Relationship between Expression of p21*WAF1/CIP1* and Radioresistance in Human Gliomas

Takashi Kokunai[1] and Norihiko Tamaki

Department of Neurosurgery, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017

The role of p21*WAF1/CIP1* (p21) in DNA repair and apoptosis following γ-irradiation remains controversial. In this study the influence of p21 on the radiosensitivity of human brain tumors was investigated. Resected tumors were stained immunohistochemically for p21. Expression of p21 in astrocytic tumors was high, but it was low in medulloblastomas, germinomas, and primary malignant lymphomas. Glioma and medulloblastoma cell lines were transfected with pcDNA/*p21* to cause p21 overexpression, then tumor-cell colony formation and apoptosis were assessed following γ-irradiation of the transfected and nontransfected cells. Overexpression of p21 enhanced clonogenic survival and suppressed apoptosis after γ-irradiation in human brain tumor cell lines with or without p53 protein deficiency. Radioresistance was acquired when p21 was overproduced in the glioma cell lines irrespective of p53 status.

Key words: p21 — Radiosensitivity — Glioma — Apoptosis — p53

The recently cloned protein p21*WAF1/CIP1* (p21) is a key downstream mediator of wild-type *p53* function in growth control and cell cycle progression,[1] forming multiple quaternary complexes with cyclins, cyclin-dependent kinases (cdk), and proliferating cell nuclear antigen (PCNA).[2–4] The p21 protein potently regulates the cell cycle by inhibiting cdk, which is required for progression from the G1 to the S phase, and also inhibits activation of DNA polymerase δ by PCNA, preventing DNA replication. Transcription of *p21* is induced by overexpression of wild-type *p53*[1,5] or by activation of *p53* after DNA damage.[6] Upregulation of p21 may also be triggered by several differentiation-inducing agents in hematopoietic and other cells through *p53*-independent pathways.[7–9]

Although p21 appears central to G1 arrest, participation of p21 in *p53*-dependent and *p53*-independent cell death remains controversial. Inhibition of p21 expression by transfection of *p21* antisense oligonucleotides has been shown to interfere with growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and to result in cell death.[10] In a tetracycline-inducible system using *p53*-deficient cells, p21 enhanced the cellular capacity to repair ultraviolet irradiation damage in reporter plasmids,[11] and HCT-116/p21 cells lacking the *p21* gene exhibited greater sensitivity to *cis*-platinum and nitrogen mustard than the parent HCT-116 cells.[12] These results suggest that p21 may participate directly in modulating nucleotide excision repair of damage caused by *cis*-platinum, nitrogen mustard, or ultraviolet exposure.

In contrast to ultraviolet irradiation or *cis*-platinum, DNA damage induced by γ-irradiation generates DNA strand breaks and other types of DNA damage that are repaired primarily by mechanisms not involving nucleotide excision repair. Another proposed mechanism of processing of DNA damage induced by γ-irradiation is apoptosis via *p53*-dependent or *p53*-independent pathways.[13–16] Recently, HCT-116/p21$^{-/-}$ cells have been found to be more readily killed by γ-irradiation than their p21$^{+/+}$ counterparts.[17] More recently, in DLD-1 human colorectal carcinoma cells, mutant *p21* lacking any cdk-inhibitory activity failed to prevent apoptosis induced by γ-irradiation or adriamycin.[18] However, another study using HCT-116 cells found that the clonogenic survival of cells exposed to γ-irradiation was not affected by p21 disruption.[19] Whether p21 protects cells against death from γ-irradiation remains to be established.

In the present study, we investigated the role of p21 in determining the radiosensitivity of human brain tumors. Glial tumors, the most common primary central nervous system tumors, are resistant to γ-irradiation.[20,21] Although *p21* is frequently overexpressed in astrocytomas, anaplastic astrocytomas, and glioblastomas,[22] p21 expression has not been examined in radiosensitive brain tumors such as medulloblastoma,[23] germinoma,[24] and primary malignant lymphoma.[25] We found greater p21 expression in radiosistant astrocytic tumors than in more radiosensitive brain tumors. We also noted that overexpression of p21 increased radioresistance in brain tumor cell lines with or without wild-type p53. Our results suggest that p21 facilitates tumor cell survival in human gliomas with DNA damage induced by γ-irradiation.

