Effects of macrophage colony-stimulating factor on folliculogenesis in gonadotrophin-primed immature rats

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The effect of macrophage colony-stimulating factor (M-CSF) on folliculogenesis and ovulation was studied. Folliculogenesis and ovulation were induced in immature female rats with a s.c. injection of equine chorionic gonadotrophin (eCG), followed 48 h later by human chorionic gonadotrophin (hCG). The ovulation rate was measured after the following treatments. (1) Graded doses of human M-CSF ($1\text{--}300 \times 10^3$ iu per rat) were administered i.p. daily for 3 consecutive days. (2) M-CSF (100×10^3 iu) was administered i.p. at designated times between 96 h before and 10 h after hCG injection. (3) Rabbit anti-human M-CSF polyclonal antibody (5 μ g) was administered into the left ovarian bursa at designated times between 49 h before and 10 h after hCG injection. In addition, the effect of M-CSF on ovarian macrophages was investigated using immunohistochemistry with mouse anti-rat macrophage monoclonal antibody, TRPM-3. The treatment with M-CSF ($> 30 \times 10^3$ iu per rat) significantly increased the ovulation compared with controls in a dose-dependent manner. This stimulatory effect of M-CSF was observed when it was administered between 96 h and 49 h before hCG injection. The ovarian intrabursal administration of anti-M-CSF antibody significantly inhibited the number of ovulated ova from the treated ovaries compared with either those from control rats or from the contralateral untreated ovaries between 24 h before and 3 h after hCG injection. The immunohistochemistry revealed that M-CSF increased the number of ovarian macrophages in growing follicles. The results suggest that M-CSF is involved in the process of folliculogenesis and that it promotes ovulation by influencing ovarian macrophages.

Introduction

Macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor 1 (CSF-1), is a glycoprotein that belongs to the family of haematopoietic growth factors (Clark and Kamen, 1987). These growth factors regulate the production of blood cells, from bone marrow progenitors to mature circulating elements (Clark and Kamen, 1987). Since M-CSF was purified to homogeneity and its genes were cloned and expressed (Stanley and Heard, 1977; Kawasaki *et al.*, 1985; Wong *et al.*, 1987; Ceretti *et al.*, 1988), diverse effects and functions have been reported. M-CSF induces proliferation and differentiation of monocyte-macrophage progenitor cells in bone marrow (Stanley and Heard, 1977; Tushinski *et al.*, 1982; Stanley *et al.*, 1983; Tushinski and Stanley, 1983), and acts on mature monocytes and macrophages to maintain their survival (Tushinski *et al.*, 1982; Becker *et al.*, 1987) and promote their essential biological functions (Tushinski *et al.*, 1982; Warren and Ralph, 1986; Wang *et al.*, 1988). M-CSF has indirect effects on cellular proliferation and differentiation through the stimu-

lation of cytokine production and release by macrophages (Baird *et al.*, 1985; Assoian *et al.*, 1987; Rom *et al.*, 1988; Fukuoka *et al.*, 1989; Higashiyama *et al.*, 1991). Furthermore, M-CSF acts as a regulator of placenta (Pollard *et al.*, 1987; Arceci *et al.*, 1989; Saji *et al.*, 1990) or trophoblast (Regenstreif and Rossant, 1989; Beauchamp and Croy, 1991) growth and differentiation, and a role in fetal development has also been suggested (Bradley *et al.*, 1971; Bartocci *et al.*, 1986; Azuma *et al.*, 1991).

Macrophages are found within the corpus luteum of several species (Bulmer, 1964; Guillim *et al.*, 1969), and are recognized as mediators that modulate its function and development (Halme *et al.*, 1985). Katabuchi *et al.* (1989) reported that macrophages are also identified within human growing follicles, and Fukumatsu *et al.* (1992) reported that they promote the proliferation of granulosa cells as local mediators in rat growing follicles.

In this study, we investigated the effect of M-CSF on folliculogenesis and ovulation and on ovarian macrophages in gonadotrophin-primed immature rats, to determine whether M-CSF and macrophages are involved in the process of folliculogenesis and ovulation.

