Keratinocyte growth factor protects epidermis and hair follicles from cell death induced by UV irradiation, chemotherapeutic or cytotoxic agents

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Summary

Owing to its potent cytoprotective properties for epithelial cells, keratinocyte growth factor (KGF) is successfully used for the treatment of chemotherapy- and radiotherapy-induced oral mucositis in cancer patients. It is therefore of major interest to determine possible clinical applications of KGF in other organs and in different stress situations and to unravel common and organ-specific mechanisms of KGF action. Here we show that KGF protects human keratinocytes from the toxicity of xenobiotics with electrophilic and oxidative properties and reduces the cell death induced by UV irradiation. In contrast to other cell types, cytoprotection of keratinocytes by KGF is not a direct anti-apoptotic effect but requires de novo protein synthesis. The in vitro findings are clinically relevant because KGF protected keratinocytes in organ-cultured human scalp hair follicles from the toxicity of the xenobiotic menadione. Moreover, injection of KGF into murine back skin markedly reduced cell death in the epidermis after UVB irradiation. This activity is dependent on FGF receptor signaling because it was abrogated in transgenic mice expressing a dominant-negative FGF receptor mutant in keratinocytes. Taken together, our results encourage the use of KGF for skin protection from chemical and physical insults.

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Key words: FGF, Keratinocyte, UV, Reactive oxygen species, Regenerative medicine

Introduction

KGF, a member of the fibroblast growth factor (FGF) family (FGF7), is an important mitogen for epithelial cells (Rubin et al., 1989). It is mainly produced by mesenchymal cells and γδ T cells in various organs, and it acts in a paracrine manner on epithelial cells. The latter express its only high-affinity receptor, a splice variant of fibroblast growth factor receptor 2 (FGFR2IIIb) (reviewed by Werner, 1998; Finch and Rubin, 2004).

Interestingly, KGF exerts a potent cytoprotective effect on epithelial cells in different organs, e.g. the gastrointestinal tract, the lung, the bladder, and murine hair follicles (reviewed by Werner, 1998; Finch and Rubin, 2004). In the lung, administration of recombinant KGF inhibited Fas-mediated apoptosis of epithelial cells (Bao et al., 2005), reduced the number of DNA strand breaks (Wu et al., 1998) and alleviated the oxidant-induced increase in cell permeability and the decrease in trans-epithelial electrical resistance (Waters et al., 1997; Chapman et al., 2002). In the murine small intestine, the epithelial damage caused by radiation and/or chemotherapy was less severe upon KGF pre-treatment, and this manipulation resulted in a remarkable reduction of the mortality rate, in reduced weight loss and in a significant increase in mucosal thickness and crypt survival (Khan et al., 1997; Farrell et al., 1998). Finally, protective effects were also reported for chemotherapy- or X-ray-induced hair loss in mice (Danilenko et al., 1995; Booth and Potten, 2000), indicating that KGF may also be cytoprotective for human skin and thus suitable for therapeutic application. This hypothesis is supported by the potent cytoprotective effect of KGF for oral epithelial cells in vivo. Thus, patients undergoing bone marrow transplantation treatment for hematological malignancies often develop severe oral mucositis as a side effect of the radio- and chemotherapy. In clinical studies, treatment of these patients with KGF before the beginning of the therapy decreased the duration and incidence of the oral mucositis owing to a better resistance of epithelial cells to the toxic insult (Spielberger et al., 2004), and recently KGF has been approved for use in these patients. The mechanisms that underlie the protective effect of KGF in vivo, have been studied in the lung and shown to depend on a direct anti-apoptotic effect mediated via phosphoinositide 3-kinase (PI3K) signaling (Bao et al., 2005; Pan et al., 2004). By contrast, the mechanisms underlying the cytoprotectivity in squamous epithelia are largely unknown.

In this study, we found a potent cytoprotective effect of KGF for human keratinocytes in vitro and in vivo, we characterized the specificity of this effect with regard to different chemical and physical insults, and we analyzed the underlying mechanisms.

Results

KGF is cytoprotective for human keratinocytes in vitro

To determine whether KGF protects human keratinocytes from cell damage induced by xenobiotics, we first established an in vitro assay. Quiescent keratinocytes were treated overnight and the next morning for an additional hour with KGF, and subsequently with different concentrations of the cytotoxic agent menadione. Two KGF treatments were performed to allow the regulation of early and late KGF target genes. The second treatment was also performed because anti-apoptotic KGF-mediated signaling pathways (PI3K and mitogen-activated kinase pathways) are still activated 1 hour after addition of the growth factor (Fig. 3A, lane 10). Menadione was chosen as a xenobiotic because it is a stable and potent ROS producer (Thor et al., 1982). Viable cells were quantified using the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay, which measures mitochondrial activity. Under these experimental conditions, KGF did not affect cell proliferation. The cell number was identical in the vehicle-treated and the KGF-treated population, but the survival of starved cells was enhanced in the presence of KGF. Thus, even in the absence of toxins, we observed higher absorption values in the MTT assay for starved KGF-treated cells compared with non-treated cells. This was taken into account for the analysis of the results (see legend to Fig. 1).

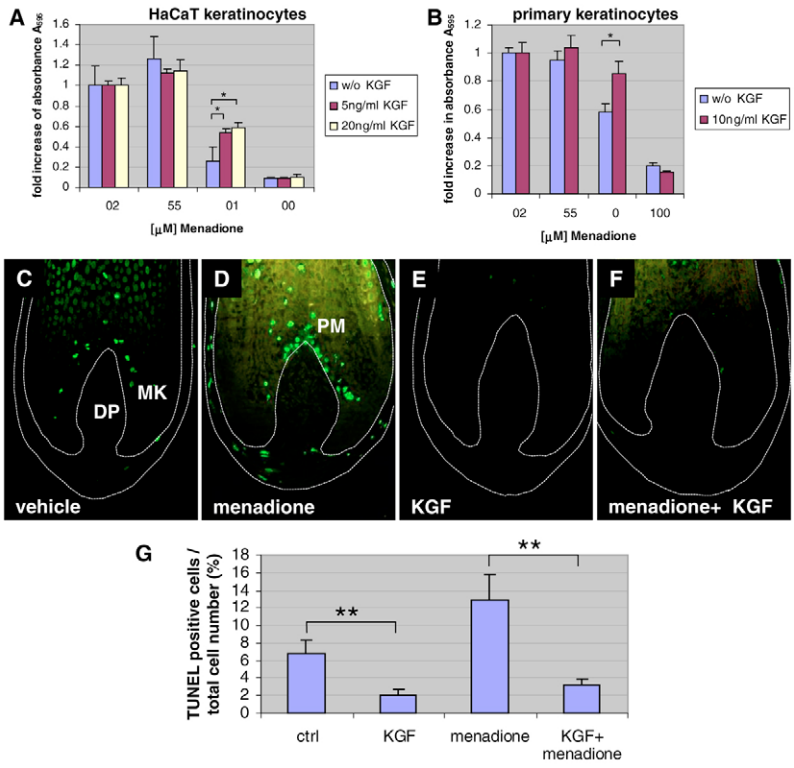

Fig. 1. KGF is cytoprotective for keratinocytes in vitro and for hair follicle keratinocytes in organ culture. (A,B) Quiescent keratinocytes were pre-treated with KGF or vehicle for 16 hours and 1 hour before the addition of menadione. Results of representative experiments of MTT assays with HaCaT keratinocytes (A) or primary human foreskin keratinocytes (B) are shown. All measurements were performed in quadruplicate and the means ± s.d. are shown. The values obtained with the non-treated cells and the KGF-treated cells without application of the cytotoxic agent were arbitrarily set as 1. This was necessary because KGF affects the survival of starved cells, i.e. although the cell number is the same in the non-treated and the KGF-treated population, the mitochondrial activity is higher in the KGF-treated cell population. (C-F) Human anagen VI follicles were isolated from scalp skin, pre-treated with 20 ng/ml KGF for 12 hours and for one additional hour with fresh KGF, and subsequently incubated with 50 µM menadione. Frozen sections from the treated hair follicles [(C) vehicle treatment; (D) 50 µM menadione; (E) 20 ng/ml KGF; (F) 20 ng/ml KGF and 50 µM menadione], were analyzed by TUNEL staining for the presence of apoptotic cells. DP, dermal papilla; MK, matrix keratinocytes; PM, precortical matrix. (G) TUNEL-positive cells and all cells (excluding the cells in the dermal papilla) were counted and the ratio between both numbers was determined (ctrl, n=10; menadione, n=9; KGF, n=11; KGF+menadione, n=12; n, number of hair follicles). Means ± s.e.m. are shown. Statistical analysis was performed using the Mann-Whitney U test. *P<0.05; **P<0.01.

