Malignant gliomas are central nervous system tumors that are frequently resistant to surgery, irradiation, and chemotherapy. Most glioblastoma patients die within 1 or 2 years of diagnosis because of ineffective salvage therapies.\textsuperscript{1-3} In recently published reports, most research has primarily addressed radiotherapy combined with adjuvant chemotherapy in the treatment of gliomas.\textsuperscript{4,5} Radiotherapy combined with temozolomide improves overall survival in newly diagnosed glioblastomas and has become a new standard of care for newly diagnosed glioblastoma patients. However, the optimal dosing schedule and duration of adjuvant treatment remain unclear, and the current role for neoadjuvant therapy is poorly defined. A better understanding of the mechanism of action of radiotherapy in the treatment of gliomas may boost research on targeted agents that can be used in combination with radiotherapy, and will help determine the optimal dosing schedule and duration of adjuvant treatment.

The cellular response to radiation is complex. As a consequence of DNA damage, cell cycle arrest occurs, allowing for DNA repair before mitosis takes place. If repair fails, several outcomes are possible: apoptosis, senescence, mitotic catastrophe, and transformation.\textsuperscript{6} Apoptotic cells demonstrate a number of distinct morphological and biochemical features, such as marginalization and condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, and, finally, formation of apoptotic bodies.\textsuperscript{7,8} Apoptosis is a characteristic mode of cell destruction and represents a major regulatory mechanism for removing abundant and unwanted cells during embryonic development, growth, differentiation, and normal

\textbf{Single-fraction $\gamma^{60}$Co radiation induces apoptosis in cultured rat C6 cells}

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BACKGROUND AND OBJECTIVES: Radiotherapy is frequently applied in the treatment of malignant gliomas, but it is unclear if radiotherapy exerts its effects via induction of apoptosis. The present study was designed to determine whether a single-fraction $\gamma^{60}$Co radiation can induce apoptosis.

DESIGN AND SETTING: In vitro cytological controlled study performed at a military medical university from October 2006 to June 2008.

METHODS: C6 cells were treated with a single fraction of $\gamma^{60}$Co radiation at various doses (0, 4, 16, and 64 Gy). The 3-(4,5)-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) assay, apoptosis assays using Annexin V-fluorescein isothiocyanate /propidium iodide or Hoechst 33258 staining, and the cell cycle assay were performed, and the expression of p53 and p21 proteins was evaluated.

RESULTS: The C6 cell numbers in the 16 Gy and 64 Gy groups were much lower than in the control group at 48, 96, and 144 hours after irradiation. The irradiated cells underwent apoptosis in a dose-dependent manner. Irradiation also impacted cell cycle progression, arresting cells in the G1 phase. The p53 protein expression was shown in both the nucleus and the cytoplasm of irradiated cells, whereas p53 was only expressed in the nucleus of control (untreated) cells. The p21 protein was expressed in irradiated cells but not in control cells.

CONCLUSIONS: Single-fraction $\gamma^{60}$Co radiation inhibited C6 cell growth by inducing apoptosis and G1 arrest, which correlated with the up-regulation of the p53-p21 pathway. The extent of apoptosis and G1 arrest was positively correlated with the dose of radiation. Better understanding of apoptosis induced by radiation therapy will help design optimal dosing schedules for radiation therapy, especially in combination with chemotherapy.
cell turnover. Failure to eliminate cells that have been exposed to mutagenic agents has been associated with the development of cancer and resistance to anticancer therapy.\(^9\)

Understanding apoptosis is often considered a key to understanding the genesis of tumors and to devising innovative strategies for malignant glioma treatment. Essential pathways regulating apoptosis are disrupted in malignant gliomas, notably the cell cycle control mechanisms regulated by the p53 and retinoblastoma proteins (pRB) and their homologs. Although apoptosis does occur spontaneously in malignant gliomas in vivo, there is little evidence that the current mode of radiotherapy exerts its effects via induction of apoptosis.

