Expression of Platelet Derived Growth Factor Receptor- Beta (PDGFR-β) in phenotypically distinct human neuroblastoma cell lines

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Abstract
Neuroblastoma cells express specific transcripts of Platelet Derived Growth Factor (PDGF) and both α and β PDGF receptor genes. The presence of PDGFR-β in neuroblastoma was confirmed by sequencing RT-PCR products that were generated using primers whose sequences matched cDNA of human PDGFR-β. There is a difference in the expression of platelet derived growth factor receptor-beta between phenotypically distinct neuroblastoma cell lines. The PDGFR-β transcripts and protein are both expressed at higher levels in the non- malignant S-type cell line compared to the malignant N-type cell line. The S-type phenotype is similar to that of Schwannian precursor cells which are known to upregulate the PDGF receptor. It seems that in neuroblastoma the expression of PDGFR-β is not an indicator of malignancy but is more likely an indicator of phenotype.

Introduction

Neuroblastoma is the most common extracranial childhood cancer and the most common tumor occurring during infancy. It is an embryonal malignancy of the sympathetic nervous system arising from neuroblasts (pluripotent sympathetic cells). In the developing embryo, these cells invaginate, migrate along the neuraxis, and populate the sympathetic ganglia, adrenal medulla, and other sites. The pattern of distribution of these cells correlates with the sites of primary disease presentation. There are three distinct neuroblastoma cell types- I-type stem cells, N-type neuroblastic/ neuroendocrine precursors, and S-type schwannian cell/ melanoblastic precursors (Ross *et.al*., 2003). The S- type cells form monolayers in culture and show contact inhibition, whereas, the N-type cells grow as mutilayers with focal aggregates in culture (Ross *et.al*., 2003).

Crucial events for the invasion and spread of tumoral cells are the ability to digest the extracellular matrix, migrate, cross blood vessel walls, and reach the circulation (Kramer

et.al., 2001). It is known that many growth factors can produce some of the characteristic features of invasive growth like proliferation, motility, and increased survival. Among these, platelet-derived growth factor (PDGF), a mitogen mostly for cells of mesenchymal origin, was shown to induce chemotaxis and actin reorganization and prevent cells from dying by apoptosis (Heldin *et.a*l., 1998). Like a variety of other neural crest-derived tumor cell lines, neuroblastoma cells express specific transcripts of PDGF genes and both ά and ß PDGF receptor genes (Matsui *et.al*., 1993). It is thought that the simultaneous presence of PDGF and its receptor genes contributes to tumor cell growth and motility (Matsui *et.al*., 1993). The biological behavior of neuroblastoma can vary, in that, some tumors regress spontaneously, whereas others progress despite aggressive treatment. Infants diagnosed during their first year of life have a good prognosis, even in the presence of metastatic disease, whereas older patients with metastatic disease fare poorly, even when treated with aggressive therapy (Ribatti *et.al.,* 2004). Unfortunately, approximately 70-80% of patients older than 1 year are diagnosed with metastatic disease, usually to lymph nodes, liver, bone, and bone marrow (Ribatti *et.al.,* 2004). Fewer than half of these patients are cured, even with the use of high-dose therapy followed by bone marrow or stem cell rescue (Ribatti *et.al.,* 2004).

Many chromosomal and molecular abnormalities have been identified in neuroblastoma. These biologic markers have been evaluated to determine their value in assigning prognosis, and some of these have been incorporated into the strategies used for risk-assignment. The most important of these biologic markers is *MYCN. MYCN* is an oncogene often expressed in neuroblastoma (Guo *et.al*., 2000). This gene is amplified in approximately 25% of de novo cases and is more common in patients with advanced-

