

# Granulosa cells as a source and target organ for tumor necrosis factor- $\alpha$

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Tumor necrosis factor (TNF- $\alpha$ ), a 17 kDa cytokine, is a product of activated macrophages which was recently shown to be produced by rat and bovine granulosa cells. In the present work, human granulosa cells derived from preovulatory follicles were used. It was demonstrated that human granulosa cells produce TNF- $\alpha$  (5–10 units/300000 cells per 15 h). This production was increased by addition of follicle-stimulating hormone or by a combination of human chorionic gonadotrophin and CSF to the culture media. TNF was also found in bovine follicular fluid and the concentration was higher in the periovulatory than mid-cycle follicles. TNF- $\alpha$  was found to increase prostaglandin F-2 $\alpha$  production by human granulosa cells ( $P < 0.001$ ). We conclude that granulosa cells are both a source and target organ for TNF- $\alpha$ .

Granulosa cell; Tumor necrosis factor; Follicle-stimulating hormone; Ovarian follicle; (Human, Bovine)

## 1. INTRODUCTION

Tumor necrosis factor- $\alpha$  (TNF), a 17 kDa cytokine, is a product of stimulated monocytes and macrophages and also produced by lymphocytes, endothelial cells and keratinocytes. Most reports on TNF have dealt with its inflammatory and anti-tumor effects [1]. Recently, rat and bovine granulosa cells have been reported to produce TNF [2,3]. Other studies have indicated a role for TNF in steroidogenesis in that it inhibits follicle-stimulating hormone (FSH) or tumor growth factor- $\beta$  induced aromatase activity and induction of progesterone synthesis by rat ovarian tissues [4,5]. The present study was conducted to determine (a) if human granulosa cells (GC) produce TNF; (b) if TNF production by follicular cells is regulated by gonadotropins; (c) the role of TNF in pre-ovulatory GC synthesis of prostanoids and progesterone; and (d) the level of TNF in follicular fluid during the cycle using bovine follicles.

## 2. MATERIALS AND METHODS

GC were obtained from follicles stimulated by human menopausal gonadotropin and aspirated during vaginal ultrasound oocyte retrieval for IVF treatment. The treatment protocol for follicular recruitment and separation of GC using Ficoll gradients was done as described previously [6]. Microscopic examination of the preparation showed that 98% of the cells present were granulosa. 300000 cells per well containing 1 ml of Dulbecco's modified Eagle medium supplemented with nutrient mixture F-12 Ham (1:1 mixture; Sigma)

serum-free media were used for incubations. The cultures were incubated overnight with or without the following compounds (dose used; range tested): FSH (25 ng/ml; 0.25–250 ng/ml; Metrodin, Teva), prostaglandin F-2 $\alpha$  (PGF-2 $\alpha$ ; 10  $\mu$ M; 1–300  $\mu$ M; Upjohn), colony stimulating factor-1 (CSF crude preparation of L929 supernatant; 20%; 5–50%), human chorionic gonadotrophin (hCG- $\beta$ ; 10 ng/ml; 5–100 ng; spec. act. 12000 U/mg; NIH), TNF (0.3–3000 pM; rbTNF; Genzyme) or 8-bromoadenosine 3',5'-cyclic monophosphate (8br-cAMP; 3 mM; 1–5 mM; Sigma). Aliquots of media were taken for analysis for TNF, progesterone and PGF-2 $\alpha$ . The TNF cytotoxicity was quantitated using 9 mm microwells at  $4 \times 10^4$  L929 mice fibrosarcoma cells/well. Test samples were applied to the cells in serial dilutions in the presence of 50  $\mu$ g/ml cycloheximide. After 12 h cell killing was quantitated by measuring the uptake of neutral red. One unit was defined as the concentration at which 50% of the cells were killed. Since TNF- $\beta$  (lymphotoxin) can cause the same cytotoxic effect, each positive sample was neutralized by coinubation for 3 h at 4°C with monoclonal anti-human TNF- $\alpha$ .

The human monocyte preparation was prepared by modification of the method of Aderka et al. [7]. Briefly, white blood cells were separated from whole blood obtained from healthy donors on a Ficoll-Hypaque cushion (Pharmacia, Sweden). The monocytes were isolated by incubating the resultant 'buffy coat' at 37°C for 1 h in tissue culture plates, followed by washing with phosphate buffer solution to remove non-adherent cells. The adherent cells (monocytes) were detached by versene and mechanically removed. After the cells were washed twice with phosphate buffer solution and media, the cells were treated the same as the granulosa cell preparations.

