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T11TS inhibits Angiopoietin-1/Tie-2 signaling, EGFR activation and Raf/MEK/ERK pathway in brain endothelial cells restraining angiogenesis in glioma model

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ABSTRACT

Malignant gliomas represent one of the most aggressive and hypervascular primary brain tumors. Angiopoietin-1, the peptide growth factor activates endothelial Tie-2 receptor promoting vessel maturation and vascular stabilization steps of angiogenesis in glioma. Epidermal growth factor receptor (EGFR) and Tie-2 receptor on endothelial cells once activated transmits signals through downstream Raf/MEK/ERK pathway promoting endothelial cell proliferation and migration which are essential for angiogenesis induction. The in vivo effect of sheep erythrocyte membrane glycopeptide T11-target structure (T11TS) on angiopoietin-1/Tie-2 axis, EGFR signaling and Raf/MEK/ERK pathway in glioma associated endothelial cells has not been investigated previously. The present study performed with rodent glioma model aims to investigate the effect of T11TS treatment on angiopoietin-1/Tie-2 signaling, EGFR activity and Raf/MEK/ERK pathway in glioma associated endothelial cells within glioma milieu. T11TS administration in rodent glioma model inhibited angiopoietin-1 expression and attenuated Tie-2 expression and activation in glioma associated brain endothelial cells. T11TS treatment also downregulated total and phosphorylated EGFR expression in glioma associated endothelial cells. Additionally T11TS treatment inhibited Raf-1 expression, MEK-1 and ERK-1/2 expression and phosphorylation in glioma associated brain endothelial cells. Thus T11TS therapy remarkably inhibits endothelial angiopoietin-1/Tie-2 signaling associated with vessel maturation and simultaneously antagonizes endothelial cell proliferation signaling by blocking EGFR activation and components of Raf/MEK/ERK pathway. Collectively, the findings demonstrate a multi-targeted anti-angiogenic activity of T11TS which augments the potential for clinical translation of T11TS as an effective angiogenesis inhibitor for glioma treatment.

1. Introduction

Malignant gliomas are highly infiltrative primary brain tumors associated with a dismal prognosis and short survival even after most aggressive therapy (Stupp et al., 2005; Wen and Kesari, 2008). Gliomas are characterized by an intensely angiogenic phenotype which makes anti-angiogenic therapeutic interventions an attractive choice for glioma treatment. Endothelial cells play a pivotal role in various steps of tumor angiogenesis including endothelial cell proliferation, migration, invasion, adhesion and tube formation (Coultas et al., 2005; Jain, 2003). Angiopoietins are a family of peptide growth factors that bind specifically with the tyrosine kinase receptor Tie-2 primarily expressed on vascular endothelial cells (ECs) (Davis et al., 1996; Maisonpierre et al., 1997). Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) primarily regulate angiogenesis and influence blood vessel remodeling, maturation and vascular stabilization steps of tumor angiogenesis (Augustin et al., 2009; Fukuhara et al., 2010; Reiss et al., 2005). Several studies have emphasized the role of Ang-1 in promoting endothelial cell migration (Audero et al., 2004; Witzenbichler et al., 1998) and inducing brain capillary endothelial cell proliferation (Kanda et al., 2005) and tubule formation (Hayes et al., 1999). Both Ang-1 and Ang-2 stimulate tyrosine residue phosphorylation of endothelial cell Tie-2 receptor (Bogdanovic et al., 2006). Previous reports have shown that activation of Tie-2 receptor significantly contributes to angiogenesis and growth of astrocytic tumors (Zadeh et al., 2004). Ang-1 mediated activation of Tie-2 triggers downstream PI3K/AKT (DeBusk et al., 2004; Kim et al., 2000) and Raf/MEK/ERK pathways (Harfouche et al., 2003; Kim et al., 2002; Yoon et al., 2003) promoting endothelial cell survival and proliferation. Since angiopoietin-1/Tie-2 signaling acts as a central regulator of angiogenesis, attempts of therapeutic targeting of the angiopoietin-1/Tie-2 axis have received increased attention in recent years as an alternative to the VEGF signaling as targets.

