Tissue transglutaminase 2 inhibition promotes cell death and chemosensitivity in glioblastomas

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Abstract

Tissue transglutaminase 2 belongs to a family of transglutaminase proteins that confers mechanical resistance from proteolysis and stabilizes proteins. Transglutaminase 2 promotes transamidation between glutamine and lysine residues with the formation of covalent linkages between proteins. Transglutaminase 2 also interacts and forms complexes with proteins important in extracellular matrix organization and cellular adhesion. We have identified the novel finding that treatment of glioblastoma cells with transglutaminase 2 inhibitors promotes cell death and enhances sensitivity to chemotherapy. Treatment with either the competitive transglutaminase 2 inhibitor, monodansylcadaverine, or with highly specific small-molecule transglutaminase 2 inhibitors, KCA075 or KCC009, results in induction of apoptosis in glioblastoma cells. Treatment with these transglutaminase 2 inhibitors resulted in markedly decreased levels of the prosurvival protein, phosphorylated Akt, and its downstream targets. These changes promote a proapoptotic profile with altered levels of multiple intracellular proteins that determine cell survival. These changes include decreased levels of the antiapoptotic proteins, survivin, phosphorylated Bad, and phosphorylated glycogen synthetase kinase 3β (GSK-3β), and increased levels of the proapoptotic BH3-only protein, Bim. In vivo studies with s.c. murine DBT glioblastoma tumors treated with transglutaminase 2 inhibitors combined with the chemotherapeutic agent, N,N'-bis (2-chloroethyl)-N-nitrosourea (BCNU), decreased tumor size based on weight by 50% compared with those treated with BCNU alone. Groups treated with transglutaminase 2 inhibitors showed an increased incidence of apoptosis determined with deoxynucleotidyl transferase–mediated biotin nick-end labeling staining. These studies identify inhibition of transglutaminase 2 as a potential target to enhance cell death and chemosensitivity in glioblastomas. [Mol Cancer Ther 2005;4(9):1293–302]

Introduction

Glioblastomas are notoriously resistant to standard radiation and chemotherapeutic treatment strategies. Current treatment strategies generally fail to achieve long-term survival. Glioma subtypes vary in their sensitivity to chemotherapy. Typically, oligodendrogliomas are more responsive to chemotherapy than astrocytomas and are associated with better survival (1). Gliomas may show mixed components with histologic characteristics that variably display astrocytoma or oligodendroglioma regions. Anaplastic astrocytomas or glioblastomas with areas with oligodendroglial features have been associated with better survival than those with pure anaplastic astrocytomas or glioblastomas (2). The mechanisms responsible for the differences in response to chemotherapy and survival associated with astrocytomas and oligodendrogliomas are not known. Differential expression of drug resistance genes in glial cell lineages are correlated increased sensitivity to N,N'-bis (2-chloroethyl)-N-nitrosourea (BCNU) in oligodendrocytes compared with astrocytes. Increased resistance of astrocytes compared with oligodendrocytes to BCNU is associated with modulation of O6-methylguanine-DNA methyltransferase and glutathione (3). Oligodendroglia-derived cell lines sensitive to chemotherapy were shown to markedly decrease expression of the antiapoptotic proteins, Bcl-xL and Bcl-2, in association with cell death after treatment with BCNU (4). Loss of chromosomal arms 1p and 19q in oligodendrogliomas have been identified as a marker for increased sensitivity to chemotherapy (5). Precise molecular alterations that determine chemosensitivity among the subgroups of gliomas are not completely understood. Therefore, identification of factors that augment sensitivity to chemotherapy is important in determining new treatment strategies in patients with glioblastomas.

Tissue transglutaminase (transglutaminase 2) belongs to a family of transglutaminase proteins that affect covalent cross-linking of proteins with formation of amide bonds between glutamine side chains and ε-amino groups of lysine residues. The action of transglutaminase 2 stabilizes proteins by conferring mechanical resistance and protection from proteolysis. Transglutaminase 2 is expressed intracellularly and secreted on the cell surface where it interacts with proteins in the extracellular matrix and aids in
anchoring cells to basement membranes. Extracellular matrix proteins are important in the maintenance of structural integrity and promoting survival. Recently, transglutaminase 2 has been identified as an important factor that interacts with fibronectin in the extracellular matrix at the cell membrane and provides survival signals and inhibits anoikis in fibroblasts (6).

