[Frontiers in Bioscience 13, 2435-2443, January 1, 2008]

Dipeptidyl peptidase IV (DPPIV), a candidate tumor suppressor gene in melanomas is silenced by promoter methylation

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1. ABSTRACT

Dipeptidyl peptidase IV (DPPIV), a serine protease is expressed by normal melanocytes but not by melanomas, the malignant counterpart. DPPIV is encoded by a gene that contains a 5 CpG island spanning a transcriptional regulatory region. Previously we have demonstrated that DPPIV abrogates growth factor independence and functions as a tumor suppressor gene in melanomas. In this study we show that loss of DPPIV occurs at RNA level and demethylating agent, 5-aza-2′-deoxycytidine (5-AZA-Cdr) treatment of DPPIV negative melanoma cell lines results in increase of DPPIV mRNA, protein, and enzyme activities. By using sodium bisulfite genomic DNA modifications, PCR, and sequencing we confirmed that DPPIV gene promoter is methylated in eight out of ten melanoma cell lines tested. Further more, 5-AZA-Cdr induced increases in DPPIV levels correlated with growth inhibition and apoptosis in melanoma cells. All together these findings suggest that frequent downregulation of DPPIV expression in melanoma can be attributed, in large part, to aberrant promoter hypermethylation and this loss of DPPIV may be a critical event contributing to melanoma development.

2. INTRODUCTION

Melanoma is a malignant tumor of melanocytes or their precursor cells. It is the leading cause of death from skin tumors worldwide (1). Dipeptidyl peptidase IV (DPPIV), a cell surface glycoprotein, is expressed by normal melanocytes but not by primary and metastatic melanomas (2,3). Our previous work has demonstrated that DPPIV is a tumor suppressor gene for melanomas (4). DPPIV is a serine protease and its proteolytic activity leads to alteration of function or degradation of bioactive peptides (5-9). DPPIV is involved in diverse biological processes including cell proliferation, differentiation, adhesion, motility, apoptosis, and immunomodulation, functions critical for controlling neoplastic transformation (5-14). Accordingly, the role for cell surface peptidases as tumor suppressor genes is being recognized (15). DPPIV is expressed in normal epithelial and endothelial cells, and its expression is lost in many types of cancers (5-11). Thus, the specific loss of DPPIV may be an important event during tumorigenesis. In support of this view, we have shown that DPPIV suppresses the malignant phenotype of lung and prostate cancer cells as well (10, 11). These data

establish a role for DPPIV in negative regulation of cancer phenotype.

The degrees of DPPIV protein levels and enzyme activities are inversely correlated with the progression of melanomas (2, 3). We have shown that restoration of DPPIV in melanoma cells leads to induction of the differentiated phenotype, suppression of tumorigenicity and re-emergence of requirements for exogenous growth factors (4). It is well established that progression of melanoma is mediated in part by increased production of growth factors (16-18). Some of these growth factors are substrates for DPPIV (5-9). Presumably, loss of this peptidase allows at least one of its biologically active substrates to provide proliferation and survival signals for melanomas.

Frequent epigenetic loss of tumor suppressor genes is strongly associated with development and progression of cancer (19-21). The methyl transferase inhibitors including the 5-AZA-Cdr are shown to inhibit the growth of tumor cells by re-activating tumor-suppressor genes. The methylation of CpG islands within gene promoters is a major epigenetic transcriptional control mechanism that is frequently dysregulated in cancer and plays a critical role in the transcriptional silencing of tumor suppressor genes (21-25). Interestingly, the 5-flanking region of the human DPPIV gene has been well characterized and is shown to contain significant numbers of CpG dinucleotide islands (26-28). However it is not known whether DPPIV, a candidate tumor suppressor gene is transcriptionally silenced by promoter hypermethylation.

In this study, we investigated the mechanism involved in loss of DPPIV expression in melanoma cell lines. Our studies show that DPPIV expression is lost at RNA level and that aberrant methylation of DPPIV gene promoter is involved in its down regulation, as indicated by transcriptional induction of DPPIV gene by 5-AZA-Cdr and bisulfite genomic DNA sequencing. These changes were accompanied by growth inhibition and apoptosis demonstrating the anti-neoplastic effects of 5-AZA-Cdr in melanomas. Over all, these results suggest that frequent downregulation of DPPIV expression in melanoma can be attributed, in large part, to aberrant promoter hypermethylation and this may be a critical event contributing to melanoma initiation and progression.