As judged by computed tomography or magnetic resonance imaging, therapeutic responses with partial or complete regression of the tumor are rare in glioblastomas. Instead, large clinical trials commonly consider the time to further tumor progression as an essential end point. This indicates that current treatments, if successful at all, merely stop the tumor growth rather than actually killing tumor cells. Fractionated radiotherapy with single doses of 2 Gy has been shown to produce objective responses in some patients with malignant gliomas, suggesting that radiation can induce death in glioma cells. However, cultured glioma cells do not appear to activate the extrinsic death receptor-dependent apoptotic pathway in response to a low dose of radiation. Single fractions of radiation up to 8 Gy each do not appear to cause apoptosis in most glioma cell lines in vitro.\(^10\)

The current study was designed to ascertain whether radiotherapy mediates its effects via induction of apoptosis in cultured glioma cells and to determine which pathway regulates this mechanism of action in malignant gliomas in vitro. Since xenografts of C6 cells (derived from a rat in which glioma is induced by nitrosourea) have been used as experimental models of glioma,\(^11\) we chose to study the effects of \(\gamma^{60}\)Co irradiation on rat C6 cells. The C6 cells were divided into 0, 4, 16, and 64 Gy groups and exposed to a single fraction of \(\gamma^{60}\)Co radiation. Following irradiation, cell proliferation, apoptosis, and cell cycle progression were evaluated. The expression of the p53 and p21 proteins were also examined to evaluate the possible pathway by which radiotherapy exerts its effects on apoptosis and the cell cycle.

### METHODS

#### Materials

C6 cells were obtained from the American Type Culture Collection (ATCC). Dulbecco’s modified Eagle’s medium (DMEM), N-2-hydroxyethylpiperazine-N’-2’-ethanesulfonic acid (HEPES), and trypsin were purchased from Gibco Co. (USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). Penicillin and streptomycin were purchased from Sigma (USA). The Hoechst staining kit was purchased from Beyotime Institute of Biotechnology (Haimen city, Jiangsu, China). The Annexin V-fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) apoptosis detection kit and DNA-Prep reagent kit were purchased from Immunotech Co. (France) and Beckman Coulter, respectively. The immunohistochemistry SABC kit and the DAB kit were purchased from Boster Co. (Wuhan, China), and 3-(4,5)-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Severa Co. (USA). Rabbit anti-p53 and mouse anti-p21 antibodies were purchased from Santa Cruz Biotechnology (USA). The fluorescence microscope was made by Leica Microsystems GmbH (Germany). The ELITE ESP flow cytometry system was purchased from Beckman Coulter (USA), and the DG3022 enzyme-linked immunosorbent tester was produced by Nanjing Huadong electrons group (China).

#### Cell culture and \(\gamma^{60}\)Co radiation

C6 cells were cultured as described previously.\(^11\) Briefly, C6 cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum and 2-mM L-glutamine in a humidified atmosphere of 95% air/5%

| Effect                          | Time (h) |
|--------------------------------|----------|
| Control                        |          |
| Apoptosis rate of C6 cells     |          |
| Control                        |          |
| 3.1 (0.5)                      | 6        |
| 3.2 (0.4)<sup>a</sup>          | 12       |
| 5.6 (0.4)<sup>ab</sup>         | 24       |
| 11.9 (0.3)<sup>ab</sup>        | 48       |
| 27.3 (0.4)<sup>b</sup>         | 72       |
| 27.1 (0.8)<sup>b</sup>         | 96       |
| 28.1 (1.4)<sup>b</sup>         |          |

<sup>a</sup>P<.01 vs. 48 hours after irradiation;  
<sup>b</sup>P<.01 vs. control.
CO₂. Cells were “passaged” every 2 to 3 days using 0.25% trypsin to detach the cells from the flasks.

Before the cell proliferation, apoptosis, and cell cycle assays, and prior to evaluating protein expression, C6 cells were exposed to a single fraction of γ-60Co radiation. The cells in the control group (0 Gy) were not irradiated. Three groups (4, 16, and 64 Gy) were exposed for different durations of time to γ-60Co radiation at the Fourth Military Hospital (Xi’an, China). The radiation dose rate was 2766.1 cGy/min, the cell density was 2x10⁵/mL, and the temperature was maintained at 25°C throughout the irradiation period. For the irradiation groups, the time when the irradiation was finished was set as time 0.

**MTT cell proliferation assay**

Cell proliferation was evaluated using the MTT cell viability assay, as previously described. MTT was added at 0 (no irradiation), 48, 96, and 144 hours after irradiation to evaluate cell viability and proliferation following the different doses of radiation. The cells were incubated for 4 hours in the presence of MTT reagent and then lysed with dimethylsulfoxide. Absorbance (A490) was measured at 490 nm. Cell growth curves were generated to compare the effects of radiation on the cells.

