SPANDIDOS PUBLICATIONS

Role of p16 and p14ARF in radio- and chemosensitivity of malignant gliomas

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Abstract. In addition to cytoreductive surgery, most patients with malignant gliomas also undergo radio- and chemotherapy. An improved understanding of the molecular genetic mechanisms underlying the radio- and chemosensitivity of gliomas may help to identify glioma patients who will benefit from aggressive and, therefore, potentially toxic adjuvant treatment. It may also allow for the development of new therapies aimed at improving the response of these tumors towards chemo- and radiotherapy. The INK4a gene products, p16 and p14ARF, have been suggested as potential regulators of glioma chemo- and radiosensitivity. We have used tetracycline controlled expression of p16 and plasmid-based p14ARF expression to study the chemo- and radiosensitivity of glioma cell lines. Ectopic p16 sensitized U-87MG cells towards treatment with vincristine and possibly also BCNU by approximately 1.5 to 2-fold, and towards ionizing radiation by a factor of 1.5. p14ARF expression was found to render U-87MG cells 2-fold more radioresistant than controls. These findings support a role for p16 and p14ARF as modulators of the radio-and chemosensitivity of gliomas. Further studies of the role of cell cycle regulators in glioma chemo- and radio-sensitivity seem warranted. We would like to point out that such candidate genes which may code for potent growth suppressors (like p16) or even toxic gene products can be successfully investigated using the approach detailed in this manuscript.

Introduction

Malignant astrocytomas account for more than 50% of primary brain tumors. The median survival for patients with glioblastoma multiforme WHO grade IV did not exceed 15 months in a recent prospective trial (1). The prognosis for patients with anaplastic astrocytomas of WHO grade III is somewhat better with a median survival of 14-36 months (2).

Surgery for glioblastomas and anaplastic astrocytomas can provide a histological diagnosis and reduce the overall tumor cell burden. The prognostic influence of the extent of the resection remains controversial (3). Involved field external beam radiotherapy with a safety margin of 2-3 cm has been shown to significantly prolong life in patients with malignant gliomas (4). The results of recent studies support the use of chemotherapy for recurrent anaplastic astrocytomas and glioblastomas (5,6). Adjuvant PCV chemotherapy (procarbazine, lomustine/CCNU, vincristine) may confer a survival advantage to patients with anaplastic astrocytomas (7,8). A modest survival benefit but a remarkable increase of midterm survivors was observed in a recently completed prospective randomized trial after radiochemotherapy with temozolomide followed by temozolomide chemotherapy (1).

Chemo- and radiosensitivity of tumors must be determined to some extent by their genetic make-up. Genetic losses of markers from chromosomes 1p and 19q may predict chemosensitivity in oligodendroglial tumors (9). Some data suggest that chromosome 1p (and 19q?) losses might also correlate with chemosensitivity in malignant astrocytic and mixed gliomas (10). Deletions of the INK4a/INK4b locus coding for the tumor suppressors and cell cycle regulators p16, p15 and p14ARF have been inversely correlated with the chemosensitivity of malignant gliomas (9). Tumor cells respond to radiation injury by a p53-dependent cell cycle arrest and DNA repair or, alternatively, undergo apoptosis (11). Hence, mutations of cell cycle regulators are attractive candidate lesions influencing the radiosensitivity of the tumor.

Disruption of the INK4a/INK4b locus is seen in more than 30% of malignant astrocytomas (12). The INK4a gene encodes the tumor suppressor, p16, which arrests cell cycle progression through inhibition of cdk4-mediated pRB phosphorylation. Alternative splicing of the INK4a gene results in the expression of a second protein (p14ARF) with cell cycle inhibitory capacities structurally unrelated to p16. p14ARF promotes the degradation of mdm2 and thus stabilizes p53 protein (13). The structurally and functionally closely related cdk4 inhibitor, p15, is encoded by the INK4b gene separated from the INK4a locus by only 30 kb (14). p16, p14ARF and their major down-stream targets, pRB and p53, play prominent

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roles in cell cycle control, DNA damage detection and repair, and apoptosis, which renders them attractive candidate regulators of radio- and chemosensitivity (13,15-17).