3. MATERIALS AND METHODS

3.1. Cell cultures and exposure to 5-AZA-Cdr

Melanocytes obtained from Clonetics (Cambrex, NJ) were grown in Clonetics MBM media containing MGM-3 growth supplement, penicillin, and streptomycin (Invitrogen, CA). Melanoma cells kindly provided by Dr. Alan N. Houghton (MEL-22a, SK-MEL 28, SK-MEL-29, SK-MEL-37, SK-MEL-188, WM-115, SK-MEL-118, SK-MEL-85, SK-MEL-19, SK-MEL-90) were grown in RPMI 1640 media supplemented with 10 % fetal bovine serum, 0.1mM non-essential amino acids, 100 U/ml penicillin, and 0.1mg/ml streptomycin in 37^0C incubator containing 5% CO_2. 5-AZA-Cdr was purchased from Sigma-Aldrich (St. Louis, MO). 5-AZA-Cdr was added to the subconfluent cultures at a final concentration of 5 μM or 2.5 μM. The cells were routinely harvested after 48 hours of culture.

3.2. Total RNA isolation and Northern blot analysis

Total RNA was extracted using the Qiagen RNA extraction kit (Qiagen Inc, Valencia, CA). Twenty micrograms of total RNA from each cell line were size fractionated in 1.2% formaldehyde agarose gels and transferred to a nylon membrane. The α-^{32}P-dCTP labeled cDNA fragments of DPPIV and β-actin were used as probes. Hybridizations were carried out overnight at 42^0C. Membranes were washed and exposed to film overnight at -70^0C.

3.3. Dipeptidyl peptidase enzymatic activity

DPPIV peptidase activity was measured by colorimetric assay using Gly-Pro *p*-nitroanilide as substrate as previously described (4).

3.4. Flow cytometry

Flow cytometric analysis was performed to determine the cell surface expression of DPPIV using FACScan (Becton Dickinson, San Jose, CA). Untreated melanocytes, melanoma cells, and the cells exposed to 5-AZA-Cdr (2.5μM or 5μM) for 48 hours were stained with primary antibody specific for DPPIV (S27 mAb). FITC-conjugated Rabbit anti mouse IgG (DAKO, Carpenteria, CA) were used as the secondary antibody. Untreated cells were analyzed in parallel as negative controls.

3.5. Semi quantitative RT-PCR

The cDNAs synthesized from total RNA (2 μg) using superscript reverse transcriptase was used as template for RT-PCR reaction. The primer set used for DPPIV amplification was 5-tca tat gac att tat gat tta-3' and 5-caa aat gag gag gca aga tca tc-3'. GAPDH amplification using the primer set, 5'-atc ttc cag gag cga gat cc-3' and 5'- acc act gac acg ttg gca gt-3' was used as control. PCR amplification was carried out in 30 cycles of denaturation (94^0C, 1min), annealing (58^0C, 30 sec), and extension (72^0C, 1 min) and the products were analyzed on 1% agarose gels.

3.6. Bisulfite Modification of genomic DNA, PCR and sequencing

We used genomic DNA sequencing technique to determine the DNA methylation status of DPPIV promoter region (29-31). Genomic DNA was modified with the CpGenome DNA Modification kit (Serological Corporation, Norcross, GA) according to manufacturer's protocol. Briefly, genomic DNA was treated with sodium bisulfite to modify unmethylated cytosine residues to uracil, while 5-methyl cytosines remain unaltered. The 317 bp promoter region spanning a transcriptional regulatory region upstream of the transcription start site of the DPPIV gene (-446 bp to -129 bp) was PCR amplified using forward 5-GTGAGGTTGGAGGAGTTGAA-3' and reverse 5-CCCGAATTAAACATTAATAAAC-3' primers that recognize bisulfite modified DNA. PCR conditions were: 3 min at 94°C, (1 min at 94°C, 1 min at 51°C, and 1 min at 72°C) for 30 cycles, and 7 min at 72°C. PCR

products were gel purified and sequenced using an automated DNA sequencer (ABI 3700 DNA Sequencer).

3.7. MTT cell proliferation assay

Cells were cultured in media containing 5-AZA-Cdr (2.5μM) for 48 hours. Untreated cells were analyzed in parallel. Cell viability was examined using a colorimetric MTT assay (ATCC, Manassas, VA). The assay was performed with triplicate samples as previously described (11).