In a number of past studies tissue transglutaminase activity has been inhibited with competitive substrates rather than enzyme inhibitors. One such agent commonly used to inhibit transglutaminase 2 activity is monodansylcadaverine. Monodansylcadaverine is a primary alkyl amine with a fluorescent dansyl group attached at the end. Structural similarity with the lysine side chain allows monodansylcadaverine to be used not only as an amine donor for the fluorescence incorporation assay of transglutaminase activity but also as a competitive substrate to inhibit cross-linking of natural substrates. More recently, highly specific small-molecule irreversible transglutaminase 2 inhibitors have been identified (7). One such class includes dihydroisoxazole inhibitors such as KCA075 and KCC009 that bind irreversibly to transglutaminase 2. These dihydroisoxazole compounds were recently tested for their ability to irreversibly inhibit transglutaminase 2. The compounds showed a high specificity for human transglutaminase 2 \[k_{inh}/k_1 > 2000 \text{ min}^{-1} (\text{mol/L})^{-1}\] but essentially no reactivity \[k < 1 \text{ min}^{-1} (\text{mol/L})^{-1}\] toward physiologic thiols such as glutathione. The identification and specificity of these highly selective agents has recently been characterized (8).

Our laboratories have recently identified that inhibition of transglutaminase 2 promotes cell death and augments sensitivity to chemotherapy in glioblastoma cells. We have examined the ability of transglutaminase 2 inhibitors to induce cell death in vitro in three glioblastoma cell lines. We investigated the mechanism of cell death induced by transglutaminase 2 inhibition. Treatment with transglutaminase 2 inhibitors resulted in a shift toward a proapoptotic profile in glioblastomas that included decreases in levels of the antiapoptotic proteins, phosphorylated Akt, survivin, phosphorylated Bad, and phosphorylated GSK-3β and an increase in levels of the proapoptotic BH-3 only protein, Bim. The combination of treatment with transglutaminase 2 inhibitors and the chemotherapy agent, BCNU, resulted in increased apoptosis in DBT cells greater than with either agent alone. The significance of these in vitro observations of the induction of apoptosis and enhanced chemosensitivity in DBT glioblastoma cells treated with transglutaminase 2 inhibitors was correlated with the induction of cell death and enhanced sensitivity to chemotherapy in vitro and with decreased tumor growth in vivo. These studies identify transglutaminase 2 inhibitors as a potential new class of agents to enhance chemotherapy in glioblastoma.

Materials and Methods

Reagents and Cell Culture

Monodansylcadaverine and BCNU (carmustine) were purchased from Sigma (St. Louis, MO). The glioma cell lines were cultured in 5% CO2 and 95% humidified air atmosphere at 37°C in complete MEM containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 2 mmol/L glutamine (all from Life Technologies, Inc., Grand Island, NY). After cells reached 60% confluence, groups were treated with vehicle (1% DMSO) or 50, 100, or 200 μmol/L monodansylcadaverine or with 0.1, 0.5, or 1.0 mmol/L KCC009 for the designated time periods. Other groups of cells were treated either alone or in combination with 6.25 μg/mL BCNU.

Synthesis of KCA075 and KCC009

N-benzoyloxycarbonyl-1-phenylalanine (0.30 g, 1.0 mmol) and 1-hydroxybenzotriazole (0.15 g, 1.1 eq) were dissolved in 2 mL DMF. 3-Bromo-5-aminomethyl-4,5-dihydroisooazole (0.18 g, 1.0 eq), prepared following a reported procedure (9), was added to the solution cooled in an ice bath followed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.23 g, 1.2 eq). After overnight stirring at room temperature, the solution was diluted with ethyl acetate and washed with saturated NaHCO3 aqueous solution and brine. The organic layer was dried over MgSO4, filtered, and evaporated under reduced pressure. The residue was purified by SiO2 chromatography to give the title compound as a white solid (0.24 g, 52%). 1H NMR (CDCl3, 200 MHz): δ = 7.34 to 7.26 (m, 8 H), 7.17 (d,2 H, J = 7.6 Hz), 6.19 to 6.09 (m,1 H), 5.21 to 5.15 (m,1 H), 5.09 (s, 2 H), 4.74 to 4.60 (m, 1 H), 4.41 to 4.36 (m, 1 H), 3.49 to 3.45 (m, 2 H), 3.26 to 3.12 (m, 1 H), 3.07 (d, 2 H, J = 6.8 Hz), 2.97 to 2.76 (m, 1 H);

