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Deregulation of cell growth and apoptosis in UV-induced melanomagenesis

Allal Ouhtit[1], Ishita Gupta[2], Rajiv L. Gaur[3], Augusta Fernando[1], Amira O. Abd El-Azim[4], Ali Eid[5], Therese M Becker[6]

[1]Department of Biological and Environmental Sciences, College of Arts & Sciences, Qatar University, Doha, Qatar, [2]College of Medicine, QU Health, Qatar University, Doha, Qatar, [3]Department of Pathology, Stanford University, CA, USA, [4]Department of Zoology, Faculty of Sciences, Mansoura University, Al Mansoura, Egypt, [5]Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon, [6]School of Medicine, Western Sydney University, Campbelltown, NSW 2560, Australia

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1. ABSTRACT

We have previously characterized the role of p16/Rb in coordinating the early events in UVB-irradiated skin. As an extension to this work, normal melanocytes and mutant p16-inducible melanoma cell models were employed to elucidate further the coordinated molecular mechanisms occurring during early UVB exposure. Our results showed that melanocytes expressed p16 only at a high UVB dose, with undetectable p53. The Bax/Bcl2 ratio increased at higher dose, indicating that the cells had selected apoptosis program. In the wt-p16 melanoma cells, while low UVB dose upregulated p16, the high dose suppressed it, and further abrogated Cdk6 but not Cdk4. Interestingly, while induction of mutant-p16 increased Cdk4, cdk6 and pRb proteins, UVB exposure did not affect this increase. More interestingly, p16 mutant cells increased their resistance to apoptosis at high UVB-dose, associated with decreased Bax and increased Bcl2 expression. Thus, mutant-p16 appears to dictate a deregulation of cell cycle and increased resistance to apoptosis in melanoma cells. Together, the data indicate a deregulation of p16INK4/Rb pathway as an early event in UVB-induced melanomagenesis.

2. INTRODUCTION

Skin cancer (SC) is the most common malignancy, and melanoma is the deadliest SC type. One of the major reasons is the direct link between the risk of SC and increased UV radiation exposure resulting from depletion of the ozone layer (1, 2). Skin cancer affects more than one million Americans every year and accounts for more than ten thousand deaths annually, which represents approximately 4% of all cancer deaths together (3). Ultraviolet radiation (UV), in particular UV-B, induces a range of effects including erythema, sun burns, immune suppression, increased photo-aging and SCs. Despite melanoma having less likely prevalence rate than non-melanoma skin cancer (NMSC), it is ranked as the most lethal human SC and its incidence has doubled in the last couple of decades (4, 5).

We and others reported that the Retinoblastoma (Rb) signaling pathway regulated by p53 and $p16^{INK4a}$ tumor suppressor genes is the key UV-target pathway disregulated in NMSCs and cutaneous malignant melanoma (CMM), respectively (6-9). The p53/Rb and the p16/Rb sub-pathways play critical roles in regulating cell cycle and apoptotic early mechanisms in response to UV exposure (6, 10-12). Intriguingly, a single UV insult to the skin tissue can selectively disrupt either p53/Rb or p16/Rb pathway or both in keratinocytes (KCs) and melanocytes (MCs). In KCs, UV-induced DNA damage results in an increase of p53 protein followed by an upregulation of its direct downstream target $p21^{WAF1}$, a cyclin-dependent kinase (Cdk) inhibitor (13, 14). The p21 protein inactivates Cdk-Cyclin complex by forming a Cdk2/A or E Cyclin/PCNA/p21complex, which prevents both Rb phosphorylation and a release of E2F transcription factor resulting in G1 arrest in order to repair DNA damage (15). If the damage is severe and not repaired, apoptosis is induced as a second preventive mechanism to get rid of damaged KCs (11, 16, 17). UV-induced apoptosis is mediated by p53 leading to up-regulation of the pro-apoptotic targets Bax and Fas, and down-regulation of its anti-apoptotic target Bcl-2 (10, 11, 18, 19). Likely, in comparison to KCs, MCs do generally possess an intrinsic resistance to apoptosis. MCs are generally characterized by a broad expression of a plethora of inhibitors of apoptosis (20), including high basal levels of the anti-apoptotic protein Bcl-2, which opposes apoptosis and increases the survival of MCs (21). However, Bax protein increase and redistribution from different compartments within the cell initiate the apoptotic response to UVB in MCs (21, 22).

