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The Investigation of a TIMP-1-modulated Glial-Derived Factor Affecting Neuroblastoma Cell Death

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THE INVESTIGATION OF A TIMP-1-MODULATED GLIAL-DERIVED FACTOR AFFECTING NEUROBLASTOMA CELL DEATH

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A THESIS SUBMITTED TO THE FACULTY OF THE NEUROSCIENCE PROGRAM IN CANDIDACY FOR THE BACCALAUREATE DEGREE WITH HONORS IN NEUROSCIENCE

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THE INVESTIGATION OF A TIMP-1-MODULATED GLIAL-DERIVED FACTOR AFFECTING NEUROBLASTOMA CELL DEATH

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ABSTRACT

The original role of astrocytes was believed to have been as a neuronal-supportive cell in the brain. It has now been discovered that they play imperative roles, from reuptake of neurotransmitters from the extracellular space to signal propagation and developmental control by the release of factors into the extracellular space. SH-SY5Y and IMR-32 cells are common neuroblastoma cell lines which model cancerous brain cells when left undifferentiated. In recent studies tissue inhibitors of metalloproteinases (TIMPs) have been implicated in neurodegenerative diseases, but their exact role in cell death is unknown. A double-blind cell culture experiment was conducted using astrocytes from wild type and TIMP-1 knockout mice to evaluate the role of TIMP-1 in neuronal cell death. Undifferentiated and differentiated SH-SY5Y cells, as well as undifferentiated IMR-32 cells, were treated with either wild type glial cell media (WT-GCM) or TIMP-1 knockout glial cell media (KO-GCM) for 24 hours and cell viability was evaluated. Undifferentiated SH-SY5Y and IMR-32 cells exposed to WT-GCM showed a significant increase in cell death when compared to cells exposed to KO-GCM. The glial conditioned media had no effect on SH-SY5Y cells differentiated using retinoic acid. Supplementation of the KO-GCM with recombinant TIMP-1 to physiological levels had no impact on cell death in SH-SY5Y undifferentiated cells, while supplementation of WT-GCM with recombinant TIMP-1 completely blocked the cell death seen following WT-GCM treatment alone. Heating of the WT-GCM completely eliminated the increased cell death produced by the WT-GCM. These results suggest that TIMP-1 may be modulating a temperature-dependent, cell media-soluble factor that is released by astrocytes and influences cell death mechanisms of undifferentiated neuroblastoma cells.
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PROJECT FOCUS

Astrocytes and other cells in the brain, such as other glia, neurons, and even cancerous cells, communicate with each other by releasing factors into the extracellular space. These factors have the ability to bind to other chemicals within the extracellular space, enter cells, or even bind to receptors on the cell membrane triggering various types of cascades. Often, to investigate these mechanisms within the brain, studies are conducted on cell cultures. An optimal in vitro model shares morphological and biochemical similarities with the cells of focus in the research project, and fortunately, there are many types of cell cultures that consist of proliferative neuroblastomal cancer cells. This study investigates the neurochemical interactions between astrocytes, neurons, and neuroblastomal cells to further elucidate the mechanisms involved with cancerous brain tumors.
1. INTRODUCTION

1.1 Astrocytes

The nervous system is formed by two major cell types: neurons and glial cells. Glial cells are subdivided into different types, with different functions: oligodendroglia, microglia, and astroglia. Based upon the first original descriptions of the cellular basis of the nervous system, neurons were swiftly recognized as the main cellular elements involved in the transfer and processing of information, as they show cellular processes that extend towards sensory organs, muscles, and glands. The concept that neurons were electrically excitable further supported this idea. It has been well established that neuronal electrical excitability is based on the expression of numerous ligand and voltage-gated membrane channels that give rise to membrane currents and membrane potential variations. Furthermore, cell signaling pathways have been recently explored in these cell types, helping to promote understanding of neurodegenerative diseases as well as brain tumor metastasis.

Until the past 30 years or so, neuroscientists believed neurons communicated only with each other, represented our ideas and thoughts, and glial cells were only the glue holding the brain together. There are a few types of glial cells, but recently the focus on is on astrocytes, as they make up 90% of the cells in the cortex. Many studies have exhibited that astrocytes are important functional elements of the synapses, reacting to neuronal activity and regulating synaptic transmission and plasticity. Accordingly, they are actively involved in the processing, transfer and storage of information by the nervous system, which tests the commonly accepted paradigm that brain function results exclusively from neuronal network activity, and suggests that
nervous system function and homeostasis actually arises from the activity of neuron-glial networks.¹

Figure 1: Santiago Ramon y Cajal’s original protoplasmic astrocyte slides impregnated by the Golgi-Cox method. a = astrocytes, bv = blood vessels, ef = endfeet, gf = gliofilaments, n = neuron, p = processes.

On the other hand, glial cells, and particularly astrocytes, were considered to simply play supportive roles, as the cells lack the long processes that connect to sensory and effector organs (Figure 1). Astrocytes have since been discovered to play relevant roles in numerous processes of the development and physiology of the central nervous...
system, such as trophic and metabolic support for neurons, neuronal survival and differentiation, neuronal guidance, neurite outgrowth, and synaptogenesis. They are also key elements in brain homeostasis, regulating the local concentrations of ions and neurotransmitters, and in control of local cerebral blood flow. There has been an influx of research focused on these previously assumed passive cells, as they have been found to perform an incredible amount of cell signaling by releasing astrocytic factors into the extracellular space, in turn affecting other cells around them. Whether these cells release neuroprotective, apoptosis-inducing, or other factors these astrocyte-mediated mechanisms are at the forefront of neuroscience research, as they may provide answers to neuroblastoma cell growth and even neurodegenerative diseases.

