

## Enhanced Type X Collagen Expression in the Extruded Nucleus Pulposus of the Chondrodystrophoid Dog

Hisanori ITOH<sup>1)</sup>, Yoshinori ASOU<sup>2)</sup>, Yasushi HARA<sup>1)</sup>, Hiroataka HARO<sup>3)</sup>, Kenichi SHINOMIYA<sup>2)</sup> and Masahiro TAGAWA<sup>1)</sup>

<sup>1)</sup>Division of Veterinary Surgery, Nippon Veterinary and Animal Science University, School of Veterinary Medicine, 1-7-1 Kyonancho, Musashino-shi, Tokyo 180-8602, <sup>2)</sup>Department of Orthopaedic Surgery, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519 and <sup>3)</sup>Department of Orthopaedic Surgery, Graduate School of Medicine, University of Yamanashi, 1110, Shiiuokato, Tamaho, Nakakoma, Yamanashi 409-3898, Japan

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**ABSTRACT.** The causes of early degeneration and calcification of the nucleus pulposus in the chondrodystrophoid dog are poorly understood, and the underlying molecular mechanism of this process has not yet been clearly defined. Type X collagen is one of the key molecules in endochondral bone growth and development, especially matrix calcification. The relationship between type X collagen and disc degeneration and calcification in chondrodystrophoid dogs has not yet been studied. We analyzed the expression of type X collagen in degeneration and calcification of the intervertebral disc in chondrodystrophoid dogs, using type X collagen immunohistochemistry. Control intervertebral discs were collected from five dogs (4 female, 1 male, average age 1.3 years, beagle breed). Degenerated intervertebral discs were surgically removed from 11 canine patients with intervertebral disc extrusion (1 female, 10 male, average age 5.1 years, dachshund breed) in Nippon Veterinary and Animal Science University. All extruded disc samples showed hypertrophic changes and clustering of cells, typical features observed in the degenerated nucleus pulposus. The relative expression of type X collagen in the degenerated nucleus pulposus ( $84.3 \pm 11.0\%$ ) was significantly increased compared to the control nucleus pulposus ( $5.4 \pm 5.4\%$ ). Our findings suggest that type X collagen might contribute to the development of degeneration or calcification in the nucleus pulposus of the chondrodystrophoid dog.

**KEY WORDS:** chondrodystrophoid dog, degenerated disc, type X collagen.

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Intervertebral disc extrusion (IVDE) is a common cause of spinal cord dysfunction in dogs [4, 25]. Disc extrusion results from rupture of the outer fibrous layers and subsequent extrusion of the nucleus pulposus into the vertebral canal. Extrusion of the nucleus pulposus may be caused by impact injury, mechanical compression of the spinal cord or both, and causes pain and paralysis which may be permanent. IVDE occurs more often in chondrodystrophoid dogs when compared with the general population [2, 5-7, 9, 11, 13]. The peak incidence of clinical disease occurs at a much earlier age in chondrodystrophoid breeds [5, 9]. The intervertebral disc of a newborn chondrodystrophoid puppy macroscopically appears glistening and gelatinous, and is similar to a disc from a non-chondrodystrophoid puppy [11]. However, changes in the nucleus pulposus occur earlier than in other breeds of dog, and by one year of age the gelatinous nucleus has been replaced almost entirely by cartilaginous tissue. With increasing age, degenerative changes and frequently matrix calcification can be observed in the nucleus pulposus. It is considered that the early intervertebral disc degeneration in chondrodystrophoid dogs is related to the high incidence of IVDE [11]. The causes of early degeneration and calcification of the nucleus pulposus

in the chondrodystrophoid dog are poorly understood, and the underlying molecular mechanism of this process has not yet been clearly defined.

Recently, it was reported that type X collagen is present in scoliotic or aged human intervertebral discs [1] and in degenerative discs [22], and that this might relate to degeneration and calcification of the intervertebral discs. Type X collagen belongs to the family of short-chain collagens. It is naturally present in hypertrophic chondrocytes of the growth plate [21, 24] and is temporally expressed during endochondral ossification. The biological function of type X collagen is thought to be to facilitate the process of calcification during endochondral ossification through the normal distribution of matrix vesicles (MV) and proteoglycans within the growth plate matrix [17]. Distribution of type X collagen is restricted in the matrix of hypertrophic chondrocytes, suggesting that it is one of the key molecules in endochondral bone growth and development, especially matrix calcification.

