Journal of Cellular Biochemistry 88:1202–1213 (2003)

A Synthetic Peptide of Link Protein Stimulates the Biosynthesis of Collagens II, IX and Proteoglycan by Cells of the Intervertebral Disc

Fackson Mwale,[1]* Caroline N. Demers,[1] Alain Petit,[1] Peter Roughley,[2] A. Robin Poole,[3] Thomas Steffen,[4] Max Aebi,[4] and John Antoniou[1]

[1]Lady Davis Institute and Department of Surgery, McGill University, 3755, Chemin de la Cote Ste. Catherine, Montreal, Quebec H3T 1E2, Canada
[2]Genetics Unit, Shriners Hospitals for Children and Departments of Surgery and Medicine, McGill University, 1529 Cedar Ave, Montreal, Quebec H3G 1A6, Canada
[3]Joint Diseases Laboratory, Shriners Hospitals for Children and Departments of Surgery and Medicine, McGill University, 1529 Cedar Ave, Montreal, Quebec H3G 1A6, Canada
[4]Orthopaedic Research Laboratory, Royal Victoria Hospital, McGill University, 687 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada

Abstract To date, there have been no reports on the effect on disc cells of the intervertebral disc (IVD) of the amino terminal peptide of link protein (DHLSDNYTLDHDRAIH) (link N) which is generated by the cleavage of human link protein by stromelysins 1 and 2, gelatinase A and B, and collagenase between His^{16} and Ile^{17}. However, link N has been shown to act as a growth factor and stimulate synthesis of proteoglycans and collagen by chondrocytes of human articular cartilage. There are also no studies on the effect of link N on type IX collagen in any tissue. In the studies reported here, a serum-free pellet culture system has been used to examine whether link N can play a role in maintaining the integrity of disc matrix, specifically at the level of matrix assembly by cells of the IVD. Using this culture system, we determined the capacity of link N to stimulate accumulation of these matrix proteins in the annulus fibrosus (AF) and nucleus pulposus (NP). Gross inspection of separate AF and NP pellet cultures in the absence of link N revealed a progressive increase in size and a transition from ''spherical'' to ''polygonal'' pellets after centrifugation. Addition of 10 ng/ml link N resulted in increased pellet sizes for both AF and NP pellet cultures. Link N increased proteoglycan, type II and type IX collagen contents with an increase in DNA content over time. This study demonstrates that link N can act directly on disc cells to stimulate matrix production, which involves increased accumulation of proteoglycan, and types II and IX collagens. This study also identifies the value of pellet cultures for studies of the IVD cells in a serum-free chemically defined medium, in which pellets can continue growing in size in response to growth factors with minimal cell loss. Link N may have value in stimulating the growth and regeneration of the damaged IVD. J. Cell. Biochem. 88: 1202–1213, 2003.

Key words: collagen; proteoglycan; link protein; pellet cultures; intervertebral disc

Degeneration of the intervertebral disc (IVD) of the spine is a common feature of the human aging population [Bogduk, 1991; Buckwalter, 1995; Dai, 2000]. Our knowledge of factors regulating the turnover, repair, and degeneration of the IVD is lacking. IVDs are composed of two major anatomic zones: the annulus fibrosus (AF) and the nucleus pulposus (NP). The AF is composed of laminated fibrous tissue wrapped around the gelatinous NP [Parke and Schiff, 1971]. The NP is composed of a lattice framework of collagen embedded in a highly hydrated gelatinous mass. IVDs are characterized by their abundant extracellular matrix and low cell density, coupled with an absence of blood vessels, lymphatic system, and nerves in all but the most peripheral annular layers. The disc

Grant sponsor: Canadian Institutes of Health Research; Grant sponsor: Canadian Orthopaedic Foundation.

*Correspondence to: Dr. Fackson Mwale, Lady Davis Institute and Department of Surgery, McGill University, 3755, Chemin de la Cote St. Catherine, Montreal, Quebec H3T 1E2, Canada. E-mail: fmwale@ldi.jgh.mcgill.ca

Received 7 March 2002; Accepted 27 November 2002
DOI 10.1002/jcb.10479

is thought to resist compressive forces by a swelling pressure that exerts tensile forces on the fibrous collagen network. The ability of disc tissue to resist compressive forces is largely due to their high content of the proteoglycan aggrecan [Tengblad et al., 1984; Hutton et al., 1998]. Aggrecan is the largest proteoglycan found in the interfibrillar matrix.

At least eight distinct collagen types have been identified in the IVD: types I, II, III, V, VI, IX, X, and XI [Eyre and Muir, 1976; Wu et al., 1987]. The AF of the IVD contains all these collagens, whereas the NP contains collagen types I, II, VI, and IX only [Adams and Muir, 1976; Eyre and Muir, 1976; Kaapa et al., 1994, 1995]. Type X collagen is present in the disc when histomorphological alterations occur consistent with degeneration [Boos et al., 1997; Aigner et al., 1998]. Types I and II collagens are important for the integrity of the disc, as they form the fibrous framework of the tissue.

Link protein is a glycoprotein that stabilizes the non-covalent interaction between an aggrecan molecule G1 domain and hyaluronate [Hardingham, 1979; Kimura et al., 1979]. Human cartilage link protein can be separated by SDS/PAGE into three components of molecular mass 48, 44, and 41 kDa, referred to as LP-1, LP-2, and LP-3, respectively [Roughley et al., 1982]. LP-1 and LP-2 are different glycosylated forms of the same intact protein core, while LP-3 is proteolytically derived from either LP-1 or LP-2 [Mort et al., 1983]. In cartilage of newborns, LP-3 is a minor component generated by stromelysin 1 [Nguyen et al., 1989]. With age, LP-3 accumulates at the expense of LP-1 and LP-2 [Mort et al., 1983]. There is an accumulation of fragmented link protein molecules in adult cartilage, resulting from cleavages within the N-terminal disulphide-bonded loop. The amino-terminal peptide of link protein (DHLSDNYTLDHDRAIH) (link N) can be generated in vitro by the cleavage of human link protein by stromelysins 1 and 2, gelatinase A and B and collagenase between His^{16} and Ile^{17} [Nguyen et al., 1993] and is released in vivo by LP-3 generation.

It is well known that isolated IVD cells express their native phenotype only in three-dimensional cultures [Maldonado and Oegema, 1992; Yung Lee et al., 2001]. Although the maintenance of disc cells in pellet cultures stabilizes the disc phenotype, the lack of nutritional support in the central area of the pellet prevents further increase in size beyond 15 days [Yung Lee et al., 2001]. This three-dimensional pellet culture system established by Kato et al. [1988] offers advantages over conventional alginate bead microspheres, as the number of cells per pellet is easy to control and is simple to perform [Hall et al., 2001; Yung Lee et al., 2001].

