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Gamma-secretase-independent role for cadherin-11 in neurotrophin receptor p75 (p75^{NTR}) mediated glioblastoma cell migration

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ABSTRACT

The p75 neurotrophin receptor (p75^{NTR}) undergoes γ-secretase-mediated regulated intramembrane proteolysis and is involved in glioblastoma cell migration and invasion. Consistent with previous reports, in this study we show that p75NTR increases U87-MG glioblastoma cell migration, which is reversed by inhibition of γ-secretase activity. However, we show that expression or stabilization of the γ-secretase-generated p75^{NTR} intracellular domain (ICD) is not sufficient to induce U87-MG glioblastoma cell migration, and that exogenous expression of p75^{NTR} ICD inhibits p75^{NTR}-mediated glioblastoma cell (U87-MG and U373-MG) migration. To identify pathways and to determine how p75^{NTR} mediates glioblastoma migration we utilized a microarray approach to assess differential gene expression profiles between parental U87-MG and cells stably expressing wild-type p75^{NTR}, a γ-secretase cleavage-resistant chimeric p75^{NTR} mutant (p75FasTM) and the γ-secretase-generated p75^{NTR}-ICD, which mimics constitutively cleaved p75^{NTR} receptor. In our microarray data analysis we identified a subset of genes that were constitutively up-regulated in wild-type p75^{NTR} cells, which were also repressed in p75^{NTR} ICD expressing cells. Furthermore, our data revealed among the many differentially expressed genes, cadherin-11 (Cdh-11), matrix metalloproteinase 12 and relaxin/insulin-like family peptide receptor 2 as constitutively up-regulated in wild-type p75^{NTR} cells, independent of γ-secretase activity. Consistent with a role in glioblastoma migration, we found that U87-p75^{NTR} cells express higher levels of Cdh-11 protein and that siRNA-mediated knockdown of Cdh-11 resulted in a significant decrease in p75^{NTR}-mediated glioblastoma cell migration. Therefore, we hypothesize that p75^{NTR} can impact U87-MG glioblastoma cell migration in a γ-secretase-independent manner through modulation of specific genes, including Cdh-11, and that both γ-secretase-independent and -dependent mechanisms are involved in p75^{NTR}-mediated U87-MG glioblastoma cell migration.

1. Introduction

Structurally, the p75 neurotrophin receptor (p75^{NTR}) belongs to the tumor necrosis factor (TNF) receptor superfamily and binds all four mammalian neurotrophins with similar affinity constants. As a co-receptor of Trk receptors, it is involved in regulation of cell cycle, survival, neurite outgrowth and differentiation through MAPK (mitogen-activated protein kinase) as well as PI-3K (phosphatidylinositol 3-kinase) signaling (reviewed in (Roux and Barker, 2002; Reichardt, 2006)). However, p75^{NTR} alone may also promote survival and cell cycle progression by activating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and Akt pathways when bound by neurotrophins (Hamanoue et al., 1999; Roux et al., 2001). With Sortilin as co-receptor (Nykjaer et al., 2004) and activated by pro-neurotrophins (Lee et al., 2001), p75^{NTR} induces apoptosis through JNK (c-Jun N-terminal kinase) induction (Harrington et al., 2002) and caspase activation through the mitochondrial apoptotic pathway (Whitfield et al., 2001; Harris and Johnson, 2001). Many signaling properties of p75^{NTR} are regulated by posttranslational modifications such as phosphorylation (Higuchi et al., 2003; Zhang et al., 2009), glycosylation (Yeaman et al., 1997; Breuza et al., 2002), and palmitoylation (Barker et al., 1994; Underwood et al., 2008). In addition, p75^{NTR} is a substrate of the ubiquitin E3 ligases TNF associated factor 6 (TRAF6) (Powell et al., 2009) and c-Cbl (Ohrt et al., 2004). Moreover, p75^{NTR} is subjected to ectodomain shedding by a disintegrin and metalloproteinase 17 (ADAM17) (Kanning et al., 2003; Weskamp et al., 2004) as well as γ-secretase-mediated intramembrane proteolysis (Jung et al., 2003). The latter results in the release of the receptor intracellular domain (ICD) into the cytoplasm, a process which has been associated with

Abbreviations: P75^{NTR}, p75 neurotrophin receptor; Cdh-11, cadherin-11; MAPK, mitogen-activated protein kinase; PI-3 K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinase; ADAM17, a disintegrin and metalloproteinase 17; ICD, intracellular domain; CTF, c-terminus fragment; MMPs, matrix metalloproteinases; PMA, phorbol 12-myristate 13-acetate; DAVID, Database for Annotation Visualization and Integrated Discovery; MMP12, matrix metallopeptidase 12; CXCL5, chemokine (C-X-C motif) ligand 5; TRAF6, tumor necrosis factor associated factor 6; EMT, epithelial-mesenchymal transition.