Several groups have, therefore, attempted to delineate systematic p16 and p14ARF effects on the chemo- and radiosensitivity in cell cultures including, to some extent, glioma cell lines. Conflicting data have been reported. In the present study, we used tetracycline-controlled p16 expression and transfection with a p14ARF expression plasmid in glioma cells to investigate a potential role for p16 and p14ARF in the chemo- and radiosensitivity of gliomas.

Materials and methods

Cell lines U-87MG-tTA-p16 and U-373MG-tTA-p16. Glioblastoma cell lines U-87MG and U-373MG were purchased from the American Type Culture Collection (ATCC). The construction and characteristics of the cell lines, U-87MG-tTA-p16 U87 and U-373MG-tTA-p16, were previously described (18). Both cell lines express no endogenous p16 protein. If grown in cell culture medium containing $2x10^{-3}$ mg/ml tetracycline, no p16 protein was detectable by Western blotting. p16 could be detected 8 h (U-373MG-tTA-p16) and 72 h (U-87MG-tTA-p16) after tetracycline withdrawal. Maximum levels were reached after 24 h (U-373MG-tTA-p16) and 96 h (U-373MG-tTA-p16), exceeding expression levels observed in HeLa and U-373MG clones still expressing p16 approximately 2-fold (18). Stable p16 expression was observed for more than 12 days in both cell lines. Adding tetracycline to the culture medium resulted in decreasing p16 levels after 12 h (both cell lines) and no detectable p16 protein after 36 h (U-373MG-tTA-p16) and 72 h (U-373MG-tTA-p16). Growing both cell lines at a tetracycline concentration of $5x10^{-6}$ mg/ml led to p16 protein levels comparable to the amount of p16 protein detected in HeLa, Hs683, glioblastoma tissue samples, and in p16 positive U-373MG clones.

Construction and characterization of p14ARF expressing U-87MG clones. A p14ARF expression plasmid based on the expression plasmid, pBabePuro, was used to transfect U-87MG cells with a lipofection protocol as previously described (18). Subclones (U-87MG-pBabePuro-p14ARF) were picked and expanded in cell culture medium containing 8 μg/ml puromycin. Control clones (U-87MG-pBabePuro) were constructed following the same protocols but using the expression vector without an insert. The subclones were tested for p14ARF expression by RT-PCR. The protocols for p14ARF RT-PCR using co-amplification of GAPDH as an internal control for mRNA integrity in order to exclude DNA contamination have been published previously (19).

Chemosensitivity testing. Chemosensitivity testing was performed with vincristine and BCNU. These agents were chosen based on their frequent clinical use, and on their cell cycle dependent vs. independent cytotoxic effects. Vincristine interrupts the assembly of the mitotic spindle in the M phase (20). Hence its cytotoxic effects strongly depend on the cell cycle phase. In contrast, the nitrosoureas, e.g. BCNU/carmustine and CCNU/lomustine are cell cycle independent agents (20). Vincristine is administered together with CCNU and procarbazine in the PCV chemotherapy protocol for malignant gliomas (7,8,20). Nitrosourea monotherapy has been used for gliomas, and the PCV protocol as well as many other polychemotherapy protocols include nitrosoureas (4,20). BCNU can also be administered intravenously and, therefore, easily in cell culture. Two other chemotherapeutic agents (procarbazine and temozolomide) commonly used for gliomas (5,6,20) act also cell cycle independent through the alkylation of cellular RNA, DNA and proteins.

