Production and Immunohistochemical Characterization of Specific Antibody to Oviductal Fluid of Miniature 'Shiba' Goat

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ABSTRACT. Monoclonal antibodies were produced against the oviductal secretory substances in 'Shiba' goats and their nature was characterized. In the ampullary region, very strong immunoreactivity with one clone (EE-1) was localized within the cytoplasm of non-ciliated cells ("secretory cells"), whereas very little or no immunoreactivity was observed in the isthmus region. This reactivity pattern was observed in both estrous and luteal phases. Immunohistochemical analysis showed the antigen to be either absent or minimally present in other organs including the alimentary canal, liver, pancreas, lung, kidney, uterus, thyroid gland, heart, spleen and spinal cord, and in oviducts of other species such as hamster, sheep and cow. The immunoglobulin isotype of EE-1 was determined as IgM with a κ light chain. The positive reactions were eliminated by treating deparaffinized sections with periodic acid or testicular hyaluronidase. Results obtained in this study suggest that ampullary secretory cells in 'Shiba' goat oviduct contain a species- and tissue-specific antigen containing hyaluronic acid or hyaluronidase-sensitive carbohydrates.—**KEY WORDS:** goat, monoclonal antibody, oviduct.

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Oviducts play some important roles in various phenomena such as sperm capacitation, acrosome reaction induction, oocyte maturation, fertilization, transportation of gametes, and early embryo development [4]. Although oviductal specific glycoproteins have been studied in some mammals such as mouse [17], hamster [3, 11, 16, 20, 25, 28, 30, 31], rabbit [10, 26, 34], cow [2, 6, 21, 39, 40], sheep [12, 24, 35], pig [8], baboon [5, 9, 37] and human [36, 38], no study has been made on caprine oviductal glycoproteins. The miniature 'Shiba' goat is native to Japan and an ideal experimental model for studies of the ruminant reproduction because of its non-seasonal breeding and genetically controlled character [22]. Monoclonal antibodies (MAbs) have been utilized to find new functional molecules in the oviduct of animals, including rabbit [10], cow [2] and hamster [27, 30]. This study was designed to produce the monoclonal antibody against the oviductal secretory substances of 'Shiba' goat and characterize its immunohistochemical nature.

MATERIALS AND METHODS

Animals: Mature 'Shiba' goats raised in the experimental farm of The University of Tokyo, Faculty of Agriculture, were used in this study. Estrus was induced by two intramuscular injections of 2 mg prostaglandin F₂ α (PGF₂ α) at an interval of 12 hr during the luteal phase. The luteal phase was determined by the existence of corpora lutea by cludoscopy. Estrus was induced about 2 days after the first injection of PGF₂ α , and goats displaying receptive behavior to males and possessing matured follicles observed by cludoscopy were regarded to be in the estrous stage.

Collection of oviductal fluids: Goats in the estrous

stage were sedated with an intravenous injection of a mixture of 10 mg/kg ketamine hydrochloride (Ketalar, Sankyo Co., Tokyo, Japan) and 0.02 mg/kg xylazine hydrochloride (Seractal; Bayer Co., Tokyo, Japan) and anesthetized with halothane inhalation. The utero-tubal junction was exposed and a catheter of 18G (Nihon Sherwood, Japan) was inserted in cranial grade way and ligated, and the another edge of the catheter was exposed out from the peritoneal cavity, and the oviductal fluid was collected continuously and kept at -20°C .

Production of hybridoma: The oviductal fluid, the concentration of protein of which was 7.2 mg/ml (OD280), was mixed with an equal volume of complete Freund's adjuvant (Difco Lab., Detroit, MI, U.S.A.). The mixture (0.1 ml) was injected subcutaneously into Balb/c mice, and thereafter 0.25 ml of the same mixture was repeatedly injected at 2-week intervals. Three days after the final injection, spleen cells (2.8×10^8) from the mice were fused with mouse myeloma cells (SP 2/0-Ag14; 4.3×10^7) in the presence of polyethylene glycol (PEG 4000, Merck, Darmstadt, Germany) as a 50% (w/v) solution in phosphate buffered saline (PBS). After fusion, the cells were distributed to the multi well dishes. After 2 weeks, supernatants from the growing cells were screened by a morphological immunoperoxidase assay on paraffin sections of Carnoy-fixed 'Shiba' goat oviduct prepared as described below. Hybridomas, the supernatant of which positively reacted with the epithelial cells of the oviduct, were cloned twice by limiting dilution, and the clones were grown in mass culture or injected intraperitoneally into Balb/c mice treated with 2, 6, 10, 14-tetramethylpentadecan to obtain ascites fluid. Culture supernatants of hybridoma and ascites fluid were used as the source of MAbs. Hybridomas were cryogenically preserved in a deep

freezer (-80°C) for future use.

