Tumor suppression and inhibition of aneuploid cell accumulation in human brain tumor cells by ectopic overexpression of the cyclin-dependent kinase inhibitor p27KIP1.

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J Clin Invest. 1996;97(8):1983-1988. https://doi.org/10.1172/JCI118631.

Research Article

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Tumor Suppression and Inhibition of Aneuploid Cell Accumulation in Human Brain Tumor Cells by Ectopic Overexpression of the Cyclin-dependent Kinase Inhibitor p27^{KIP1}

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Abstract

To investigate how overexpression of p27^{KIP1}, a downstream effector of TGF-β and a universal cyclin-dependent kinase (CDK) inhibitor could influence the malignant phenotype of malignant human brain tumor cells, an adenovirus vector system was used to transfer the human p27^{KIP1} gene (Adp27^{KIP1}) into the human astrocytoma cell line, U-373MG. Inhibition of CDK activity in Adp27^{KIP1}-infected cells was indicated by inhibition of [^{3}H]thymidine incorporation, an increase in cell doubling time and by cell cycle arrest in G_1. Notably, ectopic overexpression of p27^{KIP1} was associated with a marked decrease in the accumulation of aneuploid cells. Diminished malignant potential of Adp27^{KIP1}-infected cells was manifested by the loss of anchorage-independent growth in soft agar and by the inability to induce tumorgenesis in a xenograft model. These studies suggest that p27^{KIP1} is a tumor suppressor gene and supports the use of Adp27^{KIP1} for gene therapy of human brain tumors. (*J. Clin. Invest.* 1996. 97:1983–1988.) Key words: gene therapy • adenovirus • cell cycle • aneuploidy • brain tumors

Introduction

p27^{KIP1} was first identified as an inactive form of cyclin-dependent kinase-2 (CDK2)[1]–cyclin E after treatment of cells with transforming growth factor beta (TGF-β) or contact inhibition (1). It was purified from a cyclin E-CDK2 affinity column and had strong inhibitory activity towards cyclin E-CDK2 in histone H1 kinase assays (2). The gene encoding p27^{KIP1} was cloned by two different approaches: using protein sequence information (3) and by a two-hybrid system with CDK4-cyclin D as bait (4). The amino-terminal 60 residues of p27^{KIP1} are 44% homologous to p21$^{WAF1/CIP1}$, consistent with the many common biological features of these two proteins (4). Like p21$^{WAF1/CIP1}$, p27^{KIP1} can inhibit directly the enzymatic activity of CDK-cyclin complexes and arrest cells in G1 (5, 6). The association of p27^{KIP1} with CDK4-cyclin D or CDK2-cyclin E complexes can also block phosphorylation of CDK4 on Thr172 or CDK2 on Thr160 by CDK activation kinase (CAK) (3, 7).

Although no mutations of p27^{KIP1} have been observed to date in human cancers, the p27^{KIP1} gene has been mapped to chromosome band 12p13, a region deleted in some leukemias, (5, 8). Furthermore, p27^{KIP1} can be induced by cyclic AMP (7) and other negative regulators of cellular proliferation. Conversely, it can be down-regulated by interleukin 2 (6) and other positive regulators of cell proliferation. In addition, as a potent inhibitor of G1 cyclin-CDK complexes, p27^{KIP1} likely functions in G1-S check point control, which when lost, is associated with genomic instability and tumor progression (9). Taken together, these findings suggest that p27^{KIP1} may function as a tumor suppressor. To test this possibility, a replication-defective adenovirus vector containing p27^{KIP1} (Adp27^{KIP1}) was constructed that enabled us to investigate the effects of ectopic overexpression of this gene in human brain tumor cells both in cell culture and in a nude mouse xenograft model.

