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Tissue transglutaminase 2 expression in meningiomas

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Abstract

Meningiomas are common intracranial tumors that occur in extra-axial locations, most often over the cerebral convexities or along the skull-base. Although often histologically benign these tumors frequently present challenging clinical problems. Primary clinical management of patients with symptomatic tumors is surgical resection. Radiation treatment may arrest growth or delay recurrence of these tumors, however, meningioma cells are generally resistant to apoptosis after treatment with radiation. Tumor cells are known to alter their expression of proteins that interact in the ECM to provide signals important in tumor progression. One such protein, fibronectin, is expressed in elevated levels in the ECM in a number of tumors including meningiomas. We recently reported that levels of both extracellular fibronectin and tissue transglutaminase 2 (TG2) were increased in glioblastomas. We examined the expression of fibronectin and its association TG2 in meningiomas. Both fibronectin and TG2 were strongly expressed in all meningiomas studied. TG2 activity was markedly elevated in meningiomas, and TG2 was found to co-localize with fibronectin. Treatment of meningiomas with the small molecule TG2 inhibitor, KCC009, inhibited the binding of TG2 to fibronectin and blocked disposition of linear strands of fibronectin in the ECM. KCC009 treatment promoted apoptosis and enhanced radiation sensitivity both in cultured IOMM-Lee meningioma cells and in meningioma tumor explants. These findings support a potential protective role for TG2 in meningiomas.

Keywords

Brain tumor; Cell death; tissue transglutaminase inhibitor; Radiation

Introduction

Meningiomas are among the most common intracranial tumors with an annual incidence of approximately 6 per 100,000 [1]. Although typically considered benign and only rarely frankly malignant, management of these tumors often pose clinical challenges. Histological grades of meningiomas vary, with the majority classified as typical, WHO grade I (82%); less commonly atypical, WHO II (15%); and infrequently as anaplastic or malignant, WHO grade III (3%). The tumor doubling times vary widely ranging from 1.27 to 143.5 years

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(mean, 21.6 yr) [2]. Treatment recommendations for patients with meningiomas are based on multiple clinical considerations. Although surgery is the primary treatment modality, meningiomas may be attached to critical neurological or vascular structures. In these situations aggressive surgical resection may result in debilitating neurologic sequelae. Post-surgical follow up shows that recurrence rates invariably increase with time with an approximately 20-50% 10-year recurrence rate, irrespective of surgery and radiation treatment [3]. Stereotactic radiosurgery is an alternative treatment modality to control growth of small- or medium-sized meningiomas, although there are a number of failures, especially with higher grade atypical or anaplastic meningiomas [4,5]. Radiation treatment often does not decrease tumor mass and may be associated with radiation-induced effects in the surrounding brain. Thus an agent that sensitizes meningioma cells to apoptosis and radiation may enhance treatment efficacy in a significant group of patients with limited options in the management of their tumors.

Transglutaminase 2 (TG2, a.k.a. tissue transglutaminase) belongs to a family of enzymes that play important roles in diverse biological functions by selectively cross-linking proteins [6,7,8]. TG2, cross-links fibronectin in the ECM of organs such as brain, liver and the intestine. It has both extracellular as well as intracellular functions. Intracellular TG2 loses enzyme activity when bound to GTP [9]. Extracellular TG2 plays a crucial role in shaping the ECM by cross-linking fibronectin and related proteins. TG2 also promotes cell adhesion and motility by forming non-covalent complexes with other key proteins such as integrins and fibronectin [10].

The active site of TG2 includes a cysteine thiol group that reacts with a glutamyl substrate to form a reactive thioester intermediate, from which the acyl group is transferred to an amine substrate, such as a lysine on another protein or a polyamine. Several electrophilic functional groups have been used to design TG2 inhibitors that inactivate cysteine-dependent enzymes. We have reported that one such small molecule TG2 inhibitor, (*S*)-[3-(4-hydroxyphenyl)-2-*N*-(phenylmethyloxycarbonyl)aminopropanoic acid *N'*-(3'-bromo-4',5'-dihydro-5'-isoxalyl)methylamide (designated KCC009), promotes cell death and sensitizes glial tumors to chemotherapy. [11,12,13].

