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Vitamin D Enhances Mitogenesis Mediated by Keratinocyte Growth Factor Receptor in Keratinocytes

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Abstract The hormonally active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and keratinocyte growth factor (KGF) belong to the network of autocrine and paracrine mediators in the skin. Both were shown to modulate keratinocyte proliferation, to reverse epidermal atrophy, to increase wound healing, and to reduce chemotherapy-induced alopecia. The overlap between their activities may suggest that vitamin D exerts some of its actions by modulation of KGF activities in the skin. This notion was examined by using HaCaT keratinocytes cultured in serum-free medium in the absence of exogenous growth factors and in the presence of the EGF receptor tyrosine kinase inhibitor AG 1478 that blocks their autonomous proliferation. These cells could be stimulated to proliferate by different fibroblast growth factors (FGFs). The relative mitogenic efficacy of basic FGF, acidic FGF, or KGF was in correlation with their affinities for the KGF receptor (KGFR). Forty-eight hour co-treatment with $1,25(OH)_2D_3$ enhanced KGFR-mediated cell proliferation in a dose dependent manner. Both ERK1/2 and c-Jun N-terminal kinase (JNK) were activated by the FGFs. Treatment with $1,25(OH)_2D_3$ increased the activation of ERK but reduced the activation of JNK. Treatment with $1,25(OH)_2D_3$ increased the levels of KGFR in the presence but not in the absence of KGF, probably due to inhibition of ligand-induced receptor degradation. Inhibition of protein kinase C with bisindolylmaleimide did not interfere with the effect of $1,25(OH)_2D_3$ on KGFR-mediated ERK activation. Our results support the notion that the paracrine KGF–KGFR system in the skin can act in concert with the autocrine vitamin D system in keratinocytes to promote keratinocyte proliferation and survival under situations of stress and injury. J. Cell. Biochem. 89: 440–449, 2003.

Key words: calcitriol; fibroblast growth factor; ERK; c-Jun N-terminal kinase; protein kinase C; proliferation

Epidermal keratinocytes contain an autonomous vitamin D endocrine system. This system consists of the enzymatic machinery that converts vitamin D to its active hormonal metabolite, 1,25–dihydroxyvitamin D ($1,25(OH)_2D_3$) and of the nuclear vitamin D receptor (VDR) [Bikle and Pillai, 1993; Lehmann et al., 2001; Schuessler et al., 2001]. $1,25(OH)_2D_3$ promotes keratinocyte differentiation and modulates their proliferation. Accumulating evidence indicates that the in vivo effects of $1,25(OH)_2D_3$ or its synthetic analogs on the proliferation of keratinocytes are context specific. Whereas these compounds inhibit the hyperproliferation of keratinocytes in psoriatic skin [van de Kerkhof, 1995], they seem to promote proliferation in intact skin [Levy et al., 1994; Lutzow-Holm et al., 1996; Gniadecki et al., 1998], reverse corticosteroid-induced epidermal atrophy [Gniadecki et al., 1994] and, in some cases, enhance the rate of re-epithelialization during wound healing [Ramesh et al., 1993, Hashimoto et al., 1995; Tian et al., 1995]. In addition, active vitamin D derivatives have been shown to protect hair follicles from chemotherapy-induced damage [Schilli et al., 1998]. This finding is consistent with the complete alopecia found in patients and experimental animals with genetic defects in the vitamin D response system [Liberman et al., 1986; Kato et al., 1999].

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Keratinocyte growth factor (KGF) is a member of the rapidly expanding fibroblast growth factors (FGF) family. KGF is a specific mitogen for epithelial cells in contrast to most members of the FGF family, which exert their mitogenic activity on a variety of cell types [Rubin et al., 1995]. FGFs bind and activate receptor tyrosine kinases, which are the products of four different genes [Szebenyi and Fallon, 1999]. Further diversity of the FGF receptor family occurs through alternative splicing of these gene products. The selective binding of the different FGFs to the different receptor variants determines the specific target cells that will be activated. KGF, uniquely among the FGFs, acts only through one receptor variant, FGFR2-IIIb, also known as the KGF receptor (KGFR) [Miki et al., 1991; Ron et al., 1993a]. KGFR, on the other hand, can be activated by other FGFs, notably acidic FGF (aFGF) and to a lesser extent, basic FGF (bFGF) [Bottaro et al., 1990; Reich-Slotky et al., 1995; Shaoul et al., 1995].

