Prevention of Irradiation-induced Glioma Cell Invasion by Temozolomide Involves Caspase 3 Activity and Cleavage of Focal Adhesion Kinase

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ABSTRACT

Sublethal doses of irradiation enhance the invasiveness of human malignant glioma cells. This can be inhibited by subtoxic concentrations of temozolomide (TMZ) but not by lonidamine. Antagonism of irradiation-induced motility by TMZ is associated with the prevention of irradiation-induced αβ3-integrin, matrix metalloproteinase-2 and MT1-matrix metalloproteinase-expression. Irradiation induces focal adhesion kinase (FAK) activation by phosphorylation, whereas TMZ promotes FAK cleavage. Inhibition of caspases prevents TMZ-induced FAK processing and restores the promigratory effect of irradiation, suggesting that the resistance of glioma cells to irradiation-induced caspase processing may determine the invasive responses of glioma cells to irradiation. In contrast, DAOY medulloblastoma cells, which respond with caspase activation to irradiation alone, do not show enhanced invasiveness when irradiated.

INTRODUCTION

Excessive proliferation, disseminated tumor growth, resistance toward apoptotic stimuli, neovascularization, and suppression of anti-tumor immune surveillance are key biological features that contribute to the malignant phenotype of human malignant gliomas (1). Involved-field radiotherapy, the most important therapeutic measure, prolongs median survival for 6–8 months (2). We have recently outlined in vitro and in vivo paradigms for the local failure of radiotherapy; sublethal doses of irradiation promote the migration and invasiveness of glioma cells, which may reach the edges of the target volume of postoperative radiotherapy, escape delivery of a cumulatively lethal dose, and form the basis for locoregional relapse during or a few months after radiotherapy (3). This unexpected biological effect of irradiation involves enhanced αβ3-integrin expression, an altered profile of MMP-2 and -9 expression and activity, altered MT1-MMP and TIMP-2 expression, and an altered BCL-2/BAX ratio of glioma cells to irradiation. In contrast, TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which respond with caspase activation to irradiation alone, do not show enhanced invasiveness when irradiated. In an ongoing trial, the European Organization for Research and Treatment of Cancer examines whether TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which may reach the edges of the target volume of postoperative radiotherapy, escape delivery of a cumulatively lethal dose, and form the basis for locoregional relapse during or a few months after radiotherapy (3). This unexpected biological effect of irradiation involves enhanced αβ3-integrin expression, an altered profile of MMP-2 and -9 expression and activity, altered MT1-MMP and TIMP-2 expression, and an altered BCL-2/BAX ratio of glioma cells to irradiation. In contrast, TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which respond with caspase activation to irradiation alone, do not show enhanced invasiveness when irradiated. In an ongoing trial, the European Organization for Research and Treatment of Cancer examines whether TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which may reach the edges of the target volume of postoperative radiotherapy, escape delivery of a cumulatively lethal dose, and form the basis for locoregional relapse during or a few months after radiotherapy (3). This unexpected biological effect of irradiation involves enhanced αβ3-integrin expression, an altered profile of MMP-2 and -9 expression and activity, altered MT1-MMP and TIMP-2 expression, and an altered BCL-2/BAX ratio of glioma cells to irradiation. In contrast, TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which respond with caspase activation to irradiation alone, do not show enhanced invasiveness when irradiated. In an ongoing trial, the European Organization for Research and Treatment of Cancer examines whether TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which may reach the edges of the target volume of postoperative radiotherapy, escape delivery of a cumulatively lethal dose, and form the basis for locoregional relapse during or a few months after radiotherapy (3). This unexpected biological effect of irradiation involves enhanced αβ3-integrin expression, an altered profile of MMP-2 and -9 expression and activity, altered MT1-MMP and TIMP-2 expression, and an altered BCL-2/BAX ratio of glioma cells to irradiation. In contrast, TMZ administered during and after radiotherapy prolongs the survival of glioma cells, which respond with caspase activation to irradiation alone, do not show enhanced invasiveness when irradiated.