1.1.1 Astrocyte Roles

Astrocytes are known to play important roles in the homeostasis of the extracellular environment, providing the adequate conditions for the appropriate function of neurons and synapses. Since astrocytic processes are highly dynamic subcellular elements capable of mobility, retraction, and extension, astrocytes can dynamically shape the extracellular space, which may have a strong impact on where astrocytic-factors are being secreted and how these factors affect the cells around them, and which type of cells are being affected.

Additionally, hippocampal astrocytes have also been shown to rapidly extend and retract their processes in coordination with changes in dendritic spine location, neurotransmitter release, and even in changes involving neuronal cell death. Changes in the amount of glutamate have been shown to affect the dendritic spine locations of astrocytes (Figure 2). In rat hippocampal cultures, an increase of synaptic transmission
induced the movement of astrocytic dendrites to the synapse location, allowing for the reuptake of extracellular glutamate via the EAAT2 channel on the astrocytic dendrites.\(^2\)

The expression of the EAAT2 glutamate transporter by astrocytes has shown to be crucial in the clearance of glutamate from the synaptic cleft to terminate synaptic function.\(^7\)

This research has demonstrated the immense capacity of astrocytic mobility within dynamic, \textit{in vivo}, environments, allowing for a glimpse into the molecular dynamics of these cells.

Figure 2: The remodeling of EAAT-2 clusters associated with lateral filopodia on developing astrocytes. (A) High-magnification time-lapse sequence of a region on an astrocyte, which shows the remodeling of four filopodia and spine-like protrusions containing EAAT-2 clusters. (B) Different region of the same astrocyte showing a persistent protrusion elongating over a 15-minute period, carrying an EAAT-2 cluster at its tip.\(^7\)
1.1.2 Astrocyte Signaling

The main focus on astrocytes has been on calcium signaling between neurons and other astrocytes. This presents a novel and dynamic way in which astrocytes are able to communicate with each other, and other neurons, within the brain. Numerous studies performed during the past few years, have established the existence of bidirectional signaling between neurons and astrocytes. The calcium-based cellular excitability displayed by astrocytes can be triggered by neuronal and synaptic activity through activation of neurotransmitter receptors expressed by astrocytes. In turn, astrocyte calcium elevations stimulate the release of different substances, such as glutamate, ATP, and D-serine, which regulate neuronal excitability and synaptic transmission. These findings have led to the establishment of a new concept in synaptic physiology, the tripartite synapse, in which astrocytes exchange information with the neuronal synaptic elements.

Astrocytes not only communicate via calcium signaling, they have also been known to release several other factors such as the proteins Hevin and SPARC, that act as regulators of excitatory synaptogenesis in vitro and in vivo. Through the regulation of these proteins astrocytes control the formation, maturation, and plasticity of synapses in vivo. As mentioned earlier, astrocytes also have the capability of releasing soluble factors that aid in the differentiation of CNS stem cells. A factor known as the ciliary neutrotrophic factor is secreted from pre-existing astrocytes and has the capability of promoting astrocytic differentiation of multipotent cortical stem cells. Furthermore, astrocytes also have shown the capacity to induce neuronal differentiation by secreting bone morphogenetic proteins, which cause this functional and morphological change.
Other studies have discovered that astrocytes promote the differentiation of rat embryonic neuronal cells by releasing the chemokines: IL-4, MIP-1, KC, and RANTES. The differentiation of rat embryonic neuronal cells was promoted by treatment with astrocyte and microglia-conditioned medium. Mature astrocytes have also demonstrated the opposite phenomenon of de-differentiating into neural/stem progenitor cells following a mechanical injury. In this study it was discovered that following mechanical injury sonic hedgehog (Shh) production in astrocytes was significantly elevated, and that the incubation of astrocytes with the injured astrocyte medium caused astrocytes to gradually lose their immunophenotypical profiles, and acquire neural stem/progenitor cells characteristics.

Astrocytes also have demonstrated major neuroprotective roles in an in vitro environment when given pramipexole, a dopamine D2/D3 receptor agonist, which is used in the treatment of Parkinson’s disease. In this study cell death was induced by a proteasome inhibitor, lactacystin, on primary mesencephalic neuronal cultures and SH-SY5Y cells, a common neuroblastoma cell model. The protective effect of pramipexole against lactacystin-toxicity was found to be not a direct effect, but instead a secondary effect mediated by astrocytes. Mesencephalic astrocytes were treated with pramipexole and grown in culture. Medium from the treated astrocytes was collected and then given to SH-SY5Y cells, which were formerly treated with lactacystin. A significant reduction of cell death was seen compared to SH-SY5Y cells just treated with lactacystin, demonstrating a protective effect by the astrocytic media.

Another study demonstrated the neuroprotective effect of astrocytic media after an acute CNS lesion, which in turn is known to release neurotoxic substances. Hailer et al. (2001) treated organotypic hippocampal slice cultures with N-methyl-D-aspartate (NMDA), which resulted in a visible loss of viable granule cells, partial destruction of
the regular hippocampal cytoarchitecture and a concomitant accumulation of amoeboid microglial cells at site of neuronal damage. Astrocyte-conditioned media reduced the amount of NMDA-induced neural injury by 45.3%, diminished the degree of microglial activation, and resulted in an improved preservation of the hippocampal cytoarchitecture, yet the neurochemical mechanism underlying this reaction is unknown.12

In some situations, astrocytes are also able to promote neuronal death by secreting varying factors. Further elucidating these mechanisms can help promote the understanding of the underlying pathology of diseases as well as having the ability to take advantage of these mechanisms to promote death in unwanted cells, such as cancerous tumors. Recently, it has been discovered that astrocytes become reactive in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Huntington’s disease, Parkinson’s disease, and Alzheimer’s disease.13,14 Controlled activation of these astrocytes is considered to be beneficial to neurons, as they may exhibit differential expression of astrocytic receptors, transporters, and transmitters, yet overactive astrocytes can be harmful.15