**Apoptosis assays**

During our initial studies, we found that apoptosis in groups that had been exposed to irradiation (16 Gy) for 12 hours occurred and remained at a higher level up to 48 hours (Table 1), so the “48 hours” groups were chosen in the cell apoptosis assay. The apoptotic cells underwent chromatin condensation and nuclear fragmentation, as detected by staining with Hoechst 33258. Cells (5x10⁵) were harvested from each group after isolation using 2.5 g/L trypsin. The cells were mixed with 490 µL of binding buffer, 5 µL Annexin V-FITC and 5 µL PI were added. Then the cells were incubated for 10 minutes in the dark. The stained cells were analyzed by flow cytometry within 1 hour. Dual-parameter analysis was done to determine the percentages of normal, apoptotic, and dead cells. Each experiment was repeated at least 3 times.

**Cell cycle assay**

Groups of cells exposed to different levels of radiation 48 hours earlier were chosen for the cell cycle assay. In brief, 1x10⁶ cells were harvested from each group and suspended in 1 mL of 10% PBS diluted in water. Then the cells were mixed with a solution of 2 mL dehydrated alcohol (4°C) and sealed overnight for fixation. The solution was centrifuged, and the cells were mixed with 500 µL of DNA-Prep stain and incubated for 15 minutes at room temperature in the dark prior to the cell cycle assay by flow cytometry to determine the percentage of cells in G0/G1, G2/M, and S phases. The data were analyzed using a single-parameter DNA histogram. Each experiment was repeated at least 3 times.

**Immunohistochemical examination of p53 and p21 protein expression**

The quantitative evaluation of p53 and p21 protein expression was accomplished in the different groups 48 hours after radiation exposure using immunohistochemistry. The cells were grown to confluency on glass coverslips. The cells were first immersed in 90% ethanol for 2 hours for fixation, washed with distilled water, and then immersed in 0.5% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity, followed by rinsing 3 times in distilled water. After that, the goat serum was added, and the cells were incubated at room temperature for 20 minutes to block nonspecific antibody binding. Monoclonal antibodies for the p53 or p21 proteins were added, and the cells were incubated at 37°C for 1 hour and then washed 3 times for 2 minutes each with 10% PBS diluted in water. After that, the cells were incubated in biotin-conjugated goat anti-mouse IgG at 37°C for 20 minutes and washed 3 times for 2 minutes each with 10% PBS diluted in water. The SABC solution was then added, and the cells were incubated at 37°C for 20 minutes and washed 4 times for 5 minutes each with 10% PBS diluted in water. Finally, the cells were stained using DAB. The slides were examined by mi-
Apoptosis in cultured rat cells

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Microscopy, and the positive cell numbers were counted. A total of 5-7 randomly selected fields were examined for each group. At least 200 cells were counted, and each experiment was repeated at least 3 times. Results were scored as negative (no positive cells), mildly positive (less than 25% positive), moderately positive (less than 50% positive), or positive (more than 50% of the cells were positively stained).

Statistical analysis
Data were presented as mean (standard deviation). The statistical significance of means for the studies was determined by analyzing variance and the bivariate analysis of correlation was determined by using SPSS 11.0 (IBM Corp., Armonk, NY USA). P values for significance were set at .05.

RESULTS
Initial studies on the treatment of cells with 16 Gy for the duration 6-96 hours indicated that apoptosis was elevated by 12 hours, with maximum levels observed by 48 hours. Apoptosis levels remained stable for durations ranging from 48 to 96 hours (Table 1). Based on these data, the 48-hour time point was chosen for further studies.

Radiation inhibits cell proliferation
Figure 1 shows C6 cell growth curves after exposure to different doses of radiation. The growth curve for the control group indicates that the cells were in the log phase throughout the assay. Compared with the control group (0 Gy), the C6 cell number at the same time point in the 4 Gy group was nearly the same, while the cell numbers in 16 Gy and 64 Gy groups were much lower. In the 16 Gy group, the cell number began to fall within 48 hours after irradiation. In the 64 Gy group, the cell number 48, 96, and 144 hours after irradiation was almost the same as that just after irradiation.

Radiation arrests cell in G1 phase
Radiation also induced cell cycle arrest in C6 glioma cells. As shown in Figure 2, the percentages of non-irradiated cells (the control group) in the G0/G1, G2/M or S phase were different from those of irradiated cells. However, the percentage of cells in the G2/M phase was not significantly altered by irradiation. The percentage of cells in the G0/G1 phase increased with the dose of radiation, with a correlation coefficient of 0.9126 (r=0.9126). In the 64 Gy group, the percentage of cells in the G0/G1 phase was 81.6%. The percentage of cells in the S phase decreased with increas-
Radiation induces apoptosis

In addition to decreasing cell proliferation and viability, a single dose of radiation induced apoptosis in the C6 cells. Figures 3a-c show fluorescent images of the nuclear morphology of C6 cells in control and 64 Gy groups after Hoechst 33258 staining. The nuclei were normal in the control group (Figure 3a); whereas the nuclei became condensed or fragmented in the 16 Gy and 64 Gy groups (Figures 3b, c). The percentage of condensed or fragmented nuclei increased with the dose of radiation.