In accordance with its presumed paracrine mechanism of action in the skin, KGF is produced in the dermis and its receptor is expressed in the keratinocytes of the epidermis and the hair follicles [Finch et al., 1989, 1995]. Insight into the role of KGF in the skin came from experiments with animal model systems and in vitro reconstituted skin. Thus, its expression is markedly increased in sites of cutaneous injury [Werner et al., 1992], and targeted expression of KGF in keratinocytes resulted in hyperplasia [Guo et al., 1993]. Mice expressing dominant negative form of KGFR in keratinocytes exhibit delayed wound repair, atrophic and disorganized epidermis, slower rate of keratinocyte proliferation, and reduced number of hair follicles. The latter finding is consistent with the ability of KGF to reduce chemotherapy-induced alopecia in neonatal rats [Danilenko et al., 1995]. KGF is required for human epidermis formation in reconstituted skin [Szabowski et al., 2000]. However, KGF null mice did not show epidermal abnormalities, most likely due to functional compensation by other growth factors [Werner et al., 1994; Guo et al., 1996].

The intriguing overlap between the activities of vitamin D in intact skin and those of KGF led us to wonder whether some of the activities of vitamin D may be due to modulation of the activities of KGF via KGFR signaling pathways. The first step in the assessment of this notion is to investigate the possible cross talk between KGFR ligands and vitamin D in keratinocytes. A prerequisite for such an investigation is that it is conducted with minimal interference from other signaling systems that impact on cell fate. Cultures of primary keratinocytes do not fulfill this requirement, as they need a complex mixture of growth factors and various active ingredients in order to survive and grow in culture and to proliferate in response to growth factors. To overcome this obstacle, we established an experimental system based on HaCaT immortalized cells that are widely used as model for epidermal keratinocytes. These cells are nontumorigenic and differentiate into normal epidermis when grafted onto nude mice [Boukamp et al., 1988; Schoop et al., 1999]. In this study, HaCaT cells were rendered dependent for their survival and proliferation in culture on exogenous supply of FGFs, in the absence of serum or any other biologically active ingredients. Using these systems, we present experimental support to the notion that $1,25(OH)_2D_3$ can potentiate mitogenic signaling via the KGFR leading to proliferation of human keratinocytes.

MATERIALS AND METHODS

Tissue culture media were from Gibco BRL, Grand Island, NY; tissue culture dishes from Corning Glass Works, Corning, NY; and fetal calf serum (FCS) from Beit Haemek Industries, Beit Haemek, Israel. $1,25(OH)_2D_3$ was from Hoffmann-LaRoche, Nutley, NJ (a gift from Dr. M. Uskokovic). Recombinant human bFGF was from PeproTech, Inc., NJ. Recombinant aFGF and KGF were produced as described [Gitay-Goren et al., 1992; Ron et al., 1993b]. Tyrphostin AG 1478 was a gift from Prof. A. Levitzki, The Hebrew University, Jerusalem, Israel. Bisindolylmaleimide (BIM) was from Calbiochem-Novabiochem Corporation, San-Diego, CA. Monoclonal antibody to the dually phosphorylated ERK, rabbit anti ERK1/2 polyclonal antibody and c-Jun N-terminal kinase (JNK) polyclonal antibody recognizing JNK1 and JNK2 isoforms were from Sigma Chemical Co., St. Louis, MO. Monoclonal antibody to the dually phosphorylated JNK and monoclonal antibody to Bek (KGFR) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Peroxidase-conjugated secondary antibodies: goat anti rabbit IgG and goat anti mouse IgG, were from Jackson ImmunoResearch Laboratories Inc., West Grove, PA. Bovine serum albumin fraction

V was from ICN Biomedicals, Inc., Costa Mesa, CA. All other reagents were of analytical grade.

Cell Culture and Mitogenic Assay

The human keratinocyte cell line, HaCaT, was kindly provided by Prof. N. Fusenig, German Cancer Research Center, Heidelberg, Germany. Cells were maintained in minimal essential medium containing 0.075 mM Ca^{+2} (MEM-75) and 10% FCS in 6 cm petri dishes and sub-cultured every 3–4 days. Experiments were initiated by seeding HaCaT cells (for cell number assessment: 5,000 cells/well in 96-well plates; for Western blot analysis: 500,000 cells in 6 cm petri dishes) in MEM-75 containing 10% FCS. Twenty-four hours after seeding the medium was replaced with fresh, serum-free MEM-75 containing albumin (0.5 mg/ml), AG 1478 (0.5 μM), $1,25(OH)_2D_3$ and the appropriate FGF and cultured for 48 h. Heparin (10 μg/ml) was added to cultures containing bFGF or aFGF but not KGF to obtain optimal mitogenic responses [Reich-Slotky et al., 1994]. Ethanol, the vehicle for $1,25(OH)_2D_3$ was added to control cultures and its concentration never exceeded 0.06%. Cell number was assessed by crystal violet (CV) staining as previously described [Garach-Jehoshua et al., 1999].

