PTEN Gene Targeting Reveals a Radiation-Induced Size Checkpoint in Human Cancer Cells

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ABSTRACT

Following DNA damage, human cells arrest primarily in the G1 and G2 phases of the cell cycle. Here, we show that after irradiation, human cancer cells with targeted deletion of PTEN or naturally occurring PTEN mutations can exert G1 and G2 arrests but are unable to arrest in size. Pharmacological inhibition of phosphoinositol-3-kinase or mTOR in PTEN−/− cells restored the size arrest, whereas siRNA-mediated depletion of TSC2 in PTEN+/+ cells attenuated the size arrest. Radiation treatment potentiated Akt activation in PTEN−/− but not PTEN+/+ cells. Finally, abrogation of the size arrest via PTEN deletion conferred radiosensitivity both in vitro and in vivo. These results identify a new tumor suppressor gene-regulated, DNA damage-inducible arrest that occurs simultaneously with the G1 and G2 arrests but is genetically separable from them. We suggest that aberrant regulation of cell size during cell cycle arrest may be important in human cancer pathogenesis.

INTRODUCTION

A fundamental feature distinguishing cancer cells from normal cells is the inability to enforce cell cycle arrests. The genetic and biochemical mechanisms that enforce these arrests are known as cell cycle checkpoints and are encoded by several of the most commonly mutated tumor suppressor genes, such as p53. Numerous stimuli can induce checkpoint-dependent cell cycle arrest, including DNA damage, agents that disrupt the mitotic spindle, expression of oncogenes, and senescence. Radiation is perhaps the most well-studied inducer of cell cycle arrest (1). Irradiation of human cells is thought to result primarily in two arrests: in the G1 and G2 phases of the cell cycle.

The PTEN tumor suppressor gene is mutated in a wide variety of human cancers, most notably glioblastomas, endometrial carcinomas, melanomas, and advanced prostate adenocarcinomas (2, 3). Germline mutations of PTEN are responsible for several rare cancer predisposition syndromes, including Cowden disease, Lhermitte-Duclos disease, Bannayan-Zonana syndrome, and Proteus syndrome (4). Affected individuals frequently present with benign outgrowths known as hamartomas in multiple organ systems.

PTEN functions primarily as a lipid phosphatase and dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3), the second messenger produced by phosphoinositide-3-kinase (PI3K; ref. 5). In doing so, PTEN negatively regulates the activity of the serine/threonine protein kinase, Akt (6). Consequently, PTEN inactivation promotes cell survival and proliferation by deregulating the activity of a number of proteins, such as Bad, a pro-apoptotic Bcl-2 family member; TSC2, a known tumor suppressor; mTOR, a regulator of mRNA translation; and HIF-1α, a transcription factor subunit (7–10).

However, the phenotypic effects of PTEN inactivation in human cancer cells remain poorly defined. There is evidence that PTEN can modulate the G1 and G2 arrests, possibly through Akt or a direct interaction with p53 (11–15). Other reports suggest that PTEN may regulate cell migration (16). Most recently, PTEN has been associated with cell size control (17). This observation was first noted in mosaic eyes of Drosophila melanogaster. In Mus musculus, selective inactivation of PTEN in neurons and cardiomyocytes produced cells of increased size (18–20).

In an effort to create a suitable experimental system based on human cancer cells for the study of PTEN, we used somatic cell gene targeting to create isogenic sets of human cancer cells that differ only in their PTEN status. Phenotypic analyses of these cells and of several genetically unmodified human cancer cell lines with naturally occurring PTEN mutations revealed that PTEN controls a novel DNA damage-inducible size arrest that is distinct and genetically separable from the radiation-induced, p53-regulated G1 and G2 arrests.

MATERIALS AND METHODS

Creation of a Human PTEN Targeting Vector. A high-efficiency promoterless PTEN targeting vector was created using homologous recombination in Saccharomyces cerevisiae. In brief, an 8-kb BsmBI-SphiI fragment containing exon II of PTEN was cloned from BAC 106D15A into yeast shuttle vector YEp24 and sequenced. Next, a PCR product containing an IRES-neo* gene flanked by LoxP sites, a priming site for PCR-based identification of knockouts, restriction sites for Southern blot–based identification of knockouts, and 50 nucleotides of homology to the subcloned PTEN genomic fragment was cotransformed into S. cerevisiae with the linearized recombinant yeast shuttle vector. Successful recombinants were identified by whole-cell PCR. Recombinant plasmids were then shuttled into Escherichia coli, and their integrity was confirmed via restriction analysis and DNA sequencing. Additional technical details of this approach to human somatic cell gene targeting are discussed elsewhere (21).