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nuclear translocation of the ICD and subsequent regulation of gene transcription (Parkhurst et al., 2010), as well as induction of apoptosis (Podlesniy et al., 2006; Kenchappa et al., 2006), and inhibition of neurite outgrowth (Domeniconi et al., 2005).

Deregulation of $p75^{NTR}$ expression and signaling has been reported in several different kinds of cancer. While in prostate cancer loss of $p75^{NTR}$ has been associated with tumor progression (Krygier and Djakiew, 2002; Arrighi et al., 2010), $p75^{NTR}$ overexpression in melanoma and medulloblastoma is linked to increased invasive and metastatic potential (Marchetti et al., 2004; Marchetti et al., 2007). Indeed, $p75^{NTR}$ has been used as a marker to identify and isolate melanoma tumor stem cells (Boiko et al., 2010). In glioblastoma, the receptor was found upregulated in 85% of cases and in vitro as well as in mouse models overexpression of $p75^{NTR}$ was sufficient to induce a significant increase in cell migration and invasion of human glioblastoma cell lines (Johnston et al., 2007). Moreover, $p75^{NTR}$ is subjected to α- and γ-secretase mediated cleavage in higher-grade glioma and in invasive glioblastoma cell lines (Wang et al., 2008; Forsyth et al., 2014). Inhibition of γ-secretase activity in vitro has been shown to decreased migration and invasion of glioblastoma cell lines, while elegant in vivo studies have revealed that inhibition of γ-secretase prevents the formation of the highly invasive phenotype that is usually observed with $p75^{NTR}$ overexpression in glioblastoma (Wang et al., 2008). Similarly, γ-secretase inhibition significantly reduces spinal metastasis of medulloblastoma (Wang et al., 2010). Prognosis for glioblastoma patients is dismal, due to limited treatment options, the invasive nature of the tumors, and high recurrence rates (Kanu et al., 2009). Therefore, γ-secretase has now been suggested as a target for combined cancer therapy (Wang et al., 2008; Forsyth et al., 2014).

Many genes including those that encode $p75^{NTR}$, CD95, matrix metalloproteinases (MMPs) a disintegrin and metalloproteases (Kanu et al., 2009) cathepsins and integrins, have previously been implicated in promoting glioma migration and invasion (Johnston et al., 2007; Nakada et al., 2007; Freije et al., 2004; Kleber et al., 2008). In this study we have identified several additional genes that are involved in $p75^{NTR}$-induced U87-MG glioblastoma cell migration. Among the many differentially expressed genes, cadherin-11 (Cdh-11) is upregulated in glioblastoma cells expressing $p75^{NTR}$, independent of γ-secretase activity. Consistent with a role in $p75^{NTR}$-mediated glioma migration, glioma cells expressing $p75^{NTR}$ have higher levels of Cdh-11 and siRNA knockdown of Cdh-11 decreases migration of $p75^{NTR}$ expressing cells. Therefore, we propose that both γ-secretase-independent and -dependent mechanisms are involved in $p75^{NTR}$-mediated glioblastoma cell migration.