stage disease (Guo *et.al*., 2000). Patients whose tumors have *MYCN* amplification tend to have rapid tumor progression and a poor prognosis (Spitz *et.al*., 2004). Studies have also shown that neuroblastomas contain multiple cell phenotypes and this feature is often used to determine the prognosis of the disease. The phenotypically distinct neuroblastoma cell lines have been reported to show varying levels and differential expression of the *MYCN* gene. Higher expression of *MYCN* corresponds to a poorer prognosis. Most N and I type cell lines have higher levels of *MYCN* and some show overexpression of the *MYCN* gene. In the S-type cell line *MYCN* expression is reported to be down regulated (Spengler *et.al*., 1997). New therapeutic approaches are needed to improve the prognosis of neuroblastoma patients with high risk disease. Receptor tyrosine kinases have been proposed as potential targets for antitumor therapy because tyrosine kinase receptors play a role in angiogenesis, an essential step for tumor growth and metastasis (Beppu *et. al.,* 2004). Anti-angiogenic cancer therapies are attracting increasing attention. Neuroblastoma cells express platelet- derived growth factor (PDGF), stem cell factor (SCF), and vascular endothelial growth factor (VEGF) and their respective receptors, PDGFR, c-Kit, and Flk-1. Interest has predominantly focused on interfering with endothelial mitogens such as VEGF and FGF. However, increasing evidence also implicate PDGF receptor signaling in tumor angiogenesis.

The platelet-derived growth factor receptors (PDGFRs) belong to the receptor tyrosine kinase superfamily and are involved in regulating essential cell processes such as cell proliferation, motility, and survival (Heldin *et.al*., 1999). There are two structurally related PDGF receptors; the α and ß receptor each containing a large extracellular domain involved in growth factor binding and an intracellular region including a kinase domain

(Heldin *et.al*., 1998). Growth factor binding induces receptor homo- or hetero-dimerization at the plasma membrane, which leads to the activation of the intrinsic kinase activity. This results in the autophosphorylation of a number of tyrosine residues in the intracellular domain, which in turn act as docking sites for downstream signaling proteins containing phosphotyrosine recognition domains. PDGFRs can become oncoproteins when they are overexpressed or mutated. PDGFR-α activating mutations were found in patients with gastrointestinal stromal tumors (Heinrich *et.al*., 2003). Receptor tyrosine kinases are allosteric enzymes in equilibrium between active and inactive states. Oncogenic mutations up-regulate the tyrosine kinase activity, possibly by inducing an imbalance in favor of the active conformation of the kinase.

The aim of this project is to explore PDGFR ß as a potential target for antitumor therapy in neuroblastoma patients. By investigating whether or not there are differences in the expression of platelet derived growth factor receptor-beta between phenotypically distinct neuroblastoma cell lines and then determining if these differences can be seen on the protein level we can begin to consider that this gene may play a role in the generation of neuroblastoma.

Materials and Methods

*Cell Culture***:** The LAI-55N and LAI-5S neuroblastoma cell lines, and Hela cell line were cultured in MEM with 10% FCS, 10 U/ml penicillin and 10 µg/ml of streptomycin and incubated at 37˚C with 5% $C0_2$ and humidity.

Antibodies: An antibody to PDGFR-ß that was raised in rabbit against amino acids 958-1106 of the PDGFR-ß protein was purchased from Santa Cruz Biotechnology (958: sc-432). An anti-GAPDH antibody raised in mouse was purchased from Ambion. And an

anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (AP) and an anti-rabbit IgG secondary antibody, AP conjugated were both purchased from Promega.

RNA Isolation: Total RNA was isolated from LAI-55N, LAI-5S, and Hela cells using RNAqueous Phenol-Free Total RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's specifications. Cells were trypsinized, centrifuged, rinsed with PBS and harvested by centrifugation. Lysis/ Binding solution was added to the cells and cell debris was removed by centrifugation at 12,000g for 2-3 min. The lysates were then mixed with an equal volume of 64% Ethanol and the lysate/ethanol mixture was applied to an RNAqueous filter cartridge and centrifuged at 12,000g until all of the homogenate passed through the filter. Next, the column was washed with wash solution #1, centrifuged and the flow through discarded. The column was then washed twice with wash solution #2/3, centrifuged and the flow through discarded. Finally, the column was centrifuged for 2 min at 12,000g to remove any residual buffer. The RNA was then eluted by adding 60 µl of preheated (70˚C) ddH_2O to the column. The column was then incubated for 10 min at 70˚C and finally centrifuged at 12,000g for 1 min.