3.8. Detection of apoptotic cells by TUNEL assay

For quantitation of apoptosis, melanoma cells exposed to 5-AZA-Cdr (2.5μM) and for 48 hours were fixed and permealized in 1% parafarmaldehyde and ice cold 70% ethanol. Measurements of fragmented DNA by TUNEL assay were carried out using APOPTAG kit (Chemicon International Inc, Temecula, CA) with triplicate samples according to manufacturer's instructions. Percent apoptosis were determined using FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Untreated melanoma cells were analyzed in parallel.

3.9. Statistical analysis

For all experiments we used triplicate determinations per group. The experiments were performed in duplicate. Data are presented as the mean ± the standard deviation (S.D.), as indicated in figure legends. Significance was determined with the Student's *t*-test to compare the means between two groups. The one-way analysis of variance (ANOVA) was used to test for significance between repeated measures. A probability (P) <0.05 was considered statistically significant in all calculations.

4. RESULTS

4.1. DPPIV expression is lost in melanoma cell lines at RNA level

As an initial step we assessed the expression pattern of DPPIV at RNA and protein levels in normal melanocytes and in a panel of ten melanoma cell lines (MEL-22a, SK-MEL 28, SK-MEL-29, WM-115, SK-MEL-37, SK-MEL-188, SK-MEL-118, SK-MEL-85, SK-MEL-19, SK-MEL-90). Northern blot analysis of total RNAs isolated from same panel of melanoma cells showed undetectable levels of DPPIV mRNA, whereas the 2.3-kb DPPIV transcript was detected in melanocytes indicating that the loss of DPPIV in melanoma cells occurs at RNA level (Figure 1A). Furthermore, the levels of enzymatic activity (Figure 1B) and cell surface expression of DPPIV protein (Figure 1C) correlated strongly with the levels of DPPIV mRNA. DPPIV enzymatic activity of melanocytes ranged from 210-260 pmoles/μg protein /minute and in melanoma cell lines enzyme activities were greatly reduced to < 30 pmoles/μg protein/minute.

4.2. 5-AZA-Cdr induces expression of DPPIV

Initially, normal melanocytes and ten melanoma cell lines exposed to 2.5 μM or 5.0 μM 5-AZA-Cdr for 48 hours were examined for induction of DPPIV expression at both protein and RNA levels. Flow cytometric analysis revealed induction of cell surface expression of DPPIV in eight out of ten melanoma cell lines with the exception of SK- MEL-37 and SK-MEL-90. All of these eight melanoma cell lines showed peak induction of DPPIV expression at 2.5 μM 5-AZA-Cdr indicating the effects of methyl transferase inhibitor on DPPIV expression. There was no additional increase in DPPIV expression with 5.0 μM 5-AZA-Cdr treatment. Also, exposure to 2.5 μM or 5.0 μM 5-AZA-Cdr did not alter DPPIV expression in melanocytes. The results are shown for three representative melanoma cell lines and melanocytes (Figure 2A). The peptidase enzyme assay further demonstrated the induction of DPPIV in melanoma cells by 5-AZA-Cdr. The level of enzyme activities ranged from 140-160 pmoles/μg protein /minute and correlated well with levels of cell surface expression (Figure 2B). We further tested the induction of DPPIV mRNA by semi quantitative RT-PCR in melanocytes and melanoma cell lines before and after exposing to 5-AZA-Cdr. The results show that DPPIV expression is induced at RNA level with no difference in levels of DPPIV mRNA in melanocyte before and after treatment (Figure 3A). The quantitation of DPPIV mRNA levels in relation to GAPDH mRNA levels are shown in Figure 3B. These data strongly indicate that DPPIV gene promoter methylation is a probable mechanism involved in DPPIV silencing in melanoma.

4.3. DPPIV promoter is methylated in melanoma cell lines

In order to further confirm, we assessed the methylation status of 317 bp region of DPPIV promoter containing CpG islands located upstream of the transcription start site of the DPPIV gene. Sequencing of bisulfite modified genomic DNA PCR products revealed the presence of several methylated CpG islands in the DPPIV gene promoter in eight out of ten melanoma cell lines that showed induction of DPPIV expression by 5-AZA-Cdr. On the other hand, the DPPIV gene promoter region from normal melanocyte and control normal placental DNA did not show any methylated cytosines. The DNA sequencing data is shown for melanocyte and three representative melanoma cell lines, MEL-22a, SK-MEL-29, SK-MEL-118 (Figure 4). These results show that promoter methylation is frequently involved in silencing of DPPIV gene in melanoma cells.

