Effect of macrophages on proliferation of granulosa cells in the ovary in rats

Y. Fukumatsu^{1,2}, H. Katabuchi¹, M. Naito², M. Takeya², K. Takahashi²
and H. Okamura¹

¹Department of Obstetrics and Gynecology; and ²Second Department of Pathology, Kumamoto University Medical School, Kumamoto 860, Japan

Summary. The effect of macrophages on proliferation of granulosa cells was examined in gonadotrophin-primed immature female rats. The mouse anti-rat macrophage monoclonal antibodies TRPM-2 and TRPM-3 were used and macrophages were observed in the granulosa layer and antrum of follicles and in corpora lutea and stroma around follicles. There was no difference in distribution between TRPM-2-positive cells and TRPM-3-positive cells. Macrophages with some cytoplasmic vacuoles of various sizes were also demonstrated in growing follicles. The average ratios of macrophages to granulosa cells in preantral, antral and mature follicles were 0.008, 0.007 and 0.002, respectively. Labelling with [³H]thymidine of granulosa cells cultured with peritoneal macrophages was significantly greater and the labelling index peaked to 25.0% when the ratio of macrophages to granulosa cells was 0.01, compared with the value of 14.2% when the granulosa cells were cultured alone. This ratio of macrophages to granulosa cells was similar to that in the preantral and antral follicles *in vivo*. These results suggest that macrophages participate in promoting proliferation of granulosa cells as local mediators in growing follicles.

Keywords: macrophage; granulosa cell proliferation; immunohistochemistry; culture; rat

Introduction

Proliferation and differentiation of granulosa cells in developing follicles are modulated by gonadotrophins and ovarian steroids. Paracrine and autocrine intraovarian control mechanisms of follicular development have been suggested (Hofmann *et al.*, 1990). Epidermal growth factor and fibroblast growth factor induce the proliferation of human granulosa cells, and the former also influences progesterone production *in vitro* (Tapanainen *et al.*, 1987). Macrophages enhance progesterone production in cultured granulosa cells in mice (Kirsch *et al.*, 1981) and humans (Halme *et al.*, 1985) and thus appear to play an important role in the control of ovarian function. However, the effect of macrophages on the proliferation of granulosa cells is unknown and the presence of macrophages in growing follicles has not been demonstrated.

The present study concerned the distribution of macrophages in the follicles at various developmental stages in gonadotrophin-primed, immature ovaries in rats *in vivo* and investigated the effect of macrophages on proliferation of granulosa cells *in vitro*.

Materials and Methods

Animals

Female Wistar rats (40–50 g), 21 days old, were obtained from Kyudo Co. Ltd (Kumamoto, Japan) and kept in routine laboratory conditions. Immature female rats, 22 days old, were injected subcutaneously with 10 iu pregnant

mares' serum gonadotrophin (PMSG; Teikoku Zouki, Tokyo, Japan) at 09:00 h and 48 h later with 5 iu human chorionic gonadotrophin (hCG; Mochida, Tokyo, Japan). The rats were killed by cervical dislocation and the ovaries were removed either before the gonadotrophin priming, or at 12, 24 or 48 h after the PMSG injection. Ovaries were also removed at 12 and 24 h after the hCG injection.

Monoclonal antibodies

Two mouse anti-rat macrophage monoclonal antibodies, TRPM-2 and TRPM-3, were produced in the Second Department of Pathology, Kumamoto University Medical School (Takeya *et al.*, 1987, 1989). TRPM-2 reacts with most monocyte-macrophage populations and crossreacts with granulocytes (Takeya *et al.*, 1989). TRPM-3 binds specifically to certain rat macrophage populations, but not to peripheral blood monocytes, granulocytes or lymphocytes (Takeya *et al.*, 1987).

