Role of p53 Up-regulated Modulator of Apoptosis and Phosphorylated Akt in Melanoma Cell Growth, Apoptosis, and Patient Survival

Alison M. Karst, Derek L. Dai, Jin Q. Cheng, and Gang Li

Abstract

Malignant melanoma is an aggressive and chemoresistant form of skin cancer characterized by rapid metastasis and poor patient prognosis. The development of innovative therapies with improved efficacy is critical to treatment of this disease. Here, we show that aberrant expression of two proteins, p53 up-regulated modulator of apoptosis (PUMA) and phosphorylated Akt (p-Akt), is associated with poor patient survival. Using tissue microarray analysis, we found that patients exhibiting both weak PUMA expression and strong p-Akt expression in their melanoma tumor tissue had significantly worse 5-year survival than patients with either weak PUMA or strong p-Akt expression alone ($P < 0.001$). Strikingly, no patients exhibiting strong PUMA expression and weak p-Akt expression in primary tumor tissue died within 5 years of diagnosis. We propose a two-pronged therapeutic strategy of (a) boosting PUMA expression and (b) inhibiting Akt phosphorylation in melanoma tumor tissue. Here, we report that a recombinant adenovirus containing human PUMA cDNA (ad-PUMA) efficiently inhibits human melanoma cell survival in vitro, rapidly induces apoptosis, and dramatically suppresses human melanoma tumor growth in a severe combined immunodeficient mouse xenograft model. In melanoma cells strongly expressing p-Akt, we show that Akt/protein kinase B (Akt) signaling inhibitor-2 (API-2; a small-molecule Akt inhibitor) reduces cell survival in a dose- and time-dependent manner and enhances ad-PUMA-mediated growth inhibition of melanoma cells. Finally, we show that, by combining ad-PUMA and API-2 treatments, human melanoma tumor growth can be inhibited by >80% in vivo compared with controls. Our results suggest that a strategy to correct dysregulated PUMA and p-Akt expression in malignant melanoma may be an effective therapeutic option. (Cancer Res 2006; 66(18): 9221-6)

Introduction

Melanoma is a lethal and highly invasive form of skin cancer arising from the abnormal proliferation of epidermal melanocytes (1). Treatment of the disease is made difficult by its strong resistance to conventional cancer treatments, such as radiation and chemotherapy (2). The molecular mechanisms underlying this resistance have not been clearly elucidated. However, it is speculated that dysregulation of apoptosis may be responsible for the chemoresistant phenotype of melanoma. Apoptotic dysregulation may manifest itself in many forms: through inactivation of proapoptotic effectors, overexpression of antiapoptotic proteins, or hyperactivation of survival signaling pathways (2).

We have previously reported that expression of the proapoptotic protein p53 up-regulated modulator of apoptosis (PUMA) is significantly reduced in human cutaneous melanomas (3). PUMA is an essential mediator of cell death and plays a key functional role in the process of p53-mediated apoptosis (4, 5). PUMA is a BH3-only protein belonging to B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins that regulate apoptosis (6, 7). PUMA is a direct transcriptional target of p53, and its expression is up-regulated most notably in response to DNA damage (6, 7). Like all the BH3-only proteins, PUMA binds to and neutralizes prosurvival members of the Bcl-2 family to promote apoptosis (8). Although most BH3-only proteins interact with only a subset of prosurvival Bcl-2 proteins, PUMA targets all of them, making PUMA a particularly potent effector of apoptosis (9). In addition to neutralizing prosurvival proteins, BH3-only proteins must also activate either Bax or Bak protein to induce cell death (10, 11). Whether PUMA accomplishes this task directly or indirectly remains unresolved (12).

Aside from inactivation of proapoptotic effectors, hyperactivation of survival and/or proliferation signaling pathways is thought to contribute to the aggressive nature of melanoma. Notably, the serine/threonine kinase Akt [protein kinase B (PKB)] has been found to be constitutively activated in 43% to 67% of melanomas (13). Under normal conditions, Akt is activated indirectly via growth factor stimulation of cell surface receptors. Stimulated receptors recruit and activate phosphatidylinositol 3-kinase (PI3K), resulting in production of a second messenger molecule, phosphatidylinositol 3,4,5-trisphosphate (PIP3; ref. 14). PIP3 in turn recruits Akt to the plasma membrane, where its conformation is altered, allowing it to be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) and other kinases (14). Akt is activated by phosphorylation of its Ser$^{173}$ and Thr$^{384}$ residues (15), upon which it promotes the transcription of various cell survival and proliferation genes (16). We and others have reported that phosphorylated Akt (p-Akt) plays an important role in melanoma progression and invasion (13, 17-20).

