Molecular and Cellular Pathobiology

Sunlight UV-Induced Skin Cancer Relies upon Activation of the p38α Signaling Pathway

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Abstract

The activation of cellular signal transduction pathways by solar ultraviolet (SUV) irradiation plays a vital role in skin tumorigenesis. Although many pathways have been studied using pure ultraviolet A (UVA) or ultraviolet B (UVB) irradiation, the signaling pathways induced by SUV (i.e., sunlight) are not understood well enough to permit improvements for prevention, prognosis, and treatment. Here, we report parallel protein kinase array studies aimed at determining the dominant signaling pathway involved in SUV irradiation. Our results indicated that the p38-related signal transduction pathway was dramatically affected by SUV irradiation. SUV (60 kJ UVA/m²/3.6 kJ UVB/m²) irradiation stimulates phosphorylation of p38α (MAPK14) by 5.78-fold, MSK2 (RPS6KA4) by 6.38-fold, and HSP27 (HSPB1) by 34.56-fold compared with untreated controls. By investigating the tumorigenic role of SUV-induced signal transduction in wild-type and p38 dominant-negative (p38 DN) mice, we found that p38 blockade yielded fewer and smaller tumors. These results establish that p38 signaling is critical for SUV-induced skin carcinogenesis. Cancer Res; 73(7); 2181–8. ©2013 AACR.

Introduction

Each year, more than 1 million Americans are diagnosed with skin cancer, including 68,000 cases of melanoma (http://www.Cancer.gov/cancertopics/types/skin). Epidemiologic evidence suggests that solar ultraviolet (SUV, i.e., sunlight) irradiation is the most important risk factor for any type of skin cancer (1, 2). SUV comprises approximately 95% UVA and 5% UVB. Both UVA and UVB can cause DNA damage (3), which is considered one etiologic factor contributing to the development of skin cancer. UV irradiation can cause DNA lesions, such as DNA cyclobutane pyrimidine dimers (4). Consequently, many gene mutations have been identified in skin cancer, including mutations in p53, p16, and ras (5, 6). These gene mutations and related signal pathway activation are known to contribute to skin carcinogenesis (7, 8). Activation of UV-induced cellular signaling pathways plays a vital role in UV-induced skin tumorigenesis (9). UVB can activate PKC, ATM, Akt, extracellular signal–regulated kinase (ERK), c-jun NH2 terminal kinase (JNK), and p38 (9–14). UVA also can activate Akt, ERKs, and JNKs in HaCaT or JB6 cells (15, 16). Consequently, these kinases activate their downstream transcription factors such as NF-κB and activator protein-1 (AP-1; ref. 17). These transcription factor families are heavily involved in many cellular processes including proliferation, differentiation, and survival. On the basis of these results, inhibitors of these kinases, such as norathyriol (an ERK inhibitor; ref. 18), kaempferol (an Src and RSK2 inhibitor; ref. 14), luteolin (a PKC inhibitor; ref. 19), and caffeic acid (a Fyn inhibitor), have been suggested for UV-induced skin cancer chemoprevention (20).

Although UV activation of signal transduction pathways and their role in tumorigenesis have been intensively examined, differences in SUV-, UVA-, or UVB-induced signal transduction pathways have not been fully investigated (21). Furthermore, the primary signal transduction pathway and key molecules involved in SUV-induced tumorigenesis are not yet completely elucidated. Answers to these questions will contribute to a better understanding of UV-induced skin carcinogenesis and development of new strategies for skin cancer prevention.

