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Expression of p57^{KIP2} Potently Blocks the Growth of Human Astrocytomas and Induces Cell Senescence

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Astrocytic tumors frequently exhibit defects in the expression or activity of proteins that control cell-cycle progression. Inhibition of kinase activity associated with cyclin/cyclin-dependent kinase co-complexes by cyclin-dependent kinase inhibitors is an important mechanism by which the effects of growth signals are down-regulated. We undertook the present study to determine the role of p57^{KIP2} (p57) in human astrocytomas. We demonstrate here that whereas p57 is expressed in fetal brain tissue, specimens of astrocytomas of varying grade and permanent astrocytoma cell lines do not express p57, and do not contain mutations of the *p57* gene by multiplex-heteroduplex analysis. However, the inducible expression of p57 in three well-characterized human astrocytoma cell lines (U343 MG-A, U87 MG, and U373 MG) using the tetracycline repressor system leads to a potent proliferative block in G_1 as determined by growth curve and flow cytometric analyses. After the induction of p57, retinoblastoma protein, p107, and E2F-1 levels diminish, and retinoblastoma protein is shifted to a hypophosphorylated form. Morphologically, p57-induced astrocytoma cells became large and flat with an expanded cytoplasm. The inducible expression of p57 leads to the accumulation of senescence-associated β-galactosidase marker within all astrocytoma cell lines such that ~75% of cells were positive at 1 week after induction. Induction of p57 in U373 astrocytoma cells generated a small population of cells (~15%) that were nonviable, contained discrete nuclear fragments on Hoechst 33258 staining, and demonstrated ultrastructural features characteristic of apoptosis. Examination of bax and poly-(ADP ribose) polymerase levels showed no change in bax, but decreased expression of poly-(ADP ribose) polymerase after p57 induction in all astrocytoma cell lines. These data demonstrate that the proliferative block imposed by p57 on human astrocytoma cells results in changes in the expression of a number of cell cycle regulatory factors, cell morphology, and a strong stimulus to cell senescence. *(Am J Pathol 2000, 157:919–932)*

The most common brain tumor is the astrocytoma accounting for ~65% of all primary brain tumors. The malignant astrocytoma has a very poor prognosis primarily because of its highly proliferative and invasive nature. As with other neoplasms with increased proliferative potential, malignant astrocytomas demonstrate dysregulation of various components of the cell cycle machinery. Altered expression of positive growth regulators such as growth factors, cyclins, and cyclin-dependent kinases (CDKs), or the loss of negative regulators, including cyclin-dependent kinase inhibitors (CKIs) and the retinoblastoma protein (pRB) have all been demonstrated in malignant astrocytomas.[1,2] The CDKs phosphorylate pRB to release cells from cell-cycle arrest. In contrast with CDKs, the CKIs inhibit cyclin-CDK complexes and transduce internal or external growth suppressive signals. Accordingly, all CKIs may be construed as candidate tumor suppressor genes.

The CKIs are divided into two families, the INK4 and the CIP/KIP, which are defined on the basis of their structural homology and mechanism of action. The CIP/KIP family includes three structurally related members, p21$^{CIP1/WAF1}$,[3,4] p27^{KIP1},[5,6] and a recently isolated and cloned third member, p57^{KIP2} (p57).[7–10] These three CKIs share a common N-terminal domain for binding to and inhibiting the kinase activity of CDK-cyclin complexes. Mouse p57 consists of four structurally distinct domains, a CDK inhibitory domain, a proline-rich domain, an acidic-repeat domain, and a carboxy-terminal domain. Human p57 differs from that of mouse by virtue of sequences containing proline-alanine repeats in its internal domain. The human *p57* gene is located in 11p15.5, which frequently undergoes maternal allele loss of het-

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erozygosity in several cancers, including Wilms' tumor, and tumors associated with Beckwith-Wiedemann syndrome.[10–16]

p57 has been shown to be a potent inhibitor of several G_1 cyclin/CDK complexes, and its overexpression leads to cell-cycle arrest in G_1 phase.[10] Recently, Watanabe et al[17] showed that human p57 protein, like p21, contains proliferating cell nuclear antigen-binding domain within its C terminus that, when separated from its N-terminal CDK-cyclin binding domain, can prevent DNA replication. Hashimoto et al[18] showed that the 310 helix region of p57, but not of p21 or p27, was indispensable for the inhibition of cyclin A/CDK2 and cyclin E/CDK2 complexes. Thus, the 310 helix motif may confer a specific regulatory mechanism by which p57 differentially regulates CDK2 and CDK4 activities. p57 mRNA is expressed at high levels in embryonic tissues such as skeletal muscle, heart, lung, and brain. Interestingly, cells expressing p57 have been shown to be terminally differentiated.[10] Using multiple mutant mice, Zhang et al[19,20] showed that the CKIs p57 and p21 function redundantly to control cell-cycle exit and differentiation of lens fiber cells, placental trophoblasts, and myoblasts. As such, p57 is now thought of as a critical terminal effector of signal transduction pathways that control cell differentiation.[8,10,19] Recently, expression of p57 was shown to inhibit the conversion of conditionally immortal human mammary epithelial cells to the fully immortal state, suggesting that p57 may provide an additional barrier against indefinite proliferation.[21]

The human brain is a unique organ from a cell kinetic standpoint. Neurons become incapable of cell division in the early postnatal period. On the other hand, astrocytes retain their proliferative potential as is demonstrated in the process of reactive gliosis. Interestingly, astrocytes have the highest propensity to undergo malignant transformation of any cell type in the brain. Because p57 is expressed in cells with a high proliferative potential within embryonic brain tissue, in the present study we sought to determine the role of p57 in well-characterized, permanent human astrocytoma cell lines.

Materials and Methods

Astrocytoma Cell Lines, Culture Conditions, and Tumor Specimens

Three well-characterized malignant astrocytoma cell lines were used in this study: U 343 MG-A (U343), U87 MG (U87), and U373 MG (U373) (generous gifts of Bengt Westermark, Uppsala, Sweden).[22,23] U343 is a subclone of the original malignant astrocytoma that expresses the astrocyte differentiation marker, glial fibrillary acidic protein; it grows anchorage dependently, and is nontumorigenic in athymic mice.[24] U87 is derived from a patient with a glioblastoma multiforme, and is tumorigenic in athymic mice.[25] U373 is derived from an anaplastic astrocytoma and is also tumorigenic in athymic mice.[23] The p53 status of these three cell lines has previously and recently been determined: U87 and U343 are wild type and U373 is mutant for p53[26–28] (David Malkin, The Hospital for Sick Children, Toronto, personal communication). All astrocytoma cell lines were grown in monolayer culture in α-MEM supplemented with 10% fetal bovine serum and penicillin/streptomycin/fungizone (Life Technologies, Inc., Gaithersburg, MD) at 37°C in 5% CO_2.

Astrocytoma tumor specimens were taken at the time of craniotomy and tumor excision. A specimen of nonneoplastic human brain from a 14-year-old female was taken after craniotomy for epilepsy surgery. Human fetal brain was obtained after elective second-trimester abortion after informed consent from the parents.[29] Permission to use this material was granted by the Research Ethics Board, The Hospital for Sick Children.

Polymerase Chain Reaction (PCR)/Heteroduplex Analysis and DNA Sequencing

Five overlapping PCR fragments spanning the entire coding region of the human *p57* gene were generated using published primer pairs:[7,30] fragment I, primers 2 + 6 (TCTTCTCGCTGTCCTCTCCT + CGCCCCACCTGCACCGTCT); fragment II, primer set 3 (CTTCCAGCAGGACATGCCGCTG + TGGAGCCAGGACCGGGACT); fragment III, primer set 4 (ACTGCCTAGTGTCCCGGTC + GTCAGCGAGAGGCTCCTGG); fragment IV, primers 7 + 9 (TCAAGAGAGCGCCGAGCAG + GCGGGCCCTTTAATGCCAC); fragment V, primers 10 + 12 (TCTCCCGGCCCCCTCTCGG + CAAAACCGAACGCTGCTCTG).

