Loss of PTEN Promotes Tumor Development in Malignant Melanoma

Jill M. Stahl, Mitchell Cheung, Arati Sharma, Nishit R. Trivedi, Sumathi Shanmugam, and Gavin P. Robertson

Departments of Pharmacology [J. M. S., M. C., A. S., N. R. T., S. S., G. P. R.], Pathology [G. P. R.], and Dermatology [G. P. R.], and The Foreman Foundation for Melanoma Research [G. P. R.], The Pennsylvania State College of Medicine, Hershey, Pennsylvania 17033

ABSTRACT

Loss of tumor suppressor genes on chromosome 10 plays an important role in the development of 30–60% of melanomas; however, the identity of these genes and the mechanisms by which loss of these genes leads to tumor formation remain uncertain. The phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is one of the genes on chromosome 10 whose loss has facilitated melanoma tumor development. Mechanistically, PTEN loss led to the activation of Akt, which consequently down-regulated the apoptotic pathway of melanoma cells. In contrast, expression of PTEN attenuated Akt activation, thereby increasing sensitivity to apoptotic stimuli in cell culture and in vivo in animal models. This model demonstrated that PTEN loss is critical for melanoma tumorigenesis and allowed a dissection of the underlying mechanism by which PTEN loss facilitated melanoma tumor development. In summary, loss of PTEN reduces apoptosis and promotes cell survival, thereby favoring melanoma tumor formation. Thus, these observations provide an etiological basis for PTEN loss during the genesis of sporadic melanomas.

INTRODUCTION

Of the three forms of skin cancer, malignant melanoma carries the highest risk of mortality from metastasis (1). The primary sites of metastasis are the lungs, liver, and brain, but this aggressive cancer can invade any organ. Currently, there is no effective long-term treatment for patients suffering from the advanced stages of this disease. This is in part due to a lack of information about the genes causing melanoma and therapies targeted to correct these defects. As in other solid tumors, depletion of genetic material exceeds genomic amplifications, suggesting that loss of cancer suppressor gene function is essential for melanoma tumor development (2). Nonrandom deletion of chromosomes or subchromosomal regions in solid tumors has been used to map the sites of these putative cancer suppressor genes and has facilitated the demonstration that these sites contain functionally inactivated tumor suppressor genes; examples include INK4A/p16 at 9p21 (3, 4), RB1 at 13q14 (5), and PTEN3 at 10q23 (6–8).

Loss of cancer suppressor genes on chromosome 10 is an important process in melanoma tumorigenesis and has been reported to contribute to the development of 30–60% of noninherited melanomas (9, 10). The alteration usually entails loss of an entire chromosome 10 homologue, because it is easier for melanoma cells to lose the intact chromosome than to undergo multiple independent mutational or deletional events to eliminate cancer suppressor genes on chromosome 10 (11). Deletion of chromosome 10 has been reported in both early and advanced-stage sporadic melanomas (9, 10), and has been associated with a poor clinical outcome (12). The identity of the melanoma cancer suppressor genes on chromosome 10 remains uncertain; however, evidence from multiple groups suggests that PTEN, located at 10q23, might be important in melanoma tumorigenesis and metastasis (11, 13–24).

The PTEN gene, also known as MMAC1 and TEP1 (6–8), encodes a phosphatase of which the primary function is to degrade the products of PI3K by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3 position (25). Loss of functional PTEN from tumor cells causes accumulation of these critical second messenger lipids, which in turn increase Akt phosphorylation and activity, leading to decreased apoptosis and/or increased mitogen signaling (26–31). Reintroduction of PTEN in cells lacking this protein has been shown to lower Akt activity and induce cell cycle arrest and/or apoptosis; however, it is unknown whether PTEN functions in a similar manner in melanoma cells (32). PTEN knockout mice are not viable, whereas PTEN heterozygotes survive and develop endometrial, intestinal, thyroid, adrenal gland, and breast hyperplasia, as well as dysplasias and tumors of the skin, gastrointestinal tract, and prostate (33).

Tumor suppressor gene inactivation in noninherited cancers requires two consecutive somatic events targeting each allele through processes that involve a combination of mutation or epigenetic inactivation, and chromosomal loss, deletion, or recombination (34, 35). Unfortunately, the search for genetic lesions of PTEN in melanomas has yielded confounding results leaving uncertainty about the role PTEN plays in melanoma tumorigenesis. Mutations or deletions of PTEN have been observed in up to 60% of melanoma cell lines; however, only about 10% have been seen in uncultured tumor material (13–22). These observations have led to speculation that one of the PTEN inactivating events occurs predominantly through a mechanism other than mutation, such as by epigenetic silencing (36–38) or by altered subcellular localization (39). Alternatively, haploinsufficiency or loss of only one allele could account for the discordance in rates of LOH at 10q23 and biallelic PTEN inactivation (40). In support of these possibilities, a recent report has suggested that epigenetic silencing of PTEN in melanomas without PTEN mutation might occur in as many as 30–40% of metastatic tumors (41); however, the molecular basis of this process in melanomas remains unknown. Therefore, it is possible that epigenetic, mutational, and deletional events could account for PTEN dysfunction in as many as 40–50% of sporadic melanomas (41).

