Effects of TNF- α Injection into the Ovarian Parenchyma on Luteal Blood Vessels in Rabbits

KOICHI NARIAI, KIICHI KANAYAMA, TUYOSHI ENDO, AND AZUMA TSUKISE*

Department of Veterinary Physiology, and *Veterinary Anatomy, College of Agriculture and Veterinary Medicine, Nihon University, Fujisawa, Kanagawa 252, Japan

Abstract. It has been reported that tumor necrosis factor- α (TNF- α) derived from luteal macrophage is concerned with luteolysis. In the present study, to evaluate the correlation between TNF- α and regression of luteal blood vessels, recombinant human TNF- α (rh-TNF) was injected into the parenchyma of pseudopregnant rabbit ovaries. These injections were performed on day 7 of pseudopregnancy (functional luteal phase). Only Mg⁺⁺ and Ca⁺⁺ free phosphate buffer saline (PBS⁻) as the solvent was injected in the control group. Estimations of conditions in the luteal vessels after the injections was performed by observations of luteal vascular corrosion casts under a scanning electron microscope. Concentrations of serum progesterone before or after the injection were also assayed. In the control group, no change in the structure of luteal vessels was observed after PBS⁻ injection, but regressing blood vessels with strictures, obstructions and rugged surfaces on the vessels were observed, and also concentrations of serum progesterone decreased noticeably after rh-TNF injection. These findings suggest that TNF- α plays a role in angiolysis through luteolysis in the rabbit corpus luteum.

Key words: Tumor necrosis factor- α (TNF- α), Angiolysis, Luteolysis, Corrosion cast, Endothelial cell
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BLOOD vessels in the endocrine organs play important roles in the supply of oxygen and other substances to the organs and for the transport of hormones. It is known that a reduction in tissue function is normally accompanied by a reduction in vasculature [1]. In the regressive corpus luteum, blood flow decreases during luteolysis [2, 3], and we can also observe a dramatic reduction in the number of the luteal vessels [4]. It has been shown that luteal vascular development is coincident with growth and regression of the corpus luteum [5].

There are some studies on the mechanism of luteolysis. Prostaglandin (PG) F_{2 α} is known as the

uterine derived luteolytic factor in several species [6–9] except for primates [10–13], dogs [14, 15] and ferrets [16]. PGF_{2 α} or its analog causes a rapid and dramatic decrease in luteal blood flow and deletion of the luteal vessel [3, 4]. Azmi *et al.* [4] reported that the decline in luteal blood flow made endothelial cells of the luteal vessel disappear.

Recently, macrophage participation in luteolysis has been demonstrated [17–19]. Bagavandoss *et al.* have reported that the number of macrophages infiltrating into the corpus luteum increases during luteolysis in rabbits [18, 19], and not only PGF_{2 α} but also several kinds of cytokines produced by immunocytes play a role as luteolytic factors [18–22]. Tumor necrosis factor (TNF)- α produced by activated macrophages was discovered by Carswell *et al.* [23]. It has been demonstrated that TNF- α derived from macrophages which infiltrated into the corpus luteum plays a role in luteolysis [19, 22]. TNF- α has many biological effects in *in vivo* and *in vitro* studies. TNF- α also plays a role in

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Correspondence to: Dr. Koichi NARIAI, Department of Veterinary Physiology, College of Agriculture and Veterinary Medicine, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252, Japan

regression in blood vessels, because of the high susceptibility of endothelial cells to TNF- α [24].

In this study, we examined whether or not injection of TNF- α would induce elimination of luteal blood vessels, using a previously reported method for the preparation of ovarian vasculature corrosion casts [25].

Materials and Methods

Experimental animals

Sexually mature female Japanese white rabbits weighing between 2.8 and 3.2 kg were used. The rabbits were housed individually in 68 $\times$ 82 $\times$ 70 cm wire netting cages (R3 type; Okazaki Sangyo Co., Ltd., Saitama, Japan). The animal room was air-conditioned to 23 $\pm$ 2 $^{\circ}$ C, 55 $\pm$ 5% relative humidity, 15 air changes per hour, and illuminated for 14 h (0500–1900 h) with a 300 lux daylight fluorescent lamp. They were given commercial pellets (Labo R Stock[®]; Nihon Nosan Kogyo Co., Ltd., Yokohama, Japan) and tap water *ad libitum*.

