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Single doublecortin gene therapy significantly reduces glioma tumor volume

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Abstract

We employed lentivirus based doublecortin (DCX), as a glioma suppressor gene therapy in an intracranial glioma tumor xenograft model in nude rats. Single DCX expressing lentivirus was directly administered into the tumor on day 8 after U87 tumor cell implantation. DCX treatment significantly reduced U87 glioma tumor volume (~60%) on day 14 after DCX lentivirus injection and significantly improved median survival of tumor bearing nude rats. DCX synthesis induced neuronal markers MAP2, TUJ1 and PSA-NCAM, and the glial marker, glial fibrillary acidic protein (GFAP) in the implanted U87 glioma tumors. DCX synthesis induced GFAP that co-localized with tubulin in the mitotic stage, inhibited cleavage furrow during cytokinesis and blocked mitosis in glioma cells. DCX lentivirus infection did not induce apoptosis, but significantly inhibited expression of the proliferation marker Ki-67 and the blood vessel marker von-Willebrand Factor (vWF). U87 and other glioma cells except brain tumor stem cells (BTSCs) do not express neuronal markers or both neuronal and glial markers. DCX synthesizing glioma cells express a phenotype of anti-angiogenic BTSC-like cells with terminal differentiation that causes remission of glioma cells by blocking mitosis via a novel DCX/GFAP pathway. Direct local delivery of lentivirus based DCX gene therapy is a potential differentiation based therapeutic approach for the treatment of glioma.

Keywords

Doublecortin; glioma suppression; neuronal and glial phenotype; brain tumor stem cells; differentiation therapy

Introduction

Doublecortin (DCX), is a brain specific gene, mutated in human X-linked Lissencephaly and double cortex syndrome, and is expressed specifically in neuroprogenitor/stem cells (NSCs) and newborn neurons, but is absent in glioma cells and other brain cells such as astrocytes, microglia, oligodendrocytes and cerebral endothelial cells (Gleeson et al., 1998, Santra et al. 2009). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a tumor suppressor gene, is mutated by the most common genetic alteration of loss of heterozygosity in gliomas (Steck et al., 1997). PTEN synthesis induces DCX expression in glioma cells and suppresses glioma (Santra et al., 2006a). Loss of DCX expression in glioma therefore may underlie the effects of PTEN loss on malignancy in glioblastoma multiforme. DCX expression in U87 cells causes a loss of malignant phenotype, induces proliferation arrest, loss of migratory

potential and fails to generate tumor xenograft in immunocompromised hosts (Santra et al., 2006a).

DCX synthesis induces the neuron-specific protein MAP2 and nestin, a marker of NSC identity (Santra et al. 2009). MAP2 and nestin also inhibit tumor growth and prolong the survival of animal with tumors (Glass et al., 2005; Soltani et al., 2005). Self-renewal is the hallmark property of normal stem cells and neoplastic tissues (Reviewed in refs. Reya et al., 2001, Das et al. 2008). In leukemia and solid cancers including glioma, a small proportion of cells are phenotypically transformed to cancer stem cells (CSCs) with indefinite potential for self-renewal that drives tumorigenesis (Reviewed in refs. Reya et al., 2001, Das et al. 2008). CSCs are resistant to chemotherapy because of high expression of multiple drug transporter proteins (Reviewed in refs. Das et al. 2008, Tunici et al. 2006). All treatments of glioblastoma multiforme with surgery, radiation, and chemotherapy fail (Reviewed in ref. Das et al. 2008). According to the CSC hypothesis, only CSCs have self-renewal ability (Reviewed in refs. Das et al. 2008). CSCs restore the transit-amplifying population, even if the proliferating cancer cells are completely inhibited (Reviewed in refs. Das et al. 2008). Therapies specific for CSCs are therefore necessary to achieve tumor remission and patient survival. Differentiation therapy such as retinoic acid induces terminal differentiation of promyelocytes in leukemia leading to complete remission in 95% of patients (Reviewed in refs. Das et al. 2008, Hansen et al. 2000). Differentiation therapy can be a very effective treatment for CSCs. The genes that regulate important pathways for NSC maintenance, proliferation and differentiation are also valuable targets for inducing differentiation of brain tumor stem cells (BTSCs) (Das et al. 2008). Elevated expression of DCX in proliferating NSCs and protection of NSCs by DCX overexpression from oxygen glucose deprivation indicate that DCX is also involved in NSC maintenance, proliferation and differentiation and is a valuable mediator for inducing differentiation of BTSC-like cells (Das et al. 2008, Santra et al. 2006b, Chang et al. 2007). DCX is highly expressed in migrating neuroblasts/NSCs (Arvidsson et al. 2002).

We therefore hypothesized that DCX induces neuronal phenotypes in glioma cells and BTSC-like cells with terminal differentiation potency that cause remission of glioma. To test this hypothesis, lentivirus based DCX gene therapy was employed in the implanted U87 glioma in nude rats. We show that single DCX lentivirus gene therapy administered into an established xenografted U87 tumor significantly reduced tumor volume up to ~60% after 14 days of DCX lentivirus treatment. DCX lentivirus therapy may act as a differentiation based therapy by inducing BTSC-like cells with terminal differentiation, ceasing their proliferation and normalizing glioma cells into neuronal and glial phenotypes in xenografted glioma.

