Developing Ovarian Follicles Inhibit the Endotoxin-Induced Glomerular Inflammatory Reaction in Pseudopregnant Rats

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PROBLEM: We tested the hypothesis that developing ovarian follicles produce factors inhibiting the endotoxin induced inflammatory response.
METHOD OF STUDY: Pseudopregnant rats were treated with FSH to induced follicular development (FSH-rats). For control we used untreated pseudopregnant rats (PSP-rats) and rats in the follicular phase of the ovarian cycle (C-rats). All rats were infused with either saline or endotoxin. Three days after the infusion rats were sacrificed and kidney specimens were snapfrozen. Cryostat kidney sections were stained for the presence of monocytes, granulocytes, CD11a- and CD11b-positive cells and for ICAM-1 expression.
RESULTS: The results show that induction of follicular development in pseudopregnant rats inhibited glomerular infiltration of monocytes and CD11b$^+$ cells, while it did not affect the other parameters, i.e. glomerular granulocyte number, CD11a$^+$ cells and glomerular ICAM-1 expression.
CONCLUSION: Developing ovarian follicles produce factors inhibiting monocyte responses to endotoxin.

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INTRODUCTION

Pregnant animals are much more sensitive to the pro-inflammatory effects of endotoxin than non-pregnant animals.[1,2] This increased endotoxin sensitivity correlates well with the fact that pregnant animals exhibit a much more intense and persistent inflammatory response following endotoxin infusion than rats in the follicular phase of the ovarian cycle:[3] this persistent inflammatory response in pregnant animals is characterized by persistent infiltration of (activated) granulocytes and monocytes into the glomeruli of the kidney, which is associated with expression of adhesion molecules on the infiltrating cells (CD11b, CD11a and CD49d) and on the endothelial cells (ICAM-1 and VCAM-1).[3]

Further research in our laboratory revealed that this so-called 'pregnancy-specific' inflammatory response against endotoxin was not confined to the pregnant condition. Also pseudopregnant rats, which are rats in which a luteal phase was induced, showed an endotoxin-induced glomerular inflammatory response comparable with pregnant rats,[4] i.e. a much more intense and persistent glomerular inflammatory response after endotoxin infusion as compared with rats in the follicular phase of the ovarian cycle. Since in both pregnant and pseudopregnant rats progesterone concentrations are increased, this observation suggested that the induction of the 'pregnancy-specific' inflammatory response depends on the presence of progesterone. However, our subsequent studies excluded such a role for progesterone, since also ovariectomized rats, with or without treatment with progesterone (or estrogen), exhibited the endotoxin-induced 'pregnancy-specific' glomerular inflammatory response.[5]

Together, these experiments showed that in follicular phase rats the inflammatory response upon endotoxin is different from all other reproductive conditions tested: in this phase the endotoxin-induced glomerular inflammatory response is inhibited.

Our studies are now directed on finding the mechanism of inhibition, rather than finding pregnancy-specific hormones associated with stimulating the inflammatory response. The fact that only in the follicular phase of the ovarian cycle the endotoxin-induced inflammatory reaction is inhibited[3-5] may imply that developing ovarian follicles, which are only present in the follicular phase, produce factors inhibiting the inflammatory response. To test this we used pseudopregnant rats, which were treated with FSH, to induce follicular development, followed by infusion with endotoxin. We evaluated parameters of the glomerular inflammatory response. As control we applied untreated pseudopregnant rats and cyclic rats, which are rats in the follicular phase of the ovarian cycle.

MATERIALS AND METHODS

Experimental Animals

Female wistar rats (3–4 months; about 200 g; Harlan) were kept in a temperature- and light controlled room (lights on from 6 a.m. to 6 p.m.) with free access to food and water. Until the day of selection for the experiments, daily vaginal smears were taken to follow estrus cyclicity.