The relationship between type X collagen and disc degeneration in chondrodystrophoid dogs has not previously been studied. The dachshund and beagle are both chondrodystrophoid dog breeds that are already prone to disc disorders in adolescence [11]. It is easy to collect naturally occurring prolapsed disc material from dachshund patients, since this breed more than any other tends to suffer from intervertebral disc disease [11]. The purpose of our study is to examine the difference in expression of type X collagen between ter-

\* CORRESPONDENCE TO ITO, H., Division of Veterinary Surgery, Nippon Veterinary and Animal Science University, School of Veterinary Medicine, 1-7-1 Kyonancho, Musashino-shi, Tokyo 180-8602, Japan.  
e-mail: hisanori.itoh@gmail.com

minimal degenerated intervertebral discs and young normal intervertebral discs, and the difference between these intervertebral discs may be regarded as evidence that type X collagen relates to developing disc degeneration or maybe calcification. Therefore, we investigated the expression of type X collagen in young normal or degenerated intervertebral discs of chondrodystrophoid dogs, using type X collagen immunohistochemistry.

## MATERIALS AND METHODS

Control intervertebral discs were collected from five dogs (4 female, 1 male, average age 1.3 years, beagle breed). After euthanasia, the thoracic - lumbar disc and adjacent vertebral bodies were removed from each dog. The discs were all categorized as Stage 2 on the Bray and Burbridge scale [3, 4], with a gelatinous nucleus pulposus, distinct nuclear-annular demarcation, and normal annular lamellae. Degenerated intervertebral discs were surgically removed from 11 canine IVDE patients (1 female, 10 male, average age 5.1 years, dachshund breed) in Nippon Veterinary and Animal Science University. The samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4), decalcified in 20% ethylenediaminetetraacetic acid (EDTA, pH 7.4), embedded in paraffin and cut into 5  $\mu$ m sections. The sections were routinely stained with hematoxylin and eosin (H.E.) and safranin O.

The localization of type X collagen protein in control and degenerated intervertebral discs was examined by immunohistochemistry using a monoclonal antibody to human type X collagen (Quartett, Germany) that has cross reactivity with canine type X collagen [8]. The sections were deparaffinized in xylene, hydrated in a reversed graded series of ethanol, and incubated with 0.3% hydrogen peroxide in absolute methanol for 5 min at room temperature. After washing with PBS, the sections were treated with Proteinase K (20  $\mu$ g/ml; DakoCytomation, Glostrup, Denmark) in PBS for 10 min at room temperature. The sections were then washed in PBS, incubated in normal horse serum (1:50; Vector Laboratories, Burlingame, CA, U.S.A.) in PBS containing 0.2% Triton X-100 for 10 min at room temperature, and overlaid with the primary type X collagen antibody for 1 hr at room temperature. The following steps were carried out using the avidin-biotin complex method. The sections were incubated in the presence of biotin-conjugated mouse secondary antibody (Vector Laboratories) for 60 min at 4°C followed by addition of horseradish peroxidase-labeled streptavidin (Vectastain® Elite ABC kit; Vector Laboratories) for 30 min at room temperature according to the manufacturer's instructions. The colored reaction product was developed with 3,3'-diaminobenzidine (DAB substrate kit for peroxidase; Vector Laboratories). Finally, the sections were counterstained with hematoxylin. The relative proportions of type X collagen-stained cells per total cells were estimated, and the following designations were applied; (+), fewer than 25% positive cells; (++) , 25% to 75% positive cells; and (+++) , more than 75% positive cells. For statisti-

cal analysis, the difference in the relative numbers of type X collagen stained cells between control and patient intervertebral discs were compared by the Mann-Whitney U test. A P value of less than 0.05 was considered to be statistically significant.

## RESULTS

To evaluate the degree of intervertebral disc degeneration in the intervertebral discs of control young beagle dogs and dogs suffering from IVDE, we performed H.E. and safranin O staining. All control intervertebral discs showed strong staining of the extracellular matrix with safranin O and a cellular nest was evident, demonstrating that the discs of young beagle dogs were in the earliest stages of disc degeneration (Fig.1(a)). In contrast, the nucleus pulposus of patient intervertebral discs showed hypertrophic cells, clustering cells and weak staining of the extracellular matrix but strong staining of the pericellular region with safranin O staining, features which are observed in the nucleus pulposus of degenerated intervertebral discs (Fig.1(b)).