Follicular fluid was obtained from periovulatory and mid-cycle follicles as described by Shemesh [8].

Progesterone and prostaglandin F-2 $\alpha$  were measured by radioimmunoassay as described previously [8,9].

The Student *t*-test was performed for all data comparisons. Data are presented as means  $\pm$  SE.

## 3. RESULTS

Data presented in fig.1 demonstrate that human cultured GC secreted TNF (5–10 units/ $3 \times 10^5$  cells per

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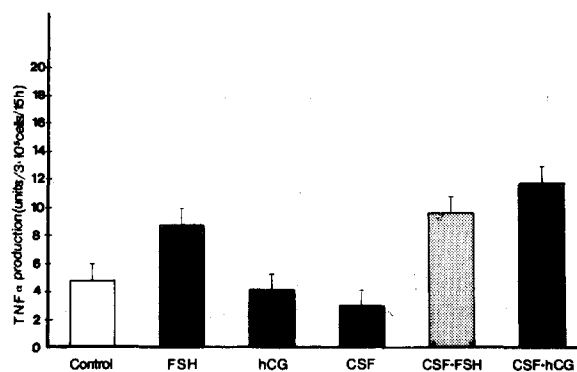


Fig.1. TNF- $\alpha$  production by cultured GC. 300000 cells were incubated for 15 h with FSH (25 ng/ml), hCG (25 ng/ml) or CSF, alone or in combination. Bars represent the mean  $\pm$  SE of 5 experiments of 5 replicates each.

15 h incubation) as determined by bioassay and verified by specific neutralization by a monoclonal antibody to TNF- $\alpha$ .

To determine the role of the tropic hormones on TNF production, GC were incubated in the presence of FSH, hCG, crude CSF preparation, PGF-2 $\alpha$ , and 8br-cAMP, alone or in combination. As can be seen in fig.1, FSH induced a significant enhancement ( $P < 0.001$ ) in TNF production over the control. In contrast, this stimulatory effect was not apparent in the presence of hCG, PGF-2 $\alpha$ , CSF or 8br-cAMP. However, the combination of the crude CSF preparation and hCG induced a significant elevation ( $P < 0.001$ ) in TNF production by the cultured GC. When isolated human monocytes were similarly incubated, FSH had no effect on TNF production.

We next examined the effect of TNF on prostanoid production. As can be seen in fig.2, TNF (300 pM) induced a 3.4-fold increase in PGF-2 $\alpha$  production by cultured GC. However in the presence of FSH, this effect was not apparent. FSH alone was found to

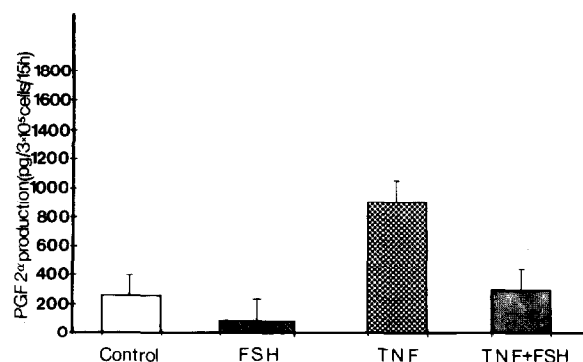


Fig.2. PGF-2 $\alpha$  production by cultured human GC. 300000 cells were incubated in tissue culture media for 15 h and the effect of addition of FSH (25 ng/ml) or TNF- $\alpha$  (300 pM), alone or in combination, was measured. Bars represent the mean  $\pm$  SE of 5 experiments of 5 replicates each.

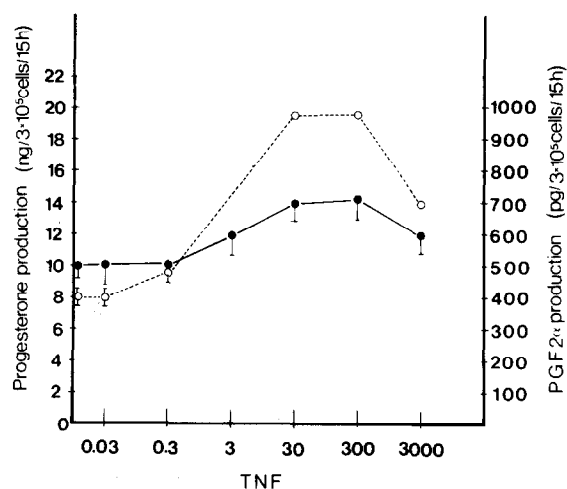


Fig.3. Dose-response curve of progesterone and PGF-2 $\alpha$  production by human GC for TNF- $\alpha$ . 300000 cells were incubated for 15 h in the presence of 0.3–3000 pM TNF- $\alpha$  and progesterone (solid lines) and PGF-2 $\alpha$  (dotted lines) production was measured. Points represent the mean  $\pm$  SE of five experiments, each of 5 replicates.

significantly ( $P < 0.001$ ) inhibit PGF-2 $\alpha$  production by the cultured GC.