MATERIALS AND METHODS

Cell cultures

Human glioblastoma U87 and human embryonic kidney (HEK) 293T cells obtained from American Type Culture Collection (Manassas, VA), human primary glioblastoma cells generously provided by Dr. Anthony N. van den Pol, Department of Neurosurgery, Yale University School of Medicine, New Haven, Connecticut, were utilized, as previously reported (Santra et al. 2009, 2006a, 2006b). All recombinant lentiviruses carrying green fluorescent protein (GFP) for control, DCX and DCX siRNA were generated by transfecting lentiviral vectors in HEK 293T cells, as previously reported (Santra et al., 2006a, 2006b). To express and knock down DCX, the glioma cells were infected with DCX and DCXsiRNA expressing lentivirus, and DCX synthesis was measured by absolute quantitative real time PCR (qPCR), as previously described (Santra et al. 2009).

Lentivirus based gene therapy

All animal experiments were performed according to protocols approved by the Henry Ford Hospital Institutional Animal Care and Use Committee. Human glioma U87 cells (5×10^5) were implanted on day 1 into the striatum of male nude rats ($n=32$) (250–300 g) at the following location: bregma +1 mm; lateral +3 mm; ventral –4 mm, as previously described (Santra et al. 2006a, 2009). Eight days later, control and DCX lentiviruses were injected once at a dose of 10^9 plaque forming units (PFU) via the same needle track where tumor cells were implanted into nude rats ($n=16$ for control and $n=16$ for treatment).

Analysis of lentivirus infection and transgene expression

Two days later after virus injection, rats ($n=4$) were sacrificed under anesthesia with ketamine (40 mg/kg) and xylazine (13 mg/kg) administered intra muscularly. The xenografted tumors were surgically resected under a microscope (OpMi 40X; Carl Zeiss Inc., Zeiss, Germany). Single-cell suspensions were prepared from whole tumors in 0.1% trypsin in PBS at 37°C for 3 hrs with gentle shaking, as previously described (Santra et al. 2009, Liu et al. 2006). The cells were immunostained with either DCX antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) for transgene expression or mouse monoclonal human specific anti-nucleolar antigen (B462) antibody (1:100, GeneTex Inc. Alton Parkway, Irvine, CA) for total tumor cell population and analyzed directly by fluorescence-activated cell sorting (FACS) in BD FACS Calibur Flow Cytometer (BD Bioscience, Qume Drive, San Jose, CA), as previously described (Santra et al., 2009, 2006a; Liu et al., 2006). Efficiency of lentivirus infection was determined by FACS analysis of GFP labeled tumor cell suspensions.

Histopathology

Rats were anesthetized and sacrificed on day 21 after tumor implantation, as previously described (Santra et al. 2009, 2006a). Perfusion of the animals, removal and fixation of brain tissue, sectioning of brain tissues into 7 coronal blocks (1 mm thick) and processing for paraffin embedded slide were performed, as previously described (Santra et al., 2009, 2006a). Serial sectioning of 6 μ m thick paraffin embedded slices was performed approximately every 1 mm. These sections were stained with hematoxylin and eosin (H&E) for light microscopic examination. In each coronal section, the area of the entire tumor was measured by tracing the respective tissue boundaries on the computer screen by using the Global Lab Image analysis program (Data Translation, Marlboro, MA, USA) and the volume was determined by multiplying the appropriate area by the section interval thickness.

Magnetic Resonance Imaging (MRI) analysis

MRI of the rat brain tumors were analyzed by using a 7 T, 20 cm bore superconducting magnet (MagneX Scientific, Abingdon, UK) interfaced to a Bruker console (Billerica, MA, USA), as previously described (Yang et al. 2007). To detect tumor, multislice T_1 -weighted images were obtained using multislice (27 slices) spin-echo (TR=1000 ms, TE=7.5 ms, 4 averages) sequences with a 0.5 mm slice thickness, 32 mm FOV and 256x192 matrix. The contrast enhanced MRI measurement included two sets of T_1 -weighted spin-echo images; one set was acquired before contrast agent Magnevist (Berlex Laboratories, Wayne, NJ; 0.1 mmol/kg IV bolus) injection and one set was acquired after Magnevist injection according to manufacturer's instruction. The volume of tumor was measured using a computerized Eigentool optical software package (Yang et al. 2007).