When we treated HaCaT keratinocytes with intermediate concentrations of menadione (50 µM) we could detect more viable cells in the KGF-treated compared with the untreated cell population (Fig. 1A). At lower menadione concentrations (25 µM), which apparently did not significantly damage the cells, and at higher menadione concentrations (100 µM), where the damage was so severe that the mitochondrial activity of the cells dropped to 10-20% of the initial activity, KGF had no obvious cytoprotective effect.

Since similar results were obtained with the immortalized, but non-tumorigenic human keratinocyte cell line HaCaT (Fig. 1A) and with primary human foreskin keratinocytes (Fig. 1B), we performed further experiments with HaCaT cells. In addition to the MTT assay, we also used readout systems, which allow the quantification of dead cells with a damaged cell membrane: determination of lactate dehydrogenase (LDH) activity in the cell supernatant and propidium iodide staining of the nuclei. The results obtained with these assays confirmed the data obtained with the MTT assay (see supplementary material Fig. S1). Under the same conditions, EGF, which is known to exhibit anti-apoptotic properties for keratinocytes (Rodeck et al., 1997; Sibilia et al., 2000), did not protect from cell death induced by menadione (data not shown). The concentration of EGF that we used (20 ng/ml) efficiently regulated the expression of EGF target genes in HaCaT cells (Braun et al., 2006) and activated Erk1/2 and PI3K in these cells (Fig. 3).

KGF reduces menadione-induced keratinocyte apoptosis in human hair follicles in situ

To verify the cytoprotective effect of KGF under more physiologically and clinically relevant conditions, a complex, highly damage-sensitive, prototypic epithelial-mesenchymal interaction system was used – the hair follicle (Paus and Cotsarelis, 1999). Maximally growing (i.e. anagen VI) hair follicles from normal human scalp skin were isolated, micro-dissected and organ-cultured (Philpott et al., 1990; Magerl et al.,

2004). They were treated overnight (12 hours) and the next morning for an additional hour with KGF, and subsequently with 50 μM menadione. As shown in Fig. 1D,G, menadione induced apoptosis in hair matrix keratinocytes, and this effect was prevented by KGF (Fig. 1F,G). In hair follicles that were not treated with menadione, KGF reduced the number of apoptotic keratinocytes in organ culture (compare Fig. 1E with 1C, quantification of apoptotic, i.e. TUNEL-positive cells in Fig. 1G). Thus, KGF is obviously a survival factor for normal hair follicle keratinocytes in situ under conditions simulating the in vivo situation and effectively counteracts the cytotoxicity of menadione in these cells.

Cytoprotection is dependent on de novo protein synthesis and does not rely on a single signaling pathway

To analyze whether the cytoprotective effect of KGF is a direct anti-apoptotic effect we first determined whether both the overnight and the 1-hour incubation with KGF are necessary to protect cells efficiently from cytotoxic damage. When added for only 1 hour, KGF was not effective at all. The incubation overnight plus 1 hour was slightly more effective compared with the overnight incubation alone, although the difference was not statistically significant (Fig. 2A). A 4-, 6- or 8-hour KGF treatment slightly enhanced survival of keratinocytes treated with 50 μM menadione in comparison with cells that were grown in the absence of KGF, however, the difference was not statistically significant (data not shown). The requirement of a rather long KGF treatment suggested that the cytoprotective effect of KGF requires de novo protein synthesis. Indeed, when the protein synthesis inhibitor cycloheximide (CHX; 10 μg/ml) was added 2 hours before KGF, the protective effect of KGF was completely abolished (Fig. 2B).

Next, we determined the signaling pathways, which could mediate the cytoprotective effect of KGF. For this purpose, we analyzed activation of PI3K, mitogen-activated protein kinase (MAPK) as well as protein kinase C (PKC) signaling pathways by analyzing the levels of phosphorylated target proteins characteristic for each pathway (phospho-Akt1/2/3, phospho-Erk1/2, phospho-p38, phospho-JNK, phospho-MARCKS) by western blotting. These pathways had previously been shown to be activated by KGF in other cell types (Portnoy et al., 2004; Zeigler et al., 1999; Le Panse et al., 1994). In keratinocytes, p38, JNK and MARCKS were not phosphorylated upon KGF treatment, whereas phosphorylation of Erk1/2 and Akt1/2/3 occurred in response to KGF (Fig. 3A, lanes 2,6,10). The levels of phosphorylated Erk1/2 and Akt1/2/3 reached a maximum at around 5 minutes. The signals decreased in intensity 25 minutes after stimulation, but were still higher compared with the control after 50 minutes.

Subsequently, we examined the roles of the PI3K and MAPK pathways in the cytoprotective effect of KGF. For this purpose, we applied the MEK1/2 inhibitors U0126 (Fig. 3A, lanes 3,7,11) and PD98059 (data not shown), the PI3K inhibitor LY294002 (Fig. 3A, lanes 4,8,12) and a combination of MEK1/2 (U0126) and PI3K (LY294002) inhibitors (Fig. 3A, lanes 5,9,13). We first determined the efficacy and specificity of these inhibitors. The loss of Akt phosphorylation in the presence of the LY294002 inhibitor (Fig. 3A, lane 4) and the inhibition of Erk1/2 phosphorylation by U0126 (Fig. 3A, lane 3) confirmed the efficacy of the inhibitors at the concentrations used. When the PI3K inhibitor LY294002 was added to the cells we did not observe a change in the levels of phosphorylated Erk1/2, p38, JNK or MARCKS (Fig. 3A, lanes 4, 8 and 12). By contrast, when the MEK1/2 inhibitor U0126 was applied, Akt1/2/3 phosphorylation was enhanced and prolonged and additionally, p38 and JNK pathways were activated (Fig. 3A, lanes 3, 7 and 11). In the presence of both inhibitors (Fig. 3A, lanes 5, 9 and 13) we detected no further changes compared with the U0126 inhibitor alone, indicating that only the inhibition of Erk1/2 signaling is compensated, but not the blockade of PI3K signaling. Therefore, both inhibitors efficiently and specifically inhibit their target signaling pathways under our experimental conditions, although compensatory effects were observed upon inhibition of MEK1/2 activation.

When we analyzed the cytoprotectivity of KGF in the presence of the LY294002 inhibitor and/or the U0126 inhibitor, we still observed a cytoprotective effect of KGF in both cases (Fig. 3B). The difference in survival between KGF-treated and untreated cells was often even more pronounced in the presence of an inhibitor, in particular in the presence of the MEK1/2 inhibitor. However, the overall absorption values in the MTT assays were decreased in the inhibitor-treated cells compared with the untreated cells. This suggests that HaCaT

