Immunohistochemical Study on Androgen Receptors in the Anterior Cruciate Ligament in Dogs

By

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Summary: Androgen is closely involved as the cause of rupture of anterior cruciate ligament (ACL) in human. In dogs, however, factors contributing to rupture of ACL remain unknown. In this study, expression of androgen receptor (AR) and histological distribution of blood vessels in ACL, and serum testosterone concentration were investigated in relation with age and sex to confirm whether canine ACL is an androgen-responsive tissue. Materials of ACL were obtained from 26 dogs: 12 young female Beagles, 2 old female mixed breeds, 9 young male Beagles, and 3 old male mixed breeds. In all canine ACL, positive AR expression was recognized in the nuclei of the fibrocytes, fibroblasts, synovial cells, and vascular endothelial cells of ACL. Expressions of AR were lesser in old males compared to the young males; however, females had no age difference in expression. Distributions of blood vessels in the synovial membrane of the ligament were fewer in old dogs both of males and females than youngs. Although distributions of vessels in the interstitium were apparently fewer in young females. Serum testosterone concentration was significantly higher in young males. Females had no age difference in the levels. From these results, it is suggested that canine ACL is an androgen-responsive tissue, and this consideration seems to closely relate to the epidemiological background that the incidence of rupture of ACL of dogs is higher in females than in males.

Introduction

Ruptured anterior cruciate ligament is one of the most important orthopedic diseases in small animal medicine. Anatomically, the anterior cruciate ligament is an important structure controlling the knee joint, originating from the caudo-medical aspect of the lateral condyle of the femur and ends at the anterior intercondylar area of the border of the tibia. It stabilizes anterior-posterior translation of the knee joint, prevents hyperextension, and controls innate rotation of the tibia [1]. Histologically, the ligament is composed of dense connective tissue with collagen fiber bundles. Between the collagen fiber bundles, rows of fibrocytes containing elongated nuclei exist sporadically as well as a small amount of loose connective tissues. Collagen, which is known as the structural protein of the collagen fibers, is synthesized by fibroblastic cells. Collagen fibril gathers to form a bundle, observed as collagen fiber. The ligament contains an extremely high amount of Type I collagen, which is 80% of the dry weight of the ligament [2, 3]. A few blood supplies exist in the loose connective tissue. There is a synovial membrane, which consists of loose connective tissue, lateral to the fibrous layer adjacent to the articular cavity, and the anterior cruciate ligament is covered with this membrane. The anterior cruciate ligament runs through the articular cavity from the femur to the tibia; therefore, it is classified as an intra-articular ligament. It, however, is covered with a synovial membrane, and therefore, it actually sits outside of the articular cavity. In the outermost layer of the synovial membrane, there are synovial cells, which are epithelial-like flat and round cells forming a single layer or several layers. The surface of the ligament is smooth and delicate and has little connection with surrounding tissues; therefore, movement is not to be
interfered [4]. In humans, a ruptured anterior cruciate ligament is more likely to occur in females than in males [5]. In females, ruptures are reported to be most likely to occur in the late luteal phase or ovulating phase [6]. Hamlet et al. (1997) reported that expression of the androgen receptors (AR) were found in the anterior cruciate ligament obtained from young males at an average age of 21.5 years, while AR expression was not found in the anterior cruciate ligament tissue obtained from older males at an average age of 47.5 years and females. On the other hand, Lovering et al. (2005) confirmed AR expression in the anterior cruciate ligament tissue from females. Others also reported that serum androgen plays an important role in the growth and development of the anterior cruciate ligament in humans [5]. In small animal medicine, it is known that a ruptured anterior cruciate ligament is more likely to occur in females than in males, and it is more likely to occur in spayed/neutered dogs than in intact dogs. On the other hand, the occurrence rate of a ruptured anterior cruciate ligament increases with age, and it occurs most frequently in dogs at the age of 6 to 10 years and with a body weight of 22 kg and above. Blood supply to the center of the anterior cruciate ligament, which is said to be the site of rupture, is reported to decline with age. As mentioned above, although androgen is considered to be closely involved in rupture of the human anterior cruciate ligament, factors contributing to a ruptured anterior cruciate ligament in dogs remain unclear, especially the involvement of androgen. Therefore, this study serves as a basic research of the anterior cruciate ligament in dogs, morphologically investigating with a focus on whether the anterior cruciate ligament is an androgen-responsive tissue, AR expression in males and females, AR expression in older and young dogs, and changes in the development of blood vessels in the center of the anterior cruciate ligament.