In most neurodegenerative diseases reactive astrocytes lose neuroprotective function and can gain neurotoxic properties. One example of how astrocytes lose their neuroprotective function is via the dysfunction of glutamate reuptake via the EAAT2/GLT1 transporter. In mice, GLT1 deficiency has been shown to lead to synaptic glutamate accumulation and subsequent excitotoxicity.16 When astrocytes become reactive during neurodegeneration they gradually lose GLT1 function and expression.17

In addition to this loss of neuroprotective function, it is speculated that reactive astrocytes also have the capability of gaining neurotoxic properties. Cultured astrocytes expressing mutant superoxide dismutase 1 (SOD1) secrete unknown neurotoxic factors
into the medium, and adding this conditioned medium to cultured motor neurons can induce cell death.\textsuperscript{17,18} In a recent study, the astrocytic reaction was further investigated, and it was discovered that these astrocytes secrete lipocalin 2, an inducible factor, that is strictly toxic to neurons in transgenic rats that express a mutant form of TAR DNA-binding protein 43 in neurons, which is an incredibly common mutation.\textsuperscript{19,20}

Although the research presented in this paper focuses on the neurodegenerative role of astrocytes within the glial-neuronal and glial-neuroblastomal environment, the examples above allow for this general principle to be extracted: factors secreted by astrocytes may have several effects depending on the target cells and the neuronal elements as well as the activated receptor subtypes, providing a high degree of complexity for astrocytic effects. This research hopes to elucidate the role of astrocyte secretion factors in a system containing human neuroblastomal cells. Cancerous, undifferentiated, cell viability within an astrocytic environment has not been studied, but furthering our understanding on this topic may help treat neuroblastomal tumors in the future.

1.2 NEUROBLASTOMA CELLS

1.2.1 Neuroblastoma

Neuroblastoma is the most common extracranial solid cancer in childhood, and the most common cancer in infancy with an annual incidence of about six hundred and fifty cases per year in the U.S. alone. It is considered a neuroendocrine tumor, arising from the primordial neural crest element of the sympathetic nervous system. These tumors may regress spontaneously, reflecting induction of apoptosis or differentiation,
or they may exhibit extremely malignant behavior. Additionally, they are insensitive to conventional cancer treatment, furthering their enigmatic qualities.\textsuperscript{21}

\subsection*{1.2.2 SH-SY5Y cells}

The SK-N-SH cell line was originally established from a bone marrow biopsy of a neuroblastoma patient with sympathetic adrenergic glangial origin in the early 1970s.\textsuperscript{22} The popular neuroblastoma cell model, the SH-SY5Y cell line, is a thrice cloned subline of the SK-N-SH cells, and has been widely used since these cells posses many biochemical and functional properties of neuroblastomal cells.\textsuperscript{23}

The SK-N-SH cell line contains cells with three different phenotypes: neuronal (N type, seen in Figure 3 A), Schwannian (S type, seen in Figure 3 B), and intermediary (I type).\textsuperscript{24} On the other hand, the SH-SY5Y cell line is homogenous, containing only the neuroblast-like cell line, with occasional S type cells (Figure 3 B). This cell line shows neuronal marker enzyme activity, specific uptake of norepinephrine, and expresses one or more neurofilament proteins. As for enzymes, the cells express low levels of tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase, as well as norepinephrine uptake transporters, and high levels of nestin, which are all markers for immature dopaminergic/noradrenergic neurons. Fortunately, these cells have the capability of proliferating in culture for long periods without contamination, making them perfect for use as an \textit{in vitro} cell model.\textsuperscript{23} When left undifferentiated, the SH-SY5Y cell line is a great model in studying brain tumor cell growth and neuroblastoma.
1.2.3 Differentiation of SH-SY5Y Cells

The differentiation of SH-SY5Y cells has been observed using a variety of treatments: all-trans retinoic acid (RA)\textsuperscript{25,26}, phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)\textsuperscript{27}, brain-derived neurotrophic factor (BDNF)\textsuperscript{28}, and dibutyryl cyclic AMP (dBcAMP)\textsuperscript{29}. All-trans RA is the most commonly used differentiation treatment in SH-SY5Y studies.\textsuperscript{26} Upon differentiation, the cells stop proliferating, become a stable population, and show extensive neurite outgrowth with morphological similarity to living neurons in the human brain (Figure 4).\textsuperscript{30} The effect of all-trans RA causes morphologically changes as early as 48 hours after treatment.\textsuperscript{31}

When differentiating SH-SY5Y cells with all-trans RA, it binds to two classes of non-steroid nuclear hormone receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs).\textsuperscript{32-34} Although RA can bind only to the RARs, activated RAR heterodimerizes with RXR and the RAR/RXR heterodimers bind to RA response
element (RARE), resulting in transcriptional activation. RA induces differentiation through regulation of the transcription of neurotrophin receptor genes, the Wnt signaling pathway, and pathways involving type II protein kinase A (PKA).

Once the cells become differentiated they possess more biochemical, structural, morphological, and electrophysiological similarity to neurons. Additionally, once differentiated using all-trans RA, the SH-SY5Y cells express a variety of neuronal-specific markers, including norepinephrine, growth-associated protein (GAP-43), receptors for neurotrophic factors, neuropeptides, neurosecretory granula, neuron-specific enolase (NSE), neuronal nuclei (NeuN), vesicle proteins such as synaptophysin, and neuronal-specific cytoskeletal proteins, including microtube associated protein (MAP), Tau, and neurofilament proteins. MAP, GAP-43, NeuN, and synaptophysin are classical makers of mature neurons. These differentiated cells also contain higher levels of dopamine-β-hydroxylase, TH, and DAT activity, which are all indicators of functional neuron differentiation.

