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Analysis of the Effect of Substrate Stiffness on the Efficacy of Fibroblast Growth Factor 2 and Bone Morphogenetic Protein 4 in Inducing Pro-Regenerative Astrocyte Phenotype: A Research Protocol

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Abstract

Introduction: Astrocytes are glial cells essential for neuronal development and repair and are thus a highly promising target for regenerative therapies for neurodegenerative disease. Neurodegeneration has been connected to the degradation of extracellular matrix (ECM) components. ECM's structural properties, such as stiffness, influence the phenotypic outcomes of growing cells. Notably, astrocytes are induced into an A2 (pro-regenerative) phenotype when grown on stiff substrates or exposed to specific signalling molecules, particularly fibroblast growth factor 2 (FGF-2) and bone morphogenetic protein 4 (BMP-4). Given that the ECM can bind and sequester growth factors, it is possible that matrix stiffness may modulate the efficacy of these molecules.

Methods: In this *in vitro* study, rat primary cortical astrocytes will be cultured on soft and stiff substrates and exposed to varying amounts of FGF-2 and BMP-4. This study aims to determine the effect of substrate stiffness on the efficacy of FGF-2 and BMP-4 in promoting pro-regenerative phenotype. Cell proliferation and glial fibrillary acidic protein (GFAP) expression are key indicators of reactive astrocytes, including pro-regenerative astrocytes. 5-bromo-2-deoxyuridine staining will be used to analyze proliferation. GFAP expression will be determined using anti-GFAP antibody conjugated with Alexa Fluor 594. Further, pro-regenerative phenotypic genes Clcf1, Tgm2, and Ptgs2 will be detected via polymerase chain reaction to differentiate from pro-inflammatory astrocytes, a separate category of reactive astrocytes.

Anticipated Results: We hypothesize there will be a greater positive correlation between the concentration of FGF-2 or BMP-4 and expression of markers of pro-regenerative phenotype under stiff substrate conditions, compared to softer substrate, thus indicating dependency or synergy between FGF-2 or BMP-4 and extracellular matrix-dependent pathways.

Discussion: The correlation between FGF-2 or BMP-4 concentration and the prominence of A2 astrocyte phenotype indicators will be graphed and reported along with a comparison of these correlations between soft and stiff substrate groups. The authors will attempt to conclude whether substrate stiffness significantly effects the activities of FGF-2 or BMP-4.

Conclusion: We hope the results of this proposed study will inform the development of neuroregenerative therapies involving astrocytes by indicating the necessity for greater focus on either the application of signalling molecules or modification of ECM.

Keywords: astrocytes; neurodegeneration; mechanotransduction; regenerative therapy; bone morphogenetic protein 4; fibroblast growth factor 2; extracellular matrix

Introduction

In recent years, neurodegeneration has been linked with the degradation and dysregulation of components of the extracellular matrix (ECM), leading to cell loss and the aggravation of diseases such as Alzheimer's disease, caused by an excessive buildup of proteins resulting in neuron death [1]. Glial cells are structural and functional support cells found in the central nervous system which are highly involved in ECM development [2,3], and are reciprocally influenced by the ECM [4]. Accordingly, glial mechanotransduction studies investigating the influence of substrate stiffness and topography, two important physical properties of ECM, are helping to advance pharmacotherapy for neurodegenerative disease [5].

Astrocytes are glial cells heavily involved in neuronal development [6], repair, and disease [7]. They are considered the most important cells for brain homeostasis [8]. As such, astrocytes are an intensely studied subject for

regenerative therapies for neurodegenerative disease [9]. Modern therapies, in contrast, typically only address disease symptoms or attempt to slow the disease progression [10]. We lack effective tools to regenerate cells or reverse the damage caused by neurodegenerative disease [10]. This is of concern considering that the burden of neurological disorders has only increased, especially with the growth in the aging population [11]. Research into regenerative therapy is urgently needed for the development of a true cure for neurodegenerative disease.

Astrocytes are induced into an A2 (pro-regenerative) phenotype when grown on stiff substrates [12]. This phenotype is characterized by increased cell proliferation, deposition of ECM [12], and support to neural circuitry via synaptic repair and axon growth [7,13]. Importantly, neurodegeneration is generally associated with a decrease in matrix stiffness, however, an abnormally stiff matrix is also indicative of a diseased state [14]. Nonetheless, A2 phenotype astrocytes may be neuroprotective particularly in diseased states involving an abnormal decrease in matrix stiffness. Various signalling molecules, including fibroblast growth factor 2 (FGF-2) and bone morphogenetic protein 4 (BMP-4), similarly induce a pro-regenerative phenotype in astrocytes [12]. FGF-2 has been shown to aid glial bridge formation in mice and zebrafish and knocking out an FGF signaling inhibitor, SPRY4, decreases gliosis after spinal cord injuries [12]. Additionally, the use of FGF-2 and BMP-4 to differentiate glial-restricted progenitors has been associated with improved recovery from spinal cord injuries [12].