Tissue Culture and Transfection. HCT116, DLD1, LNCaP, and PC3 cells were obtained from the American Type Culture Collection (Manassas, VA). U-87MG cells with inducible PTEN transgenes were provided by Maria Magdalena Georgescu (M. D. Anderson Cancer Center, Houston, TX; ref. 22). HCT116 and DLD1 cells were grown in McCoy’s 5A medium (Invitrogen, Carlsbad, CA); LNCaP cells in RPMI 1640; PC3 cells in Ham’s F-12; and U-87MG cells in Dulbecco’s modified Eagle’s medium containing 0.5 mg/mL G418, 10 μg/mL blasticidin (Invitrogen), and 1 μg/mL doxycycline (Invitrogen) for PTEN induction. All cell lines were grown in 10% fetal bovine serum (Mediatech, Herndon, VA) and 1% penicillin/streptomycin (Invitrogen) at 37°C in 5% CO2.

To obtain gene-targeted clones, HCT116 or DLD1 cells were transfected with Aat II-linearized PTEN targeting vector using LipofectAMINE (Invitrogen), following the manufacturer’s protocol. After 2 weeks of growth and selection in 96-well plates (0.6 and 1.0 mg/mL G418 for HCT116 and DLD1 cells, respectively), individual colonies were obtained, expanded, cryopreserved, and tested via PCR for the presence of a heterozygous knockout. Twenty-five percent of G418-resistant clones were knockouts. After excision of the IRES-neo* gene with adenocre, heterozygous knockout clones were then retransfected with the linearized PTEN targeting vector to delete the remaining allele. G418-resistant clones were then expanded, cryopreserved,
and tested via PCR and Southern blot for the presence of a homozygous knockou.

**Irradiation.** Subconfluent cell monolayers growing in vitro were irradiated using a J.L. Shepard Mark I 137Cs irradiator (San Fernando, CA) at ~2 Gy/min.

**Genomic Polymerase Chain Reaction, Southern Blots, and DNA Sequencing.** Preparation of genomic DNA, PCR, Southern blots, and automated sequencing were all performed using standard techniques. Taq Platinum (Invitrogen) was used for PCR, according to the manufacturer's instructions. For Southern blots, 5 μg of digested genomic DNA was separated on a 1% agarose gel and transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA). Membranes were prehybridized overnight at 60°C and then hybridized for 24 hours at 60°C using a radiolabeled, PCR-generated probe for PTEN. Blots were then washed and imaged using a phosphorimagining 445 SI (Amersham Biosciences, Piscataway, NJ).

**Western Blot and Enzyme-Linked Immunosorbent Assay.** Protein lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, probed with primary and horseradish peroxidase-coupled secondary antibodies, and visualized by ECL (Amersham Biosciences). Antibodies were obtained from Cascade Bioscience (Winchester, MA; PTEN clone 6H2.1); Cell Signaling Technologies [Beverly, MA; total Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308), total tuberin, phospho-tuberin (Thr-1462)]; and BD Biosciences (San Diego, CA; HIP-1a). Levels of phospho-Akt were quantified via enzyme-linked immunosorbent assay (ELISA), as recommended by the manufacturer (Cell Signaling Technologies).

**Measurement of Cell Size.** Cells were trypsinized in 0.5 mL added to 0.5 mL of serum-containing medium, and further diluted in 10 mL of Isoton II. Cell diameters and volumes were determined using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). At least 10,000 cells were counted per measurement.