Fragments were amplified from 500 ng of genomic DNA in PCR reactions containing 10% DMSO, 0.6 μmol/L primers, 0.25 mmol/L dNTP, and 2 units *Taq* polymerase (Life Technologies, Inc.) in the supplied buffer (2 mmol/L $MgCl_2$). Reactions were amplified by touch-down PCR with 35 sequentially linked cycles of 94°C denaturation for 30 seconds, annealing temperature for 30 seconds, and extension at 72°C for 30 seconds. Annealing was initiated at 68°C with a 1°C per 2 cycle step-down to 15 cycles at 58°C, followed by a 7-minute extension. Heteroduplex formation was achieved by denaturation at 94°C for 5 minutes and annealing at 65°C for 5 minutes. PCR products were purified using Qiaquick PCR purification columns (Qiagen Inc., Mississauga, Ontario, Canada), followed by electrophoresis in 0.4-mm thick hydrolink mutation detection enhancement gels (BioWhittaker Molecular Applications, Rockland, Maine) gels containing 15% urea and 6.25% formamide (J. T. Baker, Phillipsburg, NJ). Electrophoresis was performed in 0.6× Tris borate-ethylenediaminetetraacetic acid and run at 500 V for 17 hours before transfer to Hybond-N+ membranes (Amersham, Oakville, Ontario, Canada) and hybridization with internal primers for each PCR fragment: primer 13 (CCTTCCCAGTACTAGTGCGC), primer 6, primer 7, primer 8, and primer 11 (TCAGCAAAGCCGGCGGGGA) for fragments I, II, III, IV, and V, respectively. Samples containing heteroduplex species were directly sequenced in both orientations using the fragment specific-PCR primer pairs by Thermo Sequenase terminator cycle sequencing (Amersham) of gel-purified PCR products.

Plasmids and Transfection

The tetracycline-repressor gene expression system was used to induce expression of p57.[31] The pUHD15–1*neo* plasmid (generous gift of S. Reed, The Scripps Research Institute, La Jolla, CA) contains the *Escherichia coli* tetracycline repressor element fused to the VP16 transactivation domain of herpesvirus. This fusion protein is driven by a cytomegalovirus promoter and the vector has the neomycin resistance gene for selection. pUHD15–1*neo* (25 μg) was transfected into U 343 MG-A cells using calcium phosphate. Neomycin-resistant clones were selected in 900 μg/ml geneticin (G418; Life Technologies, Inc.) in α-MEM and stable expression of the fusion protein was determined by Western blot analysis of total cell lysates using a polyclonal antisera to VP16 (kindly provided by C. J. Ingles, Toronto, Canada). Several clones were analyzed for VP16 expression for each cell line, and the majority expressed VP16. Clones that demonstrated high-level expression of VP16 were selected for transfection with pUHD10-3 (generously provided by H. Bujard, Heidelberg, Germany). pUHD10-3 contains a multiple cloning site downstream from tandem tetracycline operator sequences and a CMV promoter. A full-length human p57 cDNA (kind gift of S. J. Elledge, Houston, TX) was inserted into the multiple cloning site of pUHD10-3, and this plasmid (25 μg) was co-transfected with *pgk*-puro (1 μg) for selection of stable lines. These clones were also maintained in 4 μg/ml tetracycline (Sigma, St. Louis, MO). Puromycin (Sigma) was used for selection at 1 μg/ml and G418 concentration was maintained at 500 μg/ml.

To induce expression of p57, astrocytoma cells were washed three times in phosphate-buffered saline (PBS) before identical medium without tetracycline was added. To screen for p57 expression, total cell lysates were extracted and Western blot analysis was performed. To determine the effect of induction of p57 expression on the growth and morphology of the astrocytoma cell clones, 2 to 5 $\times$ 10^5 cells were plated in 10-cm^2 dishes. The following day, fresh medium was added. Cell proliferation assays and flow cytometric analysis was performed as described below.

Cell Proliferation Assay

Cell growth was assayed by counting cells at defined intervals. Briefly, cells were trypsinized and resuspended in media, and an aliquot of cells was counted using a hemocytometer. Each count represented an average of three counts on three separate determinations. Cell proliferation assays were repeated in duplicate. Cell viability was determined on the basis of trypan blue exclusion as described previously.[32,33]

Flow Cytometric Analysis

To determine the proportion of cells present in a particular cell cycle phase, flow-assisted cell sorting (FACS) analysis of DNA content was performed. Briefly, 2 to 8 $\times$ 10^5 cells were trypsinized, washed in PBS, and resuspended in ice-cold 80% ethanol. Cells were kept at 4°C until propidium iodide (Sigma) DNA staining was performed. For different samples, the concentration of cells was kept equivalent. For staining, fixed cells were resuspended in propidium iodide and DNase-inactivated RNase A (Sigma) (final concentration 1 mg/ml) and were incubated for 30 minutes at room temperature in the dark. Stained cells were filtered through mesh-capped tubes and DNA content was analyzed on a Becton-Dickinson FACScan (San Jose, CA). Percent cell-cycle phase was determined using Cell Fit software (Becton-Dickinson) on three separate runs for each cell clone.

Antibodies

Antibodies to bax (N-20), p107 (SC-318), p130 (SC-317), E2F-1 (SC-193), and E2F-4 (SC-866x) were obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA); antibodies to pRB (14001A), poly(ADP ribose) polymerase (PARP) (C2-10) and p57 (65021A) were obtained from Pharmingen (Richmond, CA).

Western Blot

Total cell lysates (20 μg) prepared in 120 mmol/L NaCl, 0.5% Nonidet P-40, 50 mmol/L TrisCl, pH 8.0, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA) by semidry electrotransfer. Blots were rehydrated before immunodetection, and then were blocked in 5% skim milk dissolved in PBS with 0.1% Tween 20 (PBS-T) at room temperature for 1 hour. Primary and secondary antibody incubations were performed in PBS-T at room temperature for 1 hour. Goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies were used at 1:5,000 concentrations. Blots were immersed in enhanced chemiluminescence detection reagent (Amersham) for 1 minute; chemiluminescence was detected on Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

Western blots for p57, p107, p130, E2F1, E2F2, and pRB were obtained at multiple time points during p57 induction for the different astrocytoma cell clones. Western blots for PARP and Bax levels were obtained 8 days after p57 induction.

Immunocytochemistry

Astrocytoma cells were seeded onto glass coverslips and maintained in medium with or without tetracycline for 1 to 10 days. For senescence-associated β-galactosidase (SA-β-gal) staining, coverslips were harvested, washed in PBS, and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes at room temperature. SA-β-gal (pH 6.0) was detected as reported.[34] Coverslips were rinsed in PBS, counterstained with neutral fast red, rinsed with distilled water, and mounted onto microscope slides using mounting media (DAKO, Carpinteria, CA). In some experiments, tetracycline was added back

to the medium on day 5 after p57 induction, and the cells were then harvested for SA-β-gal staining on days 5, 7, and 10.

Morphological Analysis

The morphology of control and p57-induced U 343 MG-A astrocytoma cells was observed using a Leitz (Fluovert FS; Leica Microsystems, Heerbrugg, Switzerland) light and immunofluorescence microscope. Induced morphological changes were observed for variable periods of time. In some experiment, p57 expression was repressed after induction at various time points by adding tetracycline to the medium. Cell morphology and culture conditions were then assessed by phase microscopy.

Fluorescence Microscopy

To identify cells undergoing apoptosis, astrocytoma cells were stained with Hoechst 33258 (Sigma) and quantified by fluorescent microscopic analysis.[35] Briefly, uninduced and p57-induced astrocytoma cells were trypsinized, centrifuged, washed with PBS, and resuspended with 1% glutaraldehyde for 30 minutes for fixation. The cells were rinsed again and stained with 1 μmol/L Hoechst 33258 for 10 minutes previous to viewing under the fluorescence microscope.

