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BIOLOGY CONTRIBUTION

THE VITAMIN-LIKE DIETARY SUPPLEMENT PARA-AMINOBENZOIC ACID ENHANCES THE ANTITUMOR ACTIVITY OF IONIZING RADIATION

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Purpose: To determine whether para-aminobenzoic acid (PABA) alters the sensitivity of tumor cells to ionizing radiation *in vitro* and *in vivo*.

Methods and Materials: Cellular proliferation was assessed by WST-1 assays. The effects of PABA and radiation on tumor growth were examined with chick embryo and murine models. Real-time reverse transcriptase–polymerase chain reaction and Western blotting were used to quantify $p21^{CIP1}$ and CDC25A levels.

Results: Para-aminobenzoic acid enhanced (by 50%) the growth inhibitory activity of radiation on B16F10 cells, whereas it had no effect on melanocytes. Para-aminobenzoic acid enhanced (50–80%) the antitumor activity of radiation on B16F10 and 4T1 tumors *in vivo*. The combination of PABA and radiation therapy increased tumor apoptosis. Treatment of tumor cells with PABA increased expression of CDC25A and decreased levels of $p21^{CIP1}$.

Conclusions: Our findings suggest that PABA might represent a compound capable of enhancing the antitumor activity of ionizing radiation by a mechanism involving altered expression of proteins known to regulate cell cycle arrest. © 2006 Elsevier Inc.

Para-aminobenzoic acid, $P21^{CIP1}$, CDC25A, Apoptosis, Tumor growth.

INTRODUCTION

Although significant progress has been made in our understanding of the molecular mechanisms that contribute to tumor growth and metastasis, these important accomplishments have had limited impact on the efficacy of treatments for malignant tumors, such as melanoma and breast carcinoma. Radiotherapy is a commonly used treatment opinion for malignant human tumors. Its efficacy often depends on inherent or acquired resistance to its effects (1–6). The molecular mechanisms that contribute to the sensitivity of tumors to ionizing radiation have been extensively studied, and important new insight into these processes is accumulating (7–11). Common determinants of tumor radiosensitivity include the degree of oxygenation/hypoxia and alterations in expression and function of DNA repair proteins and cell cycle checkpoint proteins (1–11). Additional changes that are thought to regulate radiosensitivity include alterations in expression and function of cell adhesion receptors, changes in the composition and integrity of the extracellular matrix, and altered apoptotic signaling mechanisms (1–11).

Given the established role of radiotherapy in the treatment of malignant tumors, a strategy that could enhance the sensitivity of tumors but not normal tissues to ionizing radiation would be of significant therapeutic benefit. Ionizing radiation is known to cause DNA damage, including both single- and double-strand breaks (12–15). In response to DNA damage, cells can activate a variety of kinases, including ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and Rad3-related (ATR), which can lead to activation of checkpoint proteins, such as Chk2, which then can phosphorylate CDC25A, marking it for proteosome-mediated degradation (16–18). Degradation of CDC25A, as well as elevated levels of cyclin-dependent kinase (CDK) inhibitors, such as $p21^{CIP1}$, can ultimately lead to cell cycle arrest. This critical break in cell cycle progression is thought to allow time for the cell to repair DNA damage or, if the damage is too excessive, to initiate signaling pathways that

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ultimately lead to cell death by mitotic catastrophe and apoptosis (16–18). Several recent studies have suggested that disruption of normal cell cycle checkpoints leads to inhibition of cell cycle arrest and enhances the sensitivity of tumor cells to ionizing radiation (19–24). Interestingly, tumors overexpressing cell cycle regulatory proteins, such as members of the CDC25 phosphatase family, or cells that exhibit decreased levels of CDK inhibitors, such as $p21^{CIP1}$, are associated with increased sensitivity to ionizing radiation (25–27).

Clinical and experimental evidence has suggested that melanogenesis or the biochemical synthesis of melanin might also contribute to radioresistance in melanoma, given that highly pigmented melanomas often seem to be more resistant to radiotherapy than amelanotic melanoma (28–30). However, this concept still remains to be confirmed because other studies failed to show a correlation (31). In this regard, we recently made the observation that culturing melanotic B16F10 melanoma cells in basal media (Roswell Park Memorial Institute medium [RPMI]) containing elevated levels of the vitamin-like dietary supplement para-aminobenzoic acid (PABA) resulted in dramatic alterations of a number of phenotypic characteristics, including altered melanin production. Given this observation, in conjunction with the possibility that melanogenesis might impact radiosensitivity, we examined the effects of PABA on the sensitivity of tumor cells to ionizing radiation. Here we provide evidence for the first time that the vitamin-like dietary supplement PABA enhances the sensitivity of tumor cells to ionizing radiation and that this enhanced radiosensitivity is associated with increased apoptosis, elevated expression of CDC25A, and downregulation of $p21^{CIP1}$. Importantly, PABA treatment of animals bearing either melanotic melanoma or invasive mammary carcinomas resulted in a dramatic increase in the antitumor efficacy of ionizing radiation. These surprising results suggest that the ability of PABA to enhance antitumor activity of ionizing radiation was not restricted to melanotic tumors and might represent an effective new approach to selectively enhance the effects of tumor radiotherapy.

METHODS AND MATERIALS

Cells and cell culture

Murine tumor cell lines B16F10 melanoma and 4T1 breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Immortalized murine melanocytes (Melan-a) were derived from C57BL/6J black mice and were a kind gift from Dr. Seth J. Orlow (Department of Dermatology, New York University School of Medicine). All tumor cell lines were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY), high glucose supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin at 37°C with 5% CO_2. Immortalized Melan-a cells were grown in DMEM containing phorbol-12-myristate 13-acetate (0.3 μmol/L), high glucose supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin at 37°C with 5% CO_2.

Antibodies and reagents

Rabbit polyclonal antibody (C-19) directed to $p21^{CIP1}$ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antiactin antibody (MAB150IR) was purchased from Chemicon (Temecula, CA). Antimouse immunoglobulin G or antirabbit horseradish peroxidase–linked antibody was obtained from Biosource International (Camarillo, CA). Para-aminobenzoic acid was purchased from Sigma (St. Louis, MO). Stock solutions of PABA (4 mg/mL) were prepared in DMEM or phosphate-buffered saline (PBS), pH 7.0, and stored at 4°C. Optimum cutting temperature (OCT) embedding compound was from VWR Scientific Products (Bridgeport, NJ). Acetone, methanol, and ethanol were all obtained from Sigma. ApopTag apoptosis detection kit was obtained from Chemicon.