In current study, we assessed the levels of TG2 in human meningiomas. We also investigated the potential of KCC009 to sensitize meningioma cells to apoptosis after radiation treatment. The activity and protein levels of TG2 in human meningiomas were found to be markedly elevated. Immunohistological staining demonstrated TG2 co-localized with fibronectin in meningioma cells. Disruption in the assembly and disposition of fibronectin in the ECM were correlated with treatment with KCC009. KCC009 enhanced cell death and increased radiation sensitivity in meningioma cells *in vitro* and in meningioma tissue explants. These studies identify the TG2 inhibitor, KCC009, as a novel radio-sensitizer in treatment of meningiomas.

Material and Methods

Reagents and Cell Culture

KCC009 ((*S*)-[3-(4-hydroxyphenyl)-2-*N*-(phenylmethyloxycarbonyl)aminopropanoic acid *N'*-(3'-bromo-4',5'-dihydro-5'-isoxalyl)methylamide) was prepared as described earlier [13]. The IOMM-Lee human meningioma cells [14] were cultured in 5% CO₂ and 95% humidified air atmosphere at 37°C in complete MEM medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1mM non-essential amino acids, 1 mM sodium pyruvate and 2mM glutamine (all from Life Technologies, Inc. Grand Island, NY, USA). After cells reached 60% confluence, groups

were treated with vehicle (1% DMSO) or with KCC009 0.5 mM for the designated time periods.

Tissue explants studies

Meningioma tissue was directly harvested from the operating room for storage in the Tumor Repository with approval of the Washington University Institutional Review Board. Tumor specimens were evaluated with tissue explants. Meningioma tissue was directly transferred into DMEM medium and washed X2, and then cut into 4mm × 2mm × 2mm specimens. The explants were then placed in 24 well culture dishes. After waiting 10 minutes for the tissue to attach to the plate 0.5 ml of DMEM with 10% FBS was added, and the explants were maintained in a 5% CO₂/95% atmosphere.

Meningioma tissue explants were irradiated with a Pantak PMC-1000 orthovoltage x-ray generator operated at 250kV, 12 mA, with added filtration of 0.25 mm Cu and 1.0 mm Al (HVL = 1.304 mm Cu). Cells were treated in a temperature- and CO₂-regulated chamber with doses delivered at a rate of approximately 1 Gy per min.

In vitro putrescine incorporation assay

In vitro TG2 activity was assessed with an assay that measured ³H-putrescine incorporation into N,N-dimethyl casein. [15] Frozen tissue sections were allowed to thaw on ice. The tissue was then diluted into 400 µl of homogenization buffer (50 mM mannitol, 2 mM Tris, 1 mM EDTA pH = 7.2) and homogenized with approximately 35 strokes of the pestle in a 1 ml dounce tissue grinder. The homogenate was sonicated for 8 seconds using a microtip sonicator, and insoluble tissue was pelleted by pulse centrifuging.