Electron Microscopy

Uninduced and p57-induced human astrocytoma cells were harvested and lightly pelleted before fixation in 2.5% paraformaldehyde in phosphate buffer for 2 to 4 hours. Pellets were rinsed thoroughly with phosphate buffer before being postfixed with phosphate-buffered osmium tetroxide for 1 hour. The cells were then dehydrated in an ascending series of ethanols and embedded in epon-araldite via propylene oxide. After polymerization, ultrathin sections were cut on a diamond knife using an ultramicrotome and mounted on grids. The grids were then stained with ethanolic uranyl acetate and lead citrate. All specimens were viewed and photographed in a transmission electron microscope (JEOL 1200EXII; JEOL Peabody, MA).

Results

Inducible Expression of p57 Leads to G_1 Arrest and Changes in Cell Morphology

p57 was expressed under the control of the tetracycline operator (*tetO*) in U343, U87, and U373 astrocytoma cell lines constitutively expressing high levels of the tetracycline repressor protein (*tetR*)-VP16 fusion protein. Of the many clones screened for induction of p57 expression for each cell line, three clones (clone 9, $U343^{C9}$; clone 2 $U87^{C2}$; and clone 3, $U373^{C3}$) showed tightly regulated, tetracycline-dependent expression of p57 (Figure 1). Induction of p57 occurred within 24 hours for $U343^{C9}$ and $U373^{C3}$ astrocytoma cells, and by the third day for $U87^{C2}$ cells (Figure 2). High expression levels of p57 for all cell lines could be maintained for periods longer than 9 days after induction. The specimen of human fetal brain showed expression of p57, whereas the parental astrocytoma cell lines and specimen of nonneoplastic brain from a 14-year-old patient with epilepsy did not. No mutations in the *p57* gene were observed from the heteroduplex DNA detection analysis in the three astrocytoma cell lines examined and in a panel of human astrocytoma specimens (data not shown)

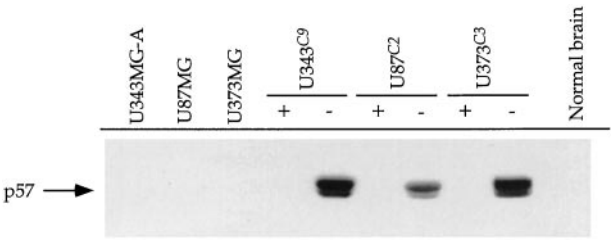

Figure 1. Western analysis of inducible expression of p57 in human astrocytoma cell lines. Before transfection with a human p57 cDNA, U343, U87, and U373 astrocytoma cell lines did not express p57. With tetracycline in the medium (+), p57-transfected astrocytoma cell clones did not express p57. However, after the removal of tetracycline from the medium (−), clones were identified with strong expression of p57 in the three astrocytoma cell lines. A specimen of nonneoplastic human brain does not express p57.

To investigate whether induction of exogenous p57 can arrest the cell cycle, we performed a proliferation assay and FACS analysis for all three p57-transfected astrocytoma cell clones. Induction of p57 rapidly blocked proliferation of $U343^{C9}$, $U87^{C2}$, and $U373^{C3}$ astrocytoma

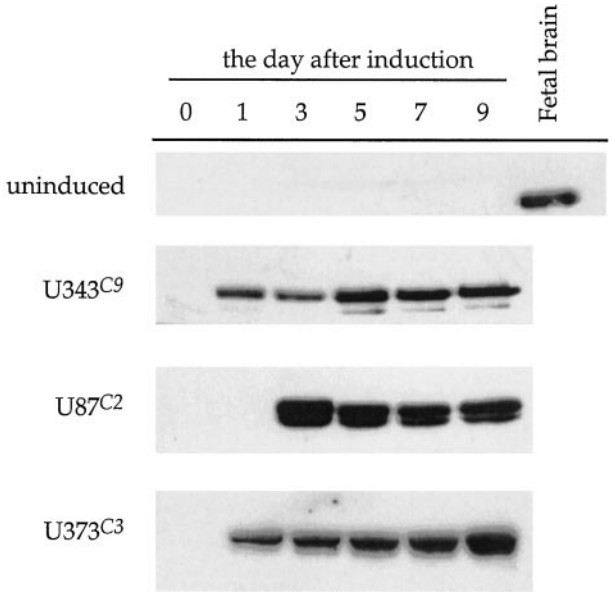

Figure 2. Temporal analysis of p57 expression in astrocytoma cell clones in the uninduced and induced states. Without induction, there is no expression of p57 in U343 astrocytoma cells (uninduced). After induction, p57 is strongly expressed in $U343^{C9}$ and $U373^{C3}$ astrocytoma cell clones on day 1, and in $U87^{C3}$ cells on day 3. High expression levels of p57 for all cell clones could be maintained for periods longer than 9 days after induction. A specimen of human fetal brain is shown to express p57.

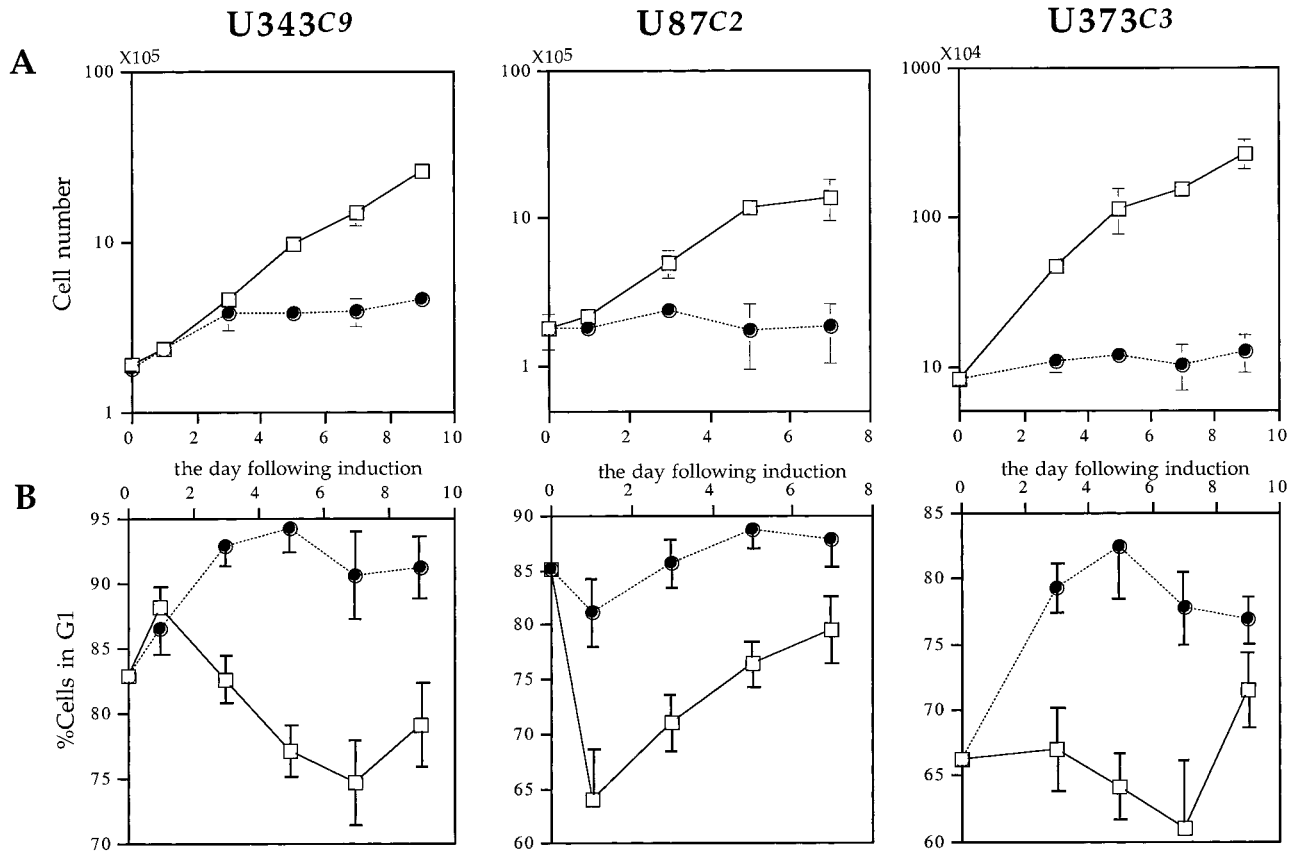

Figure 3. Growth inhibitory effects of p57 expression on human astrocytoma cell clones. **A:** Induction of p57 causes a potent proliferative block in all astrocytoma cell clones as demonstrated by growth curve analysis. **Open rectangles**, uninduced astrocytoma cells; **filled circles**, p57-induced astrocytoma cells. Error bars show the SD of three separate counts for each data point. **B:** Flow cytometric analysis demonstrates that induction of p57 causes astrocytoma cells to accumulate in G_1 phase of the cell cycle. **Open rectangles**, uninduced astrocytoma cells; **filled circles**, p57-induced astrocytoma cells. Error bars show the SD of the results of three separate FACS analyses for each astrocytoma cell clone.