Functional mapping of melanoma suppressor genes on chromosome 10 has also identified PTEN as a potentially important factor in the disease (11, 23). The involvement of PTEN in melanomas has been demonstrated using an approach termed IVLOH that involved transfer of a normal copy of chromosome 10 into melanoma cells lacking PTEN protein, and then allowing the growth-suppressing...
PTEN LOSS PROMOTES MELANOMA TUMOR DEVELOPMENT

PTEN loss promotes melanoma tumor development by disallowing the underlying mechanism by which loss of functional PTEN aids melanoma tumor development. We used melanoma cell lines expressing PTEN at normal physiological levels from an introduced chromosome 10 to show that tumor growth in animals is retarded unless, by analogy with LOH (42–44), cells inactivate the functional protein during tumor formation. Targeting PTEN in this manner demonstrated that PTEN loss led to increased Akt activation and stimulated antiapoptotic signaling in melanoma cells. Specifically, loss of PTEN reduced apoptosis rates, thereby aiding cell survival and promoting melanoma tumor development. Thus, these studies provide a mechanistic basis for PTEN loss during the genesis of sporadic melanomas.

MATERIALS AND METHODS

Cell Line, Culture Conditions, and Doubling Times. The melanoma cell line UACC 903, the creation and characterization of the microcell-mediated chromosome transfer hybrid cell lines 29, 36, and 37, as well as the growth conditions for these cell lines, have been reported previously (23). Hybrid cell lines stably maintaining the transferred chromosome 10 in tissue culture were maintained in cell culture using 15% FBS to reduce the negative effect that PTEN exerts on in vitro growth. Initially, the chromosomally heterogeneous population (from Ref. 23) was flow-sorted to isolate single cells into individual wells of a 96-well plate. These cells were then grown into mass populations in DMEM supplemented with 15% serum. The chromosome 10 donor cell line HA(10)A has been described previously (23). Human melanocytes were obtained from Clonetics (Walkersville, MD). The melanoma cell lines YUDAN-3 and SK-MEL-24 used in these studies were maintained in cell culture studies, 5 × 10^4 cells were seeded onto glass coverslips in 12-well plates and grown in DMEM supplemented with either 1% or 10% FBS for 48 h. Alternatively, cells growing in DMEM supplemented with 10% FBS were exposed to 1 μM staurosporine for 4 h before paraformaldehyde fixation. For in vivo animal studies, formalin-fixed, paraffin-embedded tumor sections were deparaffinized, rehydrated, and digested with 20 μg/ml proteinase K from Fisher Scientific (Fairlawn, NJ) and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase in a humidified dark chamber for 1 h at 37°C. Sections were counterstained with 1 μg/ml Hoechst-33258 for 10 min and mounted. A minimum of 8–10 fields were counted from three to four different tumor sections, and the number of positive cells was expressed as the percentage of apoptotic cells = (number of apoptotic cells/number of cells in each field) × 100%. Cell Proliferation Rate. The number of proliferating cells in tumor sections was measured by using a purified mouse antihuman Ki67 from Pharmingen (San Diego, CA). Formalin-fixed, paraffin-embedded tumor sections were deparaffinized and rehydrated in xylene and a graded series of ethanol, respectively. Tumor sections were incubated in 1% H_2O_2 for 10 min to quench endogenous peroxidase activity. Antigen retrieval was accomplished by incubation in a 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 98°C, followed by cooling to room temperature for 20 min. Sections were blocked for 30 min with 1% BSA, and then incubated with mouse antihuman Ki67 (dilution 1:50) for 1 h at room temperature. After washing with PBS, sections were incubated with biotinylated antivimentin IgG for 30 min, washed in PBS, incubated with peroxidase-labeled streptavidin for 30 min, and finally rinsed with PBS-T. The chromogenic reaction was carried out with 3,3-diaminobenzidine from DAKO Corporation (Carpinteria, CA) for 2–5 min and counterstained with hematoxylin. The number of Ki67-positive cells/total number of cells was counted from 8–10 fields. The mean percentage of proliferating cells is reported together with the SE for each.