The p16/Rb pathway also plays a key role in regulating cell cycle mechanisms in response to UVB-induced DNA damage (23, 24). The protein $p16^{INK4a}$ is a cell cycle regulator that specifically inhibit Cdk4/6, resulting in cyclin D-dependent phosphorylation of Rb, subsequently leading to reduced E2F driven transcription of genes necessary for S phase entry (25). In cells irradiated with low doses of UVB, p16 protein is upregulated within 12–24 hours, leading to G1 cell cycle arrest (24, 26, 27) to allow DNA repair prior to resuming the cell cycle (28). Inactivation of $p16^{INK4a}$ *via* missense mutations, deletions, or methylation, has been reported in a large number of different human tumor types including CMM (29-32), where Rb protein is no longer kept in an active form and cell replication goes unchecked.

Although human INK4a/ARF is the only genetic locus frequently lost in familial and occasionally in somatic CMM, the functional relevance of INK4a/ARF in UV-induced pathogenesis of CMM is still elusive. However, mouse models of melanoma have served as *in vivo* genetic platform where UV-induced molecular and cellular mechanisms can be investigated. These models have provided genetic evidence supporting the epidemiological link between increased risk of CMM and childhood sunburn, leading to the identification of Rb signaling pathway as the major target of UV's actions in CMM formation (8, 33).

The difference between the apoptotic early responses to UVB insult of MCs and KCs might explain why INK4a/ARF mutations predispose to CMM but not to NMSC; This provides also a molecular explanation for the link between melanoma-genesis and the regulation of cell cycle check points, in addition to their association with UV-induced DNA repair mechanisms. The high

penetrance of melanoma in families carrying germline mutations in *CDKN2A*/p16^{INK4a} suggests that melanocyte immortalization, *via* inactivation of the p16/Rb pathway, is a major step in early mechanisms of melanomagenesis. As an extension of our previous work, our aim was to study the molecular changes in the expression/function of the different sub-pathways of Rb pathway associated with UVB-induced cell cycle and apoptotic programs using normal human melanocytes as well as various melanoma cell lines that are inducible for wild and mutant p16^{INK4a}.

3. MATERIALS AND METHODS

3.1. Cell culture

Human melanocytes isolated from lightly pigmented neonatal foreskin (HEMn-LP) were purchased and cultured in medium-254 (Cascade Biologics) supplemented with Human Melanocyte Growth Supplement according to the manufacturer's recommendations. SkMel-28, a human primary melanoma cell line, was purchased from ATCC and maintained in RPMI 1640 medium, supplemented with 1% penicillin-streptomycin and 10% FBS. Melanoma cell lines; D2-parent wild-type p16^{INK4a}-inducible clone (p16wt-A4), and the N-terminal 8 amino acid duplication p16^{INK4a}-inducible clone (p16Dp1, (+24 base pairs)), that was originally established in the WMM1175 melanoma cells derived from a subcutaneous metastatic tumor of an individual with a family history of malignant melanoma (34) were provided by Dr. Therese M Becker (University of Sydney, Australia). Expression of the transgene in selected clones was induced by 4mM IPTG added to the cell culture medium.

3.2. UVB irradiation

Melanoma cell lines were pretreated with 4mM IPTG 24 hrs before irradiation. For UVB irradiation, the medium of melanocytes and melanoma cell lines was removed and washed twice with PBS. Plates were placed 20 cm far away from the light source without lids and cells were then irradiated either at 30mJ/cm^2 considered as a low dose or at 70mJ/cm^2 considered as a high dose using CL1000 Ultraviolet Cross linker, 5x 8 watt UV lamp dual bipin discharge type with an emission at 302 nm (UVP, Upland, California, USA). Immediately, cells were replenished with their own media after irradiation and the incubation was continued under the same conditions for different time points. Cells were collected for RNA and protein analyses at various time points in response to UV-irradiation (acute exposure ranging from 2 to 48 hrs or chronic exposure from 2 to 4 weeks).

For estimating the sub lethal doses of UVB-exposure, cells were exposed to different doses of UVB-irradiation ranging from 10 to 90mJ/cm^2. Cells were cultured in 96-well plates until they were 70% confluent. The growth medium was then removed and the cells were washed with PBS and later exposed to different UVB doses starting from 10mJ/cm^2 to 90mJ/cm^2. The growth medium was then replaced and cells were incubated at 37^{0}C and 5% CO_2 for 24 hours post-irradiation. The medium was removed and cells were washed twice with PBS. MTT assay (Chemicon Int.) was carried out according to the manufacturer's instructions. All experiments were performed three times and in conditions allowing at least 70% viability.

3.3. Real time-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Mini kit (Qiagen) from both irradiated and control cells according to the manufacturer's instructions. For RT-PCR analysis, 500 ng of total RNA was reverse transcribed using one step RT-PCR Qiagen kit (Qiagen). Samples were incubated in the PTC-200 Thermal Cycler for reverse transcription at 50°C for 30 min, initial PCR activation step 95°C for 5 min followed by 27 polymerase chain reaction cycles. Each cycle consisted of 95˚C for 30 seconds, 56˚C for 30 seconds, and 68˚C for 1 minute. Final annealing was at 68˚C for 10 minutes. The oligonucleotide primers used in this study are listed below in Table 1.