Western Blot Analysis

Cell extracts were prepared by scraping cells into SDS-sample buffer and boiled for 15 min. Samples were centrifuged before electrophoresis and subjected to SDS–PAGE under reducing conditions using 10% polyacrylamide gels (20 μg protein/lane). Proteins were transferred to nitrocellulose membranes and probed with the specific antibodies. Detection was carried out by horseradish peroxidase-conjugated goat anti mouse and anti rabbit antibodies and enhanced chemiluminescence. Protein content of cell extracts in sample buffer was measured by the method of Minamide and Bamburg [1990].

RESULTS

The first objective of this study was to examine the possible effects of $1,25(OH)_2D_3$ on keratinocyte proliferation induced by members of the FGF family. Our goal thus was to establish an experimental system in which keratinocyte proliferation is solely dependent on these growth factors. As was shown previously, HaCaT cells can proliferate in culture in the absence of serum or exogenous growth factors [Garach-Jehoshua et al., 1999]. This was confirmed in the present study (Fig. 1A). Twenty-four hours old HaCaT cell cultures were allowed to propagate for additional 48 h in serum-free medium and cell number was assessed by CV staining (Fig. 1A, 72 h). Cell number in these cultures was significantly higher than in parallel cultures stained before serum depletion (Fig. 1A, 24 h). Under such conditions, their proliferation is entirely dependent on autocrine EGFR ligands [Garach-Jehoshua et al., 1999] and can be blocked by the specific EGFR tyrosine kinase inhibitor AG 1478 (Fig. 1A). Addition of FGFs to HaCaT cells, treated with AG 1478, brought about a significant proliferative response. For instance, treatment with bFGF (100 ng/ml) for

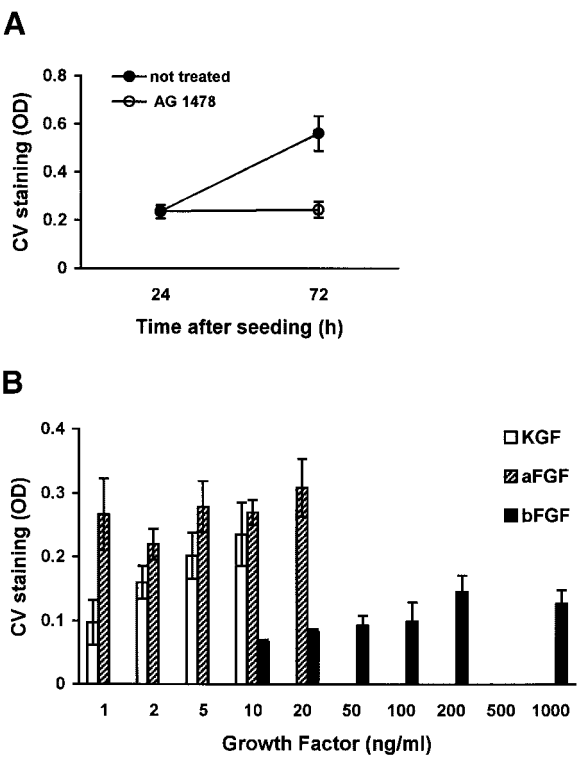

Fig. 1. Effect of FGFs on HaCaT cell proliferation. HaCaT cells were cultured in serum-free MEM-75 as described in "Methods" in the presence or absence of AG 1478 (0.5 μM). **A**: Crystal violet (CV) staining was carried out 24 h after seeding at the time of serum depletion and AG 1478 addition or 72 h after seeding. Data are presented as mean ± SEM of six independent experiments each performed with 4–5 replicate cultures. **B**: Twenty-four hours after seeding, cultures were serum depleted, treated with AG 1478 (0.5 μM) and various FGFs. CV staining was carried out 48 h after initiation of treatment and the value of CV staining of parallel cultures at the time of exposure to AG 1478 was subtracted. Results are presented as mean ± SD of five replicate cultures.

48 h increased cell number by 1.75 ± 0.08 fold (eight independent experiments, $P = 1 \times 10^{-5}$ paired t-test). We compared the mitogenic effect of three members of the FGF growth factor family, bFGF, aFGF, and KGF (Fig. 1B). It is evident that both KGF and aFGF are more potent and efficacious mitogens than bFGF, active at a ten-fold lower concentration range. These results are in agreement with previous reports describing the mitogenic response of mouse keratinocytes to these growth factors [Reich-Slotky et al., 1995; Bonneh-Barkay et al., 1997]. KGFR is the only member of the FGFR family capable of transducing KGF signaling and its affinity for aFGF is similar to that for KGF, but much higher than that for bFGF [Ornitz et al., 1996; Szebenyi and Fallon, 1999]. Thus, the results in Figure 1B strongly suggest that KGFR, previously shown to be present in HaCaT cells [Capone et al., 2000], is solely responsible for the mitogenic response of these cells to the various FGF family members. Hereafter, the different KGFR ligands were used interchangeably in the course of this study.