We created pellet cultures of disc cells isolated from different anatomical regions, i.e., AF and NP, so that we can compare the effect of link N on the biosynthesis of proteoglycan and collagens types II and IX, separately. It has been shown that link N can act as a growth factor and stimulate synthesis of proteoglycans and collagen in articular cartilage [Liu et al., 1997, 1999, 2000; McKenna et al., 1998; Dean and Sansom, 2000]. We have investigated the effects of link N on cells of the IVD, which can respond to growth factors differently than cartilage [Thompson et al., 1991]. The recent development of immunoassays provided an opportunity to study the contents of types II and IX collagens in the extracellular matrix [Hollander et al., 1996; Mwale et al., 2000]. We show that link N stimulates proteoglycan and types II and IX collagens in IVD cells. Such information may be of value in maintaining the integrity of disc matrix.

MATERIALS AND METHODS

Source of Disc

Adult bovine tails (2–4 years old) were obtained from Les Abattoirs Corbex, Inc. (QC, Canada). All IVDs were classified as non-degenerate grade I according to the grading system of Thompson et al. [1990]. A total of 12 tails were used.

Cell Isolation

Cells from 12 bovine tails (approximately 15×10^6 cells from the AF per tail and 5×10^6 cells from the NP per tail) were isolated immediately after transportation from the abattoir. The IVDs were dissected from their adjacent vertebral bodies, placed in DMEM-high glucose (Biomedia, QC, Canada), with 20 mM HEPES, and 45 mM $NaHCO_3$, pH 7.4, containing (per ml) 150 ng gentamicin, 100 μg benzyl penicillin, 100 U streptomycin, and 0.25 μg fungizone (medium A) (InVitrogen Life Technologies, Burlington, ON, Canada). Under aseptic conditions, the IVDs were separated by dissection into regions corresponding to the AF and

the NP. The AF and NP dissected into approximately 2-mm thick fragments were washed twice in medium A for 15 min. Cells were enzymatically isolated from the tissue using a sequential protease type XIV/collagenase protocol [Maldonado and Oegema, 1992; Baer et al., 2001]. Briefly, 28 and 10 g of AF and NP tissue, respectively, were incubated at 37°C with gentle agitation (75 rpm) for 1 h in medium A with bacterial protease type XIV (Sigma, Oakville, ON, Canada) at 0.2% (w/v) for the NP and 0.4% (w/v) for the AF. The tissue was then washed and a second digestion was performed overnight at 37°C and 75 rpm in washing medium (medium A without gentamicin and fungizone) supplemented with 10% fetal bovine serum (FBS) (Biomedia) and bacterial collagenase type IA (Sigma) at 0.03% (w/v) for the NP and 0.06% (w/v) for the AF. The resulting cell suspensions were passed through a 70-μm cell strainer (VWR Canlab, Ville Mont-Royal, QC, Canada) and washed twice in washing medium containing 10% FBS, and cells were recovered by centrifugation at 400×g for 6 min. Cells were counted in a hemacytometer and the viability was determined using 0.04% Trypan Blue Dye.

Pellet Cultures

The cells were cultured as a three-dimensional pellet using a method originally described by Kato et al. [1988]. In all experiments, DMEM-high glucose with 20 mM HEPES and 45 mM $NaHCO_3$ pH 7.4 was supplemented with 5 mg/ml penicillin, 10 mg/ml streptomycin, 2 mM glutamine, 50 μg/ml ascorbic acid (prepared fresh), 1 mg/ml bovine serum albumin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite. Briefly, pellet cultures were prepared by adding 1×10^6 cells in 1 ml medium to 15-ml conical polypropylene centrifuge tubes. The cells were pelleted by centrifugation at 400×g for 6 min at room temperature. The resulting cell pellets were cultured in the same centrifuge tubes, with loosened lids, at 37°C and 5% humidified CO_2. Medium was changed every 2 days, and pellets were maintained up to 20 days in medium without (control) or with 10 or 100 ng/ml of link N. Fresh peptide was added with each change of the culture medium. Before each media change the pellet was centrifuged at 400×g for 6 min to maintain the integrity of the pellet and ensure that cells were not removed to the culture medium.

Synthesis of Link N

The N-terminal peptide of link protein (DHLSDNYTLDHDRAIH) was synthesized using Fmoc (9-fluoroenylmethoxycarbonyl) chemistry, on a solid phase peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA).

Scanning Electron Microscopy (SEM) Specimen Preparation

Pellet cultures were examined by SEM to study pellet growth. The pellets were fixed by overnight immersion in 5% glutaraldehyde, 0.1 M sodium cacodylate buffer pH 7.4 at 4°C, overnight. After rinsing three times with the buffer, the preparations were stepwise dehydrated in graded ethanol (25, 50, 95, and 100%, 60 min each). The polypropylene tubes were cut close to the pellet and the dried pellets were mounted on SEM specimen stubs, and coated with gold/palladium. SEM was performed using SEM (Jeol JSM-840A) at an accelerating voltage of 10 kV. Image acquisition was performed using an EDAX Phoenix microanalysis system.

Analyses of Cell/Matrix Proteins

The contents of cell/matrix proteins were analyzed in pellets and culture media. The pellet was rinsed twice with 2 ml of PBS and frozen at −20°C until analysis. After thawing, the harvested material was digested with α-chymotrypsin followed by proteinase K [Hollander et al., 1994]. Aliquots were taken and the contents of DNA, glycosaminoglycans (predominantly from the proteoglycan aggrecan), total type II collagen content, and type IX collagen content were determined as described below.

DNA

DNA was measured using Hoechst dye [Labarca and Paigen, 1980].

Determination of Glycosaminoglycan Content

To measure sulfated glycosaminoglycans (predominantly proteoglycan aggrecan), 10-μl samples of α-chymotrypsin and proteinase K digests of pellets and media were analyzed using the 1,9-dimethylmethylene blue (DMMB) dye binding assay [Farndale et al., 1986]. The results of analyses were totaled to obtain total proteoglycan content per culture tube.

Extraction and Immunoassay of Total Type II Collagen and its Denaturation

Total type II collagen was measured by immunoassay using the COL2-3/4m epitope [Hollander et al., 1994]. This epitope is not destroyed by α-chymotrypsin or proteinase K treatment.

Immunoassay for Determination of Type IX Collagen NC4 and COL2 Domains of the α1(Chain)

These ELISAs were described recently [Mwale et al., 2000]. They were used to analyze the media, α-chymotrypsin and proteinase K extracts. The results of the analyses of α-chymotrypsin and proteinase K in the pellet and media were totaled to obtain type IX collagen content in the tube. Both the NC4 and COL2 epitopes are stable under α-chymotrypsin and proteinase K treatment. Total type IX collagen content was obtained with the COL2(IX) immunoassay.

Collagen Synthesis

Cultures were labeled with 25 μCi/ml of [^{3}H] proline (Amersham Biosciences, Inc., Baie d'Urfe, QC, Canada) for 24 h. Thereafter, corresponding media were frozen at −20°C until analyzed. Collagens were isolated from the media by 30% saturated ammonium sulfate (v/v), followed by digestion in 0.5 M acetic acid with 150 μg/ml pepsin (Sigma, P-7012) at 4°C overnight. Collagens of the cell/matrix layer, after harvesting the samples in 1 ml of TMT (10 mM Tris pH 7.5, 0.5 mM $MgCl_2$, 0.1% Triton X), were isolated by incubating with 0.5 M acetic acid containing 10 μl of a protease inhibitor cocktail (Sigma, P-8340) and 150 μg/ml pepsin (Sigma, P-7012). Collagen present in the supernatant after centrifugation (pepsin soluble collagen) was precipitated with trichloroacetic acid (15% w/v) and washed with acetone. Precipitated collagens from the media and the cell pellets (pepsin soluble and pepsin insoluble collagens) were analyzed directly by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS–PAGE) using 4–20% gradient gels, followed by autoradiography [Mwale et al., 2000]. Gradient gels were soaked in 500 ml of a 5% glycerol (w/v), 40% methanol, and 10% acetic acid for 3 h with constant gentle agitation to ensure crack-free drying.