Beyond the fact that the ECM can bind and sequester growth factors [15], it is not known whether the functions of the signalling molecules FGF-2 and BMP-4 are dependent upon mechanotransduction pathways or ECM structure. To evaluate the potential *in vivo* efficacy of these signalling molecules as neuroregenerative therapeutics, it is important to study the effects of varied matrix stiffnesses on the molecules' activity. This is especially important given that ECM remodelling and degradation occur continually in neurodegenerative conditions [1]. This research could help develop better methods of culturing stem cells or astrocytes, or lead to the research of future therapeutics that change the ECM of patients, with the effect of promoting regeneration. Ultimately, the goal of this protocol is to help open a new avenue of research into combatting neurodegenerative disease.

In this proposed *in vitro* study, rat primary cortical astrocytes [16,17] will be cultured on soft and stiff substrates to simulate soft and stiff ECM, respectively. The soft and stiff substrate groups will each consist of seven subgroups: one control subgroup and three subgroups for each of the signalling molecules FGF-2 and BMP-4. The purpose of the multiple subgroups for FGF-2 and BMP-4 signalling molecules is to investigate the effect of different concentrations of the molecule on cell phenotype and proliferation. Experiments will be performed in triplicate (i.e., three cultures for each subgroup) to account for potential anomalies.

We hypothesize greater concentrations of FGF-2 and BMP-4 will lead to higher expressions of markers of pro-regenerative phenotype and that this effect will be more pronounced on stiff substrate conditions compared to softer substrate conditions. That is to say, a given increase of FGF-2 or BMP-4 concentration would result in a greater increase in markers of pro-regenerative phenotype on stiff substrate conditions compared to softer substrate. This observation would imply synergism or dependency of the signalling molecules on increased activation of ECM-dependent pathways, such as mechanotransduction or binding of the molecules by the ECM. The results of our study will help inform the possibility of achieving a pro-regenerative astrocyte phenotype by simply increasing the concentration of pro-regenerative molecules, even in the presence of an ECM whose properties are not conducive to a pro-regenerative phenotype.

Methods

Based on the work of Wilson et al., primary rat cortical astrocytes will be cultured on poly-L-lysine (PLL)-coated CytoSoft® 6-well plates of 200 Pa and 8000 Pa to simulate soft and stiff matrices respectively [16]. These plates are available from Advanced BioMatrix, and testing may be done by the company to verify plate qualities [16]. Cortical tissue will be taken from 1-day-old Sprague-Dawley rats [17]. Cells will be enzymatically dissociated from tissue using 0.25% Trypsin–EDTA (Life Technology) and 0.016% DNase (Roche) and then maintained in Dulbecco's Modified Eagle's culture medium (DMEM) [16,17]. DMEM may be obtained from MP Biomedicals, and culture media will contain 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin–streptomycin (Life Technology) [17]. Trypsin will be removed by centrifugation at 1700 rpm for 5 minutes [16]. Cell pellets resulting from centrifugation will be resuspended and homogenized, passed through a 70 μm cell filter, repelleted, and then resuspended prior to seeding onto a tissue culture Petri dish [16]. Neurons and microglia loosely attached to the Petri dish will be separated and removed from astrocytes by vigorously shaking the Petri dish and subsequently replacing the growth media [16]. Refer to the methodology in the work of Wilson et al. for further details [16]. This process of vigorous shaking and subsequent media change will be repeated every 3 days, over the course of approximately 10 days, until cells reach confluence [17]. The cells will then be again trypsinized and cultured with DMEM prior to seeding onto a tissue culture dish [16,17]. Once cell cultures become confluent, they will be seeded onto the PLL-coated well plates of either 200 Pa or 8000 Pa stiffness [16].

Cell cultures in both the soft and stiff matrix groups will be exposed to 5 ng/mL, 10 ng/mL, or 20 ng/mL of either FGF-2 (Cell Guidance Systems) or BMP-4 (Cell

Guidance Systems) for 7 days, forming the six experimental subgroups in both the soft and stiff matrix groups [18]. Both FGF-2 and BMP-4 will be reconstituted in sterile water as per manufacturer instructions. The control subgroup in the soft and stiff matrix groups will not receive either molecule and will only receive an equal volume of sterile water. The molecule concentrations and duration of exposure are based on the work of Davies et al., who used 10 ng/mL of both FGF-2 and BMP-4 to induce the differentiation of glial cell precursors into astrocytes exhibiting regenerative qualities [18,19]. The additional concentrations not used by Davies et al. will allow us to examine the power of the molecules by analyzing the impacts of halving and doubling the concentration. It may help to better understand minimum concentrations needed for effects or to determine the point of diminishing returns. Initial cell density, growth media, and feeding schedule will be kept consistent between all cell groups [18,19].