The RT-PCR products was resolved using 1% agarose gel containing 0.2µg/ml ethidium bromide.

Table 1. Primer set for p16 gene expression by RT-PCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
p16	GCA GAG CTC GTT TAG TG	GTT CCT GTG GTT CAC TCA
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA

3.4. Immunoblotting

Western blot analysis was carried out as previously described (6). Briefly, cells were trypsinized and harvested at different time points, washed twice with ice cold PBS and then treated with RIPA lysis buffer (Santa Cruz Biotechnology, CA). Cell lysate was prepared by centrifugation at 14,000*g* for 15 min, then aliquoted and stored at -80°C. The protein content was evaluated according to the Bradford method. 60µg protein of total cell lysate was mixed with equal volume of SDS sample buffer, boiled for 5 min and subjected to 12% SDS–PAGE. Fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad). Nonspecific sites were blocked with 5% BSA in TBST buffer (0.1% Tween-20, 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.6), and followed by overnight probing with primary antibody at 4°C (dilution 1:500). The following antibodies were used: polyclonal anti-$p16^{INK4A}$: SC-468; polyclonal anti-Cdk6: SC-177, polyclonal anti-Cdk4: SC-260, polyclonal anti-Bax: SC-493, polyclonal anti-p21: SC-397, polyclonal anti-Actin SC-1616, monoclonal anti-p53: SC-126, monoclonal anti-Bcl-2: SC-7382 (Santa Cruz Biotechnology); and monoclonal anti-pRb (Pharmingen). After washing the blot three times, the membranes were incubated for one hour with horseradish peroxidase-conjugated secondary antibody (dilution 1:1000, Santa Cruz Biotechnology). The blot was washed three times and then developed using Supersignal Chemiluminescence detection kit, according to the manufacturer's protocol (Pierce, IL).

3.5. Cell cycle analysis

Cell cycle analysis was carried out according to our previous study (6). Briefly, after harvesting and spinning the cells, the resulting pellet was then fixed in ice cold 70% ethanol. Fixed cells were then centrifuged, washed and resuspended in PBS containing RNase A (1 mg/ml), and incubated at 37°C for 30 min. Propidium iodide was added to a final concentration of 1.0 mg/ml. Propidium iodide-stained cells were analyzed by a fluorescence-activated cell sorter (FACSCalibur in the UAMS Flowcytometry Core Facility, LSUHSC) followed by determination of the percentage of the cells in sub-G1, G1, S and G2/M.

4. RESULTS

4.1. Establishment of optimal UV-irradiation dose and p16-inducible expression

Prior to examining the effect of UV irradiation on the components of the p16/Rb pathway in melanocytes and melanoma cell lines, we determined the cell viability using Alamar blue assay at different UV doses, as previously described (35). As shown in Figure 1a, a dose-dependent effect was assessed in both D2-parental and p16wt-A4 wild type cells. From the analysis of dose-response curves for the two melanoma cell lines, 30 and 70mJ/cm^2 were selected as optimal low and high doses for acute UVB, respectively. Similar doses of UVB have also previously been described for melanocytes (36). From the curve, it appears that p16wt-A4 cells were more sensitive to UVB-irradiation as compared to D2-parent cells especially at doses ranging from 30 to 70mJ/cm^2 (Figure 1a). Using different concentrations of IPTG (from 0.05 to 10mM), the increase in the expression of p16 was proportional to the increase of IPTG concentration up to 6mM IPTG and then gradually declined (data not shown). We selected the concentration of 4mM IPTG to induce all melanoma cell lines for conducting the next set of experiments. The IPTG-induced p16-wt-A4 cells in particular, showed time-dependent manner of p16 expression reaching the peak after 48 hours (Figure 1b). Furthermore, in p16-A4 cells, the effect of UVB

