Ultraviolet B Irradiation Promotes Tumorigenic and Metastatic Properties in Primary Cutaneous Melanoma via Induction of Interleukin 8

Rakesh K. Singh, Mordechail Gutman, Reuven Reich, and Menashe Bar-Eli

ABSTRACT

UV radiation has been shown to play a role in the initiation of human cutaneous melanoma, but its role in the development of malignant melanoma to the metastatic state is not very well defined. Although previous studies have concentrated on the effect of UV-B on the host immune response, the effect of UV-B on the tumor cells was not elucidated. Here we show that UV-B can induce interleukin 8 (IL-8) mRNA and protein secretion in human cutaneous melanoma with negligible expression of IL-8. UV-B-induced IL-8 was constitutively expressed 60 days after irradiation in tumors implanted in mice. Induction of IL-8 was UV-B dose dependent and blocked by cyclohexamide, indicating that de novo protein synthesis is required for its expression. The UV-irradiated cells demonstrated enhanced tumorigenicity and metastatic potential in nude mice. The increase in tumorigenicity and metastatic ability could be explained by the increase in M, 72,000 type IV collagenase activity and angiogenesis attributed to the induction of IL-8 after irradiation. The acquisition of the metastatic phenotype induced by UV-B could not be attributed to abnormalities in the p53 or MTS-1 (p16INK4) genes. To the best of our knowledge, this is the first report to show that UV-B can increase the aggressiveness of human cutaneous melanoma for growth and metastasis.

INTRODUCTION

The reduction of stratospheric ozone over the past 20 years has raised concern over the possibility that additional erosion will increase danger of exposure to UVR1 (1, 2). Epidemiological data suggest that exposure to UVR plays a major role in the development of at least some cutaneous melanoma (3). The role of UV radiation in the development of human cutaneous melanoma to its metastatic state is not very well characterized. Earlier studies have demonstrated that UV irradiation can dramatically enhance the induction of primary cutaneous melanoma in mice (4, 5), and that injection of transplantable melanoma cells into chronically UV-irradiated skin resulted in an increased incidence and enhanced spread of melanoma (6, 7). These studies focused mainly on the effect of UV on the host immune response while the effect of UV-irradiation as a promoter agent on the tumor cells themselves remained unclear. UVR has been shown to produce marked pathophysiological responses in animal models. It is one of the most potent stimulators of cytokine production from different cell types in the skin (8, 9). Indeed, after UV irradiation, several cytokines have been shown to be up-regulated in epidermal cells and keratinocytes (10). These cytokines include: IL-1 (11), IL-6 (12), IL-8 (9), IL-10 (13, 14), and TNF-α (15). However, the role of UVR in mediating cutaneous melanoma progression by cytokine production has not been well defined. Malignant melanoma cells produce a variety of cytokines and growth factors either constitutively or via induction by other cytokines (16, 17) that support growth and progression directly or indirectly.

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Received 4/13/95; accepted 6/14/95.

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1 Supported in part by NIH Grant CA 41525 (M. B.-E.).
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The abbreviations used are: UVR, UV radiation; IL, interleukin; TNF, tumor necrosis factor; MTS-1, multiple tumor suppressor 1.

Recently, we showed that the expression of the multifunctional cytokine IL-8 is directly correlated with the metastatic phenotype in human melanoma cell lines (18). The weakly tumorigenic melanoma cells in nude mice did not express detectable levels of IL-8 mRNA and protein.

The purpose of this study was to investigate whether exposure to UVR can modulate the progression of human cutaneous melanoma to the tumorigenic and metastatic states. We found UVR exposure to induce IL-8 mRNA and protein secretion in a human cutaneous melanoma. UV-irradiated cells demonstrated enhanced tumorigenicity and metastatic potential in nude mice, which can be attributed to the observed increase in M, 72,000 type IV collagenase activity and angiogenesis, mediated possibly by the induction of IL-8. Our data support the notion that UVR maybe involved not only in the initiation of melanomas but in their progression to malignancy.