cells (Figure 3A). FACS analysis revealed that p57-induced astrocytoma cells rapidly accumulate in G_1 phase of the cell cycle (Figure 3B). The differences in growth rates between the induced and uninduced astrocytoma cell clones were statistically significant, and indicate that the overproduction of p57 can functionally arrest the growth of astrocytoma cell lines in G_1.

Uninduced U343^{C9} cells have a tightly packed, cobblestone appearance and resemble the morphology of the parental U343 MG-A astrocytoma cell line. Induction of p57 causes the cells to become large, round, and flat with abundant cytoplasm containing perinuclear vacuoles (Figure 4). These morphological changes are apparent by day 3, coincident with the G_1 cell cycle arrest, and are very pronounced by day 5. Uninduced U87^{C2} cells are generally bipolar in configuration, much like the parental cell line from which they are derived. After p57 induction, U87^{C2} cells became large and triangular with markedly expanded cytoplasms (Figure 4). U373^{C3} astrocytoma cells were round and flat with a ruffled peripheral plasma membrane before p57 induction. After p57 induction, these cells similarly developed an expanded cytoplasm, but maintained their overall round, flat shape (Figure 4).

Induction of p57 Alters the Expression of Cell Cycle Regulatory Factors

To determine whether the induction of p57 was associated with alterations in the expression of downstream cell-cycle regulatory proteins, we next performed Western blot analyses of the pRB and E2F family proteins (Figure 5). The expression levels of pRB and p107 were sharply reduced by day 3 after p57 induction, whereas p130 expression levels were unchanged. Expression of the E2F family proteins was also determined in p57-induced U343^{C9} cells. E2F-1 expression levels were repressed whereas no change in the levels of E2F-4 was observed.

Increased Expression of SA-β-gal in p57-Induced Astrocytoma Cells

Induction of p57 in U343^{C9}, U87^{C2}, and U373^{C3} astrocytoma cells led to the identification of SA-β-gal-positive cells (Figure 6). The generation of SA-β-gal-positive astrocytoma cells began for each cell clone by day 1 after induction and peaked between 5 to 7 days at which time

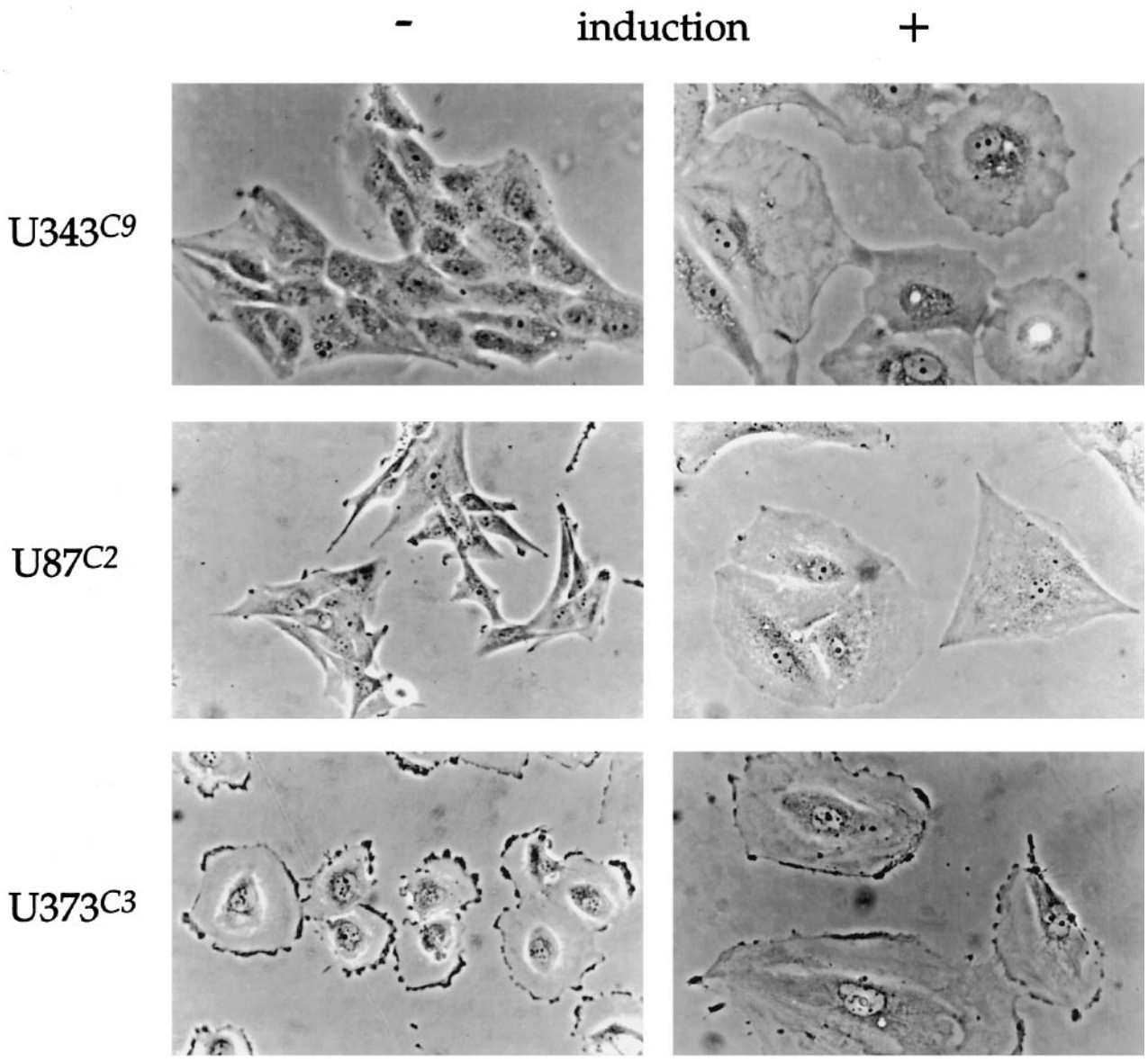

Figure 4. Morphological alterations in human astrocytoma cell clones after the induction of p57, day 5. Uninduced $U343^{C9}$ cells are characterized by their tightly packed, cobblestone appearance. The cells are cuboidal with a high nuclear:cytoplasmic ratio. After p57 expression, these cells become large, round, and flat with abundant cytoplasmic-containing perinuclear vacuoles. Uninduced $U87^{C2}$ cells are bipolar in configuration. After p57 expression, these cells become large and frequently triangular in shape with a markedly expanded cytoplasm. $U373^{C3}$ cells are characterized by being round and flat with a ruffled peripheral plasma membrane before p57 induction. After p57 expression, they also developed an expanded cytoplasm, but maintained their overall round, flat shape. Phase microscopy for all panels, ×350.

all cell clones had ~75% SA-β-gal-positive cells (Figure 7). The induction of SA-β-gal-positive cells through forced p57 expression was partially reversible as replenishment of the medium with tetracycline on day 5 led to fewer SA-β-gal-positive cells on days 7 and 10 (Table 1).