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Materials and Methods

Induction of folliculogenesis and ovulation

Immature female Wistar rats were obtained from Kyudo Co. Ltd (Kumamoto) and were housed under controlled laboratory conditions. At 09:00 h on day 23 of life, folliculogenesis was induced with a s.c. injection of 5 iu (in the experiments to test the stimulatory effect of M-CSF on a normal ovulation model) or 10 iu (in the experiments to test the inhibitory effect of anti-M-CSF antibody on a superovulation model) equine chorionic gonadotrophin (eCG; Teikoku Hormone Mfg Co. Ltd, Tokyo), and ovulation was induced 48 h later with a s.c. injection of 10 iu hCG (Mochida Pharmaceutical Co., Tokyo).

The rats were killed by cervical dislocation, and the ovaries and oviducts were removed together 24 h after hCG injection. The oviducts were separated from the ovaries, placed on a glass slide, and examined under the dissecting microscope. The number of ova released from the ovaries was counted.

Effect of M-CSF

The effects of M-CSF on folliculogenesis and ovulation were examined by administering graded doses ($1\text{--}300 \times 10^3$ iu per rat) of human M-CSF (a formulation of human urine-derived native M-CSF, generic name: Mirimostim; a gift from The Green Cross Co., Osaka) i.p. to the gonadotrophin-primed immature rats daily for 3 consecutive days (i.e. at 48, 24 and 0 h before hCG injection). Hence, the total doses of M-CSF administered were 3, 9, 30, 90, 300 or 900×10^3 iu per rat.

The most effective time of M-CSF administration was determined by administering a single injection of 100×10^3 iu M-CSF per rat i.p. to the gonadotrophin-primed rats at 96, 72, 49, 24 or 1 h before, or 3 or 10 h after hCG injection. M-CSF was dissolved in 250 μ l of 0.9% (w/v) saline. Control rats were given the same amount of vehicle containing equivalent amounts of human albumin (Sigma Chemical Co., St Louis, MO) and D-mannitol (Nakarai Tesque Inc., Kyoto), which were the other components of M-CSF used in this study.

Effect of anti-M-CSF antibody

Rats were lightly anaesthetized with ether and dorsal incisions were made in the abdominal area. Rabbit anti-human M-CSF polyclonal antibody (Genzyme Co., Boston, MA) was diluted with PBS, and 5 μ g anti-M-CSF antibody was administered into the left ovarian bursa at 49, 24 or 1 h before, or 3 or 10 h after hCG injection using a 30-gauge needle. This is an antibody against affinity-purified recombinant human M-CSF and purified IgG, and does not crossreact with human interleukin 2 (IL-2), IL-4, granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF). In control rats, the same volume of vehicle containing the equivalent amount of rabbit immunoglobulin G (Sigma) was administered intrabursally 24 h before hCG injection. In each rat, the contralateral (i.e. right) ovary was untreated and served as a control.

Immunohistochemistry

The effect of M-CSF on ovarian macrophages was determined using immunohistochemistry. M-CSF (100×10^3 iu;

$n = 5$) or vehicle solution ($n = 5$) was administered i.p. 1 h before eCG injection in five rats per treatment. Ovaries were obtained 24 h after eCG injection and fixed in 2% (w/v) periodate-lysine-paraformaldehyde for 6 h at 4°C. After washing for 4 h in PBS containing 10, 15 or 20% (w/v) sucrose, they were embedded in OCT compound (Miles, Elkhart, IN), and frozen in dry ice-acetone. At its greatest diameter, each ovary was cut into 20 semi-serial sections, 8 μ m thick, by a cryostat (Sakura Fine Technical Co. Ltd, Tokyo) and dried in air. After inhibition of endogenous peroxidase activity, the sections were incubated with mouse anti-rat macrophage monoclonal antibody, TRPM-3 (Anti-marginal metallophilic macrophage, rat; BMA Biomedicals Ltd, Augsto), for 60 min at room temperature. TRPM-3 binds specifically to certain rat macrophage populations, but not to peripheral blood monocytes, granulocytes or lymphocytes (Takeya *et al.*, 1987). After three washes with PBS for 5 min each, the sections were incubated for 60 min in avidin-biotin-peroxidase complex (Hsu *et al.*, 1981). Peroxidase activity was visualized by using 3,3'-diaminobenzidine (Sigma) as a substrate in Tris-HCl buffer (0.05 mol l^{-1} , pH 7.6) containing 0.01% (w/v) H_2O_2 . Counterstaining was performed with methylgreen. After the immunohistochemical staining with TRPM-3, the distribution and quantitative analysis of macrophages in growing follicles were performed according to the method of Fukumatsu *et al.* (1992).