Table 2 lists the percentages of apoptotic and necrotic cells as observed by staining with Annexin V-FITC/PI. The percentages of apoptotic cells in the control, 4 Gy, 16 Gy, and 64 Gy groups were 3.1 (0.5), 6.7 (0.6), 27.3 (0.4), and 36.7 (0.7), respectively. Compared with the control group, the percentages of apoptotic cells in the 16 Gy and 64 Gy groups were significantly higher (P<.01). The percentage of cell apoptosis increased with the radiation dose. The correlation coefficient was 0.856 (r=0.856), which was similar to the results of Hoechst 33258 staining. While radiation induced apoptosis, the percentage of necrotic cells was not significantly different between the control group and other groups (P>.05). The percentages of necrotic cells in the control, 4 Gy, 16 Gy, and 64 Gy groups at 48 hours after irradiation were 0.2 (0.1), 0.7 (0.5), 1.8 (0.6), and 2.0 (0.6), respectively.

Radiation affects p53 and p21 protein expression

Because p53 and its downstream target, p21, have been shown to be important for the response to radiation, we evaluated their expression in the C6 cells following exposure to different doses of radiation. As shown in Figure 4, in the control group, the p53 protein was only expressed in the nuclei of a few cells. In contrast, in the 64 Gy group, the p53 protein was not only expressed in the nucleus of more cells but was also expressed in the cell cytoplasm. Similarly, the p21 protein was not expressed in C6 control cells but was expressed in both the nuclei and the cytoplasm of C6 cells exposed to 64 Gy of radiation. Table 3 shows the percentages of the cells expressing p53 and p21 proteins in the control and 64 Gy groups. The expression levels of p53 and p21 proteins were increased in a dose-dependent manner, with a correlation coefficient of 0.889 (r=0.889).

DISCUSSION

In the present study, the C6 cell growth was depressed by large doses of γ-60Co radiation (16 Gy and 64 Gy), but not by small doses of γ-60Co radiation (4 Gy). These results are consistent with those of previous studies. However, the lower dose of radiation does appear to temporarily affect cells. As shown in Figure 1, the cell growth curve for the cells exposed to 4 Gy of radiation does not reflect steady growth. The cell number 96 hours after irradiation was lower than that of both the control cells (at 96 hours) and the 4 Gy–radiated cells (at 48 hours). Thus, γ-60Co radiation injures the C6 cells, but does not cause severe enough damage to prevent the long-term cell growth. The C6 cells are apparently able to recover from 4 Gy–radiation-induced injury, which is consistent with the findings of studies showing that even 8 Gy radiation does not lead to significant apoptosis in glioma cells.

Table 2. Percentages of apoptotic and necrotic cells in the control, 4 Gy, 16 Gy, and 64 Gy groups 48 hours after irradiation.

| Effect               | Dose of radiation (Gy) |
|----------------------|------------------------|
|                      | 0                      | 4          | 16         | 64         |
| C6 cell apoptosis    | 3.1 (0.5)              | 6.7 (0.6) | 27.3 (0.4)* | 36.7 (0.7)* |
| C6 cell necrosis     | 0.2 (0.1)              | 0.7 (0.5) | 1.8 (0.6)  | 2.0 (0.6)  |

*P<.01 vs. the control group.

Table 3. The expression of p53 and p21 proteins 48 hours after different doses of irradiation.

| Effect               | Dose of γ-60Co radiation (Gy) |
|----------------------|-------------------------------|
|                      | 0                | 4 | 16 | 64 |
| P53 protein expression (%) | 13 (4) | 18 (3) | 32 (6) | 68 (9) |
| P21 protein expression (%)   | 0    | 16 (4) | 31 (3) | 37 (6) |
Radiation, especially higher doses of radiation, led to apoptosis (as inferred from chromatin condensation, nuclear fragmentation, and translocation of phosphatidyl serine) in the C6 cells. The percentages of apoptotic cells in both Hoechst 33 258 and Annexin V-FITC/PI assays showed that the level of apoptosis increased with the dose of \( \gamma^{60}\text{Co} \) radiation, whereas the percentage of necrotic cells remained stable. These results indicate that irradiation primarily affects the C6 cell proliferation by inducing cell apoptosis.

