Fish & Shellfish Immunology 27 (2009) 549–555

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Suppression of lymphocyte proliferation by ovarian cavity fluid from the viviparous fish *Neoditrema ransonnetii* (Perciformes; Embiotocidae)

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ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form
13 July 2009
Accepted 14 July 2009
Available online 21 July 2009

Keywords:
Viviparity
Ovarian cavity fluid
Immunomodulation
Lymphocyte proliferation
Prostaglandin E_2
Pregnancy
Surfperch

ABSTRACT

As the fetus expresses paternal major histocompatibility complex molecules, viviparous vertebrates require sophisticated mechanisms to modulate maternal immunology to ensure successful pregnancy. We anticipated that ovarian cavity fluid (OCF) is likely to feature significantly in the modulation of ovarian cavity immunology. Consequently, we examined the effects of OCF upon leukocyte function in *Neoditrema ransonnetii*. OCF did not affect phagocytosis or superoxide production by phagocytes. However, OCF suppressed lymphocyte proliferation induced by ConA almost completely. As OCF contained PGE_2 at high levels during late pregnancy, we also investigated the effect of PGE_2 upon lymphocyte expansion. PGE_2 exhibited negative effects upon lymphocyte mitogenesis in a dose-dependent manner (10–1000 ng/ml). PGE_2 significantly suppressed lymphocyte proliferation when present at levels equivalent to that seen in OCF ($30.2 \pm 16.1 \sim 185.4 \pm 107.4$ ng/ml). Data indicate that PGE_2 is one of the key modulatory molecules of the maternal immune system ensuring successful pregnancy in this viviparous species.

1. Introduction

Viviparity requires highly sophisticated maternal control mechanisms in order to modulate immune responses effectively because the spermatozoa and fetuses express paternal major histocompatibility complex (MHC) antigens. It is essential for the success of internal fertilization and maintenance of pregnancy that local immune reactions are regulated. A number of studies have attempted to elucidate the immuno-modulating mechanisms which underlie pregnancy for humans and other mammalian species, and these studies have been largely successful. Such mechanisms include the restricted expression of MHC molecules on the trophoblast [1–4], presence of multiple immunoregulatory factors (e.g., cytokines, hormones, and prostaglandin E_2 (PGE_2)) [5–8], and the distinct distribution of immune cells across the maternal–fetal interface [9–15].

In stark contrast with mammalian species, this puzzle has been left almost unexplored in non-mammalian viviparous species, despite the fact that viviparity is widespread among vertebrates, with the exception of Aves and Agnatha. There are over one thousand species of viviparous fish [16]. Fish are the first vertebrate group to have developed MHC and T cell receptors (TCR), and, as a result, exhibit strict immune-rejection against non-self transplants [17–22]. Viviparous fishes are, therefore, the oldest creatures to have faced conflict between the immune system, internal fertilization and fetal growth. Understanding how fish have resolved this conflict would reveal vital information regarding the evolutionary process of viviparity in vertebrates.

Embiotocidae fish, also known as 'surfperch', possess some unique characteristics among viviparous teleosts; their gestational period is considerably long (more than 6 months), the fetus has very limited yolk reserves resulting in almost complete dependence on maternally supplied nutrients. The fish do not develop a placenta. Instead, fetuses continuously receive maternal secretions, known as the ovarian cavity fluid (OCF). *Neoditrema ransonnetii* is a common Embiotocidae fish found in Japan. Copulating behavior is observed from September to December, in Iwate, Northern Japan. Internal fertilization appears to occur from late December through to early January. Fetuses retained in the ovarian cavity ingest and vigorously absorb OCF via their hypertrophied hindgut [23]. From late July to early August, newborn are delivered. Through microscopic observation, we previously found that a large number of leukocytes exist in the ovarian cavity [24]. This indicates that inseminated sperm cells and developing fetuses are not immunologically sequestered from the maternal immune system in surfperch. Vigorous phagocytosis of the inseminated sperm cells by macrophages is evident in the ovarian lumen after coitus, and even

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doi:10.1016/j.fsi.2009.07.006

during the gestation period [24]. Nevertheless, neither an apparent immune response nor a semi-allogenic rejection appears to occur. Throughout the reproductive cycle, more than 90% of observed leukocytes in the ovarian lumen are monocytes/macrophages, while lymphocytes invariably represent only a small population. These facts suggest the presence of local mechanisms suppressing lymphocyte influx or proliferation. We therefore expected the OCF to play key roles in the immune modulation of the ovarian cavity in order to maintain pregnancy in surfperch. In the current study, we examined the immuno-modulating effect of OCF upon phagocytic functions and lymphocyte proliferation. In addition, we also studied the effect of PGE_2, a well-known inflammatory mediator and immuno-modulating agent, because we found that OCF contained high levels of PGE_2.

2. Materials and methods

2.1. Fish

Fish were collected by angling at Okirai Bay, Iwate, Japan, and were maintained under natural conditions with running sea water in fiber-reinforced plastic tanks at Kitasato University. Fish were fed a commercial diet for marine fish (Fuji-Seifun Co., Japan). To avoid any possible influences that can be induced by pregnancy-related factors, the cells used in all experiments were obtained from the males.