Vincristine serum concentrations $>10^{-4}$ mg/ml (21) and BCNU concentrations $>10^{-3}$ mg/ml (22) have been reported. BCNU but not vincristine crosses the intact blood brain barrier. However, the blood brain barrier is defective in malignant gliomas. *In vitro* chemosensitivity testing of astrocytoma biopsies identified LD50s in the range of 10^{-10} to 10^{-6} mg/ml vincristine and 10^{-4} to 10^{-3} mg/ml CCNU for sensitive tumors (23). Clinically, BCNU and CCNU are used at similar doses. We investigated vincristine/BCNU concentrations ranging from 10^{-8} to 10^{-2} mg/ml, and $3x10^{-4}$ to $3x10^{-1}$ mg/ml. Testing for statistical significance was performed at vincristine concentrations of 10^{-6} mg/ml, and BCNU concentrations of 10^{-3} mg/dl (Student's t-test).

The protocols for chemosensitivity testing were extensively optimized. Cells were seeded and grown in 96-well titer plates at variable numbers taking into account the growth suppressive effects of p16 and p14ARF, and the respective duration of the cell cycle, in order to ensure that all experiments were performed during the exponential growth phase. The cells (still expressing the transgenes) were allowed to rest after exposure to the chemotherapeutic agents in order to allow for an optimal manifestation of any chemoprotective or chemosensitizing effects. MTS assays were performed to determine the fraction of surviving cells after exposure to the chemotherapeutic agents using a commercially available kit (CellTiter 96Oc AQueous, Promega, Madison, WI, USA) and untreated cells for controls.

Specific protocols were as follows: initial cell numbers (U-373MG-tTA-p16: 5000/well, U-87MG-tTA-p16: 1000/well), tetracycline withdrawal after 24 h (U-87MG-tTA-p16 and U-373MG-tTA-p16U373), exposure to cytostatic drugs (U-373MG-tTA-p16: 48-96 h, U-87MG-tTA-p16: 96-144 h); MTS assays (U-373MG-tTA-p16: 144 h, U-87MG-tTA-p16: 168 h). U-87MG-pBabePuro-p14ARF and U-87MG-pBabePuro cells were seeded at 5000 cells/well, allowed to rest for 24 h, exposed to the chemotherapeutic agents for 48 h, and analyzed after 120 h.

Radiosensitivity testing. Radiosensitivity testing protocols were optimized to take into account the growth inhibitory effects of p16 and p14ARF. Cell lines U-87MG-tTA-p16, U373MG-tTA-p16, and U-87MG-pBabePuro-p14ARF/U-87MG-pBabePuro cells were seeded in triplicate in standard cell culture dishes at 1000-3000 cells/dish and allowed to rest for 24 h in the appropriate cell culture medium containing tetracycline as needed. Radiation was delivered by a conventional X-ray unit/tube at 200 kV, 14 mA and 2,85 Gy/min. In order to determine clonogenicity after exposure to the various radiation doses, cell colonies were fixed approximately 1 week later on the dishes, stained with hematoxylin, and the