In vitro *proliferation assays*

B16F10 and Melan-a (subconfluent and proliferating) cells were cultured with or without PABA (100 μg/mL) for 7 days. Cells were washed, trypsinized, counted, and plated at 1,000 cells for B16F10 and 10,000 cells for Melan-a per well in 96-well plates in DMEM containing 1% fetal bovine serum. For irradiation experiments, cells were irradiated with a Philips 6-MV linear accelerator (Philips Medical Systems, Bothell, WA) with full dose buildup. One hour after plating, cells were given a single dose of ionizing radiation (2 Gy for B16F10 and 10 Gy for Melan-a cells). Cellular proliferation was measured with a WST-1 tetrazolium salt cleavage assay kit (Chemicon), as has been previously described (32). In control experiments, Melan-a cells were treated with 0.01% sodium azide as a control. Cell proliferation was monitored with a microplate reader at a wavelength of 490 nm. Experiments were performed in triplicate and repeated twice with similar results.

Chick embryo tumor growth assays

The chick embryo tumor growth assay was performed as previously described with some modifications (33). Briefly, 10-day-old chick embryos (Charles River Laboratories, Wilmington, MA) were prepared by separating the chorioallantoic membrane (CAM) from the shell membrane (33). Single-cell suspensions of B16F10 melanoma cells (1.5×10^5) or 4T1 mammary carcinoma cells (5.0×10^6) cultured in the presence or absence of PABA (100 μg/mL) for 7 days were applied to the CAMs in a total volume of 40 μL of DMEM. Twenty-four hours later, the embryos were either left untreated or were irradiated with a single-fraction dose of ionizing radiation (5 Gy). Radiation was administered with a Philips 6-MV linear accelerator with full dose buildup at the tumor. A low dose rate of 0.024 cGy/min was achieved through a combination of a transmission block and an extended treatment distance. Embryos were oriented with the tumors placed at d-Max, using dose buildup plates of solid water (1.5 cm). The embryos were allowed to incubate for 6 days, with the resulting tumors resected and wet weights determined. Experiments were performed two to three times with 6–8 embryos per condition.

Murine tumor growth assay

BALB/C mice (Taconic, Hudson, NY), aged 4–6 weeks, were injected subcutaneously with 100 μL of 4T1 mammary carcinoma cells (5×10^4) or B16F10 melanoma cells (1×10^6) in PBS. Three days after tumor cell implantation, mice (harboring tumors of similar mean size) were either untreated or treated intraperitoneally (500 μg/day) daily with PABA (15 days for B16F10 and 28 days for 4T1). At 7 days after implantation, mice were either

untreated or irradiated with a single-fraction dose of 10 Gy of ionizing radiation. Irradiation was delivered with a Co^{60} source to the tumor of anesthetized mice, with lead shields to protect the rest of the body. At the end of the treatment periods (15 days for B16F10 and 28 days for 4T1), tumors were measured with calipers and the volume estimated with the formula $V = L^2 \times W/2$, where V is equal to the volume, L is equal to the length, and W is equal to the width. Experiments ($n = 6$ per group) were performed two to three times with similar results.

Immunohistologic analysis of tumor tissue

Tumors from each experimental condition ($n = 3$) grown in chick embryos were embedded in OCT embedding compound and snap frozen in liquid nitrogen. In brief, 4-μm frozen sections of the tumor tissue were fixed in 50% methanol, 50% acetone for 30 s and then stored at −80°C until use. For apoptosis analysis and quantification, sections from three individual tumors from each experimental condition were washed in PBS and then in 70% ethanol, followed by three washes with PBS, and blocked with 2.5% bovine serum albumin. All tissues were analyzed for tissue integrity by hematoxylin and eosin staining, as previously described (34). Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was performed with the ApopTag apoptosis detection kit, according to the manufacturer's instructions (Chemicon). Tumor sections were analyzed and photographed with a BX20 Olympus compound microscope (Olympus, Tokyo, Japan) fitted with a charge-coupled device digital camera. The relative levels of apoptosis were estimated by laser scanning image analysis (35) of two to three independent microscopic fields from each tumor sample from each condition. Briefly, stained tissue sections were scanned with a Kodak ID imaging system (Kodak, Rochester, NY), and the pixel density of positive staining regions (×200 microscopic fields, total of six per condition) per tumor specimen was quantified with Kodak ID version 4.0 image analysis software, as has been described previously (35).

Real-time polymerase chain reaction analysis

Tumor cells (subconfluent and proliferating) were cultured in the presence or absence of PABA (100 μg/mL) for 7 days. Total RNA was isolated with the RNeasy Kit (Qiagen, Valencia, CA), reverse transcribed to complementary DNA with random primers, using the reverse transcription system (Promega, Madison, WI) as per the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with the RedTaq PCR ready mix from Sigma. For reverse transcriptase (RT)-PCR, primer sequences for mouse CDC25A were forward 5′GAA GTT CCG CAC CAA GAG C3′ and reverse 5′GTT AAG AGT CAT CCA CGA GG3′. For control glyceraldehyde-3-phosphate dehydrogenase primers, forward 5′GAG GGG CCA TCC ACA GTC TTC3′ and reverse 5′CAT CAC CAT CTT CCA GGA GCG3′ were used. Real-time analysis, quantitative RT-PCR was carried out with an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Beta-2-microglobulin (B2M) gene was used for normalization. Primer pairs used for B2M included forward 5′ACA CTG AAT TCA CCC CCA CT3′ and reverse 5′CAC ATG TCT CGA TCC CAG TA3′, CDC25A forward 5′CAG CTC GGA TGC TAT CTT CA3′ and reverse 5′CGC CAT CCA GAA GGT CTA TG3′, and $p21^{CIP1}$ forward 5′CTT GTC GCT GTC TTG CAC-TC3′ and reverse 5′AAT CTG TCA GGC TGG TCT GC3′. Fold induction was calculated with the formula $2^{-\Delta\Delta}C_T$, where $\Delta\Delta C_T$ = target gene C_T − $B2M$ C_T (36). In turn, the $\Delta\Delta C_T$ is based on the mean ΔC_T, or the C_T difference between the treated and control cells. The C_T value is determined as the cycle at which the fluorescence signal emitted is significantly above background levels and is inversely proportional to the initial template copy number. Amplification products used through Sybergreen detection were initially checked by electrophoresis on ethidium bromide–stained agarose gels. The estimated size of the amplified products matched the calculated size for transcript by visual inspection.

Western blot analysis

Western blot analysis was performed as previously described (37). Briefly, tumor cells were cultured in the presence or absence of PABA (100 μg/mL) for 7 days and lysed in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mmol/L Tris-HCl, pH 8.0) containing protease and phosphatase inhibitors. Protein concentrations were determined with the Bicinchoninic acid (BCA) method (Bio-Rad Laboratories, Hercules, CA), and equal amounts (15–30 μg) of protein were separated on a 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gradient gel and transferred to nitrocellulose membranes. Membranes were blocked overnight in PBS Tween 20 containing 3% (vol/wt) nonfat dry milk and probed with either anti-$p21^{CIP1}$, anti-CDC25A, or for controls, anti-β1 integrin or anti-Actin antibodies at a 1:50 dilution for 1 h, followed by secondary antibody diluted 1:1,000. Proteins were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Statistical analysis was performed with the InStat statistical program for Macintosh computers. Data were analyzed for statistical significance with unpaired Student's t-tests. P values <0.05 were considered significant.