Vessel Density. The number of vessels in tumor sections was estimated using a purified rat antiamuine CD31 (PECAM-1) monoclonal antibody from PharMingen. Frozen, cryostat-cut sections were air dried for 30 min, fixed in acetone (−20°C) for 5 min, and rehydrated in PBS for 5 min. Endogenous peroxidase activity was quenched by incubation for 10 min 1% H_2O_2 in PBS. Each incubation step was carried out at room temperature and was followed by three 3-min washes in PBS-T. Sections were blocked in PBS containing 1% BSA for 30 min and then incubated with rat antimouse CD31 (PECAM-1) antibody at a dilution of 1:500 for 90 min to stain endothelial cells. Sections were then washed in PBS followed by incubation with biotinylated goat-antirat antibody for 30 min. Tumor slices were then incubated with peroxidase-labeled streptavidin from PharMingen for 30 min, and then rinsed with PBS-T. Sections were exposed to 3,3-diaminobenzidine and hydrogen peroxide chromogen substrate from Dako Corporation for up to 2 min, rinsed in distilled water, counterstained with Mayer’s hematoxylin for 1 min, then dehydrated and coverslipped with a permanent mounting medium. Vessel density was scored by counting the number of CD31-positive vessels in a 3600-μm² area of a tumor. Five different areas were counted from each of four different tumors, and the mean number of vessels is reported together with the SE for each.

Sequencing of PTEN DNA and cDNA. The procedure for extraction of high molecular weight DNA from cultured cells has been described previously (46). Total RNA was isolated from cell lines using the TRizol reagent (Life Technologies, Inc., Carlsbad, CA) and converted to cDNA (23). The DNA or cDNA was used as a template to amplify PTEN exon 4 as described previously (23, 47). The amplified PTEN exon 4 product was then sequenced on an Applied Biosystems 373XL automated sequencer (Foster City, CA).
Western Blot Analysis. Cells that were 70-90% confluent were washed once with ice-cold PBS followed by the addition of lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 5 μg/ml leupeptin. Whole cell lysates were centrifuged (≥10,000 × g) for 10 min at 4°C. Proteins were quantitated using the BCA Assay from Pierce (Rockford, IL). Thirty μg of supernatant per lane were loaded onto a NuPage gel from Life Technologies, Inc. and electrophoresed according to the manufacturer’s instructions, followed by transfer of the proteins to polyvinylidene difluoride membrane from Pall Corporation (Ann Arbor, MI). The blots were probed with the appropriate primary antibody according to each supplier’s recommendations. Antibodies were obtained from the following sources: anti-PTEN clone 6H2.1 from Cascade Bioscience (Winchester, MA); anti-Akt, anti-phospho-Akt (ser 473), and anti-Caspase-3 from Cell Signaling Technologies, Inc. (Beverly, MA); and α-enolase from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All of the secondary antibodies were conjugated with horseradish peroxidase and obtained from Santa Cruz Biotechnology, Inc. The immunoblots were developed using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech (Piscataway, NJ). In the PI3K inhibition and apoptotic studies, cells grown in DMEM supplemented with 10% FBS were treated with 10 μM LY-294002 (Alexis Biochemicals, San Diego, CA) for 24 h or 1 μM staurosporine for 4 h, respectively, before harvesting the protein lysates.

Expression of PTEN Using Adeno-associated Viral Constructs. The AAV Helper-Free System from Stratagene (La Jolla, CA) was used to create the recombinant AAV adeno-associated viruses. Constructs containing PTEN or the catalytically inactive mutant G129R both tagged with HA (generously provided by Dr. Webster Cavenee, Ludwig Institute, San Diego, CA) were transferred into the pAAV-MCS and pAAV-IRES-hrGFP vectors, and viruses were generated according to the Stratagene protocol. On the day before viral infection, 1.5 × 10⁶ cells were plated into six-well plates. After 24 h, the cells were treated for 5–6 h with permissive medium consisting of DMEM supplemented with 10% FBS, 40 mM hydroxyurea, and 1 mM sodium butyrate. Cells were then washed with prewarmed DMEM supplemented with 2% FBS. Increasing volumes of viral lysates (0 μl, 7.8 μl, 15.6 μl, 31.3 μl, 62.5 μl, and 125 μl) suspended in DMEM supplemented with 2% FBS were then added to each well to give a final volume of 1 ml and incubated at 37°C for 1–2 h. An additional 1 ml of DMEM supplemented with 18% FBS was added after the infection to bring the final serum concentration to 10%. Protein from the cells was harvested for Western blot analysis 3 days after infection. For some experiments, the amount of phosphorylated or total protein was quantitated using a Molecular Dynamics Model 100A laser densitometer (Sunnyvale, CA) and the ratio of phosphorylated Akt to total Akt protein used as an indicator of Akt activation.

Statistical Analyses. The statistical analyses of the data were performed using the unpaired Student’s t test. A P of <0.05 was considered statistically significant.