Cell proliferation and expression of glial fibrillary acidic protein (GFAP) are hallmarks of reactive astrocytes [16]. Proliferation will be measured through cell staining with 5-bromo-2-deoxyuridine (BrdU) and subsequent addition of Alexa Fluor 488 (both available from Life Technologies) conjugated to an anti-BrdU antibody for quantification of fluorescence [16]. GFAP expression will be similarly quantified using an anti-GFAP antibody conjugated to Alexa Fluor 594 [18]. To evaluate the prominence of A2 pro-regenerative reactive astrocytes specifically, as opposed to A1 pro-inflammatory reactive astrocytes [12], gene expression will be analyzed [17]. Polymerase chain reaction and subsequent spectrophotometry will be used to measure A2-like genes (Clcf1, Tgm2, and Ptgs2) and A1-like genes (Fbln5, Serping1, and Rt1-S3) [17]. Primer sequences will be obtained from Integrated DNA Technologies.

Data for cell proliferation, GFAP expression, and A1- and A2-like gene expression will be analyzed for significance using a threshold p-value of 0.05. An analysis of covariance test will be performed to compare the linear regressions of soft and stiff substrate data sets. These linear regressions will be obtained by plotting the aforementioned dependent variables (Y-axis) against the concentration of FGF-2 or BMP-4 (X-axis). Results of the analysis of covariance test will reveal the effect of substrate stiffness on the expression of pro-regenerative phenotypic markers (i.e., a comparison of Y-intercepts), as well as the effect of substrate stiffness on the change in the quantity of these markers per dose of FGF-2 or BMP-4 (i.e., a comparison of slopes).

Results

The protocol set forth is not being undertaken by the authors at this time. Rather, it is meant to serve as an inspiration and guide for future research.

Discussion

For both the soft and stiff substrate experimental groups, the correlation between FGF-2 or BMP-4 concentration and the prominence of A2 astrocyte phenotype indicators will be graphed and reported along with significance. Further, a comparison of these correlations between soft and stiff substrate groups will be illustrated graphically and reported with significance to highlight the presence or absence of an interaction between substrate stiffness and growth factor efficacy. Critically, the authors will attempt to conclude whether the hypothetical effect of substrate stiffness is of significance to the activities of FGF-2 or BMP-4 in the context of testing these molecules in a lab setting or clinically.

This research will hopefully contribute to the development of therapeutics for patients with neurodegenerative diseases. For example, by learning more about the role of ECM stiffness on pro-regenerative astrocyte phenotypes, astrocyte culturing could become more effective. Therapeutics designed to change ECM stiffness could also be developed. The research found in this protocol could open a new angle from which to approach treating neurodegenerative disease.

A limitation of this protocol is that it only addresses disease states in which the ECM is degraded, and stiffness is reduced. However, it is also possible for various components of the ECM to be upregulated, leading to disease associated with abnormally increased brain stiffness [14]. Further, the observation of the A2 pro-regenerative phenotype in astrocytes in culture does not necessarily confirm a significant regenerative function in vivo. Future research should investigate effects of the differentiated human astrocytes in co-cultures with neurons and other cells to confirm or deny inducible regenerative capabilities of astrocytes. The effect of astrocytes on ECM composition should also be investigated by developing tissue models with representative brain ECM and cellular microenvironments, as this data may reveal not only the effect of substrate and signalling molecules on astrocyte phenotype, but also the reciprocal effect of astrocyte phenotype on ECM properties [4].

Conclusions

Our proposed study aims to reveal the potential dependency of pro-regenerative astrocyte-inducing molecules FGF-2 and BMP-4 on ECM stiffness. The results inform future researchers of the possible necessity of targeted modification of ECM stiffness *in vivo* in conjunction with applying signalling molecules as a potential regenerative therapy for neurodegenerative disease. Alternatively, neural progenitor cells may be cultured on stiff matrices and implanted into the central nervous system to promote regeneration [12,19]. However, if FGF-2 and BMP-4 activity is found to be largely independent of matrix stiffness, research efforts may instead focus on the administration of growth factors like FGF-2 and BMP-4 as regenerative

therapies, which may reveal simpler, safer, and less invasive approaches to neuroregeneration than cell implantation or large-scale alteration of brain matrix properties. We hope the methodology and discussion presented herein inspires interdisciplinary approaches to regenerative therapy which consider the roles of glial support cells and mechanical properties as fundamental influencers of brain functioning.

List of Abbreviations Used
BMP-4: bone morphogenetic protein 4
BrdU: 5-bromo-2-deoxyuridine
DMEM: Dulbecco's modified Eagle's culture medium
ECM: extracellular matrix
FGF-2: fibroblast growth factor 2
GFAP: glial fibrillary acidic protein
PLL: poly-L-lysine

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent
This protocol did not require an ethics approval as it is a research proposal and is not being undertaken at this time.

Authors' Contributions
RK: made substantial contributions to the conception and design of this work, drafted and revised the manuscript critically, and gave approval of the final draft.
NS: contributed to the conception of this work, revised the manuscript critically, edited the manuscript throughout the peer-review process, and gave approval of the final draft.
RP: contributed to the conception of this work, revised the manuscript, and gave approval of the final draft.

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