Figure 4: Fluorescent SH-SY5Y cells differentiated using all-trans RA at 20x. Differentiated cells display longer neurites.
1.2.4 IMR-32 Cells

IMR-32 is another human neuroblastomal heterogeneous cell lineage: containing both N and S-type cells in culture. The predominant N-cell is a small, highly light-refractile, fibroblast-like or teardrop-shaped, neuroblast-like cell, which grows densely and often forms focal accumulation (Figure 5).\textsuperscript{39} The S-type cell is considered minor, and is relatively large, well-spread, fibroblast-like cell. The S-type cells are only known to be observed in the residual cells remaining in flasks after subcultivation procedures.\textsuperscript{39} These cells have been shown to express nestin, polysialylated acid-neural cell adhesion molecule, and neural cell adhesion molecule. This cell line also has the capability of becoming differentiated into neuronal cells using specific neurotrophic growth factors.\textsuperscript{39} IMR-32 cells represent an \textit{in vitro} model for neuroblastoma as well as cancerous brain tumors.

Figure 5: Fluorescent undifferentiated IMR-32 cells at 20x. These cells are round, and have small, and short neurites.
The proliferative nature of these neuroblastoma cells poses an experimental problem *in vitro*, as they replicate too quickly to measure a total accurate effect of chemical manipulation in experiments that have long time frames. Subsequently, the newly formed cells that just underwent cell division might be biochemically different from the older cells in the cell culture, producing varied, or inaccurate results.

### 1.3 TIMPs and MMPs

Tissue inhibitors of metalloproteinases, otherwise known as TIMPs, are considered a family of small extracellular proteins, which inhibit the enzymatic activity of matrix metalloproteinases (MMPs). MMPs are implicated in the pathophysiology of many diseases, including Alzheimer’s and Parkinson’s disease, and are extracellular, zinc-dependent endopeptidases. TIMPs are generally known to block MMP-mediated proteolysis by noncovalently binding to the MMP active site in a 1:1 stoichiometric ratio. Homeostasis is achieved by a tight balance of MMP proteolysis to TIMP expression. Many diseases of the central nervous system (CNS), such as Alzheimer’s disease, ischemic brain injury, CNS infections, Parkinson’s disease, and multiple sclerosis (MS) might be due to a dysregulation of this balance. Most articles interpret the increase of MMP/TIMP protein levels as an increase in proteolytic activity, relating to disease pathology; yet, this interpretation does not take into account TIMP signaling functions, and how they can relate to mechanisms of tissue injury or repair.

This alternative view comes from two observations: (1) the identification of functional receptors that mediate downstream signaling via TIMPs, and (2) findings which indicate that TIMPs and MMPs can be coincidentally expressed in pathology,
and sometimes from the same cells. For example, when interleukin-1β, a proinflammatory cytokine, is applied to primary glial cultures there is a large induction of MMP-3, and its inhibitor TIMP-1. Increased production of both MMPs and TIMPs within the same cellular population would abolish the activities of MMPs; and if the MMP-independent actions of TIMPs are researched, these results could reflect counter-regulation of MMPs in TIMP signaling.

All TIMPs have two basic structural domains: an N-terminal domain consisting of six conserved cysteine residues forming three disulfide loops, which possess MMP-inhibitory activity, and a C-terminal domain that also contains six conserved cysteine residues and forms three disulfide loops. TIMPs inhibit MMPs through coordination of the zinc of the MMP active site by the amino and carbonyl groups of the TIMP N-terminal cysteine residue. TIMPs are regulated at the transcriptional level by various cytokines and growth factors, resulting in tissue-specific, constitutive, or inducible expression.

1.3.1 TIMP-1 and Growth

Many distinct signaling pathways have been implicated in TIMP growth-promoting activity, including the mitogen activated protein kinase (MAPK) and adenosine 3′,5′-monophosphate (cAMP)-protein kinase A (PKA) pathways. The growth and promoting activities of TIMP-1 and TIMP-2 may require activation of Ras, by distinct pathways suggesting independent receptor mechanisms. Growth-promoting activity was only observed in the presence of free TIMPs, independent of MMP-binding or MMP inhibition.
1.3.2 TIMP-1 and Anti-apoptosis

TIMP-1 expression inversely correlates with the susceptibility to induction of apoptosis in various human Burkitt’s lymphoma cell lines. Treatment with recombinant TIMP-1, or forced expression of TIMP-1 in a TIMP-negative cell, reduces susceptibility to induction of apoptosis and suppresses caspase-3 activity. As well, TIMP-1 enhances the expression of survival and differentiation cytokines, such as interleukin-10 (IL-10), that also contribute to the anti-apoptotic effect. In human breast epithelial cells in vitro TIMP-1 also inhibits apoptosis. Bcl-2 overexpression increased the abundance of TIMP-1 protein in breast epithelial cell lines (MCF10A and MCF7), and showed no effect on MMP or TIMP-2 expression; showing that the anti-apoptotic activity of TIMP-1 is independent of MMP inhibition.

In this anti-apoptotic model system TIMP-1 activates the focal adhesion kinase (FAK)-phosphoinositol-3 kinase (PI3K) pathway to protect cells from intrinsic and extrinsic cell death.

1.3.3 TIMP-1 and Binding

The high-affinity cell surface binding of TIMP-1 to myeloid leukemia cells and keratinocytes suggested the presence of cellular binding partners. Recently, CD63, a member of the tetraspanin family, was identified as a cell-binding partner for TIMP-1 in MCF10A human breast cancer cells. Confocal microscopy confirmed the colocalization of TIMP-1 with CD63 and the β1 integrin subunit. Downregulation of CD63 with shRNA resulted in reduced TIMP-1 binding, and cell apoptosis. Furthermore, independent investigations have demonstrated that CD63 regulates PI3K, FAK, Src, and
Akt signaling pathways that have been implicated in the anti-apoptotic activity of TIMP-1 (Figure 6).\textsuperscript{46}