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[1] To whom correspondence and reprint requests should be addressed. E-mail: kokunai ksb@knc.kakogawa.or.jp

MATERIALS AND METHODS

Tissues and immunoperoxidase staining Four normal brain tissue specimens were obtained at autopsy. Tumor tissues from 5 astrocytomas, 10 anaplastic astrocytomas, 11 glioblastomas, 4 ependymomas, 12 medulloblastomas, 4 germinomas, and 3 malignant lymphomas were obtained from surgical specimens at Kobe University Hospital, fixed with 10% buffered formalin, and embedded in paraffin. Paraffin-embedded sections were deparaffinized with xylene and rehydrated in a graded ethanol series including phosphate-buffered saline (PBS), pH 7.4. Sections were incubated with 0.03% H_2O_2 in methanol for 20 min, washed with PBS for 20 min and then incubated with 5% normal goat serum in PBS for 30 min followed by monoclonal anti-p21 antibody (#SC 817, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at 37°C. They were washed with PBS for 20 min, then incubated with biotinylated goat anti-mouse IgG for 30 min at 37°C, washed again with PBS for 20 min, and incubated with avidin-biotin-peroxidase complex for 30 min at 37°C. Another wash with PBS was followed by peroxidase reaction with 0.06% diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-HCl (pH 7.0) for 5 min. Negative control sections were incubated with nonimmune mouse IgG instead of anti-p21 antibody. Three sections from each specimen were chosen for counting positive cells and calculation of the p21 positivity index. Cells were counted in groups including a total of 1000 nuclei, and numbers of stained cells were expressed as a percentage.

Cell culture Human glioma cell lines (T-98G, U-251MG, and U-87MG) were obtained from the Japanese Cancer Research Resources Bank (Tokyo). The human medulloblastoma cell line, MED-3, was developed in our laboratory from a cerebellar medulloblastoma.[26] The U-87MG-S cells were obtained from U-87MG cells by single-cell dilution cloning using 96-well cell-culture plates. These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and kanamycin sulfate (50 μg/ml) and grown in monolayer culture at 37°C. T-98G and U-251 MG cells were previously shown to contain a mutant *p53* allele in codons 237 and 273, respectively, with concomitant loss of expression of a wild-type *p53*.[27] U-87MG cells have been shown to contain a wild-type, functional *p53* allele,[27] as were MED-3 cells (unpublished data).

Gene transfer The expression vector plasmid pcDNA/*p21* contains a 492-base-pair *p21* coding region cDNA, a human cytomegalovirus promoter, an SV40 early polyadenylation signal, and a neomycin-resistance gene.[28] Cells near 70% confluence in 60-mm plates were transfected using 30 μg of lipofectin (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD) and 10 μg of pcDNA/*p21* for 24 h. At 2 days after transfection, G418

(600 μg/ml, Sigma Chemical, St. Louis, MO; cells not transfected die at this G418 concentration) was added. After pcDNA/*p21* transfection and G418 treatment, G418-resistant colonies were isolated. Selected transfectants were maintained in medium as above with G418. A transfectant with pcDNA3.1 plasmid was used as a negative control.

Analysis of *p21* mRNA with reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was prepared from cells using guanidium thiocyanate[29] and converted to a single-stranded cDNA by reverse transcriptase with oligo(dT) primer. Synthesized cDNA was used as a template in PCR with two sets of oligonucleotide primers. Human *p21* cDNA was amplified by PCR using a previously reported method.[28] The primer pairs used were 5′-ATGTCAGAACCGGCTGGGGAT-3′ and 5′-GGGCT-TCCTCTTGGAGAAGAT-3′.

The PCR reaction was carried out in a thermal cycler for 30 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 1 min at 72°C. The PCR product using the primers for the glyceraldehyde 3-phosphate dehydrogenase gene (*G3PDH*) was used as an internal control.

Western analysis of p21 Cells were dissolved with 500 μl of cell lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.01% sodium dodecylsulfate (SDS), 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), and 2 mM phenylmethylsulfonyl fluoride. Lyophilized samples were dissolved at a protein concentration of 1 mg/ml in buffer containing 2.5% SDS, 5% 2-mercaptoethanol, and 0.01 M Tris-HCl (pH 8.0), and boiled for 5 min at 100°C. SDS-polyacrylamide gel electrophoresis (PAGE) and transfer of proteins onto nitrocellulose filters were performed using standard methods. The nitrocellulose filters were blocked with nonfat milk and then incubated with anti-p21 monoclonal antibody (Santa Cruz Biotechnology). Antibody binding to the filters was detected using the Amersham ECL system (Amersham Japan, Tokyo).

Cell-cycle analysis Cell-cycle analysis was performed as previously described.[30] Cells in the exponential growth phase were labeled with 10 μM bromodeoxyuridine (BrdU) (Sigma Chemical) and incubated at 37°C for 2 h. The cells were washed twice with PBS, trypsinized, centrifuged, and washed twice with PBS/0.1% bovine serum albumin (BSA). Cell pellets (10^6 cells) were fixed with 70% ethanol for 30 min on ice. Before staining for BrdU with an anti-BrdU antibody conjugated to FITC (Becton Dickinson), the cell pellet was denatured in 2 N HCl/Triton X-100 for 30 min and then brought to pH 8.5 in 0.1 M $Na_2B_4O_7$. Cells were suspended in 0.5% Tween 20/1.0% BSA/PBS for the antibody reaction. Before analysis, cell pellets were suspended in PBS containing 5 μg/ml propidium iodide. Cells (10^5) were analyzed using a fluorescence-activated cell sorter and Lysis II software (FACScan, Becton-Dickinson).

