but important, prospective study by Boos et al. [4] showed that disc degeneration in asymptomatic adults did not predict back pain in a 5 year follow-up window. This is in contrast with 14-year-old asymptomatic adolescents with disc degeneration who were much more likely to have back pain by age 20 than MRI normal controls [5].

**Discography and computed tomography (CT) discography**

This method is unpleasant and involves injecting contrast into the disc. This shows patterns of degeneration and annular tears on plain X-ray or CT. This method also provokes pain much more in degenerate discs than in 'normal' discs. Unfortunately there is a lot of noise through difficulties in interpreting the patient’s response to the injection and the method remains controversial.

**Animal models**

There is no really good animal model of disc degeneration. Although discs are found in the spines of the earliest vertebrates, there are subtle, but important, interspecies variations. There are mammalian lines with tendencies to degenerative changes. However, for the small number of studies in this area, researchers have mainly relied on mechanically induced disc degeneration in large mammals (a small scalpel wound in the annulus); this may well not accord with humans. One problem is that humans are bipedal, another is whether or not the degenerate animal disc causes pain.

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**Expression of type II procollagens during development of the human intervertebral disc**

A. McAlinden*, Y. Zhu*, and L. J. Sandell*†

*Department of Orthopaedic Surgery, Washington University at Barnes-Jewish Hospital, Mail Stop 90-34-674, 216 South Kingshighway, St. Louis, MO 63110, U.S.A., and †Department of Cell Biology and Physiology, Washington University at Barnes-Jewish Hospital, Mail Stop 90-34-674, 216 South Kingshighway, St. Louis, MO 63110, U.S.A.

**Abstract**

Mice lacking type II collagen fail to develop intervertebral discs. The present study describes the distribution of the developmentally expressed type IIA procollagen molecule, as well as types I and III collagens, in human IV disc specimens ranging from 42 to 101 days gestation. Type IIA procollagen contains the alternatively spliced exon 2 which encodes a 69-amino-acid cysteine-rich domain. By radioactive in situ hybridization and fluorescence immunohistochemistry, we identified changes in the localization patterns of type IIA procollagen, particularly between days 54 and 101. At day 54, the developing disc was

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**What do clinicians want to know from scientists about the intervertebral disc?**

Below is a list of areas that clinicians want to know more about from scientists with respect to the intervertebral disc.

- Anatomy and microstructure;
- Mechanisms of (i) disc degeneration and (ii) cytokine production and release from degenerated and herniated discs;
- Cell products and the patterns and mechanisms of their production;
- The biochemical response to mechanical and ischaemic stress;
- Why nerves and blood vessels grow into degenerate but not normal discs;
- Genetic mechanisms involved in disc degeneration and pain production;
- Possibilities for the reversal of degenerative processes.

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**References**


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Tissue distribution of type II procollagen isoforms

Two isoforms of type II procollagen (type IIA and IIB) exist due to alternative splicing of the Col2a1 gene. Type IIA mRNA contains an additional 207 bp exon (exon 2) encoding a 69-amino-acid cysteine-rich domain present in the N-terminal propeptide of the procollagen molecule [8,9]. The shorter isoform, type IIB procollagen, is devoid of this exon 2-encoded domain. Type IIA procollagen is synthesized by pre-cartilage and non-cartilaginous epithelial and mesenchymal cells [9,10]. In particular, type IIA mRNA has been localized in the somites, notochord, neuroepithelia and pre-chondrogenic mesenchyme of mouse [9,11] and human [10,12] embryos as well as in pre-cartilaginous condensations and perichondrium during the development of avian long bones [13]. Studies using an antibody specific for the N-terminal propeptide of type IIA procollagen have shown protein localization in the extracellular matrix (ECM) of human pre-chondrogenic and epithelial tissues [14].

As pre-cartilage cells differentiate into mature chondrocytes, they switch from synthesizing type IIA procollagen to type IIB procollagen by alternative splicing of exon 2. Synthesis of type IIB procollagen, as well as the large cartilage proteoglycan aggregan, are the most reliable markers of chondrogenesis. Tissue distribution of type IIA procollagen suggests that it plays an important role during the development of cartilage and other tissues and that the exon 2-encoded domain may be responsible for this role.

