Annals of Orthopedics and Musculoskeletal Disorders

Research Article
Published: 15 Jun, 2018

Glial Cell-Derived Neurotropic Factor: Constitutive Expression in the Human Disc and up Regulation *in vitro* by Proinflammatory Cytokines

Helen E Gruber*, Brittany Jones, Emilio Marrero and Edward N Hanley

Department of Orthopedic Surgery, Carolinas Health Care System, USA

Abstract

The relationship between neurotrophins, nerves and human disc cells is still incompletely understood. GDNF has high relevance to the disc not only because past work has shown that GDNF supports survival and extension of neurites, but also because human annulus cells co-cultured with nerve cells produce GDNF. In this IRB-approved research study immune histochemistry was carried out to localize GDNF in human disc tissue and the proportion of positive cells was determined. GDNF gene expression was analyzed for human annulus cells were exposed to IL-1ß or TNF-α, and human annulus cells co-cultured with F11 nerve cells. Exogenous rhGDNF was also added to cultured annulus cells to test for a mitogenic response. Immune histochemistry verified GDNF presence in most annulus cells; the percentage of positive cells decreased significantly with increasing disc grade. In vitro production of GDNF by 3D cultured annulus cells was shown, with a significant increase in expression following exposure to IL-1ß or TNF-α ($p = 0.007$ and 0.004, respectively), and following co-culture with F11 nerve cells ($p = 0.04$). Rh GDNF (10 or 100 ng/ml) did not induce a mitogenic response. Novel findings presented here showed GDNF expression/production by annulus cells, and significant increase in its expression following exposure to IL-1ß or TNF-α, or following co-culture with F11 nerve cells. Because of its influence on nerve survival and neurite branching, findings have relevance to nerve in growth and pain in the outer annulus (where cell numbers are high), and in regions where nerves penetrate the disc via annular tears.

OPEN ACCESS

Correspondence:
Helen E Gruber, Department of Orthopedic Research and Biology, Carolinas Medical Center, USA, E-mail: helen.gruber@carolinashealthcare.org

Received Date: *01 May 2018*
Accepted Date: *11 Jun 2018*
Published Date: *15 Jun 2018*

Citation:
Gruber HE, Jones B, Marrero E, Hanley EN. Glial Cell-Derived Neurotropic Factor: Constitutive Expression in the Human Disc and up Regulation in vitro by Proinflammatory Cytokines. Ann Orthop Musculoskelet Disord. 2018; 1(3): 1011.

Introduction

Intervertebral disc cells express and produce a number of neurotrophins. These neurotrophins, which are important signaling molecules actively involved in neurite survival and outgrowth, include Nerve Growth Factor (NGF), neurotrophin 3, ROBO1, neuropilin2, and brain-derived neurotrophic factor. The relationships between disc cells which produce neurotrophins, nerve in growth, and pain production in the disc are, as yet, incompletely understood. Glial cell line-Derived Neurotrophic Factor (GDNF) was identified by Lin et al. in a rat glial cell line during their discovery that it specifically promoted the survival and differentiation of dopaminergic neurons in cultures of embryonic midbrains cells. GDNF was found to be expressed throughout the central nervous system, and in embryonic limb bud, kidney, gut, neonatal kidney, gut, lung testis, and also in non-nervous tissue such as adult lung, liver and ovary. There has been considerable interest regarding a potential role for GDNF in chronic low back pain. Madduri et al. showed a synergistic effect of GDNF and NGF on axonal branching and elongation *in vitro*, and *in vitro* GDNF supports survival of central dopaminergic neurons, motoneurons, and nociceptive sensory neurons. Functional bradykinin B1 receptors in nociceptive neurons are also now known to be up regulated by GDNF. Otoshi et al. studied the reaction of glial cells and endoneurial macrophages in DRG and their contribution to pain-related behavior after application of nucleus pulposus onto the nerve root of rats. Jung et al. used a rat model and identified an increase in GDNF gene expression in both the thalamus and Dorsal Root Ganglia (DRG) during pain induced by injection of complete Fruend's adjuvant into L5-6. Kim et al. suggested that GDNF (but not nerve growth factor) might mediate rat annulus cell-induced neurite outgrowth from rat dorsal root ganglia. Recent studies from our laboratory have shown production of GDNF by human annulus cells during co-culture with F11 nerve cells and during addition of pro inflammatory cytokines to annulus cell-nerve co-cultures, but there appear to be no direct studies to date which address the presence of GDNF in the human disc or the production of GDNF by human disc cells. Our objectives in the present work were to test for the presence of GDNF in human disc tissue using immune histochemistry, to examine changes