**Cell Cycle Analysis.** For flow cytometry, cells were fixed in 70% ethanol and stained in PBS containing 0.1% Triton X-100, 50 μg/mL RNase, and 50 μg/mL propidium iodide. DNA content was measured on a FACSsort flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using ModFit software (Verity Software House, Topsham, ME) on both linear and log scales. At least 20,000 cells were analyzed per sample. For bromodeoxyuridine (BrdUrd) incorporation, cells were pulsed with 10 μmol/L BrdUrd for 1 or 2 hours, trypsinized, and centrifuged. Using the BrdUrd Flow Kit (PharMingen, San Diego, CA), cells were then fixed and permeabilized, treated with DNase to expose BrdUrd epitopes, and stained with fluorescein isothiocyanate-conjugated anti-BrdUrd antibodies. Cells were then counterstained with propidium iodide and analyzed by flow cytometry. For the mitotic trap assay, cells were treated with 0.2 μg/mL nocodazole and immediately irradiated. At various time points, cells were collected by trypsinization, centrifuged, and simultaneously fixed and stained in a solution containing 3.7% formaldehyde, 0.5% Nonidet P-40, and 10 μg/mL Hoechst 33258 in PBS. Nuclei were visualized by fluorescence microscopy. Nuclei with condensed, evenly staining chromosomes were scored as mitotic. At least 300 cells were counted for each determination.

siRNA. PTEN+/+ HCT116 cells were transfected sequentially twice with pools of PTEN, TSC2, or negative control duplexes using LipofectAMINE 2000 (Invitrogen), as recommended by the manufacturer (Dharmacon, Lafayette, CO). Cells were irradiated (6 Gy) 24 hours after the final transfection, and their size was measured 4 days later.

**Clonogenic Survival Assay.** Equivalent numbers of exponentially growing HCT116 PTEN+/+ and PTEN−/− cells were treated with a single dose of γ radiation using a 137Cs source. Twenty-four hours after irradiation, the cells were trypsinized, counted, and plated at various dilutions in 25-cm2 tissue culture flasks. Colonies were allowed to grow undisturbed for 12 days, stained with crystal violet, and counted in a blinded fashion.

**Xenograft Growth Assay.** A total of 36 tumors were established by subcutaneous injection of 6 × 106 cells suspended in McCoy’s 5A medium in the sacral region of immunodeficient mice. Tumors were treated with a single dose of γ radiation via a 137Cs source (7.5 or 15 Gy) when approximately 50 mm3 in size. Tumor growth rate was determined by measuring three orthogonal diameters of each tumor twice a week, and the tumor volume was estimated at m/6(D1D2D3/2).

## RESULTS

**Targeted Deletion of PTEN in Human Cancer Cells.** Somatic cell gene targeting was used to create an isogenic set of human cancer cell lines differing only in the presence or absence of endogenous PTEN. HCT116 cells were initially selected because they are suitable for somatic cell gene targeting; have well-defined, intact checkpoint responses; and have two wild-type alleles of PTEN (23, 24). Of note, mutational inactivation of PTEN is found in approximately 37% of RER− and 18% of RER+ colorectal carcinomas (25, 26). The targeting strategy is depicted in Fig. 1A and described in Materials and Methods. Homozygous deletion of PTEN in multiple, independently derived clonal cell lines was confirmed by PCR, Southern blot, and Western blot analysis (Fig. 1B and C; data not shown). Isogenic PTEN+/+ and PTEN−/− cells were morphologically indistinguishable (Fig. 2A, panels a and b). These PTEN knockout cells represent, to our knowledge, the first isogenic set of human cells that differ only in the presence or complete absence of endogenous wild-type PTEN.

As expected, deletion of PTEN resulted in phosphorylation of Akt on residues Ser-473 and Thr-308 (Figs. 1C and 6; data not shown). Inactivation of PTEN also led to phosphorylation of the Akt substrate tuberin (TSC2) on Thr-1462 (Fig. 1C). Additionally, PTEN deletion led to a modest increase in the TSC2-regulated gene HIF-1α (Fig. 1C). These data demonstrate that deletion of PTEN in HCT116 cells led to biochemical modulation of the PTEN/Akt signaling pathway.

**PTEN Is Required for a Radiation-Induced Size Arrest in Human Cells.** During exponential growth, PTEN-proficient and -deficient human cells were similar in size (13.7 ± 0.7 μm and 14.5 ± 1.5 μm for PTEN+/+ and PTEN−/− cells, respectively; Fig. 2A, panels a and b, and C, panel a). To determine whether the reported effects of PTEN on cell size might be the result of an aberrant arrest, cells were measured after treatment with 6 Gy of γ radiation. Whereas PTEN+/+ cells enlarged slightly and then size-arrested, PTEN−/− cells enlarged dramatically, increasing nine times in volume by post-irradiation day 6 (Fig. 2A-D; 13.3 ± 0.6 μm and 27.5 ± 2.5 μm for PTEN+/+ and PTEN−/− cells, respectively). Similar results were obtained after treatment with 9 Gy (data not shown). To exclude the possibility that this phenotype was a clone-specific artifact unrelated to PTEN, four independently derived PTEN+/+ cell lines (parental HCT116 cells and three clonal cell lines resulting from nonhomologous integration of the targeting vector) and four independently derived homozygous knockout PTEN−/− cell lines were studied. Each of the four PTEN−/− cell lines and none of the four PTEN+/+ cell lines enlarged dramatically after irradiation (data not shown). Time course studies indicated that the PTEN−/− cells enlarged continuously after treatment, rather than size-arresting after an initial increase in size (Fig. 2D).