RESULTS

PTEN Loss Leads to Melanoma Tumor Formation. To establish whether PTEN functions as a melanoma tumor suppressor, we developed a novel chromosome-based strategy to measure the effects of PTEN expression on melanoma cell tumorigenicity. We reasoned that melanoma cells expressing PTEN at normal physiological levels from an introduced chromosome 10 could be established and maintained stably in culture under high serum conditions by stimulating cell growth and reducing apoptosis. We predicted that the growth factors present in serum would mask PTEN-mediated apoptosis by stimulating Akt activation thereby suppressing apoptosis (26–31, 48). In contrast, s.c. growth conditions in animals would be such that the presence of PTEN would retard cell growth and tumor development unless, by analogy with LOH (42–44), cells would use genetic strategies to silence the gene thereby allowing tumor development to occur. Targeting PTEN in this manner would first confirm involvement of the gene in melanoma tumor development, and second, result in the establishment of genetically related nontumorigenic and tumorigenic cell lines that could be used to determine the underlying mechanism by which PTEN regulates melanoma tumorigenicity.

To create melanoma cell lines that expressed PTEN at normal physiological levels, we used microcell-mediated chromosome transfer to introduce a normal chromosome 10 into the melanoma cell line, UACC 903. Because of a condition called uniparental disomy, this cell line contains two genetically identical copies of chromosome 10 that do not produce PTEN protein because of the same truncating point mutation (T228G) that converts a tyrosine at codon 76 in exon 4 to a stop codon, thereby severely truncating the protein (23). In addition, we reported previously that introduction of chromosome 10 into UACC 903 cells growing in culture in 5% FBS leads to breakage of the transferred chromosome by IVLOH in order for the cells to eliminate the growth-suppressing activity of PTEN (23). To overcome IVLOH for these studies, we designed a strategy to prevent fragmentation of the transferred chromosome by using serum concentrations of 15% to mask the negative effects that PTEN exerts on in vitro cell growth. Briefly, we flow sorted the initial chromosomally heterogeneous population (described in Ref. 23) to isolate single cells into individual wells. These cells were then grown into mass populations in medium supplemented with 15% FBS. STS markers that could identify polymorphic alleles in the transferred chromosome 10 were used to verify whether the intact introduced chromosome was retained in the cells, and Western blotting was used to show that these hybrid cells expressed PTEN protein (Table 1). We found that high serum concentrations could effectively mask the growth inhibitory effects of PTEN in vitro, allowing establishment of homogeneous stable cell lines containing the intact introduced copy of chromosome 10 that expressed PTEN protein. Even so, the growth rate measured as the doubling time of the cell population remained ~26% longer for the 36A hybrid cell line, doubling every 1.3 days (or 31.5 h), compared with the parental UACC 903 cell line, which doubled about every day (or 25 h). The status of the introduced chromosome 10 and PTEN protein for the parental cell line, initial unstable hybrid cell populations, and five stable hybrid cell lines derived from the initial cell populations is shown in Table 1.

To measure the biological effect of PTEN expression on melanoma tumor development, UACC 903 cells and hybrid cell lines containing the intact introduced chromosome 10 were injected beneath the skin of nude mice. Hybrid cells expressing PTEN were found to be nontumorigenic in comparison with parental UACC 903 cells lacking the protein (Table 2). In all of the cases, tumor development was dramatically reduced for the hybrid cell lines containing the introduced chromosome 10. The majority of injection sites failed to develop tumors. Because tumor development was inhibited completely.

Table 1 Status of transferred chromosome 10 and PTEN protein expression in parental UACC 903 cells and chromosome 10 hybrid cell lines

<table>
<thead>
<tr>
<th>Cell line or hybrid name</th>
<th>Retention of the intact transferred chromosome 10</th>
<th>PTEN protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental cell line</td>
<td></td>
<td>none</td>
</tr>
<tr>
<td>UACC 903</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstable hybrids cell lines&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>–&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Stable hybrids cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>36A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>36B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37B</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Heterogenous population with each cell retaining a portion of the transferred chromosome 10.

<sup>b</sup> –, unstable retention or partial expression; +, present.
in most cases, the reduced growth rate of 26% observed in vitro could not explain the lack of tumorigenicity. Approximately 30% of the injection sites did form tumors after a prolonged latency period (Table 2; Fig. 1). In these instances, tumors larger than 100 mm\(^3\) were observed between days 35 and 49, which then proceeded to develop into rapidly growing large tumor masses. The cells constituting two of these tumors, named 36A revertant 1 (36A-R1) and 36A revertant 2 (36A-R2), were re-established in culture and the tumorigenicity of these cells was re-examined (Table 2; Fig. 1). The tumorigenic potential of these cells was found to have reverted to more closely resemble that of the parental UACC 903 cell line, but even so tumor development was delayed by \(\sim 1\) week. Hence, a melanoma tumor suppressor gene on the introduced chromosome 10 was functionally inactivated during melanoma tumorigenesis.