Methods

Construction of recombinant adenoviruses. A human p27^{KIP1} cDNA (obtained from Dr. Joan Massagué, Memorial Sloan Kettering Cancer Center, New York) was inserted into the pACCMVplpa vector. The resulting plasmid was cotransfected with the pJM17 plasmid into 293 cells (adenovirus E1a-transformed human embryonic kidney cells) by calcium phosphate/DNA coprecipitation. Homologous recombination between the two plasmids resulted in a recombinant replication-defective virus containing the human p27^{KIP1} gene (Adp27^{KIP1}) (procedure described in detail in references 10, 11). Viruses from 293-cell supernatants of cultures exhibiting a complete cytopathic effect were purified by cesium chloride banding. Viral titers were determined by plaque assay (12). pACCMVplpa and pJM17 were obtained from Dr. R. Gerard (University of Texas Southwestern Medical Center). Control recombinant replication defective adenovirus containing β-galactosidase (AdβGal) was obtained from Dr. J. Alcorn (University of Texas Southwestern Medical Center).

Cell culture. The human anaplastic astrocytoma cell line,

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Received for publication 27 November 1995 and accepted in revised form 9 February 1996.

1. *Abbreviations used in this paper:* Adp27^{KIP1}, recombinant, replication-defective adenovirus containing human p27^{KIP1} (p27 or KIP1) cDNA expression cassette; AdβGal, recombinant, replication-defective adenovirus containing β-galactosidase cDNA expression cassette; CDK, cyclin dependent kinase; pfu, plaque forming unit; moi, multiplicity of infection.

J. Clin. Invest.

0021-9738/96/04/1983/06 $2.00
Volume 97, Number 8, April 1996, 1983–1988

U373MG, was obtained from the American Type Culture Collection. This cell line exhibits LOH for the p53 tumor suppressor gene and contains a point mutation in exon 8, codon 282 (CGT→CAT) (a common mutational hotspot) (13). The D-54MG glioma cell line was obtained from Dr. S. Clifford Schold (University of Texas Southwestern Medical Center). The UMSCC38 squamous cell carcinoma cell line was obtained from Dr. Tom Carey (University of Michigan). Cells were maintained at 37°C, 5% CO_2 in RPMI Medium (Richter's Medium for D-54MG cells) containing 10% fetal bovine serum, 1% penicillin and streptomycin (GIBCO BRL, Gaithersburg, MD). Cells were cultured in 60mm plates to 80% confluence and were typically infected with purified Adp27^{KIP1} or Adβgal at 9 pfu/cell for 72 h before harvesting and analysis.

Western blot analysis. Infected cells were lysed in SDS buffer (1% SDS, 25 mM Hepes, 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptin, 0.1 mM 1,10 phenanthrolin) and the lysates were cleared by centrifugation at 10,000 *g*. 30 μg of protein extracts was separated electrophoretically by 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The membranes were incubated with a polyclonal rabbit anti-human p27^{KIP1} antibody (Santa Cruz Laboratories, Santa Cruz, CA) which was visualized using an ECL detection kit (Amersham Life Science, Arlington Heights, IL) according to the recombinations of the manufacturer.

[^{3}H]Thymidine incorporation assay. [^{3}H]Thymidine incorporation into DNA was measured as described elsewhere (14) using a Packard Model 1900CA Liquid Scintillation Counter (Downers Grove, IL).

Growth curves. Adp27^{KIP1} or AdβGal-infected cells were plated at a density of 5×10^4 cells per 60-mm plate. The number of cells was counted every 24 h using a hemocytometer. Viability was determined by exclusion of trypan blue dye.

Cell cycle distribution. 5×10^5 Adp27^{KIP1}- or AdβGal-infected cells were plated in 60-mm plates overnight. The exponentially growing cells were harvested using trypsin and fixed in 70% ethanol for 16 h at 4°C. Propidium Iodide was added (100 Kunitz units/ml) with RNase A (50 μg/ml) and cell cycle distribution was measured by determination of the DNA content per cell using a Becton-Dickinson Fluorescence Activated Cell Sorter, a Lysis II program to quantify cell cycle distribution and a doublet discrimination program as described elsewhere (15).

Aneuploidy. 5×10^5 U-373MG cells plated in 60 mm dishes were treated with nocodazole (Sigma) 0.12 μg/ml for 24 h before infection with AdβGal or Adp27^{KIP1} (9 pfu/cell). Cells were incubated for 48 h, split one to three and incubated for an additional 24 h before analysis of modal chromosome content per cell by flow cytometry as described in the previous paragraph (16). Cells were exposed to nocodazole (0.12 μg/ml) throughout the assay. Dead cells were excluded from FACS analysis by gating only viable cells.