MS(ESI) : m/z = 460.1[M + H]+, 482.2[M + Na]+

In vitro Putrescine Incorporation Assay

In vitro tissue transglutaminase activity was assessed with an assay that measured 3H-putrescine incorporation into protein from cell lysates (10). Glioblastoma cells were grown in cell culture to ~70% confluence. Cells were treated with either vehicle or with various concentrations of KCC009 for 24 hours. Tissue transglutaminase activity was then assayed. Cells were washed in ice cold PBS and harvested in 50 mmol/L Tris-HCl (pH 7.5) containing 0.25 mol/L sucrose, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Lysates were spun at 10,000 × g at 4°C for 30 minutes. The assay buffer (total volume 300 μL) contained 100 mmol/L Tris (pH 8.5), 10 mmol/L DTT, 3.45 (m, 2 H), 3.26 to 3.12 (m, 1 H), 3.07 (d, 2 H, J = 6.8 Hz), 2.97 to 2.76 (m, 1 H):

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5 mmol/L CaCl₂, 0.2 mmol/L putrescine (with 0.51 μCi [³H]-putrescine), and 60 μL cell lysate. The activity was calculated as nanomoles of putrescine incorporated per hour per mg of total protein. Samples were run in duplicate.

Crystal Violet Assay
Cells were plated at a density of 7.5 × 10⁵ cells per well in 96-well microplate culture dishes. Quadruplicate wells were assessed for each time point, treatment condition, and cell type. After growing to 50% confluence, cells were fixed with 4% paraformaldehyde in PBS and stained with 1% crystal violet (BD Diagnostic Systems, Inc., Franklin Lakes, NJ) for 4 hours. The plates were washed with distilled water and allowed to air dry. The crystal violet was solubilized with 300 μL of 1% SDS per well. Absorbance was read at 570 nm with a microplate reader. Differences were assessed with a two-tailed Student’s t test for independent variables. Significance was determined with a P < 0.05.

Flow Cytometry (Fluorescence-Activated Cell Sorting)
Apoptotic cells were quantitatively identified with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences PharMingen, San Diego, CA). The cells were harvested and washed with cold PBS twice. They were then resuspended in 1× binding buffer at a concentration of 1 × 10⁶ cells/mL. One hundred microliters of resuspended cells (~1 × 10⁵ cells) were stained with 10 μL of FITC-conjugated Annexin V and 10 μL of propidium iodide reagent. After incubation in the dark for 15 minutes at room temperature, 1 mL of 1× binding buffer was added to each tube. The cells were sorted by a flow cytometry within 1 hour.

TUNEL Staining
Specimens were fixed in 4% paraformaldehyde and sectioned in 5-μm-thick sections. The tissue was then stained with terminal TUNEL. The assay labels nuclei positive that are undergoing DNA fragmentation characteristic of apoptosis. Prelabeling and labeling were done with a commercially available TUNEL kit, In situ Death Detection Kit TMR Red (BD Biosciences PharMingen) in accordance with the manufacturer’s instructions. Total nuclei were stained with Hoechst 33342 (Sigma). Slides were viewed with a Nikon fluorescent microscope and photomicrographs were analyzed with images analysis with Metamorph 6.2 software. Random images were assessed and the incidence of TUNEL-positive cells quantitated from between 3,000 and 4,000 cells per specimen. Differences were assessed with a two-tailed Student’s t test for independent variables. Significance was determined with a P < 0.05.

Western Blotting
Glioblastoma cells were grown in 100-mm dishes to an approximate confluence of 60% to 70%. Cells were washed with PBS and scraped in lysis buffer (Laemmli) from Sigma. Protein levels were determined with the Dot Metrics kit (Geno Technology, Inc., St. Louis, MO) and equivalent amount of protein (15 μg per lane) was loaded on SDS-PAGE gels. Following electrophoresis, the proteins were transferred onto Immobilon-P membranes. The membranes were blocked with 5% milk in TBS with 0.05% Tween 20 and blotted with primary antibody followed by the horseradish peroxidase–labeled secondary antibody. The reaction was developed with use of enhanced chemiluminescence plus from Amersham (Piscataway, NJ). Antibodies used for immunoblotting include anti-tubulin from Sigma; anti-Bim and anti-poly(ADP-ribose) polymerase from BD PharMingen; and anti-phospho-AKT (Ser⁴⁷³), anti-AKT, anti-survivin, anti-phospho-Bad, anti-GSK-3β, anti-caspase-7, and lamin A/C from Cell Signaling Technology (Beverly, MA).