Immunohistochemistry

The paraffin sections and cells grown in 8-well chamber slides (Lab-Tek, Nunc, Inc., Naperville, IL) were immunostained with sequential primary antibodies and secondary antibodies labeled with either fluorescein isothiocyanate (FITC) (green fluorescence) or

cyanine fluorophore (Cy3) (red fluorescence), counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (blue fluorescence) and examined under Fluorescent Illumination Microscope (Olympus IX71/IX51, Tokyo, Japan), as previously described (Santra et al., 2009, 1997). Goat antiserum for DCX, GFAP, GFP (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-class III β -tubulin (TUJ1) and mouse polysialic acid on neural cell adhesion molecule (PSA-NCAM) (1:500, Chemicon, Temecula, CA), monoclonal antibodies for MAP2 (1:1000), α -tubulin, Ki-67 (1:500; Sigma, Saint Louis, Missouri), mouse monoclonal human specific anti-nucleolar antigen (B462) antibody (1:100, GeneTex Inc. Alton Parkway, Irvine, CA), chicken antiserum for GFP (1:500, Aves Lab Inc. Tigard, Oregon), polyclonal rabbit anti-Ki-67 (1:300, Neo Markers, Fremont, CA) polyclonal rabbit anti-von Willebrand factor (vWF; 1:300; DAKO, Carpinteria, CA) as primary antibodies, and corresponding anti-goat, anti-chicken, anti-rabbit or anti-mouse secondary antibodies (1:5,000) conjugated with either FITC or Cy3 (1:5,000; Santa Cruz) were used. TUNEL staining was performed, as previously described (Santra et al. 2006b). Each experiment was repeated at least three times.

Quantitative Real-time PCR

The cDNA synthesis and real-time PCR were performed by using human, rat/mouse DCX, GFAP, vimentin, β -actin DNA primers, as previously described (Santra et al., 2006a; 2006b). DNA primers for DCX and β -actin were used, as previously described (Santra et al., 2009, 2006a). Sequences of forward and reverse DNA primers for human GFAP and vimentin are 5'-CTGCTGCTTCTTAACCCAG-3', and 5'-ACTTTGCTCGTGCCTCAGTT-3', and 5'-CCTTGAACGCAAAGTGGAATC-3' and 5'-GACATGCTGTTCTGAATCTGAG-3', respectively. Dissociation curves and agarose gel electrophoresis were used to verify the quality of the PCR products. All values were normalized to β -actin. Absolute and relative quantitative real time PCR (qPCR) was performed to measure mRNA transcripts, as previously described (Santra et al., 2009).

Immunochemical procedures

Western blot analysis was performed, as previously described (Santra et al., 2006a; 1997). For Western blot analysis, primary antibodies and their dilutions used in immunohistochemistry were utilized. Mouse monoclonal vimentin and β -actin (1:1,000–5,000; Santa Cruz Biotechnology) were also used. Donkey anti-goat, antirabbit and antimouse horseradish peroxidase (1:10,000; Jackson ImmunoResearch Labs) were used as secondary antibodies. Each experiment was repeated at least three times.

Statistical analysis

One-way ANOVA followed by Student-Newman-Keuls test was used. The values were the mean of 5 to 10 independent experiments for real-time PCR data and three independent experiments for Western blot analysis. The data are presented as mean $\pm$ SD. $P < 0.05$ is considered as significant. Kaplan-Meier approach was used to test the group difference on survival using the log rank test and to estimate the time of the median survival and its 95% confidence intervals (CI) for each group.

RESULTS

Lentivirus efficiently infects implanted glioma with the DCX+ transgene

To examine the delivery of DCX transgene into implanted glioma, rats ($n=4$) were sacrificed on day 2 after control and DCX lentivirus injection. A diagram of lentiviral vector with GFP and DCX expression cassettes (Santra et al. 2006b) used in these studies is shown (Fig. 1a). Single cell suspensions of implanted U87 tumors were immunostained and analyzed by FACS.

These data demonstrate that more than 70% of implanted U87 tumor cells were infected with control and DCX lentivirus and ~70% of infected tumor cells expressed DCX transgene (Fig. 1b–e). These data also suggest that cell suspensions of xenografted tumors were first or second generation after virus infection. At day 2 post DCX lentivirus injection, virus remained intact in extrachromosomal state within the tumor cells and transcribed both GFP and DCX equally (Book in ref. Aghi and Chiocca 2004, Reviewed in refs. Kay et al. 2001, Thomas et al. 2003). We therefore examined the effect of DCX lentivirus on glioma tumor volume in vivo. The remaining rats (n=12) were sacrificed on day 14 after lentivirus injection. Double immunostaining of tumor sections with DCX and GFP, and counter staining with DAPI revealed that DCX infected xenografted U87 cells did not grow tumor and remained within the needle track (Fig. 1f). DCX lentivirus infected tumor cells often underwent rapid differentiation, formed a tumor sphere-like morphology and did not produce tumor mass (Fig. 1f). In contrast, uninfected DCX(–) tumor cells formed a tumor mass in 14 days (Fig. 1f). Although, lentivirus carried both DCX and GFP expression cassettes, some virus infected tumor cells expressed GFP only, but not DCX (Fig. f). The lentivirus used in these studies is replication deficient (Santra et al. 2006b). After many generations of virus infected tumor cells, infected virus is no longer extrachromosomal and integrates into the genomic DNA of the chromosome of tumor cells, otherwise this virus can not express its genes (Book in ref. Aghi and Chiocca 2004, Reviewed in refs. Kay et al. 2001, Thomas et al. 2003). During integration of virus into the chromosome, DCX and/or GFP gene or their promoters sequences may be disrupted or their expressions are silenced for unknown reasons (Book in ref. Aghi and Chiocca 2004, Reviewed in refs. Kay et al. 2001, Thomas et al. 2003). As a result, GFP(+)DCX(+), GFP(–)DCX(+), GFP(+)DCX(–) and GFP(–)DCX(–) tumor cells are produced. Our data also indicate the presence of GFP(+)DCX(+), GFP(–)DCX(+) and GFP(+)DCX(–) tumor cells except GFP(–)DCX(–) tumor cells (Fig. 1f). GFP(+)DCX(–) cells tumor cells occupied more area of xenografted tumor than GFP(–)DCX(+) tumor cells (Fig. 1f). These data suggest that GFP(+)DCX(–) tumor cells proliferated as do control glioma cells and GFP(–)DCX(+) tumor cells did not proliferate.

