Experimental and Molecular Pathology 96 (2014) 225–229

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Growth and differentiation factor-5 (GDF-5) in the human intervertebral annulus cells and its modulation by IL-1ß and TNF-α in vitro

Helen E. Gruber *, Gretchen L. Hoelscher, Jane A. Ingram, Synthia Bethea, Edward N. Hanley Jr.

Department of Orthopaedic Surgery, Carolinas Medical Center, PO Box 32861, Charlotte, NC, USA

ARTICLE INFO

Article history:
Received 6 February 2014
Available online 25 February 2014

Keywords:
Intervertebral disc
Growth and differentiation factor-5
Proinflammatory cytokines
Interleukin-1ß
Tumor necrosis factor-α
Disc herniation

ABSTRACT

Growth and differentiation factor-5 (GDF-5) is a member of the TGF-ß superfamily which regulates cell division and differentiation. GDF-5 attracted high interest because of its role in skeletal development, especially in cartilaginous sites. Little is known, however, about the role of GFD-5 in disc cell biology. The present work demonstrated the immunohistologic presence of GDF-5 in human outer and inner annulus tissue. Microarray analysis of annulus cells showed significant upregulation of GDF-5 expression in herniated vs. non-herniated lumbar discs (2.14-fold change, $p = 0.021$). In vitro three-dimensional culture studies challenged human annulus cells with IL-1ß and TNF-α, two proinflammatory cytokines known to be elevated in the human degenerating disc. Exposure resulted in significant downregulation of GDF-5 during both TNF-α exposure (5.83-fold change, $p = 0.044$) and IL-1ß exposure (3.38-fold change, $p = 0.015$). In vitro findings suggest that the degenerating disc milieu, with high proinflammatory cytokine levels, may limit expression of GDF-5, resulting in limited regenerative capacity of the intact disc.

Introduction

Growth and differentiation factors are very interesting proteins which act as regulators of cell division and differentiation. Growth and differentiation factor-5 (GDF-5), and GDF-6, and GDF-7 are closely related to the bone morphogenetic proteins and form a novel TGF-ß subfamily, which acts via binding to serine–threonine kinase receptors. GDF-5 was cloned in 1994 by Hotten et al. (1994) and by Chang et al. (1994). Chang et al. named this factor cartilage-derived morphogenetic protein 1 (CDMP1), a term which is still in use today. GDF-5 is also known as lipopolysaccharide-associated protein 4 (LAP4), LPS-associated protein 4, and bone morphogenetic protein 14 (BMP14). GDF-5 binds to several BMP receptors: BMPR1A, BMPR1B, and BMPR2.

Early work by Chang et al. (1994) showed expression of GDF-5 at sites of skeletal morphogenesis, and research on the role of GDF-5 in cartilage and arthritis continues to be of high interest as reflected in recent reviews (Buxton et al., 2001; Jin and Li, 2013; Nixon et al., 2007; Pacifici et al., 2006). Genomic mutations in the *GDF-5* gene also have import due to the resulting chondrogenic dysplasias, including chondrodysplasia Grebe type, acromesomelic chondrodysplasia Hunter–Thompson type, and brachydactyly type C (for more details, see Buxton et al., 2001). Polymorphisms in the *GDF-5* gene are also now recognized to show associations with osteoarthritis (see recent citations in (Liu et al., 2013; Miyamoto et al., 2007; Miyamoto et al., 2007, 2013).

Interest in GDF-5 and intervertebral disc cell biology is a recent research topic linked to the potential therapeutic efficacy of GDF-5 in the treatment of human disc degeneration. Walsh et al. (2004) analyzed murine caudal discs exposed to static compression in vivo for 7 days and then injected with GDF-5 after a 3-week recovery period. Findings included a significant increase in disc height following GDF treatment. An analysis of nucleus pulposus tissue and cultured cells was performed by Le Maitre et al. (Le Maitre et al., 2009). These investigators identified significantly more GDF-5 positive cells in the nucleus vs. the inner annulus. Cells cultured from the nucleus and exposed to GDF-5 in vitro showed significantly increased total proteoglycan production (assaying cell matrix with conditioned media) and increased aggrecan and type II collagen gene expression levels vs. controls.

The GDF-5-deficient mouse model has provided interesting recent data on the relationship of GDF-5 to disc cell function. Maier and Harfe (2011) have studied mouse embryonic GDF-5 null mice models and reported that in situ techniques showed GDF-5 localization in the annulus, but not the nucleus, of 24-week-old mice. Work by Li et al. (2004) identified lower MRI T2-weighted signals in discs of GDF-5 null mice, and abnormal morphology on histologic examination. Proteoglycan content was decreased in the null animals. Treatment with recombinant GDF-5 results in a dose-dependent upregulation of type II collagen and aggrecan gene expression (Cui et al., 2008; Li et al., 2004).

In light of the sparse literature on the presence of GDF-5 in vivo in human discs, especially in the annulus, and the interest in the potential

* Corresponding author at: Orthopaedic Research Biology, Cannon Bldg., Room 304, Carolinas Medical Center, PO Box 32861, Charlotte, NC 28232. fax: 704 355 5620.
E-mail address: helen.gruber@carolinashealthcare.org (H.E. Gruber).

http://dx.doi.org/10.1016/j.yexmp.2014.02.005
0014-4800/

for GDF-5 in disc therapy, we analyzed human annulus tissue for immunolocalization of GDF-5 and GDF-5 expression. A second part of our study employed an in vitro experimental approach using isolated human annulus cells cultured in 3D; mRNA was isolated from control cells, or cells exposed to either IL-1-ß or TNF-α, and gene expression levels of GDF-5 were determined.