Statistical analyses

All data were presented as means $\pm$ SEM. The significance of differences between means of groups was determined by the Mann-Whitney U-test, and differences with $P < 0.05$ were considered to be significant.

Results

Effect of M-CSF

When M-CSF was administered daily for 3 consecutive days, more than 30×10^3 iu per rat significantly increased the number of ovulated ova compared with that of control rats in a dose-dependent manner ($P < 0.05$; Fig. 1).

When a single injection of 100×10^3 iu M-CSF per rat was given at the designated times between 96 h before and 10 h after hCG injection, the ovulation rate was significantly increased between 96 and 49 h before hCG injection compared with that of the controls ($P < 0.05$; Fig. 2). However, when M-CSF was given between 24 h before and 10 h after hCG injection, it had no significant effect on ovulation.

Effect of anti-M-CSF antibody

In control rats, there were no significant differences in the number of ovulated ova between the vehicle-treated ovary and the contralateral untreated ovary. There were no significant differences in the number of ovulated ova among the untreated ovaries of each group. Ovarian intrabursal administration of the anti-M-CSF antibody (5 μ g) significantly reduced the number of ovulated ova between 24 h before and 10 h after

Fig. 1. Effect of macrophage colony-stimulating factor (M-CSF) on ovulation rate in rats. *Significantly different from controls ($P < 0.05$); Number of rats (n): 16, 8, 8, 8, 9, 11 and 9 from left to right columns.

Fig. 2. Optimum time of macrophage colony-stimulating factor (M-CSF) administration on rat ovulation rate. In control rats, only the vehicle was administered i.p. 24 h before hCG injection. *Significantly different from control values ($P < 0.05$); Number of rats (n): 9, 7, 7, 8, 9, 9, 9 and 9 from left to right columns.

hCG injection compared with the number in contralateral untreated ovaries in each group (inhibition was 41.8–54.1%), and between 24 h before and 3 h after hCG injection compared with the number in vehicle-treated ovaries of control rats (inhibition was 42.7–47.4%) ($P < 0.05$; Table 1). Intrabursal injection of the antibody did not affect the number of ova shed by the contralateral untreated ovaries.

Immunohistochemistry

Ovarian follicles were classified into three groups according to the greatest diameter of follicles in semi-serial sections: preantral (< 0.15 mm), antral (0.15–0.3 mm) and mature (> 0.3 mm).

In control rats, preantral follicles were predominant in ovaries obtained 24 h after eCG injection. M-CSF did not significantly affect the proportion of the three types of follicles when it was administered 1 h before eCG, although it tended to increase the number of mature follicles.

TRPM-3-positive cells (i.e. macrophages) were found in preantral, antral and mature follicles, but not in all follicles. M-CSF significantly increased the number of follicles with TRPM-3-positive cells, the B:A ratio (the ratio of follicles with TRPM-3 positive cells to follicles per section), and the number of TRPM-3-positive cells per follicle in mature follicles ($P < 0.05$; Table 2).

