Case Report

Bilateral Ovotestes in a Female Beagle Dog

Kinji Kobayashi1, Toshihisa Fujiwara1, Tamiko Adachi1, Masatoshi Asahina1, Yoshifumi Sasaki1, Aoi Matsuda1, Tomonari Nishimura1, Toshihide Inui1, and Kazuyuki Kitamura1

1Drug Safety Research Laboratories, TANABE Seiyaku Co., Ltd., 16–89 Kashima 3-chome, Yodogawa-ku, Osaka 532–8505, Japan

Abstract: We report a case of bilateral ovotestes in a female beagle dog. This dog was used in a 4-week repeated-dose toxicity study and sacrificed by exsanguination at 6 months of age. Clinical observation, hematological examination, blood chemistry analysis, urinalysis and autopsy did not reveal any abnormal or drug-induced effects. Microscopically, seminiferous-like tissue was observed in the medulla of the bilateral ovaries. The morphological and immunohistochemical characteristics of Sertoli-like cells lining the seminiferous-like tubules corresponded to those of Sertoli cells in the testis. Myoid-like cells exhibiting positive reactions for alpha smooth muscle actin surrounded the seminiferous-like tubules. Therefore, the dog was regarded as a case of true hermaphroditism with bilateral ovotestes. PCR using DNA extracted from paraffin-embedded sections of the testicular parts of the ovotestis, uteri and spleen showed that the organogenesis of ovotestes in this case was not associated with the sex-determining region Y gene. (J Toxicol Pathol 2007; 20: 111–115)

Key words: ovotestes, true hermaphroditism, SRY, beagle

Ovotestis refers to a gonad with histology consistent with both ovarian follicles and testicular tubular elements. Such gonads are frequently found in animals with true hermaphroditism1, and canine ovotestes have been associated with XX sex reversal and true hermaphroditism2–6. Here, we report bilateral ovotestes observed in a female beagle dog with an otherwise normal appearance. The dog was purchased from Hongo Farm (Kitayama Labes Co., Ltd., Yamaguchi, Japan) and housed at Tanabe Seiyaku Co. Ltd. (Osaka, Japan). The animal was treated with a particular chemical in a 4-week repeated-dose toxicity study approved by the Animal Ethics Committee of Tanabe Seiyaku Co., Ltd. Clinical observation, hematological examination, blood chemistry analysis and urinalysis revealed no abnormalities or drug-induced effects. At 6 months of age, the animal was sacrificed by exsanguination under thiopental sodium anesthesia and an autopsy was conducted. The ovaries were fixed in 10% phosphate-buffered formalin solution and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin.

Immunohistochemistry was performed with primary antibodies, including mouse monoclonal anti-vimentin (1:200, DAKO Japan Co., Ltd., Kyoto, Japan), anti-inhibin-alpha (1:50, DAKO), anti-alpha smooth muscle actin (SMA) (used as supplied, DAKO) and anti-proliferation cell nuclear antigen (PCNA) (1:200, DAKO) antibodies. The tissue sections were boiled in Instant antigen activator agent H (neutral) (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), incubated with a 0.3% (v/v) methanol solution of hydrogen peroxide for quenching of endogenous peroxidase, and immersed in normal goat serum (used as supplied, Vector Laboratories, Inc., Burlingame, CA, USA) to prevent non-specific reactions. The antibodies were applied to the tissue sections overnight at 4°C and were subsequently detected using Histofine® Simple Stain MAX PO (MULTI) (Nichirei Co., Ltd., Tokyo, Japan) and/or Histofine® Simple Stain DAB solution (Nichirei). The seminiferous-like tubules in the ovotestes were compared immunohistochemically with seminiferous tubules in the testis of a male beagle, granulosa cell cords in the ovary of a female beagle (both of these animals were in the control group in the same toxicity study) and hyperplasia of the granulosa cell cords, which is histologically similar to our case, in the ovary of a female mongrel dog (aged 12 years old).

