Differential Sensitivity of Annulus Fibrosus and Nucleus Pulposus Cells of the Intervertebral Disc to Growth Factors and Cytokines

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INTRODUCTION: The intervertebral disc (IVD) allows motion and also functions in shock absorption and resistance to tensile and torsional forces. The function of the IVD is determined by its molecular composition. The IVD consists of two structurally distinct regions: the nucleus pulposus (NP) and the annulus fibrosus (AF). Within the AF, two areas, the outer AF (o-AF) and the inner AF (i-AF) can be further distinguished. The o-AF possesses large collagen fibers in oblique layers; the fibrocartilaginous i-AF is also rich in collagen lamellae. The central NP contains a loose matrix that is rich in proteoglycans (PGs).

To maintain this intricate structure, a balance between anabolic and catabolic processes, driven by cytokines and growth factors as well as their inhibitors, must be maintained. When the IVD degenerates, this fine balance is shifted toward catabolism, resulting in the loss of matrix molecules and the collapse of the IVD. It is believed that interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-alpha) are the two cytokines that are most involved in driving catabolism, while a variety of growth factors, including the epidermal growth factor (EGF), the insulin-like growth factor (IGF) and different bone morphogenetic proteins (BMPs), are responsible for activating anabolic processes. Among several inhibitory and regulatory molecules, we have shown that one of the PGs, biglycan, acts through the EGF receptor [1] and can balance the effects of cytokines and growth factors [2].

The purpose of this study was to delineate whether these cytokines, growth factors and regulatory molecules would act with equal efficiency on the cells that populate the structurally different areas of the IVD. These findings may be helpful in understanding which areas of the IVD are most vulnerable to catabolic attacks and, in the future, may orient efforts to targeted restoration of the matrix after IVD degeneration.

MATERIALS AND METHODS: IVD specimens were aseptically dissected from ten bovine tails purchased from the Aurora Packaging Company. IVDs were divided into four areas, the outer-AF (o-AF), the inner-AF (i-AF), the NP and the intermediate area between the AF and NP. Cells were released by sequential enzymatic digestion with pronase and collagenase and were used without passage or after a maximum of one passage in culture.

Immunocytochemistry: Cells isolated from the four areas mentioned above were plated in chamber slides, cultured overnight, and fixed with 2% paraformaldehyde. Primary antibodies to biglycan, EGF, TNF-alpha, IL-1beta and EGF receptor were applied followed by Oregon-green-labeled secondary antibodies. Signals were observed under a fluorescent microscope (Nikon Eclipse E600) equipped with MetaMorph software.

Effect of cytokines, growth factors and biglycan: IVD cells from the four areas were cultured separately in monolayers and treated for ten minutes (found to be optimal for signals for all factors used) with different concentrations of biglycan (20-80 ng/mL), EGF (0.1-50 ng/mL), IL-1 (0.01-10 ng/mL) or TNF-alpha (1-100 ng/mL). After treatment, cells were lysed and signaling molecules were detected using specific antibodies (Cell Signaling) on Western blots. Phosphorylation/activation was detected for the following signaling molecules: biglycan and EGF - Erk1/2 and Akt; TNF-alpha and IL-1 - Erk1/2 and NFkB. Signals were visualized using the ECL assay. The intensities of the protein bands on X-ray films were measured and normalized to the signal of beta-actin.

RESULTS: Immunostainings were positive for biglycan, EGF and EGF-receptor in both AF and NP areas and showed a slightly stronger presence in the AF compared to the NP. No signal was detected for IL-1 or TNF-alpha.

In cells from all four IVD regions, biglycan and EGF were found to signal through the Erk (MAPK) and the Akt (PI3K) pathways, while TNF-alpha and IL-1 activated the NFkB and Erk (MAPK) pathways. Biglycan was able to activate signals in all cells regardless of their localization in the IVD. Interestingly, EGF, which shares its receptor with biglycan, showed up to a 6-fold stronger effect on the AF than on the NP in the following order: o-AF > intermediate area = i-AF > NP (Figure 1). On the contrary, the effect of IL-1 and TNF-alpha was the exact opposite as that of EGF; they had a much stronger effect on the NP than the AF in the order of NP > i-AF = intermediate area >> o-AF. In the case of TNF-alpha (Figure 2.), a very strong signal was detected in the NP and almost no signal was detected in the o-AF.

Fig. 1. EGF signaling through Erk1/2 in cells from the o-AF, i-AF, intermediate area and NP. Please note that the y-axis is a logarithmic scale.

Fig. 2. TNF-alpha signaling through the NFkB pathway in cells from the o-AF, i-AF, intermediate area and NP. Please note that the y-axis is a logarithmic scale.

DISCUSSION: In this study, we have made several notable observations regarding the differences in sensitivity of the IVD cells to cytokines and growth factors. Although the response of the cells from the intermediate area between AF and NP were similar to that of the i-AF cells, there were significant differences among the NP, the i-AF and the o-AF cells in this respect. There are several novel findings in this study. First, the cells that reside in these structurally different compartments of the IVD preserve their differences even when isolated from their respective matrices, as demonstrated by their disparity in the signaling initiated by the effectors used in this study. Second, cytokines and growth factors may not affect all cells to the same extent. For example, EGF is very effective on the cells of the AF, especially of the outer AF, while it is less active in the cells of the NP. TNF-alpha and IL-1 act in the opposite direction, strongly targeting the NP and affecting the AF cells to a lesser extent. The outer AF cells seem to be especially resistant to these cytokines. Third, biglycan, which may serve as a regulator of both anabolic and catabolic processes [2], can activate signals in all cells with almost the same efficacy. These findings could partially be explained by the fact that these cells are programmed to establish different matrices [3]. Thus, they must possess different biological machinery, including the nature and quantity of their cell surface receptors. This was partially shown by our immunostaining for the EGF receptor.

These findings may predict which area of the IVD would be most affected by catabolic events and thus be the first to develop degeneration. Furthermore, restoration efforts can be targeted to those areas where they will be most helpful. For example, growth factors may be very effective in restoring the AF while cytokine antagonists may be more efficient in counteracting the degeneration process in the NP. The regulatory role of biglycan, the molecule that is effective on every cell of the IVD, may be important in restoring the balance in metabolism. Thus, biglycan could be beneficial for the health and repair of the IVD.

REFERENCES:

ACKNOWLEDGEMENTS: This work was supported by the NIH (PO1AR-48152).