Oligonucleotide Primer Design: Primers were designed to amplify a segment of mRNA of the human Platelet Derived Growth Factor Beta- Receptor (PDGFRβ) based on the GenBank sequence (accession number NM_002609). The sequence of the forward primer to PDGFR-β is 5'- TCCGATGGAAGGTGATTG -3' (position 2047-2063) and the sequence of the reverse primer to PDGFR-β is 5' -TAGATGGGTCCTCCTTTGG -3' (position 2369-2389). These primers were designed to amplify mRNA encoded by exons 11-13 in the juxtamembrane domain of PDGFR-β producing a 344 bp product from mRNA or a 1.89 kb product from the amplification of genomic DNA. Primers designed

to amplify a segment of mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was provided as an external control for the RT-PCR. The primers to GAPDH produced a 225 bp product when amplifying mRNA and a 2.15 kb product when amplifying genomic DNA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): cDNA was generated from 60 ηg of RNA isolated from the LAI-55N, LAI-5S, and Hela cell lines by performing RT-PCR using the PDGFRβ primers and utilizing the Qiagen 1- Step RT-PCR kit (Valencia, Ca). Added to each reaction was 3µl 5X RT buffer, 0.6 µl 10mM dNTPs, 0.75µl of 10 ρmol/µl primers, 0.6µl enzyme mix, 3µl RNA template (20ηg/µl) and the reaction was brought up to a final volume of 15µl with ddH_2O. The RT-PCR conditions were as follows: (step 1) 50˚C for 30 min, 95˚C for 15 min (step 2) 94˚C for 30 sec, 56˚C for 30 sec, 72˚C for 30 sec (50 cycles), (step 3) 72˚C for 5 min and hold at 4˚C. cDNA was generated from 2.0 ηg of RNA from the LAI-55N, LAI-5S and Hela cell lines by performing RT-PCR using the GAPDH primers utilizing the Qiagen 1- Step RT-PCR kit (Valencia, Ca) as an external control. The RT-PCR conditions were as follows: (step 1) 50˚C for 30 min, 95˚C for 15 min (step 2) 94˚C for 30 sec, 56˚C for 30 sec, 72˚C for 30 sec (25 cycles), (step 3) 72˚C for 5 min and hold at 4˚C. PCR products were analyzed on a 1% agarose gel.

PCR Purification: The RT-PCR product was purified by Rapid PCR Purification (Life Technologies). Briefly, Binding Solution (H1) was added to the amplification reaction and mixed. The mixture was then loaded on a spin column, centrifuged for 1 min at 12,000g and the flow through was discarded. Buffer H2, containing ethanol was added to the spin column and centrifuged for 2 min at 12,000g to remove any residual wash

solution. The purified PCR product was obtained following the incubation of the spin column with 30 µl of preheated (70˚C) ddH_2O for 1 min, and then centrifuging for 1 min at 12,000g.

Sequencing: Sequencing was performed using the dideoxy modified version of the Sanger Sequencing method. 50 fmol of purified PCR product was mixed with 4 µl of 10x cycling buffer, 0.2 µl of ^{33}P-dATP, 2 µl of primer and dH_2O to a volume of 30 µl. 6 µl of this master mix was then added to four tubes containing 2 µl of either ddGTP, ddATP, ddTTP, or ddCTP. These reactions were set up for both the forward and reverse primers. Next, 12 µl of mineral oil was added to each tube to prevent evaporation during the subsequent PCR reaction. The PCR conditions were as follows: (step 1) 94˚C for 3 min (step 2) 94˚C for 30 sec, 58˚C for 30 sec, 72˚C for 1 min (35 cycles), (step 3) 72˚C for 7 min and hold at 4˚C. Following the sequencing reaction, 4 µl of stop solution was added to each tube. Each sequencing reaction was heated to 94˚C for 3 min to denature the products before electrophoresing them on a denaturing polyacrylamide gel. The nucleotide sequence was visualized by autoradiography.