MATERIALS AND METHODS

Cell Line and Culture Condition. The SB-2 cell line was isolated from a primary cutaneous lesion (19) and was a gift from Dr. B. Giovanella (St. Joseph's Hospital Cancer Center, Houston, TX). The cells were maintained in culture as adherent monolayer in Eagle's MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, 2-fold vitamin solution, and penicillin-streptomycin (Flow Laboratories, Rockville, MD). All cultures were free of mycoplasma and pathogenic murine viruses (assayed by Microbiological Associates, Bethesda, MD). Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

Animals. Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

UV-B Irradiation. The procedure of Schwarz et al. (20) was used to irradiate the melanoma cells. Five × 106 cells were plated into 100-mm tissue culture dishes in 10 ml of complete medium. Medium was removed and monolayers were washed three times with PBS and overlayed with PBS. The monolayers were exposed to a radiation source (a single FS-40 sunlight bulb from Westinghouse, Bloomfield, NJ) with an output of 1.43 J/m²/s at a tube-to-target distance of 23 cm. For different doses of irradiation, monolayers were exposed to different durations. After irradiation, the cells were resuspended in complete medium. For IL-8 protein estimation, irradiated cells were trypsinized and plated in a 96-well culture plate. For RNA analysis, irradiated cells were lysed, and total RNA was isolated as described below.

ELISA for Human IL-8. IL-8 levels in cell culture-free supernatants from different treatments were determined by using an ELISA kit (Quantikine; R&D Systems, Inc., Minneapolis, MN). This assay is a quantitative immunometric "sandwich" enzyme immunoassay. A curve of the absorbance versus the concentration of IL-8 in the standard wells was plotted. By comparing the absorbance of the samples to the standard curve, we determined the concentration of IL-8 in the unknown samples.

Cyclohexamide Treatment. Cyclohexamide was purchased from Sigma Chemical Co. (St. Louis, MO). Ten μg/ml (final concentration) of cyclohexamide were added to the culture during the exposure and maintained throughout the duration of incubation.

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Northern Blot Analysis. Total cellular RNA was isolated by using TRI reagent (Molecular Research, Inc.) according to the manufacturer’s instructions. Twenty μg of total RNA/lane were electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 amp to GeneScreen nylon membrane (DuPont Co., Boston, MA) and UV cross-linked with 120,000 μJ/cm² by using a UV stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously (18). Nylon filters were washed 3 times at 55–60°C with 30 mM NaCl-3 mM sodium citrate (pH 7.2)-0.1% SDS (w/v). The cDNA probes used in these analyses were a 1.3-kb Prrl cDNA fragment corresponding to rat glyceraldehyde 3-phosphate dehydrogenase (21), a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, Kanazawa, Japan; Ref. 22), and a p53 pr4-2 cDNA probe (23). Each cDNA fragment was purified by agarose gel electrophoresis, recovered by using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled by using the random primer technique with [α-32P]-deoxyribonucleotide triphosphates.

IL-8 mRNA expression was quantitated in the linear range of the film on a Personal Densitometer by using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA). Each sample measurement was calculated as the ratio of area between the 1.8-kb IL-8-specific mRNA transcript and the 1.3-kb glyceraldehyde 3-phosphate dehydrogenase transcript.

Tumor Cell Injections. To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to 0.25% trypsin-0.02% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells, and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca2+- and Mg2+-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used.

s.c. tumors were produced by injecting 1 × 10^6 tumor cells-0.2 ml HBSS over the right scapular region. Growth of s.c. tumors was monitored by twice was determined by trypan blue exclusion, and only single-cell suspensions of supplemented medium was added. The cell suspension was pipetted to using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled by using the random primer technique with [α-32P]-deoxyribonucleotide triphosphates.

RESULTS

Induction of IL-8 mRNA by UV-B Irradiation. To assess the effect of UV-B irradiation on the progression of human primary cutaneous melanoma, we chose to work with the SB-2 cells. SB-2 cells originated from primary cutaneous melanoma (19) and are neither tumorigenic nor metastatic in nude mice (19, 27, 31, 32). In addition, SB-2 is the only cell line that exhibited negligible levels of IL-8 mRNA expression and protein secretion. All of the other 12 human melanoma cell lines analyzed (32) that originated from metastatic lesions produced and secreted IL-8, and the level of IL-8 expression was found to be correlated with their metastatic potential in vivo (18).