Results
Transglutaminase 2 Inhibition Induces Apoptosis in Glioblastoma Cells
U87 cells were treated with increasing doses of monodansylcadaverine or KCA075 25 mg/kg body weight. Treatments were continued for selected lengths of time. Subtherapeutic doses of BCNU (10 or 5 mg/kg) were given to subgroups at specified times. Tumors were removed and weighed to determine size (day 21 after implantation) or analyzed for apoptosis with TUNEL staining (day 10 after implantation). Differences between comparative groups were assessed with a two-tailed Student’s t test for independent variables. Significance was determined with a P < 0.05.

DBT Mouse Subcutaneous Tumor Model
DBT glioblastoma cells were grown with established cell culture techniques. Tumors generated after either intracranial or s.c. implantation of the DBT cells are analogous to human glioblastomas in their aggressive growth pattern, histopathologic characteristics, and immunoreactivity for glial fibrillary acidic protein (11, 12). DBT cells were grown in culture to confluence. Cells were passed by removing feeding medium, washing attached cells with CMF-HBSS (Mediatech, Herndon, VA), and then detaching the cells from the flask with 0.25% trypsin-EDTA (Life Technologies). The trypsin-EDTA was neutralized with feeding medium containing fetal bovine serum, and the suspension centrifuged at 1,000 rpm for 5 minutes. BALB/c mice (~20 g), from Charles River Laboratories (Wilmington, MA), were anesthetized with methoxyflurane per nose cone. DBT glioblastoma cells, 2 × 10⁶ in 50 μL, were injected into the s.c. tissues of each flank. In vivo research was done in accordance with the Washington University Animal Studies Committee guidelines. Tumors were allowed to establish for 1 week before initiating treatments. Mice were injected i.p. daily either with vehicle only or with transglutaminase 2 inhibitors, monodansylcadaverine or KCA075 25 mg/kg body weight. Differences were assessed with a two-tailed Student’s t test for independent variables. Significance was determined with a P < 0.05.
The effect of transglutaminase 2 inhibition was examined in three glioblastoma cell lines, U87, U138, and DBT. Treatment with monodansylcadaverine induced cell death in each of the three glioblastoma cell lines. Glioblastoma cells treated with 200 \( \mu \text{mol/L} \) monodansylcadaverine for 24 hours resulted in distinct morphologic changes among the three cell lines. U87, U138, and DBT cells became rounded and detached within 12 hours after monodansylcadaverine treatment. Morphologic evidence of cell death such as the appearance of folded, roughened cell membranes, and the release of nuclear and cytoplasmic substance appeared in cells during the first day. These changes were most striking in U138 and DBT cells and moderately present in U87 (Fig. 1B). Flow cytometry studies (fluorescence-activated cell sorting, FACS) after 200 \( \mu \text{mol/L} \) monodansylcadaverine treatment for 24 hours showed an increased incidence of apoptosis. Glioblastoma cells were incubated with 200 \( \mu \text{mol/L} \) monodansylcadaverine were harvested and stained with propidium iodide and Annexin V. All three glioblastoma cell lines showed an increase in apoptosis after treatment with monodansylcadaverine indicated by the number of cells to the right of the vertical line in the graph that stain positive for Annexin V and a decrease in viable cells indicated by cells above the horizontal line that stain positive for propidium iodide. Statistical analysis with \( \chi^2 \) determinations demonstrated significant differences between the vehicle only–treated controls and the corresponding groups treated with monodansylcadaverine (\( P < 0.001 \)).
Transglutaminase 2 Inhibition Enhances Proapoptotic Changes in Intracellular Proteins

U87 and U138 cells were treated with increasing doses of monodansylcadaverine including 50, 100, and 200 μmol/L for 24 hours. Western blots showed changes in a number of proteins associated with survival at doses of monodansylcadaverine that correlated with a loss of viable cells and increased incidence of apoptosis. Levels of phosphorylated Akt have been associated with malignancy and decreased sensitivity to chemotherapy in astrocytomas. Phosphorylated Akt promotes multiple downstream survival signals. Monodansylcadaverine treatment at 100 or 200 μmol/L resulted in decreased levels of phosphorylated Akt. Alterations in proteins downstream from phosphorylated Akt include the antiapoptotic proteins survivin, phosphorylated Bad, and phosphorylated GSK-3β. All three of these proteins are decreased after treatment with doses of 200 μmol/L monodansylcadaverine (Fig. 2). These changes are all predicted to decrease the resistance to apoptosis in the glioblastoma cells.

