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Overexpression of p75^{NTR} increases survival of breast cancer cells through p21^{waf1}

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ABSTRACT

The p75 neurotrophin receptor (p75^{NTR}) plays a critical role in various neuronal and non-neuronal cell types by regulating cell survival, differentiation and proliferation. To evaluate the influence of p75^{NTR} in breast cancer development, we have established and characterized breast cancer cells which stably overexpress p75^{NTR}. We showed that p75^{NTR} overexpression *per se* promoted cell survival to apoptogens with a concomitant slowdown of cell growth. The pro-survival effect is associated with an increased expression of the inhibitor of apoptosis protein-1 (c-IAP1), a decrease of TRAIL-induced cleavage of PARP, procaspase 9 and procaspase 3, and a decrease of cytochrome C release from the mitochondria. The anti-proliferative effect is due to a cell accumulation in G0/G1, associated with a decrease of Rb phosphorylation and an increase of p21^{waf1}. Interestingly, inhibition of p21^{waf1} with siRNA not only restores proliferation but also abolishes the pro-survival effect of p75^{NTR}, indicating the key role of p21^{waf1} in the biological functions of p75^{NTR}. Finally, using a SCID mice xenograft model, we showed that p75^{NTR} overexpression favors tumor growth and strongly increases tumor resistance to anti-tumoral treatment.
Together, our findings suggest that p75^{NTR} overexpression in breast tumor cells could favor tumor survival and contribute to tumor resistance to drugs. This provides a rationale to consider p75^{NTR} as a potential target for the future design of innovative therapeutic strategies.

1. Introduction

The p75 neurotrophin receptor (p75^{NTR}) was the first identified receptor for nerve growth factor (NGF); and it also binds all the other neurotrophins including brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and NT-4/5 as well as precursors [1–3]. Significantly, p75^{NTR} is a member of the tumor necrosis factor receptor superfamily [4], which exerts diverse functions such as the stimulation of cell survival and differentiation during neuronal development [5]. p75^{NTR} is expressed not only in nervous tissues, but also in non-neuronal normal and cancerous tissues. Particularly, p75^{NTR} has been reported to be overexpressed in some cancers such as thyroid carcinoma [6] and melanoma [7], while it is downregulated in cancers of other tissues including bladder [8], prostate [9], stomach [10] and liver [11]. p75^{NTR} may have opposite functions according to tumor types. Hence, it has been described to exert a tumor-promoting function in melanoma by favoring survival and metastasis of cancer cells [12,13], while it has been proposed as a potential tumor suppressor in other carcinomas such as prostate [9], bladder [14], stomach [10] and liver [11] cancers. In these studies, the tumor suppressor function of p75^{NTR} is associated with retardation of cell cycle progression by inducing accumulation of cancer cells in the G1 phase with a concomitant reduction of cells in the S phase of the cell cycle. We have previously shown that both p75^{NTR} and NGF are expressed in the majority of human breast tumors [15,16], but we have not observed any direct association between expression of p75^{NTR}/NGF and disease free or overall survival in breast cancer patients. However, the ratio between p75^{NTR} and NGF was reported to be of prognostic significance in breast cancers [17]. In addition, p75^{NTR} was shown to be preferentially expressed in basal-like breast carcinomas with good prognosis [18]. On the other hand, we and others have shown that p75^{NTR} is involved in NGF-stimulated cell survival in established breast cancer cell lines [19–21]. Nevertheless, the commonly used breast cancer cell lines express relatively low levels of p75^{NTR} compared to tumor biopsies [16]. To elucidate further the role of p75^{NTR} in breast cancer development and its mechanism of action, we established breast cancer cells overexpressing p75^{NTR}. We

Abbreviations: MTS, tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FBS, fetal bovine serum; TRAIL, TNF-Related Apoptosis-Inducing Ligand; FITC, Fluoresceine Iso Thio Cyanate; PARP, Poly (ADP-Ribose) Polymerase; PA, Ponasterone A; c-IAP1, cellular-Inhibitor of Apoptosis 1; XIAP, X-linked Inhibitor of Apoptosis; COX IV, Cytochrome C Oxidase IV.

