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Matrix metalloproteinase 12 is an indicator of intervertebral disc degeneration co-expressed with fibrotic markers

F.-J. Lv † ‡ § ||, Y. Peng †, F.L. Lim †, Y. Sun † ||, M. Lv ¶, L. Zhou †, H. Wang #, Z. Zheng #, K.M.C. Cheung † § || **, V.Y.L. Leung † ‡ § || *

† Department of Orthopaedics and Traumatology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China
‡ HKU Shenzhen Institute of Research and Innovation, China
§ Center for Reproduction, Development and Growth, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China
|| Stem Cell & Regenerative Medicine Consortium, The University of Hong Kong, Hong Kong, China
¶ Advanced Technology Research Institution of China Science Institution, Shenzhen, China
# Department of Spine Surgery, The First Affiliated Hospital, Sun Yat-sen University, Gaungdong, China

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SUMMARY

Objective: Recent evidence suggests a role of fibrogenesis in intervertebral disc (IVD) degeneration. We aim to explore if fibrotic genes may serve as IVD degeneration indicators, and if their expression is associated with myofibroblast activity.

Design: Transcriptional expression of fibrosis markers (*COL1A1*, *COL3A1*, *FN1*, *HSP47*, *MMP12*, *RASAL1*) were analyzed in degenerated (D) and non-degenerated (ND) human nucleus pulposus (NP) and annulus fibrosus (AF) cells, along with traditional (*SOX9*, *ACAN*) and newly established degeneration markers (*CDH2*, *KRT19*, *KRT18*, *FBLN1*, *MGP, and COMP*). Protein expression was investigated by immunohistochemistry in human IVDs, and in rodent IVDs undergoing natural ageing or puncture-induced degeneration. Co-expression with myofibroblast markers was examined by double staining on human and rat specimens. Disc degeneration severity and extent of fibrosis were determined by histological scoring and picrosirius red staining respectively.

Results: Human D-NP showed more intensive staining for picrosirius red than ND-NP. Among the genes examined, D-NP showed significantly higher *MMP12* expression along with lower *KRT19 expression*. Protein expression analysis revealed increased MMP12(+) cells in human D-IVD. Histological scoring indicated mild degeneration in the punctured rat discs and discs of ageing mouse. Higher MMP12 positivity was found in peripheral NP and AF of the degenerative rat discs and in NP of the aged mice. In addition, human D-NP and D-AF showed increased α-SMA(+) cells, indicating enhanced myofibroblast activity. MMP12 was found co-expressed with α-SMA, FSP1 and FAP-α in human and rat degenerative IVDs.

Conclusions: Our study suggests that in addition to a reduced *KRT19* expression, an increased expression of *MMP12*, a profibrotic mediator, is characteristic of disc degenerative changes. Co-expression study indicates an association of the increased MMP12 positivity with myofibroblast activity in degenerated IVDs. Overall, our findings implicate an impact of MMP12 in disc cell homeostasis. The precise role of MMP12 in IVD degeneration warrants further investigation.

* Address correspondence and reprint requests to: V.Y.L. Leung, Department of Orthopedics and Traumatology, Lab Block, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, China. Tel: 86-852-3917-6985; Fax: 86-852-2818-5210.

** Address correspondence and reprint requests to: K.M.C. Cheung, Department of Orthopaedics and Traumatology, The University of Hong Kong Medical Centre, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong, China. Tel: 86-852-2255-4341; Fax: 86-852-2817-4392.

E-mail addresses: fengjuan@hku.hk, fengjuan.lv@gmail.com (F.-J. Lv), u3002479@connect.hku.hk (Y. Peng), h0894164@connect.hku.hk (F.L. Lim), hkusunyi@connect.hku.hk (Y. Sun), lvminmin2015@163.com (M. Lv), lxzhou@connect.hku.hk (L. Zhou), wangxucheng@gmail.com (H. Wang), zhengzm1@163.com (Z. Zheng), ken-cheung@hku.hk (K.M.C. Cheung), vicleung@hku.hk (V.Y.L. Leung).

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Introduction

Low back pain is associated with lumbar intervertebral disc (IVD) degeneration[1]. Our previous study indicated a linear progression of disc degeneration prevalence with an increase in age, where 100% of subjects exhibit disc degeneration by 60 years of age[2,3]. IVD is constituted by an inner gelatinous core of nucleus pulposus (NP), an outer ring of annulus fibrosus (AF), and the capping endplates (EP). In human, the NP is initially composed of vacuolated primitive cells, or commonly called notochordal cells, which are gradually replaced by chondrocyte-like cells at around 10 years of age[4]. The chondrocyte-like NP cells share some common features of chondrocytes, such as production of pericellular matrix rich in type VI collagen[5] and extracellular matrix (ECM) rich in type II collagen and proteoglycans[6].

IVD degeneration is related to a change of the NP from a gelatinous to a fibrocartilaginous structure. A majority of investigations evaluate disc degeneration and regeneration by assessing the expression of chondrocyte markers in the NP, such as Sox9, type II collagen and aggrecan[7,8]. It is yet unclear whether these markers are ideal to indicate the degeneration/regeneration status. Recently, additional degeneration markers have been proposed. These include the upregulation of cartilage oligomeric matrix protein (*COMP*), matrix gla protein (*MGP*)[9] and fibulin 1 (*FBLN1*) gene expression[10], as well as down-regulation of keratin 18 (*KRT18*)[11], cadherin 2 (*CDH2*)[11] and keratin 19 (*KRT19*)[10] expression. In addition, disc degeneration also involves fibrotic changes of the NP with increased collagen I (*COL1A1*) and fibronectin (*FN1*)[12,13]. Our microarray study has also indicated *CD55* as a potential marker of normal NP cells (data unpublished). To date, it has not been consolidated which of these markers are the most effective in distinguishing the healthy and degenerated disc phenotype in human.