A fresh substrate solution containing 83 mM dithiothreitol, 83 mM Tris-HCl, 16.7 mM calcium chloride, 4.17 mg/ml N,N-dimethyl casein, pH = 9.0, 0.167% Triton X-100, 40 µM unlabeled putrescine dihydrochloride (ICN Biomedicals), and 20 µM [[1,4]-¹⁴C] putrescine dihydrochloride from Amersham (Piscataway, NJ, USA) was prepared, and 160 µl aliquots were pre-warmed to 37°C for 30 minutes. The putrescine incorporation assay was initiated by adding 40 µl of the tissue homogenate to the pre-warmed substrate solution. Time points were taken after 15, 30, 45, and 60 minutes of incubation at 37°C. 30 µl of the reaction mixture was spotted onto a dry 3-MM Whatman chromatography paper (approx. 1" × 1") pre-soaked in 10% trichloroacetic acid (TCA) and immediately plunged into a bath of 10% TCA. Each filter paper was soaked 10 minutes in 10% TCA, 10 minutes in 5% TCA two times, 1 minute in 50% ethanol/50% acetone, and 1 minute in acetone. The filter papers were air dried, placed in 5 ml Ready Safe Scintillation Cocktail (Beckman Coulter), and counted on a Beckman LS 3801 liquid scintillation counter. Non-washed samples were used to quantify the amount of putrescine cross-linked to dimethyl casein, and samples without homogenate added were analyzed to determine background counts. Reaction rates were calculated using linear regression. The activity was calculated as pmoles of putrescine incorporated per minute per mg of total protein. Samples were analyzed in duplicate. Protein concentrations were used to normalize the data and were determined using the Bio-Rad protein assay.

Flow cytometry (FACS)

Apoptotic cells were quantitatively identified with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA, USA). The cells were examined for incidence of apoptosis with FACS analysis with propidium iodide and annexin V as previously described [11]. The incidence apoptosis was determined with or without pre-treatment with KCC009 in IOMM-Lee meningioma cells that were irradiated with increasing doses, including 0, 5, 10, and 15 Gy. Cells were irradiated as described above.

TUNEL staining

Specimens were fixed in 4% paraformaldehyde and sectioned in 5 μ M thick sections. The tissue was then stained with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL). The assay labels nuclei positive that are undergoing DNA fragmentation characteristic of apoptosis. Pre-labeling and labeling were performed with a commercially available TUNEL kit, In situ Death Detection Kit TMR Red (BD Biosciences Pharmingen, San Diego, CA, USA) in accordance with the manufacturer's instructions. Total nuclei were stained with Hoescht 33342 (Sigma, St Louis, MO, USA). Slides were viewed with a Nikon fluorescent microscope and photomicrographs were analyzed with images analysis with Metamorph 6.2 software. Random images were assessed from twenty regions from each group and the incidence of TUNEL positive cells quantitated from between 3000 and 4000 cells per specimen. Differences were assessed with a two-tailed Student's t-test for independent variables. Significance was determined with a $P < 0.05$.

Immunohistological staining

The levels of both TG2 and fibronectin were examined in specimens from normal brain tissue and meningiomas. The tissue was collected in the operating room, snap-frozen in liquid nitrogen, and stored in the Tumor Repository. Six- μ m-thick frozen sections were cut and immunostained with a mouse anti-human TG2 antibody (1:50; NeoMarker Fremont, CA), and with rabbit anti-human fibronectin antibody (1:400; Sigma, St. Louis, MO). Subsequently, slides were incubated sequentially with anti-mice IgG conjugated with Cy2, anti-rabbit IgG conjugated with Cy3 (both from Chemicon), and with Hoechst dye 33342 (Sigma, St Louis, MO, USA). Slides were viewed with fluorescent microscopy and analyzed with Metamorph 6.2 software.

Results

TG2 activity and protein levels in human meningiomas

Meningioma tumor samples were assessed for TG2 activity included 7 typical (WHO grade I); 5 atypical (WHO grade II); 3 anaplastic (WHO grade III); and 4 from normal brain samples. TG2 activity was measured with the *in vitro* putrescine incorporation assay. The activity of TG2 from tissue samples from meningiomas were markedly elevated up to greater than 10-fold compared with normal brain tissue (Figure 1). Correlation of the subtypes of meningiomas e.g., psammomatous, transitional, or meningotheliomatous, failed to show an association with specific levels of TG2 activity (data not shown).