Discussion

There is increasing evidence that, in addition to pituitary gonadotrophins, ovarian local mediators, including growth factors and cytokines, play an important role in controlling ovarian function (Franchimont *et al.*, 1988; Chen *et al.*, 1993; Brännström *et al.*, 1993a; Tekpetey *et al.*, 1993; Marcinkiewicz *et al.*, 1994). However, little is known about the involvement of M-CSF in ovarian function. This is the first report that M-CSF

influences folliculogenesis and ovulation in rats *in vivo*. Our results showed that M-CSF increased ovulation rate in a dose-dependent manner, and that anti-M-CSF antibody decreased ovulation rate. Ideally, the route of administration of M-CSF and the antibody should be the same. Intrabursal injection for the antibody and the intraperitoneal injection for the M-CSF were chosen because repeated intrabursal injection (accompanied by repeated anaesthesia and laparotomy) damages the bursa, and because after i.p. or i.v. administration, the antibody is rapidly neutralized. In the anti-M-CSF antibody experiment, higher initial ovulation rates were induced so that an inhibitory effect of the antibody would be more obvious.

From the experiments that investigated the effective time of administration of M-CSF and its antibody, a single i.p. injection of M-CSF increased the ovulation rate between 96 h and 49 h before hCG injection, and the ovarian intrabursal administration of the antibody decreased ovulation rate between 24 h before and 3 h after hCG injection. The results indicated that M-CSF affected folliculogenesis before the hCG injection, rather than the ovulatory process after the hCG injection, and consequently, changed the ovulation rate. The most effective time of M-CSF administration was different from that of the antibody administration. This difference may be due partly to the different design of the experiments. M-CSF may not be effective at a specific time but over a broad period during folliculogenesis, for, at a lower dose, the three-stage administration of M-CSF increased ovulation rate more effectively than did a single injection.

Since ovarian stimulation was controlled completely by exogenous gonadotrophins, the possible effect of M-CSF on the hypothalamic–pituitary axis could be eliminated in our experimental rat model. M-CSF alone could not induce ovulation (data not shown), but appeared to affect folliculogenesis and ovulation in cooperation with gonadotrophins. Since the ovarian intrabursal injection of the anti-M-CSF antibody did not affect the release of ova from the contralateral untreated

Table 1. Effect of anti-macrophage colony-stimulating factor (M-CSF) antibody on ovulation rate in rats

Time of anti-M-CSF antibody administration (h after hCG)	n	Number of ova per ovary		Percentage inhibition	
		Untreated ovary	Treated ovary	Versus untreated ovary	Versus control rat
Control	10	18.4 ± 1.9	19.0 ± 1.4		
– 49	8	20.9 ± 1.1	16.8 ± 2.1	19.6	11.6
– 24	8	19.8 ± 1.3	10.4 ± 2.2	47.5*	45.3*
– 1	11	21.4 ± 1.4	10.0 ± 1.7	54.1*	47.4*
3	7	22.6 ± 2.2	10.9 ± 1.3	47.1*	42.7*
10	7	19.6 ± 1.6	11.4 ± 2.6	41.8*	40.0

Five micrograms of the antibody or vehicle was administered into the left ovarian bursa at the designated times, and the contralateral right ovary was untreated.

*Significantly different from either the contralateral untreated ovaries or the vehicle-treated ovaries of control rats ($P < 0.05$).

Table 2. Quantitative analysis of macrophages in rat growing follicles

Parameter	Preantral		Antral		Mature	
	Control	M-CSF	Control	M-CSF	Control	M-CSF
Number of follicles per section (A)	14.0 ± 1.3	13.8 ± 1.1	7.0 ± 0.6	8.2 ± 1.1	3.8 ± 0.7	4.4 ± 0.7
Number of follicles with TRPM-3-positive cells per section (B)	0.8 ± 0.6	1.8 ± 0.8	2.0 ± 0.7	3.6 ± 0.8	0.4 ± 0.2	1.8 ± 0.4*
B:A ratio	0.05 ± 0.08	0.15 ± 0.16	0.28 ± 0.19	0.43 ± 0.20	0.08 ± 0.12	0.43 ± 0.19*
Number of TRPM-3-positive cells per follicle in one section	0.4 ± 0.2	1.2 ± 0.4	1.7 ± 0.4	1.9 ± 0.8	1.6 ± 1.2	3.4 ± 0.7*

Macrophage colony-stimulating factor (M-CSF) (100×10^3 iu) or vehicle was administered i.p. to five rats in each group 1 h before eCG injection. Growing follicles were analysed in 20 semi-serial sections of ovaries obtained 24 h after eCG injection.

