UVA-Induced Cell Cycle Progression Is Mediated by a Disintegrin and Metalloprotease/Epidermal Growth Factor Receptor/AKT/Cyclin D1 Pathways in Keratinocytes

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Abstract

UVA (315–400 nm), which constitutes ~95% of the UV irradiation in natural sunlight, represents a major environmental challenge to the skin and is clearly associated with human skin cancer. Here, we show that a low, nonlethal dose of UVA induces dose-dependent cell cycle progression in human HaCaT keratinocytes. We found that UVA induced cyclin D1 accumulation, whereas siRNA knockdown of cyclin D1 blocked the UVA-induced cell cycle progression, indicating that this process is mediated by cyclin D1. UVA irradiation also induced AKT activation; when cells were incubated with phosphatidylinositol-3-OH kinase/AKT inhibitor or infected with dominant-negative AKT, cyclin D1 upregulation, cell cycle progression, and proliferation were inhibited, suggesting that AKT activation is required for UVA-induced cell cycle progression. In contrast, extracellular signal-regulated kinase (ERK) was not activated by UVA exposure; incubation with ERK/mitogen-activated protein kinase inhibitor had no effect on UVA-induced cyclin D1 upregulation and cell cycle progression. Activation of epidermal growth factor receptor (EGFR) was observed after UVA exposure. EGFR kinase inhibitor AG attenuated the UVA-induced AKT/cyclin D1 pathway and cell cycle progression, indicating that EGFR is upstream of AKT/cyclin D1 pathway activation. Furthermore, metalloprotease inhibitor GM6001 blocked UVA-induced cell cycle progression, and siRNA knockdown of a disintegrin and metalloprotease (ADAM)17 had a similar inhibitory effect, demonstrating that ADAM17 mediates the EGFR/AKT/cyclin D1 pathway and cell cycle progression to the S phase induced by UVA radiation. Identification of these signaling pathways in UVA-induced cell proliferation will facilitate the development of efficient and safe chemopreventive and therapeutic strategies for skin cancer. [Cancer Res 2008;68(10):3752–8]

Introduction

UV radiation in sunlight is clearly an important environmental factor in human skin carcinogenesis. Each year, approximately one million new cases of skin cancer are diagnosed in the United States alone, making it the most common type of cancer in this country. In animal models, UV radiation is a complete carcinogen that can both initiate and promote skin carcinogenesis, resulting in squamous cell carcinoma (SCC), basal cell carcinoma, and melanoma (1–3).

UV radiation in sunlight is composed of UVB (280–315 nm) and UVA (315–400 nm). UVA has been considered far less carcinogenic based on limited direct damage to DNA (4). However, UVA is ~20-fold more abundant than UVB in sunlight and much more UVA penetrates the epidermis and reaches the basal germinative layers (5). Recently, UVA was shown to induce p53 mutations in the basal layer of the skin, and UVA signature mutations in p53 have been detected in SCC and solar keratosis (6). Although the mechanisms may differ from those involved in carcinogenesis by UVB (7), these findings confirmed that UVA can initiate tumorigenesis in vivo. Therefore, it is possible that UVA exposure may play a greater role in the development of human skin cancers than is generally assumed. The initiation stage is a rapid process, potentially taking place within a single exposure, whereas promotion may take years to occur and is a reversible process. Therefore chemoprevention targeting signaling pathways in the promotion stage may provide higher success and feasibility (8, 9).

Cell proliferation as a consequence of cell cycle progression is the key process that leads to clonal expansion of initiated cells during tumor promotion. Cyclin D1 is a cell cycle regulatory protein that acts as a growth factor sensor to integrate extracellular signals with the cell cycle machinery, particularly during the G1 phase of the cell cycle (10). Increased cyclin D1 has been associated with mouse skin transformation (11–14). However, the mechanisms of cell cycle progression in UVA-induced tumor promotion and the role of cyclin D1 remain open questions. Understanding the signal transduction pathways by which the UVA signals to cyclin D1 and identifying the critical elements in these pathways will provide targets for chemoprevention.

The epidermal growth factor receptor (EGFR), one of the receptor tyrosine kinases (RTK), plays a pivotal role in regulating cell proliferation, differentiation, and transformation. Binding of growth factors to RTKs promotes receptor dimerization and subsequent activation, which enhances autophosphorylation of RTKs, phosphorylation of numerous cellular proteins, and recruitment of adaptor molecules, thus initiating signaling cascades, including the phosphatidylinositol-3-OH kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) pathways (15, 16). Activation of the PI 3-kinase/AKT and/or ERK pathways can lead to cell proliferation and growth by regulating the cyclin D1 level (17, 18).