Immunoglobulin subtype of monoclonal antibody: The antibodies can belong to different immunoglobulin classes and subclasses which differ in their chemical and biological properties. The immunoglobulin subtype was identified using a mouse MAb isotyping kit (Amersham International, Bucks, U.K.).

Immunohistochemistry: Adult Shiba-goats were anesthetized as mentioned above and bilateral oviducts were taken out. These oviducts were fixed with Carnoy's fixative at 4°C for 12 hr. Some 'Shiba' goats were sacrificed to collect tissues (duodenum, colon, pancreas, liver, lung, kidney, uterus, thyroid, heart, spleen and spinal cord). Fixed materials were dehydrated with ascending alcohol, embedded in paraffin, and cut into $6\text{-}\mu\text{m}$ thickness. Deparaffinized sections were treated with 0.5% periodic acid solution for 20 min to remove any endogenous peroxidase activity. After being washed in distilled water, the sections were blocked with 5% normal rabbit serum for 30 min, and incubated with MAb overnight at 4°C . After being washed in PBS, the sections were incubated with peroxidase-conjugated rabbit antibody against mouse immunoglobulin (DAKO JAPAN, Kyoto, Japan) for 1 hr. They were then washed in PBS and incubated with a diaminobenzidine color-development solution. After the development of color, the sections were washed in water and mounted for examination under a light microscope. The specificity of MAbs against the carbohydrate moieties or glycosaminoglycans of the antigens was determined using sections of antigen-positive tissues that had been pretreated with 0.5% periodic acid solution overnight at room temperature, or with a solution of chondroitinase ABC or chondroitinase AC (Seikagaku Corporation, Japan) (4.0 unit/ml) for 2 hr at 37°C , or with 0.05% testicular hyaluronidase (Boehringer Mannheim, GmbH, Germany) for 3 hr at 37°C .

RESULTS

The monoclonal antibody, EE-1, was of the immunoglobulin M class with a κ light chain, as determined by a mouse MAb isotyping kit. Immunohistochemical labeling of sections of the 'Shiba'-goat oviduct using EE-1 as the primary antibody was intense and highly specific to the ampullary region of the oviduct. A positive reaction was observed in the cytoplasm of non-ciliated cells, or secretory cells, in the ampulla (Figs. 1, 2). Very little or no immunoreactivity was observed in the isthmus region (Figs. 3, 4). This reactive pattern was observed in tissues from animals in both the estrous (Fig. 2) and the luteal phases (Fig. 5). The antigen was shown to be either absent or minimally present in other goat organs including the alimentary canal, liver, pancreas, lung (including hyaline cartilage), kidney, uterus, thyroid gland, heart, spleen and spinal cord, and in the oviducts of hamster, sheep and cow. Positive immunostaining of MAb EE-1 in the ampulla was completely eliminated after treatment with 0.5% periodic acid (Fig. 6) and hyaluronidase (Fig. 7) but

not with either chondroitinase ABC (Fig. 8) or AC (data not shown).

DISCUSSION

Although oviduct specific proteins for two other domestic ruminant species (cattle and sheep) have been extensively investigated [2, 6, 12, 21, 24, 35, 39, 40], very few data have been gathered for both functional and morphological information on the caprine oviduct, describing only the luminal surface topography studied by scanning electron microscopy [33]. Results obtained in this study suggest that ampullary secretory cells of 'Shiba' goat contain a species- and region-specific antigen recognized by MAb EE-1. This is the first report which describes the oviduct specific antigen of the goat. MAb obtained in this study will be an extremely valuable tool to investigate the ruminant reproduction, especially the gamete-oviduct interaction.