Materials and methods

Study materials were anterior cruciate ligaments obtained from 26 healthy dogs in total: 12 females Beagles of age 1 to 2 year(s), 2 female mixed breeds of age 7 to 8 years, 9 male Beagles of age 1 to 2 year(s), and 3 male mixed breeds of age 8 to 11 years. After collecting blood samples, all dogs were euthanized with intravenous injection of 0.5 mL (30 mg)/kg of Somnopentyl and anterior cruciate ligaments were obtained. Obtained tissue samples of anterior cruciate ligament were fixed in 10% neutral buffered formalin. As in a routine method, samples were then dehydrated with serially increasing concentrations of ethanol, penetrated by xylol, and embedded in paraffin to be sliced at 4 to 8 µm thickness. As in the routine method, hematoxylin and eosin (HE) staining and Alcian blue-PAS double staining were performed on the thinly sectioned specimens after deparaffinization. As in the routine method, sectioned specimens were deparaffinized and irradiated with microwave to activate antigens, and methanol containing hydrogen peroxide was added to remove intrinsic peroxidase. As for staining of AR, specimens were immune-stained with anti-AR rabbit antibody (NCL-ARp; Novocasta Laboratories, Ltd., Newcastle, U.K.) as the primary antibody, 10% healthy goat serum (Nichirei, Tokyo, Japan) as the protein blocking reagent, biotin-labeled goat anti-rabbit IgG antibody (Nichirei) as the secondary antibody, peroxidase-labeled streptavidin (Nichirei) as the enzyme reagent, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Nichirei) as the chromogenic substrate. Obtained specimens of the anterior cruciate ligament were embedded in Tissue-Tek O.C.T. compound to freeze at –80 °C after fixing in 4% paraformaldehyde (PFA). Cryostat was used to prepare frozen sectioned specimens of approximately 10 µm thickness. Sectioned specimens were mounted with Fluorescent Mounting Medium (Dako cytcmation, CA) using anti-AR rabbit antibody (Santa cruz biotechnology Inc., CA) as the primary antibody and Rhodamine-labeled goat IgG anti-rabbit antibody (MP niomedicals, Inc., OH) as the secondary antibody, and then, they were excited at 550 µm and examined using an inverted fluorescent microscope. Collected blood samples were immediately centrifuged to obtain serum to measure serum testosterone concentration. Development of blood vessels in the anterior cruciate ligament was evaluated by observing HE-stained canine specimens. Average values were obtained separately for males and females and a t-test was conducted between the groups. Diameters of the anterior cruciate ligament were measured by observing HE-stained specimens from young Beagles under Motic Images 2000 (Shimadzu Co., Kyoto, Japan). Averages were calculated separately for males and females and a t-test was conducted between the groups.

Results

Under the optical microscopic exam, anterior cruciate ligaments were composed of dense connective tissue with coarse collagen fiber bundles. In longitudinal sections of HE staining, rows of fibrocytes containing oval or elongated nuclei existed sporadically between the collagen fiber bundles (Fig. 1). In traverse sections, small bundles of collagen fibers were bundled by intestitium that consisted of small amount of loose connective tissue (Fig. 2). Although fibroblastic cells, many capillary vessels, and a small number of arterioles were observed in intestitium in young male dogs (Figs. 3a, b), few capillary vessels and arterioles were found in the intestitium in young female dogs (Fig. 4). Compared to the young dogs, older dogs had less blood vessels developed in both males and females.
Ligament areas were covered by synovial membrane, which consisted of loose connective tissue, and synovial cells were found in the outer layer of the synovial membrane (Fig. 5). In both males and females, capillary vessels bifurcating from synovial vessels were frequently found in the synovial membrane (Fig. 6). When measuring the number of vessels developed in all areas of the anterior cruciate ligament tissue, males demonstrated significantly higher levels (p < 0.01) than females (Fig. 8). When comparing diameters of anterior cruciate ligaments of young male and female Beagles, there were no significant differences between them. In addition, fibrocytes without nuclei were found more frequently in old dogs, both males and females, compared to the younger ones (Fig. 7).

In Alcian blue-PAS double staining, halo formation was obvious around nuclei of the fibrocytes, and there were often granules that stained reddish purple. These granules were found rarely in males and frequently in females (Fig. 9). The structure that stained reddish purple was granule-like to spider-web-like in young females while it was seen all over the area in older females (Fig. 10). Synovial cells stained blue.