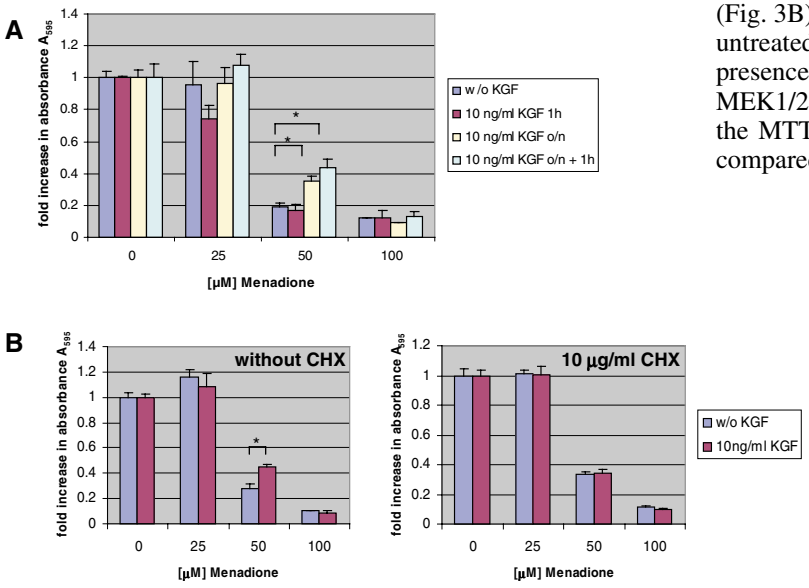

Fig. 2. The protective effect of KGF is dependent on de novo protein synthesis. (A) In cytoprotection assays (see legend to Fig. 1A) quiescent HaCaT cells were pre-treated with KGF for different periods of time (1 hour, overnight, or overnight plus 1 hour) or left untreated before the addition of menadione. (B) Results of cytoprotection assays in the absence or presence of the protein biosynthesis inhibitor cycloheximide (CHX) are shown: 2-hour treatment with DMSO before KGF addition (left panel), 2-hour treatment with 10 μg/ml CHX in DMSO before KGF addition (right panel). Results are mean ± s.d. *P<0.05 using the Mann-Whitney U test.

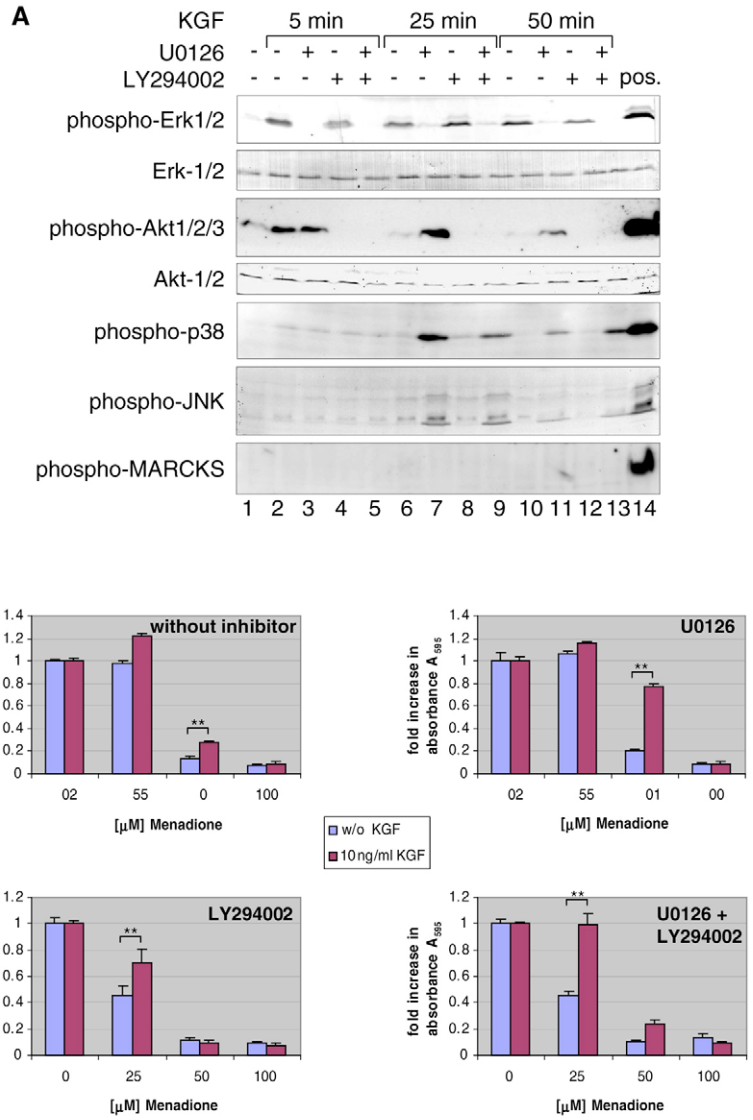

Fig. 3. The protective effect of KGF is not dependent on a single signaling pathway. (A) HaCaT keratinocytes were rendered quiescent by serum starvation (lane 1), treated with either the MEK1/2 inhibitor U0126 (lanes 3,7,11), the PI3K inhibitor LY294002 (lanes 4,8,12), a combination of both inhibitors (lanes 5,9,13) or solvent control (lanes 2,6,10), and subsequently incubated with 10 ng/ml KGF for the time periods indicated. Whole cell lysates were analyzed for the activation of Erk1/2, Akt1/2/3, MARCKS, JNK and p38 using phospho-specific antibodies. Levels of total Erk1/2 and total Akt1/2 were determined as loading controls. As positive controls (pos., lane 14), cells were treated with EGF (20 ng/ml for 5 minutes) for activation of Erk1/2 and Akt1/2/3, UVB irradiated (40 mJ/cm^2 and incubated for 45 minutes after irradiation) for activation of JNK and p38, or treated with phorbol-12-myristate-13-acetate (200 nM, 30-minute incubation) for activation of MARCKS. The results were reproduced in three independent experiments. (B) Cytoprotection assays were carried out in the absence (upper left panel) or presence of inhibitors of different signaling pathways: MEK1/2 inhibitor U0126 (10 μM, upper right panel), PI3K inhibitor LY294002 (10 μM, lower left panel), U0126 and LY294002 (10 μM each, lower right panel). Results are mean ± s.d. $^{**}P<0.01$ using the Mann-Whitney *U* test.

keratinocytes were already stressed and/or damaged by the inhibitors, especially by the PI3K inhibitor LY294002. As a consequence, the menadione concentrations at which KGF was able to protect from cell death were lower (25 μM instead of 50 μM, Fig. 3B lower panels) when cells were pre-treated with LY294002.

When we used a combination of MAPK signaling pathway inhibitors, i.e. inhibitors against Erk1/2, p38 (SB203580 or SB202190), and JNK (JNK inhibitor II) at concentrations, which had been shown to be effective in keratinocytes (Park et al., 2005; Van Dross et al., 2005), we still observed a cytoprotective effect of KGF. These results demonstrate that neither MAPK signaling nor PI3K signaling is essential for the cytoprotective effect. Rather, the latter can obviously be mediated via different signaling pathways. The evident experiment to inhibit p38 and JNK pathways, in addition to Erk1/2 and PI3K pathways, was not possible because the cells underwent apoptosis in the presence of all four inhibitors (data not shown).

KGF prevents cell death induced by chemicals with electrophilic and ROS-producing properties

We next determined the cytoprotectivity of KGF towards different chemical and physical insults (Table 1). We tested reactive oxygen species (ROS)-induced damage by using hydrogen peroxide (H_2O_2), a strong oxidant, as well as paraquat and 2,5-di-tert-butylhydroquinone (di-tBHQ), which produces superoxide anions (Suntres, 2002; Nakamura et al., 2003). Surprisingly, KGF did not protect from the cytotoxicity of these substances. Since many ROS-producing agents also have electrophilic properties, we carried out experiments with some representatives of this group, e.g. menadione and tert-butylhydroquinone (tBHQ) (Nakamura et al., 2003). Indeed, KGF potently protected keratinocytes from the toxicity of these reagents (Table 1). Remarkably, cyclophosphamide, an alkylating, DNA-cross-linking chemotherapeutic agent of the nitrogen mustard group (Brunton et al., 2006), which was used in the clinical study where KGF protected from mucositis (Spielberger et al., 2004) and its cell culture-active derivative mafosfamide, also belong to this group of toxic substances. Most interestingly, KGF also protected from cell death induced by mafosfamide, demonstrating that the protective effect is clinically relevant. Finally, we applied staurosporine, which induces apoptosis via a mitochondrial pathway (Kruman et al., 1998) or diethyl maleate, which is cytotoxic through depletion of intracellular glutathione. However, KGF failed to protect keratinocytes from the toxicity of both substances (Table 1). Taken together, KGF appears to protect predominantly from the toxicity of chemicals, which have both electrophilic and ROS-producing properties.