Next, we disrupted PTEN in DLD1 cells, a colon cancer cell line with endogenous wild-type PTEN genes and mutant p53 (27). Homozygous deletion of PTEN in DLD1 cells was demonstrated by PCR (not shown) and Western blot (Fig. 3A). PTEN deletion resulted in a 4- to 5-fold increase in phosphorylated Akt (Fig. 3B). During exponential growth, DLD1 PTEN+/+ and PTEN−/− cells were similar in diameter (Fig. 3C; 15.9 ± 0.5 μm and 15.5 ± 0.3 μm, respectively). However, 3 days after irradiation (9 Gy), DLD1 PTEN+/+ cells had arrested in size, whereas PTEN−/− cells had not (Fig. 3D; 16.0 ± 1.0 μm and 25.4 ± 1.4 μm, respectively). These data generalize our findings to a second PTEN-targeted human cancer cell line.

Finally, we sought to rule out the possibility that the size difference in irradiated PTEN+/+ and PTEN−/− cells might be secondary to polyploidization. To test this, irradiated HCT116 PTEN+/+ and PTEN−/− cells were stained with the vital dye Hoechst 33342 (10
μg/ml), flow-sorted in a FACSAria high speed cell sorter (Becton Dickinson) to separate the G1 and G2-M populations, and measured. Irradiated PTEN−/− cells in both the G1 and G2-M phases of the cell cycle were significantly larger than their isogenic, flow-sorted PTEN+/+ counterparts (G1 = 13.0 and 21.5 μm for PTEN+/+ and PTEN−/− cells, respectively; G2-M = 13.4 and 29.4 μm for PTEN+/+ and PTEN−/− cells, respectively). Furthermore, flow cytometry demonstrated that PTEN−/− cells showed no increased propensity to undergo post-irradiation polyploidization (data not shown). These experiments demonstrated that abrogation of the radiation-induced size arrest is independent of ploidy.

Maintenance of the Radiation-Induced G1 and G2 Arrests in PTEN−/− Cells. Next, a variety of experimental approaches were used to determine whether abrogation of the radiation-induced size arrest was accompanied by aberrations in the conventional radiation-induced G1 and G2 arrests. This was a particularly important point, because it remained a formal possibility that the abrogation in cell size increase after irradiation (Fig. 4A). Second, BrdUrd incorporation assays were performed to directly measure the ability of the G1 arrest to prohibit entry of G1 cells into S phase after irradiation. There was no measurable defect in the radiation-induced G2 arrest in PTEN−/− cells (Fig. 4C). Of note, Kandel et al. (14) have reported attenuation of the radiation-induced G2 arrest in HCT116 cells expressing a myristilated, activated form of Akt. It is possible that the discrepancy between the data described in Kandel et al. (14) and the data described herein are due to differences in the levels of activated Akt.

Taken together, our data have revealed no attenuation in the radiation-induced G1 and G2 arrests after deletion of PTEN in HCT116 cells. As such, the role for PTEN in controlling a radiation-induced size arrest appears to be a primary defect.

Pharmacological Restoration of the Radiation-Induced Size Arrest in PTEN−/− Cells. PI3k inhibitors were used to test whether abrogation of the radiation-induced size arrest in PTEN−/− was due to the specific loss of the PTEN lipid phosphatase activity. To do this, HCT116 PTEN+/+ and PTEN−/− cells were pretreated for 1 hour with 580 nmol/L wortmannin. The cells were then irradiated (6 Gy), cultured for 6 days in the presence of the inhibitor, and measured (Fig. 5A). Wortmannin was able to efficiently restore the size arrest, resulting in a post-irradiation size increase in PTEN−/− cells only 6% that of untreated irradiated cells. Wortmannin was nontoxic at the dose tested in this experiment. Importantly, the size of irradiated PTEN+/+ cells was virtually unaffected by wortmannin treatment. Similar results were obtained with LY294002 (data not shown). This experiment demonstrated that the elevated levels of PIP3 caused by PTEN inactivation are responsible for abrogation of the PTEN-dependent, radiation-induced size arrest.

