of 0 or 400 J per m² UVB. Immediately following irradiation, the cells were treated with 0, 1, 5, 10, or 30 µM DAPH. Cells were photographed 9 h following UVB irradiation.

Figure 3. Inhibition of erbB family receptors reduces the induction of apoptosis by UVB. HaCaT cells were irradiated with a dose of 0 or 400 J per m² UVB. Immediately following irradiation, the cells were treated with 0, 1, 5, 10, or 20 µM (A) DAPH, (B) AG 1295, or (C) combinations of AG 825 and AG 1478 as indicated. Cells were harvested 9 h following UVB irradiation and cell lysates were assayed for caspase 3 enzyme activity. Error bars signify (+/−) standard deviation and are representative of two or three different experiments. In (C), the caspase 3 specific activity from HaCaT cells treated with AG 825 or AG 1478 and irradiated with 400 J per m² of UVB was not significantly different from the caspase 3 specific activity of untreated HaCaT cells irradiated with 400 J per m² of UVB (p > 0.05, Student t test). Similarly, there was no significant difference between the caspase 3 specific activities of HaCaT cells treated with DAPH or AG 825 and AG 1478 that were irradiated with 400 J per m² of UVB (p > 0.05). There was a significant difference, however, between HaCaT cells treated with DAPH or AG 825 and AG 1478 irradiated with 400 J per m² and HaCaT cells that were untreated and irradiated with 400 J per m² UVB (p < 0.05).

concentrations of DAPH. At 10 µM DAPH, the induction of caspase 3 specific activity by UVB was reduced by 65%, and at 20 µM DAPH it was reduced by 78%. No adverse effects on HaCaT cell morphology were observed using 20 µM DAPH.

UVB has been demonstrated to activate a variety of cell surface receptors in the absence of ligand (Sachsenmaier et al., 1994; Huang et al., 1996). Therefore, the effect of DAPH on the
induction of UVB-induced apoptosis could be a generalized response to the “capping” phenomenon of cell surface receptors to UVB exposures. To examine this possibility, we used a small molecule inhibitor of the platelet-derived growth factor receptor (AG 1295) to see if the effects we described for the erbB family receptors were specific for DAPH or were more generalized to all tyrosinase-like molecules. In contrast to the results we describe for DAPH, AG 1295 had little to no effect on UVB-induced apoptosis (Fig 3B). This result implies that the effect of DAPH on UVB-induced apoptosis is specific for the inhibition of erbB family receptors.

The tyrosine kinase activity of the erbB1 receptor can be specifically inhibited by the drug AG 1478, whereas the erbB2 tyrosine kinase activity is suppressed by AG 825. If DAPH was able to suppress UVB-induced apoptosis via its ability to inhibit both erbB1 and erbB2 tyrosine kinase activities, then treating HaCaT cells with a combination of AG 1478 and AG 825 should yield similar results to DAPH. Treatment of HaCaT cells with AG 825 or AG 1478 individually resulted in a statistically insignificant decrease in UVB-induced apoptosis (Fig 3C). In contrast, when HaCaT cells were UVB-irradiated and immediately treated with a combination of AG 825 and AG 1478, there was a protection of UVB-induced apoptosis similar to that in HaCaT cells that were treated with DAPH (Fig 3C). These data demonstrate that the simultaneous inhibition of both erbB1 and erbB2 tyrosine kinase activities leads to a suppression of UVB-induced apoptosis.

The inhibitory activity of DAPH was confirmed by analyzing the tyrosine phosphorylation status of erbB family receptors. HaCaT cells were treated with DAPH for 1 h, and subsequently erbB receptors were activated by treating the cells with EGF or UVB. As shown in Fig 4, treatment of HaCaT cells with either EGF or UVB led to the tyrosine phosphorylation of erbB family receptors. Due to the potential heterodimerization of erbB1 and erbB2 receptors and their similar apparent molecular weights, we cannot distinguish between erbB1 and erbB2 receptor activation in these assays. Pretreatment of the HaCaT cells with DAPH, however, completely inhibited the activation of erbB receptors following UVB irradiation and substantially reduced the phosphorylation of erbB receptors after the addition of EGF. These data confirmed the validity of using the drugs for their intended purposes.