In the first set of experiments, we analyzed the steady state mRNA expression of IL-8 in UV-irradiated or unirradiated cells. Monolayers were irradiated with UV-B (200 J/m²) and incubated for different time periods after irradiation. Total RNA was isolated and analyzed for IL-8 expression by Northern blot analyses. Unirradiated SB-2 cells showed negligible levels of IL-8-specific mRNA transcript (Figs. 1-3, Lanes A). However, exposure to UV-B induced expression of IL-8 mRNA transcript. IL-8 mRNA transcript was observed 8 h after irradiation and was increased to higher levels at 48 and 72 h after irradiation (Fig. 1). These results, together with the data presented in Table 1, show that exposure to UV-B induced constitutive expression of IL-8 mRNA and protein. IL-8 secretion could be observed in supernatants of SB-2-irradiated cells 14 days after irradiation (Table 1), and IL-8 mRNA transcript could still be observed in tumors isolated 60 days after s.c. injections (see Fig. 3).

Effect of UV Dose on IL-8 Expression. In the next set of experiments, we analyzed whether induction of IL-8 expression depends on the dose of UVR. Cells were exposed to 100, 200, and 400 J/m² UV-B; 48 h after irradiation, the cells were analyzed for IL-8 expression by Northern blot analyses (Fig. 2). Maximum induction of the
IL-8 gene expression was observed with 400 J/m² of UV-B (Fig. 2). With this UV dose, however, we observed about 30% cell death (data not shown), and increasing the intensity of UV-B above 400 J/m² did not result in a further increase in the level of IL-8 gene expression (data not shown). Therefore, to ensure cell viability, we considered 200 J/m² as the optimum dose and continued to use it throughout our in vitro and in vivo experiments (vide infra).

**Induction of IL-8 Secretion by UV-B.** The production of IL-8 protein and its secretion to the supernatants was measured by ELISA as described in "Materials and Methods." The dose of UV-B used, 200 J/m², did not induce observable changes in cell viability as assayed by plasma membrane permeability to trypan blue; the activity in the supernatants was normalized, therefore, to total cell number. UV-B-irradiated or unirradiated cells (5 × 10⁵ cells/well) were plated on a 96-well culture plate, and culture supernatants were harvested 24, 48, 72, 96 h after irradiation. To analyze the long-term effects of UVR on IL-8 production, irradiated cells were cultured in vitro for 7 and 14 days. Twenty-four h before the termination of experiments, cultures were washed and refed with fresh media. Culture supernatants were collected and analyzed for IL-8 protein. Unirradiated, continuously cultured cells were used as control. The unirradiated cells expressed very low levels of IL-8 protein for the duration of up to 14 days in culture, whereas cells irradiated with UV-B showed induction of IL-8 secretion (Table 1). The highest level of IL-8 protein secretion was observed after 72 h of incubation (Table 1; Fig. 1) and reached a plateau 96 h after irradiation. The cells continued, however, to secrete IL-8 constitutively for at least 14 days after irradiation (Table 1).

**Effect of Cyclohexamide Treatment on UV-B-induced IL-8 Production.** To examine the mechanism of IL-8 induction in SB-2 cells, we incubated the UV-B-irradiated cells with or without cyclohexamide (10 μg/ml), which blocks de novo protein synthesis. Most of the UV-B-induced IL-8 protein level was blocked in the presence of cyclohexamide (Table 2), indicating that de novo protein synthesis is required for enhanced IL-8 production.

**Tumor Growth.** Next, we analyzed whether induced IL-8 expression in the UV-B-irradiated cells can lead to increased tumorigenicity. To that end, 1 × 10⁶ UV-B-irradiated cells (after 48 h of incubation) were injected s.c. into BALB/c nude mice. Each mouse was injected with a separate batch of irradiated cells. Tumor growth was monitored twice a week, and the tumors were harvested to analyze IL-8 expression when they have reached 1 cm in mean diameter. The results summarized in Table 3 show that unirradiated UV-B-irradiated cells were cultured for 7 and 14 days. 5 × 10⁵ cells/well were treated with UVR with or without cyclohexamide, and 5 × 10⁶ cells/well were replated into a 96-well culture plate. After 24 h of cell adherence, cultures were washed and refed with fresh media. Culture supernatants were collected and analyzed for IL-8 expression as described in "Materials and Methods."
cells did not form palpable tumors 2 months after injections; these results confirmed our data published previously (27, 31, 32). In contrast, UV-B-irradiated cells grew in all mice that received injections (100% tumor uptake), reaching 1 cm in mean diameter in 60 ± 11 (SD) days. S.c. tumors from 3 mice that received injections were harvested 60 days after injections and assayed for IL-8 gene expression by Northern blot analysis. Fig. 3 demonstrates that, 60 days after injections, s.c. tumors of irradiated SB-2 cells continued to express high levels of IL-8-specific mRNA transcript in vivo.