Single DCX lentivirus therapy significantly reduces U87 tumor volume in nude rats

To determine the effect of DCX gene therapy on glioma tumor volume, each paraffin section stained with H & E from 7 coronal blocks (A–G) of tumor-bearing cerebrum from rat brains treated with control or DCX lentivirus (n=12) was examined under the light microscope. DCX lentivirus treatment significantly inhibited U87 glioma tumor volume in nude rats (Fig. 2a, b). In a separate set of nude rats, MRI analysis was performed on day 19–21 after single control (n=7) and DCX lentivirus therapy (n=11) into the tumor of nude rat brain. MRI analysis demonstrated significant inhibition of U87 glioma tumor volume in nude rat brains after a single DCX lentivirus injection (Fig. 2c, d). After MRI analysis, these tumor bearing nude rats were allowed to survive. Kaplan-Meier plot was performed to determine the group difference on survival of tumor bearing rats by using the log rank test to estimate the time of the median survival and its 95% confidence intervals (CI) for each group. These data demonstrated that nude rats treated with a single DCX lentivirus injection significantly improved median survival up to ~45 days, compared to ~29 days in control group (Fig. 3).

Induction of DCX mediated neuronal phenotype in glioma U87 cells

DCX synthesis induces expression of the neuronal marker MAP-2 and the NSC marker nestin at the protein and mRNA levels in glioma U87 cells, as previously reported (Santra et al., 2006b). We recently found that DCX is co-expressed with the neuronal markers MAP2, TUJ1 and PSA-NCAM in the peritumor region of human and rat glioma (Santra et al., 2009). We therefore investigated the expression of MAP2, TUJ1 and PSA-NCAM after DCX lentivirus treatment in U87 implanted glioma. Our data show that GFP positive cells (~85%) coexpressed DCX and neuronal markers MAP2, TUJ1 and PSA-NCAM (data not shown). To distinguish

between the xenografted human tumor cells and non-tumor rat cells in vivo, xenografted human U87 tumors were stained with human specific anti-nucleolar antigen antibodies along with neuronal markers with MAP2, TUJ1 and PSA-NCAM antibodies. We found that U87 tumor cells (~95%) that were stained with human specific anti-nucleolar antigen antibody co-expressed DCX, MAP2, TUJ1 and PSA-NCAM in xenografted U87 tumor (data not shown). Double immunostaining data revealed that DCX positive cells (~90%) co-expressed neuronal markers MAP2, TUJ1 and PSA-NCAM (Fig. 4). These data indicate that DCX lentivirus induced MAP2, TUJ1 and PSA-NCAM in xenografted tumor. These data also suggest that DCX induces BTSC-like cells, because only BTSCs in glioma have neuronal lineage, whereas U87 cells do not express MAP2, TUJ1 and PSA-NCAM neuronal markers (Galli et al. 2004, Santra et al. 2006b).

DCX synthesis inhibits cytokinesis by inducing GFAP expression in glioma cells

DCX synthesis induces mitotic spindle catastrophe, as previously reported (Santra et al., 2009). The failure of cytokinesis causes mitotic catastrophe in the multi-nucleated giant cells (Huang et al., 2008). GFAP plays an important role for metaphase anaphase transition at the cleavage furrow (Yasui et al., 1998). To examine whether DCX regulates GFAP expression in glioma cells, qrtPCR and Western blot analysis were performed in control and DCX synthesizing U87 and primary glioma cells. Absolute qrtPCR for DCX and relative qrtPCR for GFAP and vimentin were performed to measure mRNAs, as previously described (Santra et al., 2009). DCX mRNAs were 1.15 ± 0.21 and 0.8 ± 0.15 copies mRNA/ng total RNA in DCX synthesizing U87 and human primary glioblastoma multiforme, respectively. In contrast, no DCX mRNAs were detected in control U87 and human primary glioblastoma multiforme, and DCXsiRNA treated DCX synthesizing U87 and human primary glioblastoma multiforme. Our data demonstrate that DCX synthesis significantly induced GFAP expression in U87 and human primary glioma cells at mRNA and protein levels (Fig. 5a, b). In contrast, DCX synthesis had no effect on vimentin expression in these glioma cells at mRNA and protein levels (Fig. 5a, b). DCXsiRNA treatment also reversed the DCX effect on GFAP induction in U87 and human primary glioblastoma cells (Fig. 5a, b). Double immunostaining of paraffin embedded sections of xenografted tumors after DCX lentivirus treatment revealed that the DCX positive glioma tumor cells upregulated GFAP synthesis in vivo (Fig. 5c).