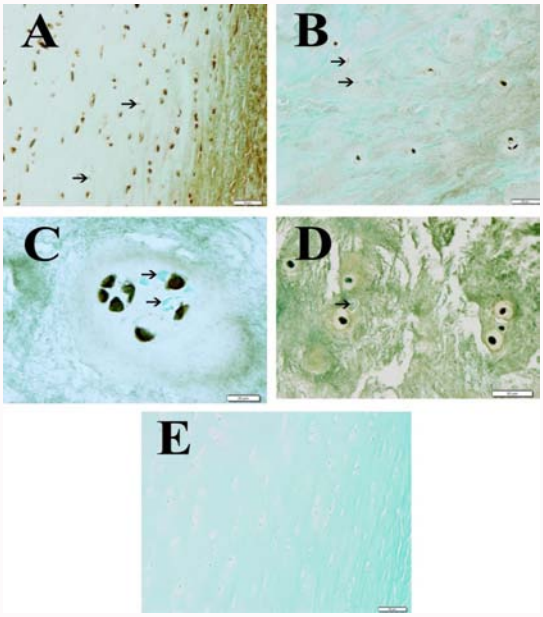

Figure 1: Immunohistochemical localization (black localization product) of GDNF in human disc tissue. A) Grade I, outer annulus control disc shows localization in almost all cells; B) Grade III surgical specimen in the deep inner annulus region shows both cells with and without localization; C) Cluster in the inner annulus showing cells with positive or negative localization in a grade III surgical disc specimen; D) Localization in annulus cells encapsulated by matrix in a grade IV surgical specimen. E. Negative control in section adjacent to that shown in A. (Arrows mark examples of cells showing no GDNF localization). (Scale bars in A, B, D and E = 50 µm. Scale bar = 20 µm in C).

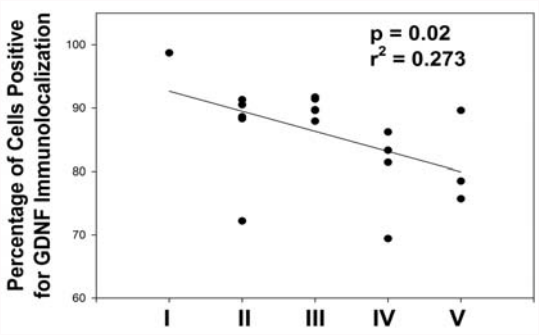

Figure 2: The percentage of cells showing immune localization of GDNF declined significantly with increasing stages of disc degeneration ($p = 0.02$; $r^2 = 0.273$).

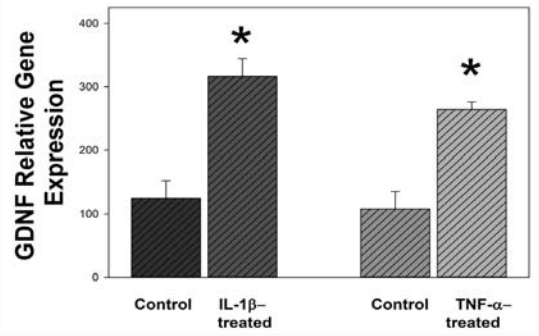

Figure 3: Mean GNDF expression levels increased significantly with IL-1ß treatment ($p = 0.007$) or TNF-α treatment ($p = 0.004$) vs. control. (Data are expressed as means ± s.e.m (4).

in GDNF gene expression during annulus cell co-culture with F11 nerve cells, to determine the effect of exogenous pro inflammatory cytokines on GDNF gene expression *in vitro*, and to determine whether rhGDNF would exert a mitogenic effect on cultured human annulus cells.

Material and Methods

Our Institutional Review Board prospectively approved these studies. Scoring of disc degeneration utilized a modification of the Thompson scoring system which incorporated author's (E.N.H.'s) radiological, magnetic resonance imaging, and surgical findings. The Thompson scoring system scores disc degeneration over the spectrum from a healthy disc (scored as a Thompson grade I) to discs with advanced degeneration (Thompson grade V). Patient specimens were derived from surgical disc procedures performed on individuals with herniated discs and/or degenerative disc disease. Surgical specimens were transported to the laboratory in sterile Minimal Essential Medium, and annulus tissue was minced and cells cultured in monolayer using previously published detailed procedures. Normal control disc tissue was obtained from the Cooperative Human Tissue Network, shipped over night in media, and processed as described here for culture establishment.