Figure 6: The multiple pathways of TIMP-1-CD63 signaling. TIMP-1 or TIMP-4 interact with the CD63 receptor, inhibiting intrinsic and extrinsic cell death pathways though activation of the FAK-PI3K pathway. These effects are mediated by activation of the extracellular regulated kinase (ERK). TIMP-1 also inhibits cell growth by suppressing cyclin D1 and upregulating p27\textsuperscript{Kip1}. The cell cycle is arrested in the G1 phase via the hypophosphorylation of pRB.\textsuperscript{46}
1.3.4 TIMP-1 and Disease

In animal models of CNS diseases, TIMP-1 has been shown to play important roles in neuroprotection, neural plasticity, and tissue repair via trophic function (Figure 7). In a recent study it has been determined that TIMP-1 can directly stimulate the differentiation of oligodendrocytes from their precursor cells.\textsuperscript{53} Elevated TIMP-1 expression in the CNS can represent an endogenous signal for remyelination. Another study demonstrated that overexpression of TIMP-1 was shown to lessen clinical disease progression and to preserve CNS myelination in a transgenic model of spontaneous demyelination.\textsuperscript{54}

Figure 7: MMP regulation of TIMP-mediated signaling. The N-terminus of TIMPs regulate MMP proteolysis, while the C-terminus interacts with cell surface receptors to initiate intracellular signaling cascades.\textsuperscript{41}
1.3.5 TIMP-1 and Glial Cells

Additionally, TIMPs and MMPs play neurochemical roles within the brain involving glial cells. TIMP-1 has a restricted expression, and is only expressed in astrocyte-enriched cultures after stimulation by IL-1β or lipopolysaccharide (LPS). LPS promotes astrocyte TIMP-1 expression through an indirect mechanism, in which LPS induces microglia to secrete a soluble factor which then stimulates astrocyte TIMP-1 expression. The addition of microglia, or conditioned medium taken from LPS-activated microglia restored astrocyte TIMP-1 expression. This effect was lost after depletion of IL-1β from the conditioned medium. These results indicate the importance of microglial-astrocyte communication in neurochemistry. The mechanism of TIMPs, specifically TIMP-1, has yet to be understood, when in the presence of astrocytes and neuroblastoma cells, involved in cell death. Furthering our understanding on these interactions could help us grasp the pathology, or even a sort of treatment, for brain cancer.
2. MATERIALS AND METHODS

2.1 Materials

1:1 Dublecco’s modified Eagle’s medium nutrient mixture F-12 with 15 mM HEPES, pyridoxine and NaHCO₃, without L-glutamine, PGS (Pen-Strep Glutamine solution containing 200 mM L-glutamine, 10,000 units penicillin g/mL, 10 mg streptomycin/mL in normal saline), all-trans retinoic acid and Hank’s Balanced Salt Solution (HBSS) were purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum was purchased from Atlanta Biologics (Lawrenceville, GA) and the Live Cell/Dead Cell Assay, containing calcein AM and ethidium, was from Invitrogen (Carlsbad, CA). Recombinant mouse TIMP-1 was obtained from R & D Systems (Minneapolis, MN). Eagle’s Minimum Essential Medium (EMEM) was obtained from ATCC (Manassas, VA). Wild type and knockout for TIMP-1 astrocytic media (WT-GCM and KO-GCM) was obtained from Dr. Stephen Crocker’s lab, including Kasey Johnson and Kumiko Ijichi at the University of Connecticut Health Center.

A stock solution of recombinant TIMP-1 was prepared by diluting the original TIMP-1 solution with 50 mM of Tris buffer, 10 mM CaCl₂, 150 mM NaCl, and 0.05% Brij-35 in deionized water to create a concentration of 100 μg/mL. The solution was sterile filtered prior to use in experiment 2. The diluted recombinant TIMP-1 was added to the wild type glial cell media, as well as the knockout glial cell media in order to raise the TIMP-1 concentration.
2.2 Cell Cultures

Human neuroblastoma SH-SY5Y cells and IMR-32 cells were purchased from ATCC (Manassas, VA). The SH-SY5Y cells were grown in 1:1 DMEM F-12 with 10% fetal bovine serum (FBS) and 1% PGS. The IMR-32 cells were grown in 1:1 Eagle’s Minimum Essential Medium (EMEM) and 10% FBS, and both of these cell cultures were grown in 75 cm² flasks and kept in an incubator at 37°C with 5% CO₂. When the cells had reached 70-80% confluence they were detached from the flask surface using trypsin and split into more flasks, or seeded on plates for use in experiments.

For experiments, cell cultures were seeded on 24-well plates (Nunc), which were coated with Nunclon Delta Surface. Plating density was kept between 1 and 2x10⁵ cells/well, which was determined by using a hemocytometer (hemocytometer 517040, Fisher Scientific, Pittsburgh, PA).

2.3 Treatment

Every two days (or 48 hours) after plating onto 24-well plates, half of the feeding media was replaced with low serum feeding media (LSFM); which consists of DMEM F-12, or EMEM for the IMR-32 cells, with 2% FBS and 1% PGS. In experiments where differentiation was induced, retinoic acid was added to the LSFM to give a concentration of 10 μM in the wells. Cells were monitored at each feeding to evaluate morphological changes associated with differentiation; and differentiation was defined as a morphological change consisting of neurite outgrowth and extension of the cell body.

For each experimental treatment the feeding media was completely removed from each well, the cells were rinsed with 400 μL of HBSS and the treatment solution for that
experiment was then applied to each well. 24 hours later cell viability was analyzed.

In addition, for the denatured media experiment (Experiment 3), the wild type glial cell media was held in boiling water for 10 minutes, with a recorded temperature of 43°C. The media changed to a slightly lighter shade of red.