DNA samples were prepared from paraffin-embedded sections of the testicular regions (medulla) in the ovotestes, of the medulla in the ovaries, uteri and spleens of the case animal and control animals, and of the testis of the control animal using DNA Isolator PS-Rapid Reagent (Wako Pure Chemical Industries Ltd., Osaka,

Received: 21 February 2007, Accepted: 18 April 2007
Mailing address: Kinji Kobayashi, Drug Safety Research Laboratories, TANABE Seiyaku Co., Ltd., 16–89 Kashima 3-chome, Yodogawa-ku, Osaka 532–8505, Japan
TEL: 81-6-6300-2958 FAX: 81-6-6300-2696
E-mail: kinji-k@tanabe.co.jp
Japan). The primer (-1) and nested primer (-2) sequences for amplification of the canine sex-determining region Y (SRY) gene on the Y-chromosome and the hypoxanthine phosphoribosyltransferase (HPRT) gene on the X-chromosome were as follows: SRY forward primer-1, 5'-GAC GAC CCA TGA ACG CAT TCT TGG-3'; SRY reverse primer-1, 5'-CTC GAA GAA TGG CCA TTT TTC GG-3'; SRY forward primer-2, 5'-GAA CGC ATT CTT GGT GTG GTC TC-3'; SRY reverse primer-2, 5'-GGC CAT TTT TCG GCT TCT GTA AG-3'; HPRT forward primer-1, 5'-CCC TCG AAG TGT TGG CTA TAA ACC-3'; HPRT reverse primer-1, 5'-TGC TTA CAA ACG TGC CTT CTC TAC-3'; HPRT forward primer-2, 5'-TGG CTA TAA ACC TGA CTG TAA GTG-3'; and HPRT reverse primer-2, 5'-CGT GCC TTC TCT ACA AAT ACT CTC-3'. The partial sequences of the canine HPRT and SRY genes were amplified for 32 cycles in a thermal cycler (PTC-200 DNA Engine™, MJ Research, Watertown, MS) using the following conditions: initial denaturation at 94°C for 2 min; 10 cycles at 94°C for 30 sec, at 58°C (HPRT)/62°C (SRY) for 30 sec; 20 cycles at 94°C for 30 sec, at 56°C (HPRT)/60°C (SRY) for 30 sec, and at 72°C for 45 sec; and a final extension step at 72°C for 1 min. The expected sizes of PCR products for the SRY and HPRT genes.

**Fig. 1.** Histopathology of the ovotestes in a beagle dog. A mass of tubular-like tissue was found in the medulla of the ovary (a). The tubular tissue was similar in structure to the seminiferous tubules and lined by spindle cells (Sertoli-like cells) with nuclei located in basal area (b). HE staining; Bar = 250 µm (a); Bar = 50 µm (b).

**Fig. 2.** Immunohistopathology for alpha smooth muscle actin (SMA) in the seminiferous-like tubules of the canine ovotestes. The cells surrounding the seminiferous-like tubules in the ovotestis (a; arrowheads) and the testis (c) were positive for SMA, whereas positive reactions for SMA were not observed around the granulosa cell cords in the ovary of a control animal (b). Bar = 30 µm.
were 132 base pairs (bp) and 94 bp, respectively. DNA sequencing analysis of the amplicons from nested PCRs obtained for the testis of the control animal was performed at Dragon Genomics Center (Takara Bio, Inc., Shiga, Japan) and showed that the products were derived from the SRY (EMBL: AF107021) and HPRT (EMBL: AY283373) genes.

Macroscopically, there were no abnormalities of the external or internal genitalia, including the ovaries, in this case, although XX true hermaphrodite dogs typically show ambiguous (partially masculinized) external genitalia. The weights of the ovaries were similar to those of female beagles in the control group. Microscopically, a mass of tubular-like tissue was noted in the medulla of the bilateral ovaries (Fig. 1a). The tubular tissue was similar to atrophied seminiferous tubules and was lined by spindle cells (Sertoli-like cells) with nuclei located in the basal area (Fig. 1b). Spermatogenic germ cells were not observed within the seminiferous-like tubules. Round or polygonal cells (Leydig-like cells) with granular eosinophilic cytoplasm and small round nuclei were scattered between the seminiferous-like tubules. In addition, there were fewer oocytes (primordial follicles) in the cortex of the ovaries in the case compared to the control animals, but primary follicles (primordial follicles) in the cortex of the ovaries in the case (Fig. 2a) and the testis. The cells lining the hyperplastic granulosa cell cords in the ovary of the control female dog (Fig. 2b) or in the hyperplastic granulosa cell cords in the female mongrel dog were positive for PCNA and vimentin, and mostly negative for inhibin-alpha. Cells exhibiting positive reactions for alpha smooth muscle actin were seen around the seminiferous-like tubules in the case (Fig. 2a) and the seminiferous tubules in the testis of the control male dog (Fig. 2c), whereas positive reactions for alpha smooth muscle actin were not observed around the granulosa cell cords in the ovary of the control female dog (Fig. 2b) or in the hyperplastic granulosa cell cords in the female mongrel dog. In addition, the Leydig-like cells between the seminiferous-like tubules were negative for PCNA and positive for vimentin. The Leydig-like cells in the testis of the control male dog were clearly positive for inhibin-alpha, and these cells showed variable affinity for anti-inhibin-alpha antibody in the case animal. The Sertoli-like cells had morphological and immunohistochemical characteristics corresponding to those of actual Sertoli cells, and myoid-like cells exhibiting positive reactions for alpha smooth muscle actin surrounded some of the seminiferous-like tubules. The results identify the tubular structures in the medulla of ovaries as seminiferous tubules, rather than normal or hyperplastic granulosa cell cords, allowing histopathological diagnosis of the lesion as bilateral ovotestes. Since true hermaphrodites have both ovarian and testicular tissues, either in the form of a combined gonad (ovotestis) or as separate organs, this case was regarded as an example of true hermaphroditism.