Immunoblot Analysis

Electrophoretic transfers to nitrocellulose membranes (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) were performed as previously described [Mwale et al., 2000]. Briefly, the PBS–3% BSA blocked membranes were incubated overnight at 4°C with anti-NC4 antibodies diluted 1:1,000 in PBS–3% BSA–Tween. After three 10-min washes in PBS–Tween, the membranes were incubated at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L) diluted 1:30,000 with PBS–3% BSA–Tween. The membranes were given 3 × 10-min washes in PBS–1% BSA–Tween before adding alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium solution from a commercial kit (Bio-Rad Laboratories Ltd.).

Statistical Analysis

All experiments were performed in triplicates. Statistical differences between the treated (10 and 100 ng/ml link N) and the control were analyzed by Statview (version 5.0.1, SAS Institute, Cary, NV) for each culture time. Results were considered significant with a $P < 0.05$.

RESULTS

Morphological Analysis of Pellet Cultures

This report determines the effect of link N on disc cells in terms of the accumulation of proteoglycan, and type II and type IX collagens in a serum-free pellet culture system. Gross inspection of IVD pellet cultures revealed a progressive increase in volume over time (data not shown). Addition of 10 ng/ml of link N resulted in an increase in pellet volume of the AF and the NP cells.

Examination by SEM of pellet cultures of the NP on day 20 revealed a compact matrix (Fig. 1A). At higher magnification of the insert in (Fig. 1A) the absence of identifiable lacunae is clearly evident (Fig. 1B). Figure 1C shows the SEM image of a NP pellet culture with 10 ng/ml of link N added. Here a marked difference in the morphology of the pellet cultures was seen where a dense "layered" matrix was produced. Figure 1D represents a higher magnification of the insert in (Fig. 1C) emphasizing the layers. Similar observations were obtained with annulus cells (data not shown).

DNA Content

DNA content showed a steady increase when both AF and NP cells were cultured in serum-

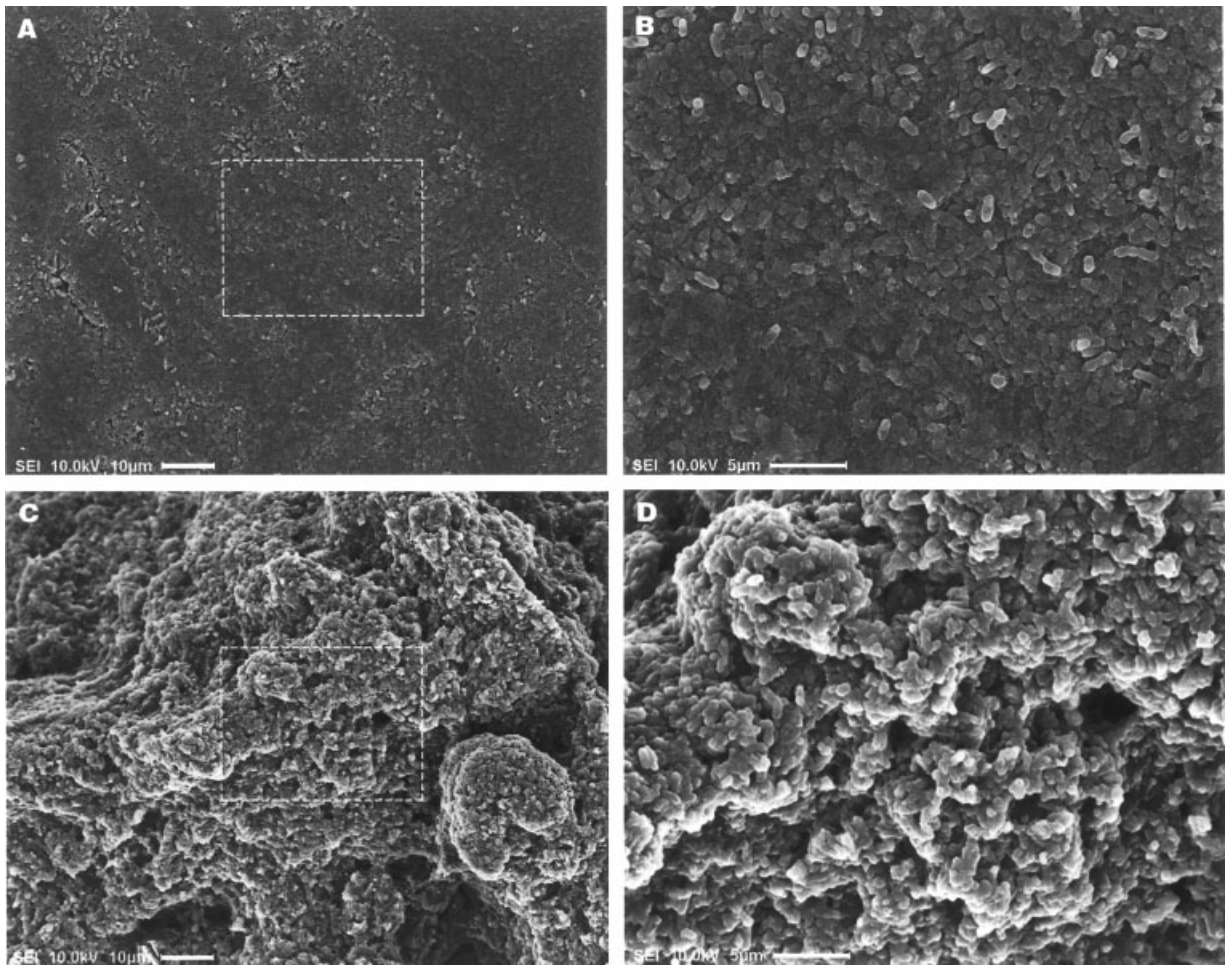

Fig. 1. Scanning electron microscopy (SEM) of the morphology of NP cell pellet cultures. **A**: Overview of the control NP cell pellet at day 20 (magnification × 1,000). **B**: Higher magnification of the insert (dotted lines) shown in (A) (magnification × 3,000). **C**: Overview of the 10 ng/ml of link N treated NP cell pellet at day 20 (magnification × 1,000). **D**: Higher magnification of the insert (dotted lines) shown in (C) (magnification × 3,000). Note the marked difference in the morphology between the ''compact'' control and ''dense-layered'' pellet cultures upon addition of link N.

free medium, but with no significant difference when link N was added (Fig. 2A,B, respectively), suggesting that the growth in size of pellets could partly be due to cell proliferation. Previously, no significant increase in DNA was shown [Chiba et al., 1997; Gruber et al., 1997; Hall et al., 2001]. Addition of link N to human articular cartilage did not alter DNA content [McKenna et al., 1998]. Similarly, prehypertrophic growth plate chondrocytes did not proliferate in this serum-free medium [Mwale et al., 2000].

