Comparison of the Efficacy of BMP6 and TGF beta-1 to Downregulate MMP 1 in Intervertebral Disc Tissues

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INTRODUCTION
Degeneration is characterized by increased degradation of the normal IVD matrix by locally produced matrix metalloproteinases (MMPs). The nature of the matrix produced in the degenerate IVDs differs from that in normal IVDs, as a consequence of switches in the production of collagen within the inner annulus fibrosus (IAF) from type II to type I and in the synthesis of proteoglycan from aggrecan to versican, biglycan, and decorin. Similar matrix changes have been reported in articular cartilage in osteoarthritis. In this disease, the body of evidence points towards these being part of a more profound change in chondrocyte biosynthesis driven by local production of IL-1 beta (β1) and tumour necrosis factor alpha (TNFα). We wanted to determine if IVD cell phenotype would be maintained and ECM synthesis stimulated by BMP-6 and TGFβ-1 despite the inhibition of matrix synthesis caused by TNFα and IL-1β. Pre-existing studies indicate that the TGFβ-1, in particular have the capabilities to influence cellular proliferation and matrix synthesis in a range of connective tissue cell types, including disc cells. Thus it is likely to have a significant role in annular repair processes. BMP-6 has known also potential to stimulate total PG synthesis in human articular chondrocytes derived from normal as well as OA joints. The purpose of this study was to compare the efficacy of combined various cytokines like BMP and TGF application with IL-1 and TNF application in porcine annulus fibrosis.

MATERIAL AND METHOD
Source of tissues
IVDs used in this investigation were obtained from lumbar disc of female domestic pigs. All procedures were conducted in by the animal facility of the University of Tennessee Health Science Center, whose animal ethics research committee also approved all experimental procedures undertaken in this study.

Cell isolation & culture
Cells from the AF tissues were isolated by 1 hour digestion at 37°C using modified F-12K medium with 5% fetal calf serum, 4.8mM CaCl² and 0.05% Pronase, followed by overnight digestion in 0.2% collagenase.

Cell culture
Cells were plated at 1x10⁶ cells/well onto 6-well plates.

Treatment of cells with cytokines and growth factor
After 3-4 days, cells were treated for 24 hours with TNFα (5ng/ml) and IL-1β (1 ng/ml) and with/without cytokines like BMP-6 (100ng/ml) and porcine TGFβ-1(10ng/ml), all treatments were performed in triplicate.

Western blotting and ELISA
Medium from cells cultured for 24 hours in serum free medium with cytokines and growth factor were collected and mixed with sample buffer, was separated by SDS/PAGE, and the proteins were transferred to PVDF membranes. Membranes were incubated overnight at 4°C with polyclonal anti MMP-1 antibody (1:1000, Biodesign). After overnight the membranes were washed and incubated with anti-rabbit antibody coupled to alkaline phosphatase (1:10,000, Amersham). Excess antibody was removed by washing and immunoreactive bands were revealed by ECF (Amersham Bioscience, USA), according to the manufacturer's instructions. The fluorescence was detected on a STORM 860 reader (Molecular Dynamics, USA). The concentration of MMP-1 in culture media was determined by ELISA. For this study, the media were desalted and concentrated ten times prior to the measurement.

Real-time RT-PCR
The mRNA was isolated from the cells using TRizol™ reagent (Invitrogen), assayed for absorbance at 260nm (Pharmacia-Amersham), then reverse transcribed using common reagents from Applied Biosystems (ABI) in a programmable thermal cycler (MJ Research). Real-time PCR (7900, ABI) using custom-designed primers and probes specific to the pig genome were performed with 50 ng of cDNA to determine the ratio of expression. The extracellular matrix genes included col-I and col-II, and proteoglycan and the proteolytic enzyme included the matrix metalloproteinase-1.

RESULTS
To investigate the mechanism of the IVD degeneration, the AF cells were treated with pro-inflammatory cytokines that are known to be expressed in degenerated IVD. Treatment with TNFα and IL-1β strongly up-regulated the expression of MMP-1 and Col-I. In contrast, the mRNA expression of col-II and aggrecan were significantly decrease [Figure 1]. Western blot analysis demonstrated increased release of MMP-1 from AFs into the culture media during treatment with TNFα and IL-1β. The secretion of MMP-1 was indicative of its augmented production within the treated AFs. MMP-1 immunoreactive bands were significantly decreased following treatment with BMP-6, TGFβ-1 and the combination of BMP-6 and TGFβ-1 [Figure 2].

DISCUSSION
In this study, we observed the effects of BMP-6, TGF, and the combination of the two on AF cells that were treated with pro-inflammatory cytokines. Cytokines treated with TNF and IL-1β without added BMP-6 or TGFβ-1 acted as controls. As shown in the [Figure 3] the combined cytokine group showed the greatest increase in aggrecan and col-II. The three experimental groups showed no significant difference in collagen type II production. The col-I and MMP-1 mRNA expression were significantly decreased in the BMP-6, TGF combined group compared to the control. The result of ELISA assay confirmed secretion of MMP-1 corresponding to the intensity of mRNA induction by the pro-inflammatory cytokines.

REFERENCE

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