*Significantly different from controls ($P < 0.05$).

ovary, diffusion from the treated ovary to the contralateral untreated ovary was minimal, and M-CSF seemed to act at the ovary.

Since macrophages have been found in atretic follicles and corpora lutea of several species (Bulmer, 1964; Guillim *et al.*, 1969), they were considered to scavenge degenerated cells in the ovary. In addition to their phagocytic function, macrophages produce and release several cytokines such as fibroblast growth factor, transforming growth factor, insulin-like growth factor I, interleukin-1, epidermal growth factor and tumour necrosis factor α (Baird *et al.*, 1985; Assoian *et al.*, 1987; Rom *et al.*, 1988; Fukuoka *et al.*, 1989; Higashiyama *et al.*, 1991; Sancho-Tello *et al.*, 1993), and modulate steroid production by granulosa-luteal cells (Azuma *et al.*, 1991). Brännström *et al.* (1993b) suggested that macrophages and neutrophilic granulocytes are involved in tissue remodelling that occurs at ovulation. Katabuchi *et al.* (1989) demonstrated that macrophages are identified not only in the corpus luteum but also in the growing follicles, and Fukumatsu *et al.* (1992) suggested that macrophages modulate follicular development by influencing the proliferation of granulosa cells. It is therefore possible that M-CSF may act on folliculogenesis and ovulation by influencing ovarian macrophages. In the study reported here, we investigated the effect of M-CSF on the changes in number of

ovarian macrophages in growing follicles by using immunohistochemistry. Although TRPM-2 is an anti-rat panmacrophage antibody and recognizes not only macrophages but also monocytes and granulocytes, and TRPM-3 binds specifically to certain rat macrophage populations and does not crossreact with monocytes or granulocytes, there is no difference in distribution or number between the TRPM-2-positive cells and TRPM-3-positive cells in follicles (Fukumatsu *et al.*, 1992). TRPM-3-positive cells can therefore be considered to represent the macrophage population alone. The present results revealed that M-CSF significantly increased the number of macrophages in mature follicles, although the increase in the number of ovarian macrophages was not significant in preantral and antral follicles. This finding suggested that the activation of macrophages may be important in the action of M-CSF on folliculogenesis, although the functional changes of ovarian macrophages were not examined.

Macrophages are known to increase the production of G-CSF and GM-CSF. Brännström *et al.* (1994) reported that the rat ovary produces GM-CSF before and during the ovulatory process and that there are temporal fluctuations in GM-CSF release with a peak in output at ovulation. In addition to M-CSF, we therefore tested the effect of GM-CSF and G-CSF on folliculogenesis and ovulation. GM-CSF and G-CSF were

administered i.p. to gonadotrophin-primed immature Wistar rats, and did not stimulate ovulation rate in our experimental model (data not shown).

M-CSF alone may stimulate the ovulation rate, but not through macrophages. Naito *et al.* (1991) reported that the number of ovarian macrophages is severely reduced in osteopetrotic (*op/op*) mutant mice, which fail in fertility and production of functional M-CSF (Pollard *et al.*, 1991). Our unpublished observation was that ovulation was inhibited in *op/op* mice. These findings support the concept that M-CSF plays an important role in the physiology of ovulation directly or through macrophages.

The gene expression of M-CSF and its receptor (*c-fms* proto-oncogene product) has been detected in the uterus and placenta of several species (Pollard *et al.*, 1987; Arceci *et al.*, 1989; Saji *et al.*, 1990). Tekpetey *et al.* (1992) reported that the mRNA encoding both CSF-1 and its receptor are present in pig granulosa cells, and Arceci *et al.* (1992) reported that the amount of mRNA encoding *c-fms* increases during the maturation of mouse oocytes and CSF-1 is detected in cumulus cells, the oviduct and uterus. These results suggest that M-CSF influences folliculogenesis through direct action on granulosa cells, and that autocrine and paracrine control mechanisms by M-CSF may regulate both follicular and placental development.

In conclusion, our results suggest that M-CSF is involved in folliculogenesis and that it promotes ovulation partly by influencing ovarian macrophages.

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