Proinflammatory Cytokines Modulate the Chemokine CCL2 (MCP-1) in Human Annulus Cells in Vitro: CCL2 Expression and Production

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Introduction: Chemokines are important secondary inflammatory mediators released in response to stimuli. They act as second-order cytokines with specialized functions in inflammation. The role of these mediators in human disc degeneration is still poorly understood. CCL2 (MCP-1, Monocyte Chemoattractant Protein-1) has been identified in disc cells (1-6). Previous studies showed that 1,25(OH)₂-vitamin D₃ inhibited annulus cell production of CCL2 (7). Here we hypothesized that exposure of human annulus cells to proinflammatory cytokines (IL-1β or TNF-α) might alter expression and production of CCL2, and tested for the presence of CCL2 in human annulus and nucleus tissue.

Methods: Following IRB approval, annulus cells were cultured from 11 patients with disc degeneration (5 Thompson grade II-III and 6 grade IV-V discs) in 3D collagen sponges and exposed for 14 days to either control conditions or 10² pM IL-1β or 10³ pM TNF-α as previously described (7). Conditioned media were assayed for CCL2 content using Procarta Cytokine Human 30 Plex Assay plates/Bio-Plex Array Reader (assay limit of detection <1 pg/ml). SAS® version 9.2 was used for analyses. Total RNA was extracted, reverse transcribed to double-stranded cDNA, subjected to two rounds of transcription, and hybridized to the DNA microarray in the Affymetrix Fluidics Station 400. GCOS Affymetrix GeneChip Operating System was used for CCL2 gene expression analysis (Affymetrix probe 216598_3p_s_at); GeneSifterTM web-based software was used for data analysis following correction for FDR. Statistical significance was determined using the student t-test or ANOVA; significance p<0.05.

Immunohistochemical studies were performed using antigen retrieval on paraffin-embedded disc specimens; the anti-MCP-1/4/eotaxin (B-2):sc-377082 antibody was employed (a mouse monoclonal antibody raised against amino acids 1-99, the full length MCP-1 in humans; Santa Cruz Biotechnology Inc) at a 1:25 dilution for 120 min. Endogenous peroxidase was blocked using 3% H₂O₂ (Sigma, St Louis, MO). The secondary reagent was Vector Immpress Reagent, Mouse (Vector Laboratories, Burlingame, CA) for 30 minutes followed by DAB (Dako) for 5 minutes. Slides were rinsed in water, counterstained with light green, dehydrated, cleared and mounted with resinous mounting media. Three grade I, 4 grade II, 3 grade III, 4 grade IV, and 3 grade V discs were utilized and digital images captured. Human adrenal tissue served as a positive control, and normal mouse IgG was used for the negative control.

Results: CCL2 levels in conditioned media showed significant differences by repeated measures ANOVA analysis (p=0.007). Data from cells from discs of all grades showed a significant increase in CCL2 media content from cells treated with IL-1β (Fig. 1A, *, p=0.016 vs. control). Levels in media from cells from all grades treated with TNF-α did not differ from controls, and were significantly lower than the IL-1β levels (p=0.003, Fig. 1A, #). Levels in media from grades IV-V disc cells treated with IL-1β showed a significant elevation vs. control (p<0.01, Fig. 1B #).
Molecular analyses showed that cells exposed to IL-1ß showed a 5.5 fold upregulation in CCL2 gene expression vs. controls, p=0.017. Cells exposed to TNF-α showed a 7.7 fold upregulation vs. controls, p=0.005.

Immunolocalization of CCL2 in human disc tissue showed the presence of many positive cells in annulus (Fig. 2A-C, spindle-shaped and round annulus cells) and in some cells in the nucleus (Fig. 2D. Cells not showing localization are marked by arrows). Figure 2E shows a negative control specimen. (Magnification bar = 20 µm).

**Discussion:** Although considerable new knowledge is being gained about the roles of cytokines within the disc, this remains a field of high research interest and clinical relevance. CCL2 is produced by several cell types, including endothelial cells, fibroblasts, muscle cells, and monocytic and microglial cells. It plays important roles in immune surveillance and modulation. The method we used here with isolated annulus cells cultured in 3D more closely mimics in vivo conditions than do monolayer cultures or disc explant cultures. The present cell 3D culture method contained only annulus cells in contrast to explant studies which might contain monocytes or other invasive cells in specimens from herniated discs. Novel data presented here show that annulus cells exposed to IL-1ß significantly upregulated their gene expression levels for CCL2, and that cells secreted significantly greater amounts of CCL2 into the culture media following proinflammatory cytokine exposure.

**Significance:** Work presented here showed novel data illustrating upregulation of CCL2 in annulus cells from grade V discs, and showed that cells exposed to TNF-α also upregulated CCL2 expression. CCL2 continues to be of interest in both research and clinical studies with respect to its role in the resorption of herniated disc tissue, and its potential role interacting with inflammatory mediators in low back pain, which is still poorly understood. We look forward to future work which will help clarify the important roles of CCL2 in disc aging and degeneration.

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**Figure 1**

![Graphs A and B](image)