Colony-forming assays in semisolid agar medium Colony-forming assay was performed as reported previously.[31] A single cell suspension was made of 10^6 cells in the exponential growth phase with or without a prior single-dose γ-irradiation. The cells were suspended in 4 ml of 0.33% Special Noble Agar (Difco Laboratories, Detroit, MI) and plated on growth medium containing 0.5% soft agar. Fourteen days after cell plating, colonies exceeding 50 cells were counted. Plating efficiencies under 0.01% were considered negative for colony formation. Experiments were performed at least three times each and pooled data were fitted to a linear-quadratic equation to obtain estimates of the survival fraction at 2 Gy (SF 2 value). Statistical analysis of differences was carried out using Student's *t* test. A ^{60}Co source was used for γ-irradiation of cells. The dose rate at the time of irradiation was 1.56 Gy/min.

DNA fragmentation For this assay, we used a modification of the protocol described by Duke *et al.*[32] Fragmentation of cellular DNA was measured 24 h after a 6 Gy dose of γ-irradiation as above. Adherent and nonadherent cells were pooled, washed in PBS, and suspended in ice-cold buffer containing 0.15 M NaCl, 10 mM Tris (pH 4.2), 2 mM MgCl$_2$, and 1 mM dithiothreitol. Nonidet P-40 (Sigma Chemical) was added to a final concentration of 0.5% (v/v), and the samples were incubated on ice for 30 min. Nuclei were isolated by centrifugation, suspended in buffer containing 0.35 M NaCl, 10 mM Tris (pH 7.4), 2 mM MgCl$_2$, and 1 mM dithiothreitol, and incubated on ice for 30 min. Nuclei were removed by centrifugation, and the supernatant was extracted with phenol and chloroform. Low-molecular-weight DNA was recovered by ethanol precipitation. Samples were suspended in 20 μl of Tris-EDTA and treated with RNAase A for 30 min prior to electrophoresis on 2% agarose gels. DNA was visualized by ethidium bromide staining.

Hoechst 33258 staining At 24 h after treatment with or without γ-irradiation, cells were fixed on dishes with methanol and stained for 10 min with Hoechst 33258 (Calbiochem, La Jolla, CA) at 0.5 μg/ml in H$_2$O. The percent-
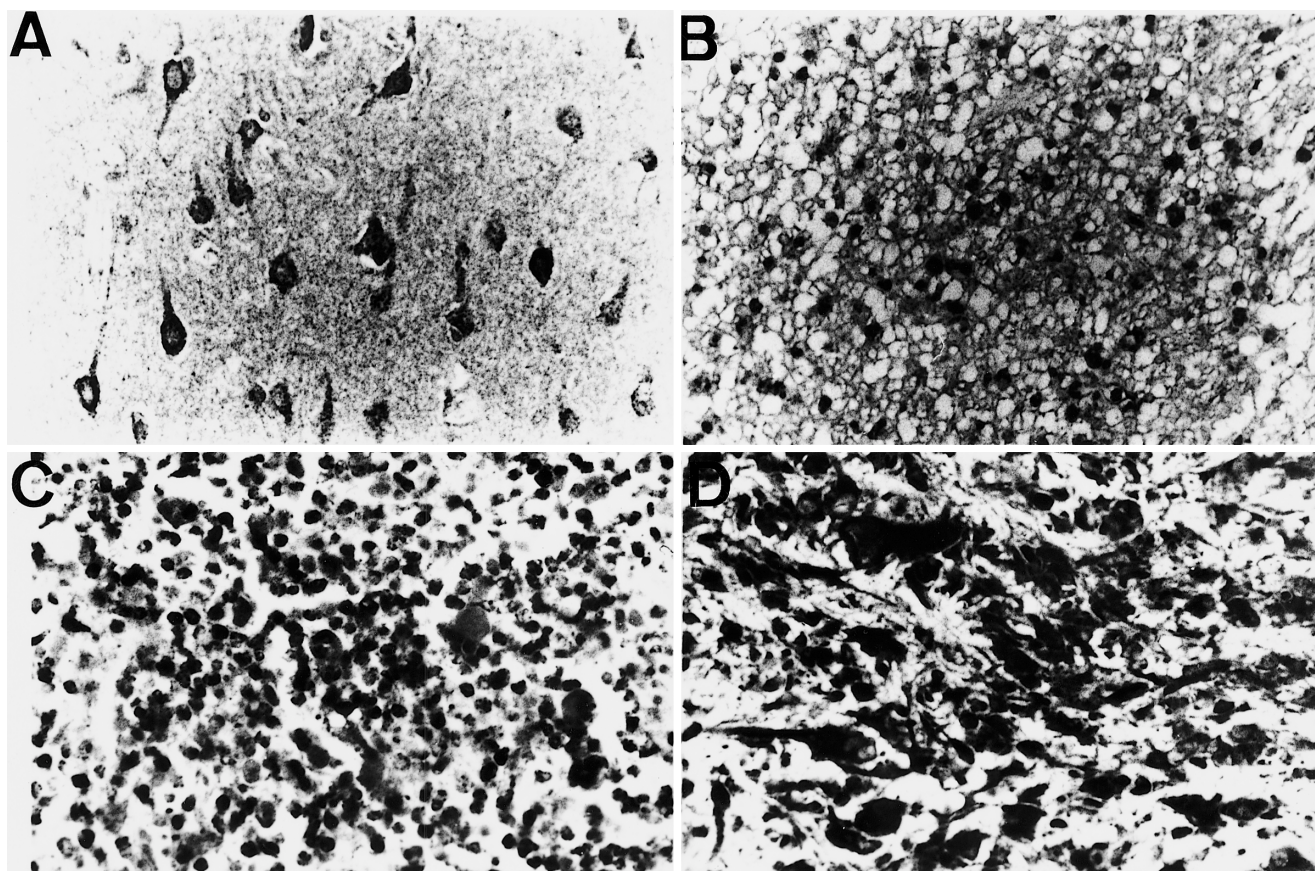