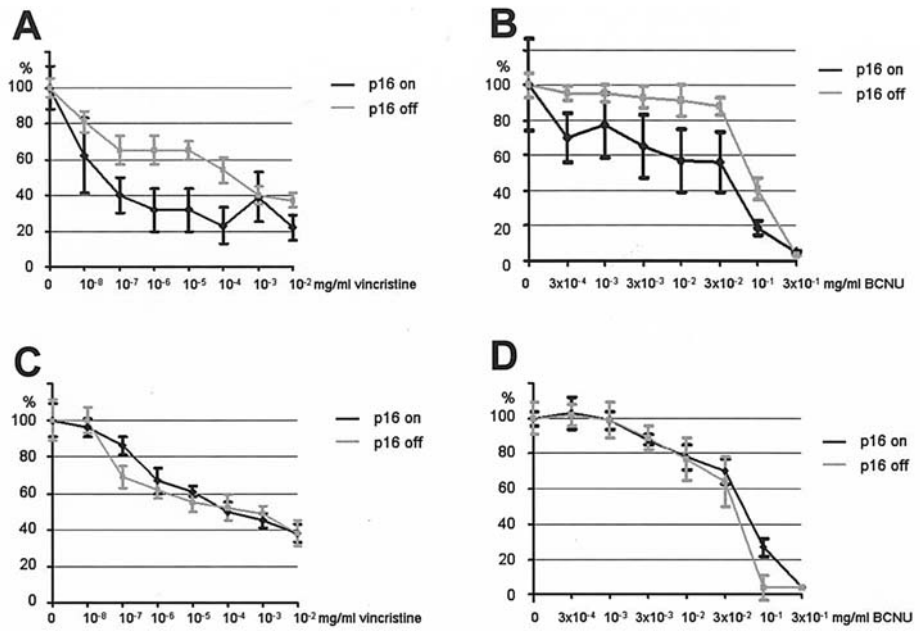

Figure 1. Results of chemosensitivity testing of glioma cell lines U-87MG-tTA-p16 and U-373MG-tTA-p16. Both cell lines conditionally express p16 under the control of a tetracycline-sensitive promoter (p16 on, normal levels of p16 expression; p16 off, no p16 expression, as assessed by immuno-blotting and comparison with cell lines harbouring intact p16 genes). Cells were seeded in multiplicates (6x) and treated with vincristine or BCNU at the indicated concentrations. The fraction of surviving cells was determined using MTS assays. Mean values and standard deviations are shown. (A) Treatment of U-87MG-tTA-p16 cells with vincristine. Ectopic p16 significantly sensitized the cells towards the chemotherapeutic agent by approximately 2-fold (at 10^{-6} mg vincristine/ml: $p<0.001$, Student's t-test). (B) Treatment of U-87MG-tTA-p16 cells with BCNU. A similar chemo-sensitizing effect of p16 is observed, but due to relatively large standard deviations results were only of marginal statistical significance (at 10^{-4} mg BCNU/ml: $p<0.05$, Student's t-test). (C) Treatment of U-373MG-tTA-p16 cells with vincristine. Ectopic p16 has no influence on the sensitivity of the cells towards vincristine. (D) Treatment of U-373MG-tTA-p16 cells with BCNU. No chemosensitizing or chemoprotective effects were observed.

colonies were counted. Statistical analysis (Student's t-test) was performed for the radiation dose, resulting in a >50% reduction of clonogenicity for both cell lines, compared in the respective experiment.

Results

Chemo- and radiosensitivity testing of U-87MG-tTA-p16 and U-373MG-tTA-p16 cells. Tetracycline-controlled p16 expression resulted in a survival disadvantage for U-87MG-tTA-p16 cells treated with vincristine, i.e. ectopic expression of p16 increased the chemosensitivity of the cell line (LD50s $3x10^{-8}$ vs. $2x10^{-4}$ vincristine mg/dl; Fig. 1A). At a vincristine concentration of 10^{-6} mg/ml, an approximate 2-fold increase in the fraction of surviving cells was noted for p16-negative cells ($p<0.001$, Student's t-test). p16 expression also seemed to increase the chemosensitivity of U-87MG-tTA-p16 cells towards BCNU by a factor of 1.5. However, these latter effects were only of marginal statistical significance (Fig. 1B; at 10^{-4} mg BCNU/ml: $p<0.05$, Student's t-test).

p16 expression did not significantly influence the sensitivity of U-373MG-tTA-p16 cells towards vincristine and BCNU. Figs. 1C and D show the results of typical experiments. Of note, U-373MG-tTA-p16 (but also U-87MG-tTA-p16) cells proved rather resistant towards BCNU.

When controlled for antiproliferative effects, expression of the p16 transgene enhanced the radiosensitivity of U-87MG-tTA-p16 cells by a factor of 1.5 (Fig. 2A; 4 Gy: $p<0.01$, Student's t-test). p16 expression did not significantly influence the radiosensitivity of U-373MG-tTA-p16 cells (Fig. 2B).