Synthesis of Extracellular Matrix: Analyses of Pellet Cultures

Proteoglycan content. Figure 3 shows the assembly of proteoglycan in pellet cultures with time. There were progressive increases in proteoglycan content throughout the culture and it peaked between days 16 and 20. Link N, when added at 10 or 100 ng/ml to pellet cultures, stimulated proteoglycan synthesis (pellet plus medium per DNA), in pellet cultures of AF cells (Fig. 3A). The assembly of proteoglycan in the AF was enhanced by the addition of 100 ng/ml of link N reaching its highest level by day 16 (19 ± 4 µg/µg of DNA).

Total proteoglycan content normalized to DNA increased significantly in the NP with time (Fig. 3B). Stimulation of proteoglycan content in the NP reached its highest level by day 16 (33 ± 3 µg/µg of DNA), and increased progressively with the addition of 100 ng/ml of link N. A similar increase was observed with 10 ng/ml of

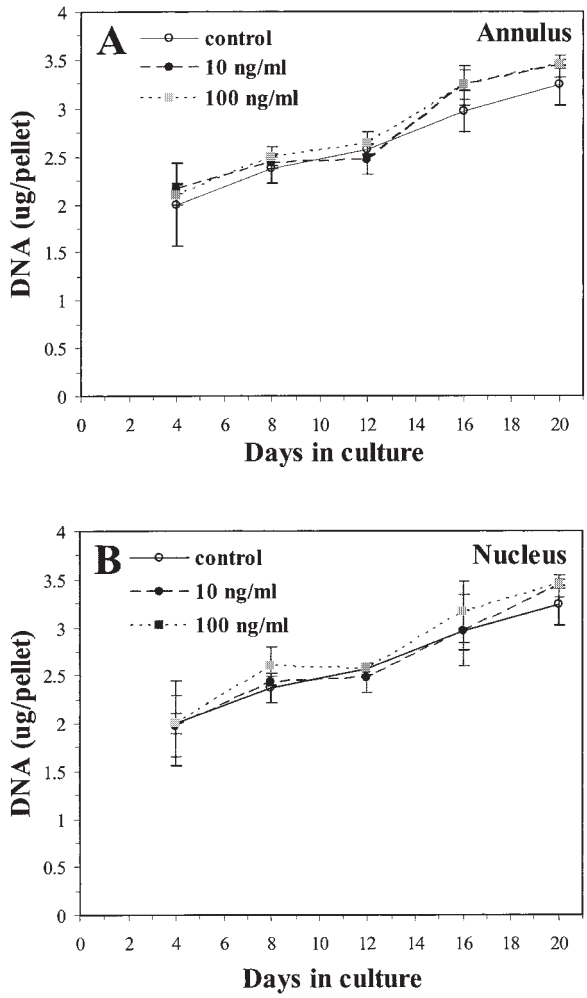

Fig. 2. Effect of link N on disc cell proliferation in pellet cultures. Annulus fibrosus (AF) cells (**A**) and NP cells (**B**) were cultured in the absence of link N or with 10 and 100 ng/ml of link N. The mean ± standard deviation of total DNA content (in triplicate) are shown in a typical experiment. Note the steady increase in cell proliferation and that link N did not significantly affect cell proliferation.

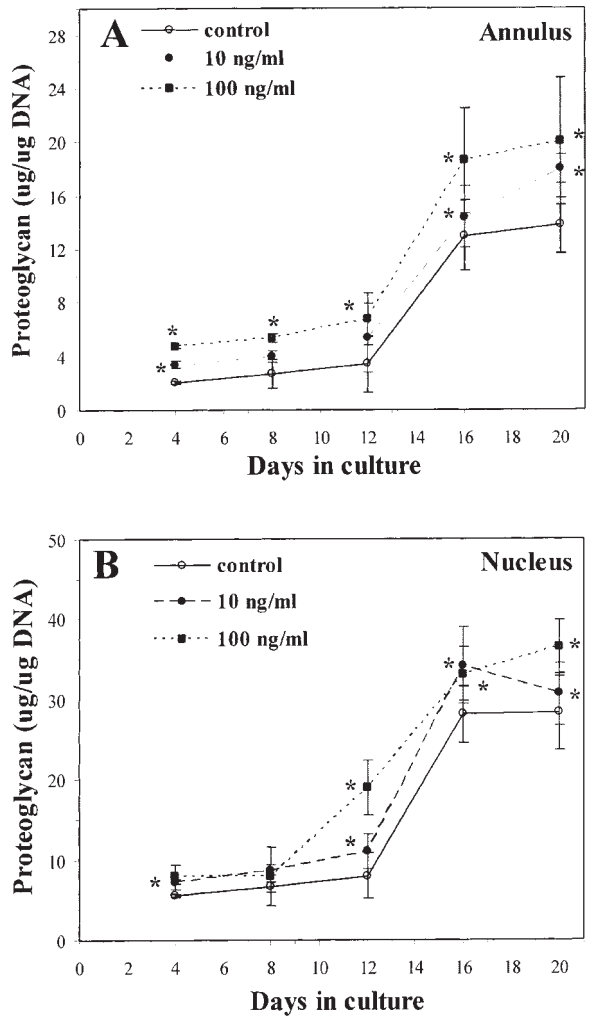

Fig. 3. Proteoglycan deposition in disc cell pellet cultures and enhancement by link N. Total proteoglycan content per DNA in pellet cultures of adult bovine AF (**A**) and NP (**B**) cultured without (control) or with 10 or 100 ng/ml of link N. The results of total α-chymotrypsin and proteinase K analyses of cell pellets and of media are shown. Results show the content of proteoglycan with the peptide or control without the peptide, and represent the mean ± standard deviation of triplicates. Asterisks indicate values significantly different from the control ($*P < 0.05$). Note increased deposition of proteoglycan and enhancement by the addition of link N.

link N with, however, a decline on day 20. The results suggest that link N can stimulate proteoglycan synthesis in cells from the AF and NP.

Previously, link N was shown to upregulate synthesis of proteoglycan from human articular cartilage by amounts ranging from 20% to as much as 270% [McKenna et al., 1998]. Link N was also found to potently stimulate the synthesis of proteoglycans with normal glycosaminoglycan chains in human articular cartilage and the macromolecular characteristics of cartilage proteoglycans did not change when synthesis was upregulated by link N [Liu et al., 1999].

Total type II collagen content. Figure 4 shows the production of type II collagen in serum-free medium monitored by the COL2-3/4m immunoassay [Hollander et al., 1994]. Type II collagen content in the AF cell cultures increased progressively with time. Previous studies have shown that link N can stimulate biosynthesis of collagen by human articular cartilage [Liu et al., 2000]. From day 12 in the AF, link N increased type II collagen content (Fig. 4A). Levels of type II collagen reached peak values on day 12, averaging 230 ± 21 pmol/μg DNA when 100 ng/ml of link N was added.