Soft agar clonogenic assay. 1×10^4 Adp27^{KIP1} or AdβGal-infected cells were plated in dishes containing 0.3% top agarose/0.6% bottom agarose as described elsewhere (17). Foci (clump size $>$ 80 μm or $>$ 50 cells) were counted after 18 d.

Tumorigenicity in nude mice. According to a protocol approved by the University of Texas Southwestern Medical Center Animal Investigational Review Board, Adp27^{KIP1}- or AdβGal-infected U373MG cells were cultured for 72–96 h, harvested and resuspended in Hank's balanced salt solution. 1×10^7 cells in 200 μl were injected subcutaneously in the right (AdβGal) and left (Adp27^{KIP1}) flanks of nu/nu female mice (Harlan, Indianapolis, IN). Mice were killed after 25 or 45 d and tumor specimens were analyzed histologically.

Histologic analysis. Tumors were fixed in formalin, embedded in paraffin, sectioned by microtome, stained with hematoxylin and eosin, and analyzed by light microscopy.

Immunoprecipitation and histone H1 kinase assays. U-373MG cells were infected with Adp27^{KIP1} or AdβGal for 3 d before being labeled metabolically using the EXPRE^{35}S^{35}S protein labeling mix (DuPont NEN, Boston, MA) for 4 h. Immunoprecipitations were performed as described elsewhere (18) using a polyclonal antibody to the COOH termini of CDK2 and a monoclonal antibody to CDC2 (Santa Cruz Laboratories, Santa Cruz, CA). One-half of each sample was analyzed by 12.5% SDS-PAGE and visualized using autoradiography. The other half of each sample was resuspended in a reaction mixture (30 μl) containing 50 mM Tris-Hcl (pH 7.4), 10 mM Magnesium Chloride, 1 mM dithiothreitol (DTT), 1 μM ATP, 5 μCi of [γ^{32}P]ATP and 0.83 mg/ml histone H1 (Boehringer-Mannheim, Indianapolis, IN). Reactions were incubated for 30 min at 30°C and stopped with 6 μl of 5× sample buffer and analyzed by SDS-PAGE and autoradiography.

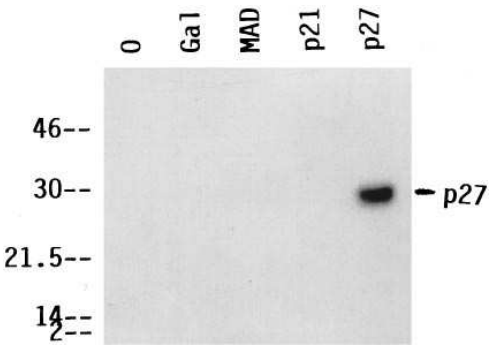

Figure 1. Western blot analysis. Total cell lysates were prepared from U-373MG cells (*0*) and those infected for 72 h with AdβGal (*Gal*), a replication-defective adenovirus containing the human MAD gene (*MAD*), a replication-defective adenovirus containing the human p21 gene (*p21*) or Adp27^{KIP1} (*p27*). 30-μg aliquots of protein extracts were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, exposed to polyclonal rabbit anti-human p27^{KIP1} antibody and detected by ECL.

Results

Antiproliferative effects of ectopically overexpressed p27^{KIP1}. To determine if ectopic overexpression p27^{KIP1} could inhibit the proliferation of an established human tumor cell line, U-373MG astrocytoma cells were infected with adenovirus constructs containing p27^{KIP1} (Adp27^{KIP1}) or β-galactosidase (Adβgal) (as a control). The U-373MG cell line is derived from an anaplastic astrocytoma tumor and contains a mutated p53 tumor suppressor gene (13).

We demonstrated previously that AdβGal exhibits 100%

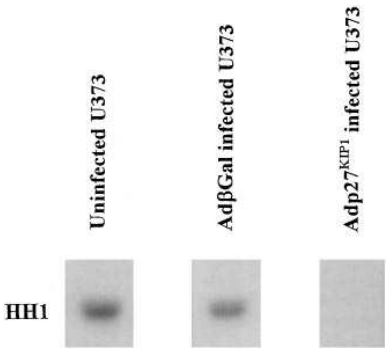

Figure 2. CDK2 Kinase activity in immunoprecipitates. Equivalent amounts of total cell lysates of uninfected U-373MG cells. AdβGal-infected U-373MG cells and Adp27^{KIP1}-infected U-373MG cells were immunoprecipitated using an anti-CDK2 antibody. Aliquots were assayed for kinase activity by quantitation of phosphorylated histone H1 (*HH1*) using a Phosphorimager.