To identify the mechanisms in UVA irradiation–induced cell proliferation, we have studied the mechanisms in cell cycle progression induced by low, nonlethal doses of UVA in HaCaT cells, a human keratinocyte line. In this study, cell cycle progression and cell proliferation were observed after UVA exposure. Cyclin D1...
was found to mediate this process, and siRNA knockdown of cyclin D1 was shown to inhibit cell cycle progression. The a disintegrin and metalloprotease (ADAM)/EGFR/AKT pathways transduced UVA signaling to cyclin D1 and induced a proliferative response in human keratinocytes. These data clearly show the mechanisms and the tumor promotional potential of UVA in human skin cells.

Materials and Methods

Cell culture and UVA treatment. HaCaT cells were cultured in 60-mm dishes with normal culture medium. The cell culture medium was replaced with 0.1% fetal bovine serum (FBS) DMEM and cultured for 24 to 36 h. Cells were exposed to UVA as described previously (19–21) after treatment, fresh medium containing 0.1% FBS was added and the cells were incubated at 37°C.

Cell cycle analysis. At predetermined time points after treatment, the cells were harvested and fixed with 5 ml of ice cold 70% ethanol. For cell cycle analysis, the fixed cells were then centrifuged (1,000 rpm; 5 min) and washed with PBS. Then, the cells were incubated with propidium iodide (20 μg/mL) containing RNase A (1 mg/mL; Sigma Chemical). The DNA content was determined by flow cytometry (Beckman Coulter) and ModFitLT software (Verity Software House, Inc.).

Adenovirus infection. The next day after the cells were seeded, empty-vector (a kind gift from Christopher Kontos, Duke University Medical Center, Durham, NC) or dominant-negative AKT (a kind gift from Kenneth Walsh, Boston University, Boston, MA) adenovirus vectors were added to the cells in a multiplicity of infection of 50. After 14 h of infection by incubation with the virus, cells were washed and fed with fresh medium. Twenty-four hours after the initiation of infection, cells were irradiated with UVA.

siRNA transfection. Cells at ~90% confluency were trypsinized and electroporated with siRNA against ADAM17 or cyclin D1 siRNA (Dharmacon) or cy3-labeled control siRNA (Ambion) with Nucleofector (Amaxa) according to the manufacturer's instructions. Briefly, one million HaCaT cells were electroporated in 100 μL Nucleofector Solution V containing 1.5 μg siRNA using program U-20. After transfection, cells were seeded in 2 ml prewarmed medium in a MatTek dish for confocal microscopy analysis or 60-mm dishes for cell cycle analysis or Western blotting.

Western blotting. Western blotting was performed as described previously (19–21). Antibodies used were as follows: cyclin D1 (Cell Signal) and p-AKT473 (Cell Signal); p-ERK (Santa Cruz); p-EGFR (BD Biosciences); and ADAM17 (Calbiochem), AKT (Santa Cruz), ERK (Santa Cruz), ERFR (Neomarker), and β-actin (Sigma).

Results

Cell cycle progression and EGFR/AKT/cyclin D1 signaling induced by UVA in human HaCaT keratinocytes. To study cell cycle progression induced by low-dose UVA exposure, human HaCaT keratinocytes were exposed to different doses of UVA from 1 to 4 J/cm². Eighteen hours after exposure, cells were harvested for cell cycle analysis. ModFit was used to quantify the cell population in the S phase (Fig. 1A). As shown in Fig. 1B, 1 J/cm² UVA exposure increased cell accumulation in the S phase, whereas 2.5 and 4 J/cm² induced significantly (P < 0.05) higher proportions of cells in the S phase (Fig. 1B). It is noteworthy that at these doses, no cell death was detected (data not shown). These data suggest that UVA-induced cell cycle progression is UVA dose dependent. When later time points were examined, including 24 and 48 h after UVA exposure, the proportion of cells in the S phase decreased at each UVA dose compared with the proportion at 18 h after UVA exposure (Fig. 1B). At 48 h after UVA exposure, most of the cells went back to the G0-G1 phase. At this time point, no significant increase in the G2-M phase was observed (data not shown). This suggests that the cells were able to finish the cell cycle and go back to the G1 phase after UVA-induced progression from G1-S phase.