To determine whether the introduced wild-type PTEN gene was specifically targeted for loss during tumor formation, DNA, RNA, and protein were isolated and compared from genetically matched 36A cells and chromosome 10 hybrid cell lines after injection into nude mice: UACC 903 cells \(\Box\), hybrid cells retaining PTEN \(\diamond\), hybrid cells that had lost PTEN \(\bigotimes\), revertant cell lines that had lost PTEN \(\bigotimes\), and revertant cell lines expressing PTEN \(\blacksquare\). Western blotting showed that both wild-type and mutant PTEN RNA expression occurred in the hybrid cell line 36A, but only mutant sequence was observed in both revertant tumorigenic cell lines. This observation was confirmed through Western blotting, shown in Fig. 2D, in which PTEN protein was detected in nontumorigenic 36A cells but was absent from the tumorigenic revertant cell lines. These results suggest that the PTEN gene in the revertant cell lines was targeted for loss of protein expression by an epigenetic, mutational, or deleterional event. Collectively, these data demonstrate PTEN is targeted for loss during melanoma tumor formation and that inactivation of the gene occurs at the DNA level.

**PTEN Loss Facilitates Cell Survival Thereby Promoting Melanoma Tumor Development.** The foregoing experiments showed a consistent relationship between PTEN loss and tumor development; therefore, subsequent studies focused on dissecting the mechanism by which *PTEN* functions as a melanoma tumor suppressor gene. Because PTEN functions as an antagonist of PI3K-mediated signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3 position, which in turn lowers Akt activity and thereby increases apoptosis rates, we reasoned that cell survival might be a critical function regulated by PTEN in

---

### Table 2. Tumor development by parental UACC 903 melanoma cells and chromosome 10 hybrid cell lines

<table>
<thead>
<tr>
<th>Cell line (number of sites injected)</th>
<th>Tumor size (mean ± SE in mm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>Parental cell line</td>
<td></td>
</tr>
<tr>
<td>UACC 903 (&gt;20)</td>
<td></td>
</tr>
<tr>
<td>Hybrids cell lines expressing PTEN</td>
<td></td>
</tr>
<tr>
<td>29A (8)</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>36A (6)</td>
<td>0(^a)</td>
</tr>
<tr>
<td>36B (20)</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>37A (8)</td>
<td>0</td>
</tr>
<tr>
<td>37B (8)</td>
<td>0</td>
</tr>
<tr>
<td>Revertant cell lines lacking PTEN</td>
<td></td>
</tr>
<tr>
<td>36A-R1 (8)</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>36A-R2 (8)</td>
<td>83 ± 8</td>
</tr>
</tbody>
</table>

\(^a\) Not available since mice were euthanized at day 21.
melanomas. To test this possibility and demonstrate a direct relationship between Akt activity and PTEN expression in this model, UACC 903 melanoma cells growing in culture were examined to determine whether cells lacking PTEN had elevated Akt activation that declined significantly after expression of PTEN protein. Initially, the levels of phosphorylated versus total Akt protein were measured by Western blot analysis in melanocytes, UACC 903 cells, and matched nontumorigenic and tumorigenic 36A cell lines containing the transfected chromosome 10 (Fig. 3A). A phospho-specific antibody (anti-Akt, Ser-473) that recognizes only phosphorylated (active) Akt indicated a dramatic reduction in the activation of Akt in hybrid cells expressing chromosomal PTEN (36A, 29A and 37A) without significant changes in the amount of total Akt protein when compared with parental UACC 903 or 36A revertant cells. Furthermore, it appeared that expression of phosphorylated Akt was lowered to amounts similar to those observed in normal human melanocytes (Fig. 3A). Also, comparison of 36A nontumorigenic and tumorigenic revertant 36A-R1 and 36A-R2 cell lines showed that PTEN loss was accompanied by increased levels of phosphorylated Akt similar to those observed in UACC 903 cells lacking the protein. To demonstrate that PTEN reduced Akt activity in a manner similar to that of LY-29004 (49), a known inhibitor of PI3K, UACC 903 cells were treated with the compound, and changes in the levels of phosphorylated versus total Akt were measured from densitometric scans of Western blots. LY-294002 treatment reduced the phosphorylated levels of Akt by approximately 60–75% compared with untreated UACC 903 cells. Thus, the observation that Akt phosphorylation were similarly decreased in 36A hybrid cells and after treatment of UACC 903 cells with LY-294002 provided evidence for a direct relationship between Akt and PTEN in this model.

