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Abstract of Dissertation

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Title : Regulation of neutrophils phagocytosis for sperm by alpha 1-acid glycoprotein (AGP) in the bovine oviduct.

（ウシ卵管における alpha 1-acid glycoprotein (AGP) を介した好中球による精子貪食の調節機構）

Abstract

The oviduct is a key component of the female reproductive tract, where essential states such as oocyte maturation, sperm capacitation, fertilization, and initial embryonic development take place. An intriguing question of oviductal function is how the oviduct mounts local immune responses against microbial pathogens, while allowing spermatozoa and the early embryo, both of which carry foreign proteins, to escape detection by the local immune system of the oviduct.

We have previously shown that polymorphonuclear neutrophils (PMNs) are present in bovine oviductal fluid under physiological conditions during the different estrous cycle. Therefore, in this study, I investigated the possible changes in the number of PMNs throughout the different stages of the estrous cycle. The bovine oviduct was very gently flushed by 200 μL PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$) without any touch from the outside but with just handing from ampulla to isthmus, and the oviduct fluid was accumulated into a 1.5 mL microcentrifuge tube. Bovine oviduct epithelial cells （BOEC） and the immune cells were separated from the oviductal fluid by 10 ml of 35% Percoll. For a total cell count, a sample of the leukocyte suspension was diluted (1:10) with 0.1% acetic acid, and mounted on a haemocytometer. To determine PMNs proportions in leukocyte populations, a 20 μl sample of the leukocyte suspension was diluted in Macs separation buffer and analyzed by flow cytometric evaluation. Neutrophils infiltrate the oviduct fluid not only during the preovulatory stage,

but also throughout the different stages of the estrous cycle, and that their numbers remain relatively constant over the estrous cycle. The average numbers of PMNs were $5–7\times10^3$ cell/oviduct flush, constituting approximately 12–16% of the total leukocyte population in the oviduct flush. The interleukin 8 (IL-8), a strong chemotactic agent for neutrophils, gene was expressed in BOECs that were freshly separated from bovine oviduct without any culture. BOECs continuously expressed IL-8 mRNA, and IL-8 gene expression was approximately 10 times higher in ampullary epithelial cells than in isthmic epithelial cells. Ampullary epithelial cells and isthmic epithelial cells were intensively stained for IL-8 by immunohistochemistry (IHC). BOECs constantly produce IL-8 throughout the whole estrous cycle. The stable production of IL-8 could be one of the factors contributing to the continuous recruitment of PMNs into the oviduct fluid and the maintenance of PMNs at a relatively constant level throughout the different stages of the estrous cycle. These results indicate that PMNs are normal constituents of the local immunological microenvironment in the bovine oviduct that protect the oviducts from potentially pathogenic microorganisms and maintain a sterile environment.

Alpha 1-acid glycoprotein (AGP) is a major acute-phase protein produced mainly in the liver. Hepatic production and serum concentrations of AGP are increased in response to systemic injury, inflammation, or infection and its effects on immunomodulation have been described. AGP mRNA is expressed in extra-hepatic organs, such as the lung, kidney, spleen, lymph node, uterus, and ovary. Therefore, I hypothesized that AGP is secreted locally in the bovine oviduct, and is involved in the regulation of the phagocytic activity of neutrophils for sperm. To test my hypothesis, I investigated, 1) the local production of AGP in the bovine oviduct; 2) the effect of AGP on the phagocytic activity of PMNs for sperm and superoxide production; and 3) the impact of AGP desialylation on the PMN phagocytosis of sperm. I have provided the first evidence for the local gene expression of AGP by bovine oviduct epithelial cells *in vitro*. The oviduct flush solutions contained AGP in the range of 20–60 ng/mL, which is much lower than that seen in cow plasma. AGP, at the detectable range of concentrations seen in the oviduct flush experiments, dose-dependently suppressed PMN phagocytosis of sperm *in vitro*. Neutrophils either directly phagocytize sperm through cell-cell attachment or entrap them with neutrophil extracellular traps (NETs), structures consisting of neutrophil nuclear DNA and associated proteins, which ensnare sperm and hinder their motility. The four-hour incubation of the PMNs with AGP (1, 10, or 100 ng/mL) prior to phagocytosis assay, resulted in a dose-dependent decrease in the phagocytosis by PMNs of sperm treated to induce capacitation. Additionally, SEM analysis demonstrated that AGP (100ng/mL)

drastically reduced NET formation, preventing sperm from being fixed and trapped by PMNs, and thus indirectly results in the suppression of PMNs phagocytosis for sperm. The ability of PMNs to release superoxide has been used as an indicator for evaluating their phagocytic activity on sperm. my results show that only in the presence of sperm did AGP (100 ng/mL) significantly suppress superoxide release by PMNs. These results suggested that the AGP secreted in bovine oviducts contributes to the protection and maintenance of sperm survival through the suppression of phagocytic activity and superoxide release by PMNs. It has been shown that NET formation may depend on superoxide release through reactive oxygen species (ROS)-generating pathways. Therefore, I hypothesize that AGP, *via* the suppression of superoxide generation, could affect the ROS-generating pathways that lead to the generation of NETs, altering the phagocytic behavior of PMNs for sperm. It is conceivable that the terminal sialic acid residues exposed on the surface of AGP block phagocytosis by binding phagocyte sialic acid-binding immunoglobulin-type lectins (Siglec). Desialylating AGP abolished an AGP hepato-protective effect and anti-apoptotic activity. Therefore, I hypothesized that the sialic acid contributes to the suppressive effect of AGP on the PMNs phagocytosis for sperm. A four-hour incubation of PMNs on the AGP-coated plates prior to phagocytosis assay resulted in a decrease in the phagocytic activity of PMNs for treated sperm. However, incubation of PMNs on the as-AGP-coated plates resulted in complete abolishment of the suppressive effect of AGP on the phagocytosis of sperm by PMNs. This phenomenon was also seen in the superoxide production following incubation of the PMNs with sperm on AGP- or as-AGP-coated plates. My results show that desialylating AGP completely abolished the AGP-suppressive effect on both PMN phagocytosis of sperm and superoxide release. Previously, we demonstrated that luteinizing hormone stimulates BOECs to secrete PGE_2, which plays a major role in suppressing the phagocytic activity of PMNs for sperm. A dose-response study was performed, in which BOECs were incubated with AGP (0, 1, 10, or 100 ng/mL) for 24 hours. My results showed that AGP stimulated PGE_2 production in BOECs *in vitro* in a dose-dependent manner. Moreover, A four-hour incubation of PMNs with the local concentration of AGP detected in oviduct flush (50 ng/mL), along with that of PGE_2 (10^{-8} M, 3.52 ng/mL), resulted in an additive effect in the suppression of the phagocytic activity of PMNs for treated sperm. Thus, these findings suggest that the AGP not only directly suppresses sperm phagocytosis but also works cooperatively with PGE_2 to suppress the phagocytosis of sperm by PMNs in the bovine oviduct.

Taken together, the results demonstrate that PMNs constantly infiltrate bovine oviducts

throughout the estrous cycle, possibly under the effects of the chemotactic cytokine, IL-8, that secreted from oviduct epithelial cells. Additionally, the present findings shed a light on the immunomodulatory functions of AGP in the bovine oviduct. It is proposed that under physiological conditions, the local AGP system in bovine oviduct may aid sperm survival through the direct suppression of the phagocytic activity of PMNs for sperm, the reduction of superoxide production by phagocytizing PMNs, and by limiting NET formation.