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Novel peptides secreted from human neuroblastoma: useful clinical tools?

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Abstract

Background: Differentially expressed neuroblastoma (NB) proteins are vital for the development of new diagnostics and therapeutics. For example, secretory NB peptides (neuron-specific enolase and chromogranins) are clinically useful. We investigated polypeptide secretion by employing proteomic technologies to analyze proteins released from cultured NB cells.

Methods: Neuroblastoma cell lines (SK-N-AS, SK-N-DZ, and SK-N-FI) were grown in serum-free media. Conditioned media from each cell line was analyzed for secreted proteins by 2-dimensional polyacrylamide gel electrophoresis. Selected polypeptides were identified by liquid chromatography–linked tandem mass spectrometry.

Results: We identified 5 polypeptides that were secreted or shed by NB. Ubiquitin, β_2-microglobulin, insulin-like growth factor binding protein–2, superoxide dismutase (copper and zinc), and heat shock cognate 70-kd proteins were secreted from NB cells, as compared with control media. Elevated levels of these proteins have been described in serum/tissues under intracellular stress and malignancies, including NB.

Conclusion: These novel secretory polypeptides may contribute to NB growth. The proteins may reveal additional tumor markers and permit putative use in the diagnosis and treatment of NB. Detection of these proteins in serum of children with NB vs controls (using 2-dimensional polyacrylamide gel electrophoresis and mass spectrometry techniques) is currently in progress.

Neuroblastoma (NB) is a neuroendocrine-related tumor that secretes a variety of regulatory peptides [1]. The expressed proteins are localized in secretory granules (core- or membrane-related), in the cytosol, or in the cellular membrane. Polypeptides commonly associated with NB include secretory granule proteins chromogranin A and

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synaptophysin and the cytosolic enzyme neuron-specific enolase [2-5]. Other selected NB secretory proteins include neural-specific protein gene product 9.5, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), gastrin-releasing peptide, galanin, somatostatin, pancreastatin, proenkephalin-derived peptides (Met-enkephalin), peptide histidine isoleucine, pituitary adenylate cyclase–activating peptide, atrial natriuretic factor, and midkine [6-18]. The spectrum of released NB-related proteins is diverse and underscores the biologic complexity involved in the regulation and modulation of this childhood tumor.

Focusing on the clinical relevance of these expressed proteins, elevated plasma concentrations of many of these neuropeptides aid in the diagnosis, correlate tumor burden, and are useful predictors of survival in NB. For example, plasma chromogranin A, VIP, and NPY have all been shown to correlate with NB tumor burden [19-21]. Recently, O'Dorisio et al [22] demonstrated the plasma levels of 6 neuropeptides (VIP, somatostatin, gastrin-releasing peptide, substance P, pancreastatin, and NPY) in healthy children and corroborated the utility of excreted peptides in plasma for diagnostic detection of NB. Taken altogether, the plasma holds a vast reserve of proteins that are useful for identifying, quantifying, and monitoring patients with NB.

Despite the arsenal of circulating plasma proteins associated with NB, the routine use of these markers is not widely used. One explanation for the underappreciation of these blood markers maybe that serum and/or plasma reveal significant inconsistencies, and standardized methods of collecting blood samples are not established. For instance, vascular endothelial growth factor is an important angiogenic stimulatory peptide essential for solid tumor growth in NB. Hormbrey et al [23] reviewed reports evaluating the presence of vascular endothelial growth factor in peripheral blood. They showed important inconsistencies existed between reports ranging from sample collection method, processing, software manipulation, and data interpretation and whether plasma, serum, or whole blood provided the best prognostic information. Although quality control studies need to address the various peptides associated with NB, the discrepancies associated with tumor markers in serum and plasma may contribute to the underuse of NB-secreted proteins in the clinical setting.

Nevertheless, the potential for serum and plasma proteins to identify disease-specific states is improving by advancing proteomic technologies. The Plasma Proteome Project is a current effort organized by The Human Proteome Organization to investigate the optimal methods of collecting and analyzing complex protein fluids such as plasma and serum [24]. In light of these efforts to characterize disease-specific markers in biofluids, we have tested the concept of *neuropeptide secretion in NB cells* by evaluating polypeptides released from NB grown in culture. Secreted proteins were resolved by 2-dimensional polyacrylamide electrophoresis (2D-PAGE) and identified by mass spectrometry.

1. Materials and methods

1.1. Cell lines and cell culture

The 3 human NB cell lines used, SK-N-AS, SK-N-DZ, and SK-N-FI, were purchased from American Type Culture Collection. Cells were initially grown as monolayers in Dulbecco's modified Eagle's medium with 4 mmol/L of L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37°C in 5% CO_2. To avoid abundant growth-related proteins present in fetal bovine serum, cell lines were gradually adapted to propagate in general purpose serum-free medium without L-glutamine (BioWhittaker, Ultraculture, Walkersville, MD). Conditioned media from exponentially growing, nearly confluent (90%) cells were collected, centrifuged to remove cell debris, and stored at −80°C until ready for protein extraction and 2D-PAGE.

1.2. Protein extraction

Conditioned media collected from NB cultures and nonconditioned media were precipitated with ethanol to both concentrate protein and remove salts that interfere with first-dimension isoelectric focusing. Samples were prepared using a modified ethanol precipitation protocol [25]. Briefly, 1 mL of conditioned/unconditioned media was combined with 9 mL of absolute ethanol, vortexed, and stored overnight at −20°C. The tubes were then centrifuged for 5 minutes at 4°C, the supernatant was discarded, and the resulting pellet was air-dried for 10 minutes. Solubilization/rehydration buffer (500 μL) (9 mol/L urea, 4% CHAPS, 2 mmol/L tributylphosphine, 0.2% [wt/vol] Bio-Lytes (Bio-Rad, Hercules, CA), 0.001% bromophenol blue) was added to the pellets, which were then vortexed and centrifuged at 5000*g* for 5 minutes at 4°C. The resulting supernatant was transferred to a new eppendorf tube, and the protein concentration was measured using a BCA Protein Assay kit (Pierce, Rockfold, Ill).

1.3. Two-dimensional polyacrylamide electrophoresis

Protein 500 μg was rehydrated into 11-cm, pH 3-10 ReadyStrips (Bio-Rad, Hercules, Calif) for 12 hours at 20°C in a PROTEAN IEF Cell (Bio-Rad) before focusing. Focusing was performed at 8000 V for 30 000 hours at 20°C. Reduction and alkylation were accomplished by following the manufacturer's instructions (Bio-Rad), and the second dimension (8% to 16% PAGE) was accomplished using a Criterion cell (Bio-Rad).

1.4. Staining, imaging, and analysis

After sodium dodecyl sulfate PAGE, the gels were fixed in 50% methanol (vol/vol) and 5% acetic acid (vol/vol) for 1 hour and subsequently stained overnight with Colloidal Coomassie Blue (CCB, GelCode Blue Stain Reagent,