MATERIALS AND METHODS

Reagents and Cell Culture. The glioma cell lines used in this study have been described (8, 9). NIH-3T3 murine fibroblasts cells were obtained from the American Type Culture Collection (Rockville, MD). DAOY medulloblastoma cells were a kind gift from Torsten Pietsch (Bonn, Germany). Cells were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine and penicillin (100 IU/ml)/streptomycin (100 µg/ml). For acquisition of NIH-3T3-conditioned medium, the cells were grown to subconfluent monolayers, washed with PBS, and incubated with serum-free DMEM for 48 h. Supernatant was harvested and stored at −20°C. TMZ (stock solution of 50 mM in H2O) was kindly provided by Schering-Plough (Kenilworth, NJ). Lonidamine CCNU (stock solution of 50 mM in H2O) was a kind gift from Bristol (Munich, Germany).

Assessment of Cell Number. Cell numbers were assessed indirectly based on nuclear staining by crystal violet. Briefly, the cell culture medium was removed, and surviving cells were stained with 0.5% crystal violet in 20% ethanol for 20 min at room temperature. The plates were washed extensively under running tap water and air-dried, and absorbance values were read in an ELISA reader at 550 nm wavelength.

Biochemical Studies. Caspase 3-like activity was measured by conversion of the fluorescent substrate Ac-DEVD-amc (12.5 µM; Bachem, Heidelberg, Germany; Ref. 10). The caspase inhibitor zVAD-fmk was purchased from Biomol (Hamburg, Germany).

Irradiation. Cells were grown to 70% confluence in DMEM and trypsinized or grown to spheroids as detailed and irradiated in a γ-cell (Cs137, Gammacell 1000; Nordion, Kanata, Canada) at 1–8 Gy. Clonogenic cell death was assessed in six-well plates at a seeding density of 500 cells/well. The cells were irradiated and further cultured for 3 weeks. The culture dishes were stained with crystal violet. Colonies of >50 cells were counted at low magnification.

Immunoblot Analysis and Immunoprecipitation. Immunoblot analysis and immunoprecipitation were performed according to standard protocols (11). For the preparation of whole cell lysates, the cells were rinsed in PBS; harvested; centrifuged at 1,200 × g; lysed in 0.1 M Tris-HCl (pH 7.2) containing 0.1% NP40, 0.1 mM EDTA, and 5 μg/ml phenylmethylsulfonyl fluoride for 40 min on ice; and centrifuged at 10,000 × g for 10 min. Protein concentration was assayed using Bio-Rad reagents with photometric analysis. For the preparation of soluble supernatant protein, the protein concentration of the supernatant was determined, and 20 μg of soluble supernatant protein were precipitated with acetone at −20°C for 12 h and pelleted at 10,000 × g for 10 min. Protein loading was ascertained by O’Neto S staining. After blocking for 1 h in PBS supplemented with 5% skim milk and 0.1% Tween 20, immunodetection of MMP-2 (M, 72,000) and -9 (M, 92,000), TIMP-2 (M, 21,000), MT1-MMP (M, 66,000; 2 μg/ml; Oncogene, Calbiochem, Schwalbach, Germany), BCL-2 (M, 26,000; Santa Cruz Biotechnology, Santa Cruz, CA), phytosphorylase residues (1 μg/ml; Sigma Chemical Co.), or FAK (M, 125,000; 2 μg/ml; Transduction Laboratories, Lexington, KY) was performed. Anti-mouse IgG (1:4000; Santa Cruz Biotechnology) and enhanced chemiluminescence (Amer sham) were used for detection.
twice for 30 min in 50 mM Tris-HCl (pH 7.5) and 2.5% Triton X-100 and incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.6), 10 mM CaCl₂, 150 mM NaCl, and 0.05% NaN₃, to allow the gelatinases to digest the gelatin structure. Gels were stained with Coomassie Brilliant Blue R-250 and destained with 90% methanol/H₂O (1:1) and 10% glacial acetic acid. Because of gelatinolytic activity, bands are visible at Mᵦ, 72,000 for MMP-2.