Abbreviations: Ang-1, angiopoietin-1; T11TS, T11 target structure; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; MEK, mitogen activated protein kinase kinase; EC, endothelial cell

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Previous studies have shown that tumor endothelial cells express epidermal growth factor receptor (EGFR) (Amin et al., 2006). EGFR signaling plays a crucial role in vessel maturation step of angiogenesis as endothelial cells produce heparin-binding EGF (HB-EGF) which triggers tumor-induced angiogenesis via activation of vascular smooth muscle cells (Arkonac et al., 1998; Iivanainen et al., 2003). EGFR is widely recognized for its central role in cell proliferation and acts as an inducer of migration and proliferation in neoplastic glial cells as well as in glioma associated endothelial cells (Brockmann et al., 2003; Lund-Johansen et al., 1990). In glioblastoma xenograft models, EGFR activation has been reported to be associated with angiogenesis (Gullick, 2009). Hence new agents targeting EGFR in glioma associated brain endothelial cells are needed to combat angiogenesis in glioma.

Proliferation and migration of endothelial cells are required for the extension of the primitive vascular network. The Ras/Raf/MEK/ERK pathway transmits downstream signals when growth factor receptors such as EGFR and Tie-2 are activated by their respective ligands. Activation of the Raf/MEK/ERK signaling cascade in endothelial cells is necessary for endothelial cell proliferation and survival, two essential requirements for tumor angiogenesis (Bullard et al., 2003; Eliceiri et al, 1998; Murphy et al., 2006). Stimulation of multiple pro-angiogenic endothelial cell surface receptors like VEGFR-2, Tie-2 and EGFR activates the canonical Raf/MEK/ERK pathway in endothelial cells (Murphy et al., 2006) Treatment of endothelial cells with angiopoietin-1 causes activation of Tie-2 receptor which promotes activation of ERK signaling cascade (Alavi et al., 2003). Hence targeting Raf/MEK/ERK cascade in glioma associated endothelial cells represent a potential anti-angiogenic intervention that abrogates signaling associated with endothelial cell proliferation. Currently available multi-kinase inhibitor Sorafenib when administered in recurrent glioblastoma patients along with daily low-dose of temozolomide shows minimal therapeutic response and trigger incidence of relapse (Strumberg et al., 2005).

Until now no clinically proven anti-angiogenic agent has been developed for effectively targeting Ang-1/Tie-2 signaling in glioma. It is widely acknowledged that multi-targeted angiogenesis inhibitors inhibiting signaling cascade associated with maturation steps of angiogenesis and endothelial cell proliferation hold promise of sustained therapeutic outcome. T11-target structure (T11TS), the membrane glycopeptide isolated from sheep erythrocyte membrane is the sheep homologue of human CD58/lymphocyte function-associated antigen-3 (LFA-3). Earlier studies from our group showed that treatment with T11TS fraction exerts remarkable immunotherapeutic and anti-neoplastic action in chemically induced rodent glioma model (Bhattacharya et al., 2013; Chaudhuri et al., 2014; Mukherjee et al., 2005; Singh et al., 2014) and also in human glioma sample (Kumar et al., 2013). Toxicological inertness of this glycopeptide T11TS been established in our laboratory where T11TS treatment showed no acute or sub-acute toxicity in mice and rats (Sarkar et al., 2007, 2010). In our recent work we reported that T11TS administration in glioma induced rats imparts anti-angiogenic action by inhibiting VEGF signaling and prosurival PI3K/AKT/eNOS pathway in glioma associated endothelial cells (Bhattacharya et al., 2013). In order to further characterize the mechanistic basis of anti-angiogenic action of T11TS, in the present study we investigated whether T11TS treatment in rodent glioma model impedes angiopoietin-1/Tie-2 signaling in glioma associated brain endothelial cells and thus interferes with the maturation phase of angiogenesis. To evaluate the regulatory action of T11TS therapy on glioma endothelial cell proliferation signaling we investigated whether T11TS can effectively modulate brain endothelial cell EGFR activation and target Raf/MEK/ERK signaling essential for cell proliferation and downstream signal transduction from both EGFR and Tie-2 receptors. In this investigation we observed that T11TS administration remarkably inhibits angiopoietin-1 and Tie-2 expression and activation in glioma associated brain endothelial cells along with concomitant inhibition of EGFR activity and downstream blockade of Raf/MEK/ERK signaling in endothelial cells.