We also demonstrated that single-fraction \( \gamma^{60}\text{Co} \) radiation also affects cell cycle progression. The percentage of cells in the G0/G1 phase increased with increasing doses of radiation, whereas the percentage of cells in the S phase decreased. These results indicate that the cells were arrested in the G1 phase. A complex system of positive and negative regulatory mechanisms governs cell cycle progression, exerting control at various checkpoints. G1 checkpoint is one of the 2-key control points in the cell cycle. Numerous pathways are involved in apoptosis and cell cycle arrest, and the p53 tumor suppressor is involved in both processes.

To understand the molecular mechanism responsible for the induction of apoptosis and G1 arrest after a high dose of single-fraction \( \gamma^{60}\text{Co} \) radiation, the expression of the p53 protein and its downstream target, p21, were measured by immunohistochemistry. There was a dose-dependent increase in the expression of p53 following \( \gamma^{60}\text{Co} \) radiation, and the protein was distributed in both the nucleus and the cytoplasm of cells. The p21 protein was only expressed after \( \gamma^{60}\text{Co} \) radiation and increased in a dose-dependent manner. These results suggest that the p53 pathway is activated in these cells and is likely to regulate their apoptosis and cell cycle progression (G1 arrest).

The p53 protein is a cell cycle control protein that senses genotoxic stress, leading to DNA damage and cell cycle arrest in the G0/G1 or G2/M phase, or leading to the induction of apoptosis. The molecular consequences of p53 activation include changes in the transcription of various p53 response genes, including p21 and BAX. Experimental transfer of p53 to glioma cells first induces the expression of p21 protein and growth arrest, and subsequently induces BAX expression and apoptosis. With respect to the cell cycle control, the p53 protein and the pRB are partially inactive in the vast majority of gliomas. Under nonreplicating conditions, the pRB binds to key transcriptional factors and sequesters them. When it is time for the cell to replicate, kinases that regulate pRB are activated and phosphorylate pRB. The phosphorylated pRB releases factors that transcriptionally activate S phase genes, permitting the transition from G1 to S phase. The p53 protein is upstream of pRB in controlling the G1 checkpoint. Following DNA damage, p53 is activated and up-regulates p21, which in turns inhibits pRB phosphorylation and prevents the transition from G1 to S phase.

Mutations to p53 are common in human brain tumors, including glioblastomas, gliomas, and astrocytomas. Loss or malfunction of the p53-mediated apoptotic pathway has been proposed as a mechanism by which tumors become resistant to chemotherapy or irradiation. In normal cells, the loss of p53 may enhance rather than decrease the vulnerability to apoptosis. However, within the process of neoplastic transformation, the loss of p53 probably allows the cell to accumulate random genetic and chromosomal aberrations without triggering the endogenous p53-controlled cell death pathway. Wild-type p53 has been shown to suppress cell transformation and neoplastic cell growth and has been proposed as a cancer therapy target. Our results indicated that wild-type p53 in C6 glioma cells was activated by irradiation and induced cell apoptosis and cell cycle arrest, which is in accordance with the previous reports on p53 gene therapy.
Our improved understanding of the mechanisms involved in apoptosis has allowed the rational design of novel therapeutic strategies. Various novel therapies targeting the p53 pathway have been studied in animals, and a few have been clinically used in the treatment of newly diagnosed glioma. An adenovirus encoding an artificial p53-based gene induced cell death in all glioma cell lines. Systemically delivered ligand–liposome–p53 gene therapeutics resulted in efficient expression of functional wild-type p53, enhancing the efficacy of conventional chemotherapy/radiotherapy. The clinical use of intracerebral p53 wild-type U87MG xenografts induced apoptosis of tumor cells in vivo and prolonged survival. Thus, the stabilization/repair of mutant p53 proteins and promotion of wild-type p53 activity is another promising approach to cancer therapy. These novel therapeutic strategies provide an opportunity to introduce a new generation of radiobiological/biological response modifiers in clinical studies. The data from our present study indicate that when p53 is present, it is possible to induce significant apoptosis in glioma cells via a single dose of radiation. This indicates that these novel p53 modulatory strategies represent a promising direction for future radiotherapy studies, and may allow for administration of less frequent radiation therapy.

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