The corresponding frozen meningioma specimens were also examined with immunohistological staining to correlate TG2 and fibronectin levels (Figure 2 A-D). The findings show no staining for TG2 and only staining in vascular structures for fibronectin in brain parenchyma (Figure 2A). In contrast, the meningioma tissue stained very strongly for both fibronectin and TG2 (Figure 2B-D). Figure 2(A-D) show examples of normal brain and representative meningioma tumors stained with fibronectin (red) and TG2 (green). Areas of co-localization of TG2 and fibronectin are depicted with yellow staining (Figure 2D). The density of fibronectin staining correlated with levels of staining with TG2 in a semi-quantitative manner as determined by a blinded examiner (data not shown). These findings demonstrate increased levels and activity of TG2 in association with a marked increase in the assembly and disposition of fibronectin in the ECM.

Immunofluorescent staining of fibronectin in meningioma cells after KCC009 treatment

IOMM-Lee meningioma cells were grown in culture and stained with antibodies to fibronectin (red). Nuclei were stained with Hoescht (blue). The meningioma cells in the

control group showed extensive disposition of fibronectin in the ECM (Figure 3A). The meningioma cells treated with KCC009 showed a failure of disposition of fibronectin in the ECM with clumping of fibronectin within the intracellular environment (Figure 3B). Similarly, meningioma tissue explants obtained from the operating room were treated with vehicle-only (Figure 3C) or with KCC009 (0.5 mM) (Figure 3D). After 24 hours meningioma cells in the tumor explants demonstrated similar changes as noted in the *in vitro* experiments. Explants treated with KCC009 showed loss of disposition and assembly of fibronectin in the ECM.

KCC009 enhances the effects of radiation in meningioma cells

We examined the ability of KCC009 to potentate the effect of radiation in IOMM-Lee cells. Cells were grown in culture to 60-70% confluence and treated with a single fraction of ionizing radiation with or without pre-treatment for 24 hours with KCC009 (0.5 mM). Figure 4A show IOMM-Lee cells 24 hours after treatment with 20 Gy radiation. The cells treated with KCC009 alone or with 20 Gy radiation alone showed no significant morphological changes. However, cells pre-treated KCC009 prior to receiving 20 Gy radiation showed dramatic morphological changes consistent with cell death. The dose-response curves of meningioma cells treated with increasing doses of radiation with or without 0.5 mM KCC009 were analyzed by FACS analysis to assess for the incidence of apoptosis. The annexin staining data show that KCC009 enhances apoptosis at multiple levels of increasing doses of radiation (Figure 4B).

The effects of radiation was then examined in meningioma tumor explants obtained from fresh tumor tissue and grown in a CO₂ incubator. The graph in Figure 5A shows that KCC009 pre-treatment increases the incidence of apoptosis in explants treated with a single fraction of 10 Gy. Corresponding photomicrographs of meningioma explants both treated with 10 Gy radiation showed that KCC009 treatment resulted in disruption of the fibronectin assembly in the ECM and loss of co-localization of TG2 and fibronectin (Figure 5B). These data show that treatment of both IOMM-Lee cells *in vitro* or treatment of meningioma tumor explants with KCC009 enhances their radio-sensitivity, as determined by apoptosis.

Discussion

We have recently reported that TG2 activity is elevated in glioblastomas compared with non-neoplastic brain [12]. Immunofluorescent studies showed increased staining of fibronectin co-localized with TG2 in the ECM in glioblastomas. Downregulation of TG2 in U87MG glioblastoma cells with RNAi demonstrated decreased assembly of fibronectin in the ECM. Treatment with KCC009 blocked the remodeling of fibronectin in the ECM in glioblastomas in both *in vitro* and *in vivo* studies [12]. This treatment also diminished levels of phosphorylated Akt and its down-stream anti-apoptotic effectors including survivin, phosphorylated BAD, and phosphorylated GSK [11]. Studies *in vivo* in mice with subcutaneous xenografts of DBT glioblastomas demonstrated that KCC009 combined with the chemotherapeutic agent BCNU, resulting in significantly decreased growth of tumors [11-13]. Based on these findings in gliomas, we further investigated the levels of TG2 expressed in meningiomas and its potential as a target to decrease resistance to apoptosis..