To confirm that PTEN was the primary factor on chromosome 10 responsible for the change in Akt activation, PTEN was ectopically expressed from adeno-associated viruses in 36A-R2 cells, and the effects on Akt phosphorylation (activity) were measured. Increasing volumes of adeno-associated viruses expressing either wild-type or catalytically inactive mutant PTEN protein (G129R) were used to infect 36A-R2 cells to determine whether ectopic PTEN expression could reduce the amount of phosphorylated (active) Akt to levels seen in the original 36A cell population. Three days after infection, expression of PTEN, as well as phosphorylated and total Akt was measured by Western blot analysis. Densitometric scans of the blots were used to quantitate the levels of phosphorylated versus total Akt at each viral concentration used to infect the cells. Plots showing Akt activation versus increasing PTEN expression are shown in Fig. 3B in which ectopic expression of PTEN or PTEN-HA in 36A-R2 cells diminished the phosphorylation (activity) of Akt. In contrast, catalytically inactive PTEN (G129R) or empty virus had minimal effects on Akt activation. These results identify PTEN as the gene on the introduced chromosome 10 that reduced the levels of active Akt in UACC 903 hybrid cells.

To provide additional evidence that PTEN is an important regulator of Akt in other melanoma cell lines in which PTEN expression is altered, the effect of ectopic PTEN expression was also measured in the YUDAN-3 and SK-MEL-24 melanoma cell lines. Changes in Akt phosphorylation (activation) of these cell lines were compared with UACC 903 cells, and the results are shown in Fig. 3C. Increasing amounts of wild-type PTEN, but not catalytically inactive G129R PTEN expression, led to decreased phosphorylation of Akt without significant changes in the levels of total Akt protein. Thus, ectopic expression of PTEN consistently reduced active Akt in melanoma cell
lines lacking functional PTEN protein, indicating that altered PTEN expression or activity plays an important role in regulating Akt activity in melanomas.

To determine whether the altered Akt activity mediated by PTEN led to changes in apoptotic signaling, the level of cleaved caspase-3 was measured after ectopic viral expression of PTEN in melanoma cells. Elevated levels of cleaved caspase-3 indicating higher levels of apoptosis were observed after expression of wild-type, but not mutant G129R PTEN in UACC 903 cells, suggesting that PTEN expression increases apoptosis in melanomas (Fig. 4A). To eliminate the possibility that viral overexpression of PTEN led to elevated levels of apoptosis, the apoptotic rates were compared between UACC 903 cells lacking PTEN protein or hybrid cells expressing chromosomal PTEN protein using the TUNEL assay. The percentage of apoptotic cells under different serum conditions and after treatment with the proapoptotic agent staurosporine are shown in Fig. 4B. No difference in apoptotic rates was observed between cells lacking or expressing chromosomal PTEN protein when grown under normal conditions in DMEM supplemented with 10% serum (Fig. 4B). This is illustrated by comparison of apoptosis rates between the genetically matched 36A and 36A-R2 cell lines that showed no significant difference in apoptosis (Student’s t test; P ≥ 0.119). In contrast, Fig. 4C shows that lowering the serum concentration to 1% for 48 h to induce apoptosis led to ~4-fold higher rates of apoptosis in PTEN-expressing cells compared with cells lacking PTEN (Student’s t test; P < 0.001). Fig. 4D shows that similar results were observed after 4 h growth in medium supplemented with 10% serum and 1 μM staurosporine, which led to 2–7-fold higher rates of apoptosis in PTEN-expressing cells versus those lacking the protein (Student’s t test; P < 0.001). This observation was confirmed by Western blot analysis of cell lines treated with 1 μM staurosporine for 4 h (Fig. 4E). Hybrid cell lines 36A2, 29A, and 37A expressing chromosomal PTEN had elevated levels of cleaved caspase-3 compared with parental UACC 903 cells or the 36A revertant cell lines after treatment with staurosporine. Collectively, these results demonstrate that expression of PTEN increases susceptibility of melanoma cells growing in culture to apoptotic stimuli.