Influence of p14ARF on the chemo- and radiosensitivity of U-87MG cells. U-87MG-pBabePuro-p14ARF and U-87MG-pBabePuro subclones showed a variable chemosensitivity towards vincristine and BCNU. The LD50s observed ranged from 10^{-7} to 10^{-6} mg/ml vincristine and 10^{-3} to 10^{-1} mg/ml BCNU for the U-87MG-pBabePuro-p14ARF clones. For U-87MG-pBabePuro subclones, we observed LD50s in the range of 10^{-6} mg/ml vincristine and 10^{-2} mg/ml BCNU. The results were normalized and combined (Fig. 3A and B). No significant influence of p14ARF on the sensitivity of the cells tested was found towards either vincristine or BCNU.

Radiation doses of 2-3 Gy resulted in a >50% reduction of clonogenicity in all U-87MG-pBabePuro-p14ARF and U-87MG-pBabePuro subclones tested. After normalization and combination of the results, a significant radioprotective effect was demonstrated for ectopic p14ARF expression (Fig. 3C). p14ARF expressing subclones proved approximately twice as radioresistant as the p14ARF negative controls (at 3 Gy: $p<0.001$, Student's t-test).

Discussion

A better understanding of the genetic basis of radio- and chemosensitivity holds the promise of identifying tumors which will respond to such treatments and thus optimize the overall outcome (24). Patients with chemo- and radiosensitive tumors could be treated more aggressively, while others could be spared ineffective and potentially toxic treatment. The increasing costs of (new) antineoplastic drugs provide further arguments for a more selective approach to chemotherapy (25).

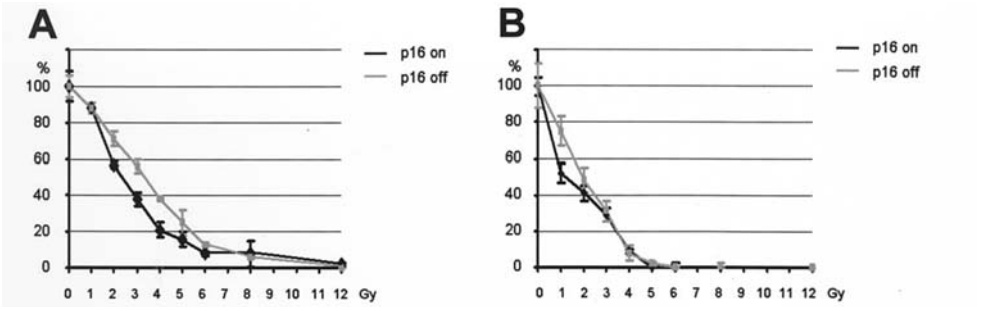

Figure 2. Results of radiosensitivity testing of glioma cell lines U-87MG-tTA-p16 and U-373MG-tTA-p16. Both cell lines conditionally express p16 under the control of a tetracycline-sensitive promoter (p16 on, normal levels of p16 expression; p16 off, no p16 expression, as assessed by immuno-blotting and comparison with cell lines harbouring intact p16 genes). Cells were seeded in triplicates and radiated as indicated. Clonogenicity was determined by counting visible colonies after staining of the cell culture dishes. Mean values and standard deviations are shown. (A) Radiation treat-ment of U-87MG-tTA-p16 cells. Ectopic p16 significantly (x1.5) sensitized the cells towards radiation treatment (at 4 Gy: $p<0.01$, Student's t-test). (B) Radiation treatment of U-373MG-tTA-p16 cells. Ectopic p16 has no influence on the radiosensitivity of the cells.