PTEN inactivation can also lead to mTOR activation (9). To test whether mTOR activation was similarly necessary for abrogation of the radiation-induced size arrest, PTEN+/+ and PTEN−/− cells were pretreated with 5 nmol/L rapamycin, irradiated (6 Gy), cultured for 6 days in the presence of rapamycin, and measured. As depicted in Fig. 5C, mTOR activation was not necessary for abrogation of the radiation-induced size arrest.
5B, treatment with rapamycin led to partial restoration of the radiation-induced size arrest in PTEN+/− cells, resulting in a post-irradiation size increase 43% that of untreated irradiated cells. Rapamycin was nontoxic at the dose tested in this experiment. Importantly, treatment with rapamycin had little or no effect on the size of irradiated PTEN+/+ cells. These data indicate that mTOR plays an important role as well.

**Attenuation of the Size Arrest by Depletion of TSC2 in PTEN+/+ Cells.** TSC2, an Akt substrate that is inactivated by phosphorylation, is known to modulate cell size in D. melanogaster (17). Deletion of PTEN in HCT116 cells resulted in an increase in phosphorylated, inactive TSC2 (Fig. 1C). As such, we considered the hypothesis that TSC2 was a required effector of the PTEN-dependent, radiation-induced size arrest. To test this, we measured the integrity of the radiation-induced size arrest after siRNA-mediated depletion of either PTEN or TSC2 in PTEN+/+ HCT116 cells. Transfection with either siRNA pool reduced the levels of PTEN or TSC2 protein by ~50% (Fig. 5C). siRNA-mediated depletion of either PTEN or TSC2 led to similar radiation-induced cell size increases (Fig. 5C; 13.74 ± 0.31 μm, 15.53 ± 0.64 μm, and 15.70 ± 0.53 μm for negative control, TSC2, and PTEN siRNAs, respectively). The magnitude of the size increases after irradiation was similar to that seen in heterozygous PTEN gene-targeted HCT116 cells, which also have approximately 50% reduction in PTEN protein (data not shown). These data demonstrate that TSC2 is required for the PTEN-dependent size arrest after DNA damage.

**Radiation Potentiates Akt Activation in PTEN−/− Cells.** We next wished to determine whether DNA damage affected the activity of the PI3k signal transduction pathway and whether these effects were modulated by PTEN. To test this, we measured levels of phosphorylated Akt by quantitative ELISA in irradiated HCT116 PTEN+/+ and PTEN−/− cells. As shown in Fig. 6, irradiation led to a dose-dependent increase in phosphorylated Akt. These data are consistent with a previous study demonstrating radiation-induced Akt activation in human endothelial cells (29). Importantly, the radiation-induced increase in phosphorylated Akt was much greater in HCT116 PTEN−/− cells than in HCT116 PTEN+/+ cells. Radiation had no effect on the levels of total Akt (Fig. 6). Identical results were obtained by Western blot analysis using phospho-specific antibodies (data not shown). Western blot analysis has ruled out the possibility that radiation-induced Akt phosphorylation is attributable to an increase in expression of the PI3k catalytic subunit (data not shown). Our results indicate that in the absence of PTEN, DNA damage leads to the accumulation of phosphorylated, activated Akt, which leads to a continuous increase in cell size despite arrest of the cell cycle.
Abrogation of the Radiation-Induced Size Arrest in Naturally Occurring PTEN-Deficient Human Cancer Cells. Next, we attempted to determine whether the radiation-induced size arrest was absent in cancer cell lines with naturally occurring mutational inactivation of PTEN. This was a particularly important point, because activating mutations of the PI3k catalytic subunit have recently been identified in a subset of colon cancers, including HCT116 cells (30). As such, it was important to demonstrate that loss of the size arrest point was not caused by the specific combination of activating mutations in PI3k and inactivating mutations in PTEN.