RESULTS

PABA enhances the growth inhibitory activity of ionizing radiation in vitro

Our previous studies have suggested that culturing malignant B16F10 melanoma cells in distinct types of basal growth media (RPMI and DMEM) dramatically alters several phenotypic characteristics of these tumor cells. In particular, the presence of elevated levels of the vitamin-like compound PABA within culture medium was shown to significantly reduce melanin levels within B16F10, causing a dramatic reduction in pigmentation (data not shown). Interestingly, clinical and experimental studies have suggested that highly melanotic melanomas might be more resistant to ionizing radiation than amelanotic melanomas (28–30). However, these observations remain controversial because other studies failed to confirm these findings (31).

Given the dramatic reduction in melanin observed in B16F10 melanoma cells cultured in the presence of PABA, we examined the impact of PABA on the growth inhibitory activity of ionizing radiation on malignant tumor cells, using the WST-1 tetrazolium salt cleavage assay, which has been previously used to quantify the effects of ionizing radiation on tumor cell growth (32). To facilitate these studies, im-

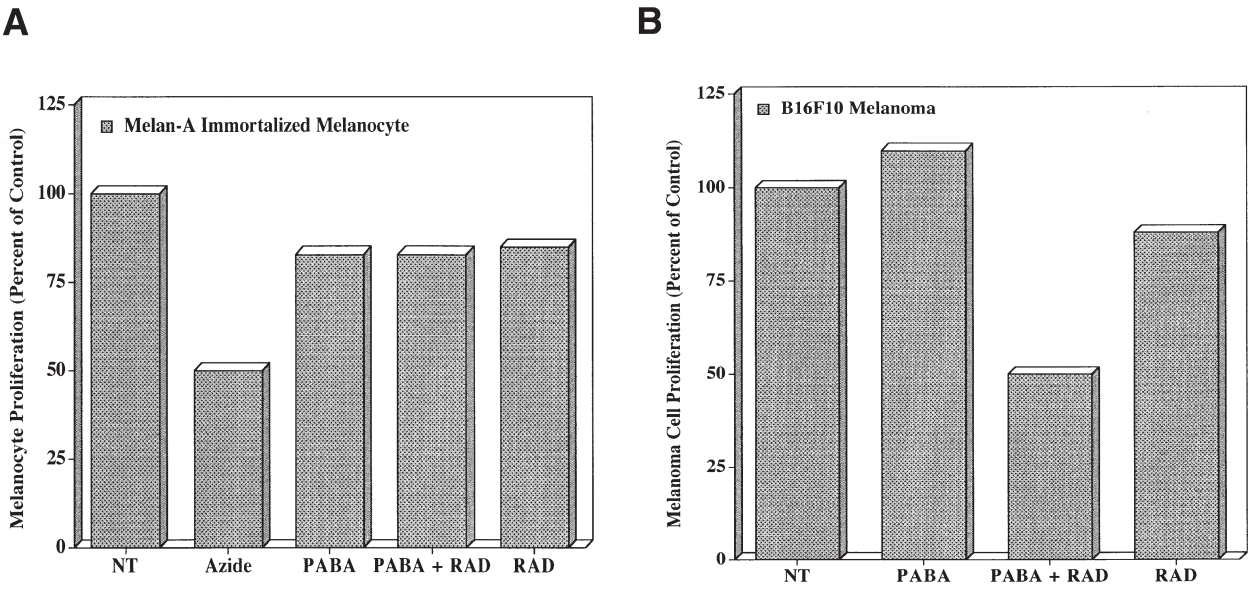

Fig. 1. Para-aminobenzoic acid (PABA) enhances the growth inhibitory activity of ionizing radiation on tumor cells. Immortalized melanocytes (Melan-a) (A) and malignant tumor cells (B16F10) (B) cultured in the presence or absence of PABA (100 μg/mL) for 7 days were harvested and resuspended in low serum (2.0%) medium. Cells were allowed to attach for 2 hours, then were either left untreated or were treated with sodium azide or with a single-fraction dose of radiation (2 Gy for B16F10 melanoma or 10 Gy for Melan-a melanocytes). Proliferation was measured with a WST-1 kit tetrazolium salt cleavage kit. (A) Quantification of melanocyte (Melan-a) proliferation in the presence or absence of PABA and with and without radiation (RAD). (B) B16F10 melanoma cell proliferation in the presence or absence of PABA and with and without radiation. Data bars indicate cell proliferation, represented as percent of control.

mortalized nontumorigenic melanocytes (Melan-a) or malignant melanotic B16F10 melanoma cells were cultured in the presence or absence of PABA (100 μg/mL) for 7 days then treated with a single suboptimal dose of ionizing radiation. As shown in Fig. 1A, neither PABA alone, radiation (10 Gy) alone, nor a combination of PABA and radiation (10 Gy) had any significant effects on nontumorigenic melanocytes. In contrast, sodium azide inhibited growth by approximately 50% as compared with control. In similar studies, PABA alone and a suboptimal dose (2 Gy) of ionizing radiation alone also had no significant effect on B16F10 melanoma cell growth (Fig. 1B). In contrast, a combination of PABA and ionizing radiation significantly inhibited B16F10 melanoma cell growth, by approximately 50%, as compared with controls. These finding are consistent with the possibility that PABA might enhance the growth inhibitory activity of ionizing radiation on malignant tumor cells *in vitro*.

PABA enhances the antitumor activity of ionizing radiation in vivo

Given the ability of PABA to enhance the growth inhibitory activity of ionizing radiation *in vitro*, we examined whether PABA could also impact the ability of radiation to inhibit tumor growth *in vivo*. To facilitate these studies we used the chick embryo tumor growth model (33, 37). Malignant B16F10 melanoma cells were either left untreated or were treated with PABA for 7 days and implanted on the CAM of 10-day old chick embryos, as described previously (37). After implantation, the embryos were either left untreated or were treated with a single dose of ionizing radiation (5 Gy), and 7 days later the resulting tumors were harvested and wet weights measured. As shown in Fig. 2A, PABA treatment had no effect of B16F10 tumor growth as compared with controls, whereas ionizing radiation significantly inhibited tumor growth, by approximately 38% ($p < 0.05$). Importantly, a combination of PABA and radiation doubled (80% inhibition) the antitumor activity as compared with radiation alone ($p < 0.05$).