Changes in proapoptotic BH3-only protein, Bim, was assessed in the two glioblastoma cell lines U87 and U138 after treatment with increasing doses of monodansylcadaverine including 50, 100, and 200 μmol/L at 24 hours. Bim levels were increased in U87 and U138 cells treated with the higher doses of monodansylcadaverine (Fig. 2). The changes in levels of the above proteins reflect an increased sensitivity toward apoptosis.

KCC009 Induces Apoptosis in U87 Cells

We initially tested the effects of inhibition of transglutaminase 2 with monodansylcadaverine. Monodansylcadaverine is a substrate competitor of transamidation and has been used as an inhibitor of transglutaminase 2 in a number of studies. However, monodansylcadaverine is not a highly specific transglutaminase 2 inhibitor. We therefore examined the effects of transglutaminase 2 inhibition with the irreversible, highly specific dihydroisoxazole small-molecule inhibitors, KCA075 or KCC009. Initial experiments with FACS studies showed that treatment of DBT cells for 24 hours with 50 μmol/L KCA075 resulted in an increase in the incidence of apoptosis (annexin-positive cells) from 9% in the control to 30% in the KCA treated. Later studies were done with the more soluble small-molecule irreversible transglutaminase 2 inhibitor, KCC009. The incidence of apoptosis in U138 cells treated with KCC009 for 24 hours increased from 2% in the controls compared with 28% in the treated groups (data not shown). Dose-response studies with KCC009 in U87 cells showed progressively striking morphologic changes consistent with cell death including detachment of cells and loss of membrane integrity (Fig. 3A). FACS studies showed an increase in the frequency of apoptosis with increased doses of KCC009: 8% (control), 29% (0.5 mmol/L KCC009), and 74% (1.0 mmol/L KCC009; Fig. 3A). Statistical analysis with χ² determinations showed significant differences (P < 0.001) between the vehicle-only treated controls and the corresponding groups treated with KCC009. These findings show that treatment of three glioblastoma cell lines with two different classes of transglutaminase 2 inhibitors resulted in apoptosis in three glioblastoma cell lines.

We further characterized the nature of cell death induced in glioblastoma cells after treatment with transglutaminase 2 inhibitors. Cells were assessed for activation of the executioner caspases. Cells were grown in culture and treated with either vehicle only or with KCC009 for 24 hours. Protein lysates were obtained and examined with Western blots. Cells treated with KCC009 showed an increase in levels of poly(ADP-ribose) polymerase, lamin A/C, and caspase-7 cleavage products (Fig. 3B). These changes are consistent with activation of executioner caspases associated with apoptosis.

The significance of changes in regulators of apoptosis after treatment with transglutaminase 2 inhibitors was assessed with addition studies. Specifically, we evaluated the effect on the incidence of apoptosis associated with the decrease levels of phosphorylated GSK-3β after treatment with KCC009. Phosphorylation of GSK-3β inhibits its activity and promotes its antiapoptotic effect. Treatment with KCC009 results in decreased levels of phosphorylated GSK-3β and therefore increases its activity. We examined the ability of the selective noncompetitive small molecule GSK-3β inhibitor, SB216763, to block GSK-3β activity and the associated increase incidence of apoptosis after treatment with KCC009. U87 cells were treated with the combination KCC009 and SB216763 and compared with those treated with KCC009 alone. Treatment with the GSK-3β inhibitor increased the levels of phosphorylated GSK-3β on Western blots (Fig. 3C) and decreased the incidence of apoptosis as determined from FACS analysis from 74% with those treated with KCC009 alone compared with 44% in those treated with KCC009 and SB216763 (Fig. 3A and C).
Measurement of Transglutaminase 2 Activity

We measured the transglutaminase 2 activity in U87 cells with the in vitro putrescine incorporation assay. The findings showed that U87 cells treated with increasing doses of KCC009 showed inhibition of transglutaminase 2 activity at a dose of 1.0 mmol/L (Fig. 4A). Western blots of U87 cells treated with increasing doses of KCC009 showed decreased levels of phosphorylated Akt at a dose of 1.0 mmol/L (Fig. 4A). The dose at which transglutaminase 2 activity was inhibited with KCC009 in U87 cells correlated with dose that resulted in decreased levels of phosphorylated Akt (Fig. 4A) and with induction of apoptosis (Fig. 3A-B). Treatment of U87 and U138 cells with KCC009 resulted in changes in levels of antiapoptotic and proapoptotic protein levels (Fig. 4B) very similar to those seen described after treatment with monodansylcadaverine. Treatment with BCNU alone at a dose of 6.25 μg/mL did not result in changes in phosphorylated Akt or downstream targets (Fig. 4B), suggesting BCNU therapy alone may work through alternative pathways.