To determine expression of type X collagen protein in control and degenerated intervertebral discs, a monoclonal antibody to human type X collagen was used for immunohistochemistry. The results of type X collagen protein expression analysis in control and degenerated intervertebral discs are summarized in Tables 1 and 2, respectively. Immunostaining of type X collagen in control intervertebral discs showed virtually no reaction in the annulus fibrosus or the nucleus pulposus (Fig. 2(a)). In two individual animals, however, the inner annulus fibrosus contained a few positive cells, most of which were only weakly stained. In control vertebral bodies adjacent to the intervertebral disc a positive reaction was observed in the cartilaginous endplate particularly at the bone-cartilage border (Fig. 2(b)). Type X collagen staining in the cartilaginous endplate was present in the interterritorial matrix and the pericellular region. Bone tissues in the vertebral bodies were not stained with the type X collagen antibody (Fig. 2(b)). Staining in the cartilaginous endplate and the bone tissues was high, with selective reactivity with canine type X collagen by the type X collagen monoclonal antibody used in our study [1, 18, 22]. Immunohistochemical analysis of type X collagen in the degenerated nucleus pulposus of patients undergoing surgery for IVDE was performed. Each degenerative nucleus pulposus showed type X collagen expression particularly in the extracellular matrix around the cells, although the extent of staining was variable (Fig. 2(c, d)). The signal was restricted to either the pericellular or territorial extracellular matrix. Enlarged or clustered cells commonly stained positive for type X collagen.

To examine differences in type X collagen expression between control and degenerated intervertebral discs, the relative proportion of type X collagen-stained cells per total cells in each nucleus pulposus was estimated. The relative expression of type X collagen in the degenerated nucleus pulposus ( $84.3 \pm 11.0\%$ ; data represents mean  $\pm$  standard

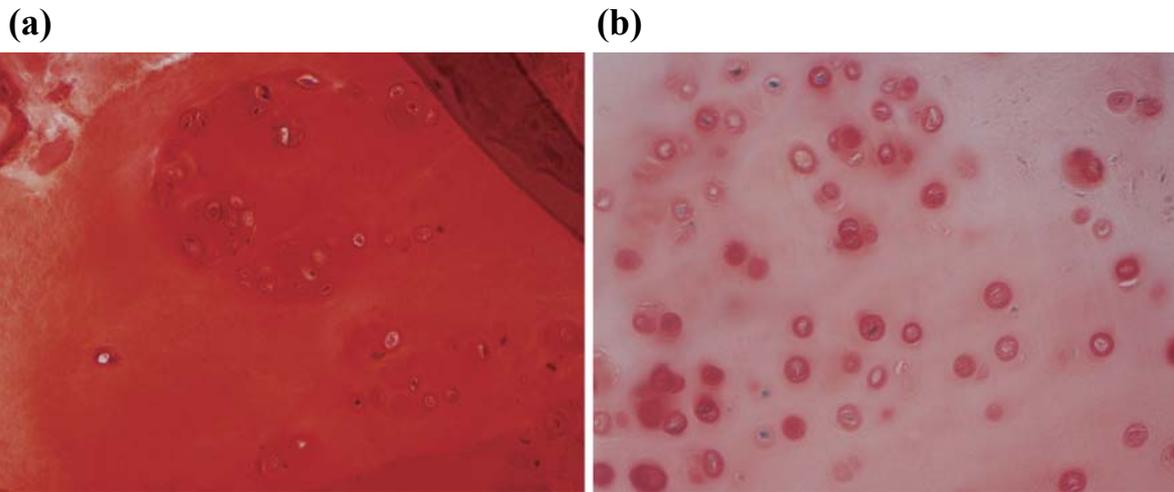


Fig. 1. Degenerative changes in the canine intervertebral disc. General structure of the nucleus pulposus of the canine intervertebral disc (female, age 1.5 years, beagle breed) is shown by safranin O (a). Degenerative intervertebral discs stained by safranin O showed clustering of hypertrophic chondrocyte-like cells (b).