Having established a culture of keratinocytes that depend entirely on the activation of KGFR for their survival and proliferation, the scene was set to examine the effect of $1,25(OH)_2D_3$ on KGFR dependent mitogenesis. HaCaT cells, treated with AG 1478, were exposed to KGF in the presence or absence of $1,25(OH)_2D_3$ (Fig. 2A). In the absence of the growth factor, cell number was slightly higher in cells co-treated with AG 1478 and $1,25(OH)_2D_3$ than in cultures treated with AG 1478 alone. This small effect may be related to the previously reported protective effect exerted by $1,25(OH)_2D_3$ on HaCaT cells exposed to various stresses [Ravid et al., 2002]. It is apparent that the mitogenic effect of KGF is markedly enhanced by co-treatment with the hormone. The enhancing effect of the hormone occurred also with bFGF and was reproduced in three independent experiments (Fig. 2B). Figure 2C shows that the enhancement of KGFR-mediated mitogenesis by $1,25(OH)_2D_3$ is dose dependent and significant at a concentration as low as 0.01 nM.

One of the early and critical steps of the mitogenic process is the activation of the mitogen-activated protein kinase (MAPK) ERK. We next examined whether the interaction between $1,25(OH)_2D_3$ and KGFR ligands was also reflected in enhanced activation of this key enzyme. We have previously shown

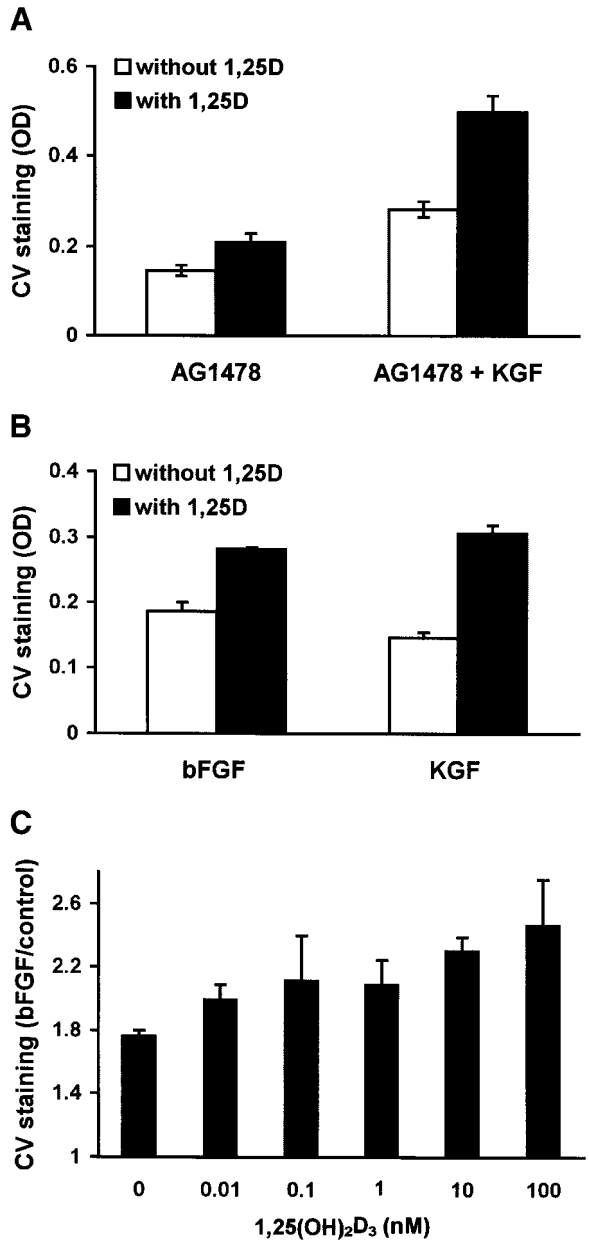

Fig. 2. Effect of $1,25(OH)_2D_3$ on KGFR ligand-induced proliferation. HaCaT cells were cultured in serum-free MEM-75 with AG 1478 and the specified KGFR ligands (KGF (4 ng/ml), bFGF (100 ng/ml)) in the presence or absence of $1,25(OH)_2D_3$ (**A** and **B**: 100 nM) for 48 h as described in "Methods". Cell number was assessed by CV staining. CV staining values of cultures containing all additives except FGF were subtracted from the respective FGF-containing cultures (**B**,**C**). Data were expressed as mean ± SEM of three independent experiments each performed with five replicate cultures (**B**), and mean ± SD of five replicate cultures (**A**,**C**).

