Mechanism of Proteoglycan Synthesis by Bovine and Human Link N

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Introduction: Link N peptide is the N-terminal region of link protein which stabilizes the interaction between aggrecan and hyaluronan in proteoglycan aggregates. In vivo, during tissue turnover, link protein is subject to proteolytic degradation by stromelysin and gelatinase A and B generating Link N peptide. It has been shown that human Link N (DHLSDNYTLDHDRAIH) can act as a growth factor and stimulate the synthesis of proteoglycans and collagen by IVD cells in vitro and improve disc height and proteoglycan levels in vivo in a rabbit model of IVD degeneration [1-3]. To date, there have been no reports on the effect of bovine Link N (DHHSNVTVDHRVIIH) on disc cells. The specific aim of this study is to compare the effects of bovine Link N (BLN) and human Link N (HLN) on bovine IVD cells in order to determine whether substitution of residues, as occurs in the BLN sequence, can alter Link N function.

Methods: Coccygeal IVDs from healthy 20-24 month old steers were obtained from a local abattoir at 2-3 hours after slaughter. The IVDs were separated from their adjacent vertebral bodies, and the NP and AF cells were isolated from the nucleus pulposus (NP) and annulus fibrosus (AF) regions by sequential digestion with 0.2% Pronase followed by 0.125% collagenase digestion as previously described [4]. After isolation, the NP and AF cells were either immediately embedded in 1.2% alginate beads (350,000 cell/bead) for proteoglycan synthesis or were plated in 6 well plates for protein extraction.

Proteoglycan synthesis: After 7 days of stabilization in complete DMEM high glucose medium, the alginate beads were placed in 24 well plates at a density of 9 beads/well and were incubated for 18 days in media supplemented with 1µg/ml of either HLN or BLN (CanPeptide, Montreal). Beads cultured in media alone for the same period of time were used as the control (CTL). The culture media with or without Link N was changed every third day for 18 days and the sulfated glycosaminoglycan (GAG, predominantly aggrecan) content of the media was analyzed using the 1,9-dimethylmethylen blue (DMMB) dye-binding assay.

Canonical SMAD-Mediated Signaling: AF and NP cells were expanded in culture medium (Dulbecco’s Modified Eagle Medium high glucose supplemented with 10% fetal bovine serum) into 6 well plates (7.5x10^5 cells/well) until reaching 80-90% confluence. The cells were pre-incubated overnight in serum-free medium, then were incubated in 1µg/ml HLN or BLN for different time points up to 6 hours. Cells incubated in medium alone were use as the control (CTL). After 6 hours of incubation, AF and NP cells were then lysed and western blots were used to measure protein expression using specific antibodies directed against P-Smad 1/5, P-Smad-2 (Cell Signaling Technology, Danvers, MA). The phosphorylation of Smad 1/5 and Smad 2 were normalized to the corresponding Smad 1 and Smad 2 total proteins.

All experiments were performed in triplicate and were repeated with three independent cultures. The effect of treatment and culture period as well as the significance of differences among the treatment groups (CTL, BLN and HLN) at each time point was assessed by repeated measures one-way ANOVA followed by Turkey's Multiple Comparison Test with a significance level of P<0.05.

Results: Most of the GAG synthesis was detected in the culture media and there was minimal retention (approx 1%) in the alginate beads. Both NP and AF cells incubated with Link N (BLN or HLN) had an increased rate of GAG release into the culture media with time. This increase was significantly higher for AF cells incubated with BLN or HLN compared to the AF control cells (p<0.001) after 9 days of incubation. On the contrary, the NP cells had a significant and consistent increase only for HLN. Western blot results revealed that HLN activates SMAD 1/5 in bovine AF cells within 5 minutes, while the activation with BLN occurred within 10 minutes, achieving maximum activation at 30 minutes (Figure 1). For both Link N supplementations, SMAD 1/5 levels in AF cells decreased to below the control levels after 6 hours. In NP cells, BLN and HLN supplementation significantly stimulated SMAD 1/5 after 30 minutes and continued to increase with time. However, for both IVD cells, HLN appeared to be more effective at SMAD 1/5 activation than BLN. In AF cells, incubation in either HLN or BLN seemed to induce a slightly increased SMAD 2 activation up to three hours. In contrast, no SMAD 2 activation was detected in NP cells incubated with Link
Discussion: BLN is also capable of stimulating GAG release in vitro in bovine IVD cells by activation of SMAD 1/5. The rapid activation within 10 minutes of SMAD 1/5 by BLN in AF cells may explain our finding that AF cells respond better than NP cells to BLN supplementation in promoting proteoglycan synthesis. Therefore in principle, BLN supplementation could also be an option for treating disc degeneration. However, HLN at the concentration of 1ug/ml is more effective at stimulating proteoglycan synthesis and can directly activate SMAD 1/5 signaling (within 5 min) in the AF, which is the main source of proteoglycan synthesis with age and degeneration. Although, both peptides have features needed for any agent designed to stimulate disc repair, HLN supplementation could be a better option for treating disc degeneration during its early stages, while the AF is still intact.

Significance: One major advantage of Link N over a growth factor such as OP-1 for therapeutic use is the large saving in cost.

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