In addition to glioblastomas, elevated levels of TG2 have been found in several neoplasms including lung [16] and breast [17] cancers, suggesting a significant role for TG2 in tumorigenesis. High expression levels of TG2 have also been associated with resistance to chemotherapy in multiple tumor cell lines [18,19]. Changes in tumor phenotype associated with drug-resistance have been associated with metastatic potential [20,21]. For example, interaction between integrin α_5 and fibronectin is necessary for metastasis of B16 murine melanoma cells [22]. TG2 in the ECM functions as a co-receptor for both integrins and

fibronectin. TG2 binds to integrins while simultaneously binding to the gelatin-binding domain (42 kilodalton fragment) of fibronectin [23,24]. This critical pro-survival interaction, mediated by TG2 between fibronectin and integrin, results in association of the p85 PI3-kinase subunit with focal adhesion kinase and activation of Akt [25-27]. Phosphorylated Akt is an up-stream effector of anti-apoptosis signals including phosphorylated BAD [28], survivin [29] and phosphorylated glycogen synthase kinase-3 [30].

The extracellular matrix is a dynamic assembly of insoluble proteins that not only provides a scaffold for cell adhesion but also presents an interface capable of transducing extracellular signals for survival, migration and proliferation to intracellular protein kinase cascades. Changes in the composition of ECM proteins are common in tumors. For example, the interactions of proteins secreted into the ECM with integrins aid in cellular adhesion and promote cell survival signals [31,32]. Cancer cells do not depend on adhesion to the extracellular matrix for survival, and therefore tend to alter the interaction between integrin and intracellular signal proteins to promote tumorigenesis [33,34]. ECM alterations in tumors also increase cellular resistance to chemotherapy and radiation [35]. Human breast cancer and lung cancer cells pretreated with fibronectin and grown on fibronectin-coated culture dishes showed improved survival after exposure to a single dose of radiation [36]. The role of fibronectin in promoting survival in meningiomas has not been previously examined.

Meningiomas are derived from arachnoid cap cells that originate from mesenchymal elements. Normal arachnoid cells secrete a number of proteins associated with adhesins and elements of basement membranes including fibronectin, laminin, and collagen IV [37]. Studies have shown that up to 98-100% of meningiomas express fibronectin in the extracellular matrix (ECM) [38]. We hypothesized that KCC009 promotes sensitization of meningioma cells to apoptosis by interfering with the binding of TG2 to fibronectin as well as TG2 catalyzed crosslinking of fibronectin. Treatment with KCC009 inhibits the disposition and assembly of fibronectin in the ECM of meningioma tumors, as demonstrated both *in vitro* with IOMM-Lee cells and in meningioma tissue explants obtained from fresh tissue from the operating room. The data support our hypothesis that KCC009 blocks the interaction of TG2 with fibronectin and is associated with the failure to deposit and assemble fibronectin into a dense linear matrix in the extracellular space (Figure 3-4). The above findings of disrupted assembly and disposition of fibronectin in meningioma cells treated with KCC009 suggests a possible mechanism of enhanced cell death.

In conclusion, we have demonstrated that a series of meningiomas including WHO grades I - III all exhibited markedly elevated TG2 activity and protein levels. These are associated with changes in the disposition and assembly of fibronectin in the ECM. Treatment with KCC009 of dissociated tumor cells grown in culture or tumor explants resulted in decreased disposition and assembly of fibronectin in the ECM. Finally, IOMM-Lee meningioma cells or meningioma tissue explants treated with radiation either alone or with prior treatment with KCC009 demonstrated that KCC009 increased the sensitivity of meningioma cells to radiation therapy. These findings support a potential role for KCC009 as a radio-sensitizing agent in the treatment of meningiomas.