Transglutaminase 2 Inhibitors Enhances Sensitivity of Glioblastoma Cells to Chemotherapy In vitro

Treatment with transglutaminase 2 inhibitors was shown to alter regulators and promote cell death in glioblastoma cells in vitro. We hypothesized that inhibition of transglutaminase 2 would sensitize glioblastoma cells to increased cell death after treatment with chemotherapy. Murine DBT cells were examined to assess the ability of treatment with transglutaminase 2 inhibitors to enhance BCNU chemotherapy in vitro. DBT cells were grown in cell culture and treated with either monodansylcadaverine alone, BCNU alone, or with the combination of monodansylcadaverine and BCNU. FACS analysis of DBT cells

Figure 3. A, photomicrographs from U87 cells treated with increasing doses of KCC009 showed changes consistent with cell death including detachment from the cell culture plate and loss of membrane integrity. FACS analysis determined the incidence of apoptosis from U87 glioblastoma treated with increasing doses of KCC009 and stained with propidium iodide and Annexin V. There was an increase in apoptosis after treatment with increasing doses of KCC009 as indicated by the cells to the right of the vertical line that stain positive for Annexin V and a decrease in viable cells as indicated by cells above the horizontal line that stain positive for propidium iodide. Statistical analysis with χ² determinations showed significant differences between the vehicle only–treated controls and the corresponding groups treated with monodansylcadaverine (P < 0.001). B, U87 cells were treated with either vehicle only or KCC009 for 24 h. Protein lysates were examined with Western blots. Cells treated with 1.0 mmol/L KCC009 showed an increase in levels of caspase-7 cleavage products, lamin A/C levels, and poly (ADP-ribose) polymerase (PARP) cleavage products. C, treatment of U87 cells with KCC009 (1.0 mmol/L) and SB216763 (10 μmol/L) compared with KCC009 (1.0 mmol/L) alone (A) showed a decreased incidence of apoptosis from 74% (KCC009 alone) to 44% (KCC009 + SB216763) as determined with FACS analysis.
that promoted apoptosis in U87 cells. This also correlated with the dose of KCC009 correlated with a major decrease in phosphorylated Akt in cells treated total Akt. There was a marked inhibition of transglutaminase 2 activity that run in duplicate. Western blots from U87 cells treated with similar KCC009 increasing doses from control: 0.1, 0.5, and 1.0 mmol/L. Samples were
dansylcadaverine or 6.25 A
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200
respectively. However, treatment with the combination of
showed an incidence of apoptosis of 32% and 22%,
Treatment of Mice with Transglutaminase 2 Inhibitors Augments Sensitivity of DBT Glioblastoma Cells to BCNU Chemotherapy in vivo
The in vivo findings that treatment with transglutaminase 2 inhibitors promoted apoptosis in DBT glioblastoma cells were extended to observations with in vivo studies. The ability of the transglutaminase 2 inhibitors, monodansylcadaverine or KCA075, to enhance chemotherapy and decrease tumor growth in glioblastoma cells were examined in mice with bilaterally implanted syngeneic DBT glioblastoma xenografts. Tumors were established s.c. for 1 week before initiating treatments. Mice were injected i.p. daily for 10 treatments with either vehicle only or with monodansylcadaverine or KCA075 at a dose of 25 mg/kg body weight. Doses of 50 mg/kg monodansylcadaverine were associated with toxicity; three of five mice died and the other two were lethargic and listless after several injections. There were no deaths in the groups treated with 25 mg/kg of monodansylcadaverine or KCA075. On days 10 and 12, specific groups of mice were conjected with vehicle only or BCNU 10 and 5 mg/kg, respectively. We chose these doses of BCNU based on results of earlier studies with the DBT mouse tumor model. BCNU given in two doses as described in the protocol above results in little or no decrease in tumor size. On day 21, tumors were dissected and weighed. The group of mice treated with vehicle only developed tumors weighing 2.43 ± 0.25 g (n = 10). The tumors from the groups treated with BCNU alone weighed 3.05 ± 0.49 g (n = 10). The group treated with the combination of monodansylcadaverine and BCNU developed tumors that weighed 1.51 ± 0.22 g (n = 8). The group treated with KCA075 and BCNU were found to have tumors that weighed 1.63 ± 0.15 g (n = 10). The tumors in the mice treated with combinations of monodansylcadaverine and BCNU or KCA075 and BCNU were significantly smaller than those from the groups treated with mono-therapy with BCNU alone or with vehicle only (Fig. 5A-B). This decrease in tumor weight was significantly different as assessed with a two-tailed Student’s t test for independent variables. Significance was determined with a P < 0.05.