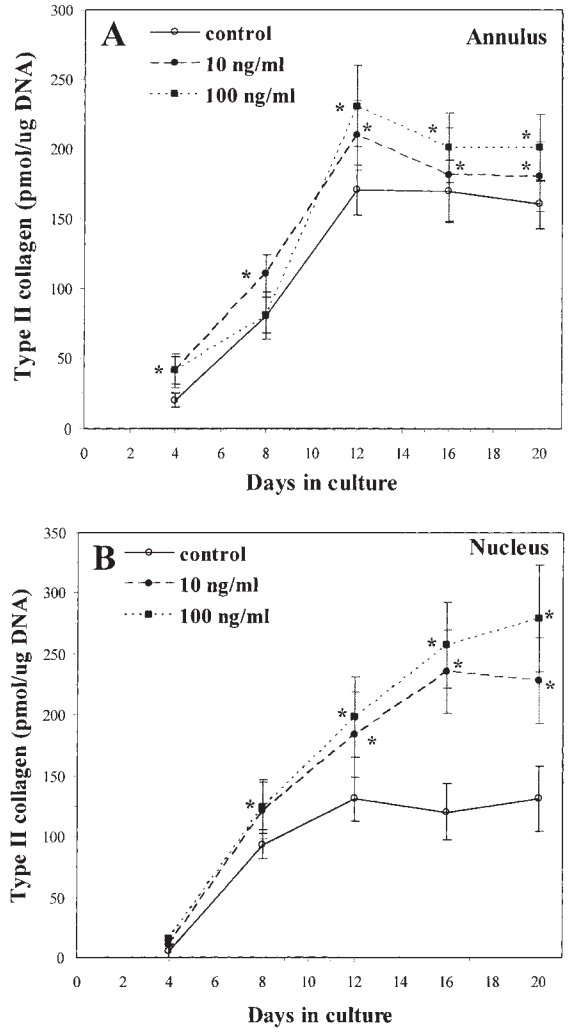

Fig. 4. Type II collagen content in cultures and enhancement by link N. Progressive accumulation of type II collagen content was observed in adult bovine AF cell (**A**) and NP cell (**B**) cultures. This was enhanced by link N. Results show the content of type II collagen per DNA with the peptide or control cultures without the peptide and represent the mean ± standard deviation of triplicates. Asterisks indicate values significantly different from the control (*$P < 0.05$).

Figure 4B shows that total type II collagen content also increased with time in cultures of NP cells. Link N enhanced this increase in type II collagen content ranging from day 12 with a mean of 184 ± 35 to 228 ± 35 pmol/μg of DNA when 10 ng/ml of link N was added and of 198 ± 33 to 279 ± 44 pmol/μg of DNA when 100 ng/ml of link was added.

Type IX collagen content. Figure 5 shows that type IX collagen content represented by the COL2 (Fig. 5A,B) and NC4 (Fig. 5C,D) domains increased in cultures of both AF (Fig. 5A,C) and NP (Fig. 5B,D). Link N stimulated type IX collagen accumulation. The increase in total type IX collagen content (COL2) in the AF was high, ranging from a mean of 0.58 ± 0.12 pmol/μg of DNA (control) to as much as 0.71 ± 0.08 pmol/μg of DNA when 10 ng/ml of link N was added and to 0.89 ± 0.07 when 100 ng/ml of link N was added on day 20 (Fig. 5A). Figure 5B shows the increase with time in total type IX collagen (COL2 domain) content in the NP. Enhancement of type IX collagen content by link N reached peak values around days 18 and 20 when 100 ng/ml of link N was added suggesting that link N can stimulate total type IX collagen synthesis in cells from the AF and the NP. Figure 5C,D shows that the long form of type IX collagen represented by the NC4 domain is also enhanced by addition of link N in both the AF and NP.

The molar ratio of type II collagen to α1(IX) COL2 collagen in the AF (Fig. 6A) was similar to that of type II collagen to α1(IX) NC4 collagen (not shown), averaging in the range of 116–235:1 (lowest values on days 4 and 20), while that of type II collagen to α1(IX) COL2 collagen (Fig. 6B) and α1(IX) NC4 collagen (not shown) in the NP averaged in the range of 22–149:1, suggesting that there are much less type IX collagen molecules in the disc compared to the growth plate. During matrix assembly, the molar ratio of type II/COL2 domain of the α(IX)-chain in the growth plate varied from 8:1 to 25:1 [Mwale et al., 2002]. The molar ratio of the COL2/NC4 domain in the NP and AF was approximately 1:1 (Fig. 5) suggesting that all molecules of type IX collagen have the NC4 domain which differs from the growth plate were the majority (90%) of type IX molecules lack the NC4 domain [Mwale et al., 2000].

Table I shows the percent increase in proteoglycan, type II collagen and type IX collagen content (COL2 and NC4 domains) in the AF and the NP cell pellet cultures, when treated with 10 and 100 ng/ml of link N, compared to the control. Enhancement by link N of type IX collagen content in the AF, measured as NC4, increased, ranging from 17 to 55%. Enhancement by link N of type IX collagen content in the AF, measured as COL2, also increased, ranging from 22 to 53%. Similar results were obtained in the NP. Enhancement of proteoglycan content by link N in the AF increased, ranging from 30 to 45%, while that in the NP increased from 8 to 28%. Type II collagen increased from 74 to 113% in the NP and 13 to 25% in the AF. This data

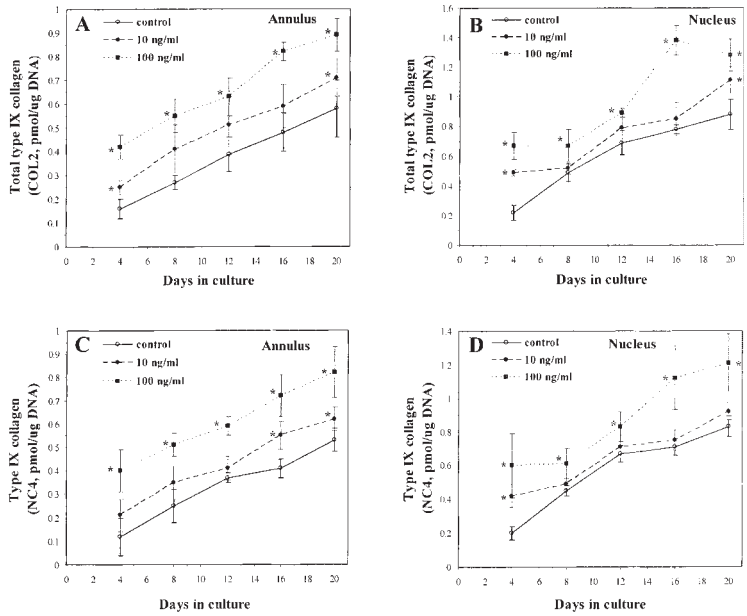

Fig. 5. Type IX collagen content in cultures and enhancement by link N. Type IX collagen content represented by the COL2 (**A**, **B**) and NC4 (**C**, **D**) domains increased in cultures of both AF (**A**, **C**) and NP (**B**, **D**) in a time-dependent manner. Results show the content of type IX collagen per DNA with the peptide or control without the peptide and represent the mean ± standard deviation of triplicates. Asterisks indicate values significantly different from the control ($*P < 0.05$).

suggests that link N can stimulate proteoglycan, types II and IX collagens in cells from the NP and the AF.