4.4. 5-AZA-Cdr inhibits proliferation and induces apoptosis in melanoma cells

We further examined the biological implications of methyl transferase inhibitors in a representative melanoma cell line (MEL-22a). Untreated melanoma cells showed small, round morphology and grew in a disorganized manner resulting in clumps of cells. On the other hand, 5-AZA-Cdr exposed melanoma cells showed striking morphological changes including enlarged and dendritic differentiated phenotype. These cells also failed to reach confluency in culture flasks. Furthermore, cell viability assay showed decreased absorbance of MTT after exposure to 5-AZA-Cdr as a sign of reduced mitochondrial function and decreased cell proliferation rate as compared to untreated cells (Figure 5A). We next determined whether an increase in apoptosis was associated with an observed

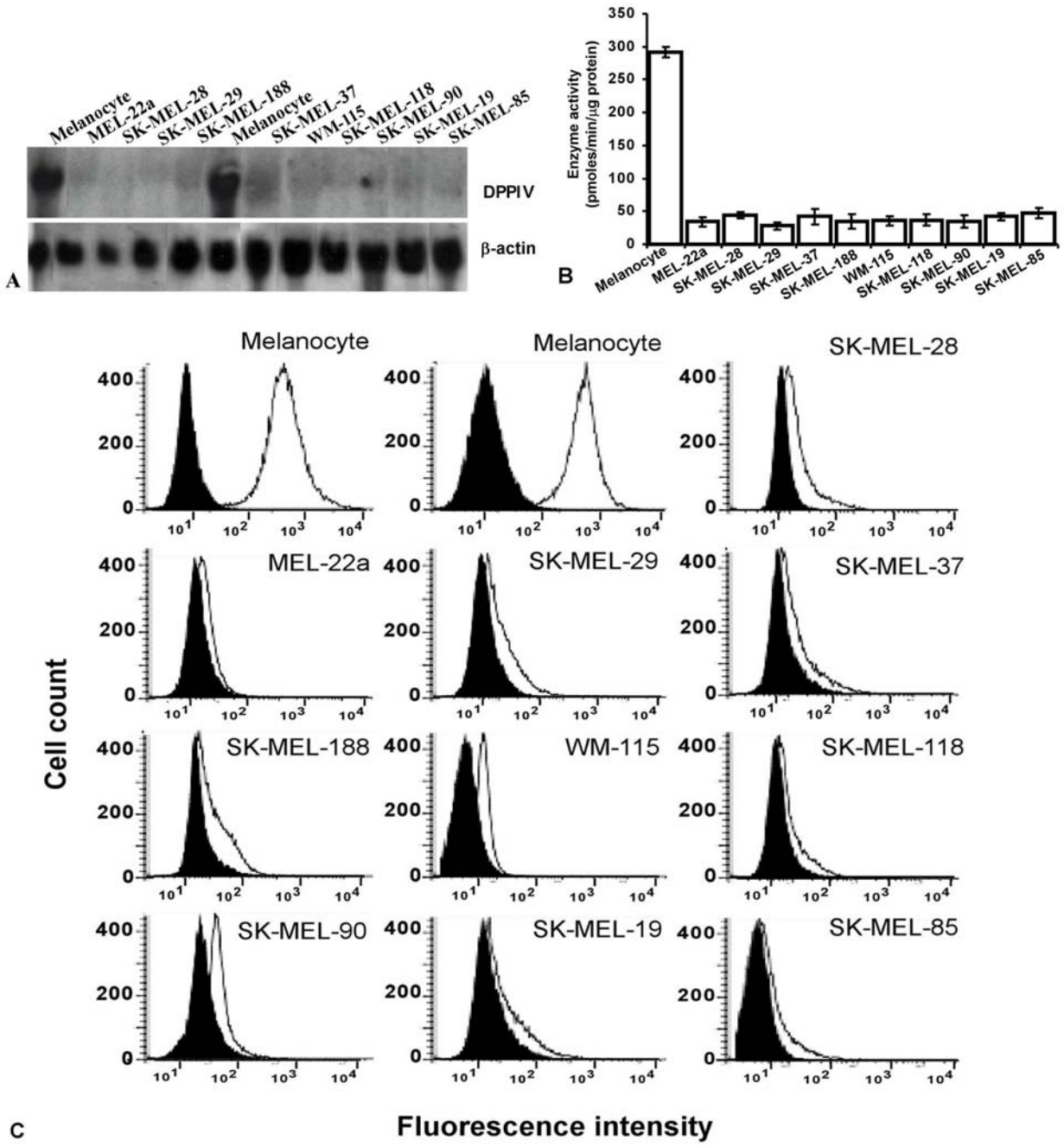

Figure 1. DPPIV expression is lost in melanoma cell lines at RNA level. A. DPPIV mRNA levels in ten melanoma cell lines (SK-MEL-28, MEL-22a, SK-MEL-29, SK-MEL-37, SK-MEL-188, WM-115, SK-MEL-118, SK-MEL-90, SK-MEL-19, SK-MEL-85), and in two normal human melanocytes were assessed by Northern blot analysis using the human DPPIV cDNA as probe. The same blot was stripped and re-hybridized with beta actin control probe to confirm that equal amounts of RNA were loaded for all samples. B. DPPIV enzymatic activity in total cell lysates obtained from above mentioned melanoma cell lines and melanocytes as measured by colorimetric assay. Results shown are mean values of DPPIV activity +/-1 SD of triplicates. C. Cell surface expression of DPPIV in above mentioned melanoma cell lines and melanocytes were examined by flow cytometric analysis. Cells were stained with the DPPIV-specific mAb S27 and fluorescein-conjugated rabbit anti-mouse secondary antibody. The Y-axis shows relative cell number and the X-axis shows the log of relative fluorescence intensity. The solid black line depicts DPPIV expression and filled histogram is control IgG1 antibody.

decrease in the number of viable cells after exposing melanoma cells to 5-AZA-Cdr. Quantitation of apoptosis using flow cytometry showed induction of significantly higher levels of apoptosis in 5-AZA-Cdr exposed melanoma cells (30-45%) compared to untreated melanoma cells (2-3%) as shown in Figure 5B. Also, Induction of