To investigate the effect of DCX on cytokinesis, the U87 and human primary glioblastoma cells were grown to mitotic stage on glass coverslips for 28 hours, fixed and double immunostained with α -tubulin and GFAP, and counterstained with DAPI. Control U87 and human primary glioblastoma cells showed distinct cleavage furrow indicating normal cytokinesis (Fig. 5d). In contrast, there were no cleavage furrows at all in DCX synthesizing U87 and human primary glioblastoma cells (Fig. 5d). DCXsiRNA treatment also reversed the DCX effect and a clean cleavage furrow reappeared in DCX synthesizing U87 and human primary glioblastoma cells (Fig. 5d). Double immunostainings revealed that GFAP and α -tubulin were colocalized in the cytoplasm in the DCX synthesizing U87 and human primary glioblastoma cells (Fig. 5d). In contrast, GFAP was reduced in control U87 and human primary glioblastoma cells (Fig. 5d). These data demonstrated that DCX inhibited cytokinesis by inducing GFAP expression and blocking mitosis in DCX synthesizing U87 and human primary glioblastoma cells. Upon exposure to differentiation cues, BTSCs that express both neuronal and glial markers undergo terminal differentiation (Ignatova et al., 2002; Singh et al., 2003; Galli et al., 2004). These data suggest that DCX synthesis induced neuronal and glial markers in glioma cells that transform into BTSC-like cells and undergo terminal differentiation by blocking mitosis via a novel DCX/GFAP pathway.

Lentivirus based DCX induces terminal differentiation in implanted glioma

We tested the hypothesis that DCX synthesis induces GFAP positive BTSC-like cells that differentiate into their terminal maturity and causes remission of glioma cells. Double immunofluorescence of implanted tumor sections with antibodies of Ki-67 as a marker of proliferation (Piccirillo et al. 2006) and GFP for infection, and counter staining with DAPI were performed. We found that almost all lentivirus infected glioma cells were positive for Ki-67 in control lentivirus treated tumor (Fig. 6a, b). In contrast, in DCX lentivirus treated tumor, Ki-67 positive cells were significantly reduced in the very dense population of lentivirus infected glioma cells compared to uninfected glioma cells (Fig. 6a, b). Double immunostainings for Ki-67 and DCX in xenografted tumor sections also revealed that Ki-67 expression was significantly reduced in DCX positive glioma cells (data not shown). These data demonstrated that DCX lentivirus infection significantly reduced proliferation in xenografted tumor (Fig. 6a,b). DCX positive Ki-67 negative glioma cells within DCX xenografted tumor resembled a tumor sphere like morphology- one of the characteristic of BTSCs (Das et al. 2008) (Fig. 6a,b).

In another experiment, the single cell suspensions prepared from xenografted glioma tumor on day 2 after control and DCX lentivirus injection were plated onto Matrigel-coated glass coverslips for 48 h and immunostained for Ki-67 and GFP and counter stained with DAPI according to the method of Piccirillo et al. (Piccirillo et al. 2006). The data were quantified by counting Ki-67 and GFP positive cells. Ki-67 positive cells were significantly reduced in DCX lentivirus treated tumors compared to control lentivirus treated tumors (Fig. 6c, d). These data confirmed that DCX lentivirus significantly inhibited proliferation of glioma cells. DCX lentivirus induced both neuronal and glial markers and inhibited proliferation in xenografted tumor. These data are also consistent with addition of differentiation cues e.g., retinoic acid, ciliary neurotrophicfactor (CTNF) and leukemia inhibitory factor, that induce terminal differentiation in BTSC-like cells expressing both neuronal and glial markers (Lee et al. 2006, Galli et al., 2004). Only BTSCs in glioma, express neuronal and glial markers (Lee et al. 2006, Galli et al., 2004). Glioma cells do not express neuronal markers and do not respond to differentiation cues for their terminal differentiation (Lee et al. 2006, Galli et al., 2004).

To examine whether these differentiated cells underwent apoptosis or not, TUNEL staining was performed in control and DCX lentivirus infected tumor. There was no TUNEL staining detected in control and DCX infected glioma tumor cells (data not shown). These data indicated that DCX gene therapy does not induce apoptosis in glioma tumor, but induces terminal differentiation of BTSC-like cells and acts as differentiation based therapy.