To determine whether the effects of PABA on the antitumor activity of radiation was restricted to melanotic tumors, such as B16F10 melanoma, similar studies were carried out with malignant mammary carcinoma cells (4T1) that lack melanin. As shown in Fig. 2B, PABA had little if any effect on 4T1 mammary carcinoma tumor growth as compared with control ($p > 0.05$). Moreover, a suboptimal dose of ionizing radiation (5 Gy) also had little effect on 4T1 tumor growth as compared with controls ($p > 0.05$). Importantly, a combination of PABA and radiation resulted in >50% inhibition ($p < 0.05$) of 4T1 mammary tumor growth. Taken together, these findings suggest that the ability of PABA to enhance the antitumor activity of radiation is not restricted to melanotic tumor cells. To confirm these findings in independent murine tumor models, B16F10 and 4T1 tumor cells were injected subcutaneously into mice. Mice were either untreated or treated daily with PABA (500 μg). After the establishment of growing subcutaneous tumors (Day 7), mice were next either left untreated or were irradiated with a single subop-

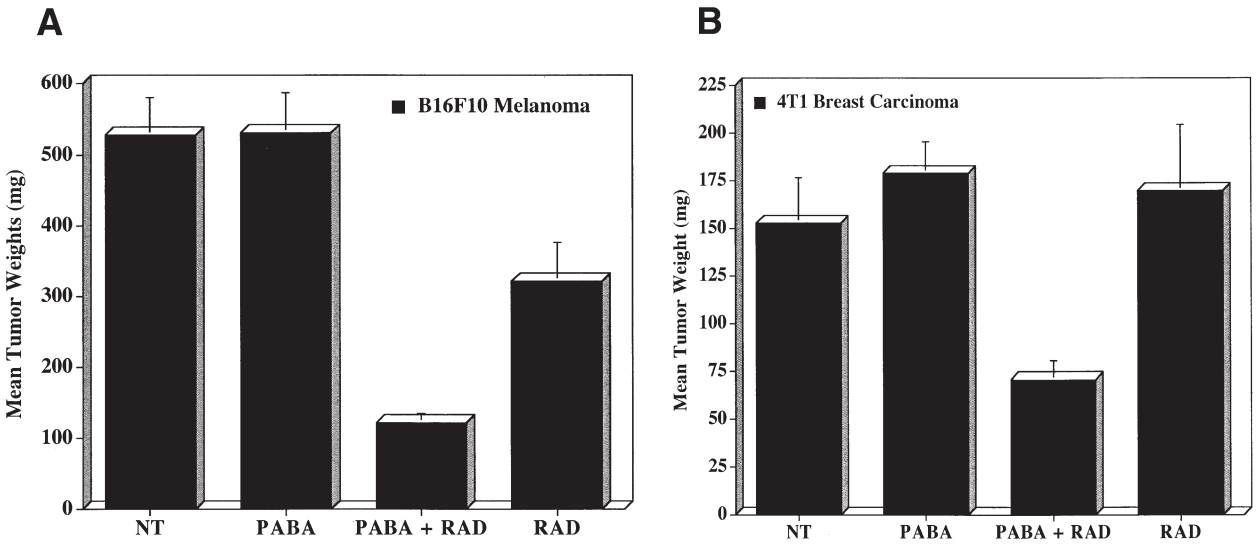

Fig. 2. Para-aminobenzoic acid (PABA) enhances the antitumor activity of ionizing radiation in the chick embryo model. B16F10 melanoma cells (A) and 4T1 mammary carcinoma cells (B) were treated with PABA (100 μg/mL) for 7 days, then seeded on the chorioallantoic membranes of 10-day-old chick embryos. Twenty-four hours later, the embryos were either left untreated or were irradiated with a single-fraction dose (5 Gy) of ionizing radiation (RAD). The tumors were allowed to grow for a total of 7 days. Tumors were resected, and wet weights were determined. Data bars represent the mean tumor weights ± standard errors from five to seven animals per condition. Experiments were completed two to three times with similar results.

timal dose (10 Gy) of ionizing radiation and tumor volumes measured 2 to 3 weeks later. As shown in Fig. 3, treatment of mice with PABA alone had minimal if any effects on either B16F10 melanoma (Fig. 3A) or 4T1 mammary carcinoma (Fig. 3B) tumor growth as compared with controls ($p > 0.05$). Moreover, the suboptimal dose of ionizing radiation also exhibited minimal effects on either B16F10 or 4T1 tumor growth ($p > 0.05$). In contrast, a combination of PABA and radiation significantly ($p < 0.05$) inhibited both B16F10 melanoma and 4T1 mammary carcinoma tumor