Differential expression of types I, II and III procollagens during IV disc development

Early studies have identified type IIA and IIB procollagen in the developing vertebral column. Type IIA mRNA was found in cells surrounding the pre-chondrogenic area that will eventually become the articular endplate of the vertebral body [10]. A later study also showed that type IIA procollagen mRNA was localized in the notochordal remnants of the vertebral column [9]. As expected, these studies showed that type IIB procollagen was primarily found in the cartilaginous tissue of the vertebral centrum (precursor area of the vertebral body). The morphology of the cells also differed depending on the collagen type synthesized: type IIA-producing cells were more

Intervertebral (IV) disc: general structure and collagen distribution

The mature IV discs are fibrocartilaginous tissues that join adjacent vertebral bodies and provide mechanical stability to the IV joints. Histologically, the structure of the mature human IV disc is well documented: there is a strong, outer annulus fibrosis containing high levels of organized collagen fibres and a soft inner nucleus pulposus consisting of a viscous proteoglycan gel with small amounts of collagen [1]. With respect to collagen, types I, II, III, V, VI, IX and XI have been found in the mature, human annulus fibrosis while only types II, VI, IX and XI have been identified in the nucleus pulposus [2–5].

The nucleus pulposus originates from the notochord, which functions to support the axis of the embryo before development of the vertebral column [6]. During early development, the annulus fibrosis consists of structurally dissimilar outer and inner regions. The outer annulus is made up of fibrous tissue whereas the inner annulus consists of both embryonic hyaline cartilage and fibrocartilage. In later fetal life, this region becomes completely fibrocartilaginous [7].

This article focuses on the distribution of type II procollagen during an early period of human fetal IV disc development. In particular, gene expression and protein localization of the two isoforms of type II procollagen will be discussed and reference will be made to the potential function of the long isoform of type II procollagen (type IIA procollagen) during IV disc development. In addition, a unique mechanism for processing type IIA procollagen by cells of the inner annulus will also be discussed.
elongated and fibroblast-like, while cells producing type IIIB were larger and more round in shape.

Studies by Oganesian et al. [14] first described the anti-IIA polyclonal antiserum that recognizes the exon 2-encoded cysteine-rich domain of type IIA procollagen. They showed distinctive staining patterns of the IIA N-terminal propeptide in the ECM of various tissues including the IV disc area.

**Figure 1**

Expression of mRNA for type IIA, type I and type III collagens in 42 (A–D) and 54 (E–H) day-old human fetal intervertebral discs

(A) Photomicrograph of frozen sections showing the dense zone (DZ) of the future IV disc, the vertebral centrum (VC) and the notochord (N). (B) Type IIA procollagen expression in the notochord and pre-chondrogenic area surrounding the vertebral centrum. (C) Type I collagen expression in cells of the dense zone and notochord. (D) Type III collagen expression in the dense zone. (E) Photomicrograph of a 54-day IV disc frozen section showing developed regions of the disc. OuA, outer annulus; InA, inner annulus; V, vertebral body; NP, nucleus pulposus; the asterisk denotes the acellular region of the notochord sheath. (F) Type IIA procollagen expression in the prechondrogenic area of the outer annulus (also shown by an arrow) and in cells of the inner annulus. (G) Type I collagen expression in the outer annulus, in tissue surrounding the vertebral body and in the nucleus pulposus. (H) Type III mRNA expression in the inner annulus and outer annulus, nucleus pulposus and vertebral body. (A) and (E) are brightfield images; (B)–(D) and (F)–(H) are darkfield images. Original magnification, × 10. Scale bar, 140 μm. Developmental Dynamics © 2001 Wiley-Liss, Inc.
Knowledge that type IIA procollagen is a developmentally expressed molecule, combined with the finding that mice lacking type II collagen fail to develop IV discs [15], led us to carry out a more detailed study of type IIA procollagen distribution during an early period of fetal IV disc development. The reader should refer to the publication of Zhu et al. [16] for more detailed information on the methodology and antibodies used. In these studies, radioactive in situ hybridization and fluorescence immunohistochemistry were used to localize collagen mRNA.

