Network: Brain Tumor Network (BTN) – Identification of Novel Diagnostic and Therapeutic Targets in Cranial Malignancies by Integrated Tumor Profiling

Project: Novel Targets for the Molecular Therapy of Gliomas

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Introduction

Background

The Laboratory of Molecular Neurooncology has established a broad repertoire of methodologies required for the identification of novel molecular targets for the treatment of gliomas and the preclinical development of strategies derived from the identification of such novel targets. In addition, we aim at generating in vitro paradigms modelling two essential biological characteristics of malignant glioma, hypoxia and infiltrative growth. These include several viability assays to characterize the mode of apoptotic and non-apoptotic cell death, retroviral and adenoviral gene transfer, gene silencing mediated by RNA interference, the assessment of migration and invasion (1,2), generation of chronic non-lethal hypoxia (2), the assessment of altered immunogenicity, and various mouse and rat glioma models (3,4). Moreover, a cooperative study with the German Cancer Research Centre Heidelberg resulted already in the identification of candidate chromosomal regions for genetic loci which determine the sensitivity to radiosensitivity and chemosensitivity in malignant glioma cell lines (5).

Project outline

The aims of the present project are to assess the effect of chronic non-lethal hypoxia (6) on the motility and radiochemosensitivity of primary glioma cells, to identify novel genes induced by chronic non-lethal hypoxia and to assess the biological role of novel candidate genes determining the malignant phenotype of gliomas

In cooperation with the project of Martina Schnölzer "Identification of Protein Biomarkers Associated with Hypoxia in Human Malignant Glioma Cell Lines Using Proteomics Technologies" 3-4 well characterized human malignant glioma cell lines exposed to chronic non-lethal hypoxia will be analysed. Extracts of nuclear proteins obtained from the various cell lines at different time-points will be separated by high-resolution 2D gel electrophoresis (2D PAGE). Computer-assisted image analysis of the gels will allow the detection of differentially expressed proteins.

Further our project is actively involved in the work of Ruthild Weber "Identification of Novel Cancer Relevant Genes by Molecular Characterization of Chormosome Translocation Breakpoints in Human Malignant Glioma Cells" by delivering long-term glioma cultures but also newly established primary gliomas. In collaboration with the Neurooncology Working Group of the German Cancer Society we perform the clinical correlations and the sampling of glioma paraffin-embedded tissues for the subproject of Stefan Jost " High Throughput Analyses of Malignant Gliomas Using Tissue Microarrays (TMAs)". The latter work has enormous implications since our sample of uniformely treated high-grade gliomas represents one of the largests clinical trials in this indication.

Results/Project Status

The first set of experiments delineated the principal role of non-lethal hypoxia for migration and invasion of malignant glioma cells. Expression analyses of candidate molecules revealed several interesting proteins regulated under hypoxic conditions with an established function in motility of malignant glioma. The further experiments performed with these candidates are exemplified with the detailed description of the antiapoptotic BCL-x_L. Parallel to these experiments, we have analyzed mRNA from normoxic and chronically, one day and 10 days, hypoxic (1% O_2) glioma cells with Affimetrix chips.

The chip-analyses have been done but candidate genes have to be confirmed by real-time PCR or Northern and Western blotting.

BCL-x_L and glioma motility

The attribution to BCL-2 family proteins of functions other than regulation of apoptosis has remained controversial. Here we propose a novel pro-invasive function of BCL-x_L separate from its anti-apoptotic activity and illustrate how altered BCL-x_L expression confers an invasive phenotype *in vivo*. In generating conditionally BCL-x_L-expressing LNT-229 glioma cell clones, we aimed at elucidating whether the enhanced migration and invasiveness observed in stably BCL-2-transfected LNT-229 cells was a selection effect or due to long-term culturing. We wanted to differentiate between the anti-apoptosis phenotype and the motility phenotype on the basis of BCL-2 family protein expression levels.

Migration and invasion are prerequisites for the neoplastic phenotype of malignant gliomas, and malignant progression is correlated with increased migratory and invasive capacity. Previously, we defined a motility pathway for malignant glioma cells that included an interaction between HGF, the membrane-cytoskeleton linker ezrin, BCL-2 and TGF-β_2. In this paradigm, expression of a dominant-negative ezrin resulted in reduced BCL-2 expression levels and significantly reduced migration and invasiveness. However, the sensitivity towards chemotherapeutics, death ligands or therapeutic irradiation remained unaltered. We postulated that subtle changes in the expression level of BCL-2 family proteins would affect motility, but might not be sufficient to alter resistance towards apoptosis. Surprisingly, the opposite was the case: we observed a BCL-x_L concentration-dependent increase in resistance to different apoptotic stimuli up to Doxycyclin (Dox) concentrations of 2 µg/ml whereas BCL-x_L expression at any level failed to influence the invasive phenotype of several LNT-229 BCL-x_L Tet-On subclones (c4.2E, c10.1D, c10.1J, and c34.2A) within the time frame of these experiments. Confirmation of these findings in several independently generated clones excluded spontaneous alterations in the long-term Dox-exposed cells to be causative for the invasive phenotype.