In this article, we sought to investigate the roles of PUMA and p-Akt expression in human cutaneous melanoma progression and patient survival and to determine whether these two molecules can be used as therapeutic targets for malignant melanoma.

Materials and Methods

Adenoviral construct. A replication-deficient (E1 and E3 deleted) recombinant adenovirus containing human PUMA cDNA under control of the cytomegalovirus promoter was constructed using AdEasy vector system from Qbiogene (Irvine, CA).
Melanoma tissue microarray. The tissue microarray was constructed as described previously (3, 17) from paraffin-embedded human melanoma biopsies obtained from the 1990 to 1998 archives of the Department of Pathology at Vancouver General Hospital (Vancouver, British Columbia, Canada). One hundred forty-six biopsies (99 primary tumors and 47 metastatic tumors) were evaluated for both PUMA and p-Akt-Ser473 immunohistochemical staining. Staining intensity was evaluated as described previously and originally scored as weak (+1), moderate (+2), or strong (+3; refs. 3, 17). PUMA staining was mostly weak or moderate, with few cores staining strongly. Conversely, p-Akt staining was mostly moderate or strong, with few cores staining weakly. Thus, to obtain categories of sufficient size for the present statistical analysis, staining intensities were reclassified as either weak or strong.

Hoechst staining. Melanoma cells were grown on coverslips infected with recombinant adenovirus containing human PUMA cDNA (ad-PUMA) or green fluorescent protein (ad-GFP) for 48 hours and stained with the nuclear dye Hoechst 33258 (Sigma-Aldrich, Oakville, Ontario, Canada). Nuclei were visualized by immunofluorescent microscopy, and the number of apoptotic cells was counted.

Subcellular fractionation. Melanoma cells were infected with ad-PUMA (or ad-GFP) for 0, 4, 8, or 12 hours. Cells were harvested and resuspended in mitochondrial isolation buffer [20 mmol/L HEPES-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA, 1 mmol/L DTT, 250 mmol/L sucrose] containing protease inhibitors. Cells were lysed by passage through a 26-gauge needle and then centrifuged at 2,000 × g for 10 minutes to remove unbroken cells, plasma membrane fragments, and nuclei. The resulting supernatant was centrifuged at 10,000 × g for 20 minutes to obtain a mitochondrial pellet. The pellet was washed with mitochondrial isolation buffer and solubilized in 30 μL of TNC buffer [10 mmol/L Tris-acetate (pH 8.0), 0.5% NP-40, 5 mmol/L CaCl2] containing protease inhibitors. The cytosolic fraction was obtained by centrifugation of the supernatant at 50,000 × g for 1 hour. Proteins were separated on 10% SDS-polyacrylamide gels, blotted onto polyvinylidene difluoride, and probed with appropriate antibodies.

Clonogenic assay. Melanoma cells infected with ad-PUMA (or ad-GFP) were seeded into 60-mm tissue culture plates at a density of 3,000 per plate. After 14 days, cells were fixed with 3.7% formalin and stained with 2% crystal violet, and the number of colonies was counted.

Sulforhodamine B cell survival assay. Melanoma cells were treated at 50% confluence with medium containing 5 to 50 μmol/L of Akt/PKB signaling inhibitor-2 (API-2) and/or ad-PUMA for 24 to 48 hours. Cell survival was determined by the sulforhodamine B (SRB) assay as described previously (21, 22).

Ad-PUMA treatment in vivo. Tumors were induced by injecting 1.5 × 10^7 melanoma cells s.c. into the right flanks of 12-week-old male severe combined immunodeficient (SCID) mice. When tumors were palpable, mice were randomized into two treatment groups. Tumors were injected intratumorally with 1 × 10^6 plaque-forming units (pfu) of either ad-PUMA or ad-GFP in a volume of 100 μL in a single pass using 30-gauge needles. Adenovirus treatments were given every 3 days until the end of the experiment. Tumor dimensions were measured using calipers. Tumor volumes were calculated according to the following formula: \( V = L \times W^2 \times \pi/6 \) (23). Experiments were stopped when the tumor burdens of ad-GFP control groups became excessive.

Ad-PUMA and API-2 combination treatment in vivo. Cells (1.0 × 10^7) were injected s.c. into the right flanks of 6-week-old male SCID mice. When tumors were palpable, mice were randomized into four treatment groups. Animals were treated with 1 × 10^5 pfu of ad-PUMA or ad-GFP delivered by intratumoral injection once every 3 days. API-2 was given daily by i.p. injection at a dose of 1 mg/kg/d (in 15% DMSO). Tumor dimensions were measured using calipers. Experiments were stopped when the tumor burdens of ad-GFP control groups became excessive.