[Garach-Jehoshua et al., 1999] and confirmed in this study (Fig. 3A) that in HaCaT cells the ERK mitogenic signaling pathway, activated by autocrine EGFR ligands, is enhanced by treat-

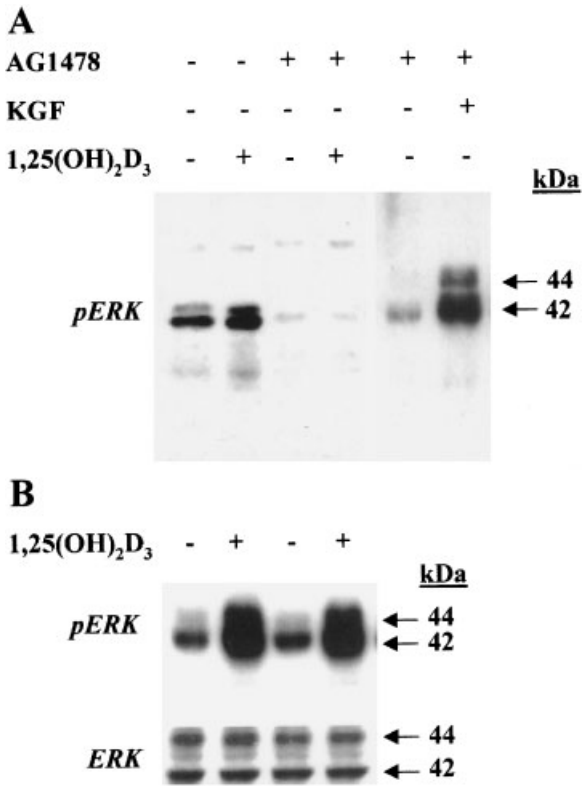

Fig. 3. Effect of $1,25(OH)_2D_3$ on ERK activation in KGFR ligand dependent HaCaT cells. HaCaT cells were cultured in serum-free MEM-75 as described in ''Methods''. **A**: Cells were cultured in serum-free medium for 48 h in the presence or absence of $1,25(OH)_2D_3$ (100 nM) and then exposed to AG 1478 (0.5 μM, 1 h) and to KGF (4 ng/ml, 10 min). **B**: Cells were cultured for 48 h in serum-free MEM-75 with AG 1478 and KGF (4 ng/ml) in the presence or absence of $1,25(OH)_2D_3$ (100 nM). Cells were harvested and cell extracts subjected to Western blot analysis with antibodies to dually phosphorylated ERK or ERK protein. The data represent multiple experiments with at least 15 independent cultures.

ment with $1,25(OH)_2D_3$. As shown in Figure 3A, the autonomous activation of ERK1/2 was practically abolished (inhibited by more than 90%) by AG 1478 whether in the presence or absence of $1,25(OH)_2D_3$. Exposure of AG 1478-treated cells to KGF restored ERK1/2 activation. Co-treatment with $1,25(OH)_2D_3$ of AG 1478- and KGF-treated cells for 48 h remarkably augmented the activation of ERK1/2 with no effect on the level of ERK protein (Fig. 3B).

We next undertook to examine whether $1,25(OH)_2D_3$ modulates the level of KGFR in HaCaT cells, and in such a case, if this effect depends on the engagement of the KGFR by its ligand. To this end, we assessed the effect of $1,25(OH)_2D_3$ on KGFR levels in autonomously proliferating HaCaT cells and cells treated with AG 1478 and KGF. This allowed us to compare

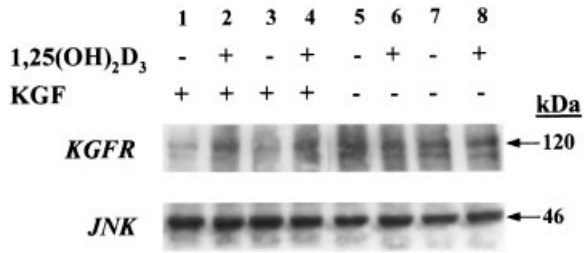

Fig. 4. Effect of $1,25(OH)_2D_3$ on KGFR levels. HaCaT cells were cultured in serum-free MEM-75 as described in ''Methods''. Cultures were treated with AG 1478 and KGF (4 ng/ml) (**lanes 1–4**) or allowed to proliferate autonomously (**lanes 5–8**) in the presence or absence of $1,25(OH)_2D_3$ (100 nM). Cells were harvested and cell extracts subjected to Western blot analysis with antibodies to KGFR. To ascertain equal protein loading, these extracts were also probed with an antibody recognizing the 46-kDa isoform of JNK that was previously shown to be unaffected by treatment with $1,25(OH)_2D_3$ [Ravid et al., 2002]. The data represent data obtained from six independent cultures.