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showed that $p75^{NTR}$ overexpression increased cell survival by inhibiting intrinsic apoptotic pathway and decreased cell proliferation by accumulating cells in the G0/G1 phase. Interestingly, the $p75^{NTR}$-induced biological effects involved $p21^{waf1}$. Moreover, $p75^{NTR}$ overexpression increased both basal tumor growth and tumor resistance to TRAIL in a SCID mice xenograft model.

2. Materials and methods

2.1. Plasmids

The ecdysone inducible system (Invitrogen™) includes 2 plasmids: pVgRXR and pIND. Plasmid pVgRXR contains the coding sequences of the modified subunits of ecdysone receptor, VgEcR and RXR, under the control of constitutive Rous sarcoma virus and cytomegalovirus promoters. Plasmid pIND contains five repeats of a modified ecdysone response element upstream from a minimal promoter. In the presence of ecdysone, an insect steroid, or synthetic analogs such as ponasterone A, the VgEcR and RXR receptors dimerize to form a functional modified ecdysone receptor that binds the modified ecdysone response element on pIND and induces the downstream gene transcription. Complementary DNA of $p75^{NTR}$ from MDA-MB-231 breast cancer cells was placed into the multiple cloning site of pIND and the obtained plasmid is named as pIND-$p75^{NTR}$. The plasmid pIND-βGal contains the β-galactosidase gene placed into the multiple cloning site of pIND.

2.2. Cell culture, transfection and generation of $p75^{NTR}$ overexpressing cancer cells

The MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection. Cells were routinely maintained in monolayer cultures in EMEM medium (Cambrex) supplemented with 10% fetal bovine serum (FBS; Perbio), 1% non-essential amino acids, 40 units/ml penicillin–streptomycin, 40 μg/ml gentamycin, and 10 μg/ml insulin for MCF-7 cells. To establish the $p75^{NTR}$ overexpressing model, MDA-MB-231 cells were first transfected with pVgRXR using Fugene (Roche) according to the manufacturer's instructions. Stably transfected clones were selected with 300 μg/ml Zeocin (Sigma-Aldrich®). The selected clones were tested for inducibility by transient transfection with pIND-βGal; the clone presenting the highest β-galactosidase activity after treatment with ponasterone A (PA; Sigma-Aldrich®) was chosen for the secondary stable transfection with pIND-$p75^{NTR}$. Secondary clones were established in the same manner as the primary clones except the cells transfected with pIND-$p75^{NTR}$ were cultured in the presence of 300 μg/ml Zeocin and 1 mg/ml G418. Levels of $p75^{NTR}$ mRNA in the selected secondary clones were then analyzed by real time PCR after PA induction. The clone presenting the highest level of $p75^{NTR}$ mRNA was chosen for further study.

2.3. Real time RT-PCR

Total RNA from cells was isolated with tri-reagent (Euromedex) and treated with DNase. Reverse transcription was performed with 1 μg of RNAs, 0.5 μg of random hexamers, 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen™) for 10 min at 25 °C, 50 min at 37 °C and 15 min at 70 °C in a final volume of 20 μl. Real time PCR amplifications were performed using a Quantitect SYBR®Green PCR kit (Qiagen) with 2 μl of 1:20 cDNA and 500 nM of primers. The primers used were as follows: for $p75^{NTR}$ transcript 5′-ACGGCTACTACCAGGATGAG-3′ and 5′-TGGCCTCGTCGGAATACGTG-3′; for $p21^{waf1}$ 5′-CACTCCAAACGCCGGCTGATCC-3′ and 5′-TGTAGAGCGGGCCTTTGAGGCCCTC-3′ and for RPLP0 (human acidic ribosomal phosphoprotein P0), which was used as a reference gene: 5′-GTGATGTGCAGCTGATCAAGACT-3′ and 5′-GATGACCAGCCCAAAGGAGA-3′. The subsequent PCR conditions were carried out in the following manner: 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. Data were analyzed using the MX4000 PCR system software (Stratagene) with the SYBRGreen option (with dissociation curves). $p75^{NTR}$ mRNA level was expressed as relative quantity compared to that expressed by control cells.