Results and Discussion

Weak PUMA expression and strong Akt phosphorylation cooperatively reduce melanoma patient survival. We have previously shown using tissue microarray analysis that weak PUMA expression in melanoma tumors correlates with poor 5-year survival of melanoma patients (3). In a subsequent study, we reported that p-Akt expression in human melanoma is strongly associated with invasion and progression and that p-Akt levels inversely correlate with melanoma patient survival (17). Based on these results, we were interested in determining whether PUMA and p-Akt have a cooperative effect on melanoma survival rates. Because both aforementioned studies were carried out on the same tissue microarray, we were able to combine the two data sets for our present analysis. To assess the combined effect of weak PUMA expression and strong p-Akt expression on disease-specific survival, Kaplan-Meier survival curves were plotted for patients whose primary tumors exhibited (a) weak p-Akt and strong PUMA, (b) strong p-Akt or weak PUMA, and (c) strong p-Akt and weak PUMA expression. We found that patients with both weak PUMA expression and strong p-Akt expression in their primary tumor tissue had significantly worse 5-year survival than patients having either strong p-Akt or weak ad-PUMA alone (\( P = 0.0069 \), log-rank test; Fig. 1A). In a combined analysis of primary and metastatic tumors, the results were even more significant; patients exhibiting both weak PUMA and strong p-Akt expression in tumor tissue again had reduced 5-year survival (\( P = 0.0004 \), log-rank test; Fig. 1B). Strikingly, there were no deaths (within 5 years) among patients exhibiting both strong PUMA and weak p-Akt expression in primary tumor tissue (Fig. 1A). Our data suggest that aberrant expression of PUMA and p-Akt may cooperatively contribute to melanoma tumor progression, resulting in worse patient prognosis. Based on these results, we hypothesized that a two-pronged
strategy, one that simultaneously addresses weak PUMA expression and hyperphosphorylation of Akt, may prove effective in treating melanoma and improving patient prognosis. To induce PUMA expression in melanoma tissue, we used an adenoviral-based gene therapy approach.

**Ad-PUMA kills melanoma cells via rapid induction of mitochondrial-mediated apoptosis.** The ability of PUMA to induce cell death has been well documented in various human cancer cell lines, including colorectal (6, 7, 24), lung (6, 7), head and neck (25), osteosarcoma (7), and glioma (26). We have previously shown that adenoviral-mediated expression of PUMA causes massive death of melanoma cells within 72 hours of infection (3). Here, we show that ad-PUMA induces morphologic changes associated with apoptosis, notably chromatin fragmentation and the formation of apoptotic bodies. In both wild-type p53 (MMRU and MMAN) and mutant p53 (Sk-mel-110 and MeWo) melanoma cell lines, infection with ad-PUMA produced a large proportion (25-45%) of apoptotic cells (Fig. 2A). In three cell lines (MMRU, MMAN, and MeWo), 37% to 45% of cells exhibited apoptotic bodies after 48 hours of ad-PUMA infection. The number was slightly lower (25%) for the Sk-mel-110 cell line because it is the most susceptible to adenoviral infection (data not shown). Consequently, apoptosis was more efficiently induced in Sk-mel-110 and many apoptotic cells had detached before *in situ* Hoechst staining was done.

PUMA reportedly induces apoptosis through the intrinsic, mitochondrial-mediated pathway (6, 7). Activation of this pathway is characterized by translocation of cytosolic Bax to the mitochondrial membrane, release of Smac/DIABLO and cytochrome c from mitochondria, subsequent activation of caspase-3 and caspase-9, and, inevitably, cell death. To confirm that our ad-PUMA construct activates this pathway in melanoma cells, we examined the cytosolic and mitochondrial protein expression of ad-PUMA-infected MMRU cells. Cells infected with ad-PUMA for 0, 4, 8, or 12 hours were subjected to subcellular fractionation to separate cytosolic and mitochondrial proteins. Western blot analysis of the protein extracts showed that PUMA was strongly