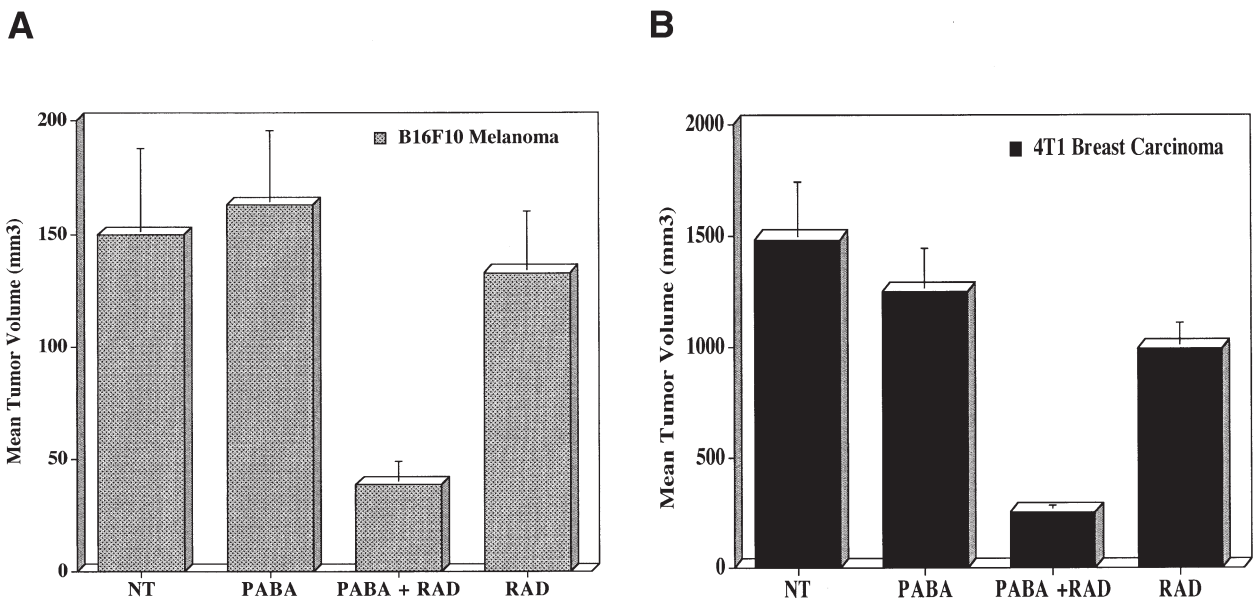

Fig. 3. Para-aminobenzoic acid (PABA) enhances the antitumor activity of ionizing radiation in a murine model. Balb/C mice were injected subcutaneously with B16F10 (A) or 4T1 (B) tumor cells. Three days later, the mice (harboring tumors of similar size) were either left untreated or were treated with PABA (500 μg /day) for 15 days (A) or 28 days (B). At Day 7, mice were either left untreated or were irradiated with a single-fraction dose (10 Gy) of ionizing radiation (RAD). On Days 15 (A) and 28 (B), tumor volumes were calculated with the formula $V = L^2 \times W/2$, where V = volume, L = length, and W = width. Data bars represent the mean tumor volumes from each experimental group ± standard errors. Six to eight animals were used for each group. Experiments were completed twice with similar results.

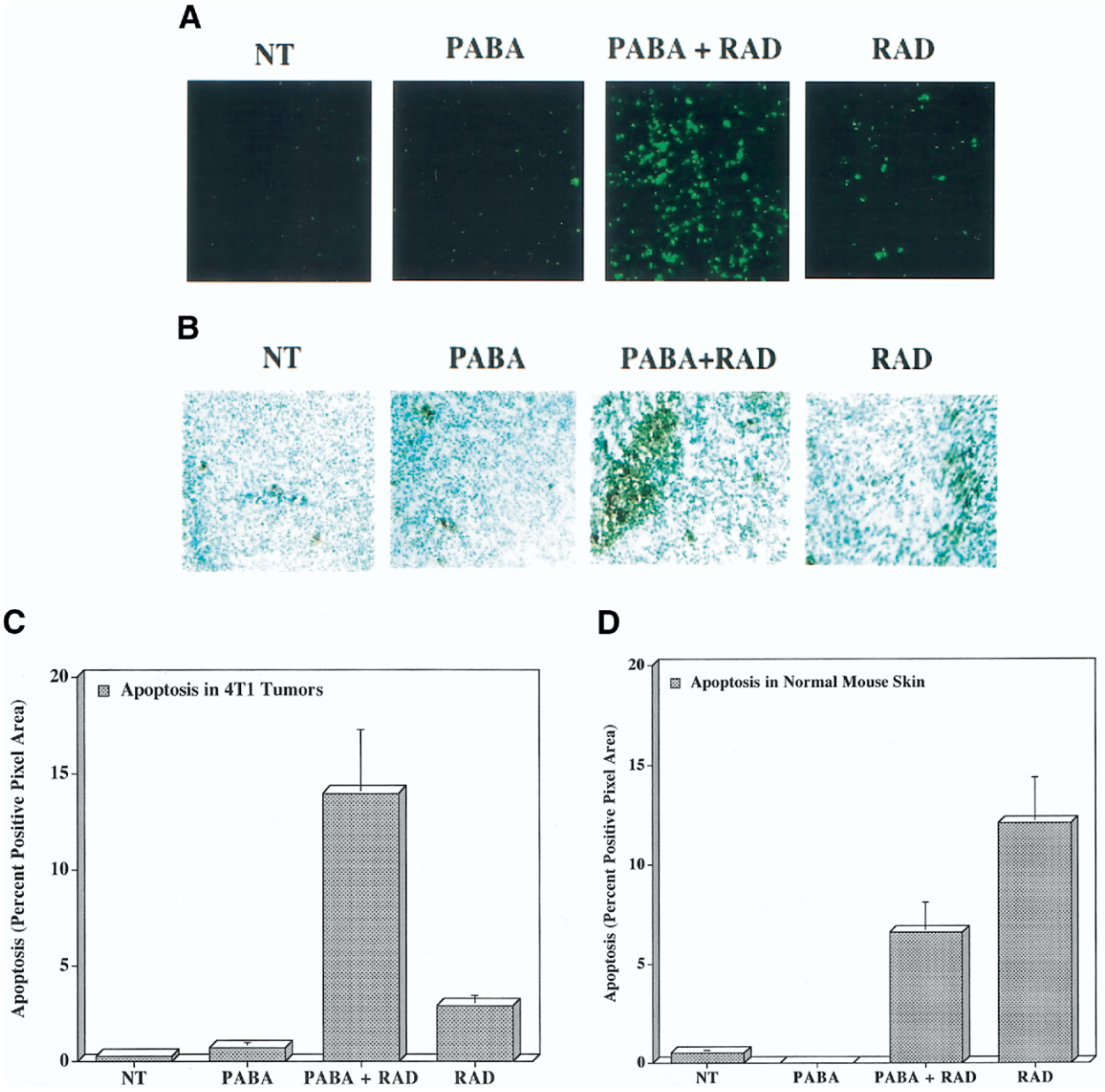

Fig. 4. Effects of para-aminobenzoic acid (PABA) on radiation-induced apoptosis *in vivo*. B16F10 melanoma (A) and 4T1 breast carcinoma tumors (B) were harvested from chick embryos, either untreated (NT) or after experimental treatments with PABA alone (100 μg/mL) (PABA), radiation alone (5 Gy) (RAD), or a combination of PABA and radiation (PABA + RAD). (A) Representative examples of B16F10 tumors from chick embryos (NT, PABA, PABA + RAD, or RAD), stained for apoptosis (green) with the ApopTag detection kit. (B) Representative examples of 4T1 mammary carcinoma tumors from chick embryos (NT, PABA, PABA + RAD, or RAD), stained for apoptosis (brown) with the ApopTag detection kit. (C). Quantification of the relative levels of apoptosis of 4T1 carcinoma tumors. Data bars represent the mean percent pixel area $\pm$ standard errors ($n = 6$ per condition). (D) Quantification of the relative levels of apoptosis within normal mouse skin after treatment of mice with radiation alone (5 Gy), PABA alone, or a combination of radiation (5 Gy) and PABA. Data bars represent the mean percent pixel area $\pm$ standard errors ($n = 6$ per condition).

growth by approximately 80% as compared with controls. Collectively, these findings confirm our previous results and suggest that PABA might significantly enhance the antitumor activity of ionizing radiation *in vivo*.