**Flow Cytometry.** For α₅β₃-integrin analysis, the glioma cells were treated as indicated, washed with PBS, incubated with trypsin for 30 s at room temperature, and harvested. Five × 10⁵ cells were incubated with 1 µg of α₅β₃-integrin mouse monoclonal antibody (Chemicon, Pomona, CA) or 1 µg of mouse IgG isotype control antibody (Sigma Chemical Co.) in 100 µl of PBS/0.1% BSA for 30 min at 4°C protected from light. The cells were washed twice with PBS and analyzed on a FACSCalibur flow cytometer using Cell Quest acquisition and analysis software (Becton Dickinson, Heidelberg, Germany). Staining intensity was quantified by calculating specific fluorescence index measures as the ratio of signal obtained with specific antibody divided by signal obtained with isotype control antibody.

**Matrigel Invasion Assay.** Invasion of malignant glioma cells through Matrigel-coated (0.78 mg/ml) membranes (Becton Dickinson) with 8-µm pores was assessed using a 48-well microchemotaxis chamber (Neuro Probe, Inc., Bethesda, MD). NIH-3T3-conditioned medium (30% v/v) was pipetted as a chemoattractant into the bottom wells. The filter membrane was placed between top and bottom chamber and equilibrated for 30 min at 37°C. Cells were trypsinized, and 50 µl of cells suspension (3 × 10⁸ cells/ml) were added in triplicate wells. Cells on the lower side of the membrane were fixed, stained in thionin and eosin solution using Diff-Quick (Dade Behring AG, Düsseldorf, Switzerland), and sealed on slides. Quantification of invasion through the coated membranes was done by counting stained cells using a microgrid.

**Glia Spheroids.** Multicellular glioma spheroids were cultured in 25-cm² culture flasks base coated with 0.75% Noble Agar (Difco Laboratories, Detroit, MI) prepared in DMEM. Briefly, 3 × 10⁶ cells were suspended in 10 ml of medium, seeded onto 0.75% agar plates, and cultured until spheroids had formed. Spheroids of ~200-µm diameter were selected for the experiments.

**Fetal Rat-brain Aggregates.** Fetal rat-brain aggregates were obtained from 18-day-old fetuses of BD9 rats. The brains were removed aseptically and minced, washed in PBS, and dissociated by serial trypsinization. Single cell suspensions were obtained and plated into agar-coated 24-well plates at an average of the cell amount of one brain per well in 2 ml of medium. After 48 h, aggregates were transferred to new plates and cultured for 19 days. Aggregates with ~200-µm diameter were used in additional experiments (13).

**Confrontation Assays.** Invasion of the glioma spheroids into fetal brain aggregates was analyzed by morphometry using the MCID digitalization system (IMAGING Research Inc., Ontario, Canada). Briefly, tumor spheroids and rat brain aggregates were transferred in triplicate to individual wells of a 96-well plate, base coated with agar. With the help of a sterile syringe and a microscope, tumor spheroids and fetal brain aggregates were placed in close contact to each other. Images were obtained at 24-hour intervals.

**Statistical Analysis.** Quantitative data were obtained for survival, invasion, α₅β₃-integrin protein levels, and caspase activity, as indicated above. The experiments were usually performed in triplicate and repeated three times. The significance of the observed effects was evaluated by t test at P < 0.05 and 0.01.

**RESULTS AND DISCUSSION**

**TMZ Reduces Invasion of Malignant Glioma Cells.** The survival of LN-18, T98G, LN-229, and U87 MG glioma cells after treatment with increasing concentrations of TMZ or CCNU was assessed in a conventional 24-h cytotoxicity assay. Only minor cytotoxicity became apparent at concentrations ≤62.5 µM for TMZ and ≤31.25 µM for CCNU. Increasing concentrations significantly reduced survival in all four cell lines (Fig. 1A, left axis, bars).**

**Drug effects on glioma cell invasion were monitored in parallel (Fig. 1A, right axis, line graphs).** The inhibition of invasion observed with CCNU corresponded to the effect predicted by the loss of cells from cytotoxicity alone, indicating that CCNU has no specific anti-invasive properties. In contrast, the TMZ-induced reduction of invasion at concentrations between 16 and 250 µM strongly exceeded the effect predicted from the effect on cell survival. To verify this phenomenon, we performed confrontation assays with glioma spheroids and aggregates generated from fetal rat brain. The invasion of the brain aggregate by the tumor cells was inhibited significantly by TMZ at 62.5 µM (Fig. 1B). These effects of TMZ on invasion do not depend on p53 or phosphatase and tensin homologue because LN-18 cells do not retain functional p53 activity and U87 MG do not harbor functional phosphatase and tensin homologue (9, 11).