Immunohistochemistry

Ovaries were fixed in 2% periodate-lysine-paraformaldehyde for 6 h at 4°C (McLean & Nakane, 1974). After washing for 4 h in phosphate-buffered saline (PBS) containing 10, 15 or 20% sucrose, they were rinsed in PBS containing 20% sucrose and 10% glycerol for 60 min, embedded in OCT compound (Miles, Elkhart, IN, USA) and frozen in dry-ice-acetone. Sections, 8 µm thick, were cut in a cryostat (Bright, Huntingdon, UK) and dried in air. After inhibition of endogenous peroxidase activity (Isobe *et al.*, 1977), the sections were incubated with each monoclonal antibody for 60 min at room temperature. After three washes with PBS for 5 min each, the sections were incubated for 60 min in peroxidase-conjugated sheep anti-mouse immunoglobulin (F(ab')₂; Amersham, UK) diluted 1:100. Peroxidase activity was visualized by using 3,3'-diaminobenzidine (Sigma, St Louis, MO, USA) as substrate in Tris-HCl buffer (0.05 mol l⁻¹, pH 7.6) containing 0.01% H₂O₂. Counterstaining was performed with methyl green.

Electron microscopy

Small pieces of fresh ovarian tissue obtained as described above were fixed in 2.5% glutaraldehyde for 2 h and subsequently fixed with 1% osmium tetroxide for 60 min. After dehydration in a graded series of ethanols, they were placed in propylene oxide and embedded in epoxy resin. Ultrathin sections were cut on an Ultratome Nova (LKB, Uppsala, Sweden), stained with lead citrate and uranyl acetate and observed in an H-300 electron microscope (Hitachi, Tokyo, Japan).

Quantitative analysis of macrophages in follicles

Ovaries were extirpated from the rats 48 h after the treatment with 10 iu PMSG, because various sizes of follicles, primary to mature, were observed in these ovaries. They were fixed and frozen as already described. Whole ovaries were cut into serial sections, 8 µm thick, which were stained immunohistochemically with TRPM-3. The numbers of granulosa cells and TRPM-3-positive cells were counted in 20 specimens of each preantral, antral and mature follicle of the greatest diameter. Ratios of TRPM-3-positive cells to granulosa cells (TR3/GC) were calculated.

In vivo labelling of granulosa cells with [³H]thymidine

At 24 h after the treatment with 10 iu PMSG, the rats were injected intravenously with 200 µCi [methyl-³H]thymidine (New England Nuclear, Boston, MA, USA). The rats were killed and the ovaries were removed 2 h after the [³H]thymidine injection. The procedures of fixation to making sections were as described in the 'Immunohistochemistry' section. The specimens were dipped in liquid emulsion, Sakura NR-M2 (Konika, Tokyo, Japan) diluted 1:2 in water and exposed for 10 days. They were developed in FD-III (Yamada *et al.*, 1990) at 20°C and stained with methyl green.

Preparation of granulosa cells

Granulosa cells were isolated from the follicles of the rats 24 h after injection with 10 iu PMSG. The ovaries were removed and dipped into PBS containing 0.02% ethylenedinitrilotetraacetic acid, pricked with a 27-gauge needle and incubated for 8 min at 37°C. Granulosa cells were isolated from the ovaries through a steel mesh by applying gentle pressure and centrifuged at 150 g for 5 min to make pellets, which were suspended in Gey's solution to destroy erythrocytes. After the second centrifugation, the pellets were suspended in RPMI-1640 (Nissui-Seiyaku, Tokyo, Japan) containing 10% fetal calf serum (FCS) and incubated in 60 mm plastic dishes (Nunc, Roskilde, Denmark) for 60 min at 37°C in 95% air and 5% CO₂ to eliminate macrophages. The non-adherent cells were used for culture with macrophages. Viability of the cells was ≥95%, as determined by trypan blue stain.

Preparation of peritoneal macrophages

Macrophages were collected from the peritoneal cavity of unstimulated 22-day-old female rats by peritoneal lavage.

