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The p75 neurotrophin receptor enhances HIF-dependent signaling in glioma

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ABSTRACT

Tumor hypoxia is associated with several features of aggressive glioma growth, including migration, invasion, and stemness. Most of the cellular adaptation to hypoxia is mediated by the hypoxia-inducible factors HIF-1α and HIF-2α, but regulation of these factors by both oxygen-dependent and –independent mechanisms in brain tumors is only partially understood. Here, we show that the p75 neurotrophin receptor ($p75^{NTR}$) is stabilized at hypoxia in murine glioma *in vivo*, as well as in primary human glioma cultures *in vitro*. Expression of $p75^{NTR}$ resulted in increased stabilization of HIF-1α and HIF-2α, and RNAi or pharmacologic targeting of $p75^{NTR}$ diminished HIF stabilization and HIF-dependent signaling at hypoxia. Consequentially, $p75^{NTR}$ inhibition resulted in decreased migration, invasion, and stemness in response to hypoxia, suggesting that $p75^{NTR}$ is a central regulator of hypoxia-induced glioma aggressiveness. Together, our findings support the literature that identifies $p75^{NTR}$ as a potential therapeutic target in brain tumors.

1. Introduction

Gliomas are the most common primary malignant brain tumors. Despite a deeper molecular understanding of the disease, survival rates have not changed dramatically in the past several decades, and high-grade glioma remains one of the most lethal cancers. Both genotypic and phenotypic intratumoral heterogeneity have been proposed as key factors underlying the lack of long-term therapeutic response to aggressive treatment. Specifically, tumor cells with stem cell characteristics have been suggested to underlie therapeutic resistance and tumor recurrence in high-grade glioma. Intriguingly, the maintenance of stem cell phenotypes appears to be regulated by the tumor microenvironment in addition to specific genetic aberrations. Glioma cells with stem cell characteristics appear to be restricted primarily to specific tumor niches, most frequently in perinecrotic (hypoxic) and/or perivascular tumor areas. It is likely that these localizations are not random: microenvironmental cues unique to the perivascular microenvironment have been demonstrated to enhance glioma stem cell characteristics [1,2], and hypoxia-inducible factors (HIF-1α and HIF-2α) elevated in perinecrotic tumor areas are central transcriptional regulators of the stem cell phenotype [3,4].

p75 neurotrophin receptor ($p75^{NTR}$ or CD271), a member of the tumor necrosis factor receptor superfamily, has been implicated in several steps of glioma tumorgenesis [5]. $p75^{NTR}$ mediates glioma invasion and progression through γ-secretase-dependent and -independent mechanisms [6,7], and its expression has been linked to stemness both in glioma and other cancers such as melanoma, breast cancer, esophageal squamous cell carcinomas, and hypopharyngeal carcinoma [8–12]. The expression of $p75^{NTR}$ with regards to intratumoral heterogeneity and the possible regulation of $p75^{NTR}$ by microenvironmental factors, are largely unknown in the context of brain tumors. However, both γ-secretase-mediated cleavage of $p75^{NTR}$ to generate a p75 intracellular domain (p75-ICD) as well as expression of $p75^{NTR}$ itself has been shown to be induced by hypoxia in other cellular contexts [13].

Here, we investigate the expression and regulation of $p75^{NTR}$ in perinecrotic tumor areas and hypoxic glioma cells. We found that $p75^{NTR}$ expression is restricted primarily to perinecrotic, hypoxic tumor areas, and that $p75^{NTR}$ protein levels are upregulated at hypoxia in primary human glioma cells. The $p75^{NTR}$ itself regulated HIF protein levels, and was required for hypoxia-induced stemness, migration, and invasion in glioma. Our data highlight the interplay between $p75^{NTR}$ and HIFs, and suggest that $p75^{NTR}$ may represent a potential molecular target to interfere with hypoxia-induced aggressive glioma phenotypes.