HaCaT cells under culture conditions in which cells proliferate at comparable rates (see Fig. 1) to avoid possible confounding effects of cell proliferation rate on KGFR levels [Marchese et al., 1997]. The results in Figure 4 show that the level of KGFR was lower in HaCaT cells treated with KGF (compare lanes 1 and 3 to lanes 5 and 7). Treatment with $1,25(OH)_2D_3$ increased KGFR levels in KGF dependent HaCaT cells, while having no effect on receptor levels in autonomously proliferating cells. Similar results were obtained with aFGF as the KGFR ligand (data not shown).

$1,25(OH)_2D_3$ was shown to increase the activity of protein kinase C (PKC) in keratinocytes [Bikle, 1996; Gniadecki et al., 1997; Bollinger Bollag and Bollag, 2001] and as PKC is known to modulate MAPK pathways [Sugden and Clerk, 1997; Schonwasser et al., 1998], it is possible that the modulatory effect of $1,25(OH)_2D_3$ on the activation of ERK is mediated by PKC. This possibility was examined by treating HaCaT cells with the specific PKC inhibitor BIM. Activation of PKC by tetradecanoyl phorbol acetate (TPA) brought about marked activation of ERK1/2. This activation was abolished in the presence of BIM, demonstrating the efficacy of this inhibitor in our experimental system (Fig. 5, compare lanes 7 and 8). In contrast to its effect on TPA-treated cells, addition of the PKC inhibitor to KGF dependent cultures caused a small increase in ERK1/2 activation (compare lane 1 to lanes 3 and 5), indicating that in this cellular context PKC exerts an inhibitory effect on the ERK pathway.

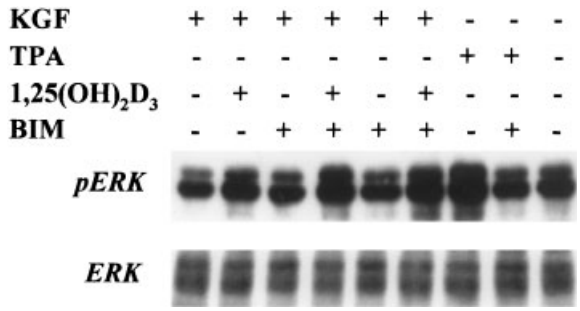

Fig. 5. The involvement of PKC in the modulation of ERK activation by $1,25(OH)_2D_3$ in KGFR ligand dependent HaCaT cells. HaCaT cells were cultured in serum-free MEM-75 as described in ''Methods''. Cultures were treated with AG 1478 and KGF (4 ng/ml) (**lanes 1–6**) or allowed to proliferate autonomously (**lanes 7–9**). The phorbol ester TPA (50 ng/ml) and the PKC inhibitor bisindolylmaleimide (BIM) (2 μM) were added 2 h before harvesting. Cell extracts were subjected to Western blot analysis with antibodies to dually phosphorylated ERK or ERK protein. The data represent one of two independent experiments.

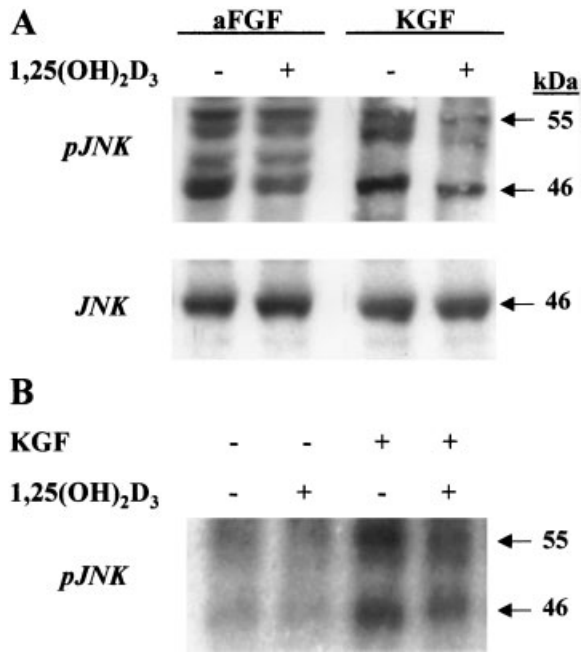

Fig. 6. Effect of $1,25(OH)_2D_3$ on JNK activation in KGFR ligand dependent HaCaT cells. HaCaT cells were cultured in serum-free MEM-75 as described in ''Methods''. Cultures were treated with AG 1478 and KGF (4 ng/ml) or aFGF (4 ng/ml) for 48 h (**A**) or allowed to proliferate autonomously (**B**) in the presence or absence of $1,25(OH)_2D_3$ (100 nM). Autonomously proliferating cultures were exposed to KGF (4 ng/ml) 15 min before harvesting (**B**). Cell extracts were subjected to Western blot analysis with antibodies to dually phosphorylated JNK or JNK protein. The data represent one of four independent cultures.