Co-culture and autoradiography with [^3H]thymidine

Granulosa cells (10^5 per well) were dispersed in RPMI-1640 medium (1 ml per well) supplemented with 10% FCS and incubated on glass chamber slides (Inter Med, Naperville, IL, USA) for 48 h at 37°C under 95% air and 5% CO_2 . The cells were then cultured with macrophages in various concentrations (10^2 , 10^3 , 10^4 and 10^5 per well) for 24 h under the same conditions. To examine the proliferative capacity of the granulosa cells, they were incubated in the medium containing 1 μCi [methyl- ^3H]thymidine ml^{-1} . After incubation for 2 h, they were fixed with 90% ethanol and dried in air. The procedures for autoradiography were as described above. They were stained with nuclear fast red. One thousand granulosa cells were examined on each chamber under a light microscope and percentages of labelled cells were calculated as the [^3H]thymidine labelling index. Cells with five or more grains on the nucleus were judged to be labelled (Kato *et al.*, 1990).

Statistical analysis

The data were presented as the mean $\pm$ SD. The significance of the differences between means of groups was determined by the Wilcoxon test.

Results

Distribution of macrophages in the ovary

In the ovaries of untreated immature rats, most of the follicles were primary and preantral. Follicles were developed by administration of PMSG. Antral follicles predominated at 24 h after the PMSG injection; every stage, primary, preantral, antral and mature, was present 48 h after the PMSG injection, although mature follicles were in the peripheral zone of these ovaries (Table 1). Corpora lutea were observed 24 h after the hCG treatment. As shown by immunohistochemistry, both TRPM-2- and TRPM-3-positive cells were observed in the granulosa layer of preantral follicles (Fig. 1a), in the granulosa layer or antrum of antral follicles (Fig. 1b) and in mature follicles (Fig. 1c), as well as in corpora lutea (Fig. 1d) and in the stroma around the follicles. These cells were round and dispersed in the follicles and round, oval or spindle-shaped in the corpora lutea and stroma. There were no differences in distribution between the TRPM-2-positive and TRPM-3-positive cells in the ovaries, or in number between the TRPM-2-positive cells and the TRPM-3-positive cells in the follicles.

Table 1. Distribution of macrophages in treated rat ovarian tissues

		Time (h) since treatment with					
		Pregnant mares' serum gonadotrophin			Human chorionic gonadotrophin		
		Untreated	12	24	48	12	24
Follicle							
Primary	—	—	—	—	—	—	—
Preantral	+	+	+	+	+	○	
Antral	○	+	+	+	+	○	
Mature	○	○	+	+	+	○	
Corpus luteum	○	○	○	○	○		+
Stroma	+	+	+	+	+		+

Macrophages were identified by immunohistochemical procedures using TRPM-2 and TRPM-3. There was no difference in distribution or number between the TRPM-2-positive cells and TRPM-3-positive cells.

+: presence of macrophages; —: absence of macrophages; ○: absence of structures.

Fig. 1. Immunohistochemistry using TRPM-3 in rats: (a) preantral follicle before injection with pregnant mares' serum gonadotrophin (PMSG), $\times 320$; (b) antral follicle 24 h after PMSG, $\times 160$; (c) mature follicle 48 h after PMSG, $\times 80$; and (d) corpus luteum 24 h after injection with human chorionic gonadotrophin, $\times 160$. TRPM-3-positive cells are present in the granulosa layer of the preantral follicle, granulosa layer and antrum of antral and mature follicle, and in the corpus luteum and stroma.

Ultrastructural features of macrophages and granulosa cells

The ample cytoplasm of macrophages in the antrum of follicles possessed lysosomal granules, an oval or slightly indented nucleus and filopodia projecting from the cell surface (Fig. 2a, b). They were large (20–30 μm) and characterized by cytoplasmic vacuoles of various sizes. The granulosa cells in these follicles had abundant ribosomes and a large nucleus, often with prominent nucleoli (Fig. 2c), suggesting that these follicles were developing. Macrophages in the corpora lutea had many lipid droplets, secondary lysosomes, or myelinoid bodies, but no cytoplasmic vacuoles

(Fig. 3a). Macrophages in the stromal tissues did not have granules or vacuoles in the cytoplasm (Fig. 3b).