We next asked whether the induction of a motile phenotype conferred by BCL-x_L was a gradual, time-dependent effect, mimicking a paradigm for malignant progression. A highly significant induction of invasiveness in trans-membrane invasion and spheroid invasion into a collagen I/fibronectin matrix was found with induction of BCL-x_L for 21 days (Fig.). Importantly, in contrast to the effects on resistance towards apoptosis, the pro-invasive phenotype conferred by long-term expression of BCL-x_L remained stable after withdrawal from Dox, that is, when BCL-x_L levels had returned to normal. To exclude that other measures of inhibiting apoptosis had the same impact on invasiveness like BCL-x_L, we analyzed long-term exposure (21 days) of c4.2E, c10.1D, c10.1J, and c34.2A cells to zVAD-fmk and did not observe any alterations in invasiveness.

Since radiation-induced glioma cell migration and invasion are inhibited by $\alpha_V\beta_3$-integrin antagonism we assessed whether over-expression of BCL-x_L altered the sensitivity of glioma cells to membrane detachment-induced apoptosis. RGD peptide binding to integrins results in detachment-induced apoptosis in several cell types. BCL-2 family proteins have been related to regulation of cell-cell contacts. However, the detachment of control cells or of c34.2A cells

expressing BCL-x_L for 3 or 21 days using RGD peptides resulted in similar rates of apoptosis, indicating that over-expression of BCL-x_L did not protect from *anoikis.*

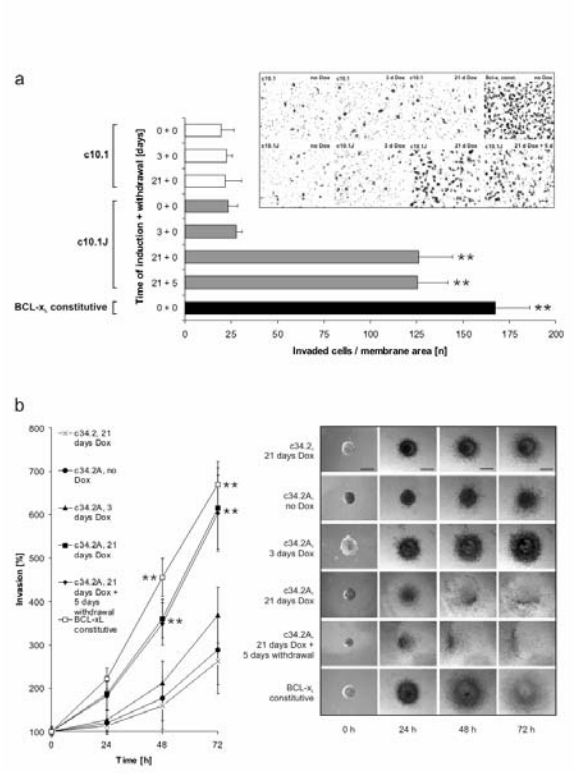

Fig 1: *a, c10.1 or c10.1J glioma cells, naïve or pre-incubated with Dox (2 µg/ml) for 3 or 21 days, with or without a subsequent withdrawal from Dox for further 5 or 21 days and with or without exposure to RO-28-2653, were analyzed for invasiveness in matrigel invasion chambers. A constitutively BCL-x_L over-expressing LNT-229 transfectant (referred to as BCL-x_L). Invading cells were counted in 5 random fields (mean and SEM, n = 3, **p < 0.01 relative to c10.1). Representative filters demonstrating BCL-x_L-dependent stimulation of glioma cell invasion are depicted in the upper right panel (at magnification x 100, insert). b, c10.1J glioma cells pre-incubated with Dox at indicated concentrations for 21 days were analyzed. c, c34.2 and c34.2A glioma cells, pre-treated as indicated, and the constitutive BCL-x_L transfectant were analyzed for invasion from preformed spheroids into a protein gel matrix consisting of collagen I enriched with fibronectin. The radial distance from the center of each spheroid was measured for 30 representative migrated cells in intervals of 24 h. Representative glioma spheroids at each time are depicted (scale bars, 400 µm; right panel).*