However, addition of the PKC inhibitor did not interfere with the ability of $1,25(OH)_2D_3$ to increase KGF-mediated stimulation of ERK. This conclusion is reached by comparing lanes 4 and 6 (in the presence of both $1,25(OH)_2D_3$ and the PKC inhibitor) to lanes 3 and 5 (in the presence of the inhibitor but in the absence of $1,25(OH)_2D_3$), excluding a mediatory role of PKC in this activity of the hormone.

In addition to the activation of ERK, binding of growth factors to their receptors results in activation of other MAPK pathways, such as the JNK pathway. Figure 6A shows the interplay between KGFR ligands and $1,25(OH)_2D_3$ on the level of JNK activation. HaCaT cells were treated for 48 h with AG 1478 and aFGF or KGF in the presence or absence of $1,25(OH)_2D_3$. As shown before in HaCaT cells, multiple bands of the activated isoforms of JNK were visualized by immunoblotting with an antibody to the dually phosphorylated enzyme [Ravid et al., 2002]. We found that the effect of the hormone on the level of activated JNK isoforms did not mirror that observed for activated ERK1/2, but rather, the level of activated JNK was decreased following treatment with $1,25(OH)_2D_3$. This decrease was not associated with reduced enzyme levels.

We have recently shown that JNK is activated upon blocking of the autonomous proliferation of HaCaT cells by AG 1478 and that this activation is inhibited by $1,25(OH)_2D_3$ [Ravid et al., 2002]. To avoid this confounding effect of AG 1478, we examined the effect of $1,25(OH)_2D_3$ on the activation of JNK by KGFR ligands in its absence. The level of dually phosphorylated JNK in autonomously proliferating HaCaT cells was negligible but was markedly increased following a brief (15 min) exposure to KGF (Fig. 6B). As in the presence of AG 1478, this activation of JNK, that was solely due to engagement of the KGFR, was also inhibited by $1,25(OH)_2D_3$.

DISCUSSION

The main objective of this research was to examine the potential for cross talk between vitamin D and KGFR ligands on the level of cell proliferation and mitogenic signaling in keratinocytes. A prerequisite for a study of an interaction between different agents is an experimental system devoid of confounding signals emanating from biologically active ingredients required for cell culture maintenance, like those present in primary keratinocyte cultures. In

addition to serum or purified growth factors, such cultures often include agents that increase intracellular cAMP or activate the glucocorticoid receptor. One of the commonly used but poorly defined constituents of such culture medium is bovine pituitary extract, that among many other active agents contains FGFs [Tanigaki-Obana and Ito, 1994]. The study of the specific cross talk between $1,25(OH)_2D_3$ and growth factors of the FGF family is practically impossible in the presence of so many ongoing signaling processes. In contrast to primary keratinocyte cultures, HaCaT keratinocytes that retained their ability to differentiate in vivo and in vitro [Boukamp et al., 1988; Garach-Jehoshua et al., 1998], can proliferate in the absence of serum or any other biologically active agents, rendering them ideally suitable for our purpose [Garach-Jehoshua et al., 1999]. The autonomous proliferation of HaCaT cells, as that of normal keratinocytes, depends on autocrine growth factors belonging to the EGFR ligand family [Garach-Jehoshua et al., 1999]. This feature creates another impediment to the study of the specific interaction between FGFs and $1,25(OH)_2D_3$ in keratinocytes: on the one hand, the mitogenic activity of FGFs is at least partly due to transactivation of the EGFR [Dlugosz et al., 1994] and on the other hand, $1,25(OH)_2D_3$ was shown to modulate EGFR dependent signaling [Garach-Jehoshua et al., 1999]. In this study, we overcame this difficulty by constructing an experimental system of keratinocytes that cannot respond to EGFR ligands and are completely dependent upon KGFR ligands for their proliferation and survival in culture.

An active KGFR was previously shown to be present in HaCaT cells [Capone et al., 2000]. The relative mitogenic efficiency of the various FGFs in our experimental system strongly indicates that the KGFR is the only FGF receptor responsible for the mitogenic action of FGFs in HaCaT cells. This experimental system enabled us to show that $1,25(OH)_2D_3$, at physiological concentrations, increases cell number in KGFR ligand-dependent keratinocyte cultures.