2.2. Collection of plasma and OCF

Fishes were anesthetized with 2-phenoxyethanol, and blood collected using heparinized syringes with a 26G needle. Following centrifugation at 700 × *g* for 15 min at 4 °C, the supernatants were harvested and stored at −80 °C to await analysis.

Following laparotomy, the ovaries were dissected and the OCF collected by a micropipette. Following centrifugation, the supernatants were preserved at −80 °C and sterilized with a syringe filter unit (pore size, 0.20 μm; ADVANTEC, Japan) before use.

2.3. Cell preparation

Blood was drawn aseptically from the caudal vein of anesthetized fish into a syringe containing 1000 IU/ml (the final dilution: 125 IU/ml) of heparin and immediately mixed with 5 volumes of 1 × Dulbecco's phosphate-buffered saline (D-PBS) (Gibco, USA). The diluted blood was then layered on 45% Percoll™ (GE Healthcare, USA) prior to centrifugation at 400 × *g* for 40 min at 20 °C. The peripheral blood leukocytes (PBL) layer at the interface was harvested and washed 3 times before use in the following experiments.

Following laparotomy, the head kidney was removed and pushed through a stainless mesh in D-PBS. The resulting cell suspension was placed on 35/51% Percoll™ and centrifuged. The macrophage-enriched cells from the 35/51% Percoll™ interface were recovered, washed with 0.01 M PBS, and resuspended in the RPMI 1640 medium (Nissui Pharmaceutical, Japan). These cells are referred to as head kidney leukocytes (HKLs) hereafter.

2.4. Phagocytosis assay

HKLs (2×10^6 cells) in RPMI 1640 were allowed to adhere to a culture coverglass (Matsunami Glass, Japan) for 24 h at 15 °C, and non-adherent cells were removed by washing with 0.01 M PBS. Cells were then maintained with 150 μl of OCF or RPMI 1640 at 15 °C for 6 h. Following washing, latex beads (L.B, 0.75 μm, Polysciences Inc, USA), opsonized with *N. ransonnetii* serum, were added to the well along with the RPMI 1640 medium. After 1 h of incubation at 15 °C and washing, the cells on the cover slip were fixed with methanol and stained with 4′-6-diamino-2-phenylindole. The number of phagocytic cells that ingested at least one particle was counted microscopically and the phagocytic activity was expressed as a percentage of the total cell counts.

Opsonic activity of OCF was also examined. L.B. were treated with OCF, serum, or RPMI 1640 medium for 1 h at room temperature. Following washing, these were resuspended in RPMI 1640 medium, and added to HKL monolayers on the cover slip. Phagocytic activity was measured as described above.

2.5. Intracellular production of superoxide anions in the macrophages

Superoxide anion production by HKLs was determined according to the method described by Chung and Secombes [25]. In brief, the HKLs (1×10^6 cells/well) were plated onto 96-well microtiter plates (AGC Techno glass, Japan) and allowed to adhere for 24 h at 15 °C. After washing with RPMI 1640, the macrophage monolayers were treated with 80 μl of OCF, male serum, or RPMI 1640 for 24 h. After another round of washing, the cells were incubated with 100 μl of NBT solution (1 mg/ml) in RPMI 1640 with or without Zymosan A (2.5×10^7 particles/well) (Sigma, USA) opsonized with *N. ransonnetii* serum. After 1 h, the NBT solution was removed and the cells were fixed with methanol. The reduced formazan within the macrophages was solubilized in KOH/DMSO for 5 min and the optical density was measured by a microplate reader (Model 550; BioRad, USA) at 620 nm.

2.6. Cell proliferation assay

Isolated PBLs were resuspended in OCF or Leibovitz's L-15 medium (Gibco) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell suspensions were incubated in 96-well microplates with or without 1 μg/well of concanavalin A (ConA, Sigma) for 72 h at 15 °C. Twenty-four hours before the end of cultivation, 5-bromo-2′-deoxyuridine (BrdU, final concentration: 10 μM) was added to the culture. BrdU uptake into nuclear DNA was measured using the Cell Proliferation ELISA kit (GE Healthcare) according to the manufacturer's instructions.

2.7. Characterization of the proliferating cells

To characterize the cells proliferating in response to ConA, we centrifuged and smeared a few of these cells onto glass slides. Following acetone fixation and blocking with 1% FBS, the cells were incubated with anti-IgM rabbit antiserum (× 10,000) [26] for 2 h at room temperature. After washing, the cells were reacted with anti-rabbit IgG goat IgG conjugated with Alexa Fluor 633 (Funakoshi, Japan) for 1 h. After washing, DNA was fragmented using DNase (Promega, USA) for 1 h at 37 °C. The cells were washed again and then exposed to 10 μg/ml of FITC-labeled anti-BrdU mouse mAb (Biomeda Corporation, USA) for 2 h.

Lectin staining with ConA was also performed in order to identify the ConA-binding cells. PBLs were fixed with 4% paraformaldehyde and smeared by cytospin. After blocking of endogenous biotin with the avidin/biotin blocking kit (Vector Laboratories, USA), cells were treated with 5 μg/ml of biotin-labeled ConA (Sigma) for 1 h and then incubated with 100 μg/ml of FITC-conjugated streptavidine (Sigma) for 30 min.

All fluorescent signals were captured by a confocal laser-scanning microscope (LSM-510META; Carl Zeiss, Germany).