2.4 Determination of Cell Viability

The cell viability assay used for analysis was the live cell/dead cell assay. The assay was then put on ice for half an hour along with HBSS. The cells were rinsed with ice-cold HBSS, and then 400 μL of the ice-cold assay solution was added to each well. The plate was covered and left in the dark, at room temperature, for 30 minutes. The cells were observed using a fluorescence microscope (Nikon eclipse TE2000-U) and through a color mosaic camera (Diagnostic Instruments Inc.) at 20x magnification. Pictures taken were observed through SPOT Advanced software (SPOT Imaging Solutions, Sterling Heights, MI). Three arbitrary fields per well were photographed using two fluorescent colors, green (live cells) and red (dead cells).

2.5 Statistics

The number of total live cells and dead cells per each field in each well were counted using Adobe Photoshop.

The data represents results from four experiments that are presented as mean values ± SEM. Statistical differences between various treatment groups were conducted using GraphPad, using a Student’s t-test.
3. RESULTS

3.1 SH-SY5Y Cell Treatment with Glial Cell Media

Treatment of undifferentiated SH-SY5Y cells with WT-GCM for 24 hours significantly increased cell death over DMEM controls as seen in Figure 8. There was no change over DMEM controls on cell death when SH-SY5Y cells were treated with KO-GCM. Overall, the wild type glial cell media increased average cell death to 15% compared to 8% for the DMEM controls and 6% for KO-GCM (Figure 8).

![Figure 8: Undifferentiated SH-SY5Y cell viability when treated with wild type or knockout for TIMP-1 glial cell media for 24 hours. DMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s t test: ** p <0.005. (n=35 DMEM pits; 33 WT GCM pits; 17 KO GCM pits)](image-url)
This increased cell death is shown in Figures 9 A-C, fluorescent pictures of the cells treated with DMEM, wild type glial cell media, and knockout glial cell media. The undifferentiated cells that were treated with the WT-GCM visually show much more clumping, compared to those treated with DMEM (Figure 9 B). On the other hand, the KO-GCM showed no significant difference of cell viability compared to that of the control, (Figures 8 and 9 C) suggesting that TIMP-1 plays a role in the apoptotic affect demonstrated by the wild type glial cell media on the undifferentiated SH-SY5Y cells.
Figure 9: Fluorescent pictures of undifferentiated SH-SY5Y cells, at 20x, treated with (A) DMEM; (B) wild type glial cell media; and (C) knockout glial cell media for 24 hours. Cells are stained using the live cell/dead cell assay. Cells stained green are live, and cells stained red are dead.

In the same set of experiments the reactions of differentiated SH-SY5Y cells towards the two glial medias (WT-GCM and KO-GCM) were investigated. There were no significant changes seen in viability over DMEM controls when the cells were given the glial medias for 24 hours as seen in Figure 10. The average cell death was much higher overall, including the DMEM treatment group, as well as the glial cell media treatments, compared to the undifferentiated cells (Figure 10).
Figure 10: Differentiated SH-SY5Y cell viability when treated with wild type or knockout for TIMP-1 glial cell media for 24 hours. DMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. (n= 26 DMEM pits; 14 WT GCM pits; 18 KO GCM pits)

Overall, these results suggest that a compound being secreted by the wild type astrocytes is having a selective effect on the SH-SY5Y cells, only inducing undifferentiated SH-SY5Y cell death, as the differentiated cells were not affected by the WT-GCM (Figure 10). Interestingly, the KO-GCM did not have the same effect on the undifferentiated, and even the differentiated cells, implying that the release of an astrocytic factor into the extracellular media depends on the presence of TIMP-1 within the astrocyte.

These results are strikingly odd, as TIMP-1 is known to have anti-apoptotic activities by suppressing caspase-3 activity, and even enhancing the expression of survival and differentiation cytokines. These experiments, which demonstrate the effect of TIMP-1 on cell survival, have only been done with differentiated types of cells,
and not undifferentiated neuroblastoma cells. The results provided instead show an apoptotic effect, due to the presence of TIMP-1 in the astrocyte, on cancerous neuroblastoma cells.

3.2 Increasing TIMP-1 in Wild Type and Knockout Glial Cell Media

In order to determine the role of TIMP-1 within this apoptotic mechanism recombinant mouse TIMP-1 (reTIMP-1) was added to the knockout glial cell media, as well as the wild type glial cell media in order to raise the concentration to supraphysiological levels. The reTIMP-1 was directly added to the two medias in order to determine if the astrocytes are releasing a factor due to the presence of TIMP-1, or if TIMP-1 is acting directly upon the undifferentiated SH-SY5Y cells to induce death.

The effect of increased levels of TIMP-1 on the undifferentiated SH-SY5Y cells was investigated by adding reTIMP-1 to both glial cell medias in order to increase the total concentrations. Assuming that the WT-GCM already contains an approximate concentration of 10 ng/mL of TIMP-1, the total concentrations were 20 ng/mL and 35 ng/mL of TIMP-1 in WT-GCM. Increasing levels of TIMP-1 in the WT-GCM for 24 hours significantly reduced undifferentiated SH-SY5Y cell death compared to the WT-GCM with no extra reTIMP-1 (Figure 11). Additionally, the increased level of TIMP-1 in the WT-GCM, significantly reduced cell death over DMEM controls.
Figure 11: Undifferentiated SH-SY5Y cell viability when treated with wild type glial cell media with supraphysiological levels of TIMP-1 for 24 hours. Wild type glial cell media has on average 10 ng/mL of TIMP-1. DMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s \( t \) test: ** \( p <0.005; *** p <0.001 \). (n=23 DMEM pits; 33 WT GCM pits; 20 WT GCM + 10 ng/mL T1 pits; 20 WT GCM + 25 ng/mL T1 pits)
Figure 12: Fluorescent picture of undifferentiated SH-SY5Y cells, at 20x, treated with wild type glial cell media plus an additional (A) 10 ng/mL of TIMP-1 and (B) 25 ng/mL for 24 hours. Cells are stained using the live cell/dead cell assay. Cells stained green are live, and cells stained red are dead.