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Figure 1.

Meningiomas tumors were assayed for TG2 activity. These included tumor tissue from 15 meningiomas including 7 typical (WHO Grade I); 5 atypical (WHO Grade II); and 3 anaplastic (WHO Grade III);, and 4 normal brain specimens. TG2 activity was quantitated with the ^3H -putrescine incorporation assay. The activity was calculated as pmoles of putrescine incorporated per min per mg of total protein. Samples were run in duplicate. All tumor samples compared with normal brain samples were significantly elevated as assessed with the Student's t-test ($P < 0.05$ in each tumor sample compared with brain tissue).

Figure 2.

Frozen meningeoma specimens and normal brain tissue were examined with immunohistological staining to correlate the levels and co-localization of TG2 and fibronectin. Normal brain tissue shown in Figure 2A shows nuclei stained blue (Hoescht) and fibronectin (red) associated with blood vessels. There was minimal or no staining brain parenchyma with either fibronectin or TG2. The representative meningeoma tissues shown in Figure B (WHO grade I) and Figure C (WHO grade III) demonstrate heavy staining for both fibronectin (red) and TG2 (green). The staining for TG2 occurred throughout the cells' cytoplasm, while fibronectin staining was noted not only in the cytoplasm, but also in dense linear strands in the ECM along the borders of cells. Overlapping areas of staining that demonstrated co-localization of fibronectin (red) and TG2 (green) stained yellow (merged photograph) shown with higher magnification in Figure D.

Figure 3.

Human meningeoma IOMM-Lee cells grown in culture (top row) and meningeoma tumor tissue explants (lower row) were evaluated after treatment with KCC009. Meningeoma IOMM-Lee cells were treated with vehicle-only (Figure 3A) or with 0.5 mM KCC009 (Figure 3B). Similarly, meningeoma tissue explants were treated with vehicle-only (Figure 3C) or with 0.5 mM KCC009 (Figure 3D). After 24 hours meningeoma cells and tumor explants were examined with immunofluorescent staining for fibronectin (red) and nuclei with Hoescht (blue). The findings show that both IOMM-Lee cells *in vitro* and meningeoma tissue explants treated with KCC009 failed to assemble dense stands of fibronectin in the ECM. Fibronectin appeared clumped within meningeoma cells treated with KCC009.

Figure 4.

Cells were treated with single fractions of radiation with or without pre-treatment with KCC009 (0.5 mM). The photographs in Figure 4A show IOMM-Lee cells 24 hours after treatment with 20 Gy radiation. The cells pre-treated with KCC009 showed morphological changes consistent with cell death. Dose-response studies shown in Figure 4B analyzed the incidence of apoptosis with FACS analysis with annexin staining after increasing doses of radiation with or without KCC009 (0.5mM) pre-treatment. The data show that KCC009 enhances apoptosis with multiple increasing doses of radiation. Matched pairs were statistically analyzed with Chi-square analysis of the FACS results from at least 17,000 cells examined in each group (*); ($P < 0.001$).

Figure 5. Meningioma tissue explants were pre-treated with KCC009 and 24 hours later treated with a single fraction of radiation (10 Gy). The subsequent incidence of apoptosis in the explants was assessed with TUNEL staining. Figure 5A shows explants pre-treated with KCC009 and a later single fraction of 10 Gy of radiation demonstrated an increased incidence of apoptosis compared with those treated with vehicle-only, KCC009 alone, or 10 Gy of radiation alone. $P < 0.05$ determined with the Student's t-test (*). The micrographs of the immunohistochemical staining for TG2 and fibronectin demonstrated in Figure 5B show that in both groups that received radiation, pre-treatment with KCC009 was associated with disruption of fibronectin disposition in the ECM and a loss of co-localization of TG2 with fibronectin.