Lentivirus based DCX inhibits angiogenesis in xenografted glioma tumor

Glioblastoma multiforme is characterized by exuberant angiogenesis, a key event in tumor growth and progression (Griscelli et al. 1998). Intratumoral angiogenesis was assessed by double immunostaining of tumor sections with antibodies of vWF as a blood vessel marker and DCX (Griscelli et al. 1998). The double immunostaining data revealed that DCX positive glioma tumor contained fewer blood vessels than DCX negative glioma tumor tissue (Fig. 6e, f). The vWF-positive vessels were quantified by counting the vessels. The results indicated a marked reduction of intratumoral vascularization within DCX lentivirus-injected glioma tumor (Fig. 6e, f).

DISCUSSION

Our present data demonstrated that single DCX lentivirus therapy significantly reduced U87 glioma tumor and improved median survival of tumor bearing nude rats. DCX synthesis induced neuronal markers such as MAP2, TUJ1 and PSA-NCAM as well as the glial marker

protein GFAP in U87 cells implanted in nude rats. These data suggest that assignment of glioma cells to neuronal and glial phenotype reduces tumor growth. Lentivirus based DCX therapy significantly inhibited intratumoral proliferation and angiogenesis that play a role in tumor growth and progression (Griscelli et al. 1998). DCX synthesizing glioma cells that expressed both neuronal and glial markers resemble BTSC-like cells with terminal differentiation potency (Lee et al. 2006). GFAP was induced after DCX transfection and co-localized with tubulin in the cytosol of U87 and human primary glioblastoma glioma cells. As a result, DCX mediated GFAP upregulation inhibits cleavage furrow and blocks cytokinesis, one of the major steps of mitosis in U87 and human primary glioblastoma cells. These data indicate that DCX synthesis induced terminal differentiation in BTSC like cells by blocking mitosis via a novel DCX/GFAP pathway. DCX synthesizing BTSC-like cells adopt neuronal and glial phenotype, obtain terminal differentiation potency, lose their self renewal potency, fail to generate tumor xenograft in immunocompromised hosts (Santra et al., 2006a) and significantly improve median survival of glioma bearing rats after DCX treatment.

DCX synthesis induces catastrophe in mitotic spindle checkpoint that leads to growth arrest in G2-M phase in cell cycle progression and inhibits glioma cell invasion in vitro, as recently reported (Santra et al., 2009). DCX is also directly involved in PTEN mediated glioma suppression (Santra et al., 2006a). DCX is thus an excellent candidate for gene therapy that can suppress glioma growth as well as glioma invasion (Santra et al., 2009). Malignant glioma is a good target for gene therapy because of its dismal prognosis since the tumor is located in an anatomically restricted area without metastases outside the central nervous system (CNS). Lentivirus efficiently infects and maintains sustained transgene expression for several months without detectable pathology (Reviewed in ref. Kay et al., 2001). We therefore employed lentivirus based gene therapy for glioma in vivo in our present nude rat model system. However, one practical limitation for a virus-based approach to therapy is virus integration into genome that creates DCX(-)GFP(+) tumor cells, although, this virus carries both DCX and GFP gene expression cassettes. Despite limitations of virus based therapy (Reviewed in ref. Kay et al., 2001), lentivirus based DCX gene therapy significantly reduced tumor volumes and improved median survival of tumor bearing nude rats after single injection of DCX lentivirus. Our current data of DCX mediated induction of GFAP expression in U87 and primary glioma cells are also consistent with previously reported data of upregulation of GFAP expression, and inhibition of proliferation and invasion in cell lines and primary cultures of malignant gliomas (Reviewed in ref. Chen et al., 1998). We found a novel mechanistic link between GFAP upregulation and glioma suppression via blocking an important step of mitosis, cytokinesis in DCX synthesizing glioma cells. Inhibition of cytokinesis in DCX synthesizing glioma cells provides additional evidence that DCX synthesis induces growth arrest in the G2-M phase of the cell cycle progression and suppresses proliferation and invasion of glioma leading to DCX mediated terminal differentiation in BTSC- like cells via a novel DCX/GFAP pathway (Santra et al. 2009). Retinoic acid also upregulates GFAP and suppressed glioma (Dirks et al.1997). Co-expression of GFAP and DCX in astrocytes and Muller glial cells mediate a de-differentiation potential after kainic acid -induced retinal degeneration (Chang et al. 2007). Retinoic acid induces differentiation in chondrocytes (Dietz and Sandell 1996). DCX positive NSCs have multi-directional differentiation in the adult rat neocortex in vivo (Tamura et al. 2007). Retinoic acid also induces differentiation of promyelocytes to their terminal maturity in leukemia and leads to complete remission in acute promyelocytic leukemia (Reviewed in refs. Das et al. 2008, Hansen et al. 2000). Retinoic acid treatment induces differentiation, a 16-fold increase in DCX expression and expression of neuronal markers, such as Map2, TUJ1 and neuron-specific enolase in human teratocarcinoma cell line NTERA-2 (Couillard-Despres et al. 2008). BTSC-like cells that express neuronal and glial markers respond to the differentiation cue retinoic acid (Lee et al. 2006). We showed that DCX mediated BTSC-like cells that respond to the DCX differentiation cue, may induce remission of glioma, markedly reduce xenografted tumor volume and significantly improve improved median survival of tumor bearing nude rats.