This attenuating affect, presented by the addition of recombinant TIMP-1, can visually be seen in comparing both Figure 12 A and B to Figure 9 B, where much more cell death is seen. Additionally, when the undifferentiated cells were treated with the WT-GCM with the additional recombinant TIMP-1, the cells were not clumpy compared to when treated with just the WT-GCM that had no recombinant TIMP-1.
Adding reTIMP-1 to the KO-GCM provided no significant changes in cell death in comparison to the KO-GCM with no additional reTIMP-1, and to the DMEM control (Figure 13). Adding extra reTIMP-1 to the knockout glial media slightly reduced cell death compared to the DMEM control from 8% to 5%.

Figure 13: Undifferentiated SH-SY5Y cell viability when treated with knockout glial cell media with supraphysiological levels of TIMP-1 for 24 hours. Knock out glial media has 0 ng/mL of TIMP-1. DMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. (n=23 DMEM pits; 20 KO GCM pits; 10 KO GCM + 10 ng/mL T1 pits; 10 KO GCM + 25 ng/mL T1 pits)
Figure 14: Fluorescent picture of undifferentiated SH-SY5Y cells, at 20x, treated with knockout glial cell media plus an additional (A) 10 ng/mL of TIMP-1 and (B) 25 ng/mL for 24 hours. Cells are stained using the live cell/dead cell assay. Cells stained green are live, and cells stained red are dead.

When the undifferentiated SH-SY5Y cells were treated with KO-GCM, with the additional recombinant TIMP-1, there was no morphological effect seen, when compared to the DMEM control and the KO-GCM without the addition of reTIMP-1 (Figure 14 A and B).
Figure 15: Undifferentiated SH-SY5Y cell viability when treated with wild type glial cell media and knockout glial cell media with supraphysiological levels of TIMP-1 for 24 hours. Knockout glial media has 0 ng/mL of TIMP-1. DMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s t test: ** p <0.005

In addition, the knockout glial cell media with a concentration of 10 ng/mL of TIMP-1, identical to that of the wild type glial cell media, showed no apoptotic effect towards the undifferentiated SH-SY5Y cells, verifying the apoptotic factor as astrocyte-secreted. Figure 15 shows that there is a significant difference in average percent cell death when comparing the cells treated with the WT-GCM, and then those treated with the KO-GCM with the same concentration of TIMP-1. This indicates that TIMP-1 is not acting alone, and the apoptotic factor, affecting the undifferentiated cells, is being secreted by astrocytes.
3.3 Undifferentiated SH-SY5Y Cell Treatment with Heated/Denatured Wild Type Glial Cell Media

Since it was established that wild type glial cell media induces apoptosis in undifferentiated SH-SY5Y cells via an astrocytic apoptotic factor, the identity of the factor, and the mechanism needs to be elucidated. Based on the assumption that the apoptotic factor is a protein, it will become denatured and inactive when boiled, inhibiting the apoptotic effect on the undifferentiated cells.

When the WT-GCM was heated to 43°C the cell death was significantly reduced compared to non-heated WT-GCM (Figure 16). These results indicate that this apoptotic factor is present within the WT-GCM, and can become easily nonactive when heated, suggesting that it is a protein or an enzyme. Additionally, the heated wild type glial cell media lowered undifferentiated cell death slightly in comparison to the knockout glial cell media.
Figure 16: Undifferentiated SH-SY5Y cell viability when treated with denatured wild type glial cell media for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s t test: ** p <0.005; *** p <0.001. (n=23 DMEM pits; 20 KO GCM pits; 30 WT GCM pits; 33 denatured WT GCM pits)
As seen in Figure 17, a fluorescent picture of undifferentiated cells treated with denatured/heated WT-GCM, one can see the difference compared to Figure 9 B, a picture of cells treated with just the WT-GCM. There is a difference in the amount of dead cells compared to live ones when they are treated with the denatured/heated media, compared to the regular wild type glial cell media, and much less cell clumping.

3.4 Undifferentiated IMR-32 Cell Treatment with Glial Media

In order to determine if this apoptotic reaction is just seen in undifferentiated SH-SY5Y cells, another type of neuroblastoma cell, called IMR-32, underwent the same glial cell media treatment. These cells showed the same morphological, and are known to have the same biochemical characteristics as SH-SY5Y cells.
Figure 18 shows that the undifferentiated IMR-32 cell death significantly increased when treated with the WT-GCM over the EMEM control, mirroring the results seen in Figure 8 with the SH-SY5Y cells. The KO-GCM, again, did not affect the undifferentiated IMR-32 cells.

![Figure 18: Undifferentiated IMR-32 cell viability when treated with wild type or knockout for TIMP-1 glial cell media for 24 hours. EMEM was used as a control, and was also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s t test: ** p <0.005. (n=72 EMEM pits; 36 WT GCM pits; 36 KO pits)](chart.png)
Figure 19: Fluorescent pictures of undifferentiated IMR-32 cells, at 20x, treated with (A) EMEM and (B) wild type glial cell media for 24 hours. Cells are stained using the live cell/dead cell assay. Cells stained green are live, and cells stained red are dead.

Figure 19 A and B demonstrate the increased undifferentiated IMR-32 cell death that occurs when the cells are treated with WT-GCM for 24 hours compared to the EMEM control. These results are not surprising, as these neuroblastoma cells have many identical characters compared to the SH-SY5Y cells that also demonstrate this reaction in the presence of wild type glial cell media.

Figure 20 summarizes the major findings of this research project, showing that the WT-GCM is inducing an apoptotic effect on both undifferentiated SH-SY5Y and IMR-32 neuroblastoma cells. These results also confirm that TIMP-1 needs to be expressed within the astrocyte in order for the apoptotic factor to be released, as the KO-GCM showed no effect on the undifferentiated cells.
It can therefore be concluded from these results that wild type astrocytes, expressing TIMP-1, are releasing an apoptotic factor that induces apoptosis in only undifferentiated SH-SY5Y and IMR-32 neuroblastoma cells. The action of the apoptotic factor can be inhibited by denaturing (boiling the media), suggesting the factor is a protein or enzyme. Additionally, the supplementation of reTIMP-1 to the WT-GCM can completely alleviate undifferentiated cell death, demonstrating the anti-apoptotic and pro-survival qualities of TIMP-1.