Experimental Lung Metastasis. We have demonstrated previously that expression of IL-8 is directly correlated with metastatic potential in malignant melanoma cells (18). Next, we analyzed the metastatic potential of UV-B-irradiated cell in an experimental lung metastasis assay. One × 10^6 cells (UV-B irradiated or unirradiated) were injected into the lateral tail vein of BALB/c nude mice. Separate batches of irradiated cells were injected into different mice. The mice were killed after 75 days. Lungs were rinsed in water and fixed in Bouin’s solution to visualize metastatic nodules. Formalin-fixed hematoxylin and eosin preparation were also made to analyze micrometastasis. We found that unirradiated cells did not metastasize to the lungs (Table 3), whereas UV-irradiated SB-2 cells formed lung metastases in 7 of 10 mice given injections (range of 1–10, median 6; Table 3). These data suggested that UV-B irradiation can lead to enhanced IL-8 production and, hence, contribute to tumor progression and metastasis.

Increase in Mr 72,000 Type IV Collagenase (MMP-2) Activity after UV-B Irradiation. The metastatic potential of tumor cells depends on proper vascularization of the tumor and its ability to degrade type IV collagen. Recently, IL-8 has been shown to be associated with angiogenesis (33). IL-8 may exert its angiogenic activity through the induction of type IV collagenase in the tumor cells. Activation of the type IV collagenase may provide a mechanism for the increase in the metastatic potential of SB-2 cells after UV-B irradiation. To test this hypothesis, we analyzed the activity of type IV collagenases (Mr 72,000 and 92,000) in SB-2 cells before and after UV-B irradiation (200 J/m^2). Supernatants from unirradiated and irradiated cells (7 and 14 days after irradiation) were analyzed for collagenase activity by zymography (26). Collagenase activity in the supernatants was normalize to cell number as described in “Materials and Methods.” The results depicted in Fig. 4A show a 2.5-fold increase in the Mr 72,000 collagenase activity at 7 and 14 days after irradiation. The activity of the Mr 92,000 collagenase remained unchanged. We next analyzed whether the increase in type IV collagenase activity is mediated by IL-8. To that end, parental SB-2 cells were incubated in the presence of different dosages of human recombinant IL-8 (h-r-IL-8), and the activity of type IV collagenase was determined. The results shown in Fig. 4B indicate that IL-8 caused an increase in the activity of Mr 72,000 but not 92,000 collagenase in a dose-dependent manner.

p53 and MTS-1 (p16⁰⁰⁰⁰ and CDKN2) Abnormalities in SB-2 cells after Irradiation. We have shown previously that p53 mutations are infrequent in human melanoma and probably do not play a major role in the acquisition of the metastatic phenotype (27). SB-2 cells express wild-type p53 (27), and exposure to 200 J/m^2 UV-B did not cause p53 mutations or changes in the levels of wild-type p53 expression. These have been determined by Northern blot analysis and by screening for mutations in exons 5–9 as we described previously (Refs. 27–30; data not shown).

Recently a candidate tumor suppressor gene on chromosome 9p21–22 has been identified as MTS-1, multiple tumor suppressor 1 (34, 35). MTS-1 encodes the protein p16 that inhibits cyclin-dependent kinase 4, cdK4. MTS-1 homozygous deletions have been reported in human melanoma cell lines (34). Our analysis of the MTS-1 gene in the SB-2 cells has revealed homozygous deletion and lack of p16.
mRNA expression. The deletion was observed in the parental SB-2 cells, however, regardless of UV-B irradiation. The observed homozygous deletion in the MTS-1 (CDKN2) gene in parental SB-2 cells, before UV-B irradiation, could not account for the acquisition of the metastatic phenotype induced by UV-B.