Protein Extraction: Cells were harvested by washing with PBS twice and detached from the flasks using EDTA- trypsin. They were then centrifuged to pellet the cells and to remove the supernatant containing the media and trypsin. Cells were washed with cold PBS. 100 µl of lysis buffer (containing 25mM Tris-phosphate, 2mM DTT, 1Mm 1,2 diaminocyclohexane tetraacetic acid, and 1% TritonX-100) per $1x10^5$ of cells was added to the cell pellet. The whole cell lysates were stored at -80˚C. The concentrations of the proteins were determined using the Bio-Rad Assay kit following the manufacture's protocol. BSA was used to construct a standard curve for this assay. 60 µg of protein was

diluted at a 1:1 dilution with 2X sample buffer and then boiled for 3 min before loading on an 8% SDS polyacrylamide gel.

Western Blotting: Proteins were resolved on an 8% SDS PAGE gel and the separated proteins were electrophoretically transferred to a nitrocellulose membrane. The membranes were incubated in 5% nonfat dry milk in Tris-buffered saline/Tween 20 buffer (TBS-Tween; 20mM Tris-HCL [pH 7.4]. 150 Mm NaCl, and 0.5%Tween 20) for 1 hour at room temperature with gentle agitation to block non specific antibody binding. The nitrocellulose membrane was cut in half based on molecular weight distribution so that each half could be exposed to a different primary antibody. The top half of the membrane was incubated at 4°C in blocking buffer to which anti-PDGFR-ß antibody (1:100 dilution) was added. The lower half of the membrane was incubated at 4°C in blocking buffer to which anti-GAPDH antibody (4 µg/µl; 1:2000 dilution in TBST) was added. Next, the nitrocellulose membranes were washed 3 times by medium agitation in TBST for 5 min each wash. An anti-mouse IgG secondary antibody, AP conjugated (1 µg/µl; 1:4000 dilution in TBST) was added to the membrane that was exposed to the anti-GAPDH primary antibody and an anti-rabbit IgG secondary antibody, AP conjugated (1 µg/µl; 1:4000 dilution in TBST) was added to the membrane that was exposed to the anti- PDGFR-ß primary antibody. The secondary antibodies were left on the membranes for 3 hours at room temperature with gentle agitation. Next, the nitrocellulose membranes were washed 3 times by medium agitation in TBST for 5 min each wash. Bound antibodies were detected with the use of a Western Blue substrate for AP (Promega). 4 ml of the substrate was added to the membrane and agitated at room

temperature until a color reaction was visible. The reaction was stopped by washing the membrane with running tap water.

Results

Expression of PDGFR-β and GAPDH transcripts in human neuroblastoma cell lines

cDNA was generated from 60 ηg of RNA isolated from the LAI-55, LAI-5S and Hela cell lines by performing RT-PCR on each sample using the PDGFR-β primers that were designed to produce a 344 bp product. These reactions were subjected to 50 amplification cycles. cDNA was generated from 2.0 ηg of RNA from the LAI-55N, LAI-5S and Hela cell lines by performing RT-PCR using primers whose nucleotide sequences matched human GAPDH encoding mRNA to show that equal amounts of mRNA were present in the RT-PCR reactions. The reactions generating cDNA of GAPDH were amplified for 25 cycles. Expression of a 344 bp product was detected in all three cell lines. Significant differences in expression were observed between the LAI-55N and the LAI-5S neuroblastoma cell lines (fig 1). The expression of the 344 bp product in the LAI-55N and the Hela cell lines looked similar to each other. Both the N type cell line and Hela cell lines are derived from malignant tumors. The expression of the 344 bp product in the LAI-5S cell line which is derived from a non-malignant cell line looked much higher compared to the two malignant cell lines. Expression of a 214 bp product (GAPDH) was also detected in all three cell lines and there were no significant differences in GAPDH expression observed between them (fig 1). RT-PCR was repeated using the LAI-55N, LAI-5S and Hela cell lines utilizing both the PDGFR-β and GAPDH primers. In these reactions the number of amplification cycles were modified to determine the saturation points of the samples (data not shown). There were no RT-PCR

products observed for the reactions that used LAI-55N and Hela mRNA as a template when the amplification cycle number was adjusted below 50, suggesting that there were very small amounts of the receptor present.