Quantitative analysis of macrophages in follicles

Ovarian follicles were classified into three stages according to the greatest diameter of follicles in serial sections: preantral (<0.15 mm), antral (0.15–0.3 mm) and mature follicles (>0.3 mm). Numbers of granulosa cells in the sections of preantral, antral and mature follicles of greatest diameter were 72.8 ± 50.2 , 531.1 ± 379.0 , 1547.7 ± 402.8 , respectively. In 20 specimens of each stage, TRPM-3-positive cells were present in six preantral, 17 antral and 16 mature follicles. Numbers of TRPM-3-positive cells per greatest diameter section of follicle were 0.6 ± 1.1 in preantral, 3.7 ± 4.9 in antral, 2.3 ± 2.0 in mature follicles. The TR3:GC ratio in the growing follicles (0.008 in preantral, 0.007 in antral) was higher than in mature follicles (0.002; Table 2).

Proliferation of granulosa cells *in vivo*

Granulosa cells in preantral and antral follicles showed strong uptake of [³H]thymidine (Fig. 4a, b). Percentages of granulosa cells labelled with [³H]thymidine were $34.0 \pm 2.7\%$ in preantral follicles and $62.6 \pm 9.3\%$ in antral follicles.

Proliferation of granulosa cells in culture with macrophages

Granulosa cells were dispersed in the culture medium containing 10% FCS. In culture, after adherence, they spread out on the glass chamber slides in a monolayer, showed a spindle or stellate shape and swelled markedly within 24–48 h. It was easy to distinguish granulosa cells from peritoneal macrophages, because the latter were rounder and apparently smaller (Fig. 5). The [³H]thymidine labelling index of granulosa cells cultured with peritoneal macrophages (10^2 , 10^3 , 10^4 and 10^5) was significantly greater (22.3 ± 1.6 , 25.0 ± 2.5 , 19.5 ± 1.6 and $19.0 \pm 1.9\%$, respectively) than that of granulosa cells cultured alone ($14.2 \pm 3.0\%$). The labelling index of granulosa cells peaked when the ratio of peritoneal macrophages to granulosa cells (macrophage:GC) was 0.01 (10^5 for granulosa cells, 10^3 for macrophages; Fig. 6). This ratio was similar to the TR3:GC in the preantral (0.008) and antral (0.007) follicles *in vivo*.

Discussion

Since the presence of macrophages in the granulosa layer of atretic follicles and the degenerating corpora lutea of rats was demonstrated by Bulmer (1964) using nonspecific esterase as a histochemical marker, macrophages have been thought to scavenge degenerated cells in the ovary. The present immunohistochemical and electron microscope observations showed the presence of macrophages not only in the corpora lutea but also in the growing follicles. To eliminate the atretic follicle, we used immature rats treated with PMSG and hCG and confirmed the quality of the growing follicles by observing their granulosa cells with an electron microscope. Although TRPM-2 is an anti-rat panmacrophage antibody and recognizes not only macrophages but also monocytes and granulocytes, and although TRPM-3 binds specifically to certain rat macrophage populations and does not crossreact with monocytes or granulocytes, we found no difference in distribution or number between the TRPM-2-positive cells and TRPM-3-positive cells in follicles. Thus, the number of TRPM-3-positive cells can be considered to represent the macrophage population alone, and the ratio of TRPM-3-positive cells to granulosa cells is interpreted as that of macrophages to granulosa cells in follicles. The ratio was higher in the growing follicles, such as preantral and antral follicles, than in the mature follicles (Table 2). These results suggest that macrophages in growing follicles modulate follicular development by influencing proliferation of granulosa cells, *in situ*.

Fig. 2. Electron microscopy of rat antral follicle. (a, b) Macrophages observed in the antrum of follicle 24 h after injection with pregnant mares' serum gonadotrophin – macrophages are 20–30 µm in diameter and characterized by the presence of cytoplasmic vacuoles; (c) granulosa cells in this follicle have abundant ribosomes and a high nucleus:cytoplasm ratio.