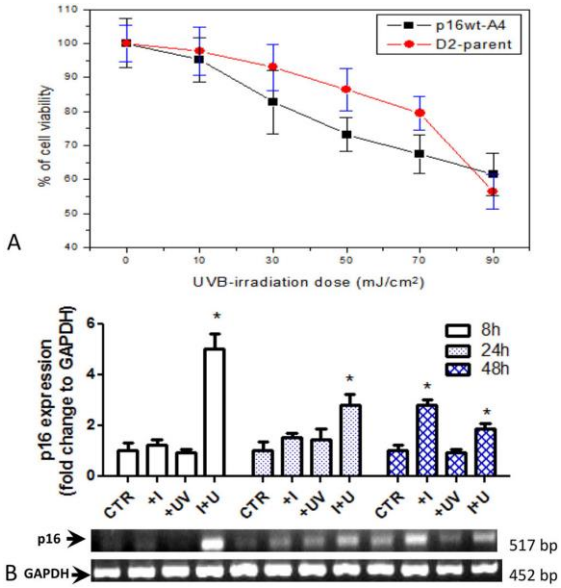

Figure 1. Optimization of UVB doses and IPTG-induced p16 expression in melanoma cells. (a) UVB-dose response curve depicts two p16 non-induced melanoma cell lines; D2-parent and wtP16-A4 irradiated with different doses of single UVB ranging from 10 to 90 mJ/cm^2 for 24 hrs. (b) Induction of P16 expression in wtp16-A4 cell line using IPTG. Cells were irradiated with single low (30 mJ/cm^2) or high (70 mJ/cm^2) doses of UVB at different time points (8-48 hrs). Cells were pretreated with 4mM IPTG (I) 24 hrs earlier, followed by low dose irradiation (UV) or treated and irradiated (U+I) in comparison to untreated cells (-). RNA was extracted from different cells and the expression of p16 was examined by RT-PCR and GAPDH was used as an internal control. * denotes significance at $p<0.05$.

30mJ/cm^2 low dose (alone or in combination with IPTG) on p16 gene expression was examined at different time points (Figure 1b), indicating a marked increase of p16 RNA expression levels at early time point (8hrs)

4.2. Effects of UVB-irradiation on cell cycle and apoptotic proteins in human melanocytes

Normal human melanocytes were exposed to UVB irradiation to determine the effect of both low and high UVB doses on the components of the p16/Rb and p53/p21pathways associated with both cell cycle and apoptosis. While UVB low dose did not induce p16 protein expression, the high dose did after 24 hrs. Cdk4 is a downstream target of p16 gene that regulates cell cycle in response to UVB irradiation. While low UVB dose increased CdK4 expression, UVB high dose decreased it, indicating a p16/cdk4 inverse relationship (Figure 2a). For the p53 pathway on the other hand, while p53 was detected only after 48 hours at UVB low dose, it was induced earlier in response to high dose (Figure 2b). In addition, Bax/Bcl2 ratio increased in response to both, low and high UVB doses, particularly at late time points, when usually apoptosis is induced (Figure 2b). These findings suggest that, at low UVB, the cells probably halted their cell cycle to allow DNA repair, while at a high UVB dose, the cells were driven to apoptosis (6). In fact, the protein expression of p53 was detected only after 48 hours at low dose of UVB-irradiation and the scenario was completely opposite at high dose (Figure 2b).

4.3. Effect of UVB on cell cycle and apoptotic proteins in wild type p16-inducible melanoma cell lines

In the wild type (WT) p16-IPTG-inducible cells, without UVB irradiation, time-point induction of p16 was parallel to the induction of Cdk4, Cdk6 and Bax (Figure 3a). In this course of induction, phosphorylated Retinoblastoma (pRb) protein expression decreased with increasing time. Both low and high UVB doses did not significantly affect the IPTG-p16 induction through time course. However, the expression of CdK4 increased after the induction of p16 which was substantial during low and high UVB exposures. Although there was a marked increase in CdK6 expression during p16-induction at low UVB exposure, at almost all time-points, the expression of CdK6 in wtp16-A4 cells were barely observed during high dose of UVB exposure (Figure 3a). The expression of Rb started to decrease early after 8hrs up to 48hrs and with IPTG treatment, it showed moderate pattern after low and high UVB exposures within most of the time-course, except after 48hrs with or without IPTG induction and/or after 8 hrs low and high UVB exposures (Figure 3a). In IPTG stimulated cells, the induction of p16 elevated the expression of pro-apoptotic protein Bax during early time points but later decreased. Interestingly, in the same cells, low UVB exposure could not induce any change in Bax expression yet high exposure