2. Materials and methods

2.1. Cell culture

The human glioma cell lines U87-MG (ATCC), U373-MG and A172 (CLS, Germany) were maintained in complete medium (minimum essential medium eagle (MEM M4655) and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, D6429). All growth medium was supplemented with 10% (vol/vol) FBS (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml). For transfection and generation of U87-MG stable expression cell lines, U87-MG cells were seeded at 2.5×10^5 cells/well in six-well plates and incubated over night at 37 °C. Cells were subjected to Lipofectamin LTX (Life Technologies) transfection with 1.2 μg of DNA per well according to manufacturer's instructions. The following day the transfection medium was replaced by complete medium supplemented with G418 (0.8 μg/ml) (Sigma-Aldrich). Cells were maintained in selection medium for a minimum of four weeks and expression of the different $p75^{NTR}$ constructs was confirmed at regular intervals. U373-MG and A172 cells were seeded at 0.2×10^6 cells per 6 cm culture dish and transfected 48 h later using Turbofect™ transfection reagent according to manufacturer's protocol (Thermo Scientific). HEK293T cells were seeded at 1.0×10^6 cells per 6 cm culture dish and transfected 6 h later using the calcium phosphate method. To evaluate effect of pharmacological inhibition of γ-secretase activity, subconfluent U87-MG or HEK293T cell cultures were pre-treated with the γ-secretase inhibitor XIX (30 nM; 4 h) (Calbiochem) in full growth medium and/or stimulated with phorbol 12-myristate 13-acetate (PMA) (200 ng/ml; 2 h) (Calbiochem) before harvest.

2.2. Plasmids and cloning

The pKS vector for expression of rat $p75^{NTR}$ was provided by Prof. Eric Shooter (Stanford University School of Medicine, California, USA). The γ-secretase cleavage-resistant $p75^{NTR}$-FasTM chimeric mutant was generated by two-step overlap PCR using the following primers; 5′-TCCTTGTTTTGTTAATTCCACTTGTATTTATATATAAGAGGTGGAACAGC TGCAAAC-3′ and 5′-GGAATTAACAAAACAAGGATGGTCAACAACCATA GGTTGTCGGTGGTGCCGCGGGTC-3′, as described (Domeniconi et al., 2005). The $p75^{NTR}$-ICD truncation mutant was created using PCR with the following primers; 5′-GTGCGAATTCGCCATGGTGGGCCTTGTGGCCT ATATTGCTTTC-3′ and 5′-GTGCGCGGCCGCTCACACTGGGGATGTGGCAG TGG-3′ and cloned into the EcoRI and NotI restriction sites of the original pKS vector (Jung et al., 2003). The $p75^{NT}$-K301R mutant was generated by site-directed mutagenesis using the following primers; 5′-CCAC CGGAGGGAGAGAGACTGCACAGCGACAGTG-3′ and 5′-CACTGTCGCTGT GCAGTCTCTCTCCCTCCGGTGG-3′. The fidelity and integrity of all expression constructs was verified by DNA sequence analysis (Macrogen).

2.3. Western blotting

Cells were washed in ice-cold PBS, then detached in PBS containing 5 mM EDTA using a cell scraper and transferred to 1.5 ml sterile Eppendorf tubes. After centrifugation and removal of supernatant, pellets were resuspended in cell lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 10 mM sodium orthovanadate and protease inhibitor mixture Complete™, Roche Molecular Biochemicals) and incubated on ice for 30 min. After centrifugation for 10 min at 13 000 rpm at 4 °C and protein concentration assessment by bicinoic acid assay (Pierce Biotechnology), proteins were resolved on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience) and analyzed by immunoblot with the appropriate antibodies as follows: rabbit polyclonal anti-human $p75^{NTR}$ 1:1000 (Promega); mouse monoclonal anti-β-actin 1:3000 (Sigma-Aldrich); mouse monoclonal anti-CDH-11 1:500 (Life Technologies). Immunoreactivity was visualized by the Odyssey Imaging System (Li-COR Biosciences) or enhanced chemiluminescense.

2.4. Scratch wound cell migration assay

U87-MG or U373-MG cell lines were seeded at 2.8×10^5 cells/well in six-well plates and grown for four days until cell monolayers reached near-confluence. Scratch wounds were inflicted using sterile P200 pipette tips and cells were washed twice with 2 ml of growth medium. After replenishing the growth medium, images were taken of defined areas of the wound immediately after (0 h) and 8 or 10 h after inflicting the scratch. The area of the scratch wound was determined using the polygon tool of the software ImageJ (www.imagej.net). The relative area covered by cells within 8–10 h was calculated using the 0 h and 8 or 10 h time point images of the same wound area. A minimum of six image sets was analyzed per experiment and experiments were replicated at least three times. Statistical significance was determined using Student's two-tailed t-test, one-way ANOVA followed by Tukey's Multiple Comparison Post-Test, or two-way ANOVA followed by Bonferroni post-test In case of treatment with γ-secretase inhibitor, cells were treated with 30 nM XIX or DMSO over night before inflicting the scratch wound and after washing the cell monolayer twice, fresh