Immunohistochemical localization of GDNF in human disc tissue

Paraffin sections were cut at 4µm, collected on PLUS slides (Cardinal Health, Dublin, OH) and dried at 60°C. Sections were deparaffinized in xylene (Cardinal) and rehydrated through graded alcohols (AAPER, Shelbyville, KY) to distilled water. Endogenous peroxidase was blocked using 3% H_2O_2 (Sigma St Louis, MO). Sections were incubated for one hour with anti-GDNF (ab119473, a rabbit polyclonal antibody to GDNF) (Abcam, Cambridge, MA) at a 1:100 dilution (Dako, Antibody Diluent). Slides were treated with Vector ImmPress anti-rabbit IgG reagent for 30 minutes and DAB (Dako) for 5 minutes. Slides were rinsed in water, counterstained with light green, dehydrated, cleared and mounted with resinous mounting media. Rabbit Super Sensitive Negative Control (Biogenix, Fremont, CA) was used as the negative control; human brain tissue was used as a positive control. The number of cells positive for immune localization of GDNF was determined and the percentage of positive cells analyzed for its relationship with the Thompson grade of that disc tissue. The mean number of cells scored/specimen was 269 + 101 (15) (mean + S.D. (n)) with a range of 60 to 507.

Measurement of the Effects 0f Hrgdnf on Cell Proliferation

Annulus cells were plated into 96-well plates at a cell plating density of 5,000cells/well. Cells were exposed to 0 ng/ml (control), 10 ng/ml or 100 ng/ml hrGDNF (#212-GD, R&D Systems, Minneapolis, MN) (doses shown to be mitogenic to SH-SY5Y cells by Hirata and Kiuchi). Cell proliferation was measured using the CCK-8 method Cell Counting Kit-8) (Sigma) with a 4-hour incubation assessment using a micro plate reader (Spectramax Plus 384 Micro plate Reader, Molecular Devices, Sunnyvale, CA). Assays were run in triplicate and results averaged.

Determination of the Production of GDNF by 3D Cultured Human Annulus Cells

Annulus cells were established in monolayer culture, and expanded for use in 3D culture in a collagen sponge as previously

described. Cells were cultured in 3D for 14 days in MEM20, with no media changes over the last 5 days to allow metabolite accumulation. Medium samples were then harvested, frozen at -70° C, thawed and GDNF levels (pg/ml) were determined using the L-series Array (L-507) (Ray Biotech, Inc., Norcross, GA) read on a Molecular Devices GenePix Personal 4100 Scanner (Molecular Devices, Inc., Sunnyvale, CA). Extracted densitometry values were analyzed using the Q-Analyzer Software for QAH-GF-1 from Ray Biotech, Inc. for protein quantification. GENF levels of detection were 6.7 - 8,000 pg/ml. Assays were run in duplicate, results were averaged and data were expressed as relative signal intensity.

Gene Expression of GDNF in 3D Cultured Human Annulus Cells Exposed To Pro Inflammatory Cytokines

Annulus cells were established in monolayer culture and expanded for use in 3D culture in as described above. Annulus cells from a grade II, a grade III and three grade IV discs were used. Cells were cultured in 3D for 14 days with media changes under control conditions (minimal essential medium plus 20% FBS) or under experimental conditions with addition of 10^{-2} pM IL-1beta (IL-ß) or 10^{3} pM TNF-alpha (TNF-α). Doses used had been determined by earlier studies. RNA was harvested from cells at the end of the experiment and gene expression studies were performed using microarray analysis. Total RNA (100 µg) was harvested, reverse transcribed, amplified, labeled, fragmented and hybridized to the Affymetrix human X3P microarray chips. The GCOS Affymetrix GeneChip Operating System (version 1.2; Affymetrix, Santa Clara, CA) was used to determine gene expression levels of GDNF (Affymetrix probe set NM_000514).

Determination of GDNF Expression in Annulus Cells Co-Cultured With F11 Nerve Cells

Details of this experimental design have been previously published but gene expression patterns in the annulus cells have not been published. Briefly, annulus cell co-culture 4-day studies were performed using a cell well insert system with F11 cells in monolayer on top of the insert and human annulus cells in Minimal Essential Media with 20% FBS (MEM20) on the plastic well bottom. F11 and annulus cells were seeded at a density of 12,500cells/cm^2. Control cells were incubated in the MEM20 media. At study completion, RNA was harvested from cells at the end of the experiment and gene expression studies were performed using microarray analysis. Total RNA (100 µg) was harvested, reverse transcribed, amplified, labeled, fragmented and hybridized to the Affymetrix human U133 microarray strips. The GCOS Affymetrix GeneChip Operating System (version 1.2; Affymetrix, Santa Clara, CA) was used to determine gene expression levels of GDNF (Affymetrix probe NM_000514) with correction for false discovery rate (FDR).

