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Differential response of neuroblastoma cells to TRAIL is independent of PI3K/AKT☆

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Abstract

Background: In many human tumor cells, tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) induces apoptosis through caspase activation, whereas activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway prevents apoptosis. We hypothesized that inhibition of PI3K/Akt would increase TRAIL-induced apoptosis in neuroblastoma cells.

Methods: SK-N-AS, SH-SY5Y, and IMR-32 neuroblastoma cells were cultured with either standard media or media with PI3K/Akt inhibitor for 24 hours. These cells were then exposed to 100 ng/mL of TRAIL for 90 minutes and harvested. Cells either underwent flow cytometric analysis of apoptosis, had protein extracted for Western blot, had RNA extracted for reverse transcription–polymerase chain reaction, or had cell lysates analyzed for caspase-3, -8, and -9.

Results: Baseline expression of TRAIL receptors and Akt varied among the cell lines. Inhibition of PI3K/Akt decreased caspase-3 activation in the AS and SY cells, but did not alter TRAIL-induced apoptosis in any of the cell lines. Activity of caspase-8 and -9 was also unaffected by PI3K/Akt attenuation.

Conclusions: Inhibition of the PI3K/Akt pathway does not increase the sensitivity of neuroblastoma cell lines to TRAIL-induced apoptosis. Neuroblastoma is unique in that activation of the PI3K/Akt pathway is either not essential to its TRAIL resistance or counteracted because of the multiple repetitive pathways of TRAIL resistance.

Neuroblastoma, a childhood tumor of neural crest origin, is the most common extracranial tumor of childhood. In children less than 1 year of age, metastatic disease possessing favorable biological markers responds well to therapy and may even regress spontaneously [1,2]. However, metastatic disease in children older than 1 year responds poorly to both aggressive medical and surgical interventions and has a dismal prognosis. As little progress has been made in improving outcome, the 5-year survival for patients with advanced-stage disease ranges from 18% to 30% [3]. Researchers have attempted to determine the mechanisms responsible for the dichotomous behavior of neuroblastoma with the goal of creating improved treatments for the aggressive form of this tumor.

One of the cornerstones of malignant cell transformation is a dysregulated increase in cell number, occurring either through increased cellular proliferation, decreased apoptosis, or a combination of both. Apoptosis, or programmed cell

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death, is defined by typical biochemical and morphological characteristics, such as cellular shrinkage, nuclear DNA fragmentation, and membrane blebbing [4,5]. Tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) leads to apoptosis through the binding with specific cellular receptors. TRAIL is unique in that it induces apoptosis in transformed, malignant cells while sparing normal host tissue [6,7]. In neuroblastoma, apoptosis is associated with more benign tumors, whereas aggressive tumors are associated with resistance to programmed cell death. There have been a number of studies linking the presence of apoptosis with favorable outcome with this cancer [8-10]. As apoptosis may play a role in spontaneous tumor regression or maturation, our ability to manipulate this process in neuroblastoma cells may lead to improved outcomes.

PI3K is a heterodimeric lipid-modifying enzyme consisting of p85 and p110 catalytic subunits that phosphorylate Akt [11]. Phosphorylation of AKT results in activation, which in turn suppresses apoptosis and promotes the cell cycle [12,13]. In fact, PI3K/Akt is thought to be one of the most effective anti-apoptotic survival pathways, acting through several mechanisms including the inactivation or the downregulation of pro-apoptotic proteins and the increased expression of several anti-apoptotic proteins [14,15].

We wished to investigate the possible role that the PI3K/Akt pathway might play in neuroblastoma TRAIL sensitivity. TRAIL has been demonstrated to induce the rapid phosphorylation of Akt in certain neuroblastoma cell lines (SK-N-MC) [16]. In addition, this same TRAIL-sensitive line had enhanced TRAIL-induced apoptosis with PI3K/Akt inhibition [17]. For this reason, we hypothesized that the specific inhibition of the PI3K/Akt signaling pathway would enhance TRAIL-induced apoptosis in neuroblastoma cell lines.