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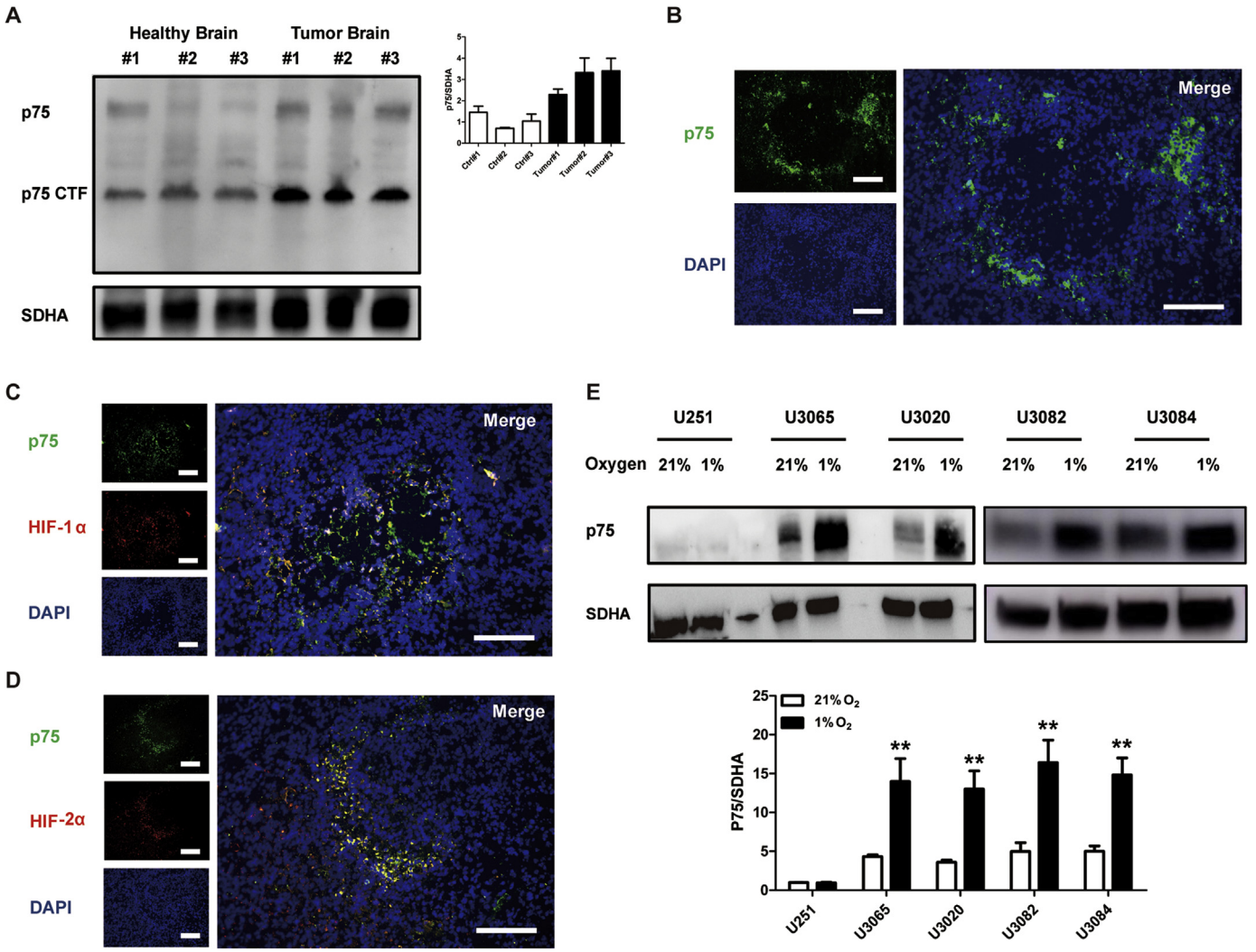

Fig. 1. Enhanced p75^{NTR} expression at hypoxia in glioma *in vivo* and *in vitro*. A. Protein expression of full-length and carboxyterminal fragment (CTF) p75^{NTR} in healthy and PDGFB-induced murine glioma brains as measured by western blot. Succinate dehydrogenase complex, subunit A (SDHA) is shown as loading control. Expression levels (full-length) were quantified and relative expression is plotted in bar graphs, normalized to average normal brain expression levels. B. Immunofluorescent staining of perinecrotic areas in PDGFB-induced murine glioma for p75^{NTR} (green) and DAPI (blue). Scale bars represent 50 μm. C-D. Immunofluorescent staining of PDGFB-induced murine glioma for p75^{NTR} (green), HIF-1α (red) or HIF-2α (red), and DAPI (blue) in perinecrotic areas. Scale bars represent 50 μm. E. p75^{NTR} protein expression in different glioma cell lines analyzed by western blot. Cells (U251, U3065, U3020, U3082, and U3084) were grown at 21% or 1% oxygen condition for 24 h. Data represent three independent experiments and are expressed as means ± SEM, n = 3. $**P < 0.01$.

2. Material and methods

2.1. Reagents

The following regents were used in this study: EGF, basic FGF (bFGF) (PeproTech, NJ); N2, B27, DMEM, DMEM/F12 (Life Technology); Accutase (Thermo Fisher Scientific) Polyornithine (Sigma-Aldrich, St. Louis, MO); Laminin-521 (Biolamina, Sweden); Xtreme gene 9 (Roche); HiPerFect (Qiagen); Ro 08–2750 (p75^{NTR} antagonist) (Santa Cruz, Dallas, TX). Small interfering RNAs: non-targeting (D-001810–01-20), *HIF1A* (LQ-004018–00-0002,), *EPAS1* (LQ-004814–00-0002), *NGFR* (J-009340–08-0002) from GE Dharmacon. Plasmids: RFP-empty vector, RFP-p75 full length vector were kind gifts from Prof. Chao's Lab; all plasmids were verified by sequencing. Antibodies: NGFR (SC-13577, Santa Cruz; p75 antibody kindly provided by prof. M. Chao); HIF-1α (NB100–479, Novus Biologicals, Littleton, CO); HIF-2α (ab199, Abcam, Cambridge, MA); SDHA (ab14715, Abcam).

2.2. Generation of murine glioma

Generation of PDGF-induced murine gliomas using RCAS/tv-a has been previously described [15]. DF-1 cells (10^5 cells in 1 μL PBS) transfected with *RCAS-PDGFB* [16] were injected into the left hemisphere of the cerebral cortex of neonatal *Ntv-a Ink4a/Arf*$^{-/-}$ mice from an entry point of the skull using a 10-μL gas-tight Hamilton syringe. Mice were monitored daily and euthanized upon appearance of symptoms of glioma. All animal procedures were approved by the Malmö-Lund Ethical Committee (Dnr. M186-14) and the use of laboratory animals was conducted in accordance with European Union directive on the subject of animal rights.

2.3. Immunofluorescent labelling of tumor cryosections

Tumor tissue from mouse brains was embedded in OCT and snap-frozen in pre-cooled isopentane. The frozen material was stored at −80 °C until sectioning. Tumor tissue was cut into 5 μm sections and placed on glass slides. Sections were dried at room temperature for 30 min and fixed in pre-cooled acetone. After permeabilization in 0.3% Triton X - 100