Response of Astrocytoma Cells to p57 Induction

After p57 induction, we detected a statistically significant decline in viable cells in $U343^{C9}$ and $U373^{C3}$ astrocytoma cells as determined by trypan blue dye exclusion from days 3 to 7 (Figure 8). p57 induction had a greater effect on reducing the number of viable cells among $U373^{C3}$ astrocytoma cells than among $U343^{C9}$. No such decline in cell viability was apparent for $U87^{C2}$ astrocytoma cells.

Hoechst 33258 staining of all three astrocytoma clones revealed rare abnormalities in nuclear configuration among $U87^{C2}$ and $U343^{C9}$ cells, but multiple fragmented micronuclei for $U373^{C3}$ astrocytoma cells induced to express p57 (Figure 9). Approximately 15% of $U373^{C3}$ nuclei were abnormal after Hoechst 33258 staining of p57-induced cells (Figure 10). Electron microscopy of p57-induced and uninduced astrocytoma cells confirmed the findings of the Hoechst 33258 staining analysis. Unlike p57-induced $U87^{C2}$ and $U343^{C9}$ astrocytoma cells which showed a preponderance of cells with normal ultrastructural features, many p57-induced $U373^{C3}$ cells were characterized by

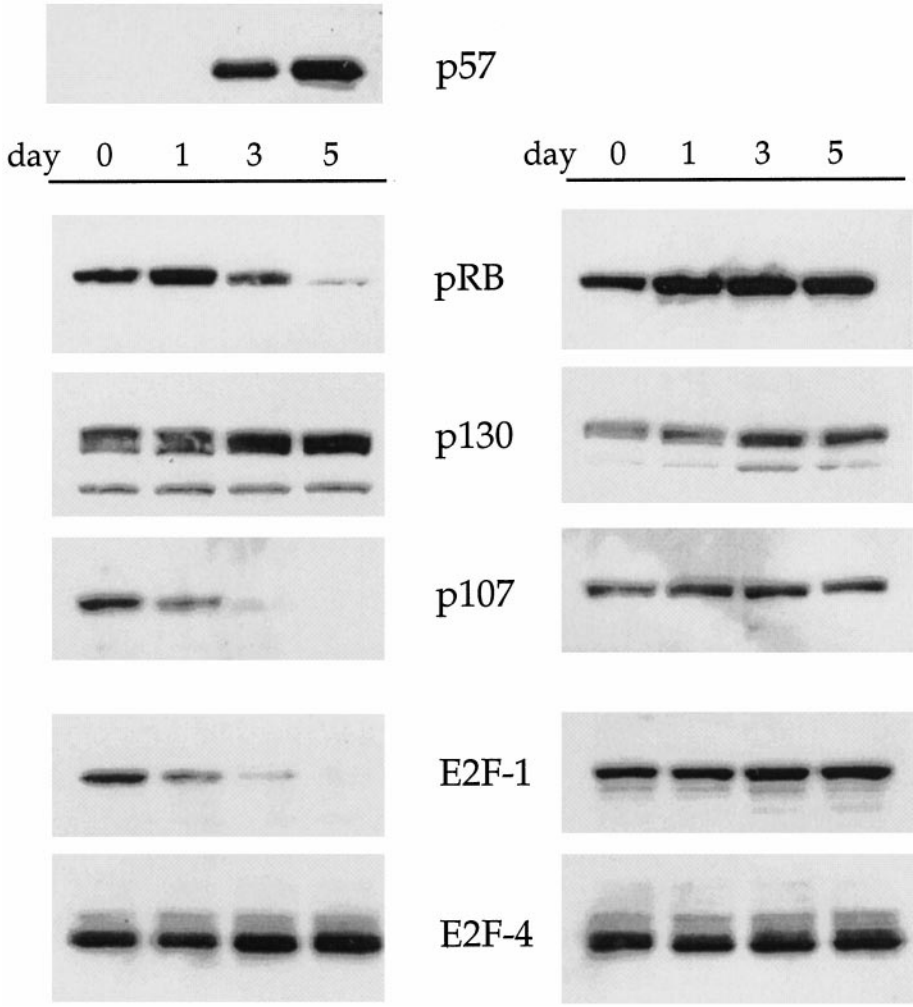

Figure 5. Expression of pRB- and E2F-family proteins in uninduced (**right**) and p57-induced (**left**) U87^{C2} human astrocytoma cells. Without induction of p57, expression levels of pRB- and E2F-family proteins are not significantly altered throughout a 5-day time interval. However, after induction of p57, decreased levels of pRB, p107, and E2F-1 are observed. pRB is shifted to a faster migrating, hypophosphorylated form. Levels of p130 and E2F-4 are unchanged in this analysis throughout the 5-day time interval.

convolution of nuclear and cytoplasmic outlines, apoptotic bodies, and compaction and margination of nuclear chromatin (Figure 11).

Western analysis of uninduced and p57-induced astrocytoma clones for Bax (Figure 12) revealed no change in expression levels. However, induction of p57 among astrocytoma cell clones led to diminished PARP expression (Figure 12). The reduction in PARP expression was particularly marked for U373^{C3} cells.

Discussion

We have shown that the inducible expression of p57 in three different permanent astrocytoma cell lines has a profound effect on their proliferation and morphology. Within 3 days after induction of p57, U343^{C9}, U87^{C2}, and U373^{C3} astrocytoma cells were rapidly growth arrested and accumulated in G_1 phase of the cell cycle. After the induction of p57, pRB and the pRB family protein, p107, are diminished; pRB is shifted to a faster migrating, hypophosphorylated form. Likewise, E2F-1 is repressed whereas E2F-4 levels are unchanged after induction of p57. The inducible expression of p57 in all three astrocytoma cell lines led to the identification of SA-β-Gal-positive cells which accumulated during the first week of induction such that ~75% of all cells in culture were positive. Although equivalent numbers of cells expressed this marker of cell senescence in each astrocytoma cell line, interestingly a population of U373^{C3} astrocytoma cells responded to p57 induction by following an apoptotic pathway as determined by Hoescht 33258 staining and electron microscopy.

Previous studies have shown that *p57* is a gene which undergoes genomic imprinting. Genomic imprinting is a process that results in the expression of only one allele of a gene depending on its parental origin. As such, it is thought to play an important role in embryonal development. Genomic imprinting may also play a role in certain pediatric tumors such as Wilms' tumor as a highly selective loss of maternal alleles for the Wilm's tumor gene is found not uncommonly in this tumor type.[36] In fact, a potential role for p57 in tumorigenicity was postulated on