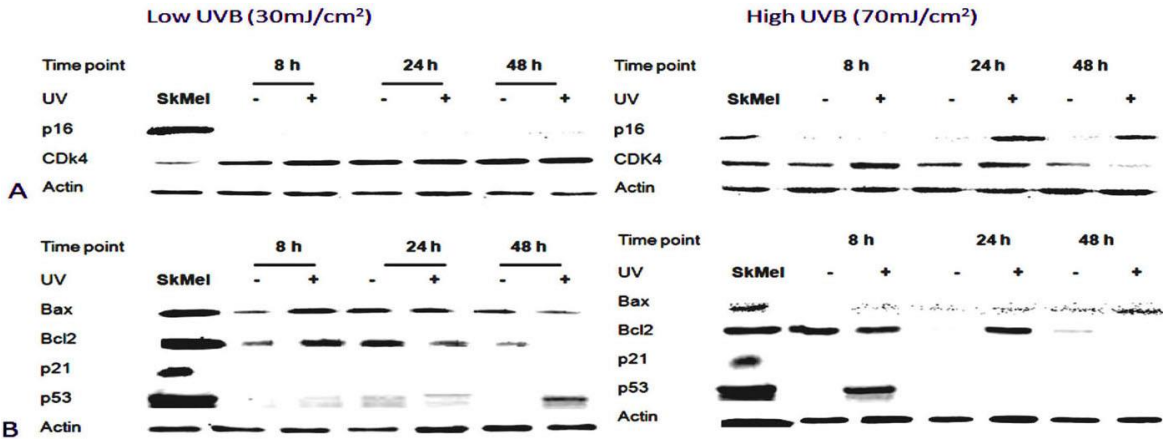

Figure 2. Effect of UVB irradiation on p16 expression, cell cycle check points and apoptotic proteins in human menlanocytes. Cells were exposed to single low (30mJ/cm^2) or high (70mJ/cm^2) doses of UVB-irradiation at different time points (8-48hrs). Protein lysate was collected and Western blot was performed to examine the expression of (a) p16INK4a, (b) cell cycle proteins and pro-apoptotic proteins in melanocytes using β-actin as a loading control. All experiments were repeated at least twice.

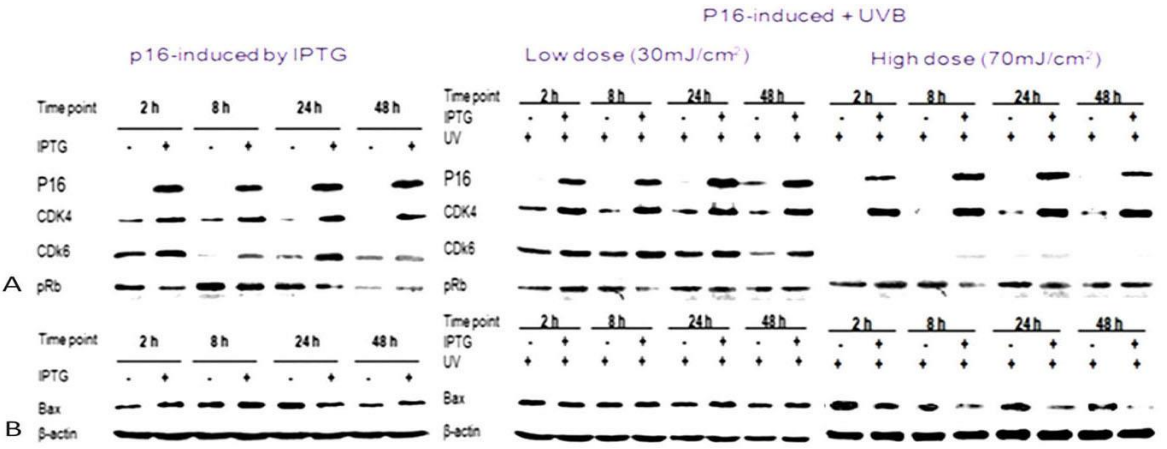

Figure 3. Validation of p16 protein expression in response to UVB-irradiation (30 and 70 mJ/cm^2) after p16^{INK4a} induction by IPTG. WM1175-wtp16-A4 cells were 24hrs pretreated with 4mM IPTG and then irradiated with UVB at different time points (2, 8, 24 & 48hrs). The expression of (a) cell cycle and (b) pro-apoptotic on protein levels was examined using Western blot analysis. All experiments were repeated at least twice.

brought out the expression of Bax to a lower level (Figure 3b). Flow cytometric analysis showed that the induction of p16wtA4 via IPTG as well as UVB, hindered the cells to enter S phase especially after 8 hrs (Figure 4). However, UVB could not inhibit S Phase at late time points (Figures 4a and b). Meanwhile, there was marked increase in the percentage of cells that underwent apoptosis especially when treated with UVB and/or IPTG at late time point (Figures 4a and b).