Pseudopregnancy was achieved by electrical stimulation of the cervix uteri at 5 p.m. on pro-estrus and on 3 p.m. on estrus.[6] This day was designated as day 0 of the experiment. A normal pseudopregnancy lasts for about 10 days.[6]

On day 0 of pseudopregnancy, or on diestrus in cyclic rats, rats received a permanent jugular vein cannula according to the method of Steffens et al.[7]

Follicular development in pseudopregnant rats was induced by daily (day 3–7) intraperitoneal injections of 10 IU FSH (Metrodin; Organon, Oss, The Netherlands) per rat in 0.2 mL of saline. Control rats were injected with 0.2 mL of saline alone. Follicular development was assessed by microscopic inspection of the ovaries, while corpus luteum function was assessed by measurement of progesterone according to the method of de Jong et al.[8]

Endotoxin (1.0 μg/kg bw; *Escherichia coli* 0.55: B5; Whittaker MA Bioproducts Inc., Walkersvill, MD, USA) was dissolved in 2 mL saline and infused via the jugular vein cannula into conscious rats according to standard methods.[3]

Experimental Protocol

Three groups of rats were used in this study, cyclic rats (C-rats; $n = 10$), pseudopregnant rats (PSP-rats; $n = 10$) and pseudopregnant treated with FSH (FSH-rats; $n = 10$).

In FSH rats, FSH treatment was started on day 3 and lasted until day 7. Injections were given at 9 a.m. Both FSH rats and PSP rats were infused with either endotoxin solution or saline alone on day 5 (10 a.m.) and were killed three days after the infusion, i.e. on day 8. C rats were infused on diestrus 2 and also killed 3 days later.

At sacrifice, specimens of the left kidney as well as the ovaries were snap-frozen. Kidney specimens were prepared for immunohistological staining as described above. Ovaries were prepared for staining with hematoxylin and eosin to check for follicular development.

Demonstration of Glomerular Inflammation

Antibodies. Monoclonal antibody against rat granulocytes (HIS 48; Pharmingen, San Diego, CA, USA); monoclonal antibody against rat monocytes (ED-1; Serotec, Oxford, UK); a monoclonal antibody against rat ICAM-1 (1A29; Genzyme, Cambridge, MA, USA); a monoclonal antibody against rat CD11a (WT.1; Genzyme); a monoclonal antibody against rat CD11b (WT.5; Seikagaku Corp., Tokyo, Japan).

Immunohistology. Cryostat kidney sections (4 μm) were stained for the presence of granulocytes, monocytes, CD11a, CD11b and ICAM-1 as previously described.[3] In brief: sections were fixed in acetone, and after pre-incubation with normal rabbit serum, they were incubated with the primary antibody for 30 min. Peroxidase conjugated secondary antibody (rabbit-anti-mouse; Dako A/S, Glostrup, Denmark) was used. The secondary antibody was visualized with 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St Louis, MO, USA) and hydrogen peroxide (Sigma). Control sections, omitting the primary or secondary antibody were consistently negative.

Evaluation of kidney sections. Two kidney sections of each animal were scored by light microscopy by two independent observers in the following ways:

- Sections stained for the presence of monocytes, granulocytes, CD11a en CD11b positive cells were quantified by counting the total number of positive cells in 100 glomeruli per section. The mean of the two sections was taken and results are expressed as mean number of positive cells per glomerulus.
- Sections stained for ICAM-1 were semiquantitatively graded by scoring a total of 100 glomeruli

per section using an arbitrary scale from 1 to 4 as described previously[3] (1, no staining; 2, weak staining; 3, moderate staining; 4, bright staining). The mean of the two sections was taken and results are expressed as mean ICAM-1 expression per glomerulus.

Statistics

Results are expressed as mean ± S.E.M. Differences between groups were evaluated using the Mann–Whitney U-test and considered significant if $P < 0.05$.

RESULTS

Plasma Progesterone Concentrations and Ovarian Follicles

Plasma progesterone concentrations were not affected by endoxin treatment. Progesterone concentrations were significantly increased in PSP-rats and FSH-rats when compared with C-rats. We found no difference between PSP-rats and FSH-rats (C-rats: 30 ± 7 nM; PSP-rats: 125 ± 11; FSH-rats: 135 ± 15). Histological evaluation of the ovaries revealed development of ovarian follicles in all rats treated with FSH.

