Introduction

Cytokines are intercellular signalling proteins secreted by lymphohaemopoietic and other cell lineages, and their pivotal roles in the processes of inflammation, immunity and tissue remodelling are well recognized. Cytokines have been implicated in numerous interactions with gonadotrophins and growth factors to influence a variety of reproductive processes, including ovarian function (Adashi, 1990) and embryonic growth (de Moraes and Hansen, 1997; Martal et al., 1997; Robertson et al., 1999). Resident and infiltrating leukocytes are the major source of cytokines in the ovary, although they are also produced by ovarian somatic cells, including luteal, stromal, thecal and granulosa cells.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is synthesized within the ovary. The rate of synthesis displays temporal fluctuations across the oestrous cycle with peak values reached around the time of ovulation (Brännström et al., 1994a). A specific role for GM-CSF in development of corpora lutea has been identified (Jasper et al., 2000). GM-CSF has been located in follicular compartments including the thecal layer, which is the most prominent follicular site of GM-CSF mRNA and protein synthesis, and in follicular fluid (Zhao et al., 1995; Jasper et al., 1996, 1997; Tamura et al., 1998). Whether granulosa cells and the oocyte in developing follicles express GM-CSF remains contentious (Zhao et al., 1995; Jasper et al., 1996; Tamura et al., 1998). Gonadotrophins and ovarian steroid hormones may regulate GM-CSF production. Oestradiol promotes GM-CSF mRNA expression and protein secretion in uterine epithelial cells, whereas progesterone has an inhibitory effect (Robertson et al., 1996). Both plasma and follicular fluid concentrations of GM-CSF are increased significantly in individuals undergoing ovarian hyperstimulation compared with naturally cyclic individuals (Jasper et al., 1996).

Effect of granulocyte–macrophage colony-stimulating factor deficiency on ovarian follicular cell function


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Granulocyte–macrophage colony-stimulating factor (GM-CSF), a cytokine secreted by lymphohaemopoietic and other cell lineages, is known to influence ovarian cyclicity and embryo development. The aim of this study was to examine the effect of GM-CSF on ovarian follicular cell function using GM-CSF-deficient (GM –/–) mice. Immature GM –/– and GM +/+ mice were stimulated with eCG, and cumulus–oocyte complexes and mural granulosa cells were collected 48 h later. Expression of GM-CSF receptor (GM-CSFR) α and β mRNA subunits by cumulus–oocyte complexes and mural granulosa cells was examined using RT–PCR. Cumulus–oocyte complexes from both genotypes were found to express mRNA for the GM-CSFRα-subunit only, while the mural granulosa cells expressed both the α and β receptor subunits. Cumulus–oocyte complexes recovered from GM –/– mice had approximately twice the number of cumulus cells per cumulus–oocyte complex than did those of GM +/+ mice (P < 0.05), even though the growth-promoting activity of denuded GM –/– oocytes was found to be equivalent to that of wild-type oocytes. GM-CSF deficiency was associated with marginally increased DNA synthesis in cumulus cells and significantly (P < 0.05) lower progesterone production by mural granulosa cells recovered from GM –/– compared with those recovered from GM +/+ mice. The addition of rec-mGM-CSF in vitro did not affect DNA synthesis in either cell type or progesterone production by mural granulosa cells, irrespective of GM-CSF status. There was no effect of GM-CSF deficiency on the capacity of FSH and insulin-like growth factor I to stimulate DNA synthesis in cumulus–oocyte complexes (approximately 15- and threefold, respectively) and in mural granulosa cells (approximately two- and threefold, respectively). Taken together, these data show that GM-CSF influences events associated with follicular maturation in mice. The effects of GM-CSF are not exerted directly in granulosa or cumulus cells, but appear to be mediated indirectly, perhaps through the agency of steroidogenesis-regulating secretions of local macrophage populations residing in the theca.
GM-CSF acts via a cell surface receptor–ligand complex comprising two cytokine-specific α-subunits, two signal transducing β-subunits and two ligand molecules (Bagley et al., 1997), the β-subunit being common to receptors for interleukins 3 and 5. In the absence of β-subunit, GM-CSF binds GM-CSFRα with low affinity and, in some cells, this may lead to improved glucose transport (Ding et al., 1994). However, high affinity ligand binding is conferred by the β-subunit, which results in rapid intracellular tyrosine phosphorylation required to elicit the full range of GM-CSF-dependent effects on cell proliferation and differentiation (Duronio et al., 1992). GM-CSF receptor α-subunit (GM-CSFRα) expression occurs in both haemopoietic and non-haemopoietic cell lines, whereas it is generally thought that the β-subunit (GM-CSFRβ) is limited to haemopoietic and endothelial lineages. The GM-CSFRα-subunit is located in cells throughout the ovary (Jasper et al., 1997), its expression increasing in theca externa and granulosa cells with increasing follicle size (Zhao et al., 1995). As would be expected, there is prominent expression of the GM-CSFRβ-subunit within the vascular regions of the ovary, including the corpus luteum and thecal layer of the follicle (Zhao et al., 1995; Jasper et al., 1997). However, the GM-CSFRβ-subunit has also been located within the avascular mural granulosa cells, where expression was found to increase with follicular development (Zhao et al., 1995).