Fig. 1. Expression of p21 in normal brain (A), astrocytoma (B), anaplastic astrocytoma (C), and glioblastoma (D) demonstrated by immunohistochemical staining with anti-p21 antibody. Original magnification, ×400.

age of cells containing apoptotic nuclei was determined by fluorescence microscopy at a magnification of ×400. Cells with three or more condensed chromatin fragments were considered apoptotic. For each sample 500 to 700 cells were counted.

RESULTS

Expression of p21 in human brain tumors In this study the 4 normal brain samples showed p21 expression. p21-positive cells were seen mainly among neurons and glial cells (Fig. 1), but not vascular endothelial cells. The 49 brain tumors examined all showed varying degrees of p21 expression. Astrocytic tumors, such as astrocytoma, ana-

Table I. p21 Expression in Normal Brain and Brain Tumors

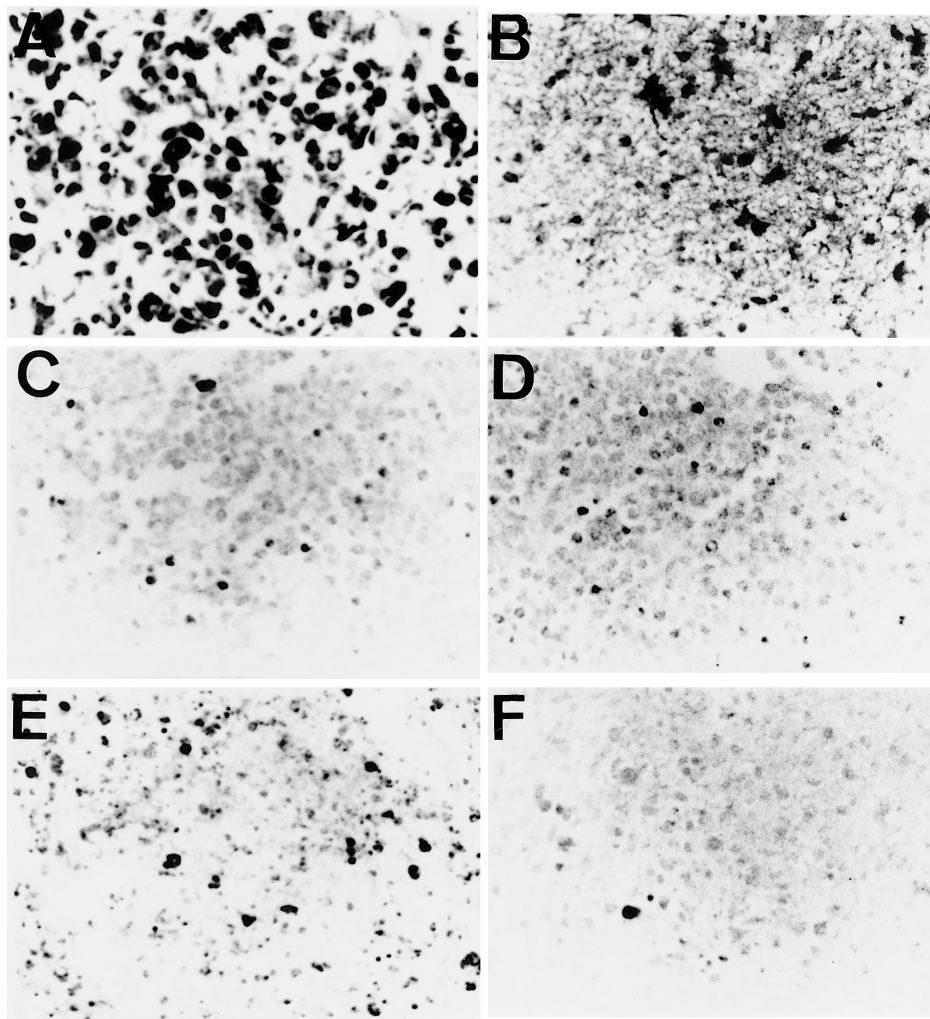| Tissue	No. of cases	Positivity index of p21(%)[a]
Normal brain	4	58.15±4.88
Astrocytoma	5	66.92±5.78
Anaplastic astrocytoma	10	71.97±9.92
Glioblastoma	11	77.41±9.03
Ependymoma	4	52.85±9.38
Medulloblastoma	12	15.21±6.28
Germinoma	4	6.48±2.37
Malignant lymphoma	3	9.85±2.52