Fig. 3. Electron microscopy of macrophages in (a) the corpus luteum in rats 24 h after injection with human chorionic gonadotrophin – they have many lipid droplets and secondary lysosomes; and (b) the stromal tissue 24 h after injection with pregnant mares' serum gonadotrophin – no granules or vacuoles in the cytoplasm.

Table 2. Quantitative analysis of granulosa cells and macrophages in rat follicles

	Preantral	Antral	Mature
Diameter of largest follicle section (mm)	<0.15	0.15–0.3	>0.3
Number of follicles examined	20	20	20
Number of follicles in which TRPM-3-positive cells were present	6	17	16
Number of granulosa cells (GC)*	72.8 ± 50.2	531.1 ± 379.0	1547.7 ± 402.8
Number of TRPM-3-positive cells (TR3)*	0.6 ± 1.1	3.7 ± 4.9	2.3 ± 2.0
TR3:GC	0.008	0.007	0.002

*Mean ± SD.

Fig. 4. Autoradiography with [³H]thymidine *in vivo* in rats: (a) preantral follicle, × 360; and (b) antral follicle, × 180.

Preantral and antral follicles predominated 24 h after the PMSG injection and the granulosa cells in these follicles showed a high proliferative capacity *in vivo* (Fig. 4). To examine the effect of macrophages on granulosa cell proliferation *in vitro*, we obtained granulosa cells 24 h after the PMSG treatment from the ovaries and cultured them with unstimulated peritoneal macrophages. The [³H]thymidine labelling index of cultured granulosa cells was significantly greater in the culture with macrophages than in the culture of granulosa cells alone. The labelling index was greatest when the ratio of macrophages to granulosa cells was 0.01; this value is similar to the ratio of TRPM-3-positive cells to granulosa cells in the growing follicles *in vivo*. These results imply that macrophages have a mitogenic effect on the granulosa cells.

The mechanism whereby macrophages promote granulosa cell proliferation remains unclear. Macrophages are known to secrete various cytokines, including interleukin 1, which stimulates

Fig. 5. [^3H]thymidine autoradiography of 10^5 rat granulosa cells cultured with 10^3 macrophages. Macrophages (arrowheads) can be easily distinguished, because they are smaller and rounder than granulosa cells. Granulosa cells labelled with [^3H]thymidine have numerous grains on the nucleus; $\times 360$.

Fig. 6. [^3H]thymidine labelling index of rat granulosa cells (GC). Values are mean $\pm$ SD. * $P < 0.01$ compared with control value in granulosa cells cultured alone.

granulosa cell growth (Fukuoka *et al.*, 1989) and is present in human ovarian follicular fluid (Khan *et al.*, 1988). Macrophages are also known to produce growth factors, such as platelet-derived growth factor (Shimokado *et al.*, 1985), fibroblast growth factor (Baird *et al.*, 1985), insulin-like growth factor I (Rom *et al.*, 1988), transforming growth factors β (Assoian *et al.*, 1987) and α

(Rappolee *et al.*, 1988) and heparin-binding epidermal growth factor (Higashiyama *et al.*, 1991), all of which control cell function through the paracrine or autocrine mechanisms. Of these, fibroblast growth factor and epidermal growth factor have been reported to induce granulosa cell growth (Tapanainen *et al.*, 1987). Proliferative responses in cells are generally induced by two types of growth factor: a competence factor and a progression factor (Wharton *et al.*, 1982). From the results of our study and those of others, it appears that macrophages may promote proliferation of granulosa cells with two or more of the factors listed above.