Since UV irradiation is a major environmental challenge for cells of the skin that can induce both keratinocyte apoptosis and necrosis (Schwarz, 2005), we also investigated the effect

Table 1. Cytotoxic agents that have been tested

Cytotoxic reagent	Toxicity	Protective effect of KGF	KGF-sensitive dose*
H_2O_2	ROS	–	
Paraquat	ROS	–	
di-tBHQ	ROS	–	
Menadione	ROS + electrophile	+	50 μM
tBHQ	ROS + electrophile	+	200 μM
Mafosfamide	ROS + electrophile	+	100 μM
Diethyl maleate	Glutathione depletion	–	
Staurosporine	Apoptosis inducer	–	
UVA and UVB irradiation	ROS, DNA damage, apoptosis	+	10 J/cm^2 (UVA) 50 mJ/cm^2 (UVB)

*The ability of KGF to protect cells from the cytotoxic insult and the doses of toxic reagents that are sensitive to KGF treatment are indicated.

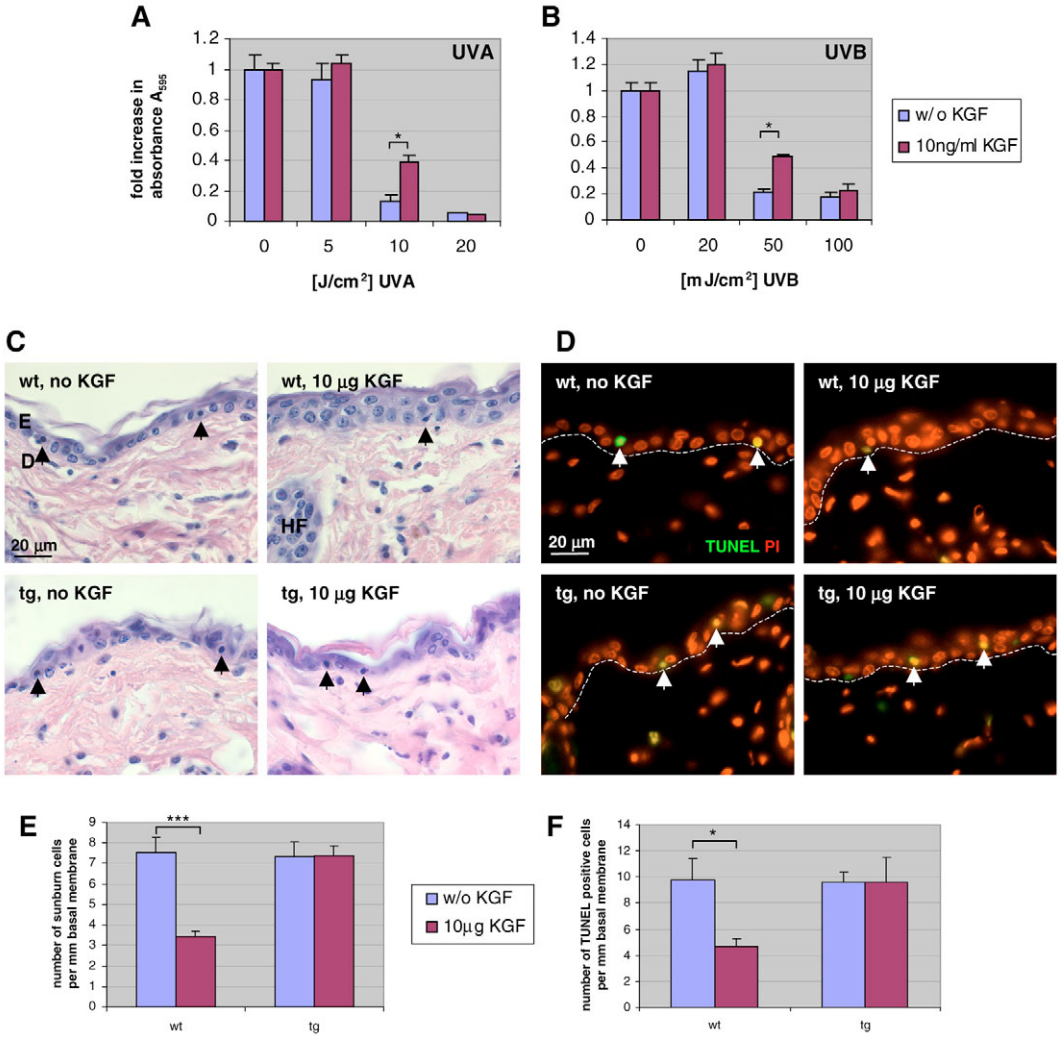

Fig. 4. KGF protects keratinocytes from UV-induced cell damage in vitro and in vivo. (A,B) Quiescent HaCaT cells were treated with KGF or vehicle 16 hours and 1 hour before irradiation with different doses of UVA (A) and UVB (B) as indicated. UV-irradiated cells were subjected to MTT assay 24 hours after irradiation. (C) The shaved back skin of transgenic mice expressing a dominant-negative FGFR2IIIb (tg) and their wild-type littermates (wt) was irradiated with 100 mJ/cm^2 UVB. One group of mice of each genotype received a subcutaneous injection of 10 μg KGF 24 hours before irradiation (n=8 for each genotype), whereas the control group (n=7 for each genotype) was only injected with the solvent (0.5% BSA in PBS). Representative histological pictures of each treatment group and genotype are depicted. The arrows indicate sunburn cells. E, epidermis; D, dermis; HF, hair follicle. (D) Apoptotic cells (arrows) were detected by TUNEL staining (green). Propidium iodide (PI) staining (red) was used to visualize the nuclei. The basement membrane is indicated with a dashed line. Four animals were used of each genotype for vehicle control and five animals of each genotype for KGF injections. Sunburn cells (E) or TUNEL-positive cells (F) were counted and their number per mm basement membrane was calculated. For quantitative analyses in E and F one section from each animal was analyzed; ten pictures were taken from each section. Results are mean ± s.d. For statistical analysis the Mann-Whitney U test was used. *P<0.05; ***P<0.001.

of KGF on keratinocytes irradiated with doses of UVA and UVB, which are within the physiological range. Interestingly, KGF-treated cells were more resistant to UVA- and UVB-induced damage compared with non-treated cells (Fig. 4A,B). Consistent with the data obtained with menadione (Fig. 1A), KGF was effective at intermediate doses of UVA and UVB. It had no effect at UV doses that do not severely harm the cells and at doses that significantly damage the majority of the cells. In agreement with the menadione data (Fig. 2A), a single 1-hour KGF treatment was not sufficient for KGF to exert its protective effect against UV irradiation (data not shown).

KGF protects keratinocytes from UVB-induced cell death in vivo

Finally, we determined the relevance of the cytoprotective effect of KGF for keratinocytes in vivo. We injected 10 μg KGF or vehicle control subcutaneously into the backs of mice. After 24 hours, mice were irradiated with 100 mJ/cm^2 UVB. After additional 16-20 hours, the tissue was removed and processed for histological analysis. In the KGF-treated mice we observed a hyperthickened epidermis (Fig. 4C, upper panel) as expected from the known mitogenic activity of the growth factor (Rubin et al., 1989). Most interestingly, we found that KGF injection significantly decreased the number of 'sunburn' cells, apoptotic epidermal keratinocytes morphologically characterized by a pycnotic nucleus and a shrunken cytoplasm (Daniels et al., 1961) (Fig. 4C,E). The same result was obtained when we performed TUNEL assay, an independent biochemical method to assess the number of apoptotic cells (Fig. 4D upper panel, F). Thus, in addition to its recognized mitogenic effect, KGF is able to promote survival of UV-irradiated keratinocytes in vivo.