Here, we first used phospho-protein kinase array analysis to identify the potential signaling pathways induced by SUV irradiation. The results indicated that the p38-related signal transduction pathway was markedly affected by SUV treatment. To investigate the role of p38 in SUV-induced tumorigenesis, wild-type and p38 dominant-negative (DN) mice were used. Our results indicated that compared with wild-type control mice, p38 DN mice exhibited fewer and smaller tumors when exposed to chronic SUV. In conclusion, our results indicated that p38 signaling plays an important role in SUV-induced skin carcinogenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-12-3408

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Materials and Methods

Materials

Chemical reagents, including Tris, NaCl, and SDS, for molecular biology and buffer preparation were purchased from Sigma-Aldrich. Antibodies for Western blot analysis were from Cell Signaling Technology, R&D Systems, or Santa Cruz Biotechnology. The Human Phospho-Kinase and Human Phospho-MAPK Arrays were obtained from R&D Systems.

Solar UV, UVB, and UVA irradiation systems

The solar UV resource was UVA-340 lamps purchased from Q-Lab Corporation. The UVA-340 lamps provide the best possible simulation of sunlight in the critical short wavelength region from 365 nm down to the solar cutoff of 295 nm with a peak emission of 340 nm (22). The percentage of UVA and UVB of UVA-340 lamps was measured by a UV meter and was 94.5% and 5.5% respectively. UVB irradiation was conducted in a chamber with a transiluminator emitting UVB light photons and fitted with an Eastman Kodak Co. Kodacel K6808 filter to eliminate all wavelengths below 290 nm (23). The UVA source was a Phillips TL100w/10R system from Ultraviolet Resources International. UVA irradiation was filtered through 6 mm of plate glass, eliminating UVC and UVB light below 320 nm (16).

Cell culture and UV irradiation

Normal human skin keratinocytes, N/TERT-1 and N/TERT-2G cells, were generously provided by Dr. James G Rheinwald (Harvard Medical School, Boston, MA). These cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available to ensure that all cell-based experiments were completed. N/TERT-1 and N/TERT-2G cells are hTERT-immortalized human keratinocyte cell lines derived from different batches of the same cell line. They still maintain the ability to differentiate (24). N/TERT-1 and N/TERT-2G cells were maintained at low density in keratinocyte serum-free media (sfm; Invitrogen) supplemented with 0.2 ng/mL EGF, 0.4 mmol/L Ca\(^{2+}\), 5.5% bovine pituitary extract (BPE), 5 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. When 90% confluent, cells were starved for 24 hours with F-12K medium without serum and then irradiated with UVA, UVB, or SUV in the same medium. The cells were harvested and proteins visualized by Western blotting or protein kinase array.

Western blotting analysis

For Western blotting, cells (1 \(\times\) 10\(^6\)) were cultured in 10-cm dishes for 24 hours. The cells were then cultured with F-12K medium without serum for 24 hours and then treated with various doses of UVA, UVB, or SUV in the same medium. The protein concentration was determined, and lysate proteins (30 \(\mu\)g) were subjected to 10% SDS-PAGE. After transferring proteins, membranes were incubated with a specific primary antibody at 4°C overnight. Protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridization with a horseradish peroxidase–conjugated secondary antibody.

Protein kinase array analysis

Analysis of phosphorylated proteins by human phospho-kinase-activated protein kinase (MAPK) and human phospho-kinase arrays was conducted following the manufacturer’s instructions. Briefly, cell lysates (500 \(\mu\)g) were collected and incubated with each array at 4°C overnight on a rocking platform shaker. The cell lysate was removed the next day, and arrays were washed 3 times with washing buffer. Arrays were incubated with the primary antibody solution for 2 hours at room temperature and washed 3 times with washing buffer. The second antibody solution was added to the array and incubated on a rocking shaker for another 1 hour. The array was washed 3 times with washing buffer, and protein spots were visualized using a chemiluminescence detection kit. The density of the each duplicated array spot was assessed using the ImageJ computer program (v.1.37v, NIH), and the density was calculated by subtracting the background and PBS negative control. The fold change was obtained by comparing SUV-treated samples with the untreated control (indicated as a value of 1).