Because the foregoing experiments demonstrated that PTEN sensitized melanoma cells to apoptotic stimuli in vitro, subsequent studies focused on establishing whether increased apoptosis was the mechanism underlying PTEN-mediated tumor inhibition in vivo. Twenty million cells of the nontumorigenic (36A and 36B) or 1 million of the tumorigenic cell lines (UACC 903 and 36A-R2) were injected s.c. into 4–6-week old female nude mice to temporally and spatially match tumor development. Tumor cell masses developing in parallel from each cell type were then harvested every 2 days, starting at day 2 and ending at day 12, and the rates of apoptosis, growth, and vascular development were compared. A comparison of the number of apoptotic cells in tumor masses 4 days after injection into nude mice is shown in Fig. 5A. At each time point, more apoptotic cells were...
observed in the hybrid 36A and 36B tumor masses than in tumors formed from the UACC 903 or 36A-R2 cell lines. The percentage of apoptotic cells at 2-day intervals for each cell line is quantified in Fig. 5B. In general, PTEN-expressing cells (36A and 36B) had 3–6-fold higher rates of apoptosis compared with cells lacking PTEN protein (UACC 903 and 36A-R2). However, day 2 is the most significant time point for these comparisons, because cells have just been introduced to the in vivo environment and are reflecting the stimuli experienced in the new environment. Comparison of the apoptotic rates between the genetically matched cell lines 36A and 36A-R2 at each time point were found to be statistically significantly different from one another (Student’s t test; P < 0.001). Although apoptotic rates for all of the cell lines declined to constant levels by day 8, the rates of apoptosis for PTEN-expressing cells remained 2–3-fold higher than cell lines lacking the protein. In contrast to the differences in apoptosis, the rate of growth (Fig. 5C) and vascular development (Fig. 5D) were not found to be significantly different at day 2. These observations led to the conclusion that elevated levels of apoptosis were inhibiting tumor development by melanoma cells expressing PTEN. In additional support of this conclusion, Fig. 4C shows that whereas the rate of cell growth for cell lines lacking PTEN increased steadily from day 4 onwards, the number of dividing cells remained constant in tumor masses established from cells expressing PTEN protein (Student’s t test; P > 0.05). In addition, Fig. 4D showed that the number of vessels present in the tumor masses remained relatively constant for all of the cell lines to day 6 after which a steady increase was observed in tumors developing from cells lacking PTEN compared with cells expressing PTEN protein. Thus, these results confirm that the regulation of Akt-mediated apoptosis by PTEN is critical in melanoma tumor development and that PTEN loss reduces apoptosis rates, thereby aiding melanoma tumorigenesis.

DISCUSSION

This study demonstrates the biological role that PTEN plays in melanoma tumorigenesis by exploiting chromosome transfer technology together with cancer cell evolution during tumor development to demonstrate the relationship between PTEN loss and melanoma tumor development. Because tumor development only occurred with a corresponding loss of PTEN expression, this model demonstrates that altered PTEN pathway signaling in melanomas is not just an in vitro phenomenon but important in melanoma tumor development. The melanoma cell line UACC 903 provided an ideal tool for establishing this model, because it had lost a copy of chromosome 10 and duplicated the remaining one that had a mutated PTEN gene (23). Therefore, both copies of the PTEN gene had an identical truncating point mutation leaving cells devoid of PTEN protein. The transfer of a normal copy of chromosome 10 into UACC 903 cells by microcell-mediated chromosome transfer resulted in PTEN protein levels at near to normal physiological levels. In addition, the PTEN gene was under normal regulatory control with the RNA undergoing appropriate processing although only a single functional copy of the gene was present. Therefore, this model also overcame the growth-inhibitory limitations often encountered after high ectopic expression of tumor suppressor genes.
Fig. 5. Measurements of apoptosis, proliferation, and angiogenesis in melanoma tumor masses differing in PTEN expression. 

A, comparison of TUNEL-positive cells in tumor masses derived from UACC 903 parental cells, 36A and 36B cell lines expressing chromosomal PTEN, and the tumorigenic revertant 36A-R2 cell line lacking PTEN protein 4 days after injection into nude mice; magnification, ×200. 

B, apoptotic rates for each cell line were measured at 2-day intervals using TUNEL assays on formalin-fixed paraffin sections of the tumors. 

C, percentage of proliferating cells measured at 2-day intervals by scoring the number of Ki67-positive cells in formalin-fixed paraffin sections of the tumors. 

D, blood vessel density was measured at 2-day intervals by counting the number of CD31-positive vessels in frozen tumor sections. The data shown represent the mean of counts from two to four separate tumors; bars, ±SE.
genes in cancer cells using an active promoter element (23, 51). Thus, this is a biologically relevant model where PTEN loss occurs during tumor development in vivo and not as a consequence of evolution in tissue culture.

Although PTEN expression was at normal physiological levels in the hybrid cells, the levels were sufficient to exert a negative effect on melanoma cell growth in culture. This was evidenced by the genetic instability or IVLOH that we have reported previously after transfer of chromosome 10 into UACC 903 cells (23). The genetic heterogeneity of the cells undergoing IVLOH made them unsuitable for studying the role PTEN plays in melanoma tumor development (11). To overcome this obstacle, the negative effect that PTEN exerts on cell growth in culture was masked using growth factors present in serum to stimulate PI3K (48). As predicted, this led to activation of Akt (26–30) and resulted in antiapoptotic signaling (31). Masking IVLOH using growth factors present in serum resulted in the establishment of cell lines stably retaining the intact introduced chromosome. In contrast, introduction of these cells into an in vivo environment unmasked the effects of serum; therefore, cell growth and tumor development were retarded unless the protein expression was lost. Isolation of the cells that lost PTEN during tumor formation resulted in the establishment of genetically matched cell lines that could be used as tools for dissecting the signaling mechanisms leading to the evolution of these cells. Thus, these cell lines were used to confirm the involvement of PTEN in melanoma tumorigenesis resulting in a model to demonstrate PTEN regulation of melanoma tumor development.