Canine ovotestes have been reported in cases of XX sex reversal and true hermaphrodites. Peripheral karyotype analysis was not performed in the current case, but PCR primers targeting the SRY gene were used to judge whether the animal had a Y chromosome. Since the SRY gene was not amplified in PCR using DNA samples isolated from paraffin-embedded sections of the spleen, it was judged that the case animal did not have a Y chromosome.

Phenotypic, gonadal, and molecular studies have led to several hypotheses for the developmental mechanism of ovotestes, including genetic chimeraism due to double fertilization or an exchange of cells between dizygous twins of different sex; mitotic non-disjunction on the Y chromosome at the first two cleavage divisions; translocation Y-chromosomal sequences, including the SRY gene, onto an X chromosome; and hidden mosaicism with Y-bearing cells in the gonads. We evaluated SRY hidden gonadal mosaicism in this case by SRY gene amplification using DNA samples isolated from paraffin-embedded sections. As a result, although a HPRT gene was not found in the ovotestes or uterus (Fig. 3). Therefore, we concluded that the SRY gene was not associated with the development of ovotestes in this case.

Expression of SRY might initiate testis induction by up-regulating SRY-related high-mobility group box 9 (Sox9).
expression. The timing of SRY and SOX9 expression is consistent with a role in testis determination in dogs, as well as in humans, sheep and pigs. However, SOX9 is up-regulated by loss of WNT4 in XX gonads, even if SRY is absent, and loss of WNT4 gives rise to masculinization of the XX gonad. There were fewer oocytes in the cortices of the ovaries in XX sex reversal cases, compared with the control. Since it is thought that WNT4 acts through follistatin to maintain germ cell survival in the ovary, WNT4 deficiency may induce a decrease in oocytes. It has also been reported that exogenous androgen administration to a pregnant bitch caused true hermaphroditism with the presence of bilateral ovotestis and remnants of the Wolffian duct system in all female pups. Because WNT4 overexpression inhibits testosterone synthesis by repressing steroidogenic factor 1/ beta-catenin synergy, loss of WNT4 in a female may enhance testosterone synthesis. Aromatase is a catabolizing enzyme that controls the androgen/estrogen ratio by irreversible conversion of testosterone into estradiol, and aromatase inhibitors administered before sexual differentiation of the gonads can induce transdifferentiation from ovarian medullary cords into testicular cords/tubes, differentiation of the gonads can induce transdifferentiation.

The authors would like to thank Dr. Mitsuru Kuwamura of Osaka Prefectural University for his kind gift of paraffin-embedded sections of the ovary of a female mongrel dog. We are also grateful to all attendees of the Kansai Conference on Toxicologic Pathology for the histopathological review.

Acknowledgements: The authors would like to thank Dr. Mitsuru Kuwamura of Osaka Prefectural University for his kind gift of paraffin-embedded sections of the ovary of a female mongrel dog. We are also grateful to all attendees of the Kansai Conference on Toxicologic Pathology for the histopathological review.

References

2. Melnyczek JR, Dambach D, Prociuk U, Jezik PF, Henthorn PS, Patterson DF, and Giger U. Sry-negative XX sex reversal does not seem to be located in regions containing WT1, DMRT1, GATA4, FO2G, LHX1, SF1, SOX9 or Lhx9. Mutation of the WNT4 gene is a further possible candidate as a cause of canine SRY-negative XX sex reversal, and it remains to be seen whether loss of WNT4 occurred in the current case. Quantitation of plasma sex hormones and aromatase activity in the gonad may also shed light on the cause of canine ovotestes.

Kobayashi, Fujiwara, Adachi et al.