**TMZ Prevents Irradiation-induced Up-Regulation of α₅β₃-Integrin- and MMP-2 and MT1-MMP Expression.** To further investigate the potential clinical relevance of the observations summarized in Fig. 1, we used the paradigm of induction of glioma cell...
invasion by sublethal irradiation (3). As reported previously, sublethal irradiation at 1 and 3 Gy led to a dose-dependent increase in glioma cell invasion (Fig. 2A). Parallel experiments were performed in the presence of TMZ or CCNU. We chose concentrations that were approximately equi-effective in the survival assay (Fig. 1A, left axis).

In all cell lines, the proinvasive action of irradiation was almost nullified by TMZ, whereas no such effect was observed with CCNU (Fig. 2A and data not shown). For comparison, we also analyzed whether TMZ or CCNU altered the sensitivity of LN-229 cells to irradiation-induced clonogenic cell death. Irradiation followed by exposure to TMZ or CCNU at the same concentrations did not reduce clonogenic survival compared with irradiation alone (Fig. 2B). Next, we determined whether TMZ-induced inhibition of glioma cell invasion was associated with a modulation of the expression of αvβ3-integrin. As expected, the expression of αvβ3-integrin protein in LN-229 cells was enhanced by irradiation at 1 or 3 Gy (Fig. 2C, top panel; Ref. 3). TMZ did not reduce the constitutive αvβ3-integrin expression but significantly diminished the induction of αvβ3-integrin by irradiation (Fig. 2C, top panel). Further, irradiation promoted the accumulation of MMP-2 and MT1-MMP and the loss of TIMP-2 protein, and TMZ interfered with these irradiation-induced changes, too (Fig. 2C, bottom panel).

Caspase-3-dependent Cleavage of FAK As a Possible Mechanism of TMZ-induced Inhibition of Invasion. The next series of experiments sought to examine whether the effects of irradiation or their modulation by TMZ involved the activation of caspases. Irradiation alone did not induce the processing of caspase 3 or the generation of DEVD-amc cleaving activity, indicating caspase activity. In contrast, TMZ induced the cleavage of caspase 3 to the M, 20,000 active fragment in LN-229 glioma cells and a 50% increase in caspase 3-like activity either in unirradiated or irradiated cells (Fig. 3A). Caspase 3 cleavage and caspase 3-like activity were inhibited by preincubation of the cells with the caspase inhibitor zVAD-fmk. FAK is a caspase 3 substrate that is involved in the regulation of apoptosis and cell motility (14). FAK acts as a receptor-proximal bridging protein that links growth factor receptor (epidermal growth factor receptor and platelet-derived growth factor receptor) and integrin (αvβ3) signaling pathways (15). FAK may also play a pivotal role in the concanavalin A-dependent secretion of MMP-2 (16). Therefore, we analyzed whether FAK expression was altered in response to TMZ. Immunoprecipitation indicated the cleavage of FAK into the M, 85,000 form and, hence, inactivation in response to TMZ. FAK cleavage was unaltered by concomitant irradiation but was prevented by zVAD-fmk, suggesting that TMZ-mediated FAK cleavage is caspase dependent (Fig. 3A). To support the significance of FAK cleavage in inhibiting the effects of irradiation, we confirmed that irradiation increased the tyrosine phosphorylation of FAK, indicating activation of FAK (17). The next step was to establish a link between TMZ-dependent modulation of the effects or irradiation and TMZ effects on caspase activity. The activation of caspases appeared to be essential for the anti-invasive effect because zVAD-fmk nullified the inhibitory effect of TMZ on the constitutive and irradiation-enhanced invasive potential of LN-229 cells (Fig. 3C). Because MMP is essential for the establishment of an invasive phenotype, we assessed changes of MMP-2 protein expression and activity in response to irradiation and TMZ and their modulation by zVAD-fmk. Fig. 3D shows that zVAD-fmk reverts the reduction of irradiation-enhanced MMP-2 protein expression and activity mediated by TMZ.