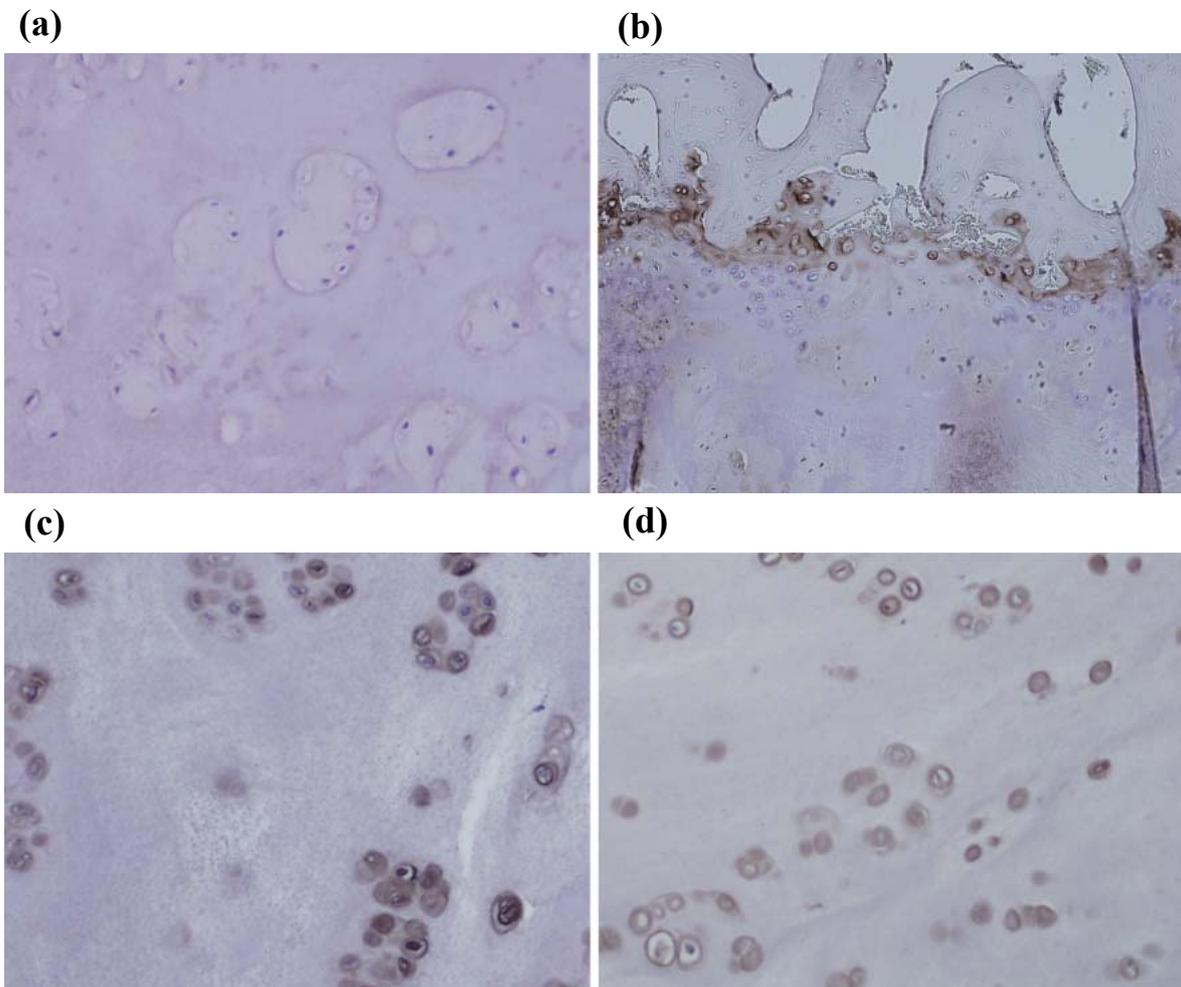


Fig. 2. Expression patterns of type X collagen in canine intervertebral disc. The nucleus pulposus, cartilaginous endplate (a, b; female, age 1.5 years, beagle breed), and degenerative nucleus pulposus (c; male, age 7.7 years, d; male, age 4.9 years) were immunostained using type X collagen antibody. Type X collagen was present in the cartilaginous endplate and the degenerative nucleus pulposus in the interterritorial matrix and pericellular region, but not in the nucleus pulposus at 1.5 years.

Table 1. Properties of control intervertebral discs

Age	Sex	Disc Level	Type X collagen expression			
			Endplate	Outer annulus	Inner annulus	Nucleus
1.0	Female	T12/T13	+++	-	-	-
1.5	Male	T13/L1	+++	-	+	-
1.0	Male	T13/L1	+++	-	-	-
2.0	Male	T13/L1	+++	-	+	-
1.5	Male	T13/L1	+++	-	-	-

This table indicates the age, sex, collected disc level and type X collagen expression of control intervertebral discs. (+), fewer than 25% positive cells; (++) , 25% to 75% positive cells; and (+++), more than 75% positive cells.