2.4. Western blot analysis

Extraction of total protein as well as protein from mitochondrial and cytosolic fractions was performed before Western blot analysis, as previously described [22]. Primary antibodies used are as follows: antibodies against $p75^{NTR}$ (1:2000, Promega), $p21^{waf1}$ (1:500), cleaved caspase 3 (1:1000), caspase 8 (1:1000), caspase 9 (1:1000), COX IV antibody (1:1000), cyclin D1 (1:500), cyclin D3 (1:500), cyclin E (1:500), E2F(1:500), $p27^{Kip1}$ (1:500), phospho-Rb Ser795 (1:500), Rb (1:500), $p15^{INK4B}$ (1:500), $p16^{INK4A}$ (1:500) from Cell Signaling Technology®, antibodies against PARP (1:200) from Santa Cruz Biotechnology®, antibodies against c-IAP and Bid (1:500, R&D System®), anti-XIAP (1:500, BD Transduction™), anti-β-actin (1:5000, Sigma-Aldrich®) and anti-cytochrome C (1:5000, Millipore™).

2.5. Immunocytochemistry

Cells (1×10^4) were grown on cover slips and treated with 10 μM PA for 24 h in culture medium containing 10% FBS. Cells were then fixed with 4% paraformaldehyde for 30 min (4 °C) and after two washes, they were permeabilized and blocked in PBS containing 1% BSA and 100 mM glycine for 45 min at room temperature. Cover slips were incubated with FITC coupled anti-p75 antibody (1:25, Cedarlane Laboratories®) overnight at 4 °C in PBS–1% BSA. After several washes in PBS, cover slips were mounted in Mowiol. Observation was performed on a Zeiss LSM 510 confocal microscope (488 nm excitation for FITC).

2.6. Apoptosis detection and cell survival assay

Cells were stained with 1 μg/ml Hoechst 33342 for 15 min at room temperature in the dark before fixation with cold methanol (−20 °C) during 20 min. The apoptotic cells, exhibiting condensed and fragmented nuclei, were counted under a Leica fluorescence microscope in randomly selected fields. A minimum of 500–1000 cells was examined for each condition, and results were expressed as a ratio of the total number of counted cells. Cell viability was determined by MTS assay (Promega) according to the user's manual.

2.7. Cell growth assay and cell cycle analysis

2.7.1. Cell growth

Cells (5×10^4) were plated in 35 mm dishes and cultured in EMEM containing different concentrations of serum. Cell numbers were evaluated after 72 h by using a cell counter (Beckman Coulter).

2.7.2. Cell cycle analysis

Cells were trypsinized and washed twice with PBS. They were subsequently stained by Cell Cycle Coulter reagent (Beckman Coulter). Briefly, pelleted cells (1×10^6) were mixed with 50 μl of reagent A (15 s, 20 °C, under vortex agitation) and 950 μl of reagent B. After incubation for 2 h at 4 °C in the dark, cell cycle was analyzed with a Beckman FACS analyzer.

2.8. siRNA inhibition

siRNA oligonucleotides targeting $p21^{waf1}$ 5′-GGACCUGUCACUGUCUUGUACCC-3′ and 3′-GGGUACAAGACAGUGACAGGUCC-5′, $p75^{NTR}$ 5′-AUGCCUCCUUGGCACCUCC-3′ and 5′-GGAGGUGCCAAGGAGGCAU-3′ or