The ability of transglutaminase 2 inhibitors to enhance the incidence of apoptosis in tumors after treatment with chemotherapy in vivo was assessed with TUNEL staining. A group of mice were implanted with DBT glioblastoma cells and tumors were allowed to develop s.c. for 6 days. Mice were then injected i.p. daily for four treatments with either vehicle only, monodansylcadaverine, or KCA075 at a dose of 25 mg/kg body weight. One group was treated with vehicle only, and all of the remaining groups were injected with 10 mg BCNU /kg body weight 24 hours before sacrifice. The groups were assessed for the incidence of apoptosis with TUNEL staining; vehicle only (0.7 ± 0.06%; n = 3), vehicle only + BCNU (1.0 ± 0.3%; n = 3); KCA075 + BCNU (2.3 ± 0.2%; n = 4), and monodansylcadaverine + BCNU (1.6 ± 0.1%; n = 3). Those treated with transglutaminase 2 inhibitors showed a significantly greater incidence of apoptosis compared with the vehicle-only group as determined with a two-tailed Student’s t test for independent variables. Significance was determined with a P > 0.05 (Fig. 6C).

Due to technical reasons, we were unable to quantify tumor-associated transglutaminase 2 activity. However, in experiments with control mice dosed with comparable doses of bromo-dihydroisoxazole inhibitors (30–60 mg/kg, i.p.), >80% of transglutaminase 2 activity in small intestinal mucosa was blocked at 1 hour after administration. Thus, the observed pharmacologic effect of KCA075 on flank tumors is likely to be due to transglutaminase 2 inhibition.

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5 M. Siegel, unpublished results.
Discussion
Among the multiple cellular functions of transglutaminase 2 are examples of transglutaminase 2 activity that are correlated with induction of apoptosis or conversely with promotion of cell survival. Expression of transglutaminase 2 has been associated with apoptosis during development with formation of the interdigital web and myoblasts (13) and during embryo implantation and postpartum involution of the uterine epithelium (14). Transglutaminase 2 expression increases significantly in response to apoptotic stimuli in a number of cell types. Alternatively, cultures with primary thymocyte cells isolated from transglutaminase 2 knockout mice seem more sensitive to cell death (15). Activity of transglutaminase 2 is necessary to allow the protective effect of retinoic acid. Inhibition of transglutaminase 2 with monodansylcadaverine converts retinoic acid from a differentiating agent to an apoptotic agent (16). Epidermal growth factor up-regulated activity of transglutaminase 2 in breast carcinoma cells was associated with increased survival. In the same study, down-regulation of transglutaminase 2 activity with monodansylcadaverine or with a dominant-negative form of transglutaminase 2 enhanced sensitivity of breast carcinoma cells to treatment with doxorubicin (17).

A key protein associated with survival in glioblastomas after chemotherapy is phosphorylated Akt. Inactivation of Akt and its downstream survival targets results in enhanced sensitivity to chemotherapy (18). Elevated levels of phosphorylated Akt via the phosphatidylinositol 3-kinase pathway have been implicated in the pathogenesis of glioblastoma and in their resistance to chemotherapy (19, 20). Up to 80% of glioblastomas express elevated levels of Akt (21). Activation of Akt pathway is associated with progression of anaplastic astrocytomas to glioblastomas (19). Down-regulation of the Akt pathway with an adenovirus containing MMAC/PTEN (Ad-MMAC) resulted in induction of anoikis in glioblastoma cells (22). Western blots show that apoptosis induced with inhibition of transglutaminase 2 in U87 and U138 cells are correlated with decreased levels of phosphorylated Akt. Changes downstream from Akt induce decreased levels of the antiapoptotic proteins phosphorylated Bad (23), survivin (24), and phosphorylated GSK-3β (25). These changes are all predicted to decrease the resistance to apoptosis in the glioblastoma cells. The significance of these changes, specifically decreased levels of phosphorylated GSK-3β, was evaluated by treating cells with the selective GSK-3β inhibitor, SB216763. Cells cotreated with KCC009 and SB216763 showed 40% less apoptosis compared with those treated with KCC009 alone.