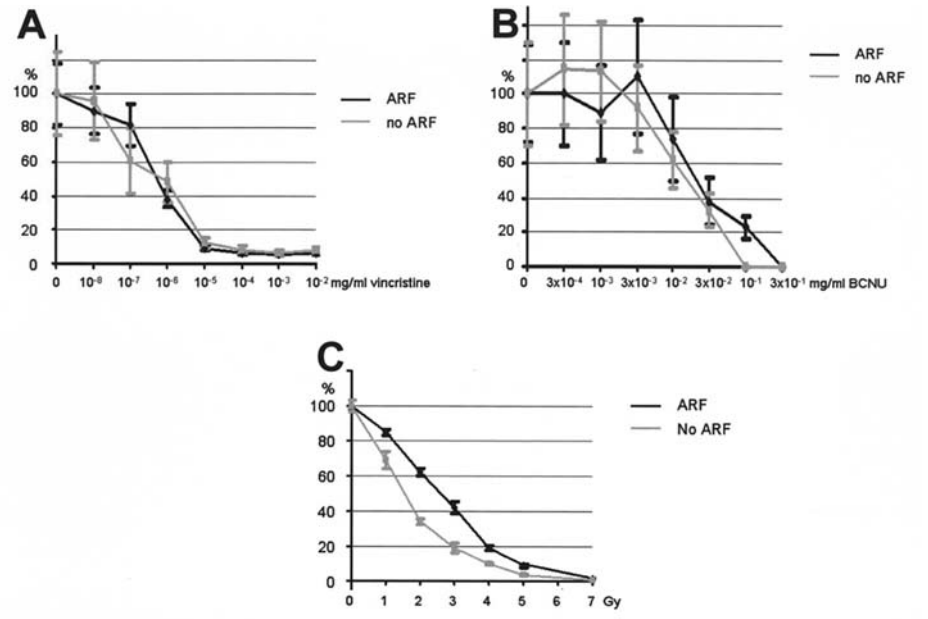

Figure 3. Results of chemo- and radiosensitivity testing of U-87MG glioma cells. After transfection with a p14ARF expression plasmid (pBabePuro-p14ARF) and a control vector (pBabePuro), three p14ARF positive (ARF) and three p14ARF negative subclones (no ARF) were expanded and analysed for their chemo- and radiosensitivity as described in Materials and methods. The results were normalized and combined. Mean values and standard deviations are shown. (A) Vincristine treatment. Ectopic p14ARF did not significantly influence the sensititvity of U-87MG-pBabePuro-p14ARF cells towards vincristine, when compared with p14ARF negative controls (U-87MG-pBabePuro). (B) BCNU treatment. No significant effects were observed. (C) Radiation treatment of U-87MG cells. p14ARF expression rendered cells significantly more radioresistant when compared to controls (at 3 Gy: $p>0.001$, Student's t-test).

Substantial data points to an important role for the tumor suppressors, p16 and p14ARF, in the development of malignant gliomas (12). Their role in cell cycle control renders them attractive candidates for regulators of chemo- and radio-sensitivity. In this study, we have analyzed a potential role for the tumor suppressors, p16 and p14ARF, in the chemo- and radiosensitivity of two glioma cell lines.

p16 expression was found to sensitize U-87MG-tTA-p16 cells towards vincristine and possibly also BCNU. However, the results were only of marginal statistical significance. The fractions of surviving cells differed by approximately 2-fold at vincristine concentrations probably achievable *in vivo*. No chemosensitizing effects were seen for p16 in U-373MG-tTA-p16 cells. Ectopic expression of p14ARF in U-87MG cells did not significantly alter the sensitivity of these cells towards vincristine and BCNU. When controlled for its growth inhibitory effects, ectopic p16 expression increased the radiosensitivity of U-87MG-tTA-p16 cells. Again, no effects were seen in U-373MG-tTA-p16 cells. A radio-protective effect was found for p14ARF expression in U-87MG cells.