To delineate the mechanisms and pathways involved in UVA-induced cell cycle progression from the G1-S, we exposed cells to UVA at different doses, harvested them at different times, and analyzed protein samples by Western blot. Cyclin D1 expression, AKT phosphorylation, ERK phosphorylation, and EGFR phosphorylation were determined at different time points after UVA exposure or kept in the dark.
As shown in Fig. 1C, UVA exposure (1, 2.5, or 4 J/cm²) increased cyclin D1 accumulation and AKT phosphorylation at 3 hours after the exposure. In contrast, ERK activation remained unchanged after UVA exposure compared with ERK phosphorylation in the dark. These data suggest that low-dose UVA induced cyclin D1 expression and activated AKT but had no effect on the ERK/mitogen-activated protein kinase (MAPK) pathway.

To further identify the upstream signaling, we determined the level of EGFR phosphorylation with and without UVA exposure. As shown in Fig. 1C, EGFR phosphorylation in the cells kept in the dark was low, whereas UVA exposure increased the phosphorylation of EGFR in a dose-dependent manner with 2.2-fold of EGFR phosphorylation at UVA (4 J/cm²) as much as that of dark samples. These data indicated that EGFR is activated by a low dose of UVA.

To determine whether the activation of AKT and EGFR and the increase in cyclin D1 level are time dependent, cells were exposed to UVA (4 J/cm²) or kept in the dark. At 3, 6, or 9 hours after exposure, cells were harvested for Western blot analysis. The activation of AKT and EGFR and the increase in cyclin D1 were detected at 3, 6, and 9 hours after UVA exposure to a similar extent (Fig. 1D). These findings indicate that AKT and EGFR activation and cyclin D1 accumulation upon UVA radiation are sustained.

**Role of cyclin D1 in UVA-induced cell cycle progression.** Cyclin D1 plays a key role in cell cycle progression, especially the...
G1-S progression (10). To determine whether the cyclin D1 accumulation observed in Fig. 1 is responsible for cell cycle progression, HaCaT cells were transfected with siRNA against cyclin D1 or negative control siRNA and then exposed to UVA or kept in the dark.

Although keratinocytes, including HaCaT cells, are difficult to transfect, an 95% transfection efficiency of siRNA was achieved by electroporation as described in the Materials and Methods (data not shown). When HaCaT cells were transfected with siRNA against cyclin D1, a knockdown of 75% was observed (Fig. 2A), whereas transfection alone or with negative siRNA had no effect on cyclin D1 expression. A similar knockdown was seen with cells exposed to UVA (Fig. 2A, right).

As shown in Fig. 2B, UVA irradiation at 2.5 J/cm² induced 19% of the cells in the S phase, similar to cells without transfection (Fig. 1). In comparison, cells transfected with siRNA against cyclin D1 showed only a 12% cell proportion in the S phase after the same dose of UVA exposure (Fig. 2B), indicating that cyclin D1 up-regulation plays an important role in cell cycle progression from the G1-S phase.

Role of AKT activation in UVA-induced cell cycle progression. To determine whether AKT activation is involved in the cell cycle progression induced by UVA exposure, cells were incubated with the PI3K inhibitor LY-294002 (LY; 10 μmol/L) to block AKT activation. The presence of LY inhibited UVA-induced AKT phosphorylation and cyclin D1 accumulation (Fig. 3A), indicating that AKT activation mediates cyclin D1 up-regulation. Compared with cells incubated with EV, infection with A− increased the AKT level, confirming the expression of viral mutant protein in the cells. Overexpression of dominant AKT inhibited UVA-induced AKT activation (Fig. 3C). Compared with cells infected with EV, infection with A− significantly (P < 0.05) reduced the cyclin D1 level and the proportion of cells in the S phase after UVA exposure (Fig. 3D). These data indicate that UVA-induced cyclin D1 accumulation and cell cycle progression require AKT activation.

Role of ERK signaling in UVA-induced cell cycle progression. To determine whether ERK signaling is involved in UVA-induced cell cycle progression, cells were incubated with the ERK inhibitor PD98059 (PD; 20 μmol/L) after UVA exposure. Pretreatment with PD blocked ERK phosphorylation, although it had no effect on cyclin D1 accumulation induced by UVA exposure (Fig. 3A).

We also determined cell cycle progression and cell proportion in the S phase when cells were infected with empty vector (EV) or dominant-negative AKT (A−) adenovirus (22). The dominant-negative mutant adenoviral AKT (A−) expressing the mutant AKT (T308A, S473A) construct cannot be activated by phosphorylation. Infection with A− causes overexpression of mutant AKT, which competes with endogenous wild-type AKT for activation and therefore inhibits AKT activation. As shown in Fig. 3C, infection with A− increased the AKT level, confirming the expression of viral mutant protein in the cells. Overexpression of dominant AKT inhibited UVA-induced AKT activation (Fig. 3C). Compared with cells infected with EV, infection with A− significantly (P < 0.05) reduced the cyclin D1 level and the proportion of cells in the S phase after UVA exposure (Fig. 3D). These data indicate that UVA-induced cyclin D1 accumulation and cell cycle progression require AKT activation.