PABA enhances apoptosis in irradiated tumors in vivo

To begin to assess the potential mechanisms by which PABA might enhance the antitumor activity of radiation, we examined B16F10 and 4T1 mammary carcinoma tumors grown in chick embryos from each experimental condition. Malignant tumors were resected, washed, and snap frozen. Frozen tumor sections from each experimental condition were analyzed for the relative levels of apoptosis by TUNEL staining with the ApopTag apoptosis detection system (38, 39). As shown in Figs. 4A and B, tumors from control animals and animals treated with PABA alone and

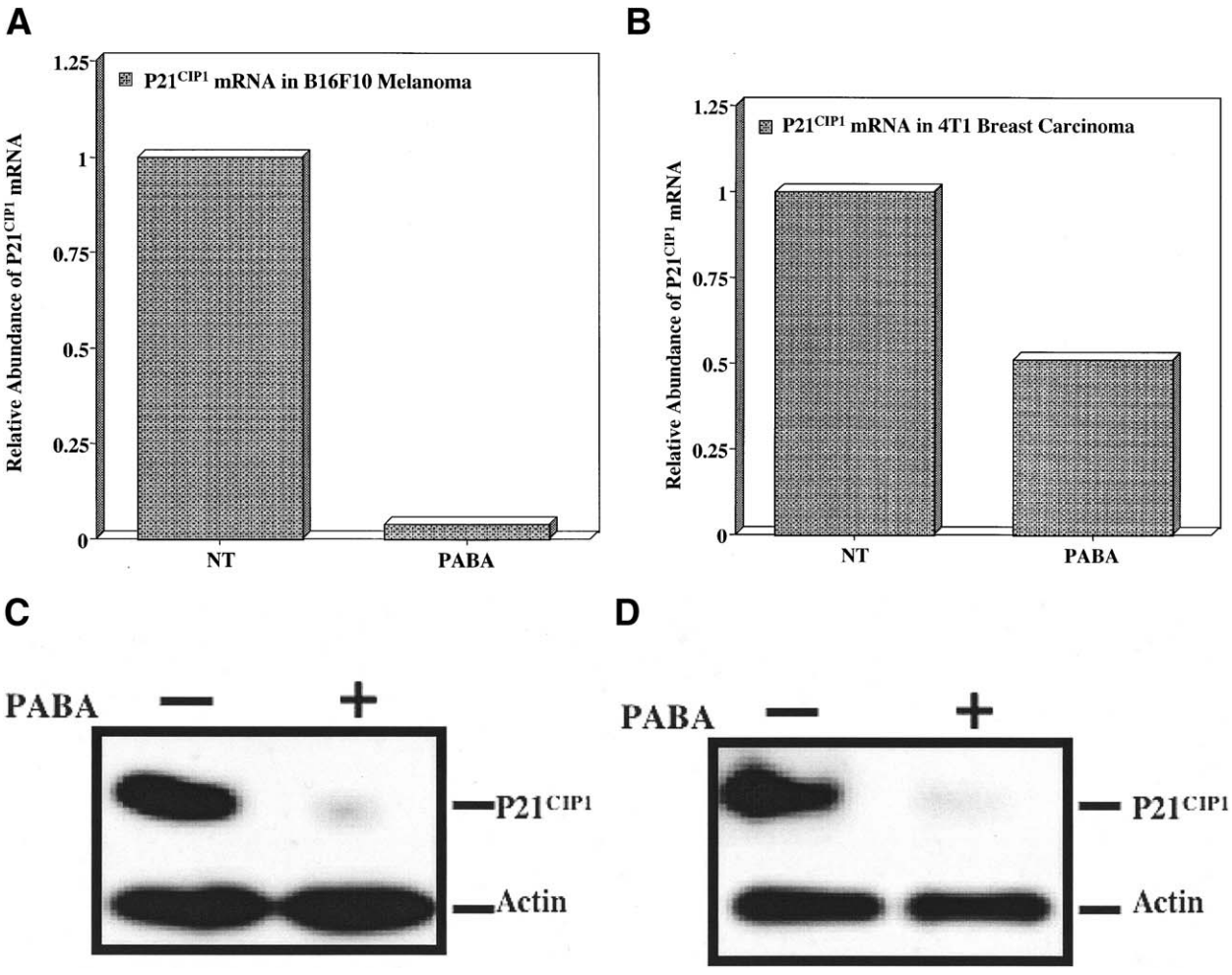

Fig. 5. Para-aminobenzoic acid (PABA) reduces the expression of the cyclin-dependent kinase inhibitor $p21^{CIP1}$. 4T1 mammary carcinoma cells (A, C) and B16F10 melanoma cells (B, D) were treated with PABA (100 μg/mL) for 7 days and either RNA isolated or whole cell lysates prepared. The relative levels of $p21^{CIP1}$ mRNA were examined by real-time quantitative reverse transcriptase–polymerase chain reaction (A, B) or Western blot (C, D). Experiments were completed at least twice with similar results. NT = untreated.

radiation alone exhibited random and scattered apoptotic staining cells. Interestingly, tumors from animals treated with a combination of PABA and radiation exhibited a distinct increase in the relative levels of apoptotic staining as compared with controls (35, 39). To quantify the relative changes in apoptotic staining, tumor sections from each experimental condition were analyzed for apoptosis, and quantification was carried out by laser scanning image analysis to estimate the relative staining by pixel density, as described previously (35, 36). The apoptotic staining was characterized by increased numbers of scattered TUNEL-positive cells, as well as by enhanced numbers of clustered TUNEL-positive foci within the tumor. Although apoptotic staining within the various 4T1 carcinoma tumor sections did exhibit variation, quantification of the relative levels of apoptosis within animals treated with a combination of PABA and radiation suggested a 70–80% increase in apoptosis as compared with animals treated with radiation alone, as measured by laser scanning image analysis (Fig. 4C). In similar studies, the relative levels of apoptotic staining were also examined within normal mouse skin from identically treated control animals that were not injected with tumor cells. As shown in Fig. 4D, little if any change in the relative levels of apoptosis within normal mouse skin was observed between nonirradiated, PABA-treated, and untreated mice. Importantly, whereas radiation alone caused a significant increase in the relative levels of apoptosis in the normal skin, a combination of PABA and radiation failed to enhance the apoptosis observed within normal mouse skin, suggesting a selective effect of PABA on enhancing tumor-associated apoptosis (Fig. 4D). Taken together, these findings suggest that PABA might enhance the antitumor activity of ionizing radiation in part by a mechanism involving increased apoptosis.