Statistical analyses

Standard statistical analyses were performed with InStat (GraphPad Software, Inc., San Diego, CA) including paired t-tests and ANOVA. Data are presented as means + s.e.m. (n), and a two-tailed P value of less than 0.05 was considered statistically significant. When data were not normally distributed, nonparametric statistics were used. Spearman's correlation coefficient was calculated to test for the association of Thompson grade with the proportion of cells positive for GDNF immune localization in annulus tissue.

Statistical analysis of microarray data

GeneSifterTM web-based software was used to analyze microarray data. Using GC-RMA (Robust multi-array average), Affymetrix ".cel" files were uploaded to the GeneSifterTM web site, normalized, and corrected for False Discovery Rate (FDR). Statistical significance was determined using Student's t-test (2 tailed, unpaired) and significance was set at $p < 0.05$). Fold change was set at 2.0 for False Discovery Rate (FDR) correction.

Results

Demographic features of patients from whom disc tissue was obtained, and use of disc tissue and cultured cells in the work described here are specified. Immunohistochemical analyses of 18 annulus specimens showed that GNDF was present in most cells in the human annulus, especially in the outer annulus of discs from young subjects (Figure 1A). In the inner annulus, some cells did not show localization (Figure 1B), and large cell clusters were noted to contain cells with or without GDNF localization (Figure 1C). Cells with encapsulating matrix material also showed GDNF localization (Figure 1D). (Figure 1E) shows the absence of localization product in a negative control specimen adjacent to the section shown in 1A). The proportion of annulus cells with positive localization was also quantified. The proportion of cells showing immune localization of GDNF declined with increasing stages of disc degeneration (Figure 2). Spearman rank correlation analysis showed $p = 0.02$ and an r^2 value of .273 for this association. Additional studies described below utilized cultured human annulus cells in analyses of production of GDNF *in vitro*, analysis of gene expression of 3D cultured cells exposed to pro inflammatory cytokines, monolayer cells co-cultured with F11 nerve cells, and lastly to determine if rhGDNF exerted mitogenic effects on disc cells in monolayer culture. Three-D cell culture studies were used to directly confirm secretion of GDNF into the media by annulus cells. Since we found no correlation of GDNF media levels and disc grade, there does not appear to be an effect due to the stage of disc degeneration from which cells were isolated (GDNF levels produced by grades I and II cells (1227.3 + 260.4 (4)) v_s. grade IV cells (1186.3 + 301.8 (3))). In order to explore the relationship between two important pro inflammatory cytokines produced by human disc cells (IL-1ß and TNF-α) and GDNF, we also measured GDNF gene expression levels. GNDF gene expression was significantly increased in cells exposed to either IL-1ß ($p = 0.007$) or TNF-α ($p = 0.004$) (Figure 3). We next examined GDNF expression levels in cells co-cultured with F11 nerve cells. Our recent work showed that co culture of human annulus cells and F11 nerve cells with added IL-ß or TNR-α significantly increased neurite lengths compared to controls, and increased levels of BDNF, NT3 and GDNF. In the present study, we examined GDNF gene expression in these co cultured annulus cells and found significantly increased expression in more degenerated cells from Thompson grade IV and V discs compared to that levels in healthier grade I, II and III cells (fold difference = 1.09; $p = 0.04$). Our final studies examined whether exogenously administered rhGDNF could exert a mitogenic effect on human lumbar annulus cells in monolayer culture. One-way ANOVA analysis showed that there was no significant difference in mean cell numbers between control (14,650 + 3,286 (12)), and cells exposed to 10 ng/ml rhGDNF (13,973 + 2.199 (12)) or 100 ng/ml rhGDNF (12,809 +2,589 (12).

Discussion

The relationship between neurotrophins produced by disc cells and how these neurotrophins influence nerve in growth in the disc is an important area of current research. Recently Binch et al. have shown that nerve in growth can occur in the human disc in the absence of blood vessels, a result which suggests that nerves do not