Role of ERK signaling in UVA-induced cell cycle progression. To determine whether ERK signaling is involved in UVA-induced cell cycle progression, cells were incubated with the ERK inhibitor PD98059 (PD; 20 μmol/L) after UVA exposure. Pretreatment with PD blocked ERK phosphorylation, although it had no effect on cyclin D1 accumulation induced by UVA exposure (Fig. 3A). As
shown in Fig. 4B, the presence of PD had no effect on UVA-induced cyclin D1 up-regulation and cell cycle progression, implying that ERK signaling is not involved in this process.

**Role of EGFR activation in UVA-induced cell cycle progression.** To determine whether EGFR activation is required for the UVA-induced AKT/cyclin D1 pathway and cell cycle progression, cells were incubated with the EGFR inhibitor AG1478 (AG; 1 μmol/L). The presence of AG blocked EGFR phosphorylation, AKT activation, and basal ERK phosphorylation (Fig. 5A). As compared with cells incubated with vehicle alone, AG significantly (P < 0.05) reduced the cell proportion in the S phase (Fig. 5B). EGF (10 ng/mL), an EGFR ligand, induced 30% of cells in the S phase compared with 8% in control cells and increased EGFR phosphorylation, AKT phosphorylation, and cyclin D1 accumulation (data not shown). These findings indicate that EGFR activation is required for the activation of the AKT/cyclin D1 pathway and cell cycle progression from the G1-S phase induced by UVA radiation.

**Role of ADAM17 in UVA-induced cell cycle progression.** We have found that EGFR is activated by UVA exposure at a lower nonlethal dose. However, the mechanism of EGFR activation is unknown. The members of (ADAM) family transactivate EGFR in vivo and in vitro (23). ADAM17, a member of the ADAM family, plays an essential role in activating EGFR pathways compared with other members of the ADAM family (23–26). To determine whether metalloproteases are involved in the EGFR activation and cell cycle progression induced by low-dose UVA irradiation, we incubated cells with the metalloprotease inhibitor GM6001 (GM; 25 μmol/L) and then exposed them to UVA or kept them in the dark. Pretreatment with GM inhibited EGFR phosphorylation induced by UVA exposure and reduced the basal EGFR phosphorylation level (Fig. 6A). As compared with cells incubated without GM, the presence of GM reduced the proportion of cells in the S phase significantly (P < 0.05) after UVA exposure (Fig. 6B).

To determine the specific involvement of ADAM17 in the observed cell cycle progression, we used siRNA against ADAM17. Transfection by siRNA reduced ADAM17 expression by ~90% (Fig. 6C) and reduced EGFR phosphorylation induced by UVA exposure (Fig. 6C). UVA exposure caused an increase in S-phase cell proportion when cells were transfected with control/negative siRNA (Fig. 6D), similar to the result without transfection (Fig. 1). As compared with negative siRNA, siRNA against ADAM17 attenuated the cell proportion in the S phase significantly (P < 0.05; Fig. 6D). These data show that ADAM17 mediates EGFR activation and the cell cycle progression induced by UVA exposure.

**Discussion**

In the present work, cell cycle progression in response to low-dose UVA irradiation was investigated using the HaCaT human keratinocyte cell line. HaCaT cells are spontaneously immortalized through p53 mutation, a modification similar to that initiated in cells during skin carcinogenesis, thereby providing a valuable model system for the investigation of tumor promotion events associated with skin carcinogenesis (27). Using this model, we have shown that a nonlethal dose of UVA irradiation induces cell cycle progression and cell proliferation, and that this process is mediated via cyclin D1. In this process, the ADAM/EGFR/AKT pathway transduced the UVA signal to cause cyclin D1 up-regulation and cell proliferation, which might be equivalent to inducing tumor promotion in skin carcinogenesis.