During melanoma tumor development, it is common for an entire copy of chromosome 10 to be lost from the melanoma cells (2, 10). This occurs because multiple tumor suppressor genes residing on this chromosome must be lost in unison during tumor progression, and this is more efficiently accomplished by chromosome segregation than by multiple mutational and/or deletional events (11). Loss of the intact chromosome has hindered dissection of the role that the individual genes on chromosome 10 play in melanoma tumor development. However, this model has overcome that obstacle by allowing the biological consequence of a single gene to be dissected. Clearly, tumor development in vivo occurs after loss of PTEN protein from melanoma cells, thereby demonstrating the importance of this gene in melanoma tumorigenesis. Whereas the tumorigenic potential of the melanoma cells reverted to more closely resemble that of the parental UACC 903 cell line after PTEN loss, tumor development remained delayed by ~1 week because of retention of a second cancer suppressor gene located at the tip of the short arm at 10p15.3 (11). Therefore, the biological effect of each gene could be measured using this model.

Ectopic expression of PTEN using adeno-associated viruses verified that the model was biologically accurate and that PTEN regulated the same signaling pathways as PTEN expressed from the transferred chromosome 10 present in hybrid cell lines. Hence, this model has allowed the effect of the PTEN tumor suppressor gene to be measured individually and in combination with the others on chromosome 10, thereby confirming PTEN involvement in melanoma tumorigenesis.

The regulation of cell survival and cell death is an essential process in tumor development (50, 52). The balance of signals that promote or retard cell survival defines tissue homeostasis, and mutated cells that have escaped the constraints of normal growth regulation can lead to tumor development (50, 52). In this report, we have shown that PTEN is one factor regulating melanoma tumor development by controlling Akt-mediated cell survival. In melanocytes, PTEN expression blocks Akt activation, thereby regulating normal cellular apoptotic signaling. In contrast, PTEN dysfunction in melanomas leads to up-regulation of Akt signaling, thereby reducing apoptosis rates. Thus, melanoma cells lacking functional PTEN protein are better suited for surviving in the in vivo tumor environment. Therefore, the model we developed demonstrates the role of PTEN in melanomas by showing that restoring functional PTEN protein resulted in reduced Akt signaling and consequently prevented tumor growth in animal models.

This model has enabled a better understanding of the role PTEN dysfunction plays in melanoma tumor development by supporting and extending existing models for PTEN involvement in melanoma tumor progression. Although studies of cultured melanoma cells have found deletions or mutations of PTEN in up to 60% of cell lines, only 10% of patient tumors contain inactivating mutations. However, additional studies have suggested that other mechanisms leading to inactivation of PTEN such as epigenetic alteration or altered subcellular localization might play a more prominent role in melanomas. Taken together, loss of functional PTEN may occur in as many as 40–50% of sporadic melanomas (13–22, 41). For those melanomas expressing catalytically active PTEN at normal physiological levels, it is possible that other members of the PTEN signaling cascade could have altered activity or might be functionally inactivated. This raises the possibility that other potentially important, as yet unidentified, alterations in proteins upstream or downstream of PTEN play an equally important role in cutaneous melanomas lacking PTEN alteration. Future studies will determine whether other components of the pathway are also altered.

Furthermore, the results presented here suggest that PTEN replacement by viral administration may prove to be a useful and important therapeutic approach for inducing apoptosis in melanomas (50, 52). In support of this possibility, a recent report has suggested that adeno-viral-mediated PTEN gene therapy may be a promising treatment for melanoma tumors, even in those that express normal PTEN protein (53). In conclusion, this study establishes the biological significance of PTEN in the development of sporadic human malignant melanomas by demonstrating the importance of this gene in melanoma tumor formation and the underlying mechanism by which its loss aids this process.

ACKNOWLEDGMENTS

We thank Dr. Lakshman Sandrasegaran for helpful discussions and for critical reading of this manuscript, Melissa Zimmerman for technical assistance, and Dr. Elliot Vessel for proofreading of this manuscript.

REFERENCES

PTEN LOSS PROMOTES MELANOMA TUMOR DEVELOPMENT


Loss of PTEN Promotes Tumor Development in Malignant Melanoma


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/11/2881

Cited articles
This article cites 51 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/11/2881.full#ref-list-1

Citing articles
This article has been cited by 31 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/11/2881.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.