PABA alters the expression of the cyclin-dependent kinase inhibitor $P21^{CIP1}$

To gain a more in-depth understanding of the potential mechanisms by which PABA might enhance the sensitivity of tumor cells to ionizing radiation, an Affymetrix-based

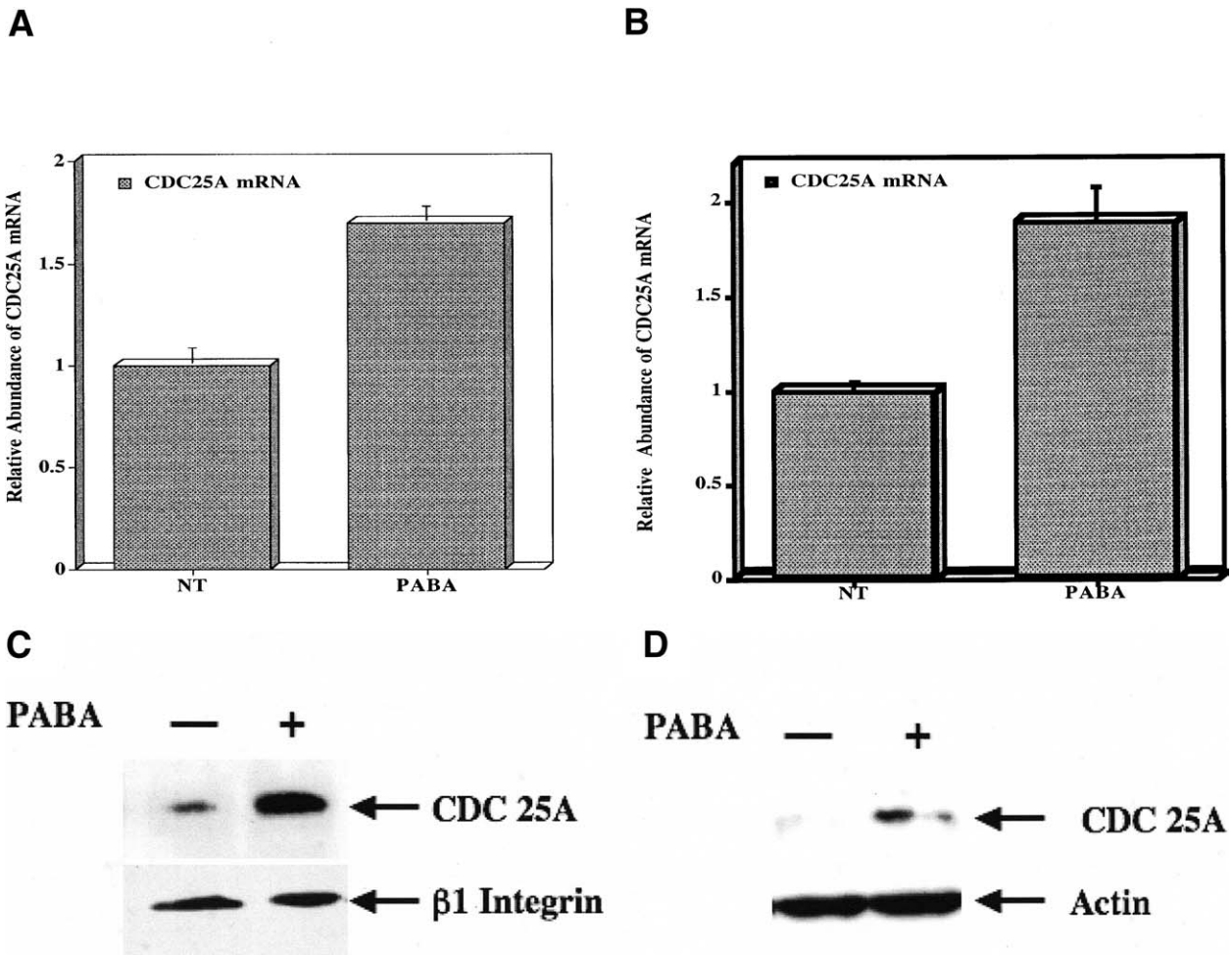

Fig. 6. Para-aminobenzoic acid (PABA) increases the expression of the cell cycle checkpoint control protein CDC25A. 4T1 mammary carcinoma cells (A, D) and B16F10 melanoma cells (B, C) were treated with PABA (100 μg/mL) for 7 days and either RNA isolated or whole cell lysates prepared. The relative levels of CDC25A mRNA were examined by real-time quantitative reverse transcriptase–polymerase chain reaction (A, B) or Western blot (C, D). Experiments were completed at least twice with similar results. NT = untreated.

differential microarray analysis (Affymetrix, Santa Clara, CA) was performed on untreated and PABA-treated tumor cells. A number of genes were shown to be differentially expressed after PABA treatment, and thus we chose to focus on those genes that might impact radiosensitivity. In this regard, numerous studies have indicated that regulation of cell cycle checkpoint proteins can impact the sensitivity of tumor cells to ionizing radiation (21–27). Interestingly, microarray analysis suggested that the CDK inhibitor $p21^{CIP1}$ was significantly downregulated in B16F10 melanoma cells after PABA treatment. To confirm the PABA-induced alterations in expression of $p21^{CIP1}$ at both the messenger RNA (mRNA) and protein levels, real-time quantitative RT-PCR and Western blot analysis was carried out. As shown in Fig. 5A, treatment of B16F10 melanoma cells with PABA resulted in >80% reduction in the relative levels of $p21^{CIP1}$ as compared with controls. Importantly, in similar studies carried out with 4T1 mammary carcinoma cells, an approximately 50% reduction in $p21^{CIP1}$ was also observed (Fig. 5B). To confirm the ability of PABA to reduce the expression of $p21^{CIP1}$ at the protein level, Western blotting was performed on both B16F10 melanoma cells (Fig. 5C) and 4T1 mammary carcinoma cells (Fig. 5D) treated in the presence or absence of PABA. As shown in Figs. 5C and 5D, treatment of either B16F10 or 4T1 tumor cells with PABA resulted in a >80% reduction in the levels of $p21^{CIP1}$, as quantified by laser scanning densitometry.

PABA alters the expression of the cell cycle checkpoint control protein CDC25A

Interestingly, previous studies have suggested that inhibiting cell cycle checkpoint controls in tumor cells can significantly enhance the sensitivity of these cells to ionizing radiation (21–27). In fact, studies have suggested that increased expression of members of the CDC25 family of checkpoint control proteins might enhance the sensitivity of tumors to radiation (20, 23, 24, 26). In this regard, microarray analysis suggested that CDC25A was upregulated by approximately twofold after PABA treatment in B16F10 melanoma cells (data not shown). To confirm the PABA-

induced alterations in expression of CDC25A, real-time RT-PCR was carried out. As shown in Fig. 6, treatment of 4T1 carcinoma cells (Fig. 6A) or B16F10 melanoma cells (Fig. 6B) with PABA resulted in an approximately 1.7-fold to nearly twofold increase in the relative levels of CDC25A mRNA as compared with controls. To confirm the ability of PABA to increase the expression of CDC25A at the protein level, Western blotting was performed on both B16F10 melanoma cells (Fig. 6C) and 4T1 mammary carcinoma cells (Fig. 6D) treated in the presence or absence of PABA. As shown in Fig. 6, treatment of either B16F10 (Fig. 6C) or 4T1 (Fig. 6D) tumor cells with PABA resulted in significant increases in the relative levels of CDC25A as compared with no treatment. Taken together, these results are in good agreement with our previous microarray analysis and suggest that whereas PABA might decrease $p21^{CIP1}$, it increases expression of CDC25A in these tumor cells.