The increase in cell number was associated with modulation of two MAPK pathways, an increase in ERK and a decrease in JNK activation. Whereas activation of ERK is associated with cell cycle progression and enhanced activity of survival pathways [Robinson and Cobb, 1997], activation of JNK can lead to cell cycle arrest and cell death [Ip and Davis, 1998; Leppa and Bohmann, 1999]. The balance between the output of the two MAP kinase cascades is considered to be a major determinant of cell fate [Robinson and Cobb, 1997]. Enhanced activity of ERK coupled to reduced activity of JNK may thus lead to an increase in both cell proliferation and survival of keratinocytes. It is noteworthy that treatment with $1,25(OH)_2D_3$ increased cell number and ERK activation also in autonomously proliferating HaCaT cells that are dependent on signaling via another receptor tyrosine kinase, the EGFR [Garach-Jehoshua et al., 1999]. However, activation of JNK was not observed in these cells under these conditions. The inhibitory effect of $1,25(OH)_2D_3$ on JNK activation by KGFR ligands seems to be one example of a more general phenomenon. We have previously shown that treatment of HaCaT cells with $1,25(OH)_2D_3$ inhibited JNK activation by various different stimuli [Ravid et al., 2002]. It is interesting to note that $1,25(OH)_2D_3$ attenuated JNK activation induced by two opposite stimuli, growth factor activation of receptor tyrosine kinase, on the one hand, and inhibition of receptor tyrosine kinase activity, on the other hand [Ravid et al., 2002].

Availability of cell surface growth factor receptors may be a limiting factor for the mitogenic response of their ligands. Here, we found that KGFR levels in KGF dependent HaCaT cultures increased as a result of treatment with $1,25(OH)_2D_3$, a finding that may account for both the enhanced activation of ERK and the mitogenic activity induced by KGFR ligands. It could be argued that the increase in KGFR levels is secondary to the well documented prodifferentiating activity of $1,25(OH)_2D_3$ [Bikle and Pillai, 1993], since it was previously shown that KGFR levels in keratinocytes increase with differentiation [Marchese et al., 1997; Capone et al., 2000]. This explanation is unlikely in this case, since there was no detectable effect of $1,25(OH)_2D_3$ on KGFR levels in autonomously proliferating cells that were treated with the hormone in the absence of FGFs for the same time period.

As stated above, the effect of $1,25(OH)_2D_3$ on KGFR levels was observed only in the presence of its ligand. Similarly to other growth factors, FGFs are internalized through binding to their receptors. Receptor endocytosis results in down regulation of cell surface receptors and the receptor ligand complexes are subsequently

degraded within the cells [Belleudi et al., 2002]. A possible explanation consistent with our results is that treatment with $1,25(OH)_2D_3$ inhibits ligand-mediated KGFR degradation and that this action accounts for the increased mitogenic potency of FGFs.

Numerous studies have suggested that activation of PKC has a role in the action of vitamin D in keratinocytes. The hormone was shown to activate PKC by inducing the biosynthesis or directly activating phospholipase C and phospholipase D [Bollinger Bollag and Bollag, 2001; Xie and Bikle, 2001]. Moreover, treatment with the PKC inhibitor BIM inhibited some actions of $1,25(OH)_2D_3$ in keratinocytes [Gniadecki et al., 1997]. Stimulation of FGF receptors also leads to activation of phospholipase C and PKC [Szebenyi and Fallon, 1999] that is known to result in ERK activation [Sugden and Clerk, 1997; Schonwasser et al., 1998]. On the other hand, phosphorylation of FGF receptor by PKC was shown to attenuate signaling initiated by this receptor [Le Panse et al., 1994; Gillespie et al., 1995]. Here, we show that inhibition of PKC resulted in enhanced activation of ERK by KGF. This finding accords with a previous report that inhibition of PKC enhanced the mitogenic action of KGF in human keratinocytes [Le Panse et al., 1994]. On the other hand, inhibition of PKC did not have any effect on the modulatory action of $1,25(OH)_2D_3$, allowing us to rule out the possibility that enhanced ERK activation in $1,25(OH)_2D_3$-treated KGF dependent cells is secondary to PKC activation.

The underlying question of this study was whether the overlap between the activities of vitamin D and KGF in the skin may be due, at least in part, to the synergistic interaction between these two agents. Our results support the notion that such a mechanism may indeed be operative in vivo, and that the paracrine KGF–KGFR system in the skin can act in concert with the autocrine vitamin D system in keratinocytes to promote keratinocyte proliferation and survival under situations of stress and injury.

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