SUV-induced skin carcinogenesis

Skin carcinogenesis in mice was induced using UVA-340 lamps (Q-Lab Corporation). SKH-1-p38 Wt and SKH-1-p38 DN hairless mice were developed and provided by Dr. G. Tim Bowden (University of Arizona Cancer Center, Tucson, AZ) and propagated and maintained at The Hormel Institute (Austin, MN). Mice were maintained under conditions based on the guidelines established by Research Animal Resources, University of Minnesota. The wild-type and transgenic mice were identified by PCR (Supplementary Fig. S1) using the K14 forward primer (5-AAG CAG TCG CAT CCC TTT CC-3) and reverse primer (5-ACA GGT TCT GGT ATC GTT C-3). The mice were divided into 4 groups of 20 animals each (6-week-old, with an average body weight of 25 g). For untreated control groups, SKH-1-p38 Wt and SKH-1-p38 DN mice were not irradiated with SUV. For the experimental SUV-treated groups, SKH-1-p38 Wt and SKH-1-p38 DN mice were initially treated with an SUV dose of 30 kJ UVA/m\(^2\)/1.8 kJ UVB/m\(^2\) twice a week. The dose was increased by 10% each week until week 6. At week 6, the dose reached 48 kJ UVA/m\(^2\)/2.9 kJ UVB/m\(^2\), and this dose was maintained from weeks 6 to 15. At 15 weeks, SUV exposure was discontinued, and tumor growth was monitored an additional 15 weeks. A tumor was defined as an outgrowth of > 1 mm in diameter that persisted for 2 weeks or more. Tumor numbers and volume were recorded every week until the end of the experiment. Half of the samples were immediately fixed in 10% neutral-buffered formalin (NBF) and processed for hematoxylin and eosin (H&E) staining and immunostaining. The other half was frozen for Western blot analysis.

Statistical analysis

All quantitative data are expressed as means ± SE or SD as indicated. One-way ANOVA was used for statistical analysis. A probability of \(P < 0.05\) was used as the criterion for statistical significance.
Results

SUV activates JNKs in a time- and dose-dependent manner

JNK activation is an indicator of cellular stress and JNK-induced phosphorylation of the transcription factor c-Jun and the subsequent expression of c-Jun–induced genes mediate JNK-induced apoptosis (25). Previously, we reported that JNKs were activated by both UVA and UVB (26, 27), and therefore, we first determined whether SUV could also induce JNK activation. To examine the dose and the time point at which SUV...
might stimulate a response in normal keratinocytes, we chose a TERT-immortalized normal human keratinocyte line (N/TERT-1) and treated the cells with different doses of SUV and examined the phosphorylation of JNKs and their downstream target, c-Jun. The results indicated that the phosphorylation of JNKs was increased at a dose of 60 kJ UVA/m²/3.6 kJ UVB/m² (Fig. 1A, top). Higher doses (90 kJ UVA/m²/5.4 kJ UVB/m²) caused the cells to undergo apoptosis within 3 hours. We thus determined the optimal time point after exposure to SUV (60 kJ UVA/m²/3.6 kJ UVB/m²) at which JNKs were clearly activated and cells maintained a healthy condition. Results indicated that JNKs and downstream c-Jun could be activated as early as 15 minutes after SUV irradiation and sustained up to 4 hours, at which time the phosphorylation level began to decrease (Fig. 1A, bottom). To identify the major MAPK pathway induced by SUV in keratinocytes, we conducted a parallel phospho-MAPK array assay on cells treated with SUV, UVA, or UVB irradiation. The doses of UVA and UVB were the same UV doses observed in SUV. Before the MAPK array assay, we determined that the cellular response to UVA, UVB, and SUV irradiation. UVB (3.6 kJ/m²) or SUV (60 kJ UVA/m²/3.6 kJ UVB/m²), but not UVA, could activate JNKs (Fig. 2A, top). Interestingly, we found that ERK phosphorylation (T202/Y204) was decreased after UVA, UVB, or SUV treatment (Fig. 2A, top). We then used the same cell lysates to conduct a phospho-MAPK array assay. The results indicated that SUV induced increased phosphorylation of p38α by 5.78-fold, MSK2 by 6.38-fold, and HSP27 by 34.56-fold compared with the untreated control. The pattern induced by SUV was similar to the induction by UVB. Phosphorylation of p38α increased 5.88-fold, MSK2 7.65-fold, and HSP27 20.67-fold. In contrast, induction by UVA was weaker in that phosphorylation of p38α increased 1.71-fold, MSK2 0.86-fold, and HSP27 3.44-fold. Phosphorylation of JNKs was also increased by 2.08-fold after SUV treatment (Fig. 2A and B).