Further, we decided to gain some insight into the potential mechanism of how link N stimulates collagen biosynthesis by analyzing collagen synthesis rates on protein level, rather than on collagen amounts deposited as they would tell us whether our reported effect is owing to enhanced collagen synthesis or retarded degradation. To specifically analyze the collagens, the [^{3}H] proline-labeled media were precipitated by the addition of $(NH_4)_2SO_4$ and analyzed by autoradiography [Mwale et al., 2000]. Using this approach, quite variable amounts of collagen bands appeared to be synthesized at different stages of the culture (Fig. 7). Large amounts of type II collagens were detected in the media and this was enhanced by the addition of link N. Type II collagen (~120 kDa) began to be predominantly expressed in the media at early time points. While there was a large range in the size of proteins synthesized, particularly prominent bands were evident at 200, 120, 102, and 78 kDa. To specifically analyze type IX collagen a Western blot using anti-NC4 antibodies was performed [Mwale et al., 2000]. Figure 8 shows that type IX collagen was detected in the AF as well as the NP, and this was enhanced by the addition of link N. These results suggest that the observed stimulation by link N is due to enhanced collagen synthesis and not due to retarded degradation.

DISCUSSION

Previous studies have shown that the link protein amino terminal peptide DHLSDNYTLDHDRAIH (link N), which can be removed by proteolysis from the N-terminus of the link protein of cartilage proteoglycan aggregates, can act as a growth factor and stimulate synthesis of proteoglycans [McKenna et al., 1998] and collagens [Liu et al., 2000] in human articular cartilage. There have been no studies of the effects of the peptide on cells of the IVD or its effect on specific collagens such as types II and IX.

Here we show that link N at a concentration of 10 and 100 ng/ml can stimulate matrix assembly in cultures of the NP and AF by increasing production/accumulation of proteoglycans and collagens II and IX, while having no effect on cell division. These results of enhanced matrix assembly are shown by the morphological analyses. The concentrations of link N used were chosen because earlier experiments with human articular cartilage demonstrated that significant stimulation was achieved at a

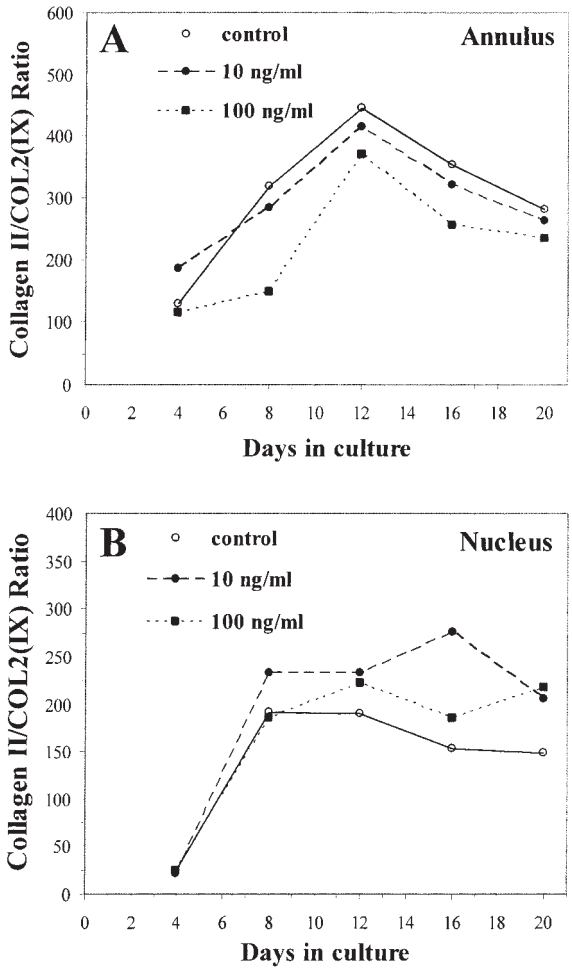

Fig. 6. Effect of link N on type II/IX molar ratios. Analyses of the molar ratios of total type II to total type IX contents measured as the COL2 domain for adult baine AF cell (**A**) and NP cell (**B**) cultures. Data shown of type II/type IX collagen molar ratios are means of pellet cultures for the experiments described in Figures 4 and 5.

concentration of link N that varied between 10 and 1,000 ng/ml, with optimum stimulation at 100 ng/ml of link N [McKenna et al., 1998; Liu et al., 2000]. Assuming a 1:1 ratio of link protein to aggrecan and the molecular weight of the peptide to be 1,760 Da, with that of link protein being 49 kDa, we calculate that addition of 100 ng/ml of link N corresponds to approximately 3.6% of total peptide content of link protein in the disc or culture at day 20 suggesting that the concentrations used are in the physiological range.

Our results suggest that degradation products of link protein generated by matrix metalloproteinases can "feed-back" and stimulate matrix assembly. They may, therefore, have a regulatory role in maintaining matrix integrity in the IVD. Since link protein is present in normal disc, with and without its N-terminal peptide [Donohue et al., 1988; Liu et al., 1991], the peptide must be generated extracellulary within the disc. Its concentration would be dependent on the rate at which link protein was synthesized and the degree of proteolysis. Therefore, as previously postulated, link N could be a primary indicator to disc cells that matrix degradation was taking place, signaling to the cells to increase synthesis of matrix components to compensate for degradation.

We have also shown that the contents of collagen types II and IX, and proteoglycans were significantly greater in cultures of the NP than the AF. Previously, it was found that type II collagen accounts for approximately 82% of the total collagen in the NP but only 64% of the total collagen in the AF of the human L5-S1 IVD [Antoniou et al., 1996]. These data are similar

TABLE I. Summary of the Effect of Link N at Day 20 on Proteoglycan, Type II Collagen, and Type IX Collagen Content COL2 and NC4 Domains in the Annulus Fibrosus (AF) and Nucleus Pulposus (NP)

	Percent increase (compared to control)			
	NP		AF	
	10 ng/ml link N	100 ng/ml link N	10 ng/ml link N	100 ng/ml link N
Proteoglycan	7.7	28.2	30.4	44.9
Type II collagen	74	113	12.7	25.3
NC4(IX)	10.8	45.8	17.0	54.7
COL2(IX)	26.1	45.5	22.4	53.4

Results are shown as the percent increase in matrix components of treated pellet cultures, compared to the control, for the experiments described in Figures 2–5.