**Figure 2**

**Immunolocalization of type IIA procollagen in 54-101 day-old human IV disc specimens**

Antibodies recognizing the N-terminal propeptide (IIA), the fibrillar domain (IIF) and the C-terminal domain (IIC) were used to localize type II procollagen. Localization of (A) IIA and (B) IIF in the extracellular matrix of a 54 day-old IV disc. Confocal imaging showed that these domains are co-localized in the inner annulus (IA) and vertebral bodies. Only the N-terminal propeptide was found in the nucleus pulposus (NP), OA, outer annulus. (C) Localization of IIC in the cytoplasm of inner annulus cells in a 54 day-old specimen and (D) IIF staining in a serial section of the disc sample shown in (C). Localization of (E) IIA and (F) IIF in the IV disc of a 72 day-old specimen. Localization of (G) IIA and (H) IIF in the inner annulus of a 101-day-old IV disc. Arrow denotes the acellular region of the notochord sheath. All photographs are confocal images. (A), (B), (E) and (F) were at x 20 magnification; scale bar, 118 μm. (C)–(E) and (H) were at x 40 magnification; scale bar, 8 μm. Developmental Dynamics © 2001 Wiley-Liss, Inc.
and protein in fetal IV disc specimens ranging from day 42 (embryonic stage 17) to day 101 (week 14.5) of gestation.

As early as 42 days of gestation, before appearance of the annulus fibrosis and nucleus pulposus (Figure 1A), type IIA mRNA is present in cells of the pre-chondrogenic area surrounding the vertebral centrum, as well as in cells of the notochord and those surrounding the notochord in the dense zone (pre-IV disc area; Figure 1B). As expected, cells in the vertebral centrum were shown to express type IIB procollagen while type I and type III collagen mRNA was localized mainly to the dense zone (Figures 1C and 1D).

At 54 days gestation, the IV disc region has a clearly defined nucleus pulposus and annulus fibrosis, with distinct inner and outer regions (Figure 1E). Here, type IIA procollagen mRNA is found in the pre-chondrogenic area that will form the articular endplate, as well as in cells of the inner annulus and nucleus pulposus (Figure 1F). Type I collagen mRNA is now localized primarily to the cells of the outer annulus and the fibrous tissue surrounding the vertebral centrum as well as in cells of the nucleus pulposus (Figure 1G). Type III collagen mRNA has a more widespread expression than type I collagen at day 54, being found in cells throughout the inner and outer annulus, nucleus pulposus and vertebral body (Figure 1H).

Protein localization of types I and III procollagen in the developing IV disc at 54 days gestation is similar to the distribution pattern found at the mRNA level. However, positive staining is also noted in the matrix of the notochord sheath at this stage. Type IIA procollagen was analysed using different antibodies against three potentially functional domains of the molecule: (1) the cysteine-rich domain in the N-terminal propeptide; (2) the major collagen fibrillar domain and (3) the C-terminal propeptide domain. Figure 2(A) shows localization of the type IIA N-terminal propeptide in the inner annulus, vertebral bodies and nucleus pulposus. With the exception of the nucleus pulposus, the staining pattern of the major collagen fibrillar domain is similar to that shown for the IIA N-terminal propeptide (Figure 2B). Confocal imaging showed co-localization of these domains and suggests that type IIA pN procollagen (the major collagen fibrillar domain attached to the N-terminal propeptide, and devoid of the C-terminal domain) is present in the inner annulus and vertebral bodies during this time. Figure 2(C) shows that the C-terminal domain is never present in the extracellular matrix, unlike the major fibrillar domain (Figure 2D), but is cleaved from the procollagen molecule inside the cell. Thus, at this phase of development, intracellular C-proteinase enzymes are active in removing the C-terminal domain while N-proteinase activity appears to be non-functional or absent. The following section discusses a change in the mechanism type IIA procollagen processing at a later stage of IV disc development.