Immobilization of the erbB1 receptor also suppresses UVB-induced apoptosis. In order to confirm the results obtained using the pharmaceutical inhibitor DAPH, the activity of the erbB1 receptor was also inhibited through the use of neutralizing antibodies prior to UVB irradiation. As erbB2 homodimers have not been described to occur in keratinocytes, the use of a neutralizing antibody to erbB1 should suppress the activation of both erbB1 and erbB2 due to stearic hindrance of erbB1 mobility in the plasma membrane. This mechanical inhibition of the erbB1 receptor will effectively inhibit both the erbB1 homodimers and erbB1:erbB2 heterodimers. Following treatment with the neutralizing antibody, the induction of caspase 3 activity by UVB was reduced by 70% (Fig 5), whereas irrelevant control antibodies had no effect on UVB irradiation. UVB-induced apoptosis could also be suppressed by pretreating the HaCaT cells with neutralizing antibodies to erbB2. These data imply that erbB1:erbB2 heterodimers play a substantial role in UVB-induced apoptosis, but further studies are required to more completely delineate their relationship. Nevertheless, the inhibition of the erbB family receptors using two different mechanisms resulted in the suppression of UVB-induced apoptosis.

Suppression of apoptosis by DAPH is specific for UVB. Apoptosis can be induced in cells by a variety of different types of factors. To determine if the effect of DAPH on UVB-induced apoptosis was a generalized response to apoptotic factors or if it was specific for UVB, we used an alternative method to induce apoptosis in HaCaT cells. The tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in HaCaT cells through the activation of the death receptors DR4 and DR5 (Kothny-Wilkes et al, 1998; Southall et al, 2001). HaCaT cells were treated with DAPH or the neutralizing anti-erbB1 antibody for 30 min prior to the addition of TRAIL. As seen in Fig 6, the addition of DAPH or the neutralizing antibody did not influence the induction of apoptosis by TRAIL. This suggests that the suppressive effects of DAPH on the induction of apoptosis are limited to a protective response specifically to UVB.

**DISCUSSION**

Exposure of HaCaT keratinocytes to UVB leads to the induction of apoptosis in a time- and dose-dependent manner, as measured

![Figure 4](image_url)  
**Figure 4.** DAPH inhibits UVB-dependent erbB family receptor tyrosine phosphorylation. HaCaT cells were pretreated with 10 μM DAPH or left untreated for 60 min. The keratinocytes were then untreated, treated with 20 ng per ml EGF, or exposed to 400 J per m² of UVB. Cells were harvested 15 min following EGF or UVB treatment and cell lysates were assayed for erbB1 tyrosine phosphorylation. Equal loading of each cell lysate was confirmed by stripping each immunoblot and reprobing with erbB1-specific antibodies.

![Figure 5](image_url)  
**Figure 5.** Neutralizing antibodies to erbB1 and erbB2 suppress UVB-induced apoptosis. HaCaT cells were untreated or treated with 1 μg per ml neutralizing anti-erbB1 antibody, 1 μg per ml neutralizing anti-erbB2 antibody, or 1 μg per ml nonspecific mouse IgG antibody for 30 min. The cells were then irradiated with a dose of 0 or 400 J per m² UVB. Cells were harvested 9 h following UVB irradiation and cell lysates were assayed for caspase 3 enzyme activity. Error bars signify (+/−) standard deviation and are representative of two to three different experiments.
Figure 6. The role of erbB family receptors in apoptosis is specific for UVB. HaCaT cells were untreated, treated with 10 μM DAPH, or treated with 1 μg per ml neutralizing anti-erbB1 antibody for 30 min. The cells were then irradiated with a dose of 400 J per m² UVB or treated with 40 ng per ml TRAIL. Cells were harvested 9 h following the induction of apoptosis and cell lysates were assayed for caspase 3 enzyme activity. Error bars signify (+/−) standard deviation and are representative of two or three different experiments.

by increases in caspase 3 activity. Maximal induction of UVB-induced apoptosis in our experimental set-up occurred at a UVB dose of 400 J per m², 9 h following irradiation. Treatment of HaCaT cells with either a pharmaceutical or a physical inhibitor of the erbB receptor family, however, could substantially inhibit the induction of apoptosis. The protective effect on apoptosis by the erbB receptor inhibitors was specific for UVB-induced apoptosis because the inhibitors had no effect on the induction of apoptosis by TRAIL.