Glomerular Inflammation

Infiltration of inflammatory cells. Numbers of monocytes per glomerulus can be observed in Fig. 1A. This figure shows that in C-rats we found no effect of endotoxin upon glomerular monocyte number at 3 days after the infusion. In PSP-rats the number of monocytes per glomerulus is significantly increased after endotoxin infusion as compared with saline infusion. Induction of follicular development affects glomerular monocyte number after endotoxin infusion in PSP-rats: in FSH-rats no effect of endotoxin upon glomerular monocyte number was found at 3 days after the infusion.

The number of granulocytes per glomerulus after endotoxin or saline infusion can be seen in Fig. 1B. While no effect of endotoxin upon glomerular granulocyte number can be observed in cyclic rats, in PSP-rats the number of granulocytes per glomerulus is significantly increased at 3 days after endotoxin infusion as compared with saline infusion. No effect of FSH treatment to induce follicular development can be observed upon endotoxin-induced glomerular granulocyte number.

Expression of adhesion molecules. Glomerular ICAM-1 expression can be observed in Fig. 2A. We found no effect of endotoxin upon glomerular ICAM-1 expression in C-rats. In PSP-rats, glomerular ICAM-1 expression 3 days after endotoxin infusion is significantly upregulated as compared with 3 days after saline infusion. Also in FSH rats, glomerular ICAM-1 expression is significantly upregulated 3 days after endotoxin

Fig. 1. Mean (±S.E.M.) number of monocytes (A) or granulocytes (B) per glomerulus in PSP-rats, FSH-rats and C-rats after infusion of endotoxin (solid bars) or saline (open bars). *Significantly increased from saline-treated rats (Mann–Whitney U-test, $P < 0.05$).
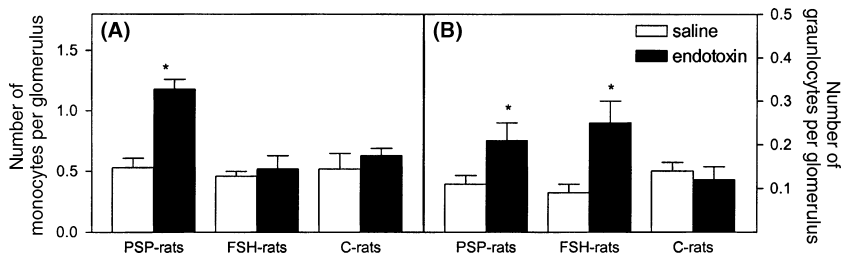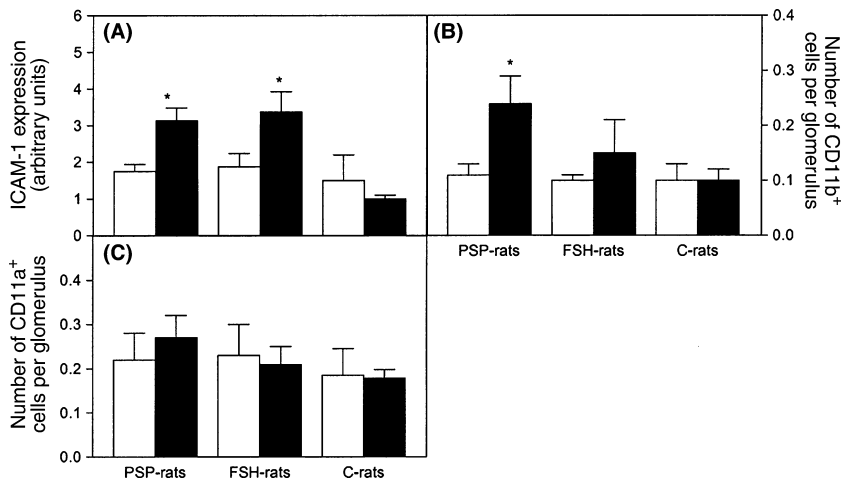