Table 2. Properties of patients' discs

Age	Sex	Grade	Disc Level	The period from the symptom to the operation	Type X collagen expression Degenerated nucleus
4.9	Male	4	L1/L2	5	+++
5.0	Male	2	L2/L3	7	++
5.4	Female	4	T11/T12	20	++
3.8	Male	3	T12/T13	2	++
4.2	Male	2	T13/L1	50	++
5.9	Male	4	T12/T13	10	+++
4.2	Male	3	T12/T13	4	+++
7.7	Male	3	T12/T13	6	+++
3.6	Male	4	T12/T13	2	+++
5.9	Male	3	T12/T13	2	++
5.5	Male	3	T11/T12	4	+++

This table indicates the age, sex, neurological deficit grade by Schulz, extrusion disc level and type X collagen expression of each patient. (+), fewer than 25% positive cells; (++) , 25% to 75% positive cells; and (+++), more than 75 % positive cells.

deviation) was significantly increased compared to the control nucleus pulposus ( $5.4 \pm 5.4$  %).

## DISCUSSION

It has been previously reported that the nucleus pulposus of scoliotic or elderly human intervertebral discs [1] and degenerative human discs [22] show high type X collagen expression, while a young nucleus pulposus barely expresses type X collagen, indicating that expression of type X collagen relates to progression of disc degeneration in the human nucleus pulposus. In this study, the degenerated nucleus pulposus of canine IVDE patients had high expression of type X collagen, while young beagles did not, which suggests that type X collagen may be one of the key molecules related to progression of intervertebral disc degeneration in chondrodystrophoid breeds of dog. Human intervertebral discs naturally express type X collagen in old age, while expression is seen in the discs of chondrodystrophoid dogs in middle age. Thus early expression of type X collagen might relate to early progression of intervertebral disc degeneration in chondrodystrophoid dogs.

In this study, we compared dachshund and beagle breeds, because both breeds are categorized as chondrodystrophoid dog breeds and the pathological changes seen in the degen-

erated discs of both breeds are very similar [11]. In this study, the ratio of males to females differed between the groups. The majority of previous reports indicate that both sexes are at equal risk of developing disc degeneration [15, 19, 20, 23], indicating that sex does not relate to the process of intervertebral disc degeneration. The difference in the sex ratio might not influence our results. Clinical and basic science observations of patients with disc herniation have demonstrated that prolapsed disc materials evoke more severe inflammation than do non-prolapsed disc materials [12, 16]. In this study, because all samples from the patient group were of extruded disc material, samples are likely to have been directly exposed to the host immune system and to evoke severe inflammation. For this reason, it can be considered that exact evaluation of the natural changes involved in disc degeneration cannot be performed in prolapsed disc materials. However, because the duration of extrusion was only short and, to our knowledge, type X collagen does not influence inflammation, the use of extruded disc material might not affect our results.

Several studies indicate that molecular changes occur in the extracellular matrix in the process of intervertebral disc degeneration in chondrodystrophoid dogs, which may explain the remarkable qualitative and quantitative differences in the collagen and proteoglycan content of the annu-

lus fibrosus and nucleus pulposus between non-chondrodystrophoid [7] and chondrodystrophoid [6] dogs. The nucleus pulposus of the chondrodystrophoid disc at birth contains up to 12-fold more collagen than proteoglycan [6]. Although already high, the collagen content continues to increase rapidly, and by 11 months of age the nucleus pulposus at all spinal levels contains on average 25% collagen content [6]. This contrasts with the nucleus pulposus of non-chondrodystrophoid dogs in which the collagen content remains below 5% for most of the dog's life [7]. These significant differences in collagen content suggest that changes in collagen content are important in the degenerative processes of the nucleus pulposus in chondrodystrophoid dogs.

Chondrogenesis is an important process in endochondral ossification [10, 14]. The initial stage of endochondral ossification is the differentiation of mesenchymal cells to chondroprogenitors, as well as the expression of cartilage-specific matrix proteins, including collagen II, IX and XI and aggrecan. The chondroprogenitors then proliferate and undergo the process of hypertrophic maturation. The hypertrophic chondrocytes begin to express hypertrophic chondrocyte markers, which include type X collagen and alkaline phosphatase. The final stage of endochondral ossification involves cartilage matrix calcification, vascular invasion, and ossification. Interestingly, type II collagen and aggrecan, the normal matrix components of the nucleus pulposus of intervertebral discs, decrease with intervertebral disc degeneration [4], and our data show enhanced expression of type X collagen in the degenerative nucleus pulposus with cell hypertrophy and clustering in the chondrodystrophoid dog. The similarities of the changing process in both endochondral ossification and nucleus pulposus degeneration indicate that calcification of the disc might relate to the endochondral ossification process.

In conclusion, type X collagen expression is significantly increased in chondrocytes of the degenerative nucleus pulposus in chondrodystrophoid dogs. Type X collagen might therefore contribute to the development of degeneration or calcification in the nucleus pulposus of the chondrodystrophoid dog. However the precise function and the factors governing expression of type X collagen remain unclear.

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