DISCUSSION

Evidence from both basic research and clinical studies suggests that successful treatment of human tumors with ionizing radiation is hampered by inherent or acquired radioresistance, as well as by dose-dependent cellular damage to surrounding normal tissues (40–43). Recent studies have provided crucial molecular insight into the mechanisms by which tumor cells might acquire resistance and escape radiation-induced cell death (1–11). In fact, alterations in DNA repair, cell cycle checkpoint controls, genetic mutations leading to altered apoptotic signaling, and modulation of key cell cycle regulatory proteins, such as p53, $p21^{CIP1}$, and CDC25A, are among some of the important alterations contributing to radiosensitivity (1–15). Thus, a concerted effort has been undertaken to develop novel approaches to selectively enhance the sensitivity of tumor cells but not normal tissue to the damaging effects of ionizing radiation.

Recent work has indicated that ionizing radiation can facilitate proteosome-mediated degradation of CDC25A as well as enhance the expression of CDK inhibitors, such as $p21^{CIP1}$. These radiation-induced alterations promote cell cycle arrest and provide irradiated cells the critical time needed to effect repairs of radiation-induced DNA damage (44–49). Depending on the extent of DNA damage and the repertoire and function of DNA damage sensors, such as ATM and ATR, as well as repair proteins such as BRCA-1 and BRCA-2, crucial signaling pathways are activated to either initiate DNA repair or trigger cell death. Given these facts, selective approaches to modulate these critical molecular processes might provide effective strategies to enhance the efficacy of radiotherapy in treating malignant human tumors. However, because of the ubiquitous nature of these signaling cascades, targeting molecules within these critical pathways might be associated with certain toxicities. Despite the potential drawbacks of these types of approaches, novel compounds that disrupt the ATM pathways are currently being evaluated in human clinical trials (50).

Given the emerging molecular understanding of the coordinated roles that cell cycle modulators and DNA repair mechanisms play in response to ionizing radiation, a strategy that selectively disrupts these essential pathways in tumor cells but not normal cells would have obvious appeal for clinical use. Interestingly, recent studies have suggested that certain dietary supplements and botanical extracts, such as green tea and caffeine, might impact the sensitivity of tumors to ionizing radiation by a mechanism involving alterations in cell cycle control or induction of apoptosis in endothelial cells (51, 52). Although some progress has been made in gaining a molecular understanding of how these compounds function *in vitro*, few of these compounds have been translated into useful therapies in the clinic. In the present study, we provide the first evidence that the vitamin-like dietary supplement PABA, once a common component of sun screens, can potently enhance the growth inhibitory effects of ionizing radiation on malignant tumor cells *in vitro*, with little if any effect on nontumorigenic melanocytes. These observations are consistent with the possibility that PABA might enhance the antitumor activity of ionizing radiation while exhibiting minimal activity on normal tissues. Interestingly, our studies suggest that PABA failed to enhance the radiation-associated apoptosis in normal mouse skin *in vivo*. Importantly, treatment of tumor-bearing animals with PABA alone or a suboptimal dose of ionizing radiation had little effect on tumor growth. In contrast, a combination of PABA and a suboptimal dose of ionizing radiation led to a 50–80% inhibition of both malignant B16F10 melanoma and 4T1 mammary carcinoma tumor growth in two independent animal models. These observations indicate that the ability of PABA to significantly enhance radiosensitivity is not restricted to a single tumor type. Moreover, the PABA-enhanced, radiation-induced antitumor activity was accompanied by elevated levels of apoptotic staining throughout the tumor tissues. Importantly, PABA alone failed to enhance the radiation-associated apoptosis observed in the skin of normal mice. These findings are consistent with the possibility that PABA might have a selective effect on enhancing apoptosis in tumor tissue. However, additional studies on other normal tissues would be required to confirm significance of these findings in broader applications.

A number of recent studies have provided compelling evidence that $p21^{CIP1}$ plays a critical role in determining the ultimate fate of cells with DNA damage, as well as in regulating apoptosis (53–55). Moreover, recent evidence also suggests that $p21^{CIP1}$ plays a critical role in the ability of paclitaxel and HER2/Neu antagonists to induce apoptosis (53–55). Several additional studies confirm that reduced levels or functional inactivation of $p21^{CIP1}$ by altered subcellular localization are associated with enhanced radiosensitivity within several tumor types (53–55). In fact, using antisense strategies to reduce expression of $p21^{CIP1}$, investigators have demonstrated enhanced apoptosis and radiosensitivity in both mammary carcinomas and glioblastoma (53–55). To this end, we provide evidence that PABA dramatically reduces expression of $p21^{CIP1}$ at both the

mRNA and proteins levels. Our findings are consistent with the possibility that PABA might enhance tumor cell radiosensitivity in part by a mechanism involving reductions in the levels of $p21^{CIP}$. In addition, inhibition of cell cycle arrest by upregulation of the expression of CDC25A or prevention of CDC25A degradation has been shown to enhance radiosensitivity (18, 20–26). These important findings are also in good agreement with our observation and are also consistent with the possibility that the PABA-induced upregulation of CDC25A contributes to the enhanced tumor radiosensitivity observed in our studies. Although the mechanisms by which PABA alters $p21^{CIP1}$ and CDC25A expression are not completely understood, it would be interesting to speculate that PABA and/or one or more of its three major physiologic metabolites might modulate expression of specific transcription factors, such as c-Myc, which is known to enhance expression of CDC25A and repress expression of $p21^{CIP1}$. Further studies are currently under way in our laboratory to address these issues and to demonstrate a direct functional requirement for alterations of both CDC25A and $p21^{CIP1}$ for the ability of PABA to enhance tumor radiosensitivity.

Interestingly, PABA has been evaluated in several human clinical trails for the treatment of connective tissue diseases, such as arthritis and scleroderma, and has been shown to be associated with relatively few toxicities when administered at <12 g per day (56). Our studies, taken together with previously published findings concerning the relatively low toxicities associated with PABA administration in humans, combined with the roles of $p21^{CIP1}$ and CDC25A in regulating cell cycle arrest and tumor radiosensitization, suggest that PABA might represent a clinically useful modulator of tumor radiotherapy. This potential modulator of radiotherapy might selectively enhance the antitumor activity of ionizing radiation by a mechanism involving inhibition of cell cycle arrest within tumor cells harboring DNA damage and thereby potentiate cell death by mitotic catastrophe and/or apoptosis.

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