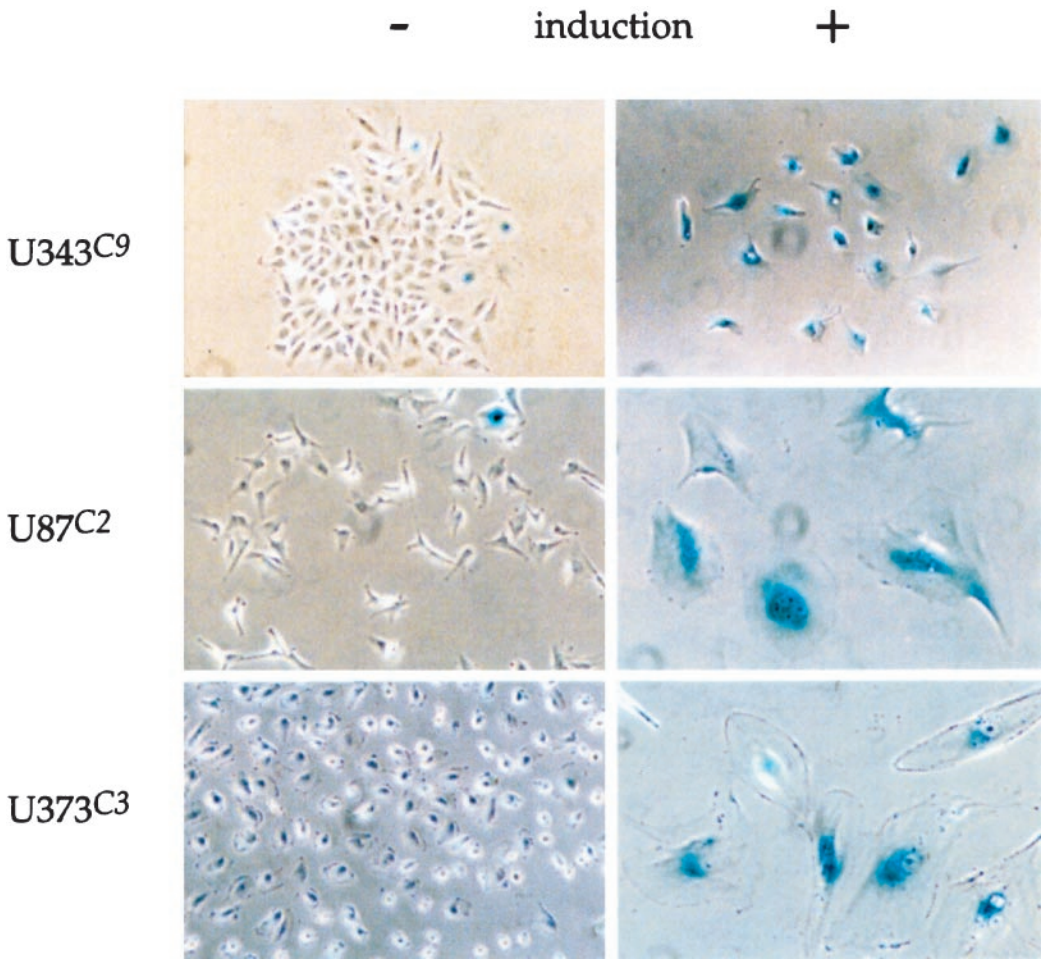

Figure 6. Induction of SA-β-gal-positive cells after p57 induction. Uninduced astrocytoma cell clones (**left**) demonstrated rare cells positive for the SA-β-gal marker. After induction of p57 (day 5), the majority of astrocytoma cells are seen to be positive for SA-β-gal (**right**). Phase microscopy for $U87^{C2}$ and $U373^{C3}$ p57-induced cell panels, ×250; phase microscopy for all other panels, ×125.

the basis of its chromosomal mapping to 11p15, a region which frequently demonstrates loss of heterozygosity in a number of common cancers in adults such as lung, breast, and bladder carcinoma, as well as Wilms' tumor in children.[10–16,37] Not all attempts to manipulate p57 expression levels have resulted in tumor suppression or formation. For example, $p57^{-/-}$ transgenic mice have delayed differentiation without tumor formation.[38,39] Although a previous report suggested that germline deletions in the proline-alanine-rich (PAPA-repeat) region of p57 are associated with increased risk of a variety of cancers, including breast cancer, a recent report by Li et al[40] failed to substantiate this observation in the context of breast cancer patients. In our study, p57 expression was documented within human fetal brain. However, none of the astrocytoma cell lines expressed p57, and none contained mutations in the *p57* gene. Absence of *p57* mutations in the human astrocytomas examined here and within a variety of other cancer types[30,40,41] suggest that other mechanisms of transcriptional or posttranslational silencing must be involved in the loss of p57 protein expression in astrocytic tumors and other cancers. Two possible mechanisms of gene inactivation include methylation in the promoter region of the *p57* gene and histone deacetylation. Recently, Shin et al[41] demonstrated that formation of inactive chromatin through histone deacetylation is a general mechanism for inactivation of both *p21* and *p57* genes in gastric cancer cells, and that methylation of the promoter region of the *p57* gene occurred in five of eight gastric cancer cell lines as an alternative pathway for inactivation of *p57*.

It is becoming clear that a common feature of cancer cells is the abrogation of cell-cycle checkpoints, either by aberrant expression of positive regulators such as cyclins and CDKs, or the loss of negative regulators, including CKIs and pRB. It has previously been suggested that transformation of glial cells into malignant astrocytic tumors also involves significant dysfunction of this cell cycle-control machinery.[1,2] Pedram et al[42] have shown that fetal rat diencephalic astrocytes can be stimulated to progress through G_1/S phase by the endogenous neuropeptide, endothelin-3. Atrial natriuretic peptide was inhibitory for cell proliferation, and induced the expression of p57 among other CKIs. In fact, multiple CKIs were shown to be necessary to restrain cell-cycle progression in astrocytes, an observation that may have relevance for inhibition of human astrocytoma cells.

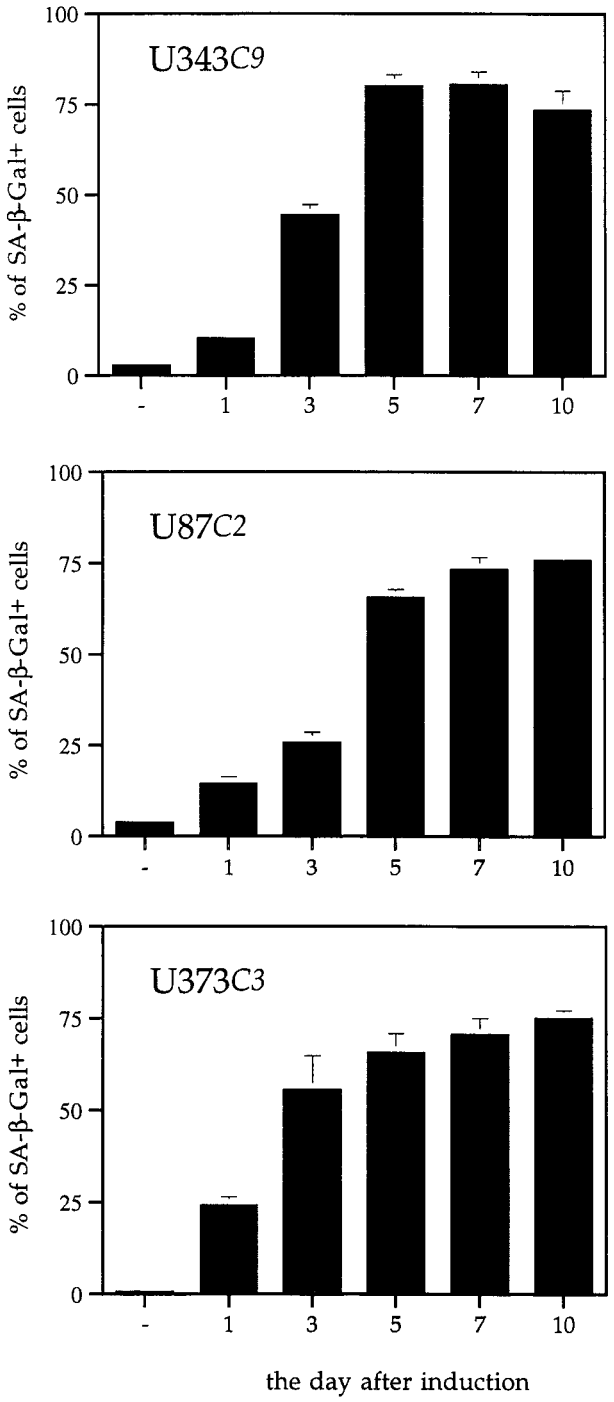

Figure 7. Generation of SA-β-gal-positive cells over time after p57 induction. Uninduced astrocytoma cells have rare SA-β-gal-positive cells. After p57 induction, there is an increase in the number of SA-β-gal-positive astrocytoma cells throughout time such that by day 7 ~75% of p57-induced cells express the marker.