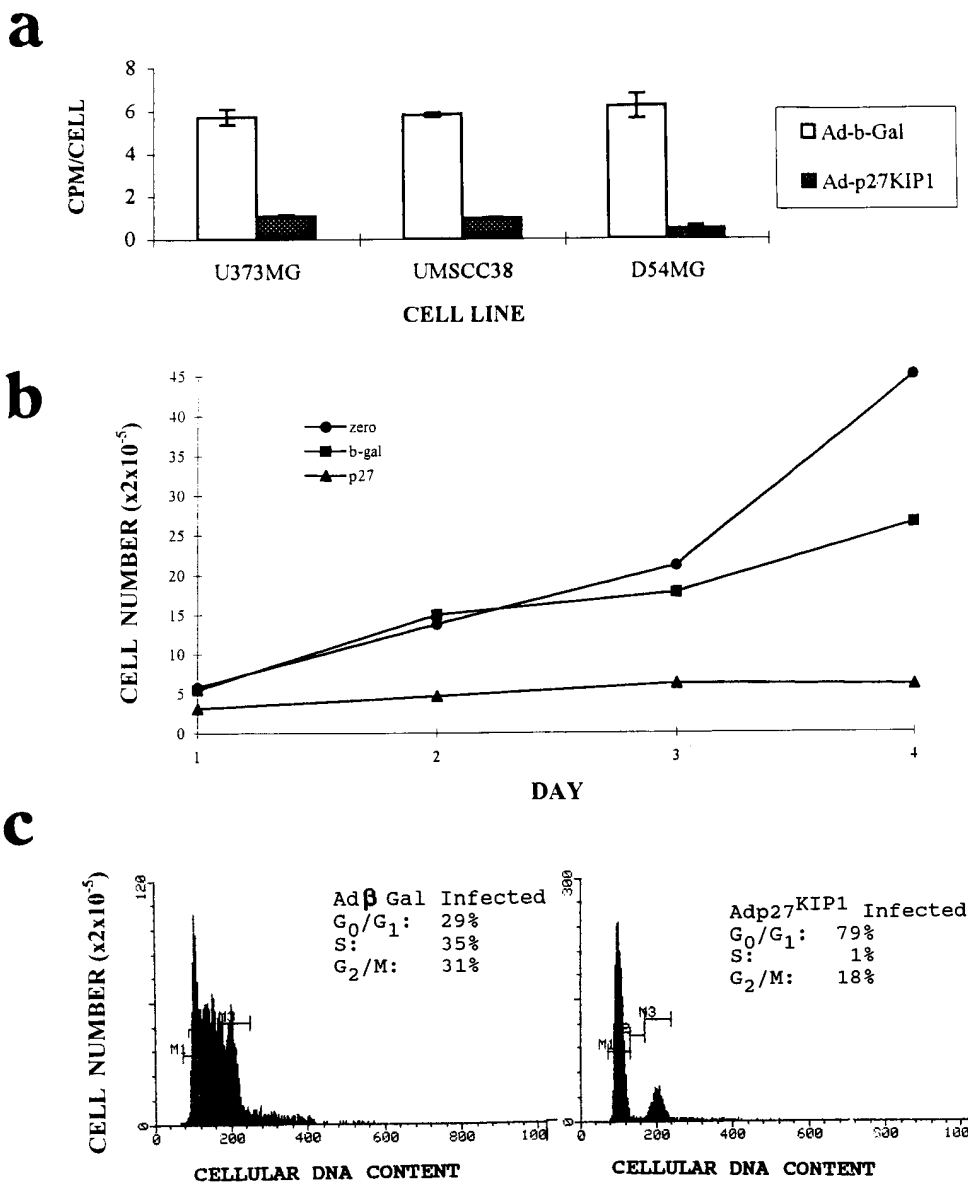

Figure 3. (*a*) [^{3}H]Thymidine incorporation in AdβGal- and Adp27^{KIP1}-infected U-373MG, UMSCC38, and D54MG cells. Subconfluent U-373MG, UMSCC38, and D54MG cells were infected with Adp27^{KIP1} (*solid bars*) or AdβGal (*open bars*). Experiments were performed in triplicate. The average amount of [^{3}H]thymidine incorporation in cpm (and SEM) is indicated at an moi of 9 pfu/cell for both viruses. (*b*) Growth curves of Adp27^{KIP1}- and AdβGal-infected cells. The numbers of cells in equivalent subconfluent cultures of uninfected U-373MG cells (●) and cells infected with Adp27^{KIP1} (▲) or AdβGal (■) were counted daily for 4 d. The average doubling time (from three experiments) of Adp27^{KIP1}-infected cells was 69.5±2.5 h compared with 32±2 h for uninfected or Adβgal-infected cells. (*c*) Cell cycle distribution in Adp27^{KIP1} and AdβGal infected cells. Cell cycle distribution was measured in infected cells that were cultured at low density (4×10^5 cells/60-mm plate) and fixed in ethanol, stained with Propidium Iodide and separated by FACS using a doublet discrimination program. A histogram of the distribution of the cells is demonstrated. The percent of cells in G_1, S and G_2/M is indicated on the right.

infectivity and persistence of expression for greater than 30 d by measuring β-galactosidase activity (11). Abundant expression of p27^{KIP1} protein in U-373MG cells infected with Adp27^{KIP1} was confirmed by Western blot analysis of total cell extracts; endogenous p27^{KIP1} was not detectable in these cells by this method (Fig. 1).