**Figure 3.** The p38α signaling pathway is strongly activated by UVB or SUV irradiation. A, N/TERT-1 cells were cultured with keratinocyte sfm medium and starved for 24 hours. The cells were irradiated with UVA (60 kJ/m²), UVB (3.6 kJ/m²), or SUV (60 kJ UVA/m²/3.6 kJ UVB/m²). Protein extracts (500 µg) were used for a phospho-kinase array analysis. Array spots were visualized using an ECL kit. B, the density of each duplicated array spot (A) was measured as described in Materials and Methods. The graph shows the fold change in SUV-induced phosphorylation of JNKs, c-Jun, p38α, MSK1/2, and HSP27 compared with untreated control (i.e., value of 1). Data are shown as an average of duplicate samples. C, cell lysates (30 µg) from A were subjected to 10% SDS-PAGE. Protein bands were visualized using a chemiluminescence detection kit after hybridization with a horseradish peroxidase–conjugated secondary antibody.

**p38α activation is the dominant SUV-induced signaling transduction pathway**

MAPKs play an important role in UV-induced skin cancer (28). To identify the major MAPK pathway induced by SUV in keratinocytes, we conducted a parallel phospho-MAPK array assay on cells treated with SUV, UVA, or UVB irradiation. The doses of UVA and UVB were the same UV doses observed in SUV. Before the MAPK array assay, we determined that the cellular response to UVA, UVB, and SUV irradiation. UVB (3.6 kJ/m²) or SUV (60 kJ UVA/m²/3.6 kJ UVB/m²), but not UVA, could activate JNKs (Fig. 2A, top). Interestingly, we found that ERK phosphorylation (T202/Y204) was decreased after UVA, UVB, or SUV treatment (Fig. 2A, top). We then used the same cell lysates to conduct a phospho-MAPK array assay. The results indicated that SUV induced increased phosphorylation of p38α by 5.78-fold, MSK2 by 6.38-fold, and HSP27 by 34.56-fold compared with the untreated control. The pattern induced by SUV was similar to the induction by UVB. Phosphorylation of p38α increased 5.88-fold, MSK2 7.65-fold, and HSP27 20.67-fold. In contrast, induction by UVA was weaker in that phosphorylation of p38α increased 1.71-fold, MSK2 0.86-fold, and HSP27 3.44-fold. Phosphorylation of JNKs was also increased by 2.08-fold after SUV treatment (Fig. 2A and B). Consistent with the Western blotting results, the array results indicated that phosphorylation of ERKs (T202/Y204) was decreased, along with its downstream kinases RSK1 (Ser 380) and RSK2 (Ser 386). The phosphorylation pattern of the various p38 isoforms, including p38δ, p38γ, p38δ, was not as clear as the phosphorylation of p38α after SUV irradiation (Supplementary Fig. S2).
Solar UV activates the p38α-signaling pathway in a time-dependent manner