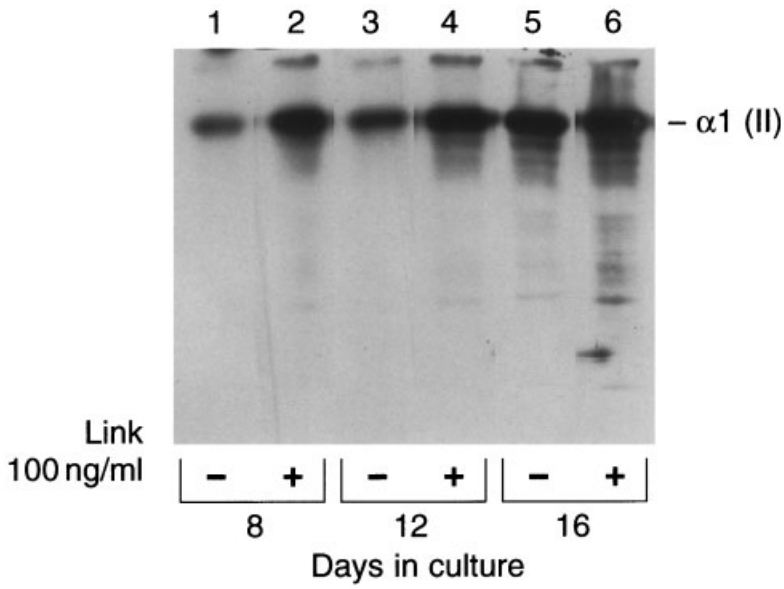

Fig. 7. Effect of link N on collagen synthesis in NP cell pellet cultures. To specifically analyze the collagens, the [^{3}H] proline-labeled media were precipitated by the addition of $(NH_4)_2SO_4$ and analyzed by autoradiography [Mwale et al., 2000]. Protein bands present on the autoradiogram were compared with the location of labeled standards.

to those of Eyre and Muir [1974], who found that in adult human IVDs more than 85% of the collagen in NP was type II collagen, whereas in the AF, type II collagen represented 50–65% of total collagen [Adams et al., 1977]. The relatively high content of type VI collagen observed in bovine NP [Wu et al., 1987] may account for a significant proportion of the remaining collagen.

Previously, it was found that human IVD cells seeded in pellet cultures at 5×10^5 grew in size and stabilized at a diameter of approximately 3 mm after 2 weeks. In this study, NP and AF cells from adult cows were separated and 1×10^6 cells were cultured to ensure sufficient tissue formation for all our analyses on the same sample. We found that if the pellets are maintained in the described manner with centrifugation at each media change the compact pellets continue to grow in size. However, if we did not centrifuge, the pellets grew in size only for the first 7–10 days (data not shown), probably due to decreased nutritional support in the center of the pellet [Yung Lee et al., 2001]. Alternative possibilities are that centrifugation might force unwanted waste product out of the pellet or that centrifugation compresses the pellet, which then swells up as new medium is added, in a similar manner that loading regulates protein synthesis [Koob et al., 1992; Puustjarvi et al., 1994]. Another explanation is that centrifugation provides a mechanical stimulus by compacting cells.

Previous studies have shown that in the adult, link protein content is lower in the disc compared to autologous articular cartilage [Pearce et al., 1989]. The AF contains more extractable link protein relative to tissue wet weight than the NP, and the nuclear link protein, at least in adolescents, contains a greater proportion of the smallest link protein. Such changes in the quantity and structure of the disc link proteins may affect the availability of the link peptide and, therefore, responses to it. Collectively, these results demonstrate that link N can at physiological concentrations stimulate the synthesis of matrix molecules in the IVD. This information may be relevant to the degeneration of IVD seen in aging since with a reduction in content of link protein the potential for this anabolic feedback loop would be reduced. It also presents an alternative method to stimulate disc growth and regeneration.

ACKNOWLEDGMENTS

This study was funded by the Canadian Institutes of Health Research (to JA) and the Canadian Orthopaedic Foundation (to FM). The

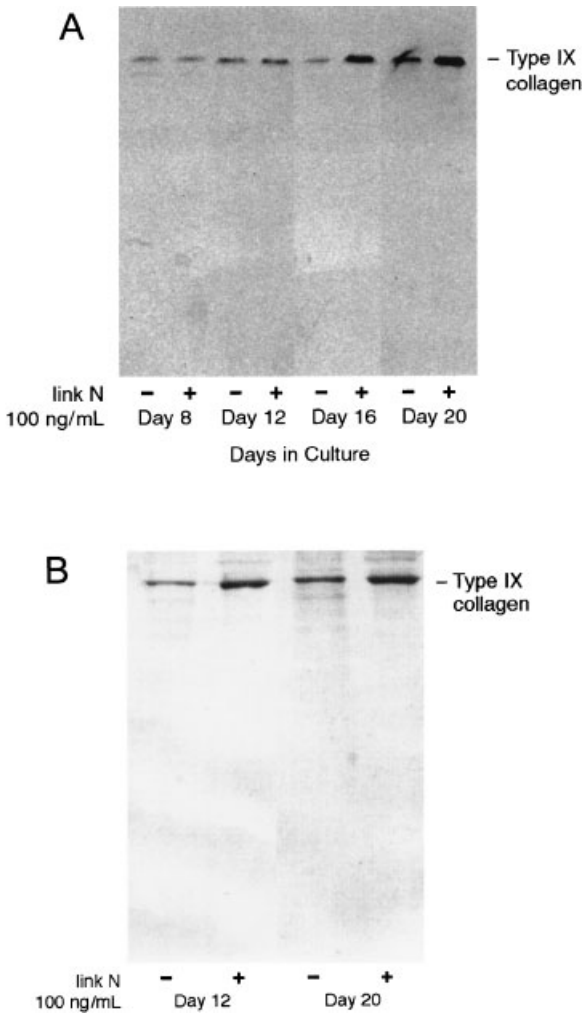

Fig. 8. Confirmation of the enhancement of link N on the synthesis of type IX collagen in AF (**A**) and NP (**B**) cell pellet cultures by Western blotting. To confirm for the presence of type IX collagen, Western blotting using anti-NC4 antibodies was performed as previously described [Mwale et al., 2000].

technical assistance of Elisa de Miguel with the preparation of synthetic peptides is gratefully acknowledged. Helen Campbell kindly assisted with the SEM. We thank Linda for kindly assisting in the preparation of figures.

REFERENCES

Adams P, Muir H. 1976. Qualitative changes with age of proteoglycans of human lumbar discs. Ann Rheum Dis 35:289–296.

Adams P, Eyre DR, Muir H. 1977. Biochemical aspects of development and ageing of human lumbar intervertebral discs. Rheumatol Rehabil 16:22–29.

Aigner T, Gresk-otter KR, Fairbank JC, von der Mark K, Urban JP. 1998. Variation with age in the pattern of type X collagen expression in normal and scoliotic human intervertebral discs. Calcif Tissue Int 63:263–268.

Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, Poole RA, Aebi M, Alini M. 1996. The human lumbar intervertebral disc: Evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. J Clin Invest 98:996–1003.

Baer AE, Wang JY, Kraus VB, Setton LA. 2001. Collagen gene expression and mechanical properties of intervertebral disc cell-alginate cultures. J Orthop Res 19:2–10.

Bogduk N. 1991. The lumbar disc and low back pain. Neurosurg Clin N Am 2:791–806.

Boos N, Nerlich AG, Wiest I, von der Mark K, Aebi M. 1997. Immunolocalization of type X collagen in human lumbar intervertebral discs during ageing and degeneration. Histochem Cell Biol 108:471–480.