a) No. of positive nuclei/1000 nuclei (mean±SD).

Fig. 2. Expression of p21 in medulloblastoma (C and D), malignant lymphoma (E), and germinoma (F); compare with the higher levels of expression in anaplastic astrocytoma (A) and gliosis (B). Immunohistochemical staining with anti-p21 antibody, original magnification, ×400.
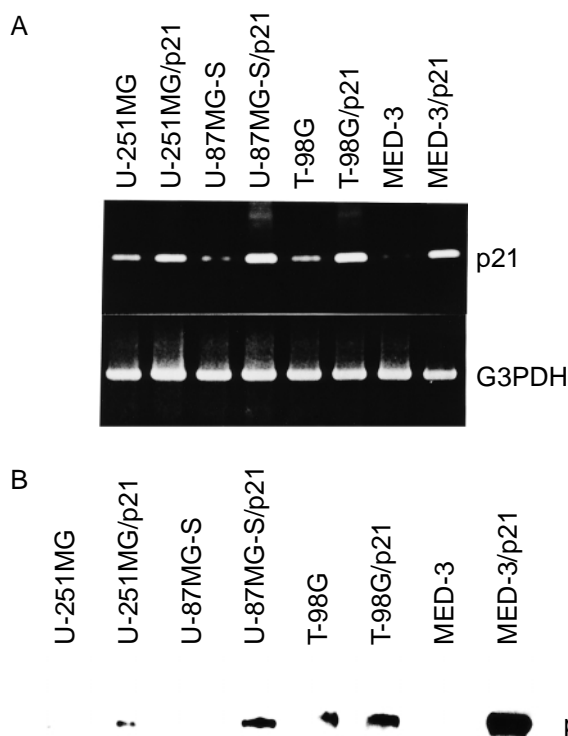
A

B

Fig. 3. Expression of *p21* mRNA and p21 protein in parent cells and *p21* transfectants. A: *p21* mRNA bands after amplification. Products generated using glyceraldehyde 3-phosphate dehydrogenase primers were used as an internal control. B: expression levels of p21 protein in parent cells and *p21* transfectants analyzed by gel electrophoresis and western blotting.

Table II. Flow-cytometric Analysis of G1 Phase in the Cell Cycle in Parent Cells and Their *p21* Transfectants

Cells	% G1 phase (mean±SD)	% S phase	% G2M phase
MED-3	12.8±1.0	44.3±2.23	42.9±1.72
MED-3/p21	64.2±6.36[a]	20.2±1.75	16.2±6.37
U-87MG-S	31.8±6.25	41.5±3.25	29.3±8.56
U-87MG-S/p21	63.8±7.22[b]	15.1±3.4	18.3±3.84
U-251MG	50.7±1.51	30.0±4.93	21.7±6.17
U-251MG/p21	66.6±4.57[b]	18.5±5.55	13.9±9.66
T-98G	60.3±3.33	20.8±6.96	17.7±7.32
T-98G/p21	72.0±2.36[b]	11.0±1.59	16.7±3.54

a) Significantly different from parent cells ($P<0.01$ by Student's *t* test).
b) Significantly different from parent cells ($P<0.05$ by Student's *t* test).

plastic astrocytoma, and glioblastoma (Fig. 1) showed high positivity indices for p21 (means, 58.15, 66.92, and 71.97%, respectively; Table I). The positivity index of p21 in astrocytic tumors was significantly higher than in normal brain ($P<0.05$). No relationship was seen between positivity index for p21 and histologic maligancy of astrocytic tumors ($P>0.05$). In contrast, radiosensitive tumors such as medulloblastoma, germinoma, and primary malignant lymphoma (Fig. 2) showed low positivity indices for p21 (15.21, 6.488, and 9.85%, respectively; Table I). The positivity indices for radiosensitive tumor type were significantly different from those of astrocytic tumors ($P<0.01$). These results suggest a relationship between p21 expression and radiosensitivity.