Evidence indicating a functional role for GM-CSF in reproduction has been provided by studies with GM-CSF knockout (GM −/−) mice, which exhibit impaired reproductive capacity, having 25% smaller litters at weaning than wild-type mice owing to fetal death late in gestation and early in postnatal life (Robertson et al., 1999). Furthermore, the developmental competence of GM −/− preimplantation embryos is slightly impaired with both a delay in blastocyst formation and a reduction in the number of blastomeres on day 4 of pregnancy (Robertson et al., in press). Processes occurring during early embryogenesis may account for this diminished developmental competence and deficiencies in folliculogenesis leading to oocyte defects may also contribute.

Although GM −/− mice exhibit an ovulation rate comparable with that of wild-type mice, their oestrous cycle is longer and the number of activated macrophages within the ovarian stroma and theca is reduced at the time of ovulation (Jasper et al., 2000). These findings, together with observations that GM-CSF and its receptors are expressed selectively within various follicular cell lineages and that both subunits of the GM-CSF receptor are upregulated in granulosa cells during follicular development, indicate that GM-CSF may play a role in folliculogenesis. Therefore, this study was undertaken to examine the effects of GM-CSF on mouse follicular cell proliferation and secretory function in vitro.

**Materials and Methods**

**Animals and collection of cells**

This study used mice homozygous for a disrupted GM-CSF gene (GM −/−), produced through deletion of the first exon of the GM-CSF gene using gene targeting techniques in embryonic stem cells (Stanley et al., 1994). Control mice (GM +/+ ) were generated by backcrossing GM −/− females with wild-type SV129 males. Genotypes were confirmed using PCR, as described by Robertson et al. (1999). Mice were maintained at The Queen Elizabeth Hospital at 23°C on a 14 h light:10 h dark cycle with pelleted food and water available ad libitum. This study was approved by local Animal Ethics Committees and was conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Immature female mice (3–5 weeks old; GM +/+ and GM −/−) were primed with 5 iu equine chorionic gonadotrophin (Intervet, Castle Hill, NSW) and killed approximately 48 h later by cervical dislocation. Reproductive tracts were removed aseptically and placed in 25 mmol Hepes-buffered tissue culture medium-199 1× (H-TCM; ICN, CA) supplemented with 2 mmol sodium pyruvate 1× (Sigma, St Louis, MO) and a macromolecule (see details below). Ovaries were trimmed of fat and mesentery and rinsed before collection of cells.