BTSCs are extremely resistant to all treatments and lethal for glioma patients (Reviewed in refs. Das et al. 2008). BTSCs are tumor initiating and have self-renewal and multilineage differentiation potential (Das et al. 2008, Lee et al. 2006). Therapy specific for BTSCs is important for the treatment of glioblastoma multiforme. BTSCs derived from primary glioblastoma multiformes bear remarkable phenotypic similarity to human NSCs and appear to be the tumorigenic component of at least a subset of human glioblastoma multiformes (Lee et al. 2006). Although, BTSCs bear striking similarities to normal NSCs, BTSCs lack one of the major NSC markers- DCX (Lee et al. 2006, Santra et al. 2006a). In contrast, DCX is not only highly expressed in proliferating normal NSCs, but also protects NSCs from oxygen glucose deprivation (Santra et al. 2006b). Boosting of this NSC marker DCX in glioblastoma multiforme ceases their proliferation, restores terminal differentiation in BTSC-like cells with neuronal and glial lineages and causes remission in glioma bearing nude rats.

BTSC-like cells have the potential for multilineage differentiation and clonogenicity (Lee et al. 2006). Upon exposure to differentiation conditions (10% FBS), BTSCs show markers of multiple different neural fates, including glial (GFAP) and neuronal (TUJ1) cell lineages, although sometimes in aberrant patterns (Ignatova et al., 2002; Singh et al., 2003, 2004; Galli et al., 2004). Only, GFAP and TUJ1 positive BTSC-like cells respond to differentiation cues (Lee et al. 2006, Galli et al., 2004). Glioma cells that are weakly positive or negative for GFAP and TUJ1 do not respond to differentiation cues (Lee et al. 2006). We found that DCX synthesis induced GFAP and TUJ1 in glioma U87 cells. DCX synthesis may make glioma cells more susceptible to terminal differentiation by inducing both glial and neuronal lineages. Our data demonstrated that lentivirus based DCX gene therapy inhibited proliferation by inducing terminal differentiation of infected glioma cells. When DCX lentivirus treatment induces expression of neuronal and glial markers in glioma cells, these glioma cells may become BTSC-like cells that respond to the DCX differentiation cue and undergo terminal differentiation (Lee et al. 2006, Galli et al., 2004, Chang et al. 2007). BTSCs in glioblastoma multiforme reside in a niche in which vascular endothelial cells secrete factors that promote BTSC proliferation and self renewal (reviewed in ref. Das et al. 2008). Inhibition of vascular endothelial cells in glioblastoma multiforme disrupts BTSC niche, impairs self-renewal and markedly reduces tumor growth (reviewed in ref. Das et al. 2008). Inhibition of vascular endothelial cells in DCX lentivirus infected glioma cells in tumor may abolish the niche of BTSC like cells for self-renewal and thereby inhibit tumor growth.

In summary, our results suggest that single lentivirus based DCX gene therapy reduces U87 tumor volume by 60% and significantly improves median survival of tumor bearing nude rats. Ectopic DCX expression may induce lineage switching of proliferating glioma cells into self renewal deficient cells with phenotype similar to BTSCs that may be reprogrammed to terminally differentiated normal cells (Lee et al. 2006, Heyworth et al. 2002, Reviewed in ref. Gurdon and Melton 2008). The clinical value of differentiation based therapy has been best demonstrated in the use of retinoic acid for the treatment of acute promyelocytic leukemia (Reviewed in refs. Das et al. 2008, Hansen et al. 2000). BTSC-like cells only respond to differentiation cues when they express GFAP (Lee et al. 2006). Similarly, synthesis of DCX and GFAP causes dedifferentiation of astrocytes and Muller glial cells in injured rat retina (Chang et al. 2007). DCX synthesis not only induces neuronal markers, but also induces the glial marker GFAP, blocks mitosis and inhibits intratumoral proliferation leading to terminal differentiation in glioma cells via a novel DCX/GFAP pathway, and causes remission of glioma cells. DCX lentivirus treatment does not induce apoptosis in xenografted tumor, but inhibits intratumoral angiogenesis, impairs self-renewal potency and markedly reduces tumor growth. DCX may therefore be a differentiation-based therapy for the treatment of glioma in the BTSC compartment. Lentivirus based DCX gene therapy as a novel approach of differentiation-based treatment for glioma warrants further investigation.