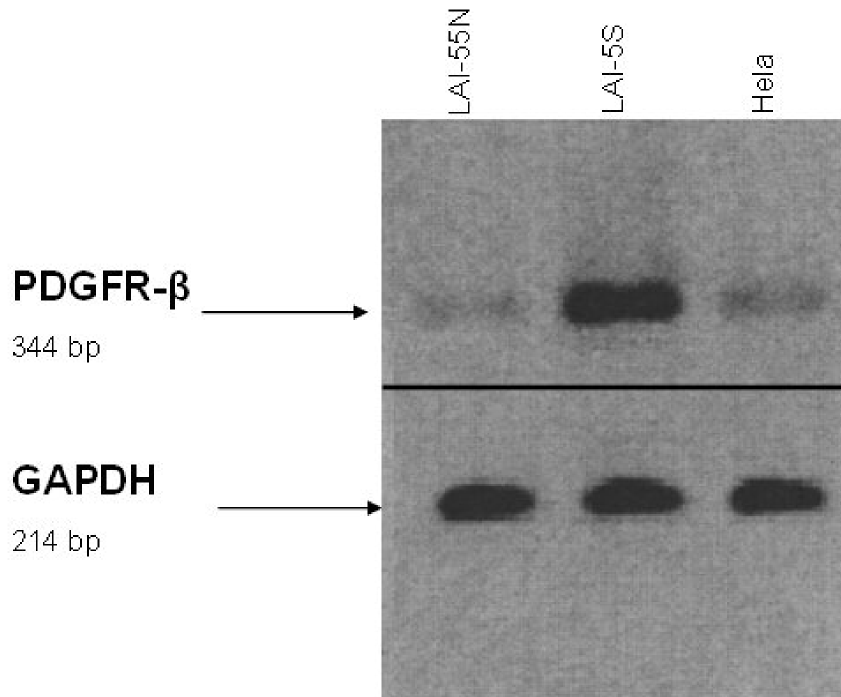

Figure 1. Expression of human PDGFR-β and GAPDH transcripts in neuroblastoma. 60 ηg of total RNA from the LAI-55N, LAI-5S , and Hela cell lines was subjected to RT-PCR amplification using primers whose sequences matched a region of human PDGFR-β cDNA. The sequence of the forward primer to PDGFR-β is 5'-TCCGATGGAAGGTGATTG -3'(position 2047-2063) and the sequence of the reverse primer is 5' TAGATGGGT CCTCCTTTGG -3 (position 2369-2389) accession number NM_002609. These RT-PCR reactions were subjected to 50 cycles of amplification. 2 ηg of total RNA from the LAI-55N, LAI-5S, and Hela cell lines was subjected to RT-PCR amplification using primers whose sequences matched a region of the human GAPDH gene. The sequence of the forward primer to GAPGH correspond to 100-119 and the reverse sequence corresponds to 308-327 of accession number NM_002046. These RT-PCR reactions were subjected to 25 cycles of amplification. Sizes of the RT-PCR products were revealed in relation to a 100 bp ladder.

Sequence analysis of generated 344 bp RT-PCR product

mRNA isolated from the LAI-55N neuroblastoma cell line was subjected to RT-PCR using primers whose nucleotide sequences matched human PDGFR-β encoding mRNA. The resulting cDNA product was sequenced to verify that the primers amplified the

correct regions and that the band that was resolved using gel electrophoresis corresponded to the 344 bp region of PDGFR-β that the primers spanned. The ensuing sequence was read and aligned to the cDNA sequence of human PDGFR-β in GenBank (accession number NM_002609) using the ClustalW Formatted Alignment program from MacVector. The alignment confirmed that the 344 bp band that was resolved using gel electrophoresis had homology to a region of human PDGFR-β cDNA and that this region matched the sequence that was spanned by the primers that were designed (fig 2). Therefore, the expression of the 344 bp product observed in fig.1 does represent a differential expression of PDGFR-β between the cell lines derived form malignant tumors (LAI55N and Hela) versus the cell line derived from a non malignant tumor (LAI-5S).