In human astrocytic tumors, many reports have demonstrated alterations in the expression and activity of cell-cycle regulatory proteins, especially the loss of p16 expression.[43–48] We have previously shown that induction of p16 leads to a cell-cycle block in the U343 human astrocytoma cell line.[49,50] To our knowledge, however, alterations in the p57-cyclin/CDK complexes-pRB/E2F pathway have not been described previously in malignant astrocytomas. Our data demonstrate for the first time that induction of p57 in p57-negative human astrocytoma cell lines can potently block the proliferation and alter the morphology of three different human astrocytoma cell

Table 1. Percent SA-β-Gal-Positive U343 Astrocytoma Cells

	Day 5	Day 7	Day 10
p57-uninduced, tetracycline placed in medium on day 1	1.5 ± 0.8	3.4 ± 1.6	3.4 ± 1.2
p57-induced, tetracycline removed from medium on day 1	80.0 ± 3.2	80.4 ± 3.4	73.3 ± 5.4
p57-uninduced, tetracycline added back to medium 5 days after p57 induction	77 ± 4.2	38.7 ± 7.1	18.7 ± 2.3

The induction of p57 expression in U343 astrocytoma cells led to the generation of a high percentage of cells which stained positively for SA-β-gal when compared to p57-uninduced cells. When tetracycline was added back to the medium on day 5, the percentage of cells which stained positively for SA-β-gal decreased suggesting that the senescent state was at least partially reversible.

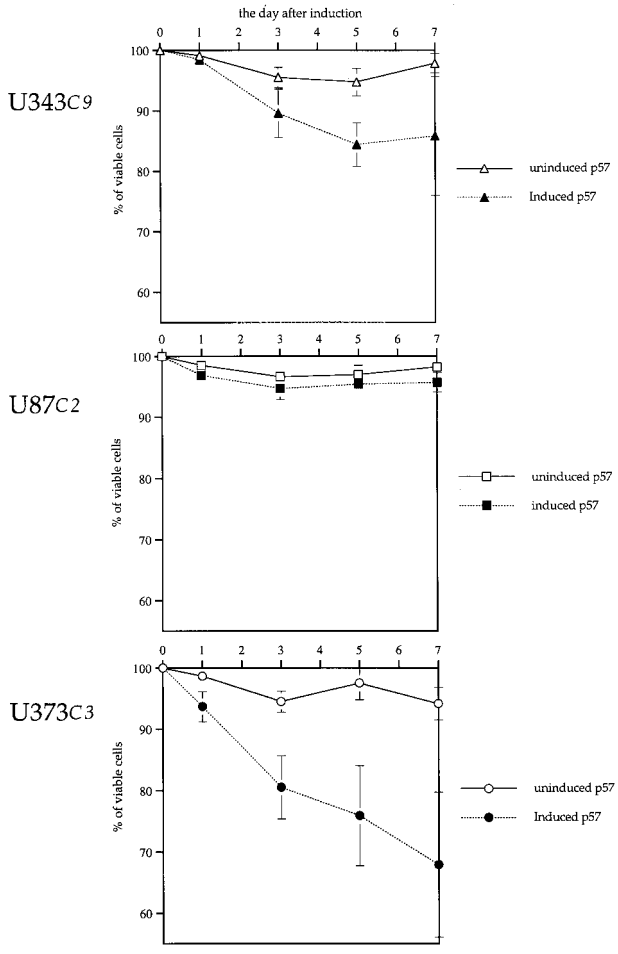

Figure 8. Viability of human astrocytoma cells after p57 induction. After p57 induction, U343^{C9} and U373^{C3} astrocytoma cells responded by having diminished numbers of viable cells as determined by trypan blue dye exclusion when compared to uninduced cells. U87^{C2} astrocytoma cells showed no loss in cell viability after p57 induction.

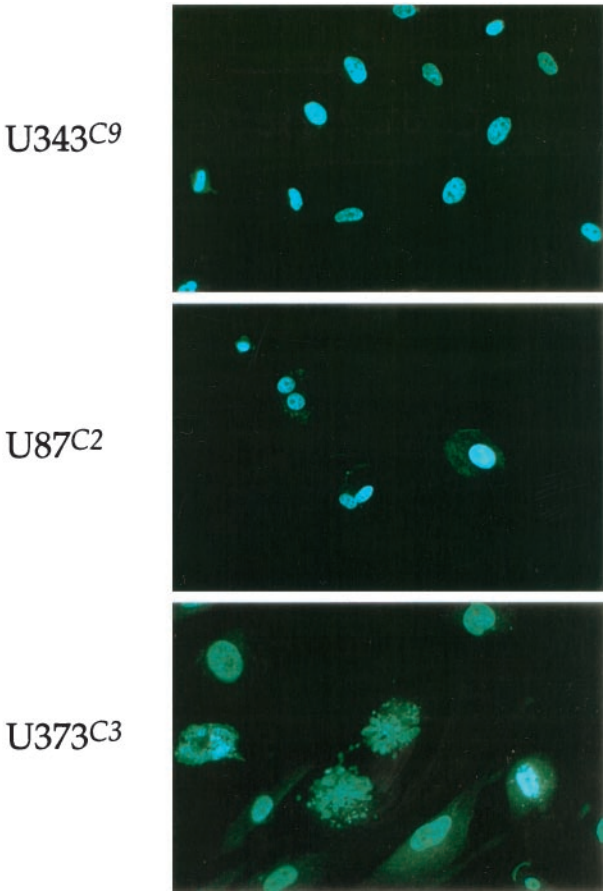

Figure 9. Hoechst 33258 staining of p57-induced human astrocytoma cell clones, day 5. Nuclear integrity was unaltered in U343^{C9} and U373^{C2} astrocytoma cells. However, U373^{C3} cells were characterized by exhibiting fragmentation of the nucleus in ~15% of cells. Fluorescence microscopy, ×200.

lines. This p57-induced cell-cycle arrest is accompanied by alterations in the expression and activity of a number of cell-cycle regulatory proteins.

As examples, our data demonstrate the presence of a potential autoregulatory mechanism which may control the levels of expression of members of the pRB/E2F

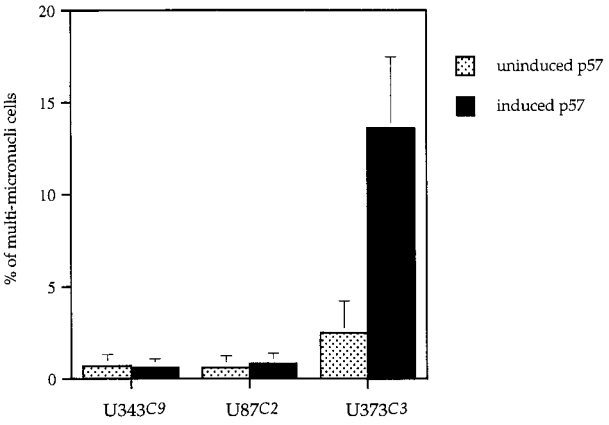

Figure 10. Number of cells demonstrating micronuclear fragmentation by Hoechst 33258 staining. Unlike U343^{C9} and U87^{C2} astrocytoma cell clones which showed no increase in numbers of cells with nuclear fragmentation after p57 induction, ~15% of U373^{C3} astrocytoma cells demonstrated micronuclear fragmentation.