In immunohistochemical findings, all nuclei from fibrous and fibroblast cells (Fig. 11), vascular endothelial cells (Fig. 12), and synovial cells (Fig. 13) were positive for AR expression in both males and females. Synovial cells were strongly positive, fibroblast and vascular endothelial cells were moderately positive, while males showed stronger positives. Compared to younger males, older males showed weaker AR expression (Fig. 14), and there was no difference by age in females (Fig. 15).

In AR immunostaining by immunofluorescence antibody technique, all nuclei from fibrocytes, fibroblast cells, vascular endothelial cells, and synovial cells were positive for AR expression (Figs. 16–21). Males showed stronger...
H. Ohno et al.

AR expression than females.

In serum biochemical findings, males showed significantly higher serum testosterone concentration ($p < 0.01$) compared to females (Fig. 22). Although older males showed slightly lower testosterone concentration than the younger males, there was no difference by age in females (Fig. 23).

Discussion

Since AR protein was expressed in all the nuclei of fibrocytes, fibroblast cells, synovial cells, and vascular endothelial cells of the anterior cruciate ligament in male and female dogs, the canine anterior cruciate ligament is considered an androgen target tissue, as with the human anterior cruciate ligament. Canine AR protein consists of 907 amino acid residue. It is a nucleoprotein with a predicted molecular weight of 98.7 kDa. Since it is localized within the nucleus, it is classified into the ligand-dependent intranuclear receptor superfamily and it is reported to exist along the X chromosome [6, 7, 8].

In the human anterior cruciate ligament, androgen target cells are reported to be fibrocytes, fibroblast cells, synovial cells, and vascular walls [5, 6].

Upon comparison of the AR protein expression and serum testosterone concentration between males and females, males showed stronger AR protein expression and significantly higher serum testosterone concentration than females. From above, it was considered that AR

Fig. 4. Young female dog: HE staining
Vascular structure is rarely observed in the stroma. $\times 600$

Fig. 5. Young female dog: HE staining
Synovial cells are found in the outermost layer of the synovial membrane near the ACL. $\times 600$

Fig. 6. Young female dog: HE staining
Many vessels were found in the synovial membrane. $\times 600$

Fig. 7. Old male dog: HE staining
Compared to the younger dogs, fibrocytes without nuclei (arrowhead) were more frequently observed. $\times 600$
Androgen receptors in the ACL of dogs

Protein expression is regulated by androgen and the anterior cruciate ligament is a testosterone-dependent tissue. Major ligands for AR protein in the human body include testosterone and dihydrotestosterone. In rats, AR protein is expressed as moderately to strongly positive in the prostate, vesicular gland, coagulating gland, epididymis, and testes in males, and negatively to moderately positive in the vagina, uterus, and ovaries in females. While male rats show weakly positive expression in the liver, females are reported to show negative or weaker positive expression than males [9]. Testes secrete a large amount of androgen, mainly as testosterone, as well as a small amount of estrogen. On the other hand, ovaries secrete a large amount of estrogen and a small amount of androgen; therefore, androgen is widely known as a male hormone and estrogen as a female hormone. In ruptured anterior cruciate ligament in dogs, a ruptured anterior cruciate ligament is known to occur more frequently in spayed females than in intact females and more in neutered males than in intact males [10, 11]. Therefore, since removal of sex glands in dogs increases the likelihood of a ruptured anterior cruciate ligament, it was suggested that sex hormones possess some effects on the anterior cruciate ligament.

AR protein is expressed more weakly in old male dogs than in younger ones, and there was no difference in expression by age in females. Old male dogs had lower serum testosterone concentrations than younger males, and there was no difference by age in females. This indi-

Fig. 8. The number of blood vessels developed in the ACL. The number of males is significantly greater compared to females. (p < 0.01).

Fig. 9. Young female dog: Alcian blue-PAS double staining
Granular or spider web-like structure (arrow head) are formed in the perinuclear halos. ×600

Fig. 10. Old female dog: Alcian blue-PAS double staining
The structures in the perinuclear halos are stained reddish brown (arrow head). ×600
Fig. 11. Young male dog: Immunostaining using AR antibody. Nuclei of fibrocytes and fibroblasts shows positive for AR expression. ×300

Fig. 12. Young male dog: Immunostaining using AR antibody. Nuclei of endothelial cells of capillary vessels and arterioles in the stroma are positive for AR expression (arrowhead). ×600

Fig. 13. Young male dog: Immunostaining using AR antibody. Nuclei of the synovial cells (arrow) and endothelial cells (arrowhead) are positive for AR expression. ×600

Fig. 14. Old male dog: Immunostaining using AR antibody. Nuclei of the fibrocytes and fibroblasts are weakly positive for AR expression compared to that of young male (Fig.11). ×300