To determine if ectopically overexpressed p27^{KIP1} protein was biologically active, total cell lysates were immunoprecipitated using a polyclonal anti-cyclin-dependent kinase 2 (CDK2) antibody. Next, CDK2 kinase activity in the immunoprecipitates was measured using a histone H1 kinase assay. As compared to uninfected cells, AdβGal-infected cells exhibited minimal effect on CDK2 activity, while Adp27^{KIP1}-infected cells reduced CDK2 activity below detectable limits (Fig. 2).

To determine if ectopic overexpression of p27^{KIP1} affected cellular proliferation, [^{3}H]thymidine incorporation was measured in Adp27^{KIP1}-infected U-373MG cells and compared with AdβGal-infected and uninfected cells. While control adenovirus (i.e., AdpβGal) infection had a modest inhibitory effect on [^{3}H]thymidine incorporation, Adp27^{KIP1} infection was associated with a greater than fivefold decrease in [^{3}H]thymidine incorporation (Fig. 3 *A*).

Further evidence for inhibition of proliferation was obtained by measuring the rate of cell division of Adp27^{KIP1}-infected U-373MG cells as compared with AdβGal-infected or uninfected cells. The average doubling time (from two separate experiments) of Adp27^{KIP1}-infected cells was 69.5±2.5 h compared with 34±2 h for uninfected- or AdβGal-infected cells (Fig. 3 *b*).

To determine if the anti-proliferative effects of Adp27^{KIP1} were tumor cell-type specific, two additional human tumor cell lines UMSCC38 (human squamous cell carcinoma) and D-54MG (human glioma) were similarly tested. [^{3}H]Thymidine incorporation and the rate of cell division was inhibited equivalently by Adp27^{KIP1} in these cell lines as well (Figure 3 *a* and data not shown).

Previous studies demonstrated that increased expression of p27^{KIP1} is associated with G1 cell cycle arrest (2). Accordingly,