The proapoptotic BH3-only protein, Bim, was identified independently after screening a cDNA expression library with a Bcl-2 probe (26) and after experiments with a two-hybrid yeast assay with Mcl-1 as bait (27). Members of the BH3-only protein group all share the biological function of promoting Bax-dependent apoptosis via mitochondrial release of cytochrome c and initiation of downstream cell death programs (28). The role of Bim in modulating tumor responses to chemotherapy is not well understood. Loss of Bim function was associated with resistance to apoptosis after exposure to the chemotherapy agent, taxol, in purified pre-T cells (29). Lovastatin treatment resulted in extensive cell death in the glioblastoma cell lines, U87 and U251. Lovastatin-induced death was associated with significantly increased levels of the proapoptotic protein, Bim (30). We noted an increase expression of Bim in both U87 and U138 cells treated with transglutaminase 2 inhibitors in association with apoptosis.

The alkylating agent, BCNU, has been frequently used in treatment of patients with glioblastomas for >30 years. Clinical studies have shown that BCNU chemotherapy and radiation therapy improves survival compared with radiation treatment alone. In a group of 358 patients with malignant gliomas treated with BCNU in addition to radiation therapy and compared with those treated with radiation therapy alone, the addition of BCNU to the treatment regimen improved median survival by 15 weeks (31). Responses to chemotherapy are difficult to accurately
predict. Chemosensitivity testing in vitro shows variable responses between individual cell lines and particular drugs agent tested. There has however been a general correlation between chemosensitivity testing in vitro compared with in vivo responses (32, 33). Tumors resistant to chemotherapy have developed mechanisms that inhibit apoptotic pathways. Identifying agents that reverse these antiapoptotic factors in resistant neoplasms may render these tumor cells sensitive to chemotherapy (34). The concept was shown in colon carcinoma cells by reversing chemotherapy resistance mediated by the transcription factor, nuclear factor-κB. The use of inhibitors of nuclear factor-κB promotes chemosensitivity and enhances apoptosis. These studies suggest that therapies that target antiapoptotic mechanisms may augment chemotherapy in typically resistant neoplasms (35). The data presented in the current report supports the hypothesis that inhibition of transglutaminase 2 sensitizes glioblastoma to chemotherapy. The in vitro observations after treatment of cells with transglutaminase 2 inhibitors showed they are capable of promoting apoptosis or sensitizing glioblastoma cells to chemotherapy. It was hypothesized that targeting survival promoting pathways such as activated Akt and modulating downstream effectors would sensitize glioblastoma cells to chemotherapy. As proof-of-principle, we examined the ability of transglutaminase 2 inhibitors to augment chemosensitivity with an in vivo mouse model. Compared with BCNU monotherapy, combination chemotherapy (with BCNU and transglutaminase 2 inhibitors) in mice harboring syngeneic DBT glioblastoma xenografts led to substantial reduction of tumor weights after a series of 10 treatments over a 3-week period. Treatment with BCNU and transglutaminase 2 inhibitors resulted in a 50% decrease in tumor weight compared with treatment with BCNU alone. Treatment of mice with the combination of transglutaminase 2 inhibitors and subtherapeutic dose of BCNU resulted in...
a significant increase of apoptotic cells. The data suggest that the findings in vitro may be translated to animal models. Additional experiments with the DBT s.c. tumor model have been completed that examined the transglutaminase 2 inhibitor, KCC009. These data showed similar results as with treatment with monodansylcadaverine and KCA075 (8).

In conclusion, we identified the novel finding that inhibition of transglutaminase 2 promotes cell death in glioblastoma cells. We examined the effects of the competitive transglutaminase 2 inhibitor, monodansylcadaverine and the irreversible small-molecule transglutaminase 2 inhibitors, KCC009 and KCA075 on multiple glioblastoma cell lines. Transglutaminase 2 inhibition resulted in a change toward a proapoptotic profile in glioblastoma cells that include decreased levels of phosphorylated Akt and its downstream targets survivin, phosphorylated GSK-3β, and phosphorylated Bad, and increased levels of Bim. Use of a mouse model showed that treatment of s.c. DBT glioblastoma cells with combination of monodansylcadaverine and BCNU or with combination of KCA075 and BCNU decreased tumor growth by 50% and significantly increased the incidence of apoptosis. These studies identify transglutaminase 2 inhibitors as a new class of agents that may augment the sensitivity of glioblastoma cells to apoptosis and chemotherapy.

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References

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