Fig. 15. Young female dog: Immunostaining using AR antibody. Nuclei of the fibrocytes and fibroblasts are weakly positive for AR expression compared to that of young male (Fig.11) ×300

Fig. 16. Young male dog: Immunofluorescence staining using AR antibody. Nuclei of the fibrocytes and fibroblasts are strongly positive for AR expression. ×200
Androgen receptors in the ACL of dogs

This study also revealed that AR protein is expressed in the nuclei of vascular endothelial cells. In addition, males had more numbers of blood vessels distributed in the ligament than females. And, older dogs had less number of blood vessels distributed in the anterior cruciate ligament in both males and females. Therefore, it is considered that androgen might affect vascular endothelial cells of the anterior cruciate ligament, regulating blood flow. Changes in the development of blood vessels by sex and age are

cates that age affects the expression of AR protein in the anterior cruciate ligament in male dogs although it does not affect females, and there was a correlation with serum testosterone concentration. In human anterior cruciate ligament, AR protein is not expressed in older males with decreasing secretion of androgen. It is reported that it does express in males in puberty to maturity, when androgen is actively secreted [5].

Fig. 17. Young male dog: Immunofluorescence staining using AR antibody
Nuclei of synovial cells (arrowhead) are strongly positive for AR expression. ×20

Fig. 18. Young male dog: Immunofluorescence staining using AR antibody
Nuclei of the vascular endothelial cells in the stroma (arrowhead) are strongly positive for AR expression. ×20

Fig. 19. Young female dog: Immunofluorescence staining using AR antibody
Nuclei of fibrocytes and fibroblasts (arrowhead) are weakly positive for AR expression than those of young males. ×200

Fig. 20. Young male dog: Immunofluorescence staining using AR antibody
Nuclei of synovial cells (arrowhead) were weakly positive for AR expression than those of young males. ×10

Fig. 21. Young female dog: Immunofluorescence staining using AR antibody
Nuclei of vascular endothelial cells in the stroma (arrowhead) were weakly positive for AR expression than those of young males. ×20
similar to epidemiological background of canine ruptured anterior cruciate ligament; therefore, it was considered that blood flow might affect the strength of the anterior cruciate ligament. In canine anterior cruciate ligament, the largest numbers of blood vessels exist at the attachment site at the femur, and it is reported that the number of blood vessels decreases as it goes toward the center of the ligament [12]. In another study, it is said that the reason for fewer vessels in the center of the anterior cruciate ligament was that anterior and posterior cruciate ligaments entwine themselves and become twisted, causing vessels to compress [13]. Others reported that age is one of the factors for lack.

Fig. 22. Serum testosterone concentrations
Compared to females, males had significantly higher serum testosterone concentration (p < 0.01).

Fig. 23. Serum testosterone concentrations by ages
Although there is no age difference in females, the concentration in old males is lower than in young males.
of blood supply to the center of the anterior cruciate ligament. In canine coronary arteries, testosterone induces remarkable vasodilation [14], and in naturally occurring hypertensive rats, vasodilation induced by testosterone can be decreased by removing vascular endothelial cells [15]. In addition, the incidence rate of rupture increases with age; it occurs most frequently between the ages of 7 and 10 years in the canine ruptured anterior cruciate ligament [16]. One of the factors was that as collagen fibers of the canine anterior cruciate ligament become hyalinized progressively with age, tension of the ligament is said to regress gradually in dogs at age 5 years and older. A ruptured anterior cruciate ligament is known to occur more frequently in females than males [16]. In addition, the fibrocytes without nuclei was frequently observed in older dogs, both male and female, compared to the younger ones. This is similar to loss of nuclei in fibrocytes, which is an histopathological characteristic of a ruptured anterior cruciate ligament; therefore, older dogs might more likely be affected than younger dogs [17, 18].

When Alcian blue-PAS double staining was conducted, reddish purple granules were found in the area close to the halo around the nuclei of the fibrocytes more frequently in females than in males. And, the entire area was stained reddish purple in older female dogs. Considering the report that says halo formation near the nuclei of the fibrocytes might be involved in the rupture of the anterior cruciate ligament, as well as epidemiological background of the ruptured anterior cruciate ligament, the granule, which was stained with Alcian blue-PAS double staining where the halo was observed, should be investigated [17].

In conclusion, AR protein is expressed in the canine anterior cruciate ligament; therefore, it was considered to be an androgen target tissue. Expression of the AR protein is considered to be affected by sex, age, and serum testosterone concentration.

References