AKT activation has been shown to be one of the key molecular determinants of keratinocyte transformation (28). AKT has been shown to increase the up-regulation of cyclin D1 in AKT-transformed keratinocytes via both transcriptional and posttranscriptional processes (17). In our study, we show that AKT activation is mediated by the EGFR pathway after UVA exposure. The accumulation of cyclin D1 by UVA exposure could be due to both transcriptional and posttranscriptional regulation by AKT activation. Knockdown of cyclin D1 expression reduced the G1/S transitions induced by UVA exposure, demonstrating that cyclin D1 plays an important role in this process. Regardless of the precise regulation mechanisms, it seems that for cyclin-D1–mediated cell
cycle progression induced by UVA exposure, AKT activation is required. In addition to cyclin D1, other factors might be involved in UVA-induced cell cycle progression because knockdown of cyclin D1 did not block cell cycle progression completely. Multiple mechanisms including the blockade of Chk1 by phosphorylation at Ser280 by EGFR/AKT activation (29) may play important roles in UVA-induced cell proliferation. It is possible that AKT activation as a central signaling pathway mediates the proliferative effect of UVA through several downstream targets. We are currently in the process of identifying those targets to understand the complex mechanisms of UVA-induced cell proliferation and skin carcinogenesis.

The ERK/MAPK pathway is neither activated by low-dose UVA exposure nor involved in UVA-induced cell cycle progression, although EGFR activation was observed in this study. We previously detected sustained and prolonged ERK/MAPK activation after apoptosis-inducing UVA but not low-dose UVA exposure (21). It seems that ERK signaling is clearly UVA dose dependent, which is expected because the low dose of UVA used in this study did not induce cell death. High-dose UVA-induced ERK activation protected cells from cell death and allowed more cells to survive (21).

The involvement of EGFR activation was first seen in the activation of EGFR after UVA irradiation and further confirmed by the inhibitory effect of the EGFR kinase inhibitor AG on cell cycle progression. We have previously shown that a lethally high dose (24 J/cm²) did not induce EGFR phosphorylation (20). EGFR activation clearly seems to be UVA dose dependent and occurs only at a low UVA dose. The level of EGFR activation in this study did not cause EGFR down-regulation, as seen by the failure of the EGFR level to decrease after UVA exposure (data not shown). This moderate EGFR activation is sufficient to activate the downstream AKT/cyclin D1 signaling, and transduce a proliferative signal to induce cell cycle progression.

ADAM proteins are membrane-anchored metalloproteases that process and shed the ectodomains of membrane-anchored growth factors, cytokines, and receptors (23). Tumor cells frequently produce autocrine growth factors and are often highly proliferative, motile, and invasive (30). ADAMs can activate EGFR by shedding the EGFR ligand (31). During EGFR transactivation, an upstream signal acts through a G-protein–coupled receptor to activate the EGFR ligand (31). ADAM17-mediated transactivation of EGFR by amphiregulin seems to be important for tumor cell growth and migration (25).

Recently, it has been reported that environmental stress signaling, including oxidative and osmotic stress, is mediated by ADAM proteases and hearin-binding epidermal growth factor (32). UVC exposure stimulated MAGP cleavage via the metalloprotease pathway (33). In this work, we observed that UVA induces cell cycle progression and that ADAM protease is involved because the metalloprotease inhibitor GM inhibits cell cycle progression after UVA exposure. Furthermore, siRNA knockdown of ADAM17 attenuated EGFR activation and the cell cycle progression induced by UVA, indicating that ADAM17 was involved in the signal transduction for UVA irradiation to the EGFR/AKT/cyclin D1 pathway and cell cycle progression. The incomplete inhibition of EGFR activation and G1-S transition by siRNA against ADAM17 compared with the metalloprotease inhibitor GM suggests that other mechanisms may contribute to UVA-induced EGFR activation. A recent study has shown that the oxidative inhibition of protein tyrosine phosphatase λ by UV results in the activation of EGFR (34). In addition, other ADAM members and/or metalloproteases including ADAM 10 may also be involved in UVA-induced EGFR activation and G1-S transition. The precise mechanisms of EGFR/AKT activation by ADAM17 and the potential role of other metalloproteases are under further investigation in our lab.

In summary, a nonlethal dose of UVA has been shown to induce cell cycle (the G1-S) progression of human HaCaT keratinocytes, mediated by increased cyclin D1. The ADAM/EGFR/AKT pathway is required for UVA-induced cyclin D1 up-regulation and cell cycle progression. Given the complexity and interactions of this signaling pathway, it is entirely possible that other pathways may also play a role in UVA-induced cell proliferation. This study provides compelling evidence that UVA alone, at low, nonlethal doses, has the potential to be a human skin tumor promoter. The acquisition of proliferation signaling in UVA-irradiated keratinocytes may be an important factor in the formation of premalignant skin lesions including actinic keratoses and malignant SCC in the clinical setting. Identification of the fundamental mechanisms of the effect of UVA on tumor promotion will facilitate the development of safe and efficient chemopreventive and therapeutic strategies for skin cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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