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**Figure 2.** Ad-PUMA inhibits human melanoma cell survival via rapid induction of mitochondria-mediated apoptosis. A, left, Hoechst staining of MMRU cells infected with ad-GFP or ad-PUMA for 48 hours; right, percentage of cells exhibiting apoptotic bodies after 48 hours of ad-PUMA infection in two wild-type p53 (MMRU and MMAN) and two mutant p53 (Sk-mel-110 and MeWo) melanoma cell lines. B, Western blot analysis of MMRU cells infected with ad-PUMA for indicated times. C, left, clonogenic assay of MMRU cells infected with GFP or ad-PUMA. Cells were infected for 4 hours and then reseeded at low density. Right, percentage of colonies formed in two wild-type p53 (MMRU and MMAN) and two mutant p53 (Sk-mel-110 and MeWo) melanoma cell lines.
Our results indicate that ad-PUMA indeed inhibits melanoma tumor growth \textit{in vivo}. However, the growth-inhibitory effect observed \textit{in vivo} was not as strong as that seen in colony formation assays \textit{in vitro}. We speculate that two factors may contribute to this apparent discrepancy: (a) insufficient distribution of virus throughout tumors and (b) leakage of virus from tumor tissue. In our study, tumors were injected with adenovirus in a volume of 100 \( \mu \)L in a single pass using 30-gauge needles as described previously (27). Although a single intratumoral injection may be adequate for the delivery of replication-competent adenoviruses, which can "spread" from cell to cell, this administration method may not have sufficiently distributed our replication-deficient ad-PUMA construct throughout the tumor, especially as tumor volumes increased. Additionally, we suspect that some viruses may have "leaked" from tumors, reducing infection efficiency. To circumvent these technical problems, we propose that a conditionally replicating adenoviral vector under the control of a melanocytic tissue-specific promoter be used to improve the efficiency of PUMA gene delivery to melanoma tumor tissues.

\textbf{Ad-PUMA and API-2 cooperatively inhibit melanoma cell growth.} We next hypothesized that the inhibitory effects of ad-PUMA on melanoma cell growth could be enhanced by eliminating prominent cell survival and/or proliferation signals from melanoma cells. Based on our finding that p-Akt is associated with melanoma invasion, progression, and poor prognosis (17), we sought to determine whether combining p-Akt inhibition with ad-PUMA treatment would be of therapeutic value. Western blot analyses of the malignant melanoma cell lines MMRU and MMAN revealed that both cell lines strongly express activated Akt phosphorylated on Ser473 (Fig. 4A and B). To eliminate this survival/proliferation signal, we treated cells with the small-molecule Akt pathway inhibitor API-2. This compound specifically inhibits Akt phosphorylation and kinase activity without altering the activity of related kinases, such as PI3K, PKD1, protein kinase C, serum- and glucocorticoid-inducible kinase, protein kinase A, signal transducers and activators of transcription 3, extracellular signal-regulated kinase 1/2, or c-Jun NH2-terminal kinase (28). Treatment of melanoma cells with API-2 expressed in mitochondria at 8 hours after infection (Fig. 2B), showing the ability of ad-PUMA to rapidly induce gene expression. PUMA expression was accompanied by translocation of cytosolic Bax to the mitochondria, release of Smac and cytochrome \( c \) from mitochondria into the cytosol, and cleavage of procaspase-3 and procaspase-9 (Fig. 2B). These data confirm that ad-PUMA induces melanoma cell death \textit{via} mitochondrial-mediated apoptosis.

To determine whether ad-PUMA suppresses long-term survival of melanoma cells, clonogenic assays were done. We found that infection with ad-PUMA significantly impaired the abilities of both wild-type p53 (MMRU and MMAN) and mutant p53 (Sk-mel-110 and MeWo) melanoma cells to form colonies over a 14-day period (Fig. 2C). Ad-PUMA infection inhibited colony formation by 80% to 90% compared with an ad-GFP control virus.

Ad-PUMA inhibits growth of melanoma tumor xenografts. In light of the potent cell killing ability of ad-PUMA \textit{in vitro}, we hypothesized that ad-PUMA would be highly effective at inhibiting melanoma tumor growth \textit{in vivo}. Melanoma tumors are notoriously resistant to both chemotherapy and radiotherapy and thus are difficult to treat. Ad-PUMA gene therapy may be an effective alternative to conventional treatments that rely on cytotoxic DNA-damaging agents to induce apoptosis. To test the efficacy of ad-PUMA \textit{in vivo}, we used a SCID mouse model, in which tumors were induced by s.c. injection of MMRU (\( n = 6 \)) or MMAN (\( n = 10 \)) human melanoma cells. Once tumors were palpable, mice were randomly assigned to either ad-PUMA or ad-GFP treatment groups. Virus (1 × 10\(^8\) pfu) was given by intratumoral injection every 3 days. As shown in Fig. 3A and B, melanoma tumors injected with ad-PUMA grew more slowly than those injected with ad-GFP-negative control virus. After 60 days of treatment, MMRU tumors treated with ad-PUMA were 60% smaller than controls (\( P < 0.01; \) Fig. 3C). MMAN tumor growth was also inhibited; ad-PUMA-treated tumors were 40% smaller than controls after 52 days of treatment (\( P < 0.05; \) Fig. 3D). The 20% difference in treatment efficiency between cell lines may be attributed to the fact that MMAN cells exhibit lower susceptibility to adenoviral infection than MMRU (data not shown).
inhibited their Akt phosphorylation levels (Fig. 4A and B). Furthermore, API-2 inhibited MMRU and MMAN cell survival in a dose- and time-dependent manner (Fig. 4C and D). At the lowest dose tested (5 μmol/L), 48 hours of API-2 treatment inhibited MMRU and MMAN cell survival by 35% and 45%, respectively, compared with treatment with vehicle alone.