**Reverse transcription–polymerase chain reaction**

Mural granulosa cells (MGCs) and completely enclosed cumulus–oocyte complexes (COCs) from GM −/− and GM +/+ mice were examined for expression of α and β GM-CSF receptor subunit mRNA and for mouse leukocyte common antigen (mLCA; CD45) mRNA by RT–PCR. COCs and MGCs were collected from large antral follicles excised manually with considerable care to minimize the likelihood of contamination with blood-borne leukocytes. These follicles were devoid of stroma and interstitial tissue but had an intact theca layer. For comparative purposes, RNA was also extracted from MGCs collected from ovarian surface follicles punctured in such a way as to allow leukocyte contamination. After collection in H-TCM supplemented with 5% (v/v) fetal calf serum (Trace Biosciences, Castle Hill, NSW), COCs and MGCs were washed twice, transferred to Eppendorf tubes (approximately 50 COCs per tube) on ice, lysed in Tri Reagent (Sigma), and snap-frozen in liquid N2 before storage at −80°C. Total cellular RNA was extracted using a modified Tri Reagent protocol, where 20 μg glycogen (Boehringer Mannheim, Castle Hill, NSW) was added to each sample before precipitation for 48 h at −80°C. All samples were treated with DNase using reagents from Promega (Madison) to eliminate potential contamination by genomic DNA. Each reaction consisted of 5 μl DNase I, 100 μl RNase inhibitor, 10 μl of 10× DNase buffer in a final volume of 97 μl.

RNA was reverse transcribed using random hexamers (Boehringer Mannheim) and a Superscript II Reverse Transcription kit (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. A negative RT control substituting water for reverse transcriptase was included in each reaction. PCR amplification used reagents supplied in a HotStarTaq DNA polymerase kit (Qiagen, Hilden). Each reaction consisted of 5 μl Qiagen 10× buffer, pH 8.7, MgCl2 (1.5 mmol β-actin 1×; 2 mmol mLCA 1×; 2 mmol GM-CSFRβ 1×; 2.5 mmol GM-CSFRα 1×), 10 μl Q solution (mLCA only), 0.4 mmol 1× of each of dATP, dCTP, dGTP and dTTP, HotStarTaq DNA polymerase (1 μl β-actin,
1 IU mL LCA, 1 IU GM-CSFRβ, 0.5 IU GM-CSFRα, 0.2 μmol 3’ and 5’ primers, 1 μl cDNA, made up to a final volume of 50 μl with ultra pure water (Biotech, Bentley, NSW) and overlayed with 50 μl mineral oil (Sigma). The negative control included in each reaction consisted of substituting water for cDNA. Primer pairs designed to bracket an intron were identical to those used previously for GM-CSFRα and GM-CSFRβ (AIC2B) (Robertson et al., 2000) and β-actin (Robertson et al., 1996). Mouse LCA (CD45) 5’ primer (5’-GGCTCTTCAGAGACCCA-3’) corresponds to the cDNA sequence from nucleotides 330 to 347, and mLCA 3’ primer (5’-GTGCTGACATTGGAGGTG-3’) is anti-sense to the cDNA sequence from nucleotides 610 to 627 (Genebank accession number M22455) yielding an amplified product of 298 bp. Initial activation of the enzyme at 95°C for 15 min was followed by PCR amplification of the GM-CSFR sequences with 42 cycles at 94°C for 1 min (denaturation), 60°C for 45 s (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 7 min. The mLCA sequence was amplified in a similar manner, with an annealing step of 62°C for 1 min with 41 cycles. Beta-actin was amplified with annealing at 58°C for 1 min and 30 cycles. PCR products were analysed by electrophoresis using a 2% (w/v) agarose (ICN) gel containing 15 μg ethidium bromide (Boehringer Mannheim) in Tris borate EDTA (TBE) and visualized with a Kodak 120 digital camera over an ultraviolet light box. PCR product size was determined by comparison with Hpa II digested pUC19 (Bresatec, Adelaide, SA). The 325 bp GM-CSFRβ bands were excised from gels, purified and sequenced to verify their identity.