Fig. 2. (A) Mean (±S.E.M.) glomerular ICAM-1 expression in arbitrary units in PSP-rats, FSH-rats and C-rats after infusion of endotoxin (solid bars) or saline (open bars). Mean (±S.E.M.) number of CD11b$^+$ cells (B) and mean number of CD11a$^+$ cells (C) per glomerulus in PSP-rats, FSH-rats and C-rats after infusion of endotoxin (solid bars) or saline (open bars). *Significantly increased from saline-treated rats (Mann–Whitney U-test, $P < 0.05$).

infusion as compared with saline infusion, indicating that developing ovarian follicles do not affect endotoxin-in-induced glomerular ICAM-1 expression.

The number of CD11b^{+} cells (Fig. 2B) was significantly affected by induction of follicular development: only in PSP-rats, not in FSH-rats or in C-rats, the number of CD11b^{+} cells was significantly increased after endotoxin infusion as compared with saline infusion.

No effect of neither endotoxin nor induction of follicular development was observed upon the number of CD11a^{+} cells per glomerulus (Fig. 2C).

DISCUSSION

In the present study, we successfully induced follicular development in PSP-rats by daily FSH injections. This treatment did not affect luteal function as judged by unchanged progesterone levels. In line with our previous study, PSP-rats showed the characteristics of the 'pregnancy-specific' glomerular inflammatory response after endotoxin infusion.[4] The treatment of pseudopregnant rats with FSH significantly decreased some of the parameters of the glomerular inflammatory response, notably it inhibited the endotoxin-induced infiltration of monocytes into the glomeruli as well as the number of CD11b positive cells. Glomerular granulocyte number and glomerular ICAM-1 expression after endotoxin were unaffected by this treatment.

This study confirms our previous study in which we showed that developing ovarian follicles inhibited the endotoxin-induced inflammatory response in pregnant rats, in particular the glomerular monocyte infiltration.[9] The inhibition by FSH is most likely an indirect effect, since in ovariectomized rats, FSH levels are elevated (because of the lack of negative inhibition on FSH production by the ovaries). Yet in these animals the endotoxin-induced glomerular monocyte infiltration is not inhibited as in FSH treated PSP rats.[5] Therefore, we conclude that FSH induced ovarian follicles to grow and start producing anti-inflammatory factors which inhibit monocyte responses to endotoxin. The nature of the putative factor(s) and the mechanism by which the factor(s) affect monocytes remain unknown at present, we are however currently investigating this.

In contrast to monocytes, granulocytes are not affected by putative anti-inflammatory factors. This corroborates our previous study in which we have shown that the persistent endotoxin-induced glomerular granulocyte infiltration was the result of increased progesterone levels.[5] Indeed the present FSH treatment did not affect plasma progesterone concentrations and thus it did not affect luteal function. Moreover, the fact that induction of follicular development did not affect endotoxin-induced ICAM-1 expression in the present study, suggest that also endothelial cells are not under the influence of the putative follicular factor(s).

From the above, it seems plausible that follicular factors exert a direct effect on monocytes. This confirms our previous observations that in the rat monocytes may be directly affected by developing ovarian follicles.[10] In this study, we found that monocyte TNFα production, the main effector of the endotoxin response,[11] after *ex vivo* stimulation of whole blood with endotoxin was decreased in rats in the follicular phase of the ovarian cycle as compared with pregnant and pseudopregnant rats.[10] This observation was not restricted to experimental animals, since human follicular phase monocytes produce less TNFα after stimulation with endotoxin than luteal phase monocytes.[12]

Although the relationship between reproduction and immunology has been recognized for a long time now,[13–15] the present data present a new view on the regulation of immune responses by the reproductive condition: anti-inflammatory factors produced by developing ovarian follicles. Although differences exist in immune responses between pregnant and non-pregnant females of many species,[3,16] this is usually attributed to increased sex hormone concentrations during pregnancy.[14,15] In this paper, we show a different mechanism by which the ovaries control immune responses.

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