To determine whether the observed cytoprotective effect of KGF in vivo is mediated via its high affinity receptor, we also performed the experiment with transgenic mice expressing a dominant-negative mutant of FGFR2IIIb under the control of the keratin 14 promoter in basal cells of the epidermis and in the outer root sheath of hair follicles (Werner et al., 1994). These cells also express the endogenous receptor (Danilenko et al., 1995) (our unpublished data). The dominant-negative FGFR mutant inhibits signaling by all FGFs that bind to this receptor, including KGF (Werner et al., 1994). In contrast to wild-type mice, we observed no significant difference in the number of sunburn cells when we compared vehicle- and KGF-injected transgenic littermates (Fig. 4C lower panel, E). This result was verified by TUNEL assay (Fig. 4E lower panel, F). In the absence of KGF treatment, however, we found no significant difference in the number of sunburn cells between wild-type and transgenic mice, demonstrating that the levels of endogenous KGF receptor ligands are not sufficient to exert a cytoprotective effect. Thus, pharmacological application of KGF appears to be a powerful strategy for skin protection under stress conditions.

Discussion

In this study we identified KGF as a potent cytoprotective growth factor for murine and human keratinocytes in vitro and in vivo. KGF protected keratinocytes from UVA and UVB irradiation as well as from the toxicity of chemicals with electrophilic and ROS-producing properties (results summarized in a schematic drawing, Fig. 5). Since these chemical and physical insults frequently challenge the skin, our findings encourage the pharmacological use of KGF as a powerful strategy for skin protection under various stress conditions.

KGF protects keratinocytes from the toxicity of ROS-producing chemicals with electrophilic properties

Unexpectedly, KGF did not protect keratinocytes from chemicals that induce apoptosis via a mitochondrial pathway or via glutathione depletion. Moreover, the cytotoxicity of H_2O_2, a direct oxidant, was also not altered by KGF pre-treatment. This contrasts with the results of previous studies where KGF prevented H_2O_2-induced damage in epithelial cells from the lung (Wu et al., 1998; Waters et al., 1997; Chapman et al., 2002) and the retina (Geiger et al., 2005). Therefore, KGF appears to mediate its cytoprotective effect in a cell-type-specific manner. Under our experimental conditions, KGF only protected keratinocytes from cell death induced by substances that produce ROS by redox cycling and simultaneously have electrophilic properties and reactive Michael reaction functions. It is well known that electrophilic agents activate the transcription factor Nrf2 (NF-E2 related factor 2) at the posttranslational level. Nrf2 is a potent cytoprotective transcription factor, which induces the expression of various ROS-detoxifying enzymes and other antioxidant proteins (reviewed in Itoh et al., 2004). Interestingly, we also identified

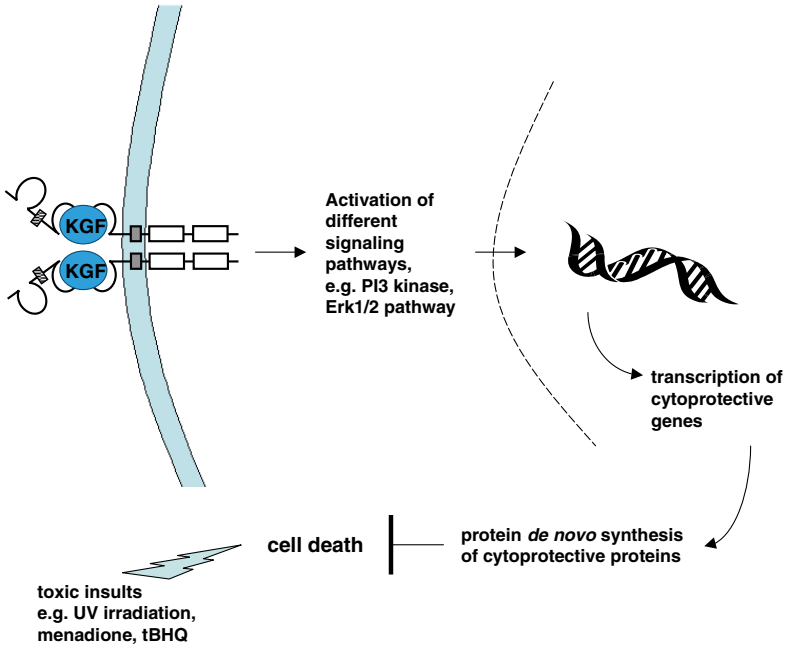

Fig. 5. Proposed mechanism of the cytoprotective effect of KGF. The cell-damaging effects of UV irradiation and of xenobiotics with electrophilic and oxidative properties are alleviated by KGF. Upon binding of KGF to its receptor signaling pathways are activated, which induces the expression of cytoprotective genes. A higher activity of cytoprotective proteins renders a cell more resistant against the cellular damage caused by UV irradiation, menadione, tBHQ and other substances.

Nrf2 in a search for KGF target genes (Braun et al., 2002). These findings suggest that KGF induces the expression of Nrf2, which in turn is activated by electrophilic toxins. As a result, cytoprotective proteins are expressed, which reduce the toxicity of the electrophilic components. Preliminary results from our laboratory suggest that inhibition of Nrf2 action in keratinocytes indeed reduces the cytoprotectivity of KGF, but only to a certain extent. Thus, additional proteins are likely to be required for the full cytoprotective effect and this needs to be explored in more detail in the future.

The cytoprotective effect of KGF for keratinocytes requires de novo protein synthesis and does not rely on a single signaling pathway

Previous studies showed that protection of alveolar epithelial cells from oxidative injury is a direct anti-apoptotic effect of KGF, which is mediated via PI3K signaling (Pan et al., 2004). By contrast, in keratinocytes the mechanism of KGF action does not rely on the pro-survival PI3K signaling cascade – rather the cytoprotective activity of KGF requires de novo synthesis of proteins that are regulated via multiple signaling pathways, probably including the PI3K and MAPK pathways. This reflects the situation in the oral mucosa and intestine, because the best effect of KGF in mucositis patients was achieved when KGF was first applied 1-3 days before the onset of treatment (Spielberger et al., 2004). The proteins that are produced upon KGF treatment and which mediate the cytoprotectivity, are as yet unknown, but are likely to include ROS-detoxifying enzymes because KGF reduced the number of oxidized proteins in menadione-treated keratinocytes (data not shown). One of these enzymes is probably peroxiredoxin 6, a previously identified KGF target in keratinocytes (Frank et al., 1997), which is also upregulated in the intestinal mucosa upon treatment of rodents with KGF (Farrell et al., 2002). Interestingly, constitutive overexpression of peroxiredoxin 6 in the epidermis of transgenic mice had a similar protective effect on keratinocytes as observed here for KGF. Thus, epidermal keratinocytes from mice overexpressing peroxiredoxin 6 were more protected against oxidative damage induced by ROS and UV irradiation in vitro and in vivo (Kümin et al., 2006). The upregulation of ROS-detoxifying enzymes suggests that KGF can prevent the initial damage of cellular macromolecules by ROS through the rapid detoxification of these aggressive agents, resulting in enhanced survival of the affected cells. The hypothesis that KGF treatment reduces the extent of DNA damage is supported by preliminary results obtained in the hair follicle organ culture model, where KGF pre-treatment strongly reduced the menadione-induced accumulation of p53 protein (E.B. and R.P., unpublished data).

KGF protects keratinocytes from UV-induced cell death in vitro and in vivo

KGF also protected keratinocytes from cell death induced by UVA or UVB irradiation. It remains to be determined whether electrophilic products of lipid peroxidation, such as 4-hydroxy-2-nonenal (Feng et al., 2004), which are generated in response to UV irradiation, are involved in the protective effect of KGF under these circumstances or if KGF-induced UV protection involves different mechanisms. For example, it has recently been shown that UVB irradiation induces FGFR2IIIb phosphorylation and activation via the generation of ROS, followed by receptor endocytosis (Marchese et al., 2003; Belleudi et al., 2005). This UVB-induced FGFR2IIIb activation caused apoptosis and cell-cycle arrest (Belleudi et al., 2005). As a consequence, KGF-mediated receptor downregulation reduced the UVB-induced apoptosis, indicating that receptor downregulation underlies the protective effect of KGF towards UVB. In this case, however, one would also expect a protective effect of KGF towards ROS-producing agents such as H_2O_2, since a KGF-induced receptor downregulation should reduce the rate of H_2O_2-induced apoptosis. In our studies, however, KGF did not protect from H_2O_2-induced cytotoxicity. In addition, other observations of the present study argue against a role of receptor downregulation in the protective effect of KGF: (1) the protective effect required de novo protein synthesis and (2) a 1-hour KGF pre-treatment, which already induces receptor downregulation (Belleudi et al., 2005), had no cytoprotective effect. Furthermore, one would expect that mice expressing a dominant-negative FGFR2IIIb mutant in the epidermis are directly protected against UV irradiation, because they cannot initiate FGFR2 signaling in response to UVB. However, this was not the case in our experiments (see Fig. 3). Independently of the underlying mechanisms of action, we show that the protective properties of exogenous KGF towards UV irradiation are also relevant in vivo. Whether this is beneficial or deleterious for the tissue is under debate. On the one hand it prevents severe tissue damage, but on the other hand it may also enhance the risk of skin cancer development, because cells with damaged DNA that fail to undergo apoptosis are more prone to malignant transformation (Kraemer, 1997).