**Cell culture conditions and experimental design**

Large antral follicles in intact ovaries were ruptured using a 27 gauge needle and MGCs and COCs were squeezed gently from follicles and pooled according to genotype. MGCs and COCs were collected in H-TCM supplemented with sodium pyruvate and 0.2 mg polyvinyl alcohol ml–1 (PVA, Sigma) and cultured in bicarbonate-buffered TCM (B-TCM) with PVA, sodium pyruvate, 100 μIU Penicillin G ml–1 (Sigma) and 100 μg streptomycin sulfate ml–1 (Sigma). Pooled cells from three mice were washed twice in each of H-TCM and B-TCM, before groups of 10–15 COCs or 10,000 undissociated MGCs were transferred to individual wells of 96-well flat-bottomed microplates for approximately 2 min in H-TCM plus PVA, and then denuded oocytes were rinsed twice in B-TCM and added to cultures of either –/– or +/+ MGCs at a concentration of 31 oocytes per 125 μl well (0.25 oocytes μl–1). This experiment was replicated on five occasions.

**Experiment 2: effect of GM-CSF on cumulus cell DNA synthesis in vitro**

A 2 × 2 factorial experiment was designed to determine the effect of GM-CSF on cumulus cell DNA synthesis in vitro. COCs collected from both genotypes were assigned to wells containing either (i) 0 (control), or (ii) 10 ng recombinant mouse GM-CSF ml–1 (R & D Systems, Minneapolis, MN). Eleven replicates of this experiment were carried out using 29 GM –/– and 31 GM +/+ mice.

**Experiment 3: dose response of GM-CSF on cumulus cell DNA synthesis in vitro**

A 2 × 4 factorial experiment was conducted to determine whether the concentration of GM-CSF in vitro affected cumulus cell DNA synthesis. COCs from both genotypes were cultured in one of the following treatments: (i) 0 (control), (ii) 0.1, (iii) 1, or (iv) 10 ng GM-CSF ml–1. Six replicates of this experiment were carried out using 14 GM –/– and 16 GM +/+ mice.

**Experiment 4: genotype × hormone interactions in cumulus–oocyte complexes**

A 2 × 3 factorial experiment was conducted to investigate the interaction between GM-CSF status and insulin-like growth factor I (IGF-I) and FSH on cumulus cell DNA synthesis. COCs were cultured in one of the following treatments: (i) control, (ii) recombinant human IGF-I (Gro-pep, Adelaide, SA; 10 ng ml–1), or (iii) purified pig FSH (pFSH; UCB Bioproducts, Braine-l’Alleud; 10 ng ml–1), or (iv) IGF-I and pFSH. This experiment was replicated on four occasions using 12 GM –/– and 12 GM +/+ mice.

**Experiment 5: genotype × hormone × cytokine interactions in mural granulosa cells**

A 2 × 2 factorial experiment was conducted to determine the effect of IGF-I, FSH and GM-CSF on granulosa cell DNA and progesterone synthesis in GM –/– and GM +/+ mice. Treatments consisted of: (i) control, (ii) IGF-I (50 ng ml–1), (iii) pFSH (10 ng ml–1), (iv) GM-CSF (10 ng ml–1), (v) IGF-I and pFSH, (vi) IGF-I and GM-CSF, (vii) pFSH and GM-CSF, and (viii) IGF-I and pFSH and GM-CSF. Each treatment was conducted in duplicate and the experiment was replicated eight times.
Determination of number of cells

MGCs were dissociated as described by Luciano et al. (2000). Briefly, cells were incubated for 5 min in H-TCM supplemented with 9.1 mmol EGTA l⁻¹ (Sigma), pelleted, resuspended, incubated for a further 10 min in H-TCM with 2.1 mmol EGTA l⁻¹ and 0.5 mmol sucrose l⁻¹ (BDH Chemicals, Kilsyth, Vic), pelleted, resuspended in a known volume of H-TCM 199 plus 0.2% (w/v) BSA (Sigma) and finally counted using a haemocytometer. Complexes were collected and cultured for 24 h in B-TCM with 5% (v/v) fetal calf serum (Trace Scientific, Clayton, Australia) and 10 ng ml⁻¹ pig FSH (Vetrapharm, London, ON) before dissociation and counting.