Figure 20: Undifferentiated SH-SY5Y and IMR-32 cell viability when treated with wild type or knockout for TIMP-1 glial cell media for 24 hours. Solid colored bars represent SH-SY5Y cells and striped bars represent IMR-32 cells. DMEM and EMEM were used as a control, and were also given to the cells for 24 hours. Height of the columns indicates percentage of dead cells; error bars represent SEM. Student’s t test: ** p <0.005.
4. DISCUSSION

The wild type glial cells are releasing an apoptotic factor that is only inducing apoptosis in undifferentiated neuroblastoma cells. The factor is believed to be an enzyme or protein, that is temperature-dependent, and has the capability of binding to receptors on the outside of the cell, or even entering the cell inducing an apoptotic pathway.

The astrocytes, only when in the presence of TIMP-1, are releasing a factor that induces stress on the undifferentiated cells. The WT-GCM is only invoking stress on the undifferentiated cells, demonstrating their biochemical differences compared to the differentiated cells. Since the undifferentiated cells have not morphologically and biochemically chosen their cellular path, they have many different receptors that are sensitive towards specific chemicals, making them susceptible to chemical responses.25

On the other hand the differentiated dopaminergic SH-SY5Y cells show completely different biochemical and morphological properties, which is why they might not demonstrate the same effect towards the WT-GCM.25 The results demonstrate that the excess recombinant TIMP-1, when added directly to the WT-GCM, inhibits the apoptotic effect seen in the undifferentiated cells. One theory might be that the wild type astrocytes only release an apoptotic factor, which affects only undifferentiated cells, and this factor can be inhibited by excessive TIMP-1 when it is added directly to the WT-GCM. The apoptotic factor could possibly have the same structure and biochemical properties as an MMP, making it a great candidate to be blocked by the additional recombinant TIMP-1 in the media. The apoptotic factor binds to the N-terminus of TIMP-1, causing inhibition of the factor. This therefore prevents the apoptotic factor
from binding to a receptor on the undifferentiated cells, or even entering the cell, inhibiting cell death.

Undifferentiated SH-SY5Y cell death is reduced when additional reTIMP-1 is added to the WT-GCM, even when compared to the DMEM control. The additional reTIMP-1 within the WT-GCM may be acting as an anti-apoptotic factor by suppressing the normal activity of caspases and increasing survival cytokines, causing the DMEM control wells to have higher cell death compared to wells with additional TIMP-1. These results pose a paradox for the action TIMP-1: when available at normal levels in astrocytes it causes a release of an apoptotic factor, but when in excess it acts as an anti-apoptotic agent.

Tumor protein 53, also known as p53, is commonly known to cause apoptosis in cells that have undergone major stressors, such as DNA damage induced by UV, IR, and chemical agents, oxidative stress, osmotic shock and ribonucleotide depletion. Within the undifferentiated cells p53 might become activated due to stress, brought upon by the WT-GCM. p53 activation causes the upregulation of three multidomain proapoptotic proteins: Puma, Noxa, and Bax. All three of these proteins, when upregulated, disrupt the outer mitochondrial membrane causing the activation of cytochrome c, inducing apoptosis. When the excess reTIMP-1 was added to the WT-GCM it could bind to the stressor inducing this apoptotic reaction, preventing p53 from becoming activated within the cell, inhibiting the apoptotic effect. In addition, the recombinant TIMP-1 could also bind with p53 inhibiting the apoptotic cascade that it induces. With this effect, the TIMP-1 acts as an anti-apoptotic factor by binding with p53, but it also has the capability of enhancing pro-survival cytokines, which might account for the alleviated undifferentiated cell death.
The CD63 receptor, which is known to be located on cancer cells, binds with TIMP-1 and inhibits intrinsic and extrinsic cell death pathways through activation of the FAK-PI3K pathway. The apoptotic factor released by astrocytes could have the ability to bind to the CD63 receptor and block its action, preventing TIMP-1 from binding, and initiating this pathway. When the reTIMP-1 is added to the media it could knock out this factor, and then activate this pro-survival pathway, which is unwanted in cancerous cells. When there is no TIMP-1 available, such as in the KO-GCM, the apoptotic factor is not released, so this effect is void.

Another theory comes from new research on the TRAIL (TNF-related apoptosis-inducing ligand) death receptor 5, which has been only found in various cancer cells. TRAIL binds to DR4 and DR5 receptors, which both activate apoptosis through intrinsic factors. Once TRAIL binds the Fas-associated death domain is activated (FADD). FADD recruits initiator caspase-8 through its N-terminal death-effector domain, which then form death-inducing signaling complex (DISC). This pathway induces apoptosis via caspase activation.

The apoptotic factor being released by the wild type astrocytes and is in the WT-GCM could closely resemble a TRAIL protein; therefore it could act as a ligand to death receptors located on the undifferentiated cells. When reTIMP-1 is added to the WT-GCM, it could block this apoptotic factor from binding to the DR4 or DR5 receptors, attenuating cell death. This new research shows promising results, as the death receptor is only located on cancer cells, allowing us to just target unwanted cells within the body.
In order to further investigate these mechanisms proposed the activation of p53 could be blocked via chemical alterations, the CD63 receptor could be knocked out, and death receptors could be blocked. Using these proposed methods one can narrow down the apoptotic pathway seen in the undifferentiated cells treated with the wild type glial cell media. Once this is done, this mechanism can be taken advantage of in order to only selectively target apoptosis in cancerous cells.
REFERENCES