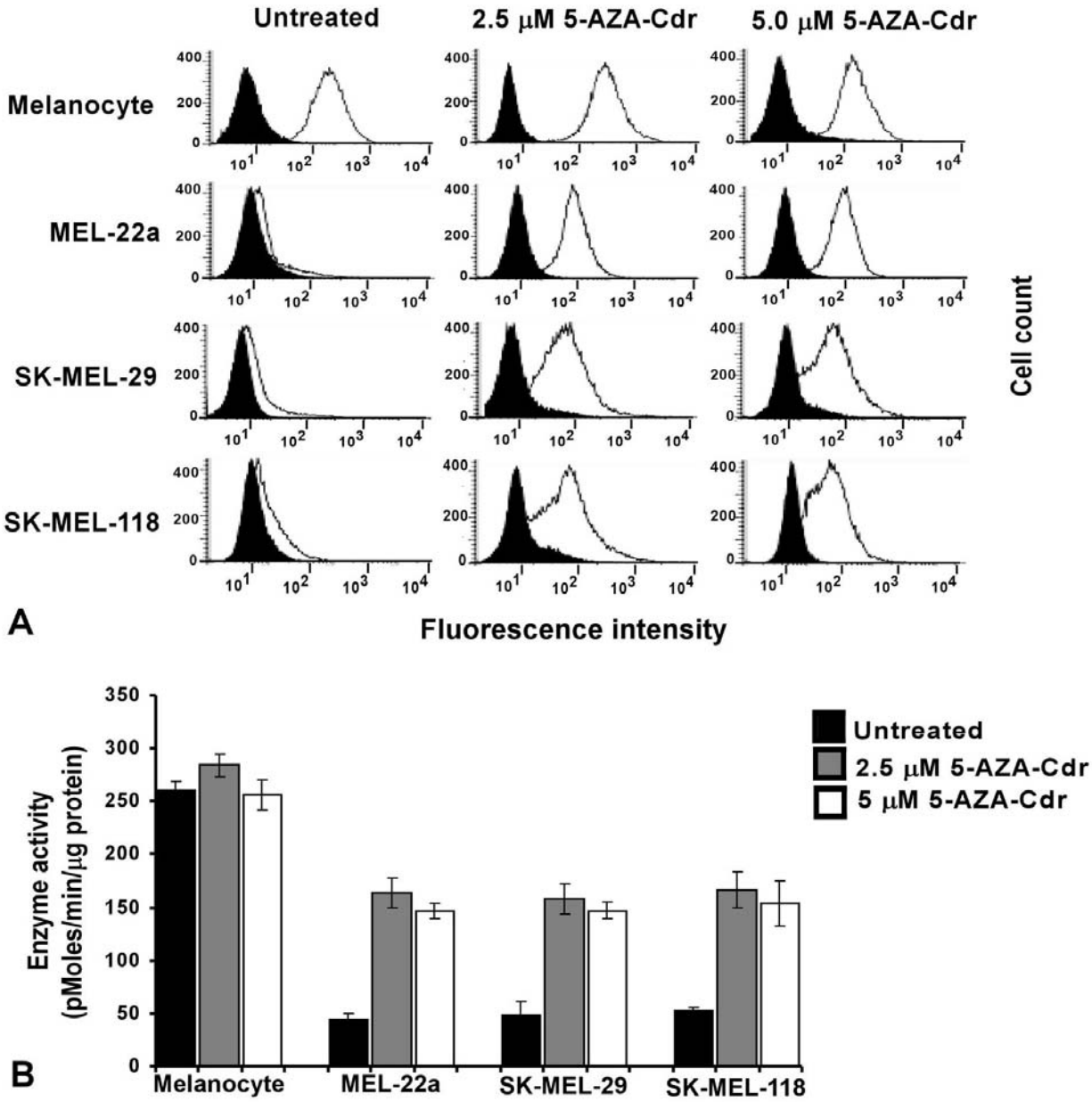

Figure 2. Induction of DPPIV cell surface expression and enzyme activities by 5-AZA-Cdr in melanoma cells. A. Cell surface expression of DPPIV in melanoma cell lines (MEL-22a, SK-MEL-29, SK-MEL-118) and in melanocytes was determined by flow cytometry. Untreated and 5-AZA-Cdr exposed cells were stained with the DPPIV-specific mAb S27 and fluorescein-conjugated rabbit anti-mouse secondary antibody. The Y-axis shows relative cell number and the X-axis shows the log of relative fluorescence intensity. The solid black line depicts DPPIV expression and filled histogram is control IgG1 antibody B. DPPIV enzymatic activity in total cell lysates obtained from untreated and 5-AZA-Cdr exposed melanoma cells. Cell lysates were prepared and DPPIV activity was determined as described in Materials and Methods. The data for three representative melanoma cell lines and a melanocyte are shown.

apoptosis was evident as revealed by changes in cell morphology, nuclear condensation, and DNA fragmentation. TUNEL negative (untreated) and positive (5-AZA-Cdr exposed) melanoma cells are shown in Figure 5B panels, a and b. These data indicate that inhibition of the melanoma cell growth by 5-AZA-Cdr is associated with induction of apoptosis.

5. DISCUSSION

Decreased levels of DPPIV protein are frequently observed in human melanoma tissues and cell lines (2-4). However, the mechanism underlying loss of DPPIV gene expression in melanoma is not known. The silencing of DPPIV leading to activation of bioactive peptides may