Assessment of cell cultures

Culture supernatants were assayed for progesterone content using a radioimmunoassay kit (Ortho-Clinical Diagnostics, Doncaster, Vic) in accordance with the manufacturer’s instructions. The kit, which uses a ¹²⁵I-labelled progesterone tracer, has a sensitivity of 0.25 pmol ml⁻¹ and an intra-assay coefficient of variability of 5.4%. After culture, COCs and MGCs were harvested using a Tomtec Harvester onto a filtermat and incorporated [³H]thymidine was quantified using a Wallac microbeta scintillation counter. [³H] counts were used as a measure of incorporation of [³H]thymidine into follicular cell DNA and hence as an indicator of DNA synthesis.

Data analysis

Data were analysed using multifactorial ANOVA analysis (SAS Institute Inc., Cary, NC) with differences between treatment groups in the amount of [³H]thymidine incorporated and progesterone produced determined using a Waller–Duncan K ratio t test. A Student’s t test was performed to determine differences in COC cell counts between genotypes.

Results

RT–PCR of GM-CSFRα and β mRNA

COCs from both genotypes were found to express mRNA for the GM-CSFRα-subunit, but not the GM-CSFRβ-subunit. However, the MGC populations from both genotypes expressed mRNA for both GM-CSF receptor subunits (Fig. 1). The identity of the GM-CSFRβ PCR product was confirmed by sequencing (> 97% homology with sequence AIC2B; GenBank accession number M34397). Both cell types were examined for possible leukocyte contamination by screening for mLCA mRNA expression. COCs and MGCs collected from dissected follicles from GM –/– and GM +/+ mice did not express mRNA for the mLCA. In preliminary experiments, the mLCA RT–PCR protocol was sufficiently sensitive to detect one leukocyte in 10 000 fibroblasts (data not shown), indicating that leukocyte contamination was negligible. Conversely, MGCs (but not COCs) collected from follicles punctured from whole ovaries were found to express mRNA for mLCA, consistent with there being a considerable number of leukocytes within this cell preparation.

Determination of number of cells in cumulus–oocyte complex

COCs of GM –/– mice had almost twice the number of cumulus cells than did the COCs of GM +/+ mice (means of 969 and 510, respectively, P < 0.05; Fig. 2).

Experiment 1: effect of genotype on denuded oocyte-stimulated mural granulosa cell DNA synthesis in vitro

Because GM –/– COCs have a larger cumulus cell mass than do +/+ COCs, it was postulated that GM –/– oocytes may have a greater growth-promoting activity than wild-type oocytes. Denuded oocytes from both genotypes significantly (P < 0.001) stimulated DNA synthesis in MGCs approximately 11-fold above control amounts. GM –/– oocytes stimulated [³H]thymidine uptake by MGCs to the same extent as wild-type oocytes, regardless of whether the MGCs originated from GM –/– or GM +/+ mice (Fig. 3).

Experiments 2 and 3: effect of GM-CSF on cumulus cell DNA synthesis in vitro

There was a notable effect of GM-CSF deficiency on COC DNA synthesis, with COCs from GM –/– mice exhibiting approximately twice the amount of [³H]thymidine incorporation as COCs from GM +/+ mice (P = 0.08; Fig. 4). The variable nature of COCs and the high degree of variance among experiment replicates may account for the low statistical significance. Within each experimental replicate, differences observed between each treatment were similar, although raw values between experiments varied significantly (P < 0.05). Counts within each experiment were expressed as a percentage of the GM +/+ control group to reduce the degree of inter-experimental variation. Addition of rec mGM-CSF at a concentration of 10 ng ml⁻¹ did not affect the amount of [³H]thymidine incorporated into the COCs of either genotype, whether calculated from the raw or the adjusted data (Fig. 4). Likewise, addition of GM-CSF in vitro at various concentrations (0.1, 1 or 10 ng ml⁻¹) did not alter [³H]thymidine incorporation into COCs, irrespective of GM-CSF status (Table 1).