Accordingly, flow cytometry for the activated β-integrin chain and expression of $\alpha_5\beta_1$- or $\alpha_v\beta_3$-integrin expression were unaltered by long-term BCL-x_L expression and invasion of long-term induced cells did not vary amongst different substrates. These experiments made a relevant contribution of integrins to the motile phenotype unlikely.
cDNA array analysis was then used to decipher whether the invasive phenotype conferred by BCL-x_L resulted from altered gene expression. Interestingly, these arrays linked the BCL-x_L effect to increased bio-availability of TGF-β_2 and MMPs. The loss of invasiveness of LNT-229 cells stably expressing small interfering TGF-$\beta_{1/2}$ RNA has been linked to the loss of MMP activity. Over-expression of BCL-x_L for 21 but not for 3 days leads to a persistent induction of MMP-2, TGF-β_2 and membrane-bound MT1-MMP, remains to be elucidated. A direct transcriptional activity has not yet been ascribed to BCL-x_L and may be unlikely because of the prolonged time course. Alterations in integrin expression and activity have been excluded. MMP-2 and MT1-MMP were co-induced after expression of BCL-x_L for 21 days. In contrast to the significant reduction of invasiveness, inhibiting TGF-β_2 or MMP-2 did not alter the sensitivity of control or BCL-x_L-inducible cells to CD95L/CHX-induced cell death, confirming two independent pathways to invasiveness or survival controlled by BCL-x_L. Previously, BCL-x_L-induced down-regulation of type 1 inositol 1,4,5-trisphosphate receptor had been linked to reduced T cell antigen receptor ligation-induced Ca^{2+} flux in transgenic murine T cells and lower inositol 1,4,5-trisphosphate mediated Ca^{2+} release capacity in microsomes[25]. This altered Ca^{2+} flux might influence the motility of glioma cells. However, in our cDNA arrays, we did not observe any relevant regulation of type 1 inositol 1,4,5-trisphosphate receptor.
Importantly, over-expression of BCL-x_L also resulted in massive invasiveness of otherwise poorly invasive LNT-229 glioma cells *in vivo*. This occurred without an increase in the tumor volume, suggesting that the pro-invasive effect of BCL-x_L is more relevant *in vivo* than the pro-survival effect. Further, the effect is sustained despite withdrawal from Dox *in vivo.* Interestingly, MMP inhibition displays a specific MMP-neutralizing effect both *in vitro* and *in vivo*.

Summary

In summary, we delineate a novel pathway of BCL-x_L-induced invasiveness *in vitro* and demonstrate the induction of satellite formation of a non-invasive cell line in an intracranial xenograft model as a result of enhanced BCL-x_L expression. We illustrate the central roles of TGF-β_2 and MMP in these processes whereas altered sensitivity to *anoikis* or integrin expression or activity of MAPK/ERK were probably irrelevant for the newly-characterized 'motility phenotype'. Essentially, we have elucidated a novel consequence of long-term BCL-x_L over-expression independent from its anti-apoptotic function, establishing a unique paradigm for malignant glioma progression (7).

Outlook

In brief, adenoviral infection of human glioblastoma cell lines with cDNA of progression- or radiochemosensitivity-associated candidate genes and generation of siRNA to knock down overexpressed candidate genes are planned. We are testing for differential radiochemosensitivity, migration and invasion, T cell lysis, tumorigenicity in these modified sublines. We are going to use adenoviral infection of human glioblastoma cell lines with cDNA of hypoxia-induced genes and test pharmacological, viral or siRNA-based strategies to reverse the phenotype of malignant progression or reduced radiochemosensitivity.

Lit.: ***1.*** *Wick W, et al. Ezrin-dependent promotion of glioma cell clonogenicity, motility and invasion mediated by BCL-2 and TGF-β_2. J Neurosci 2001;21:3360-8.* ***2.*** *Wick W, et al.. Prevention of irradiation-induced glioma cell invasion by temozolomide involves caspase-3-activity and cleavage of focal adhesion kinase. Cancer Res 2002;62:1915-9.* ***3.*** *Friese MA, et al. MICA/NKG2D-mediated immunogene therapy of experimental gliomas. Cancer Res 2003;15:8996-06.* ***4.*** *Fulda S, et al. Smac agonists sensitize for TRAIL/Apo2L- or anticancer drug-induced apoptosis and induce regression of human glioma xenografts. Nat Med 2002;8:808-15.* ***5.*** *Weber RG, et al. Chromosomal imbalances associated with response to chemotherapy and cytotoxic cytokines in human malignant glioma cell lines. Int J Cancer 2001;91:213-8.* ***6.*** *Wick A, et al. Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. J Neurosci 2002;22:6401-07.* ***7.*** *Weiler et al. BCL-x_L: time-dependent dissociation between modulation of apoptosis and invasiveness in human malignant glioma cells. Cell Death and Differ in press.*