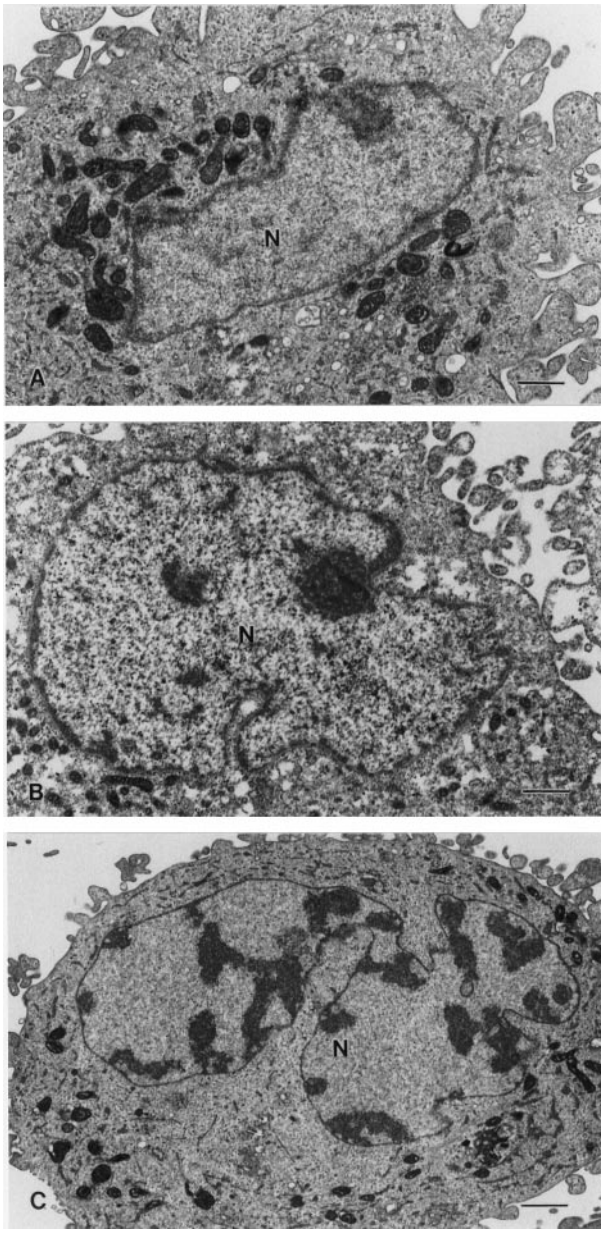

Figure 11. Ultrastructural features of p57-induced human astrocytoma cells. **A:** Typical nucleus (N) from U343C9 cells. **B:** Typical nucleus (N) from U87C2 cells. **C:** Apoptotic nucleus (N) from U373C3 cells. Approximately 15% of these cells were found to contain nuclei similar to this with dense peripheral chromatin and bizarre shapes. Scale bars, 1 μm.

family proteins. It has been shown that both pRB and p107 have E2F binding sites in their promoter regions, and E2F-1 also contains E2F binding sites in its promoter region. pRB phosphorylation is critical for control of cell-cycle progression in G_1.[51,52] After pRB phosphorylation, E2F, freed from the repressive effects of pRB family proteins, are then able to activate transcription of genes required for S phase progression.[52–54] It seems reasonable that pRB-E2F-1 complexes repress activated transcription from the pRB and p107 promoters. The repression of these factors suggests a model where initially, pRB and E2F family proteins form complexes that aggressively block transcription of factors required for cell-

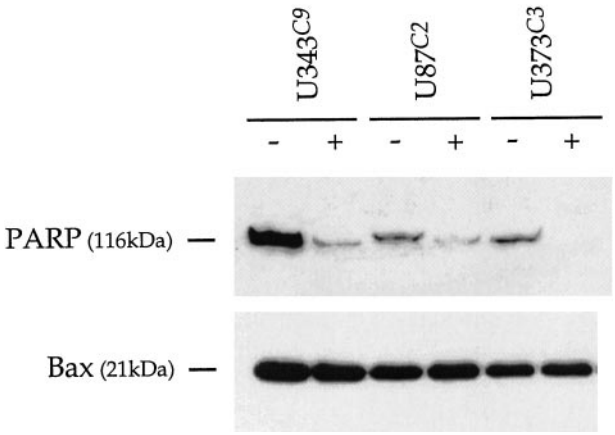

Figure 12. Western analysis for PARP and Bax in uninduced and p57-induced astrocytoma cell clones. There is no change in Bax expression with p57 induction. After p57 induction, PARP levels are observed to decrease. This is most marked for U373^{C3} astrocytoma cells. No cleavage products for PARP were observed.

cycle progression. These same complexes are also responsible for inhibiting expression of the pRB and E2F family proteins, loss of the latter ensuring that E2F-dependent cell-cycle progression is not possible. Other investigators have previously demonstrated that different pRB family proteins show distinct binding specificities for different E2F family proteins.[50,55–61] pRB seems to associate specifically with E2F-1, E2F-2, and E2F-3,[59] whereas p107 binds E2F-4[56,61] and p130 binds E2F-4 and E2F-5.[58,61] In p57-induced, growth-arrested U343 cells, the expression of pRB and E2F-1 is repressed, and pRB becomes quantitatively hypophosphorylated.

We also demonstrate here that p57-induced astrocytoma cells undergo a change in morphology—cells becoming large and flat and having abundant cytoplasm. This morphological change is reminiscent of the phenotype exhibited by human osteosarcoma cell line, SAOS-2, after exogenous expression of pRB.[16,30,62–64] In the presence of pRB, SAOS-2 cells become flat and round with a greatly expanded cytoplasm in their growth-arrested state. These flat cells resemble senescent primary fibroblasts after extended *in vitro* passage. In a report by Uhrbom et al,[34] ~40% of U1242 MG astrocytoma cells induced to express p16 developed a senescent cell phenotype at 7 days as determined by SA-β-gal staining. In our study, ~75% of astrocytoma cells were SA-β-gal-positive 7 days after p57 induction.

Cellular or replicative senescence is a state of permanent growth arrest and altered cell function after a finite number of cell divisions. Cellular senescence is thought to be a tumor suppressive mechanism, and a contributing factor in aging.[65,66] Three features distinguish senescent from presenescent cells: a block-to-cell proliferation, increased resistance to apoptotic death, and changes in differentiated functions.[65] Although our data suggest that inducible p57 expression causes a cell senescent phenotype among astrocytoma cells, the effect of p57 on the generation of SA-β-gal cells was at least partially reversible. Of the several growth regulatory transcriptional modulators known to be repressed in senescent cells, the repression of E2F-1 after p57 induction holds particular significance in our study on human astrocytoma cells.

Although senescent cells are thought to be resistant to apoptotic cell death, one of the astrocytoma cell lines induced to express p57, U373^{C3}, exhibited a population of cells which underwent apoptosis as determined by Hoechst 33258 staining for micronuclear fragmentation and electron microscopy. Why this particular cell line responded in a different manner to p57 induction is an interesting yet unanswered question. Apoptosis is a genetically encoded cell death program defined by typical morphological and biochemical changes.[67] Although ultrastructural characterization of nuclear and plasma membrane alterations remains one of the most important determinants of apoptosis, Hoechst 33258 staining for fragmented nuclei has also been used in a number of studies.[68–74] Apoptosis has been shown to be induced in human astrocytomas after ionizing radiation and treatment with DNA-damaging agents.[75–79] Several apoptosis-related molecules are involved in astrocytoma cell death including bcl-2, interleukin-1-β-converting enzyme, and p53.[80–85]

Interestingly, of the three astrocytoma cell lines examined in this study, U87 and U343 express wild-type p53 whereas U373 is mutant for p53.[26,27,80–86] As there have been several cancer cell systems in which apoptosis has been demonstrated in p53-inactivated cells,[87–96] it is conceivable that the apoptosis observed in U373^{C3} cells after p57 induction occurs in a p53-independent manner. We showed that Bax levels were unchanged but PARP levels were decreased without cleavage formation after p57 induction in all cell clones. In response to DNA damage, PARP activity increases, resulting in poly-(ADP) ribosylation of many nuclear proteins, including PARP itself. In cells which have become activated to undergo apoptosis, the 116-kd PARP protein becomes cleaved by caspase-3 producing 85-kd and 25-kd fragments and resulting in loss of normal PARP function.[97,98] Although we did not observe cleaved fragments of PARP in any of the astrocytoma cell clones examined here, down-regulation of PARP without cleavage product formation was observed for U343^{C9}, U87^{C2}, and U373^{C3} astrocytoma cells. This phenomenon has been described previously in replicatively senescent fibroblasts.[99] Our inability to detect cleaved PARP fragments in U373^{C3} in particular may relate to the small number of cells that are undergoing apoptosis compared to the large number of cells that continue to express the senescent cell phenotype.

In summary, we have shown that inducible expression of p57 in three different astrocytoma cell lines is a strong stimulus against cell proliferation and for cell senescence. Ongoing studies in the laboratory are attempting to determine precisely what role p57, in conjunction with other CKIs, may have in inhibiting the growth of human astrocytic tumors *in vivo*.

Acknowledgment

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