To determine whether API-2 and ad-PUMA could act synergistically to inhibit melanoma cell survival, MMRU cells were infected with ad-PUMA (or ad-GFP) and then treated with 20 μmol/L API-2 for 48 hours. The cells were analyzed by SRB assay to determine the percentage of surviving cells. We found that API-2 treatment enhanced ad-PUMA-mediated cell death in an additive manner compared with treatment with the vehicle DMSO (Fig. 5A). Overall, these data are consistent with the hypothesis that weak PUMA expression combined with strong p-Akt expression promotes melanoma cell survival. It is not unexpected that combining PUMA and p-Akt in Melanoma Patient Survival.
ad-PUMA and API-2 treatments had an additive, but not synergistic, effect on cell survival because PUMA and Akt function in two distinct signaling pathways and are not known to interact with each other. To determine whether API-2 could augment ad-PUMA-induced apoptosis of melanoma cells, we analyzed cells by flow cytometry following combination treatment with ad-PUMA and API-2. Although other groups have reported that Akt inhibition induces apoptosis in various mammalian cell lines (28–31), API-2 did not seem to induce a significant degree of apoptosis in the melanoma cell lines we tested (data not shown). This suggests that API-2 may function primarily to inhibit growth rather than promote apoptosis in human melanoma cells.

**Ad-PUMA and API-2 cooperatively inhibit growth of melanoma tumor xenografts.** To determine whether API-2 could enhance ad-PUMA-mediated inhibition of melanoma tumor growth, we used a SCID mouse tumor xenograft model. Animals bearing MMRI tumors (N = 20) were randomized into four groups and treated with either (a) ad-PUMA and API-2, (b) ad-PUMA and DMSO (vehicle), (c) ad-GFP (control virus) and API-2, or (d) ad-GFP and DMSO as described in Materials and Methods. As expected, ad-PUMA significantly inhibited MMRI tumor growth (Fig. 5C and D). After 54 days of treatment (18 viral injections), tumors treated with ad-PUMA were 57% smaller than those treated with ad-GFP (P < 0.005, t test). API-2 also dramatically slowed MMRI tumor growth rates; after 54 days of treatment, tumors dosed with API-2 were 46% smaller than those treated with DMSO vehicle (P = 0.005, t test). When API-2 and ad-PUMA treatments were combined, however, a marked enhancement of growth inhibition was observed; 54 days of the combination treatment (ad-PUMA and API-2) yielded tumors that were 81% smaller than the negative control group (ad-GFP and DMSO; P < 0.001, t test). Our data indicate that inhibiting p-Akt expression clearly augments the inhibitory effect of ad-PUMA on melanoma growth in vivo. This suggests that combining a p-Akt inhibitor drug with ad-PUMA gene therapy may be of significant therapeutic value in treating malignant melanoma.

In summary, we have presented the first evidence that PUMA and Akt may together play an important role in melanoma progression and patient survival. Our data perhaps explain why melanoma is both highly invasive and resistant to chemotherapy; an inability to express PUMA could severely impair p53-dependent apoptosis, while at the same time hyperphosphorylation of Akt may promote aggressive tumor growth. Our novel finding that combined dysregulation of PUMA and p-Akt expression results in poorer prognosis than aberrant expression of either protein alone provides a strong rationale for dual therapeutic targeting of PUMA and Akt in melanoma. In further support of this principle, we have shown that p-Akt inhibition enhances the inhibitory effect of ad-PUMA on melanoma cell survival in vitro and melanoma tumor growth in vivo. Thus, by reversing the "weak PUMA and strong p-Akt" expression pattern in melanoma tissue, we can effectively induce cell death and suppress proliferation. These results, taken together, form the basis for a novel and potentially more effective therapeutic strategy in the treatment of malignant melanoma.

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**References**

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