The protective effect of KGF towards UVB irradiation in vivo was shown to depend on FGFR signaling, because it was abrogated in transgenic mice with a blockade in FGFR signaling in basal keratinocytes owing to expression of a dominant-negative FGFR2IIIb mutant (Werner et al., 1994). Interestingly, no difference in the extent of UVB damage was seen in these mice in the absence of exogenous KGF. This finding is consistent with our result that – in contrast to many other situations where KGF expression is upregulated upon epithelial injury (Werner et al., 1992; Zeeh et al., 1996; Baskin et al., 1997; Charafeddine et al., 1999; Adamson and Bakowska, 1999) – enhanced KGF expression upon UV irradiation could be detected neither in murine skin nor in cultured fibroblasts (our unpublished data).

Therapeutic potential of KGF for the prevention of skin damage?

An important finding of our study was the demonstration that KGF protects keratinocytes from the cytotoxicity of mafosfamide, the cell culture active derivative of cyclophosphamide, which is used as a chemotherapeutic agent in the above-mentioned clinical studies (Spielberger et al., 2004). These in vitro findings are important for the in vivo situation because we demonstrated a potent cytoprotective effect of KGF for normal human scalp hair follicle keratinocytes grown in organ culture, where they continue to produce a hair shaft at an almost in-vivo-like rate (Philpott et al., 1990; Bodó et al., 2005). Thus, it is clinically promising to explore whether KGF, ideally applied topically via follicle-targeting liposomes, protects anagen hair follicles in patients treated with cyclophosphamide or related chemotherapeutic

drugs. The finding that KGF protected against hair loss in mice treated with the chemotherapeutic agent cytosine arabinoside (Danilenko et al., 1995) further encourages such clinical tests.

Taking into account the similarities in the cellular damage caused by UV irradiation and ionizing radiation, e.g. oxidative stress and DNA damage, pharmacological doses of KGF may also be suitable for the prevention of epithelial cell injury induced by X-rays or γ-irradiation. Indeed, exogenous KGF increased hair follicle survival after X-ray irradiation (Booth and Potten, 2000) and enhanced thymic recovery after sublethal γ-irradiation (Alpdogan et al., 2005) in mice. The results presented in the above-mentioned studies as well as our data strengthen the usefulness of KGF as a pharmaceutical agent for epithelial cell protection and suggest the use of KGF or KGF-inducing agents for the protection of exposed parts of the skin from UV- or ionizing-radiation-mediated damage. KGF seems to be appropriate for use in patients because recent reports provide evidence for anti-tumorigenic rather than pro-tumorigenic properties of KGF/FGFR2IIIb signaling (Finch and Rubin, 2004; Bernard-Pierrot et al., 2004). This is clearly an advantage compared with other growth factors with anti-apoptotic potential, such as EGF, which was shown to promote tumor growth (Bazley and Gullick, 2005).

Materials and Methods

Cell culture

The human keratinocyte cell line HaCaT (Boukamp et al., 1988) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland) including 10% fetal calf serum (Amimed-BioConcept, Allschwil, Switzerland). Human foreskin keratinocytes were cultured in K-SFM (Invitrogen, Basel, Switzerland) including epidermal growth factor (EGF) and bovine pituitary extract. KGF was kindly provided by AMGEN (Thousand Oaks, CA) or purchased from R&D Systems (Minneapolis, MN, USA).

Animals and UV source

Transgenic mice expressing a dominant-negative FGFR2IIIb mutant in the epidermis were described previously (Werner et al., 1994). Mice were housed and fed according to federal guidelines and all procedures were approved by the local veterinary authorities of Zurich, Switzerland.

The UVB source was a Medisun HF-54 lamp (Schulze & Boehm, Huerth, Germany) equipped with six UVB-TL/12 bulbs (9 W each; Philips, Amsterdam, The Netherlands), which emit UVB in the range of 280-350 nm with a peak emission at 312-315 nm, and with six UVA-TL/08 bulbs (9 W each; Philips), which emit UVA in the range of 315-400 nm with a peak emission at 350-352 nm.

In vitro cytoprotection assay

20,000 HaCaT cells were seeded into 96-well plates, grown to confluence and starved in serum-free medium for 24 hours (for subsequent UV irradiation medium without phenol red was used). For cytoprotection assays with human foreskin keratinocytes, 5000 cells were seeded in 96-well plates, grown to ~70% confluence, and starved in serum-free medium (KBM2 medium without supplements, Cambrex) for 48 hours. Subsequently, KGF was added (10 ng/ml) and cells were incubated overnight. The next morning KGF was added again at the same concentration. After a 1-hour incubation, different concentrations of cytotoxic agents were added and incubated for 6 hours: diethyl maleate (200-5000 μM), hydrogen peroxide (100-2000 μM), menadione (25-100 μM), paraquat (100-2000 μM), staurosporine (0.5-50 μM), *tert*-butylhydroquinone (tBHQ; 100-400 μM) or 2,5-di-*tert*-butylhydroquinone (di-tBHQ; 100-1000 μM) (all from Sigma). Alternatively, cells were irradiated with UVA (5-20 J/cm^2) or UVB (20-100 mJ/cm^2) and incubated for 24 hours.

To study the involved signaling pathways, individual inhibitors or combinations of inhibitors were added before KGF was applied: 10 μg/ml cycloheximide (Sigma), 2 hours before KGF; 10 μM U0126, 10 μM PD98059, 10 μM LY294002, 10 μM SB203580, 1 μM SB202190 or 10 μM JNK inhibitor II (all from Calbiochem), 1 hour before KGF. Control cells were treated with the solvent DMSO.

For MTT proliferation and cytotoxicity assays (Mosmann, 1983) 20 μl of a saturated MTT (methylthiazolyldiphenyl-tetrazolium bromide, Sigma) solution (5 mg/ml) in 1× PBS were added and incubated for an additional hour (HaCaT) or 2 hours (primary keratinocytes). The supernatant was removed, the blue formazan dye was dissolved in 100 μl of 0.04 M HCl in isopropanol, subsequently, 100 μl of water were added and the absorbance at 595 nm was determined.

Lactate dehydrogenase (LDH) assay

100 μl cell supernatant were transferred into a new microtiter plate. 20 μl of freshly prepared 26 mg/ml lactate in 10 mM Tris-HCl, pH 8.5, 20 μl of 2 mg/ml 2-p-iodophenyl-3-nitrophenyl tetrazolium chloride (INT; dissolved in DMSO at a concentration of 20 mg/ml, 1:10 dilution in PBS) and 20 μl NAD$^+$ (3 mg/ml NAD$^+$, 13.5 U/ml diaphorase, 0.03% BSA, 1.2% sucrose in PBS) solution were added and samples were incubated for 20 minutes. The reaction was stopped with 20 μl of 16.6 mg/ml sodium oxymate and the absorbance at 490 nm was measured.

Propidium iodide staining

For propidium iodide staining 100 μl of trypsinized cells (cell suspension with approximately 4-6×10^5 cells/ml) and 100 μl of a 4 μM propidium iodide solution in PBS were combined, mixed, incubated for 10 minutes and measured in a fluorescent microplate reader with excitation and emission at 493 nm and 630 nm, respectively.