Buckwalter JA. 1995. Aging and degeneration of the human intervertebral disc. Spine 20:1307–1314.

Chiba K, Andersson GB, Masuda K, Thonar EJ. 1997. Metabolism of the extracellular matrix formed by intervertebral disc cells cultured in alginate. Spine 22:2885–2893.

Dai LY. 2000. Disc degeneration in patients with lumbar spondylolysis. J Spinal Disord 13:478–486.

Dean MF, Sansom P. 2000. Link peptide cartilage growth factor is degraded by membrane proteinases. Biochem J 349:473–479.

Donohue PJ, Jahnke MR, Blaha JD, Caterson B. 1988. Characterization of link protein(s) from human intervertebral-disc tissues. Biochem J 251:739–747.

Eyre DR, Muir H. 1974. Collagen polymorphism: Two molecular species in pig intervertebral disc. FEBS Lett 42:192–196.

Eyre DR, Muir H. 1976. Types I and II collagens in intervertebral disc. Interchanging radial distributions in annulus fibrosus. Biochem J 157:267–270.

Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 883:173–177.

Gruber HE, Fisher EC, Jr., Desai B, Stasky AA, Hoelscher G, Hanley EN, Jr. 1997. Human intervertebral disc cells from the annulus: Three-dimensional culture in agarose or alginate and responsiveness to TGF-beta1. Exp Cell Res 235:13–21.

Hall RA, Lee JY, Pelinkovic D, Cassinelli EH, Usas A, Gilbertson LG, Huard J, Kang JD. 2001. A three-dimensional pellet culture system for human intervertebral disc cells: Initial characterization and potential for tissue engineering. 47th Annual Meeting, Orthopaedic Research Society, February 25–28, 2001, San Francisco, CA 0891.

Hardingham TE. 1979. The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem J 177:237–247.

Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR. 1994. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 93: 1722–1732.

Hollander AP, Heathfield TF, Liu JJ, Pidoux I, Roughley PJ, Mort JS, Poole AR. 1996. Enhanced denaturation of the alpha (II) chains of type-II collagen in normal adult human intervertebral discs compared with femoral articular cartilage. J Orthop Res 14:61–66.

Hutton WC, Toribatake Y, Elmer WA, Ganey TM, Tomita K, Whitesides TE. 1998. The effect of compressive force applied to the intervertebral disc in vivo. A study of proteoglycans and collagen. Spine 23:2524–2537.

Kaapa E, Zhang LQ, Muona P, Holm S, Vanharanta H, Peltonen J. 1994. Expression of type I, III, and VI collagen mRNAs in experimentally injured porcine intervertebral disc. Connect Tissue Res 30:203–214.

Kaapa E, Han X, Holm S, Peltonen J, Takala T, Vanharanta H. 1995. Collagen synthesis and types I, III, IV, and VI collagens in an animal model of disc degeneration. Spine 20:59–66; (discussion 66–67).

Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. 1988. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci USA 85:9552–9556.

Kimura JH, Hardingham TE, Hascall VC, Solursh M. 1979. Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma. J Biol Chem 254:2600–2609.

Koob TJ, Clark PE, Hernandez DJ, Thurmond FA, Vogel KG. 1992. Compression loading in vitro regulates proteoglycan synthesis by tendon fibrocartilage. Arch Biochem Biophys 298:303–312.

Labarca C, Paigen K. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102:344–352.

Liu J, Roughley PJ, Mort JS. 1991. Identification of human intervertebral disc stromelysin and its involvement in matrix degradation. J Orthop Res 9:568–575.

Liu H, McKenna LA, Dean M. 1997. An N-terminal peptide from link protein stimulates synthesis of cartilage proteoglycans. Biochem Soc Trans 25:427S.

Liu H, McKenna LA, Dean MF. 1999. The macromolecular characteristics of cartilage proteoglycans do not change when synthesis is up-regulated by link protein peptide. Biochim Biophys Acta 1428:191–200.

Liu H, McKenna LA, Dean MF. 2000. An N-terminal peptide from link protein can stimulate biosynthesis of collagen by human articular cartilage. Arch Biochem Biophys 378:116–122.

Maldonado BA, Oegema TR, Jr. 1992. Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. J Orthop Res 10:677–690.

McKenna LA, Liu H, Sansom PA, Dean MF. 1998. An N-terminal peptide from link protein stimulates proteoglycan biosynthesis in human articular cartilage in vitro. Arthritis Rheum 41:157–162.

Mort JS, Poole AR, Roughley PJ. 1983. Age-related changes in the structure of proteoglycan link proteins present in normal human articular cartilage. Biochem J 214:269–272.

Mwale F, Billinghurst C, Wu W, Alini M, Webber C, Reiner A, Ionescu M, Poole J, Poole AR. 2000. Selective assembly and remodelling of collagens II and IX associated with expression of the chondrocyte hypertrophic phenotype. Dev Dyn 218:648–662.

Mwale F, Tchetina E, Wu CW, Poole AR. 2002. The assembly and remodeling of the extracellular matrix in the growth plate in relationship to mineral deposition and cellular hypertrophy: An in situ study of collagens II and IX and proteoglycan. J Bone Miner Res 17:275–283.

Nguyen Q, Murphy G, Roughley PJ, Mort JS. 1989. Degradation of proteoglycan aggregate by a cartilage metalloproteinase. Evidence for the involvement of stromelysin in the generation of link protein heterogeneity in situ. Biochem J 259:61–67.

Nguyen Q, Murphy G, Hughes CE, Mort JS, Roughley PJ. 1993. Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein. Biochem J 295: 595–598.

Parke WW, Schiff DC. 1971. The applied anatomy of the intervertebral disc. Orthop Clin North Am 2:309–324.

Pearce RH, Mathieson JM, Mort JS, Roughley PJ. 1989. Effect of age on the abundance and fragmentation of link protein of the human intervertebral disc. J Orthop Res 7:861–867.

Puustjarvi K, Lammi M, Helminen H, Inkinen R, Tammi M. 1994. Proteoglycans in the intervertebral disc of young dogs following strenuous running exercise. Connect Tissue Res 30:225–240.

Roughley PJ, Poole AR, Mort JS. 1982. The heterogeneity of link proteins isolated from human articular cartilage proteoglycan aggregates. J Biol Chem 257:11908–11914.

Tengblad A, Pearce RH, Grimmer BJ. 1984. Demonstration of link protein in proteoglycan aggregates from human intervertebral disc. Biochem J 222:85–92.

Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IK, Bishop PB. 1990. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine 15:411–415.

Thompson JP, Oegema TR, Jr., Bradford DS. 1991. Stimulation of mature canine intervertebral disc by growth factors. Spine 16:253–260.

Wu JJ, Eyre DR, Slayter HS. 1987. Type VI collagen of the intervertebral disc. Biochemical and electron-microscopic characterization of the native protein. Biochem J 248:373–381.

Yung Lee J, Hall R, Pelinkovic da L, Cassinelli E, Usas A, Gilbertson L, Huard J, Kang J. 2001. New use of a three-dimensional pellet culture system for human intervertebral disc cells: Initial characterization and potential use for tissue engineering. Spine 26:2316–2322.