4.4. Effect of UVB-irradiation on cell cycle and apoptotic proteins in mutant p16INK4a-inducible clone p16Dp1 (with +24 base pair duplication)

Induction of p16 by IPTG was identified in mutant p16 Dp1 but only two bands were observed; one band at regular size (16 kDa) and the second band was present at a slightly higher level (18 kDa) as compared to control SkeMel cells (Figure 5a). The

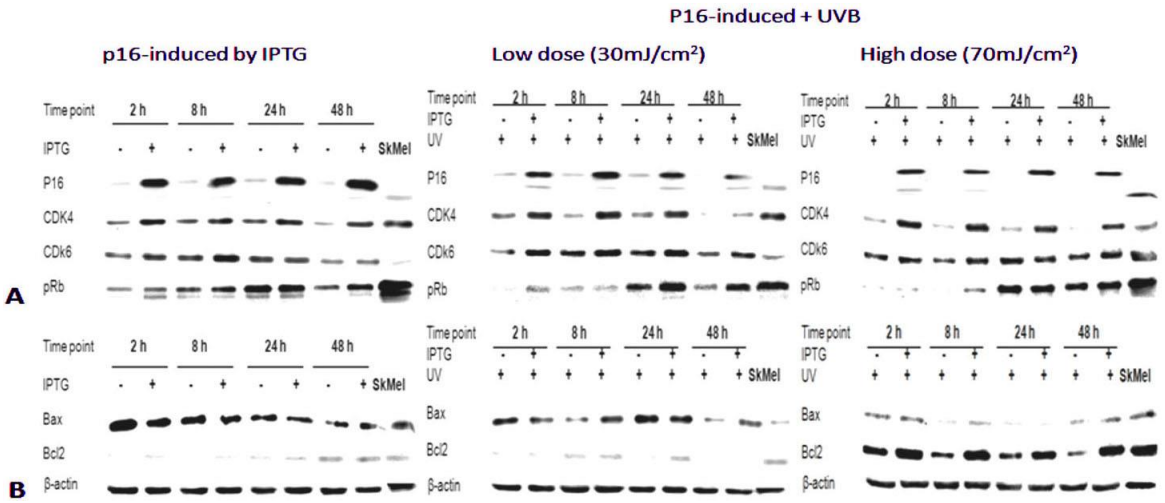

Figure 4. FACS analysis showing the effect of UVB-irradiation on cell cycle checkpoints in wild and mutant melanoma cell lines. A. The percentages of Pre-G0 and S phases after p16INK4a induction. Wild (WMM1175-p16wt-A4) and mutant (+24bp Dp1) melanoma cells were 24hrs pretreated with 4mM IPTG and then irradiated with UVB at low (LUV) or high (HUV) doses for 2, 8, 24 & 48 hours. (a) Cells were collected, stained with propodium iodide and then examined by flowcytometry. (b) Representative cell cycle diagrams showing the percent of cells cycle checkpoints after 8hrs of UVB exposure. All experiments were repeated at least twice.

response of mutant Dp1 cells to UVB exposure was investigated along with cell cycle and apoptotic related proteins to determine whether their patterns of expression would be affected due to impaired p16. The expression of cell cycle proteins Cdk4/6 and pRb increased upon induction of p16, while UVB exposure did not affect this increase significantly when compared to the wild type p16 A4 cells (Figure 5a). Pro-apoptotic protein Bax showed a marked consistent decrease up to 24 hrs and then returned to the basal level after 48hrs of p16 induction. Anti-apoptotic protein Bcl2 did not show any remarkable difference after treating +24 bp Dp1 cells with IPTG (Figure 5b). More interestingly however, Bcl2 was considerably increased during the time course of high dose UVB exposure (Figure 5b). Furthermore, p16 induction and UVB revealed no notable difference in the percentage of cells count during S-phase at early time points (Figure 4). Noteworthy, p16 induction exhibited evident increase in cell count during pre-G0 phase especially after 48hrs of exposure, indicating apoptosis induction (Figure 4).

5. DISCUSSION

Multiple lines of evidence, including our previous work have unveiled the pivotal role of Rb-signaling pathway, which is regulated by p53 and $p16^{INK4a}$ in UVB-induced skin cancer model (6, 8, 10, 11, 37). We have previously characterized the normal protein expression patterns of the cell cycle (p21 and Rb) and apoptosis (bax and Bcl2)-associated genes, which are regulated by both p53 and p16 pathways in coordinating the normal early responses to a single UVB-irradiation in human skin. As a continuation to this work, the present study tested the hypothesis that the occurrence of $p16^{INK4A}$ mutation deregulates apoptosis mechansims, and initiates UV-induced melano-magenesis. Pursuant to this goal, normal melanocytes and a panel of p16-induced cell lines, including wild-type $p16^{INK4a}$-inducible clone p16wt-A4, and N-terminal 8 amino acid duplication $p16^{INK4a}$-inducible clone p16Dp1 (+24 base pairs), were used as experimental cell models in the present study. These particular cell models were established to elucidate the molecular mechanisms that underpin UV-induced deregulation of apoptosis.