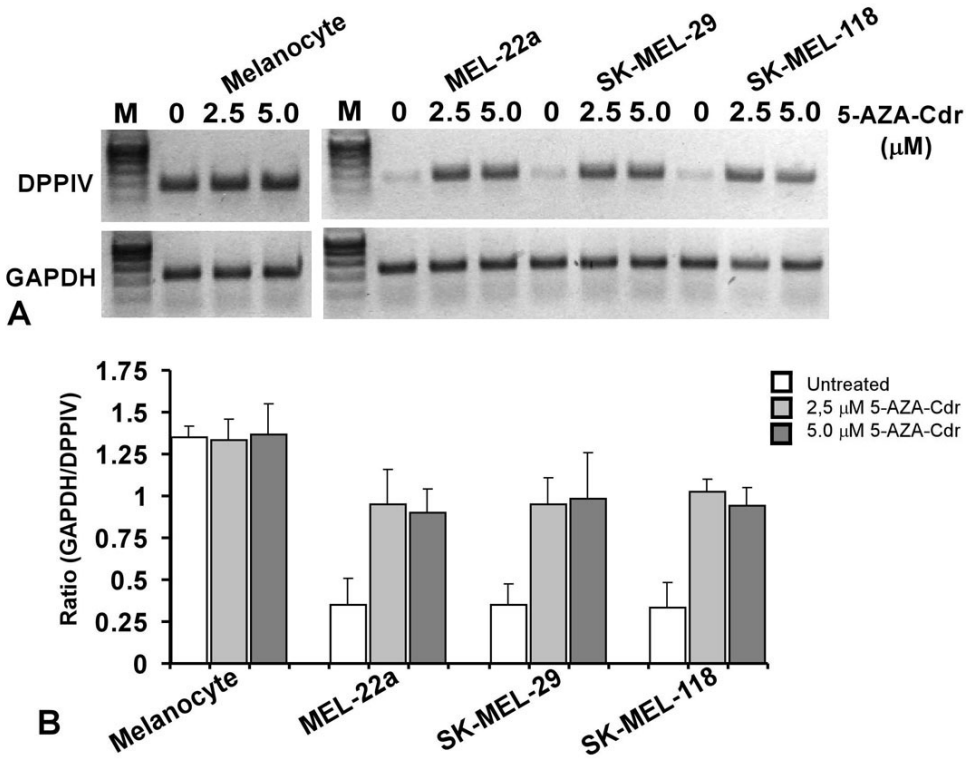

Figure 3. Induction of DPPIV mRNA by 5-AZA-Cdr in melanoma cells. A. Semi quantitative RT-PCR analysis of DPPIV mRNA expression in melanoma cell lines (MEL-22a, SK-MEL-29, SK-MEL-118) and in melanocytes in presence or absence of 5-AZA-Cdr. DPPIV mRNA was amplified from untreated and 5-AZA-Cdr exposed melanoma cells. Amplification of GAPDH mRNA was used as control to assess amounts of cDNA used in each reaction. B. quantitation of DPPIV mRNA levels as compared with mRNA levels of house keeping gene GAPDH.

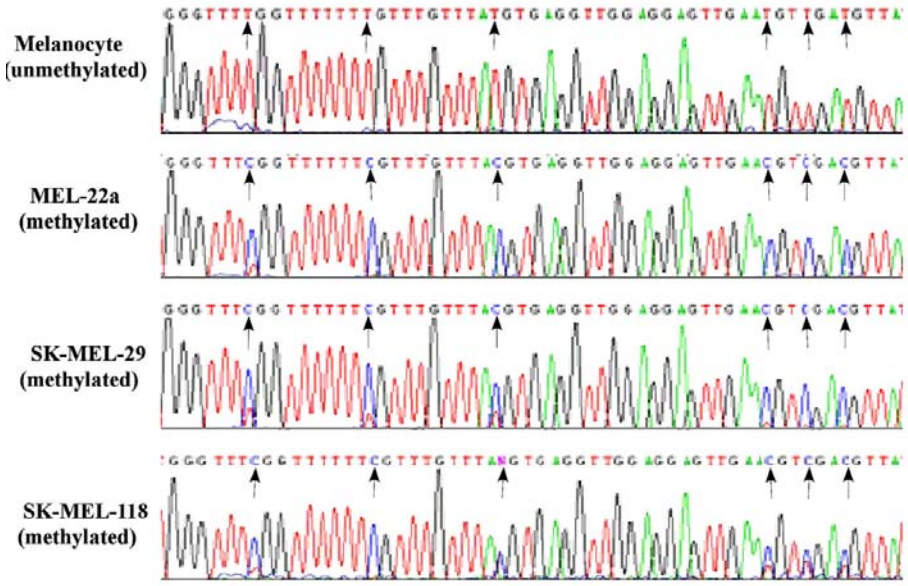

Figure 4. Sequencing of bisulfite modified genomic DNA to assess DPPIV promoter methylation. Representative electropherograms are shown for a melanocyte cell line and three melanoma cell lines, MEL-22a, SK-MEL-29, SK-MEL-118. Methylated cytosines are shown by arrows.