References

- Assouan, R.K., Fleurdelys, B.E., Stevenson, H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R. & Sporn, M.B. (1987) Expression and secretion of type β transforming growth factor by activated human macrophages. *Proceedings of the National Academy of Sciences USA* **84**, 6020–6024.
- Baird, A., Mormede, P. & Bohlev, P. (1985) Immuno-reactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. *Biochemical and Biophysical Research Communications* **126**, 358–364.
- Bulmer, D. (1964) The histochemistry of ovarian macrophages in the rat. *Journal of Anatomy* **98**, 313–319.
- Fukuoka, M., Yasuda, K., Taii, S., Takakura, K. & Mori, T. (1989) Interleukin-1 stimulates growth and inhibits progesterone secretion in cultures of porcine granulosa cells. *Endocrinology* **124**, 884–890.
- Halme, J., Hammond, M.G., Syrop, C.H. & Talbert, L.M. (1985) Peritoneal macrophages modulate human granulosa-luteal cell progesterone production. *Journal of Clinical Endocrinology and Metabolism* **61**, 912–916.
- Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. & Klagsbrun, M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936–939.
- Hofmann, G.E., Scott, R.T., Brzyski, R.G. & Jones, H.W. (1990) Immunoreactive epidermal growth factor concentrations in follicular fluid obtained from *in vitro* fertilization. *Fertility and Sterility* **54**, 303–307.
- Isobe, S., Nakane, P.K. & Brown, W.R. (1977) Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements to study the peroxidase-labelled antibody method for application to study of human intestinal mucosa. *Acta Histochemica et Cytochemica* **10**, 167–171.
- Kato, T., Takeya, M., Takagi, K. & Takahashi, K. (1990) Chemically induced transplantable malignant fibrous histiocytoma of the rat: analysis with immunohistochemistry, immunoelectron microscopy and [3 H]thymidine autoradiography. *Laboratory Investigation* **62**, 635–645.
- Khan, S.A., Schmidt, K., Hallin, P., Di Pauli, R., De Geyter, C.H. & Nieschlag, E. (1988) Human testis cytosol and ovarian follicular fluid contain high amounts of interleukin-1-like factor(s). *Molecular and Cellular Endocrinology* **58**, 221–230.
- Kirsch, T.M., Friedman, A.C., Vogel, R.L. & Flickinger, G.L. (1981) Macrophages in corpora lutea of mice: characterization and effects on steroid secretion. *Biology of Reproduction* **25**, 629–638.
- McLean, I.W. & Nakane, P.K. (1974) Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *Journal of Histochemistry and Cytochemistry* **22**, 1077–1083.
- Rappolee, D.A., Mark, D., Banda, M.J. & Werb, Z. (1988) Wound macrophages express TGF- α and other growth factors *in vivo*: analysis by mRNA phenotyping. *Science* **241**, 708–711.
- Rom, W.N., Basset, P., Fells, G.A., Nukiwa, T., Trapnell, B.C. & Crystal, R.G. (1988) Alveolar macrophages release an insulin-like growth factor I-type molecule. *Journal of Clinical Investigation* **82**, 1685–1693.
- Shimokado, K., Raines, E.W., Madtes, D.K., Barrett, T.B., Benditt, E.P. & Ross, R. (1985) A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell* **43**, 277–286.
- Takeya, M., Hsiao, L. & Takahashi, K. (1987) A new monoclonal antibody, TRPM-3, binds specifically to certain rat macrophage populations: immunohistochemical and immunoelectron microscopic analysis. *Journal of Leukocyte Biology* **41**, 187–195.
- Takeya, M., Hsiao, L., Shimokawa, Y. & Takahashi, K. (1989) Heterogeneity of rat macrophages recognized by monoclonal antibodies: an immunohistochemical and immunoelectron microscopic study. *Journal of Histochemistry and Cytochemistry* **37**, 635–641.
- Tapanainen, J., Yamamoto, M., Leinonen, P.J., Jaffe, R.B. & Tapanainen, P. (1987) Regulation of human granulosa-luteal cell progesterone production and proliferation by gonadotrophins and growth factors. *Fertility and Sterility* **48**, 576–580.
- Wharton, W., Gillespie, G.Y., Russell, S.W. & Pledger, W.J. (1982) Mitogenic activity elaborated by macrophage-like cell lines acts as competence factor(s) for BALB/c 3T3 cells. *Journal of Cellular Physiology* **110**, 93–100.
- Yamada, M., Naito, M. & Takahashi, K. (1990) Kupffer cell proliferation and glucan-induced granuloma formation in mice depleted of blood monocytes by strontium-89. *Journal of Leukocyte Biology* **47**, 195–205.

Received 7 August 1991