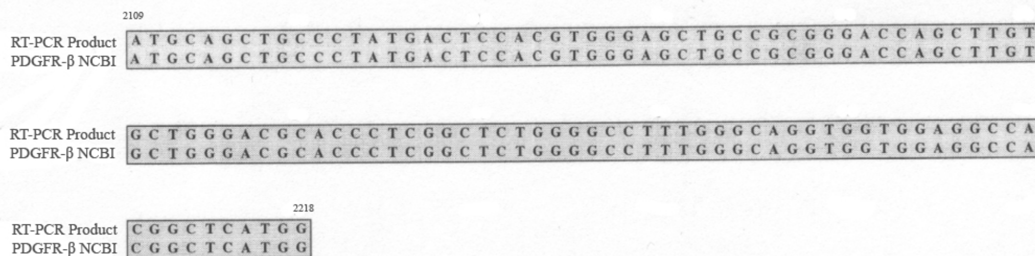

Figure 2. Sequence analysis of the generated RT-PCR product revealing 100% sequence identity to human PDGFR-β (position 2109-2218 accession number NM_002609. The RT-PCR product was purified, sequenced, and aligned to the cDNA sequence of human PDGFR-β in the GenBank NCBI database using the ClustalW Formatted program.

Expression of PDGFR-β and GAPDH protein in neuroblastoma.

60 µg of protein extracted from the LAI-55N, LAI-5S cell line and Hela cell lines were resolved with an 8% SDS polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with antibodies against human PDGFR-β and GAPDH. Western blot analysis using the anti-PDGFRβ antibody revealed that expression of PDGFR-β was detectable in only the LAI-5S neuroblastoma cell line (fig 3). Moreover, the anti-PDGFR-β antibody detected both the mature glycosylated (180 kDa) and immature unglycosylated (165 kDa)

form of the PDGF-β receptor. Antibody recognition to both forms of PDGFR-β was confirmed by Santa Cruz Biotechnology. There was no expression of PDGFR-β observed in the LAI-55N and Hela cell lines, whereas the expression of PDGFR-β in the LAI-5S cell line was very high. Expression of GAPDH was detected in all three cell lines (a 35 kDa band was observed) (fig3) and there were no significant differences in the expression of GAPDH observed between the cell lines. These results suggest equal amounts of protein were loaded onto the gel and that the differential expression of PDGFR-β protein observed between the cell lines is valid.

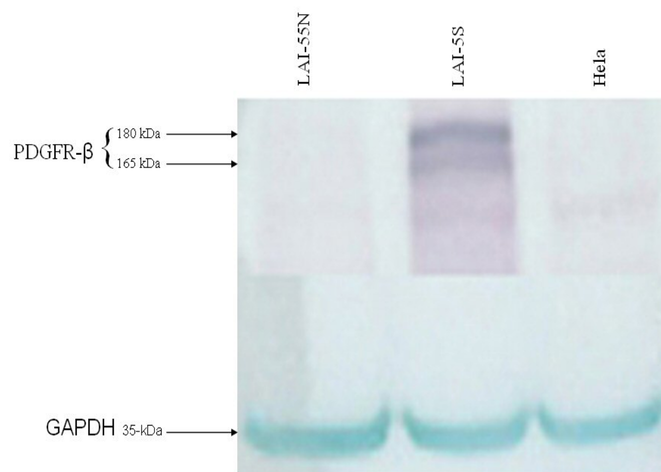

Figure 3. Expression of PDGFR-β protein (180 kDa glycosylated mature and 165kDa nonglycosylated immature) and GAPDH (35 kDa) proteins in neuroblastoma cell lines. 60 μg of protein from the LAI-55N, LAI-5S, and Hela cell lines were resolved on an 8% SDS PAGE gel, transferred to a nitrocellulose membrane, and probed with anti-PDGFR-β and anti-GAPDH antibodies. Sizes of recognized proteins were revealed using a prestained molecular weight marker.