Characterization of human brain tumor cell lines transfected with *p21* gene Investigations of the role of p21 in the radiosensitivity of brain tumors were conducted in two human astrocytoma cell lines with mutant-type *p53*, U-251MG and T-98G cells, and in two wild-type *p53*-positive cell lines, U-87MG-S and MED-3. U-251MG and

T-98G cells showed higher basal levels of p21 expression than those seen in U-87MG-S and MED-3 cells, suggesting that p21 expression in human brain tumor cells is *p53*-independent. In stable *p21* transfectants, *p21* mRNA (Fig. 3A) and p21 (Fig. 3B) were confirmed to be overexpressed. These transfectants with an exogenous *p21* gene showed growth delay and phenotypic differentiation.[28]

Cell cycle alterations after transfection of *p21* (Table II) Cell cycle patterns were analyzed in parent cells and in transfectants using a BrdU incorporation assay.[30] Intrinsic sensitivity of cells to γ-irradiation has been reported to be a function of cell cycle phase at exposure. In some studies,[33–35] cells were more sensitive in the G2/M and early S phases. Mean fractions of cells in the G1 phase for MED-3 and U-87MG-S were 12.8% and 31.8%, respectively. In contrast, the G1 phase in lines representing radioresistant tumors, U-251MG and T-98G, accounted for 50.7% and 60.3% of cells, respectively. Fractions of transfectants in the G1 phase were much larger than in the radiosensitive parent cells. In radioresistant lines, MED-3/p21 and U-87MG-S/p21, G1 fractions were 64.2% and 63.8%, respectively; U-251MG/p21 and T-98G/p21 cells had G1 fractions of 66.6% and 72.0% respectively, which are elevated compared to the parent cells.

Decreased sensitivity to γ-irradiation after transfection with p21 Clonogenic survival curves are shown in Fig. 4. The SF 2 values ranged from 0.061 to 0.92 in brain tumor cell lines, as shown in Table III. MED-3 and U-87MG-S cells were more radiosensitive than U-251MG and T-98G cells. The SF 2 values of MED-3/p21 and U-87MG-S/p21 cells were 0.69 and 0.47, respectively, differing significantly from those in the parent cells ($P<0.01$). In radioresistant cell lines, SF 2 values in transfectants with *p21* cDNA differed from those of the parent cells ($P<0.05$).
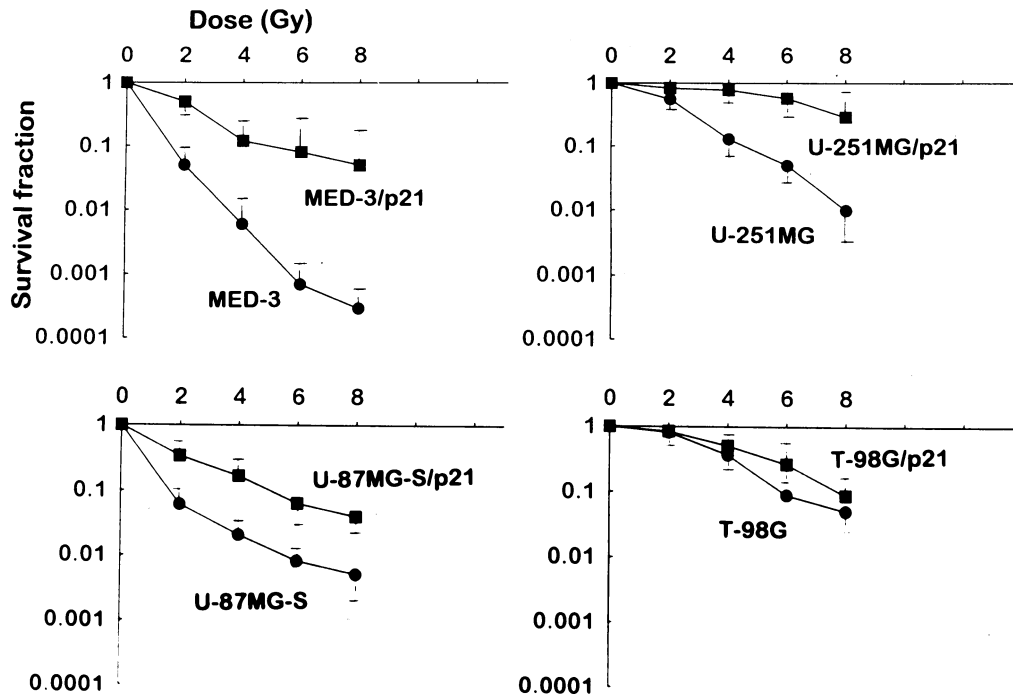
Apoptosis induced by γ-irradiation While a DNA frag-

Fig. 4. Survival curves of parent cells and *p21* transfectants after γ-irradiation.