Our results showed that p16 transcript was induced at a higher dose of UVB in normal melanocytes whereas the expression of CdK4, a downstream target of p16, was inhibited. It has been

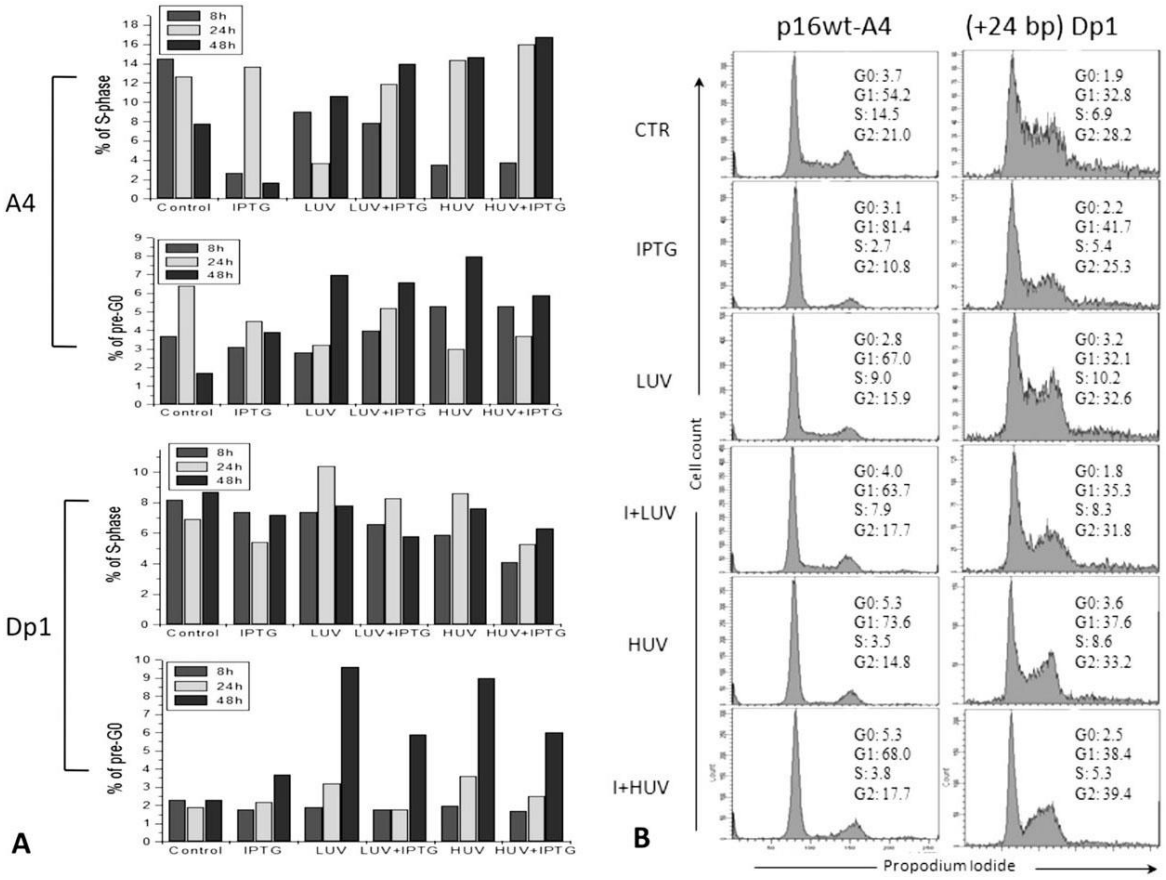

Figure 5. Effect of UVB-irradiation on mutant WMM1175-p16Dp1 melanoma cells. Cells were 24h pretreated with 4mM IPTG and then exposed to either 30 or 70 mJ/cm^2 UVB-irradiation at different time points (2, 8, 24 & 48hrs). Cells were collected and examined for (a) cell cycle and (b) pro-apoptotic protein expressions in mutant WMM1175-p16Dp1 melanoma cells that contain N-Terminal 8 amino acids duplication in p16. All experiments were repeated at least twice.

reported that binding of p16^{INK4a} to CdK4 is accompanied with cyclin kinases inactivation (34, 38). In response to low UV dose, p53 expression was observed after 48 hours, while p53 showed no expression at high UV dose. This phenomenon indicates that unlike keratinocytes, melanocytes are resistant to apoptosis even without showing any basal expression level of p21 (20). Also, wild-type melanocytes are resistant to UV-induced apoptosis than genetically modified melanocytes (39). Instead of the melanocytes showing a high Bax/bcl2 ratio at high doses, the ratio was observed at low doses of UVB, indicating cells being sensitive to apoptosis. UVB-induced melanocytes up-regulate Bcl-2 and Bax at mRNA level without showing any change at the protein level although other alternative apoptotic signaling pathways were reported (21).