Although the exact pathway by which UVB induces apoptosis has yet to be elucidated, clues to the mechanism are beginning to emerge. It should not be too surprising that many of the pathways that have been identified are specific for certain types of cells. For example, treatment of primary NHK with hepatocyte growth factor/scatter factor will abrogate UVB-induced apoptosis via phosphatidyl inositol-3 kinase and Akt activation (Mildner et al., 2002). Even though the Akt pathway is functionally intact in HaCaT cells, hepatocyte growth factor/scatter factor does not provide any protection from UVB-induced apoptosis. There are some similarities in the UVB response of HaCaT cells and NHK, however. Inhibition of the stress-response mitogen-activated protein kinase p38 leads to a partial suppression of UVB-induced apoptosis and that these results have implications for UVB exposure of actively proliferating keratinocytes in the epidermis.

sensitization of UVB-induced apoptosis (Stoll et al., 1998; Peus et al., 2000; Jost et al., 2001a). These results appear to be in contrast to the data presented in this paper, which describe a protective effect of erbB receptor inhibition of UVB-induced apoptosis. It has also been reported that inhibitors of cellular tyrosine kinase activity in HaCaT cells will prevent UVB-induced apoptosis (Fukunaga et al., 2001). This dissimilitude of results can be resolved by the difference in the inhibitors used in each of the studies. In this paper, a pharmaceutical inhibitor that inactivates both the erbB1 and erbB2 receptors was used. Previous studies describing a protective function for the erbB1 receptor have used highly specific small molecules that specifically inhibit the erbB1 receptor activity without affecting erbB2 receptor activity. The importance of these results is evident as UVB has been reported to cause the tyrosine phosphorylation of both the erbB1 and erbB2 receptors. erbB1:erbB2 heterodimerization has been described in other epithelial cells and is known to alter erbB1 function (Mathuswamy et al., 1999; 2001; Zhang et al., 2002). Hence, we hypothesize that the dual specificity of DAPH on erbB1 and erbB2 tyrosine kinase activity alters the response of the HaCaT cells to UVB irradiation in a manner that is distinctly different from inhibition of erbB1 alone.

The analysis of erbB receptor function in keratinocytes, and specifically HaCaT cells, is only just beginning. Similar to NHKs, HaCaT cells uniformly express the erbB1, erbB2, and erbB3 family members (Marques et al., 1999). Following ligand binding, heterodimerization of all combinations of these three receptors has been described. It is still unclear, however, what the consequences of heterodimerization are. Heterodimerization of erbB1:erbB2 can lead to changes in cellular localization, substrate interactions, and receptor stability compared to erbB1 homodimers. For instance, Zhang et al. (2002) have shown that erbB1 homodimers lead to raf recruitment to the cell membrane and raf activation, followed by rapid dissociation of raf and erbB1 from the membrane. In contrast, erbB1:erbB2 heterodimers also lead to raf membrane recruitment and activation, but raf and the erbB receptors are maintained at the cell surface. Interestingly, we have preliminary evidence that UVB activation of erbB receptors remain cell surface associated and remain activated for extended periods of time (personal communication). Therefore, we are actively pursuing the possibility of UVB-induced heterodimerization of erbB1 and erbB2.

In conclusion, we have presented unique data that suggest that the inhibition of the erbB family of tyrosine kinase receptors protects HaCaT cells from UVB-induced apoptosis. We further propose that both the erbB1 and erbB2 receptor tyrosine kinase activity must be inactivated to observe the inhibition of UVB-induced apoptosis and that these results have implications for UVB exposure of actively proliferating keratinocytes in the epidermis.

REFERENCES


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