Western blot analysis

Cells were seeded in 6-cm dishes and treated as described above for cytoprotection assays. Cells were harvested before and at different time points after KGF addition and lysed with 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 9.5 M urea, 2 mM EDTA, 0.5 mM AEBSF, 1 mM $Na_2P_2O_7$, 1 mM Na_3VO_4). Lysed cells were scraped off the dish and the lysates were sonicated. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Antibody incubations were performed in 5% non-fat dry milk in TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) or in 5% BSA in TBS-T (anti-phospho-p38, anti-phospho-MARCKS). The following antibodies were used: anti-phospho-Erk1/2, Thr202/Tyr204 (Cell Signaling Technology, Beverly, MA; diluted 1:2000), anti-total-Erk1/2 (K-23, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:500), anti-phospho-Akt1/2/3, Ser473 (Santa Cruz; diluted 1:1000), anti-Akt1/2 (N-19, Santa Cruz; diluted 1:1000), anti-phospho-p38, Thr180/Tyr182 (Cell Signaling; diluted 1:1000), anti-phospho-JNK, Thr183/Tyr185 (Cell Signaling; diluted 1:1000), anti-MARCKS, Ser152/156 (Cell Signaling; diluted 1:1000).

Subcutaneous injection of KGF into mice and UVB irradiation

Mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). The backs of the mice were shaved and 10 μg KGF dissolved in 100 μl 0.5% BSA in PBS or 100 μl 0.5% BSA in PBS, respectively, were subcutaneously injected at a marked point at the dorsal midline. 24 hours later, mice were irradiated with 100 mJ/cm^2 UVB. 16-20 hours after UVB exposure, mice were sacrificed and 1 cm^2 of skin around the injection site was removed. For histological analyses the skin biopsies were fixed overnight in 1% acetic acid 95% ethanol and embedded in paraffin. Sections (7 μm) were stained with hematoxylin/eosin. Sunburnt cells were identified by their distinctive histological appearance (isolated keratinocytes with pycnotic nuclei and shrunken cytoplasm), counted and their number per mm basement membrane was determined.

Cytoprotection assays with human hair follicle organ cultures

Human anagen VI follicles were isolated from scalp skin obtained from females undergoing routine face-lift surgery. All experiments were performed according to Helsinki guidelines. Hair follicles were isolated as described (Philpott et al., 1990; Magerl et al., 2004; Bodo et al., 2005) and maintained in 24-well plates in serum-free Williams' Medium E (Biochrom, Cambridge, UK) supplemented with 2 mM L-glutamine (Invitrogen), 10 ng/ml hydrocortisone (Sigma), 10 μg/ml insulin (Sigma) and 1% antibiotic/antimycotic mixture (100×, Invitrogen). Hair follicles were incubated overnight (5% CO_2, 37°C), then KGF (20 ng/ml) was added and incubated for 12 hours. The medium was subsequently replaced by fresh medium supplemented with 20 ng/ml KGF. After a 1-hour incubation, menadione was added to a final concentration of 50 μM and incubated for a further 6 hours.

Terminal dUTP nick-end labelling (TUNEL) assay

For detection of apoptotic cells, TUNEL assays were performed as described (Bodo et al., 2005) using the ApopTag fluorescein in situ apoptosis detection kit (Intergen, Purchase, NY). After stopping the enzyme reaction, sections were incubated with FITC-conjugated anti-digoxigenin antibody (ApopTag kit) and counterstained with DAPI. TUNEL assays of UVB-irradiated skin were performed with acetic acid/ethanol (1%/95%)-fixed paraffin sections using the TUNEL assay kit from Roche (Basel, Switzerland).

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References

Adamson, I. Y. and Bakowska, J. (1999). Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. *Exp. Lung Res.* **27**, 605-616.

Alpdogan, Ö., Hubbard, V. M., Smith, O. M., Patel, N., Lu, S., Goldberg, G. L., Gray, D. H., Feinman, J., Kochman, A. A., Eng, J. M. et al. (2005). Keratinocyte growth factor (KGF) is required for post-natal thymic regeneration. *Blood* **107**, 2453-2460.

Bao, S., Wang, Y., Sweeney, P., Chaudhuri, A., Doseff, A. I., Marsh, C. B. and Knoell, D. L. (2005). Keratinocyte growth factor induces Akt kinase activity and inhibits Fas-mediated apoptosis in A549 lung epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288**, L36-L42.

Baskin, L. S., Sutherland, R. S., Thomson, A. A., Nguyer, H. T., Morgan, D. M., Hayward, S. W., Hom, Y. K., DiSandro, M. and Cunha, G. R. (1997). Growth factors in bladder wound healing. *J. Urol.* **157**, 2388-2395.

Bazley, L. A. and Gullick, W. J. (2005). The epidermal growth factor receptor family. *Endocr. Relat. Cancer* **12**, S17-S27.

Belleudi, F., Leone, L., Aimati, L., Stirparo, M. G., Cardinali, G., Marchese, C., Frati, L., Picardo, M. and Torrisi, M. R. (2005). Endocytic pathways and biological effects induced by UVB-dependent or ligand-dependent activation of the keratinocyte growth factor receptor. *FASEB J.* **20**, 395-397.

Bernard-Pierrot, I., Ricol, D., Cassidy, A., Graham, A., Elvin, P., Caillault, A., Lair, S., Broet, P., Thiery, J. P. and Radvanyi, F. (2004). Inhibition of human bladder tumour cell growth by fibroblast growth factor receptor 2b is independent of its kinase activity. Involvement of the carboxy-terminal region of the receptor. *Oncogene* **23**, 9201-9211.

Bodó, E., Biro, T., Telek, A., Czifra, G., Griger, Z., Toth, B. I., Mescalchin, A., Ito, T., Bettermann, A., Kovacs, L. et al. (2005). A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am. J. Pathol.* **166**, 985-998.

Booth, C. and Potten, C. S. (2000). Keratinocyte growth factor increases hair follicle survival following cytotoxic insult. *J. Invest. Dermatol.* **114**, 667-673.

Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106**, 761-771.

Braun, S., Hanselmann, C., Gassmann, M. G., auf dem Keller, U., Born-Berclaz, C., Chan, K., Kan, Y. W. and Werner, S. (2002). Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. *Mol. Cell. Biol.* **22**, 5492-5505.

Braun, S., Mauch, C., Boukamp, P. and Werner, S. (2006). Novel roles of NM23 proteins in skin homeostasis, repair and disease. Oncogene doi: 10.1038/sj.onc.1209822.

Brunton, L. L., Lazo, J. S. and Parker, K. L. (2006). Section IX: Chemotheraphy of neoplastic diseases. In *Goodman and Gilman's Pharmacological Basis of Therapeutics* (ed. L. Brunton, D. Blumenthal, I. Buxton and K. Parker), pp. 1315-1403. New York: McGraw-Hill.

Chapman, K. E., Waters, C. M. and Miller, W. M. (2002). Continuous exposure of airway epithelial cells to hydrogen peroxide: protection by KGF. *J. Cell Physiol.* **192**, 71-80.

Charafeddine, L., D'Angio, C. T., Richards, J. L., Stripp, B. R., Finkelstein, J. N., Orlowski, C. C., LoMonaco, M. B., Paxhia, A. and Ryan, R. M. (1999). Hyperoxia increases keratinocyte growth factor mRNA expression in neonatal rabbit lung. *Am. J. Physiol.* **276**, L105-L113.

Daniels, F., Brophy, D. and Lobitz, W. C. (1961). Histochemical responses of human skin following ultraviolet irradiation. *J. Invest. Dermatol.* **37**, 351-357.

Danilenko, D. M., Ring, B. D., Yanagihara, D., Benson, W., Wiemann, B., Starnes, C. O. and Pierce, G. F. (1995). Keratinocyte growth factor is an important endogenous mediator of hair follicle growth, development, and differentiation. Normalization of the nu/nu follicular differentiation defect and amelioration of chemotherapy-induced alopecia. *Am. J. Pathol.* **147**, 145-154.