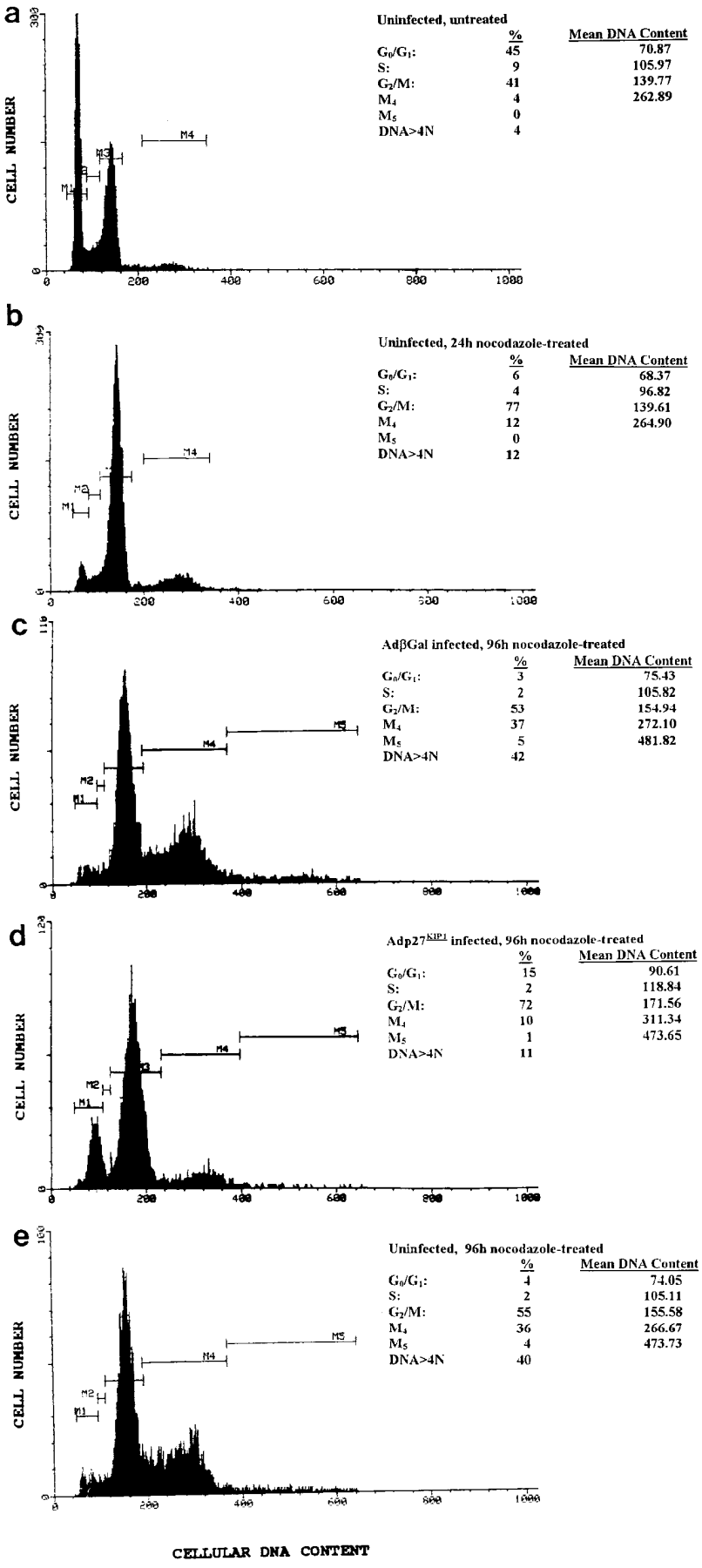

Figure 4. Effects of p27^{KIP1} on the accumulation of aneuploid cells. (*a*) Uninfected U-373MG cells not treated with nocodazole. (*b*) Uninfected U-373MG cells 24 h after treatment with nocodazole (0.12 μg/ml). After 24 h treatment of nocodazole, cells were infected with (*c*) AdβGal or (*d*) Adp27^{KIP1} for 48 h. (*e*) Uninfected, nocodazole-treated cells after 96 h. Cells were split at low density (4×10^5 cells/60-mm plate) 24 h before cell cycle distribution was measured by FACS. A histogram of the distribution of these cells is demonstrated. The percent of cells in each population M1 (G_0/G_1), M2 (S), M3 (G_2/M) M4 (aneuploid, DNA content tetraploid) or M5 (aneuploid, DNA content octoploid or greater) is indicated on the right. In all experiments, the mean fluorescence indicative of the DNA content per cell for each peak was similar as demonstrated by the mean values on the right. In each case, the experiment was repeated three times with similar results.

the cell cycle distribution of exponentially growing cultures of Adp27^{KIP1}-infected cells, AdβGal-infected cells and uninfected cells were determined by flow cytometry (Fig. 3 *c*). Seventy-nine percent of Adp27^{KIP1}-infected cells were in G_1 as compared with 29% of AdβGal-infected cells. The cell cycle distribution of uninfected cells was the same as that of AdβGal-infected cells (Fig. 3 *c*).