Induction of pseudopregnancy

Pseudopregnancy was induced in the rabbits by mating with vasoligated mature males and by injection of 75 iu of human chorionic gonadotropin (hCG; Gonatropin[®], Teikoku hormone Mfg. Co., Ltd., Tokyo, Japan) into the ear vein. The day of this treatment was designated as day 0 of pseudopregnancy (D0).

TNF- α injection into the ovarian parenchyma

We used recombinant human TNF- α (rh-TNF; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for injection into the ovary. 1 $\times$ 10⁴ units of rh-TNF was dissolved in 1 ml of Ca⁺⁺ and Mg⁺⁺ free phosphate buffer saline (PBS⁻).

On D7, all of the animals examined were laparotomized with a lower abdominal incision under general anesthesia with 20 mg/kg of sodium pentobarbital; Nembutal[®] (Abbot Laboratories, North Chicago, IL, USA) i.v., and macroscopic observation of both the ovaries was performed. In cases in which functional corpora lutea existing at the time of the observation, 0.1 ml of rh-TNF solution was subsequently injected into the parenchyma of

the ovary (TNF group). In the control group, 0.1 ml of PBS⁻ only as the solvent of the rh-TNF solution was injected into the parenchyma of the ovary in the same manner as in the TNF group. Three rabbits were examined in the TNF group and three in the control group.

Preparation of ovarian vascular corrosion casts

On the day following rh-TNF or PBS⁻ injection (D8), ovarian vasculature corrosion casts were prepared. The method of preparation of the corrosion cast was based on that in reported studies [25, 26]. That is, after general anesthesia with Nembutal[®], the animals were perfused slowly through the abdominal aorta with physiological saline and with a low viscosity methacrylate casting medium; Mercocox[®] (Dainippon Ink Co., Ltd., Tokyo, Japan). The perfused ovaries were removed and placed for 3 h in a water bath (60 $^{\circ}$ C) until polymerization. The ovaries were corroded overnight or longer in 20% of NaOH solution, and were washed overnight in running tap water. The blood vascular casts of the ovaries were air-dried and then were sputtered with gold after mounting on aluminium stubs.

Observations of the casts

The observations of the casts were performed under a Hitachi S-2100 scanning electron microscope (SEM), operating at 5–10 kV.

Progesterone assays

Blood samples for the assay of serum progesterone were collected from the ear artery at D0, D7 (before rh-TNF solution or PBS⁻ injection) and D8 (before preparation of ovarian vascular casts) in both groups. Each serum sample was extracted with ethylether, and the extracts were radioimmunoassayed without chromatography, as described by Den *et al.* [27]. The antiserum to progesterone-3-CMO-BSA was purchased from Cosmo Bio. Co., Ltd. (Tokyo, Japan). Cross-reactivity values for the antiserum for 5 α -pregnanedione, pregnenolone, 20 α -hydroxyprogesterone, 17 α -hydroxyprogesterone and other steroidal hormones were 12.5%, 2%, 0.2%, 0.01% and less than 0.01%, respectively. The coefficients of variation for both intra- and inter-assay were less than 10%.

Statistical analysis

The data on the serum progesterone was statistically analyzed by analysis of variance followed by Student's *t*-test or Cochran-Cox test. Differences of $P < 0.05$ were considered to be statistically significant.

Results

Changes in the luteal blood vessels after rh-TNF injection

We could observe angiogenesis as the luteinization in D7 (before rh-TNF injection) corpus luteum (Fig. 1A and 1B). There were large solid blood vessels (Fig. 1A) and some sinusoidal capillaries (Fig. 1B). On D8 of the control group (the day after PBS⁻ injection), the structure of the blood vessels in the corpus luteum was almost unchanged (Fig. 2A and 2B), but on D8 of the TNF group, large blood vessels regressed (Fig. 3A), the strictures and obstructions of the luteal capillaries (Fig. 3B) and leakages of casting medium from the capillaries (Fig. 3A) were observed. Casts with rough surfaces were also observed (Fig. 3B and 3C) against D8 of the control group (Fig. 2C). These changes in the capillaries occurred only in the corpora lutea. Follicular and ovarian parenchymal blood vessels had not changed after rh-TNF injection.

Changes in serum progesterone concentration after rh-TNF injection

Changes in the serum progesterone concentration after rh-TNF injection are shown in Table 1. In both groups, the serum progesterone concentration was increased by D7. But on D8, as the day after rh-TNF injection, the level of serum progesterone decreased dramatically in the TNF group. In the control group, the functional luteal phase was maintained at D8.