Matrix metalloproteinase 12 (MMP12) is a matrix degradation enzyme. It degrades various types of ECM proteins and is known to associate with tissue fibrosis[14]. Our previous study indicates that the modulatory effect of mesenchymal stem cells (MSCs) on IVD degeneration involves a down-regulation of MMP12[15]. In this study, we aimed to explore the expression levels of MMP12 along with a series of candidate genes in non-degenerated vs degenerated human NP, so as to determine if MMP12 is an effective indicator of IVD degeneration. The analysis included the conventional chondrogenic markers, putative disc degeneration markers, and known markers associated with fibrosis. We further examined MMP12 protein expression in a rat model of induced disc degeneration and ageing mouse, and tested if it is linked to the activity of myofibroblasts, the major effector cells of fibrosis.

Methods

Patient samples

Human IVD were collected from twelve patients with disc degeneration (graded III–IV at the Schneiderman scale) undergoing discectomy, and from nine patients undergoing corrective scoliosis surgery (as non-degenerative control) in the local children hospital in Hong Kong, and in First Affiliated Hospital of Sun Yat-sen University in Guangzhou with informed patient consent and corresponding institutional review board (IRB) approval. The NP donor information was listed in Table I.

Human NP cell isolation and culturing

Among the human NP specimens we harvested, five batches of scoliotic NP and five batches of degenerated NP were subjected to cell isolation. Briefly, the NP was first carefully dissected from the other compartments of the IVD based on the tissue tone and softness, and then verified being lack of lamella structures under light microscope by experienced staff within 8 h of tissue collection. The dissected NP specimen were cut into small pieces and washed in PBS, before centrifuged at 800 × g for 5 min. They were then digested in three volumes of 0.25% (w/v) pronase solution in serum-free DMEM medium (Life Technologies Ltd., HK) at 37°C for 1 h with agitation, followed by incubation of three volumes of 0.6 mg/ml collagenase type II solution (Worthington Biochemicals, Lakewood, N.J., US) in DMEM containing 10% fetal calf serum (FCS) for 8 h. The isolated cells were filtered and expanded in monolayer in DMEM medium supplemented with 10% FCS, 1% penicillin/streptavidin, 1% L-glutamine, and 0.4% fungizone in a 37°C humidified incubator. Medium was refreshed twice a week and the cells were sub-cultured when they reached 90% confluency.

Our previous study has demonstrated that alginate 3D culture could mimic *in vivo* environment and enrich committed NP cells[16], therefore expanded NP cells were embedded into alginate before gene expression study. For culture in alginate beads, NP cells at passage 2 were resuspended in 1.2% (w/v) alginate (Sigma–Aldrich Company Ltd., US) solution in 0.9% sterile sodium chloride at a concentration of 1×10^6 cells/ml. The cell suspension was passed through a 21-gauge needle into a 102-mmol/L calcium chloride solution where each drop was immediately transformed into a semisolid microspheric bead. After 15 min of incubation at RT to complete the polymerization, the beads were washed and cultured in DMEM culture medium for 1 week before subjected to mRNA analysis.

Gene expression analysis

Five batches of scoliotic and degenerated human NP cells, respectively, were analyzed for gene expression of selected markers. RNA was isolated by Trizol (Life Technologies Ltd., HK) after the cells were released from alginate by incubating in dissociation buffer (55 mmol/L sodium citrate, 30 mmol/L disodium EDTA, and 0.15 mol/L sodium chloride, PH6.8) on ice for 10 min and washed twice in PBS. The quantity and quality of RNA was assessed by Agilent 2100 bioanalyzer. Reverse transcription of RNA (with high RNA integrity number, RIN>7) to cDNA was performed using a High Capacity RNA to cDNA kit (Applied Biosystems, US). Quantitative real-time PCR (qRT-PCR) was performed on a Step-one Plus system with 5 ng cDNA (Applied Biosystems, US) using Taqman primers. The expression of the following genes were examined: *SOX9* (Hs00165814_m1), *ACAN* (Hs00153936_m1), *CDH2* (Hs00983056_m1), *CD55* (Hs00892618_m1), *FBLN1* (Hs00972609_m1), *MGP* (Hs00179899_m1), *KRT19* (Hs00761767_s1), *KRT18* (Hs01941416_g1), *COMP* (HS00164359_m1), *COL1A1* (Hs00164004_m1), *COL3A1* (Hs00943809_m1), *FN1* (Hs01549976_m1), *HSP47* (Hs00241844_m1), *RASAL1* (Hs00183013_m1) *and MMP12* (Hs00899662_m1) (all from Life Technologies Ltd.). *GAPDH* (Hs03929097_g1) was used as endogenous control. The relative quantification was achieved by comparative CT method.

Disc degeneration and ageing models

A rat model of induced IVD degeneration was established by physical trauma to the disc as previously described[17] with approved animal license from Department of Health, The Government of The Hong Kong Special Administrative Region, and CULATR approval from the Committee on the Use of Live Animals in Teaching and Research in HK. Lewis rats at 3–6 months was anaesthetized and underwent spine radiography to determine the location of target discs to be punctured. Under anaesthesia, a longitudinal incision was made between caudal discs levels 4–7 in the tail. After making