Farrell, C. L., Bready, J. V., Rex, K. L., Chen, J. N., DiPalma, C. R., Whitcomb, K. L., Yin, S., Hill, D. C., Wiemann, B., Starnes, C. O. et al. (1998). Keratinocyte growth factor protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. *Cancer Res.* **58**, 933-939.

Farrell, C. L., Rex, K. L., Chen, J. N., Bready, J. V., DiPalma, C. R., Kaufman, S. A., Rattan, A., Scully, S. and Lacey, D. L. (2002). The effects of keratinocyte growth factor in preclinical models of mucositis. *Cell Prolif.* **35** Suppl. 1, 78-85.

Feng, Z., Hu, W. and Tang, M. S. (2004). Trans-4-hydroxy-2-nonenal inhibits nucleotide excision repair in human cells: a possible mechanism for lipid peroxidation-induced carcinogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 8598-8602.

Finch, P. W. and Rubin, J. S. (2004). Keratinocyte growth factor/fibroblast growth factor 7, a homeostatic factor with therapeutic potential for epithelial protection and repair. *Adv. Cancer Res.* **91**, 69-136.

Frank, S., Munz, B. and Werner, S. (1997). The human homologue of a bovine non-selenium glutathione peroxidase is a novel keratinocyte growth factor-regulated gene. *Oncogene* **14**, 915-921.

Geiger, R. C., Waters, C. M., Kamp, D. W. and Glucksberg, M. R. (2005). KGF prevents oxygen-mediated damage in ARPE-19 cells. *Invest. Ophthalmol. Vis. Sci.* **46**, 3435-3442.

Itoh, K., Tong, K. I. and Yamamoto, M. (2004). Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic. Biol. Med.* **36**, 1208-1213.

Khan, W. B., Shui, C., Ning, S. and Knox, S. J. (1997). Enhancement of murine intestinal stem cell survival after irradiation by keratinocyte growth factor. *Radiat. Res.* **148**, 248-253.

Kraemer, K. H. (1997). Sunlight and skin cancer: another link revealed. *Proc. Natl. Acad. Sci. USA* **94**, 1-14.

Kruman, I., Guo, Q. and Mattson, M. P. (1998). Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. *J. Neurosci. Res.* **51**, 293-308.

Kümin, A., Huber, C., Rülicke, T., Wolf, E. and Werner, S. (2006). Peroxiredoxin 6 is a potent cytoprotective enzyme in the epidermis. *Am. J. Pathol.* **169**, 1194-1205.

Le Panse, R., Mitev, V., Lebreton, C. and Coulomb, B. (1994). Modulation of epidermal growth factor and keratinocyte growth factor effects on human keratinocyte growth by protein kinase C inhibitor, GF109203X. *Biochem. Biophys. Res. Commun.* **204**, 1081-1087.

Magerl, M., Paus, M., Farjo, N., Müller-Röver, S., Peters, E. M., Foitzik, K. and Tobin, D. J. (2004). Limitations of human occipital scalp hair follicle organ culture for studying the effects of minoxidil as a hair growth enhancer. *Exp. Dermatol.* **13**, 635-642.

Marchese, C., Maresca, V., Cardinali, G., Belleudi, F., Ceccarelli, S., Bellocci, M., Frati, L., Torrisi, M. R. and Picardo, M. (2003). UVB-induced activation and internalization of keratinocyte growth factor receptor. *Oncogene* **22**, 2422-2431.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.

Nakamura, Y., Kumagai, T., Yoshida, C., Naito, Y., Miyamoto, M., Ohigashi, H., Osawa, T. and Uchida, K. (2003). Pivotal role of electrophilicity in glutathione S-transferase induction by tert-butylhydroquinone. *Biochemistry* **42**, 4300-4309.

Pan, Z. Z., Devaux, Y. and Ray, P. (2004). Ribosomal S6 kinase as a mediator of keratinocyte growth factor-induced activation of Akt in epithelial cells. *Mol. Biol. Cell* **15**, 3106-3113.

Park, H. J., Kim, H. J., Lee, J. H., Lee, J. Y., Cho, B. K., Kang, J. S., Kang, H., Yang, Y. and Cho, D. H. (2005). Corticotropin-releasing hormone (CRH) downregulates interleukin-18 expression in human HaCaT keratinocytes by activation of p38 mitogen-activated protein kinase (MAPK) pathway. *J. Invest. Dermatol.* **124**, 751-755.

Paus, R. and Cotsarelis, G. (1999). The biology of hair follicles. *N. Engl. J. Med.* **341**, 491-497.

Philpott, M. P., Green, M. R. and Kealey, T. (1990). Human hair growth *in vitro*. *J. Cell Sci.* **97**, 463-471.

Portnoy, J., Curran-Everett, D. and Mason, R. J. (2004). Keratinocyte growth factor stimulates alveolar type II cell proliferation through the extracellular signal-regulated kinase and phosphatidylinositol 3-OH kinase pathway. *Am. J. Respir. Cell Mol. Biol.* **30**, 901-907.

Rodeck, U., Jost, M., Kari, C., Shih, D. T., Lavker, R. M., Ewert, D. L. and Jensen, P. J. (1997). EGF-R dependent regulation of keratinocyte survival. *J. Cell Sci.* **110**, 113-121.

Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. and Aaronson, S. A. (1989). Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA* **86**, 802-806.

Schwarz, T. (2005). Biological effects of UV radiation on keratinocytes and Langerhans cells. *Exp. Dermatol.* **14**, 788-789.

Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F. M., Schlessinger, J. and Wagner, E. F. (2000). The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. *Cell* **102**, 211-220.

Spielberger, R., Stiff, P., Bensinger, W., Gentile, T., Weisdorf, D., Kewalramani, T., Shea, T., Yanovich, S., Hansen, K., Noga, S. et al. (2004). Palifermin for oral mucositis after intensive therapy for hematologic cancers. *N. Engl. J. Med.* **351**, 2590-2598.

Suntres, Z. E. (2002). Role of antioxidants in paraquat toxicity. *Toxicology* **180**, 65-77.

Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A. and Orrenius, S. (1982). The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J. Biol. Chem.* **257**, 12419-12425.

Van Dross, R. T., Hong, X. and Pelling, J. C. (2005). Inhibition of TPA-induced cyclooxygenase-2 (COX-2) expression by apigenin through downregulation of Akt signal transduction in human keratinocytes. *Mol. Carcinog.* **44**, 83-91.

Waters, C. M., Savla, U. and Panos, R. J. (1997). KGF prevents hydrogen peroxide-induced increases in airway epithelial cell permeability. *Am. J. Physiol.* **272**, L681-L689.

Werner, S. (1998). Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine Growth Factor Rev.* **9**, 153-165.

Werner, S., Peters, K. G., Longaker, M. T., Fuller-Pace, F., Banda, M. J. and Williams, L. T. (1992). Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl. Acad. Sci. USA* **89**, 6896-6900.

Werner, S., Smola, H., Liao, X., Longaker, M. T., Krieg, T., Hofschneider, P. H. and Williams, L. T. (1994). The function of KGF in morphogenesis of epithelium and re-epithelialization of wounds. *Science* **266**, 819-822.

Wu, K. I., Pollack, N., Panos, R. J., Sporn, P. H. and Kamp, D. W. (1998). Keratinocyte growth factor promotes alveolar epithelial cell DNA repair after H_2O_2 exposure. *Am. J. Physiol.* **275**, L780-L787.

Zeeh, J. M., Procaccino, F., Hoffmann, P., Aukerman, S. L., McRoberts, J. A., Soltani, S., Pierce, G. F., Lakshmanan, J., Lacey, D. and Eysselein, V. E. (1996). Keratinocyte growth factor ameliorates mucosal injury in an experimental model of colitis in rats. *Gastroenterology* **110**, 1077-1083.

Zeigler, M. E., Chi, Y., Schmidt, T. and Varani, J. (1999). Role of ERK and JNK pathways in regulating cell motility and matrix metalloproteinase 9 production in growth factor-stimulated human epidermal keratinocytes. *J. Cell Physiol.* **180**, 271-284.