Discussion

It is already well known that PGF_{2 α} is involved in luteolytic processes in several species of mammal [6–9]. It has also been reported that luteal endothelial cells are damaged by the decline in luteal blood flow due to the effects of PGF_{2 α} [4]. And TNF- α derived from activated macrophages damages the endothelial cells [24, 28–30]. Sato *et al.* [24] demonstrated that TNF- α caused endothelial cells to be damaged morphologically and inhibited proliferation of the endothelial cells. TNF- α makes endothelial cells produce a variety of PGs and platelet activating factor (PAF) [28]. These PGs [31] and PAF [32] cause breaking up of the luteal capillaries with damage to the endothelial cells, and also cause intravascular coagulation. The surfaces of vascular corrosion casts are shown as a replication of the inner wall of the blood vessels. Rugged surfaces of the capillaries and strictured

Fig. 1. Scanning electron micrographs of blood vascular casts of the D7 corpus luteum (functional luteal phase, before rh-TNF or PBS⁻ injection). A: Some large solid blood vessels were observed. $\times 30$. B: Some sinusoidal capillaries were observed. An arrowhead shows angiogenesis. $\times 500$.

Fig. 2. Scanning electron micrographs of blood vascular casts of the D8 control (the day after PBS⁻ injection) corpus luteum. A: Remaining large blood vessels. $\times 50$. B: Some solid sinusoidal capillaries have also remained. $\times 500$. C: The inner wall of the luteal blood vessels is smooth. $\times 1500$.

Fig. 3. Scanning electron micrographs of blood vascular casts of the D8 corpus luteum of the TNF group (the day after rh-TNF injection). A: Large blood vessels regress (arrows), and leakages of casting medium from capillaries are also observed (arrow heads). $\times 40$. B: Arrowheads show several constrictions and obstructions of the luteal capillaries. $\times 800$. C: Higher magnification of Fig. 3B. Rugged inner surfaces of the luteal capillaries are shown. $\times 2000$.

Table 1. Effect of rh-TNF injection on serum progesterone concentration (n=3)

Group	Day of pseudopregnancy		
	D0	D7 ^a	D8
TNF	0.88 $\pm$ 0.11	12.57 $\pm$ 1.76	1.29 $\pm$ 0.23*
Control	0.51 $\pm$ 0.13	11.54 $\pm$ 0.92	8.97 $\pm$ 0.19

Values are the mean $\pm$ SEM. a: Blood sampling was performed before rh-TNF or PBS⁻ injection. *: $P < 0.01$ (significantly lower level than the control, as determined by Student's *t*-test).

or obstructive capillaries were observed in this study. In a recent study by us, similar changes in the luteal capillaries were also observed at the end of pregnancy and pseudopregnancy in rabbits (unpublished). It is considered that these phenomena on the luteal vessels are due to injury of the endo-

thelial cells caused by the effects of TNF- α . It is well known that the macrophage is as the main source of TNF- α [23]. As Bagavandoss *et al.* [18, 19] reported, the number of macrophages which infiltrated into the corpus luteum and participated in TNF- α activity increased in the luteolysis stage. Yamada *et al.* [33] reported that the luteal capillaries strangulated at the end of the luteal phase. In view of previous reports and the results of the present study, it can be assumed that the changes in the luteal blood vessels in luteolysis were caused by the direct effects of TNF- α or the effects of PG produced by TNF- α stimulation. On the other hand, concentration of serum progesterone decreased noticeably on the day following rh-TNF injection. TNF- α has a cytotoxic effect on luteal cells, and makes luteal cells synthesize PGs [22]. We consider that the decline in the progesterone concentration is due to the effects of TNF- α on luteal cells and on regression of luteal vessels.

These changes in the blood vessels were found specifically in the corpora lutea in this observation. For example, TNF- α administration or induction cause angiolytic locally in also tumoral tissue such as Meth A fibrosarcoma [34]. It has been considered that the tumoral blood vessels were specifically regressed by synergism of TNF- α and humoral factors released from tumoral cells with TNF- α stimulation [34]. Similarly we can consider that synergism of the direct effect of TNF- α and PGs produced by luteal cells make the blood vessels regress in the corpus luteum.

In conclusion, we found that the injection of rh-TNF into the parenchyma of the ovary in the functional luteal phase caused degeneration of the

luteal vessels with a decrease of progesterone production. These findings suggest that TNF- α produced by luteal macrophages activated at the end of the luteal phase may play a role in angiolytic in the luteolytic process.

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