"follow" blood vessels as previously believed. This new finding adds a new perspective on the relationship between annulus cells and nerves. Here we present novel data on GDNF, a neurotrophin shown here to be produced by disc cells, and which we have previously shown to be involved in significantly increasing neurite lengths during annulus cell-F11 nerve cell co culture. Strengths of the present work include direct immunehistochemical demonstration that GDNF is present *in vivo* in the annulus with significantly fewer immune positive cells with increasing stages of disc degeneration ($p = 0.02$), and direct measurement of GDNF secreted by annulus cells in their cell medium. We also showed up regulation of GDNF gene expression by two important pro inflammatory cytokines known to be present in the aging/degenerating disc, IL-1ß and TNF-α. Annulus cells from a variety of disc grades were tested to measure whether GDNF was mitogenic. We also showed that annulus cells co-cultured with F11 nerve cells showed significant up regulate of GDNF gene expression. One limitation of this work was that we only investigated pro inflammatory cytokine levels of 10^2pmol/L for IL-1ß and of 10^3pmol/L for TNF-α. Future studies would be important to evaluate different dose levels, and to test the effects of additional types of pro inflammatory cytokines on GDNF expression and production. The present study presented novel immune cytochemical data demonstrating the presence of GDNF in the human inner and outer annulus, and cell culture analyses demonstrated production of GDNF in 3D cultured annulus cells, a significant up regulation of GDNF gene expression *in vitro* in cells exposed to the pro inflammatory cytokines IL-1ß or TNF-α, and in annulus cells co-cultured with F11 nerve cells. Our work also showed that GDNF did not exert a mitogenic influence on cultured human annulus cells at the doses tested.

Our finding that GDNF gene expression is up regulated in the presence of IL-1ß or TNF-α *in vitro* is important from several perspectives. These pro inflammatory cytokines play important roles in disc degeneration and have effects which enhance axonal outgrowth. Abe et al have shown that pro inflammatory cytokines stimulate the expression of NGF by human disc cells, and these cytokines also now have recognized roles in the generation and maintenance of joint pain by their action on nociceptive nerve cells. The role of GDNF in exerting a neuro protective effect, and in promoting survival and sprouting of nerve cells have been verified in several previous studies; such findings raise interest in the utility of GDNF in the treatment of neurological disorders. Several previous studies have explored the mitogenic capacity of GDNF in non-disc cells. Yang and Han determined that GDNF was mitogenic for cultured mouse immature Sertoli cells via its receptor subunit NCAM and ERK1/2 signaling pathway. GDNF induced proliferation (but not differentiation) in cultured neuro blastoma cells in the studies of Hirata and Kiuchi. Neuronal and glial cells from neural crest-derived precursors showed age-dependent differences in response to GDNF; proliferation of nestin-expressing precursor cells was seen at E12 but not E14-16 stages. Motor neurons produced from mouse embryonic stem cells showed a mitogenic effect to GDNF in work by Cortes et al., and enteric neural crest cells also showed proliferation in response to GDNF as shown by Mwizerwa et al. Our work with human annulus cells, however, did not show a mitogenic response to doses of either 10 or 100 ng/ml hrGDNF vs. control proliferation levels; these GDNF doses had previously been shown to be mitogenic for SH-SY5Y cells by Hirata and Kiuchi. It is interesting to note that Capelle et al. found measurable levels of GDNF (~90pg/ml) in spinal fluid specimens from a group of patients with chronic nociceptive lumbar back pain. The mean, however, did not differ significantly from levels from patients with neuropathic pain who had had previous spinal surgery. It is important to note that most studies to date assume that GDNF levels result from nerve activity, since the disc production of neurotrophins is still not widely recognized. Thus previous studies which target GDNF as a potential agent for controlling normal and pathologic pain may or may not have direct relevance to the disc. BDNF and GDNF expression has been noted in discrete small- to medium-sized DRG neurons which appear to be a subpopulation of calcitonin gene-related peptide (CGRP) neurons. GDNF has also been noted to up regulate function bradykinin B1 receptors in nociceptive neurons. Previous studies of pain-related genes expressed by disc cells in our lab showed immune localization and gene expression of both CGRP and bradykinin B1 proteins. Taken as a whole, considerable evidence suggests that expression of GDNF represents multiple function of this neurotrophin within and outside of the nervous system. Findings presented here have potential direct clinical relevance *in vivo* related to nerve in growth and pain in the outer annulus (where disc cell numbers are high), and in regions where nerves penetrate annular tears. We look forward to future studies which will clarify the role of GDNF in disc degeneration and its participation in signaling nerve in growth and survival in the disc.

Acknowledgement

The authors have no conflicts of interest to disclose.

Research funds from the North American Spine Society were received in support of portions of this work. The authors wish to thank Natalia Zinchenko for assistance with immune histochemistry, Nury Steuerwald, PhD. and Judy Parsons in the Molecular Biology Core for assistance with microarray analysis, Letitia Bullock for assistance with cell culture, and to acknowledge the support of the Brooks Back Pain Research Endowment for general laboratory support.