First, we studied PC3 cells, a genetically unmodified human prostate cancer cell line with mutational inactivation of PTEN (31). Three days after irradiation (9 Gy), PC3 cells had dramatically enlarged from 16.7 ± 0.3 μm to 33.1 ± 2.1 μm (Fig. 7A and B), as though they were deficient in the PTEN-dependent radiation-induced size arrest. Pretreatment with LY294002 (10 μmol/L) or rapamycin (5 nmol/L) was able to efficiently restore the size arrest, resulting in a post-irradiation size increase only 20% and 22% that of untreated irradiated cells, respectively (data not shown). LY294002 and rapamycin were nontoxic at the doses tested in this experiment and had no effect on the size of unirradiated PC3 cells (data not shown).

Second, we studied LNCaP cells, another genetically unmodified human prostate cancer cell line with mutational inactivation of PTEN (2). Three days after irradiation (6 Gy), LNCaP cells had enlarged from 15.2 ± 0.1 μm to 18.3 ± 0.5 μm (Fig. 7C), as though they were deficient in the PTEN-dependent radiation-induced size arrest. Pretreatment with LY294002 (10 μmol/L) or rapamycin (5 nmol/L) restored the size arrest, resulting in a post-irradiation size increase only 0% and 16% that of untreated irradiated cells, respectively. LY294002 and rapamycin were nontoxic at the doses tested in this experiment and had no effect on the size of unirradiated LNCaP cells (data not shown).

Third, we wished to determine whether PTEN controlled a radiation-induced size arrest in U-87MG cells, a glioblastoma cell line with endogenous mutant PTEN genes. To test this, we studied genetically modified versions of U-87MG cells with tetracycline-inducible wild-type or mutant PTEN transgenes (22). Three days after irradiation (9 Gy), U-87MG cells had enlarged from 16.3 ± 0.1 μm to 19.0 ± 0.1 μm, as though they were deficient in the PTEN-dependent radiation-induced size arrest. Induction of the wild-type transgene led to a restoration of the size arrest, resulting in a post-irradiation size increase only 26% that of cells without transgene induction (Fig. 7D). In contrast, induction of a mutant PTEN transgene was unable to restore the arrest. In the absence of transgene induction, pretreatment with LY294002 (50 μmol/L) or rapamycin (1 nmol/L) partially restored the size arrest, resulting in a post-irradiation size increase 41% and 55% that of untreated irradiated cells, respectively (data not shown). LY294002 and rapamycin were nontoxic at the doses tested in this experiment and had no effect on the size of unirradiated U-87MG cells (data not shown).

These experiments demonstrate that a variety of human cancer cell lines with naturally occurring PTEN mutations display an aberrant, PI3k-regulated, radiation-induced size arrest.

Radiosensitivity in PTEN<sup>−/−</sup> Cells. It has been suggested that mutational inactivation of DNA damage-inducible cell cycle arrest genes may underlie the radiosensitivity found in many types of human cancer (32). As one example, mutational inactivation of the rad9 gene leads to loss of radiation-induced cell cycle control and confers radiosensitivity (33). Therefore, we hypothesized that abrogation of the PTEN-dependent size arrest would confer sensitivity to radiation therapy. To test this, eight isogenic HCT116 PTEN<sup>+/+</sup> and PTEN<sup>−/−</sup> cell lines were treated with various doses of γ radiation and studied via clonogenic survival assay. As depicted in Fig. 8A and B, PTEN<sup>−/−</sup> cells were approximately an order of magnitude more sensitive to radiation than isogenic PTEN<sup>+/+</sup> cells. Next, we established isogenic tumors derived from PTEN<sup>+/+</sup> and PTEN<sup>−/−</sup> HCT116 cells and treated them <i>in vivo</i> with 0, 7.5, or 15 Gy of radiation. As shown in Fig. 9A–C, PTEN<sup>−/−</sup> tumors were significantly more sensitive to radiation than their isogenic PTEN<sup>+/+</sup> counterparts.

These data demonstrate that loss of PTEN function results in...
increased radiosensitivity both in vitro and in vivo. Furthermore, they provide additional experimental confirmation for the proposed relationship between aberrant radiation-induced arrests and radiosensitivity in human cancer. Our results are consistent with a recent report that treatment of the PTEN-null human A172 glioblastoma cell line with wortmannin leads to radioresistance, but they are inconsistent with other reports suggesting that inhibition of PI3k causes radiosensitivity (34, 35). Additional genetic studies seem warranted and may provide further clarification.