Table III.   Radiation Dose-cell Survival Fraction at 2 Gy (SF 2 Value) for the Cell Lines in Clonogenic Cell Survival Assay

Cells	SF 2 value (mean±SD)
MED-3	0.061±0.008
MED-3/p21	0.69±0.177[a]
U-87MG-S	0.077±0.008
U-87MG-S/p21	0.47±0.106[a]
U-251MG	0.35±0.106
U-251MG/p21	0.89±0.035[a]
T-98G	0.48±0.099
T-98G/p21	0.92±0.014[a]

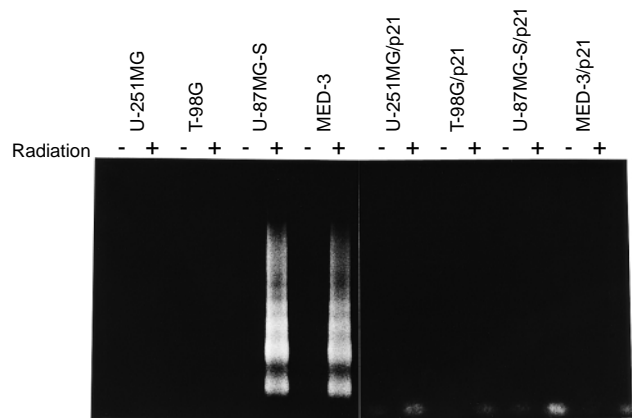*a*) Significantly different from parent cells (*P*<0.05 by Student's *t* test).

Fig. 5. DNA fragmentation analysis in MED-3, U-87MG-S, U-251MG, and T-98G cells, and in *p21* transfectants exposed to a 6 Gy dose of γ-irradiation.

mentation assay (Fig. 5) revealed DNA ladder formation in radiosensitive MED-3 and U-87MG-S cells after γ-irradiation, induction of p21 overexpression resulted in a marked reduction of DNA ladder formation in MED-3 and U-87MG-S cells following γ-irradiation. Quantitative analysis of apoptotic cells using Hoechst 33258 staining showed more apoptotic nuclei in MED-3 and U-87MG-S cells than in radioresistant cells after γ-irradiation (Table IV). The p21-overexpressing cells showed much greater inhibition of γ-irradiation-induced apoptosis than the parent cells, especially among radiosensitive lines (Fig. 6).

DISCUSSION

Expression of p21 in brain tumor tissues   This study investigated the role of p21 in the radiosensitivity of human brain tumors. We found high p21 expression in radioresistant astrocytic tumors, but low expression in radiosensitive medulloblastomas, germinomas, and primary malignant lymphomas.

Table IV. % Apoptotic Nuclei in Parent Cells and Their *p21* Transfectants Analyzed Using Hoechst 33258 Staining

Cells	% apoptotic nuclei (mean±SD)
MED-3	61.3±5.79
MED-3/p21	12.9±0.57[a]
U-87MG-S	45.0±1.47
U-87MG-S/p21	12.9±1.47[a]
U-251MG	19.6±2.77
U-251MG/p21	13.3±1.44[a]
T-98G	17.2±2.40
T-98G/p21	10.8±1.37[b]

a) Significantly different from parent cells (*P*<0.01 by Student's *t* test).
b) Significantly different from parent cells (*P*<0.05 by Student's *t* test).

Fig. 6. Nuclear morphology analysis of MED-3 (A) and MED-3/p21 (B) cells visualized by Hoechst 33258 staining.

No previous reports have analyzed the relationship between p21 expression in tumor tissue and radiosensitivity. In our study, a striking inverse relationship was evident between p21 expression and radiosensitivity in human brain tumors. It has been reported that p21 expression is absent in most fetal tissues, in contrast to adults.[36] Generally, fetal tissues are thought to be more radiosensitive than adult tissues. Jung *et al.*[22] have reported that p21 was elevated in most gliomas tested, regardless of histologic grade, and suggested that both *p53*-dependent and *p53*-independent mechanisms are involved in p21 expression. Similarly, we found no relationship between positivity index for p21 and histologic maligancy of astrocytic tumors. These results suggest that p21 expression in human astrocytic tumors is partially *p53*-independent.