Methods

Clinical study population

Experimental study of human disc specimens was approved prospectively by the authors' Human Subjects Institutional Review Board at Carolinas Medical Center. The need for informed consent was waived by the ethical board since disc tissue was removed as part of routine surgical practice. Scoring of disc degeneration utilized the Thompson scoring system; this system scores disc degeneration over the spectrum from a healthy disc (Thompson grade I) to discs with advanced degeneration (grade V, the most advanced stage of degeneration) (Thompson et al., 1990). Patient specimens were derived from surgical disc procedures performed on individuals with herniated discs and degenerative disc disease. Surgical specimens were transported to the laboratory in sterile tissue culture medium. Care was taken to remove all granulation tissue and to sample only disc tissue. Non-surgical control donor disc specimens were obtained via the National Cancer Institute Cooperative Human Tissue Network (CHTN); they were shipped overnight to the laboratory in sterile tissue culture medium and processed as described below. Specimen procurement from the CHTN was included in our approved protocol by our human subjects Institutional Review board.

Immunolocalization of GDF-5 in the human annulus

Paraffin sections were cut at 4 μm, 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. Slides were treated with proteinase K, 2 μg/ml (Sigma, St Louis, MO) for 15 min. Endogenous peroxidase was blocked using 3% H_20_2 (Sigma, St Louis, MO). Sections were incubated for 1 h with anti-GDF-5 (A-10) (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution. GDF-5 (A-10) is a mouse monoclonal antibody specific for an epitope mapping between amino acids 375–408 within an internal region of human GDF-5. The secondary antibody was 4 + biotinylated universal goat link (Biocare Medical, Concord, CA) for 10 min followed by 4 + streptavidin HRP Label (Biocare) for 10 min and DAB (Dako) for 5 min. Slides were rinsed in water, counterstained with light green, dehydrated, cleared, and mounted with resinous mounting media. Human adrenal tissue served as the positive control. Mouse IgG (Dako, Carpinteria, CA) was used as a negative control.

Human annulus cell culture

Annulus cells were established in monolayer culture (Gruber et al., 1997) and expanded for use in 3D in a collagen sponge as previously described (Gruber et al., 2006). Annulus cells from a grade II disc, a grade III, and 3 grade IV discs were utilized. 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 IL-1beta (10^{-2} pM) or TNF-alpha (10^3 pM). Doses used were determined in previous studies (Gruber et al., 2012; Gruber et al., 2013). RNA was harvested from cells at experiment termination.

Microarray analyses

For analysis of human discs, tissue was snap frozen in liquid nitrogen, pulverized (BioPulverizer, BioSpec Products, Inc., Bartlesville, OK, USA), and homogenized via the FastPrep-24 instrument (MP Biomedicals L.L.C., Santa Ana, CA, USA).

Total RNA (100 μg) was harvested, reverse transcribed, amplified, labeled, fragmented, and hybridized to the Affymetrix human U133 X3P microarray chips. The GCOS Affymetrix GeneChip Operating System (version 1.2, Affymetrix, Santa Clara, CA 95051) was used for determining gene expression levels of GDF-5 (gene identifier NM_000557) and its receptor bone morphogenetic protein receptor, type II (serine/threonine kinase) (gene identifier g704361_3p_a_at).

Statistical analysis of microarray data

The 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.

Results

Molecular and immunohistochemical studies on GDF-5 in disc tissue

Studies to show the constitutive expression of GDF-5 in human intervertebral discs were performed on disc tissue using microarray analyses and immunohistochemistry. Demographic data on the groups of patients used in immunolocalization and molecular work are shown in Tables 1 and 2, respectively.

Immunohistochemical localization of GDF-5 revealed positive presence in all outer annulus cells of Thompson grade I discs (Fig. 1A), and in the majority of cells in more degenerated discs (Fig. 1C). Localization was also present in the majority of cells in the inner annulus of healthier and more degenerated discs (Fig. 1D). Both spindle-shaped and rounded cells showed localization, as well as some cells present in large clusters (Fig. 1E). (Fig. 1F shows a representative negative control image).

Microarray analysis did not detect any significant differences in expression levels of GDF-5 in more degenerated grades III, IV, and V discs compared to healthier grades I and II lumbar discs. A significant upregulation of GDF-5 expression was seen, however, in analyses of herniated vs. non-herniated lumbar discs (2.14-fold change, $p = 0.021$). A less strong but still significant upregulation was also seen in data for one of the GDF-5 receptors, BMP protein receptor, and type II (serine/threonine kinase) (BMPR2) in the analysis of more degenerated Thompson grade IV and V discs vs. healthier grade I, II, and III discs (1.67-fold change, $p = 0.021$).

In vitro studies on the expression of GDF-5 following exposure to proinflammatory cytokines

Our studies also include the use of an in vitro model to test annulus cells grown in 3D culture, a technique that may more closely mimic the in vivo disc than does monolayer cell culture (Gruber et al., 2012; Gruber et al., 2010). In these experiments, annulus cells were grown either under control conditions or exposed to IL-1ß or TNF-α, two proinflammatory known to play important roles in disc degeneration (Hoyland et al., 2008; LeMaitre et al., 2005; LeMaitre et al., 2007).

Analysis of expression levels of GDF-5 in cultured cells showed a significant downregulation in cells exposed to TNF-α (5.83-fold change, $p = 0.044$) and IL-1ß (3.38-fold change, $p = 0.015$) (Fig. 2).

Discussion

In this study, we describe our findings on immunohistochemical localization of GDF-5 at the protein level in the annulus of human discs and our identification of significantly upregulated GDF-5 expression in cells of herniated vs. non-herniated discs. This work with annulus tissue