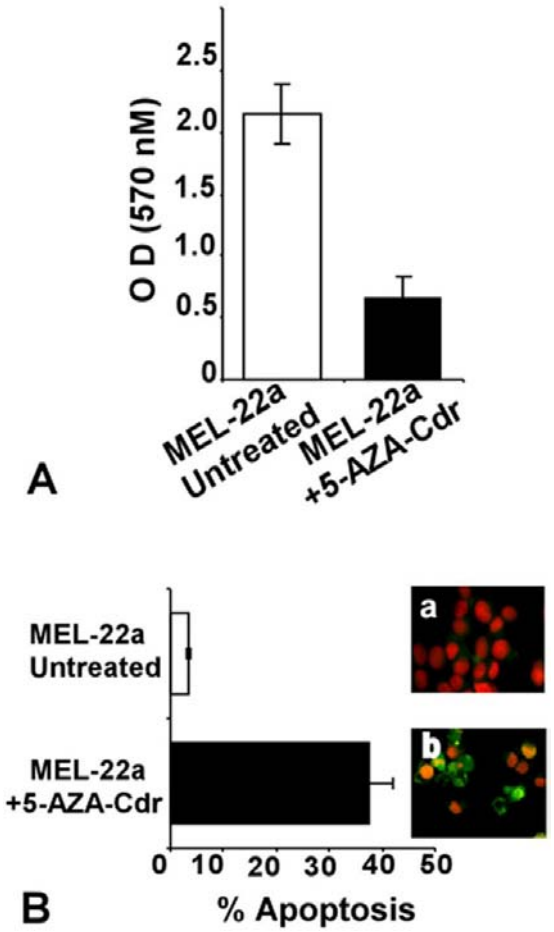

Figure 5. The effects of 5-AZA-Cdr on proliferation and apoptosis of melanoma cells. A. The rates of cellular proliferation of untreated and 5-AZA-Cdr exposed MEL-22a cells were assessed by MTT assay as described in Materials and Methods. Optical densities (O D) correlating with the number of viable cells were read at 570 nm. O D values are expressed as mean +/-1 SD of triplicates. B. For apoptosis assays, melanoma cells (MEL-22a) were grown without or with 5-AZA-Cdr and for 48 hours. Fixed cells were washed and measurements of DNA nicks were carried out by TUNEL assay using the APOPTAG kit. Percent apoptosis was calculated by FACScan analysis. Results are presented as mean % apoptotic (TUNEL-positive) cells with +/- 1 SD. The untreated (panel a), and 5-AZA-Cdr and exposed (panel b) MEL-22a cells were observed under an inverted Nikon fluorescence microscope following TUNEL staining.

provide a selective growth and survival advantage for melanocytic cells progressing to a malignant phenotype. In support of this view, we have previously demonstrated that DPPIV abolishes the growth factor independence and abrogates the tumorigenic potential of melanoma cells (4).

Increasing attention is being directed towards the involvement of epigenetic events in the progression of many cancers that do not affect DNA sequence, but may lead to changes in gene expression. Epigenetic events including DNA methylation play a key role in normal development and are important for establishing the correct program of gene expression. Disruption in this program can lead to aberrant patterns of gene expression and loss of anti-cancer checkpoints. Thus, to date several genes have been reported to be dysregulated in many cancers including melanoma by aberrant DNA methylation (32-36). Indeed clinical concepts for epigenetic therapies are currently being developed by using demethylating agents for the treatment of leukemias and other tumors (39-42).

In the present study, we show that the loss of DPPIV occurs at RNA level, and selective inhibition of methyl transferases using 5-AZA-Cdr results in transcriptional induction of DPPIV gene. These data indicate that the molecular mechanism of DPPIV silencing is likely to involve the promoter methylation. The bisulfite modification of genomic DNA followed by PCR and sequencing further established that DPPIV gene promoter is frequently methylated in melanoma cell lines. Importantly, exposure of melanoma cell lines to 5-AZA-Cdr was associated with decreased rate of cell proliferation and increased apoptosis, thus demonstrating their anti-neoplastic effects on melanomas. These observations parallel our previous work that ectopic expression of DPPIV suppresses the malignant phenotype of melanoma cells (4) and support the notion that absence of DPPIV confers growth advantage to melanocytic cells. In accordance with our data, loss of another cell surface peptidase, Neutral Endo Peptidase (NEP), a tumor suppressor gene for lung and prostate cancers occurs through promoter methylation (37-38) suggesting that silencing of these peptidases is an important step during cancer initiation and progression.

Altogether, our findings show that the DPPIV gene is transcriptionally inactivated by promoter methylation in malignant melanoma and support a role for DPPIV, at least in part regulating melanoma initiation and progression.

6. ACKNOWLEDGEMENT

This work was supported in part by Department of Microbiology and Molecular Genetics, Lake Champlain Cancer Research Organization (LCCRO), Vermont Cancer Center (VCC) at the University of Vermont. We thank Timothy Hunter, Mary Lou Shane, and Scott Tighe of VCC and Vermont Genetics Network (VGN) for their technical help in DNA sequence and flow cytometric analysis. We thank Drs. Alan N. Houghton, Cedric Wesley, David Nanus, Marcus Bosenberg, and Vishwanath Muthuswamy for helpful discussions.

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Abbreviations: 5-AZA-Cdr, 5-aza-2′-deoxycytidine, DPPIV, Dipeptidyl peptidase IV, TUNEL; TdT-mediated dUTP-biotin nick-end labeling.

Key Words : Dipeptidyl peptidase IV, melanoma, Tumor Suppressor Gene, Promoter Methylation

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