1. Materials and methods

1.1. Cell cultures

We used human neuroblastoma cell lines with known MYCN amplification states. The cell lines and their respective MYCN oncogene amplification states are as follows: nonamplified SK-N-AS ([AS] CRL-2137, American Type Culture Collection [ATCC], Manassas, Va) [39], 1 copy of SH-SY5Y ([SY] CRL-2266, ATCC) [39], and amplified IMR-32 ([IMR] CCL-127, ATCC) [39,40]. The rationale for choosing these particular cell lines is to allow us to make comparisons between cells lacking and possessing MYCN amplification, corresponding to a clinical situation that would compare tumors with good and poor prognosis, respectively.

Human neuroblastoma cell lines, SK-N-AS (ATCC), SH-SY5Y (ATCC), and IMR-32 (ATCC), were cultured as previously described [18-20]. Briefly, the cells were placed in standard growth medium consisting of Eagle's minimal essential medium (Invitrogen, Carslbad, Calif) supplemented with a 1% antibiotic/antimycotic containing penicillin, streptomycin, and amphotericin B (Sigma, St Louis, Mo), and with 10% fetal calf serum (Sigma). Neuroblastoma cell lines were plated (3×10^5 cells/well) in 6-well plates and maintained at 37°C in a humidified incubator with 95% O_2 and 5% CO_2 until they reached 80% confluence. Subsequently, media were replaced with either standard media or standard media with a specific PI3K inhibitor (LY294002, Cell Signaling, Beverly, Mass), at a concentration of 20 μmol/L, and cultured for an additional 24 hours. Next, these cells were exposed to either 0 or 100 ng/mL recombinant human TRAIL (R&D Systems, Minneapolis, Minn) for 90 minutes, creating 4 treatment groups: control, TRAIL (exposure to TRAIL only), LY (exposure to LY294002 only), and LY+TRAIL (exposure to both LY294002 and TRAIL). To remove the cells from the culture plates, trypsin 0.25% with EDTA 0.1% was placed with the cells for about 30 seconds to make a single cell suspension. The cells were then washed with PBS and the cell number was determined. All experiments were carried out at least in triplicate.

1.2. Reverse transcription and polymerase chain reaction

1.2.1. Rationale

We wished to demonstrate the relative abundance of mRNA present in the 3 neuroblastoma cell lines for the 4 known TRAIL receptors: the 2 functional receptors, DR4 and DR5, and the 2 decoy receptors, DcR1 and DcR2.

1.2.2. Total RNA isolate

The total RNA of the groups was extracted using TRIZOL reagent (Invitrogen) according to the supplier's instructions. RNA was quantitated by optical density measurements at 260 and 280 nm using a spectrophotometer.

1.2.3. Synthesis of cDNA

For the first-strand synthesis of cDNA, 5 μg of RNA was used in a 20-μL reaction mixture using a cDNA Cycle Kit (Invitrogen) according to the supplier's instructions. Resulting reverse transcription (RT) products were diluted 10 times and stored at −20°C until later use.

1.2.4. Reverse transcription–polymerase chain reaction

The polymerase chain reaction (PCR) primers were synthesized using the Nar Software for the functional TRAIL receptors, DR4 and DR5, and for β-actin according to human DNA sequences, and according to the noted references for the decoy TRAIL receptors, DcR1 [21] and DcR2 [22]. The following primers were used: DR4: forward 5′-ACTTTGGTTGTTCCGTTGCTGTTG-3′, reverse 5′-GGCTTTCCATTTGCTGCTCA-3′ (PCR product, 214 bp); DR5: forward 5′-TGGAACAACGGGGACAGAACG-3′, reverse 5′-GCAGCGCAAGCAGAAAAGGAG-3′ (PCR product, 347 bp); DcR1: forward 5′-GAAGAATTTG GTGCCAATGCCACTG-3′, reverse 5′-CTCTTGGACT TGGCTGGGAGATGTG-3′ (PCR product, 612 bp); DcR2: forward 5′-CCCCCGGCAGGACGAAGTT-3′, reverse 5′-CTCCTCCGCTGCTGGGGTTTT-3′ (PCR product, 418 bp);