To further identify the primary signal transduction pathway induced by SUV, UVA, or UVB, we used a human phospho-kinase array comprising 46 kinases, including the kinases from the phospho-MAPK array. The results indicated that SUV increased phosphorylation of p38α by 2.53-fold, MSK1/2 by 3.67-fold, CREB by 2.67-fold, and HSP27 by 3.36-fold compared with control. Similarly, UVB increased phosphorylation of p38α by 2.36-fold, MSK1/2 by 3.97-fold, CREB by 2.40-fold, and HSP27 by 2.54-fold. In contrast, UVA had little effect (e.g., p38α = 1.01-fold, MSK2 = 1.00-fold, CREB = 1.22-fold, and HSP27 = 0.91-fold; Fig. 3A and B). To determine whether the phospho-MAPK array and phospho-kinase array data are reliable, we used the same cell lysates for Western blot analysis. SUV and UVB, but not UVA, could strongly activate p38, MSK1/2, and CREB (Fig. 3C). Besides the p38-related pathway activation, we also found that SUV or UVB induced phosphorylation of several other kinases and transcription factors including paxillin (Y118), Src (Y419), PLCγ-1 (Y783), Fyn (Y402), Yes (Y426), STAT1 (Y701), STAT3 (Y705), STAT4 (Y693), and STAT5β (Y699; Supplementary Fig. S3). To further confirm that the p38α signaling pathway dominates the cellular response to SUV in keratinocytes, we conducted the phospho-kinase array assay over a time course, harvesting cells at 15, 60, or 180 minutes after irradiation. Again we found that, compared with other kinases, p38α signaling was dramatically changed in a time-dependent manner (Fig. 4A; Supplementary Fig. S4). The Western blotting data for the same batch of cell lysates was consistent with the results of the array analysis (Fig. 4B).

A dominant-negative inactive p38 increases the incidence of SUV-induced skin tumorigenesis in SKH-1 hairless mice

The p38 protein plays a functional role in UVB-induced carcinogenesis (29, 30). To further investigate the role of p38 in SUV-induced skin carcinogenesis, we exposed p38 domain-
negative (DN) and wild-type SKH-1 hairless mice to SUV for 15 weeks and then stopped treatment and observed tumor growth for an additional 15 weeks. The results indicated that tumors began to emerge at week 27 but the p38 DN mice exhibited fewer and smaller tumors compared with wild-type mice (Fig. 5A–C). The average number of SUV-induced tumors per mouse was significantly decreased in SKH-1 p38 DN mice compared with wild-type mice (\( P < 0.05 \); Fig. 5B). The size (mm\(^3\)) of tumors in SUV-treated mouse skin was also significantly decreased in SKH-1 p38 DN mice (\( P < 0.05 \); Fig. 5C). Skin and tumor samples were processed for H&E staining. After treatment with SUV, epidermal thickness in wild-type mice was increased by edema and epithelial cell proliferation, whereas SKH-1 p38 DN mice showed a much smaller increase in epidermal thickness and inflammation (Fig. 5D).