**Processing of type IIA procollagen during early IV disc development**

Fibrillar collagens are synthesized as procollagens containing globular extension peptides at both the N- and C-termini. By using type I procollagen as a model system, it is generally believed that the propeptides are removed during secretion from the cell [17]. The N-terminal propeptide is removed by procollagen N-proteinase and the C-terminal propeptide is removed by the C-proteinase/bone morphogenetic protein (BMP)-1 [18-20]. However, pN-procollagen (procollagen containing the N-terminal domain, but not the C-terminal domain) of types I and IIII collagen have been observed in embryonic tissues, indicating that the procollagen N-proteinase is either non-functional or not synthesized by cells in some rapidly growing tissues [21].

Type IIA pN-procollagen is present in the ECM of the inner annulus of the developing IV disc at day 54 (Figures 2A and 2B). However, the collagen-processing mechanism changes during differentiation of the cells in the inner annulus. On days 72-101 of development, N-proteinases, synthesized by the inner annulus disc cells, cleave the type IIA N-terminal propeptide before or during protein secretion. As a result, only the major fibrillar domain of type I collagen is secreted into the matrix of the inner annulus and the N-terminal propeptide is retained inside the cells, as shown in Figure 2(E) (day 72) and Figure 2(G) (day 101). Figures 2(F) and 2(H) show the ECM staining pattern of the collagen fibrillar domain in serially sectioned disc specimens. This suggests that, in these inner annulus cells, removal of the N-terminal propeptide is in contrast with that found during chondrogenesis. That is, during chondrogenesis, chondroprogenitor cells synthesize type IIA procollagen while maturation into chondrocytes results in type IIB synthesis by alternative splicing of exon 2. In the inner annulus, the form of collagen formed is consistently type IIA procollagen. The N-terminal
Figure 3
Differential expression and processing of type IIA collagen in the developing IV disc

(A) Localization of type IIA pN-collagen in the ECM is represented by the shaded area. Type IIA N-terminal propeptide not attached to the fibrillar domain is represented by X in the notochord (N) and nucleus pulposus (NP). Note that expression was also found in the vertebral bodies (V), but this diagram focuses on changes in expression in the developing IV disc area only. Type IIA pN-collagen is localized throughout the dense zone (DZ) at 42 days and by day 54 is concentrated in the ECM of the inner annulus (InA) and endplate region between the IV disc and vertebral body. By day 72, type IIA propeptide is localized in the cytoplasm (represented by black dots) of the inner annulus and is no longer detected in the ECM. OuA, outer annulus. (B) Schematic showing the change in type IIA procollagen processing by the inner annulus cells (C) between 54 and > 72 days of development of the IV disc. F, fibrillar domain. Developmental Dynamics © 2001 Wiley-Liss, Inc.

Potential function of type II collagen and the type IIA procollagen N-terminal propeptide during IV disc development and repair
Type II procollagen in notochord regression and normal disc development

Among the tissues that express type IIA procollagen, the notochord has one of the most diverse functions during vertebrate development. The early notochord is a rod-like structure of mesodermal origin that plays an important role in dorsoventral patterning of both the neural tube and somitic mesoderm. In normal disc development, cells of the notochord gradually disappear between the areas of the vertebral bodies and accumulate in the centre of the IV disc area that will eventually form the nucleus pulposus. This cell-free notochord that is visible within the cartilaginous vertebral bodies is eliminated during ossification. In the col2a1-null mice, the anlagen of the vertebral bodies and the IV discs develop abnormally [15]. This defect is associated with enlargement of the vertebral bodies, no endochondral ossification, and the expression of abnormal collagen fibrils. The notochord persists as a rod-like structure without signs of expansion (to form the IV disc area) or regression (between the vertebral bodies). It is thought that this inhibition of notochord regression is due to a lack of a tight collagen network that contributes to restraining the swelling pressure of the proteoglycan aggre-
gates, thereby increasing the internal pressure within the vertebral bodies. Consequently, it is believed that this mechanical stimulus on the notochord induces the migration of cells towards the presumptive IV disc area [22,23]. Type II collagen in cartilage of the null mice is replaced by continued synthesis of types I and III collagens. Although these replacement collagens are able to form a cartilage-like tissue, they are not able to support the regulation of notochordal reorganization leading to the formation of the IV disc and dismantling of the notochord.