Effects of p27^{KIP1} on the accumulation of aneuploid cells. Mutation of the p53 tumor suppressor gene deregulates the G1-S check point rendering cells unable to dwell in G1 and repair DNA damage. This contributes to genomic instability manifested by aneuploidy and gene amplification, leading to the generation of genetically altered cells, some of which may be selected out and become malignant (19–21). Not surprisingly, we observed that p53-mutated U-373MG cells accumulated a population of aneuploid cells after long term passage in vitro (J. Chen and P. Nisen, unpublished observations). The accumulation of aneuploid cells can be exaggerated by treating cells with nocodazole, an agent that disrupts assembly of the mitotic spindle apparatus and causes mitotic arrest (16). To determine if ectopic overexpression of p27^{KIP1} could inhibit the accumulation of aneuploid U-373MG cells, subconfluent exponential cultures were treated with nocodazole and were subsequently infected with AdβGal or Adp27^{KIP1}. The distribution of uninfected, non-nocodazole-treated cells demonstrates four peaks: M1 (G_0/G_1, 45%), M2 (S, 9%), M3 (G2/M, 41%), and M4 (cells with DNA content > 4N, 4%) (Fig. 4 *a*). 24 h after nocodazole treatment, 12% of uninfected U-373MG cells were in the M4 peak corresponding to aneuploid cells with a DNA content tetraploid or greater (Fig. 4 *b*). 72 h after subsequent infection with AdβGal and continued exposure to nocodazole, 42% of the cells were aneuploid with DNA content per cell ranging from tetraploid (M4) to octaploid or greater (M5) (Fig. 4 *c*). This result was similar to the percentage of aneuploid cells in uninfected cells but incubated with nocodazole for 96 h (Fig. 4 *e*). A doublet discrimination program was used to exclude the possibility that the M4 and M5 peaks were due to cell aggregates. In contrast, only 10% of cells infected with Adp27^{KIP1} were aneuploid (Figure 4 *d*); these cells may have developed aneuploidy during nocodazole treatment in the 24 h before Adp27^{KIP1} infection (compare with Fig. 4 *b*). These findings indicate that Adp27^{KIP1}-infected cultures will not accumulate additional aneuploid cells in the presence of nocodazole. Similar results were obtained when U-373MG cells were separately infected with AdβGal and Adp27^{KIP1} for 48 h before treatment with nocodazole for 24–48 h (data not shown).

Effects of p27^{KIP1} on tumorigenicity. To determine whether p57^{KIP1} could affect the malignant potential of human tumor cells, the effects of Adp27^{KIP1} infection on the malignant phenotype of U-373MG cells was examined in vitro and in vivo.

As a measure of tumorigenicity in vitro, loss of anchorage-independent growth as manifested by focus formation in a soft agar clonogenic assay was measured in the U-373MG, UMSCC38 and D-54MG cell lines. In all cases, focus formation was decreased > 100-fold in Adp27^{KIP1}-infected cells as compared with Adβgal-infected cells or uninfected cells (Table I).

To investigate the malignant potential of Adp27^{KIP1}-infected cells in vivo, a nude mouse xenograft model was utilized. Female nu/nu mice were injected subcutaneously with equivalent amounts (1×10^7 cells) of adp27^{KIP1}-infected and AdβGal-infected U-373MG cells on opposite flanks. In all eight mice tested, large tumors formed on the side injected with AdβGal-infected cells, but no tumor formed on the side injected with Adp27^{KIP1}-infected cells after 45 d of observation (Fig. 5).

Table I. Focus Formation in Adp27^{KIP1}-infected cells

Cell Type	AdβGal-infected	Adp27^{KIP1} infected
U-373MG	153+/−29	1+/−1
D-54MG	719+/−6	33+/−2

Discussion

Ecotopic overexpression of p27^{KIP1} using a replication-defective adenovirus delivery system in a p53-mutated human astrocytoma cell line markedly affected cellular proliferation as measured by increased [^{3}H]thymidine incorporation, increased cell doubling time and the accumulation of cells in G1. The anti-proliferative effect of p27^{KIP1} is likely due to its ability to inhibit CDKs leading to accumulation of cells in G1. This was supported by our finding that CDK kinase activity was strikingly down-regulated in Adp27^{KIP1}-infected cells.