Discussion

SUV or sunlight has been recognized as an environmental factor in the development of melanoma and non-melanoma skin cancers for many years. SUV comprises approximately 95% UVA and 5% UVB. Both UVA and UVB can activate signal transduction pathways related to cell survival (16, 21). However, under normal sunlight conditions, both UVA and UVB irradiate skin. The identification of the cellular signaling pathway(s) evoked by SUV and their role in skin carcinogenesis needs to be further addressed. Here, UV-340 lamps were used to mimic SUV and to irradiate human N/TERT-1 normal keratinocytes. We found that SUV (60 kJ UVA/m\(^2\)/3.6 kJ UVB/m\(^2\)), which is equal to 15 minutes of sun exposure in Austin, Minnesota in midsummer from 2 to 4 PM, can activate p38\(\alpha\) and JNKs (Figs. 1 and 2).
MAPKs play a major role in UV-induced skin cancer development (28). A phospho-MAPK array analysis indicated that p38α and its downstream target proteins, MSK2 and HSP27, were strongly activated by UVB and SUV (Fig. 2A and B). However, other isoforms of p38, including p38β, p38γ, and p38δ, were not affected (Supplementary Fig. S2). Moreover, phospho-kinase array and Western blotting data also clearly indicated that the p38α signaling pathway was strongly activated by UVB and SUV irradiation (Figs. 3A–C and 4A and B). These data provide firm evidence that p38α signaling is the pathway most strongly activated by SUV. Besides p38α signaling, JNK signaling was also activated after SUV irradiation and its role in SUV-induced skin carcinogenesis needs to be elucidated in future studies. Interestingly, the kinase activation pattern was similar in UVB- and SUV-induced signaling, which implies that UVB plays a major role in SUV-induced signaling. Surprisingly, we found that ERK phosphorylation (T202/Y204) was decreased in UVA-, UVB-, or SUV-treated N/TERT-1 cells. This phenomenon has also been observed in UV-irradiated human lung adenocarcinoma ASCT-a-1 cells. The ERK activation by UV might be related to the activation of the JNKs/Forkhead box O (FOXO) transcription factors (31). JNK activation of c-Jun can result in the transcription of ERK phosphatases, which are involved in ERK inactivation (32). Interestingly, ERK activation can be observed in JB6 and HaCaT cells (16, 18, 33). ERK activation in these cell lines likely involves EGFR receptor (EGFR) activation by UV (33–35).

Besides the MAPKs, several other kinases and transcription factors, including Fyn, Src, and PKC (14, 19, 20), have been reportedly activated by UVB irradiation. Here, we also found that paxillin (Y118), Src (Y419), PLCγ1 (Y783), Fyn (Y402), Yes (Y426), STAT1 (Y701), STAT3 (Y705), STAT4 (Y693), and STAT5β (Y699) were activated by SUV and UVB irradiation (Supplementary Figs. S3 and S4).

The p38 signal transduction pathway has been implicated in both suppression and promotion of tumorigenesis. Inhibition of p38 can reduce acute UVB-induced inflammation and chronic UVB-induced skin cancer (9). The p38-regulated/activated protein kinase, a substrate of p38, plays an important role in Ras-induced senescence and tumor suppression (36). However, activation of p38 is also critical for the anti-proliferation effect of β-elemene (37). These reports suggest that the role of p38 in tumorigenesis may be cell-specific and tissue-dependent. Here, p38 DN SKH-1 mice were used to investigate the role of p38 in SUV-induced skin cancer. The results indicated p38 DN mice developed less skin tumors and exhibited slower tumor growth than wild-type mice (Fig. 5), which is consistent with the signaling activation induced by UVB. These data indicated that p38 is dramatically activated by SUV and plays a functional role in SUV-induced skin carcinogenesis. Besides p38, the mechanism probably involves p38 downstream substrates, MSK, CREB, and HSP27, which are also important in carcinogenesis (38, 39). Furthermore, p38 also regulates AP-1 and its target gene cox-2, which also play a role in 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and carcinogenesis (40).

In conclusion, our data show that the p38α signal transduction pathway is markedly activated by SUV irradiation and p38α plays a vital role in chronic SUV-induced skin cancer. Our work also suggests that p38α might be a promising target for skin cancer chemoprevention.

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

Authors’ Contributions
Conception and design: K. Liu, Y.-Y. Cho, G.T. Bowden, Z. Dong
Development of methodology: K. Liu, Y.-Y. Cho, W. Ma, S. Li, Z. Dong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-Y. Cho, W. Ma, K. Yao, S. Li, Z. Dong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Liu, A.M. Bode, S. Li, Z. Dong
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Acknowledgments
The authors thank Tonya Poorman for assistance in submitting our manuscript.

Grant Support
This work was supported by The Hormel Foundation and NIH grants CA027502, CA077646, CA120888, ESO04548, and R37 CA081064.

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Received August 31, 2012; revised December 26, 2012; accepted January 8, 2013; published OnlineFirst February 4, 2013.

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Cancer Res 2013;73:2181-2188. Published OnlineFirst February 4, 2013.