**DISCUSSION**

A common theme in cancer research has been the role of tumor suppressor genes such as p53 in control of the DNA damage-induced G1 and G2 arrests. Here, we build on that theme by identifying a separate radiation-induced size arrest in human cells and by demonstrating its dependence on PTEN, a commonly mutated tumor suppressor gene (Fig. 10). To our knowledge, the existence of such a DNA damage-inducible size arrest has neither been postulated nor demonstrated in any organism. We show that this arrest is dependent on PIP3, TSC2, and mTOR. Furthermore, we demonstrate that abrogation of the size arrest leads to radiosensitivity both in vitro and in vivo.

A number of recent studies performed in D. melanogaster and in mammalian systems have examined a related point: the biochemical basis of the coordination of cell cycle progression with cell growth (36, 37). The conclusion of these studies—that cell cycle progression and cell growth are coordinated but genetically separable—is consistent with the data presented herein.

We believe that it is appropriate to refer to PTEN as a "cell size checkpoint." Hartwell explains that a checkpoint is present where (1) a dependent relationship between two processes exists, (2) relief of dependence can be demonstrated by mutation in a gene, and (3) the gene is nonessential (38). The role of PTEN in enforcing the radiation-induced size arrest meets each of these criteria. During cell cycle arrest, a dependent relationship exists between the ability of the cell to arrest in the G1 or G2 phase of the cell cycle and the ability to arrest growth of the cell soma (Figs. 2–4). Relief of this dependence can be
demonstrated by mutational inactivation of PTEN (Figs. 2–4). Last, PTEN is nonessential (Figs. 1–3).

This new role for PTEN in controlling cell size during radiation-induced cell cycle arrest is likely related to the recently demonstrated role for PTEN in regulating the size of both cardiomyocytes and neurons (but not mouse embryo fibroblasts or stem cells) from tissuespecific PTEN knockout mice (18, 19, 20). It is intriguing to us that the two stimuli currently known to induce a size increase in PTEN−/−

Fig. 6. Radiation-induced Akt phosphorylation in PTEN−/− cells. Two independently derived HCT116 PTEN+/+ and PTEN−/− cell lines were treated with 0, 6, or 9 Gy radiation, cultured for 24 hours, harvested, and studied via ELISA and Western blot to measure the levels of phospho-Akt (P-Akt; Ser-473) or all Akt (pan-Akt).

Fig. 7. Generalizability. A, light microscopy of unirradiated PC3 cells (a) and PC3 cells (b) three days after treatment with 9 Gy radiation. Scale bar = 200 μm. PC3 (b) and LNCaP (c) cells were measured during exponential growth or 3 days after irradiation (9 or 6 Gy, respectively) with or without LY294002 (10 μmol/L) or rapamycin (5 nmol/L). D, U-87MG cells were measured 3 days after irradiation (9 Gy) with or without induction of either a wild-type or mutant PTEN transgene.

Fig. 8. Radiosensitivity of PTEN−/− cells. A and B, clonogenic survival assay of HCT116 PTEN+/+ and PTEN−/− cells. Equivalent numbers of four PTEN+/+ clones (●) and four independently derived PTEN−/− clones (□) were treated with various doses of γ radiation. Each experiment was performed at least in triplicate, and the values of all four PTEN+/+ or PTEN−/− cell lines were averaged after adjusting for plating efficiency. The mean number of colonies ± SEM is shown. Examples are shown in A.
cells (radiation and terminal differentiation) are similar to two of the most potent inducers of p53 responses (DNA damage and senescence; refs. 39 and 40). We speculate that somatic inactivation of size checkpoint control may, together with loss of other checkpoints, enable cells to continue proliferating in the face of these types of stimuli. Further study of such potential relationships seems warranted.

In addition to these findings, the HCT116 and DLD1 PTEN knockout cells represent, to our knowledge, the only isogenic set of human cells that differ solely in the presence or the complete absence of endogenous wild-type PTEN. As such, they may prove useful not only for studying the role of PTEN in cancer pathogenesis, but also for anticancer drug discovery targeting the PTEN pathway (41).

In conclusion, we have identified a genetic mechanism that enforces an arrest in cell size during cell cycle arrest (modulation of PI3K signaling by PTEN), and we demonstrate that it is distinct from the mechanisms that enforce DNA damage-inducible G1 and G2 arrests. We suggest that abrogation of the DNA damage-induced size checkpoint may contribute to the pathogenesis of human cancer.

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REFERENCES

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