In addition to the important structural role during IV disc development, type II collagen is likely to function in a similar way during the repair process in degenerated discs. Type II collagen gene expression was found to be up-regulated in experimentally induced degeneration of the rabbit IV disc [24]. Although not distinguished in these studies, it is highly likely that the form of type II procollagen synthesized by cells in these degenerated discs is that of type IIA procollagen. The N-terminal propeptide of type IIA procollagen may also play a functional role during repair as will be discussed in the section below.

**Growth factor regulation by type IIA procollagen N-terminal propeptide**

The cysteine-rich domain encoded by the alternatively spliced exon 2 present in the N-terminal propeptide of type IIA procollagen is homologous with BMP-binding domains present in chordin from *Xenopus* [25] and sog from *Drosophila* [26]. Both of these proteins bind to BMPs in the ECM and regulate their ability to bind to cell receptors [27,28]. We have previously shown that the exon 2-encoded domain of type IIA procollagen binds to BMP-2 and transforming growth factor-β in vitro [29]. Consequently, we hypothesized that during cell differentiation, type IIA procollagen N-terminal propeptide may bind to and regulate BMPs and that this is an important function in skeletal morphogenesis. In *vivo*, *Xenopus* type IIA procollagen has been shown to function similarly to chordin in that the ventralizing activity of BMPs was inhibited, resulting in dorsalization of *Xenopus* embryos [30]. This paradigm for BMP regulation may also be important during the development of the IV disc and regression of the notochord. To our knowledge, there have been no reports on the localization of BMPs in the notochord or IV disc area during development. Recent unpublished data from our laboratory have identified BMP-2 in the cells of the vertebral body, inner annulus and nucleus pulposus during early development (day 50) of the human embryonic IV disc (Y. Zhu, A. McAlinden and L. J. Sandell, unpublished work). Thus it is likely that BMPs and other growth factors are crucial for normal development of the vertebral column.

Type IIA pN-procollagen is the only form of type II collagen synthesized and deposited by cells of the notochord/nucleus pulposus and inner annulus during early morphogenesis, rendering it a favourable candidate for the regulation of BMP activity in these areas. Our finding that the type IIA N-terminal propeptide is removed from the matrix of the inner annulus after day 54 of development may suggest that its function as a growth factor regulator is no longer required. However, it remains to be established as to whether the intracellular source of the IIA N-terminal propeptide detected in the inner annulus cells at later stages of development may also function to bind to intracellular BMPs as they are synthesized, thus preventing their secretion from the cell.

To further extend the BMP regulation paradigm, it is known that chordin is subject to cleavage by the astacin proteinase, xolloid [31], resulting in disruption of the BMP–chordin complex and subsequent activation of BMP. Similarly, recent studies have shown that the N-terminal propeptide of type IIA procollagen (produced recombinantly in our laboratory [32]) can be cleaved by matrix metalloproteinases (MMPs) including MMP-3 (stromelysin), MMP-7 (matrilysin), MMP-9 (gelatinase B), MMP-13 (collagenase 3) and MMP-14 (MT-MMP-1) [33]. If type IIA N-terminal propeptide binds to BMP *in vivo*, then it is possible that a mechanism exists for release and activation of the growth factor by MMP cleavage. Although MMPs have not been analysed during IV disc development, it is likely that they are present and function to maintain the catabolic/anabolic state of the tissue. In addition, MMPs have been identified in degenerated disc specimens and it is believed that processes occurring during development are replicated during degeneration as a means to attempt to repair the tissue.

Thus, in addition to its role in development, type IIA N-terminal propeptide will probably play an important role during IV disc repair. BMP-2/4 and its receptor have been identified in degenerated discs in mice, particularly within the mature annulus fibrosis [34]. Of the MMPs that were found to cleave the IIA N-terminal pro-
peptide [33]. MMP-3 and MMP-9 have been found in the degenerated disc [35–38]. In addition to the accepted function of MMPs in degrading the IV disc matrix, they may also have an anabolic effect by cleaving BMP-binding proteins such as type IIA procollagen, thus activating the growth factor to induce anabolic effects.

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