2. Materials and methods

2.1. Reagents and antibodies

N′-N′-ethylnitrosourea (ENU), Diethylaminoethyl (DEAE) cellulose, fetal bovine serum, collagenase, deoxyribonuclease, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), poly-L-lysine, mounting media with DAPI (Fluoroshield) and nitrocellulose membrane were purchased from Sigma-Aldrich, USA. Dispase (BD Biosciences), Percoll (Pharmacia Biotech) and p-nitroblue tetrazolium chloride (Calbiochem) were procured from respective suppliers. M199 culture media, Bovine Albumin Fraction V and antibiotics like penicillin and streptomycin were purchased from Gibco BRL (USA). Monoclonal primary antibodies specific to Ang-1, Tie-2, p-Tie-2 (Tyr992), EGFR, Raf-1, MEK-1, p-MEK-1 (Thr286), ERK-1/2 and p-ERK-1/2, CD31 (PECAM-1) and CD 34 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The primary antibody specific to p-EGFR (Tyr1068) was purchased from Cell Signaling Technology (Beverly, MA, USA). All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and grouping

Healthy newborn Druckrey rat pups 2–3 days old of both sexes were maintained. The animals six in each group were weaned at 30 days of age and housed in separate cages at 22 °C in a 12 hour light/darkness cycle. Animals were fed with autoclaved feed pellets and water ad libitum. The animals were grouped into five groups.

Normal: Age matched normal healthy adult control rats.
ENU: 5 month old glioma bearing rats treated intraperitoneally (i.p.) with acute dose of ENU when 3 to 4 days of age.
ET1: 5-month-old ENU-treated rats injected (i.p.) with the first dose of T11TS.
ET2: 5-month-old ENU-treated rats injected (i.p.) with the first and second doses of T11TS at an interval of 6 days.
ET3: 5-month-old ENU-treated rats injected (i.p.) with first, second and third dose of T11TS at an interval of 6 days each.

Animal maintenance and experiments were performed in accordance with protocols approved by Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiment on Animal (CPCSEA), Government of India.

2.3. Induction of glioma with ethylnitrosourea

N′-N′-ethylnitrosourea (ENU) was freshly prepared (10 mg/ml in sterile PBS, pH adjusted to 4.5) and injected intraperitoneally (i.p.) to newborn rat pups (3–5 days old) at an acute dose of 80 mg/kg body weight as described previously (Bhattacharya et al., 2013; Mukherjee et al., 2004). Induction of glioma was achieved at 100% success and gliomas were most pronounced in the 5th month (post-injection) which is the chosen month of animal procedure (Mukherjee et al., 2004).

2.4. Isolation of T11TS and its administration in animals

T11TS was isolated from sheep erythrocyte membrane using DEAE-cellulose column chromatography as described previously (Bhattacharya et al., 2013; Singh et al., 2015). The third elute fraction exclusively contains T11TS and was selected as the fraction of choice. The first dose of 1 ml of T11TS (0.4 mg/kg body weight) from elute fraction III was injected intraperitoneally to 5 month old glioma induced adult rats which was followed by a second booster dose of 1 ml on the sixth day and a third booster dose on the 12th day