A recent work on glycoproteins in sheep oviductal fluid has indicated the presence of a novel substance, subunit size of Mr 80,000–90,000, that is present for 3 to 6 days of each estrous cycle, the period coinciding with that of a high rate of fluid flow and the presence of embryos in the oviduct [35]. Fractions of oviductal flashings obtained from estrous ewes have been shown to contain a 90–92 kDa periodic acid-Schiff (PAS)-positive protein that binds to sperm. This estrus-associated 90–92 kDa protein has been shown to be localized in the zona pellucida, blastomere membranes, and in the perivitelline space of early cleavage-stage sheep embryos [12]. Murray [23] demonstrated, by light and electron microscopic immunocytochemistry, the localization of this protein in the secretory granules in the non-ciliated cells of the ampulla. Gerena and Killian [13] separated two oviduct-specific proteins (47 kDa and 80–95 kDa) in the bovine oviductal fluid. This 80–95 kDa protein appeared for 3–4 days at or near the time of ovulation. Abe *et al.* [2] obtained MAbs which were produced against materials obtained from the bovine oviduct. These MAbs reacted strongly with the supra-nuclear cytoplasm of oviductal epithelial cells of cows in the follicular phase. Immunohistochemical labeling of sections of the 'Shiba'-goat oviduct using EE-1 as the primary antibody was intense and highly specific to the ampullary region of the oviduct. The positive reaction was observed in the cytoplasm of non-ciliated cells, or secretory cells, in the ampulla, but not in the isthmus. The secretory cells in the ampulla are postulated to play a more important role than do those in the isthmus region of the oviduct of the sheep [14]. Fertilization and early embryonic development take place in the ampulla. Smith and Yanagimachi [32] speculated in the hamster that ampullary and fimbrial secretions play an important role in oocyte maturation, cumulus cell dispersion, and fertilization, but that isthmus secretions facilitate sperm capacitation and transport and maintenance of cleaving embryos. Thus, the isthmus, the site of sperm storage, may not synthesize or release the molecule which is recognized by

Fig. 1. Positive immunostaining of MAb EE1 is shown in the ampullar region of the oviduct obtained from a goat in estrus. $\times 520$.

Fig. 2. High magnification of a part of Fig. 1. Arrow heads indicate ciliary cells that do not show positive reaction against MAb EE1. Arrows show secretory cells with intense immunostaining of MAb EE1. $\times 1,600$.

Figs. 3. and 4. Low (Fig. 3: $\times 520$) and high (Fig. 4: $\times 1,040$) magnification of the isthmus region of the oviduct of a goat in estrus. No immunoreactivity is observed.

Fig. 5. Immunohistochemical staining of MAb EE1 in the ampullary epithelia in a pregnant goat. $\times 260$.

MAb EE-1. This MAb will be a useful probe to study the functional significance of the ampullar in the oviduct of the goat.

Oviductal serotory glycoproteins have been shown to be associated with the zona pellucida and/or the perivitelline spaces of ovulated ova and developing embryos in the mouse [17], golden hamster [1, 16, 20, 25, 29], sheep [12], pig [7, 14] and baboon [5]. It will be worth studying whether MAb EE-1 is associated with the zona pellucida and/or embryos. These further studies using this MAb will

elucidate critical functions of the oviductal glycoproteins.

It is speculated that the antigens recognized by MAb EE-1 are secretory components of oviductal fluid. It is still unknown whether or not MAb EE-1 reacts with what kind of the secretory components of oviductal fluid. Pretreatment with periodic acid or hyaluronidase completely eliminated immunostaining of MAb EE-1 in the oviduct. These results suggest that MAb EE-1 may recognize the carbohydrate moieties or glycosaminoglycans including hyaluronic acid. Lee and Ax [18] demonstrated the

Fig. 6. Positive immunostaining of MAb EE-1 in the ampullar region is completely eliminated after treatment with 0.5% periodic acid. $\times 1,040$.

Fig. 7. Positive immunostaining of MAb EE-1 in the ampullar region is completely eliminated after treatment with hyaluronidase. $\times 1,040$.

Fig. 8. Immunohistochemical staining of MAb EE-1 is not disturbed with treatment of chondroitinase ABC. $\times 1,040$.

existence of hyaluronic acid and chondroitin sulfate in the bovine oviduct. Glycosaminoglycans lead to a significant increase of acrosome reactions in sperm *in vitro* [19], but their role in secretions of the female reproductive tract remains to be established. Positive reaction of MAb EE-1 disappeared by treatment with hyaluronidase. However, this antibody did not react with cartilage matrix which contained a large amount of hyaluronic acid. From these aspects, it is speculated that the epitope recognized by MAb EE-1 is composed of hyaluronidase-sensitive carbohydrates specifically expressed in the caprine oviduct. Further characterization of the antigen would be still necessary.

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