**DISCUSSION**

In this study we demonstrated that SB-2 human melanoma cells, which are not tumorigenic in nude mice, expressed negligible levels of IL-8 mRNA and protein. Upon exposure to UV-B, however, IL-8 mRNA and protein production were induced to the constitutive expression state. High levels of IL-8 mRNA transcript could still be observed in tumors isolated 60 days after s.c. injections (Fig. 3). Induction of IL-8 production in SB-2 cells increased their tumorigenic and metastatic potential in nude mice.

Several possible mechanisms for the induction of IL-8 gene expression in the UV-irradiated SB-2 cells should be considered. Our data suggested that UV-B irradiation "switched on" the IL-8 gene to be expressed constitutively, and that inhibition of de novo protein synthesis by cyclohexamide completely blocked IL-8 stimulation, suggesting that new protein synthesis is required for the induction of IL-8 in these cells. Different autocrine and/or paracrine stimulation of other cytokines induced by UV-B may contribute to this up-regulation. Studies have shown that IL-1 or TNF-α can very rapidly induce IL-8 in variety of cell types (36-37). In fact, responsive elements for IL-1 and TNF-α have been found in the 5’ flanking region of the IL-8 gene. The promoter region of the IL-8 gene was also shown to contain an AP-1-binding site (38), which interacts with a dimeric transcriptional activator composed of c-jun and c-fos (39). UV-B may induce IL-8 expression through the induction of c-jun/c-fos and their binding to the AP-1 site. This speculation is supported by the fact that UV-B enhanced rapid and transient c-jun expression in mouse skin, as well as immediate early c-fos expression in mouse epidermal cells (40, 41). The mechanisms regulating induction of IL-8 in SB-2 cells remain to be elucidated. They might involve one of the above-mentioned mechanisms or be caused by unknown mechanisms regulated by UV-B irradiation.

The enhanced or constitutive expression of IL-8 in melanoma progression has a major role in pathogenesis because of its multifunctional effects. IL-8 has been shown to be an angiogenic molecule (33), an autocrine growth factor for melanoma cells (18), and an inducer of haptotactic migration in melanoma cells (42). We have shown that constitutive expression of IL-8 in different human melanoma cells correlated with their metastatic potential in nude mice (18). Our data suggested that UV-B-irradiated SB-2 cells grew and formed s.c. tumors in all mice injected, and they metastasized to the lungs in 70% of the mice given injections. This induction of tumorigenic and metastatic potential in a human cutaneous melanoma might be attributed to IL-8 expression and its angiogenic properties. In addition, IL-8 has recently been shown to be capable of activating phospholipase D in human neutrophils (43). Phospholipase D, in turn, is involved in the intracellular signal transduction mechanisms that mediate induction of gelatinase A (MMP-2) by laminin in tumor cells (44). IL-8, therefore, can cause an increase in type IV collagenase activity and, hence, contribute to invasion and metastasis. Indeed, our data showed an increase in M₈, 72,000 type IV collagenase activity after UV-B irradiation of the melanoma cells. This increase in collagenase activity could be attributed directly to the effect of IL-8 because activation of type IV collagenase could also be achieved when human recombinant IL-8 was added to the parental SB-2 cells (Fig. 4B). We cannot, however, rule out the possibility that UV-B irradiation can cause other genetic changes that may contribute to enhanced tumorigenicity and metastasis. These genetic changes are yet to be defined.

p53 and MTS-1 (p16LNKA and CDKN2) abnormalities were ruled out in our studies as a possible mechanism for the acquisition of the metastatic phenotype in SB-2 cells after UV irradiation. Mutations in p53 were, however, observed to be induced by sunlight during the development of squamous cell carcinoma of the skin (45). In this model, UV-B acted first to induce p53 mutations, and subsequently to select for clonal expansion of the p53-mutated cells. Our studies suggested that in addition to its role in initiation, UVR maybe involved in the acquisition of the metastatic phenotype in melanomas by induction of cytokine production. Understanding the mechanism involved is important to providing a means for pharmacological intervention in the process. We hope that additional studies will elucidate some of the relationships between UV radiation and the progression of human melanoma and suggest other means to manipulate the consequence of exposure to UV irradiation.

**ACKNOWLEDGMENTS**

We thank Dr. Isaiah J. Fidler and Dr. Margaret L. Kripke for critical review and Patherine Greenwood for expert preparation of this manuscript.

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