Glioma cell lines have been previously used to study a potential role for p16 in chemosensitivity (26,27). Plasmid-based and adenoviral p16 overexpression, respectively, were found to protect glioma cells against treatment with ACNU, cisplatin, paclitaxel and topotecan. In support of these findings, a correlation between chemoresistance and p16 expression in 12 glioma cell lines was recently reported (28). Iwadate *et al* used astrocytoma biopsy samples harboring p16 and p14ARF

to show a somewhat similar relationship between 6 expression and increased sensitivity towards antimetabolites (29). On the other hand, and in line with our results obtained with U-87MG-tTA-p16 cells treated with vincristin, a p16-mediated increase in chemosensitivity has been demonstrated in several non-glioma cell lines (30-32).

Ectopic expression of p16 has been used by Miyakoshi *et al* (33) and Hama *et al* (34) to investigate a potential role for p16 in glioma radiosensitivity. Similar to our findings for U-87MG-tTA-p16 cells, these authors report a radiosensitizing effect mediated by p16. Their findings are supported by studies in other non-glioma cell lines (35-37).

p14ARF had not been widely investigated previously as a potential determinant of chemo- or radiosensitivity. p14ARF expression can increase the resistance of p53 defective HT-1080 fibrosarcoma cells to antimetabolites such as methotrexate (38). Gao *et al* reported an increase in radiosensitivity after plasmid based ectopic expression in lung cancer cell lines (39). In the study by Iwadate *et al* (29), p14ARF losses correlated with the sensitivity of astrocytoma biopsy samples towards antimetabolites. p14ARF is a negative cell cycle regulator critically depending on p53 function. p53 plays a major role in apoptosis, DNA damage control and, therefore, chemo- and radiosensitivity (13,15). We have, therefore, utilized U-87MG cells in our p14ARF study, which harbor an intact p53 gene. In contrast, the p53 gene in U-373MG cells is mutant and functionally defective (40). This could explain why U-373MG cells proved generally rather chemoresistant and no significant chemo- and radiosensitizing effects were seen following the ectopic expression of p16 in these cells.

Conflicting data regarding the role of p16 in (glioma) chemo- and radiosensitivity may have resulted in part from the use of cells overexpressing p16. p16 overexpression leads to cell cycle arrest (13), which clearly does not reflect clinical reality. Another problem encountered by most investigators is the introduction of collateral genomic alterations during plasmid-based and adenoviral gene transfer. In addition, the minute efficiency of all transfection techniques for plasmids currently used makes selection for successfully transfected cells necessary. This is a particularly important issue when the gene analyzed codes for a growth suppressor or a toxic gene product.

Inducible expression of transgenes may solve these problems. We employed a tetracycline sensitive promoter to express p16 at levels comparable to cells containing intact INK4a genes as evidenced by Western blotting. We acknowledge that the effects of p14ARF expression in U-87MG cells may have to be studied using the same method, for the reasons outlined above. However, the availability of the p14ARF expression plasmid at the beginning of this study provided a short-cut which allowed for the acquisition of the data detailed above. Tetracycline-controlled p16-expression was also used by Prabhu *et al* (41) to investigate the role of p16 in chemosensitivity. They demonstrated differential effects for p16 in osteosarcoma cells treated with etoposide and taxol, or epirubicine. Stone *et al* found that IPTG controlled ectopic p16 expression increased the resistance of melanoma cells towards vinblastine, methotrexate, and cisplatin (42).

In summary, the results of this investigation support a role for the INK4a gene products, p16 and p14ARF, in glioma chemo- and radiosensitivity. However, the effects observed in this study were comparatively small, and restricted to one of two cell lines. This may limit the clinical relevance of these data. Nevertheless, the apparent role of cycle regulators in the radio- and chemosensitivity of malignant gliomas warrants further study. Examining the influence of specific genes and their mutations on radio- and chemosensitivity in the controlled environment of a cell culture will form an important part of such research. Only *in vitro* studies will allow for the development of new therapeutic strategies; e.g. modulation of chemo- or radiosensitivity by gene transfer (43). Investigating gene products with strong antiproliferative effects in cell culture poses specific challenges. However, these problems may be overcome by following the approach outlined in this manuscript (i.e. using tetracycline controlled expression).

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