To determine the possible role of TNF in steroidogenesis, progesterone production by the cultured GC was assayed. In the presence of TNF (fig.3) there was significant elevation in progesterone production ( $P < 0.05$ ). However, the increase in progesterone production by TNF was of the same magnitude as that induced by PGF-2 $\alpha$  alone (data not shown). The stimulation of both PGF-2 $\alpha$  and progesterone production by TNF appears to be biphasic as the highest dose used (3000 pM) was inhibitory.

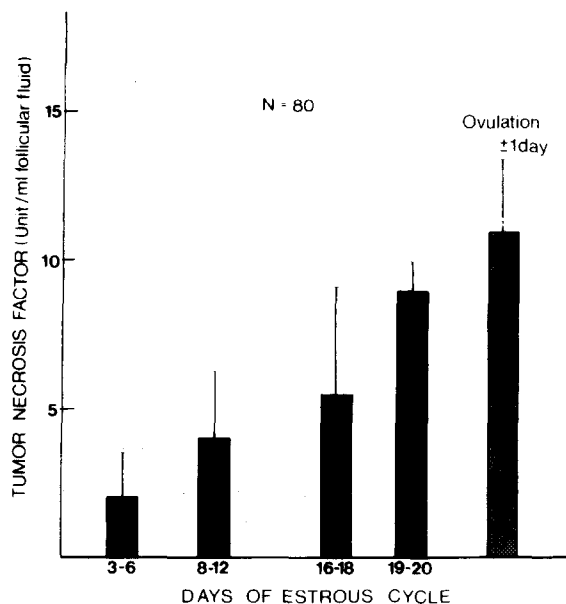


Fig.4. Concentration of TNF in bovine follicular fluid derived from early mid-cycle and periovulatory follicles (mean  $\pm$  SE).

In order to determine the extent of TNF production throughout the cycle, bovine follicular fluid was used. As can be seen in fig.4, measurable amounts of TNF were found in follicular fluid from mid-cycle ovaries (8–12 days). The activity in the periovulatory follicle was significantly higher ( $P < 0.05$ ) than that in the mid-cycle ovaries. The activity was still high in fluid remaining in the follicular cavity in the 24 h after ovulation (corpora hemorrhagica present).

#### 4. DISCUSSION

Data presented indicate that human GC produce TNF and that this production is regulated by FSH. Furthermore, although CSF or hCG alone had no effect on TNF production, the combination of the two increased TNF production significantly. These data document for the first time a role of gonadotropins and CSF in the regulation of TNF by the human preovulatory follicular GC. The function of TNF production by GC is unknown. However, since we have also found [3] that prior to ovulation TNF increases prostaglandin production by both bovine granulosa and theca cells, it is likely that TNF plays a role in the induction of the preovulatory increase of PG synthesis by GC. This is supported by the data showing that the bovine follicular fluid concentrations of TNF increased from mid-cycle to the periovulatory period. On the other hand, the significance of the effect of TNF in increasing granulosa progesterone production is not clear. Although this effect was statistically significant, it may well have been due to the increased prostanoid synthesis which by itself can increase progesterone production by the GC.

The effect of TNF on PGF-2 $\alpha$  was biphasic. This could explain why FSH, which increased TNF production, paradoxically decreased PGF-2 $\alpha$  production. Alternatively, since it has already been reported [1] that prostanoids have a negative feedback on TNF production by macrophages, it is possible that part of the effect of FSH in increasing TNF production is the result of its suppression of PGF-2 $\alpha$  production.

The combined effects of hCG and CSF on TNF production are intriguing but lack an obvious explanation.

It is possible to speculate that hCG may act by inducing CSF receptors and the CSF is responsible for the TNF increase. Support for this concept emerges from the work of Pollard et al. [10] who have shown an increase in CSF secretion by rat endometrial tissue at the time of ovulation.

The field of growth factors and cytokines and their effects on reproduction is a rapidly growing new area of investigation. It appears that there is a complex interplay between the growth factors such as CSF, the cytokines and the gonadotropins [11,12]. In addition, the present work shows that cytokines such as TNF- $\alpha$  can act as an autocrine hormone in that it is produced by its target organ, the follicular granulosa.

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