We have previously determined and confirmed here (Fig. 3A) that chemotherapy, but not irradiation, induces caspase activation in glioma cells. Further, in contrast to irradiation, exposure to various chemotherapeutics, such as vincristine, doxorubicine, or 1-β-D-arabinofuranosylcytosine, fails to promote migration or invasion of LN-229, U87 MG, or LN-18 glioma cell lines (data not shown). The differential effects of irradiation on acute survival, clonogenic survival, and caspase activity are likely to determine whether a biologically and clinically relevant modulation of migration and invasion by irradiation occurs. Figs. 2, A and B and 4A and our earlier data (3)
show that glioma cells are highly resistant to acute cytotoxic effects even of high doses of irradiation, reduced clonogenic survival at doses of 2–6 Gy, and enhanced migration and invasion in the dose range of 1–50 Gy (Fig. 4A) but never exhibit an increase in caspase activity. These data indicate counterproductive effects of irradiation in the
clinically relevant dose range of 2–4 Gy in human malignant glioma cells. For comparison, we performed similar experiments with DAOY medulloblastoma cells, another type of malignant tumor considered more radiosensitive than glioma. Irradiation at 6 and 12 Gy resulted in a reduction of the number of invading cells that corresponded to the reduced number of surviving cells (Fig. 4B). We next asked whether the reduced invasiveness reflected merely the decreased number of surviving cells or whether there were cell death-independent mechanisms of inhibition of invasiveness. In contrast to glioma cells (Fig. 2C; Ref. 3), MMP-2 protein expression in DAOY cells was reduced at 6 and 12 Gy, indicating that there might be a specific anti-invasive effect exerted by irradiation in these cells (Fig. 4C). Because we had proposed a link between caspase activation mediated by TMZ and prevention of irradiation-induced invasion in LN-229 cells, we next asked whether irradiation, which failed to induce invasiveness in DAOY medulloblastoma cells, might instead promote caspase processing activity and FAK cleavage. Fig. 4D documents that this was indeed the case. Further, inhibition of caspase activity by zVAD-fmk reversed survival and invasion (Fig. 4E).

What are the implications of these observations for the efficacy of radiotherapy and our understanding of the molecular effects triggered by irradiation in a cell type-specific manner? The proinvasive effects of sublethal irradiation are associated with enhanced αβi-integrin, MMP-2, MT1-MMP, and decreased TIMP-2 expression (Figs. 2C and 3). All of these changes were shown here to be prevented by TMZ (Figs. 2C and 3A), suggesting that TMZ interferes with an upstream signaling event triggered by irradiation. TMZ also inhibited the constitutive invasion of unirradiated cells, without altering integrin or metalloproteinase expression or activity (Figs. 2C and 3B). These changes may, therefore, not be essential to the action of TMZ. In contrast, the caspase inhibitor zVAD-fmk reverted the anti-invasive effect of TMZ both on constitutive and irradiation-induced invasion. Both in unirradiated and irradiated cells, TMZ promoted the cleavage of FAK in a caspase-dependent manner, suggesting that FAK cleavage is the critical mediator of the anti-invasive effects of TMZ. FAK is required for integrin-dependent signaling and modulates cellular adhesion, migration (18), and survival (19). Recently, FAK has been found to integrate growth factor and integrin signals to promote the migration of fibroblasts (15).

In summary, this study provides evidence for a novel mechanism by which TMZ exerts its antitumoral action, i.e., inhibition of glioma cell invasion. The clinical relevance of our data will be determined by the results of European Organization for Research and Treatment of Cancer trial 26981 (Concomitant and adjuvant TMZ and radiotherapy for newly diagnosed glioblastoma multiforme: A randomized Phase III study) and specifically by the evaluation of the mode of recurrence in the different arms of this trial.

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