The effect of p21 overexpression on radiosensitivity in glioma cells Transfection of *p21* cDNA induced stable overexpression of p21 in brain tumor cells with mutant-type p53 (U-251MG and T-98G), as well as in those with wild-type p53 (U-87MG-S and MED-3). High levels of p21 expression enhanced clonogenic survival and caused suppression of apoptosis after γ-irradiation, irrespective of the tumor cell line's p53 status. Radiosensitive U-87MG-S and MED-3 cells showed low basal p21 levels despite having wild-type p53, while radioresistant U-251MG and T-98G cells showed relatively high p21 expression. Recently, some p53 mutants found in tumors have shown a subtle transcriptional defect affecting bax induction, but not p21 induction.[37, 38] Some *in vivo* and *in vitro* studies have shown that p21 expression is actively controlled through *p53*-independent pathways.[39–41] The suggestion was made that some p53 mutants retain the ability to activate p21-mediated G1 arrest and protection from apoptosis, but not bax-mediated apoptosis. Expression of *p21* mRNA and the corresponding protein appeared to be *p53*-independent in human brain tumor cells.
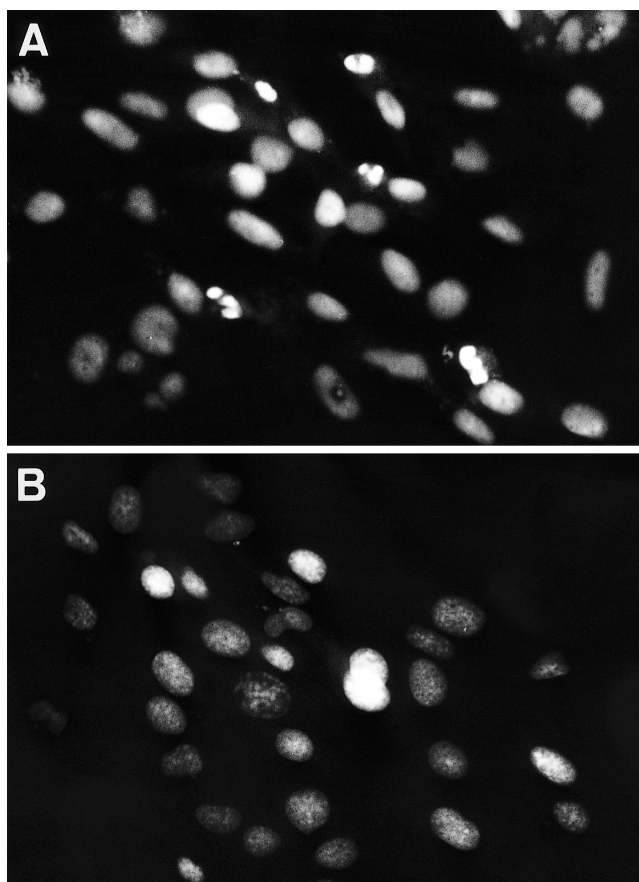
We observed that overexpression of p21 induced G1 arrest and prevention of γ-irradiation-induced apoptosis, suggesting that DNA repair occurs during the G1 phase and that the increasing effect of p21 on radioresistance may result from cell cycle arrest in the G1 phase. A recent study has shown that cells deficient in p21 displayed a markedly different response to DNA-damaging agents than cells with an intact p21 checkpoint. Cells with intact p21 entered stable arrest following DNA damage by γ-irradiation or chemotherapeutic agents, while cells with a defective p21 response underwent apoptosis which apparently was induced by uncoupling between mitosis and the S phase after DNA damage. Cells without functioning p21 continued to undergo DNA synthesis in the absence of mitosis, culminating in apoptosis.[17] However, Shim *et al.*[42] have recently reported an inhibitory effect of p21 on c-Jun N-terminal kinase (JNK), a key regulatory protein in multiple stress-activated pathways. As JNK has recently been implicated in apoptosis, its interaction with p21

raises the intriguing possibility that p21-mediated inhibition of apoptosis may be linked directly to its inhibitory effect on JNK. More recently, p21 has been shown to permit repair of DNA damage derived from [1,3-bis(2-chloroethyl)-1-nitrosourea or *cis*-platinum, as well as protecting glioma cells from chemotherapy-induced apoptosis. The p21 protein was suggested to participate in nuclear factor κB-dependent gene expression through p300, as well as in the expression of chemoresistance genes.[43, 44] Further, the Fas-initiated death signaling was endogenously prevented by p21-binding to procaspase 3, resulting in cell survival.[45]

In summary, we think that p21 has various inhibitory functions against apoptotic signals, including those derived from γ-irradiation, and p21 may promote survival of glioma cells exposed to a broad range of DNA-damaging agents; the p21 status of gliomas might predict general responsiveness to such agents. Furthermore, p21 may represent an important new target for radiosensitization protocols, possibly involving antisense oligonucleotides directed against *p21*.

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