Discussion

This study revealed that PDGFR-β mRNA is present in neuroblastoma. By sequencing we have confirmed that the 344 bp RT-PCR product generated is in fact an amplification product of PDGFR-β. The results of the western blot corresponded to the results of the RT-PCR experiment in which the expression of PDGFR-β was higher in the nonmalignant LAI-5S cell line as compared to the malignant LAI-55N and Hela cell lines.

These preliminary experiments were meant to explore PDGFR-ß as a potential target for antitumor therapy in neuroblastoma patients. The difference in the expression of platelet

derived growth factor receptor-beta between phenotypically distinct neuroblastoma cell lines is significant because these receptors, if activated, can couple with chemotactic and neurotrophic signal transduction pathways resulting in neuronal growth and migration. Thus, receptor expression could be crucial in tumor development by conferring a selective growth advantage to the cancer cells. However, this study found that PDGFR-β was expressed at higher levels in the non- malignant S-type cells as compared to the malignant N-type cells. These results may be explained because epithelial growth factor receptor expression (EGFR) is generally higher in S-type neuroblastoma cultures (Rettig *et.al*., 1987) leading to the assumption that PDGFR is involved in this autocrine loop. More important to note is that the S-type phenotype is similar to that of a Schwannian/ glial/ melanoblastic precursor cell (Ross *et.al*., 2003) and that several studies show that Schwann cells and their precursors express PDGFR. Neuroblastoma tumors consist of two main cell populations, neuroblastic/ganglionic cells and Schwann cells, and the ratio of these cell types varies according to tumor maturation. Immature neuroblastoma tumors are composed of undifferentiated neuronal cells and a paucity of Schwannian stroma, whereas larger, ganglion-like cells and abundant Schwannian stroma are seen in maturing neuroblastoma tumors (Chlenski et.al., 2003). It has been speculated that Schwann cells influence neuroblastoma tumor growth by secreting molecules that serve as anti-proliferative and pro-differentiation factors for neuronal cells (Chlenski et.al., 2003). Early postnatal Schwann cells in the rat have been found to express both PDGF and the PDGF-receptor, which have been implicated as a potential mechanism for autocrine mediated survival (Winseck *et.al*., 2002). Developing mammalian Schwann cells eventually become axon-independent and use IGF-1, IGF-2, PDGF-BB, NT-3 and their

respective receptors as part of an autocrine mechanism to regulate their own survival (Winseck et.al., 2002). After axon elimination in the chick embryo at E16, Schwann cells upregulate the PDGF receptor which is likely to be a component of autocrine-mediated survival in more mature Schwann cells, similar to the situation in rodents (Winseck et.al., 2002). Lobsiger et.al., in 2000 reported that PDGFR-α and PDGFR-β are expressed in freshly isolated Schwann cell precursors, and that PDGF-BB is able to rescue a subpopulation of these cells from apoptotic cell death *in vitro*.

Due to the similarity of the S-type neuroblastoma phenotype to that of a Schwannian/ glial/ melanoblastic precursor cell (Ross *et.al*., 2003) the high expression of PDGFR-β observed in S-type neuroblastoma cells compared to N-type neuroblastoma cells observed in this study is logical. It seems that in neuroblastoma the expression of PDGFR-β is not an indicator of malignancy but is more likely an indicator of phenotype.

Future Studies

It is necessary to repeat these experiments in more phenotypically distinct neuroblastoma cell lines in order to validate the results of this study. The expression levels of PDGFR-α in neuroblastomas may also be another interesting subject of investigation. Determining whether or not PDGFR plays a role in diverting the malignant process of neuroblastoma is also important to elucidate.

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