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Fig. 1. Lentivirus efficiently infected implanted glioma tumor cells and induced DCX transgene expression

The map (a) indicates diagram of lentiviral vector with GFP and DCX expression cassettes. FACS analysis of single cell suspensions of implanted U87 tumor from nude rats treated with lentivirus for 2 days, indicates U87 cells (black lines) stained with human specific anti-nucleolar antigen antibody as total cell populations (100%) in control (b) and in DCX lentivirus treated (c, d) rats, GFP (+) cells (green lines) in control (b) and in DCX treated (c) rats, and DCX (+) cells (green lines) in DCX treated (d) rats. Bar graph (e) indicates FACS analysis of lentivirus infection and DCX transgene expression in xenografted tumors treated with lentivirus for 2 days. Paraffin sections (f) of xenografted U87 tumor treated with DCX lentivirus for 14 days were double immunostained with Cy3 labeled DCX antibody and FITC labeled GFP antibody, and counter stained with DAPI. Arrows indicate the needle track for U87 cells and virus injection. Asterisks represent tumor. Non-tumor areas are marked with "O". Arrow head indicates tumor sphere like morphology. Scale bar of images = 100μm.

Fig. 2. DCX lentivirus injection reduces glioma tumor volume in nude rats

One 6 μ m thick paraffin slice from each 7 coronal paraffin blocks (from A to G) of tumor-bearing cerebrum from rat brains treated with control lentivirus (Control) and DCX expressing lentivirus (DCX) were stained with H&E for light microscopic examination (a) and quantitative measurements of glioma U87 tumor volume (b). Sequence of coronal images (13–20) from 27 images (c) of whole tumor bearing nude rat brains and quantitative measurements of glioma U87 tumor volume (d) were analyzed by MRI after control and DCX lentivirus (DCX) treatment. Arrows indicate the glioma tumors. Asterisk indicates significance values ($P < 0.05$).

Fig. 3. DCX lentivirus treatment significantly improves median survival of tumor bearing nude rats by Kaplan-Meier approach

Top panel indicates Kaplan-Meier plot of the group difference on survival using the log rank test to determine the time of the median survival and its 95% confident intervals (CI) for each group. Bottom panel represents that tumor bearing nude rats treated with DCX lentivirus significantly improves median survival up to 45 days with 95% CI 44 to 47 days, compared to 29 days median survival and 95%CI 26 to 29 days observed in control group ($p < 0.01$).

Fig. 4. DCX synthesizing cells express neuronal markers in xenografted tumors

Paraffin sections of implanted U87 tumor bearing brain treated with control and DCX lentivirus for 14 days were double immunostained with FITC labeled MAP2, PSA-NCAM and TUJ1 antibodies and with Cy3 labeled DCX antibody and counterstained with DAPI. Arrows indicate the merged images (mer) of MAP2, TUJ1 and PSA-NCAM with DCX. Scale bar of images = 20 μ m.

Fig. 5. DCX synthesis inhibits cytokinesis by inducing GFAP expression in glioma cells

Bar graph (a) indicates relative qrtPCR analysis of total RNA extracts from control (C), DCX synthesizing (D) and DCXsiRNA treated DCX synthesizing (S) U87 and human primary glioblastoma (PGM) cells for GFAP and vimentin. In Western blot, the protein bands were immunoblotted with either GFAP or vimentin or DCX from control, DCX synthesizing and DCXsiRNA treated DCX synthesizing U87 and human primary glioblastoma (PGM) cells, and detected by enhanced chemiluminescent. The loading of samples was normalized with β -actin. (c) Paraffin sections of control and DCX lentivirus treated xenografted tumor for 14 days were immunostained with FITC labeled GFAP antibodies and with Cy3 labeled DCX antibody and counterstained with DAPI. Arrows indicate the merged images of GFAP with DCX. Scale bar of images = 20 μ m. (d) The control (U87 and PGM), DCX synthesizing (UDCX and PDCX) and DCXsiRNA treated (siRNA) DCX synthesizing U87 and PGM cells were double immunostained with FITC labeled α -tubulin and Cy3 labeled GFAP and counterstained with DAPI. Left column indicates the phase contrast images. Arrows indicate the cleavage furrow. Scale bar of images = 5 μ m.

Fig. 6. DCX lentivirus infection inhibits proliferation and angiogenesis in xenografted glioma tumor
 For proliferations, double immunostainings of paraffin sections from control and DCX lentivirus treated implanted U87 tumors for 14 days with FITC labeled GFP antibody and Cy3 labeled Ki-67 antibody and counterstainings with DAPI (a, 100 μ m scale bar) were quantified by counting Ki67 positive cells in GFP positive cells (100%) (b). In another experiment for proliferation, the single cell suspensions were plated onto Matrigel-coated glass coverslips from xenografted glioma tumor on day 2 after control and DCX lentivirus injection, immunostained for Ki-67 and GFP, and counter stained with DAPI (c, 50 μ m scale bar), and quantified by counting Ki-67 positive cells in GFP positive cells (100%) (d). To analyze angiogenesis in control and DCX lentivirus treated xenografted U87 tumor for 14 days, double immunostainings of paraffin sections with Cy3 labeled DCX antibody and FITC labeled vWF antibody and counterstainings with DAPI (e, 50 μ m scale bar) were quantified by vWF positive vessels in control tumor cells (100%) as well as in DCX positive cells (100%) (f). Asterisk indicates significance values ($P < 0.05$).