In our experiments, we first showed that the IPTG-p16 inducible systems function properly as confirmed by RT-PCR and Western blot analyses mRNA and protein levels, respectively. The next step was to examine cell cycle check point proteins, using Cdk4, Cdk6 and phosphorylated pRb, in addition to apoptotic pathway using Bax and Bcl2 protein markers. Early induction of p16 in wild-type p16-A4 melanoma cells revealed upregulation of Cdk4/Cdk6, but down-regulation of pRb in a time-dependent manner. When p16 was induced, it effectively competes with cyclin D1 for Cdk4 binding, leading to inactive p16^{INK4a}-CDK4 complexes. Consequently, pRb remained in its hypophosphorylated active form, repressing E2F activity, inhibiting both, cell cycle and replication (34, 40). Since wt-p16-A4 cells are p53 and p21-naïve cells, the p16 pathway is dominant for

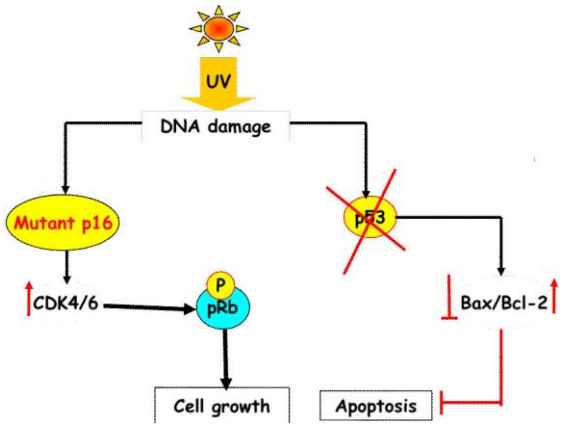

Figure 6. Schematic representation of effects of p16, CDK4/6, pRB and Bax/Bcl2 on UV induced DNA damage impacting the cell growth and apoptosis.

inducing cell-cycle arrest at G_1/S phase in response to low dose of UVB while at high dose the cells cycle is disrupted as indicated by the hypophosphorylation of pRb and downregulation of Bax. We further analyzed the deregulation of cell cycle in wt-p16 cells after induction of p16 or UVB exposure by flowcytometry. When p16 was induced by IPTG, G_1 phase was arrested and S phase was drastically reduced as shown in other previous studies (27, 31, 41). Similar results were also obtained at high dose of UVB exposure as described before (27, 41, 42), which explains the critical role of p16 in cellular response to UVB exposure.

The induced N-terminal 24-bp duplication mutant cells have no effect on cell cycle related proteins at high UVB exposure in comparison to wild-type $p16^{INK4a}$-A4 melanoma cells. Despite mutant-p16 cells having the same binding property to CdK4/CdK6; this complex may reduce the phosphorylation of pRb (34). The anti-apoptotic properties of mutant cells were predominant at high dose of exposure as indicated by the decrease in Bax and the increase in Bcl2 expression. Previous reports indicated that the redistribution of Bax from different cellular compartments play significant roles in inducing cell apoptosis rather than protein up-regulation (21). Deregulation of $p16^{INK4a}$-CdK4 complex is a critical step in melanomagenesis as indicated by CDKN2A mutations in familial melanoma, which selectively blocks the binding of p16 in Cdk4 but not Cdk6 (43). Recent study demonstrates that point mutations in p16 gene are common in familial melanoma and these mutations are critical, not only because of cell cycle regulation but also in controlling oxidative stress within the cell (44).

Finally, it is worth mentioning that in wild-type p16-induced melanoma cells which lack p53 gene, the early response to UVB exposure is mainly regulated through p16/Rb pathway. However, in p16-mutant melanoma cells the cell cycle check points were deregulated and DNA repair was bypassed. Also, these cells were directed to apoptosis at low doses while at high dose they were directed to uncontrolled cell division, suggesting this event as the first step of melanoma formation. Our study UVB *in vitro* model is an intricately interwoven process demonstrating p16 as a key regulator gene in the process of melanomagenesis. Further experiments using *in vivo* p16-inducible systems are ongoing in our laboratory to better understand the role of p16 in melanomagenesis in impairing cell cycle checkpoints and deregulation of apoptosis in response to chronic UVB exposure (Figure 6).

6. ACKNOWLEDGMENTS

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Abbreviations: CdK: Cyclin-dependent Kinase, pRb: Phosphorylated Retinoblastoma, Rb: Retinoblastoma, SkC: Skin Cancer, UV: Ultraviolet, WT: Wild Type

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Send correspondence to: Allal Ouhtit, Department of Biological and Environmental Sciences, College of Arts and Sciences, Qatar University, Doha, Qatar, Tel: 00974-4403-7572, Fax: 00974-4403-4531, E-mail: aouhtit@qu.-edu.qa