Ectopic overexpression of p27^{KIP1} was also associated with a marked decrease in the accumulation of aneuploid U-373MG astrocytoma cells. While this may be due to enhanced genomic stability, it may also be explained by a p27^{KIP1}-induced selection against proliferating aneuploid cells due to the induction of G1 arrest. However, the inhibition of aneuploid cell accumulation of p27^{KIP1}-infected cells cannot be explained exclusively by cell cycle arrest since nocodazole treatment, which causes mitotic arrest, did not inhibit aneuploidy in AdβGal-infected U-373MG cells. Similar observations have been made in nocodazole-treated murine fibroblasts that differ only with respect to p53 status; p53-mutated cells exhibited aneuploidy whereas p53-wild-type cells did not (6). The effect of p53 on genomic stability is presumably mediated through cell cycle checkpoint control (9, 19), possibly via the p53 transcriptional

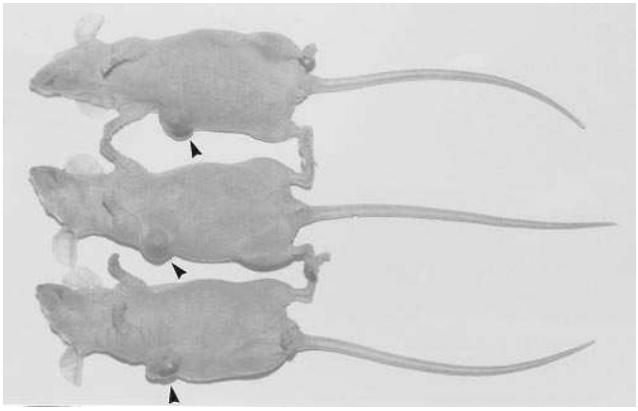

Figure 5. Tumor formation in nude mice. Equivalent numbers (1×10^7) of AdβGal- and Adp27^{KIP1}-infected U-373MG cells were injected subcutaneously in the right (AdβGal) and left (Adp27^{KIP1}) flanks of nu/nu female mice. Photograph representative of nine mice (25 d after injection) in which a tumor formed at the site injected with AdβGal- (*arrowheads*) but not Adp27^{KIP1}-infected cells.

target, p21$^{WAF/CIP1}$, which functions similarly to p27^{KIP1} (22, 23). In this context, ectopic overexpression of p27^{KIP1} may complement the cell cycle checkpoint defect in p53-mutated cells such as U-373MG.

Finally, ectopic overexpression of p27^{KIP1} in U-373MG astrocytoma cells was associated with a loss of anchorage independence and by the inability to generate tumors in a murine xenograft model. These findings combined with unique features of brain tumors, suggest a potential treatment modality for malignant brain tumors. Although adenovirus (or any other current delivery system) cannot selectively deliver genes to tumor cells, inadvertent ectopic overexpression of p27^{KIP1} should not be deleterious to adjacent nonreplicating postmitotic normal brain cells. Furthermore, although malignant brain tumors are generally amenable to at least partial neurosurgical resection, they are typically fatal due to local recurrence that cannot be controlled or prevented by conventional chemoradiography. Conceivably, Adp27^{KIP1} (or a construct similar to it) could be applied to a site after surgical resection and inhibit proliferation of residual tumor cells but not be harmful to normal brain. However, such an approach would be predicated on the development of nonimmunogenic adenovirus vectors which currently do not yet exist.

Acknowledgments

We wish to thank Drs. Robert Gerard and Joe Alcorn for their assistance with the adenovirus vectors, Dr. Joan Massagué for generously providing the p27^{KIP1} cDNA and Dr. John Kettman, Jr. for his extensive assistance with flow cytometry and critical analysis of our data. P.D. Nisen was supported by National Institutes of Health grant CA60173, American Cancer Society grant EDT-96, the Lowe Foundation and the Klion Family Fund for Pediatric Research.

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