Experiment 4: genotype × hormone interactions in cumulus–oocyte complexes

A comparison was conducted between the COCs of the two genotypes in their response to IGF-I and FSH, factors
known to be important in follicular cell mitogenesis. No genotype interaction with either hormone was observed as assessed by \[^3\text{H}\] incorporation. IGF-I increased the relative amount of cumulus cell DNA synthesis in both genotypes approximately threefold above control amounts (Fig. 5), while the addition of FSH significantly \((P < 0.05)\) increased \[^3\text{H}\] counts approximately 15-fold in both genotypes. An interaction between IGF-I and FSH was evident, with the two factors combined resulting in significantly \((P < 0.05)\) lower \[^3\text{H}\]thymidine incorporation than in COCs cultured in the presence of FSH alone.

**Experiment 5: genotype \(\times\) hormone \(\times\) cytokine interactions in mural granulosa cells**

There was no significant effect of GM-CSF genotype on the amount of \[^3\text{H}\]thymidine incorporated into granulosa cells,
The present study was undertaken to determine whether GM-CSF can influence follicular functions by evaluating the effects of GM-CSF on isolated follicle cells in vitro and by comparing the responses of cells isolated from GM-CSF-deficient (GM –/–) and wild-type (GM +/+ ) mice to various factors in vitro. Although there were clear differences in the numbers of cumulus cells per COC between GM –/– and GM +/+ mice, no mitogenic effect of GM-CSF was evident when factor was added in vitro to either cumulus cells or MGCs of either genotype, despite the presence of GM-CSF receptors. This finding indicates that GM-CSF plays some role in the differentiation and maturation of follicular cells in vivo but that any effect is mediated indirectly, perhaps through the agency of an intermediate cell population.

Previous research has demonstrated the expression of GM-CSF and its receptors in ovarian cells (Zhao et al., 1995; Jasper et al., 1997; Tamura et al., 1998). The present study demonstrated that COCs only express the α-subunit of the GM-CSFR. Thus, COCs are unlikely to respond to GM-CSF, although there are some reports that the free α-subunit can facilitate glucose uptake and thereby promote viability and proliferation in certain cell lineages (Ding et al., 1994). In contrast, MGCs were found to express both the α- and the β-subunits. This finding was somewhat unexpected because expression of the β-subunit is normally restricted to haemopoietic cells. Contamination with leukocytes was proven to be unlikely in view of the absence of mLCA mRNA expression in MGC preparations. This finding is in agreement with a previous report that human MGCs express the GM-CSFRβ-subunit (Zhao et al., 1995). Differential expression of growth factor receptors is not unexpected, since MGCs differ substantially from cumulus cells in many other respects. In direct contrast to cumulus cells, MGCs are slow growing, highly steroidogenic cells (Armstrong et al., 1996; Li et al., 2000), expressing, for example, high concentrations of LH receptor mRNA transcripts, which are rarely found in cumulus cells (Camp et al., 1991).

GM-CSF deficiency was found to be associated with an increase in the number of cumulus cells per COC, and a tendency for a greater degree of cumulus cell DNA synthesis when COCs were cultured in vitro. When differences in cumulus cell [3H]thymidine incorporation were normalized regardless of the type or combination of hormone or cytokine added (Fig. 6). Both FSH and IGF-I alone significantly (P < 0.05) increased the amount of granulosa cell DNA synthesis approximately two- and threefold, respectively. Treatment of granulosa cells with a combination of both IGF-I and FSH resulted in a fivefold increase in the amount of DNA synthesis in both genotypes (P < 0.05). The IGF plus FSH effect was additive and was significantly (P < 0.05) greater than the effect of either factor alone (Fig. 6). The addition of GM-CSF in vitro did not influence any of the observed effects or interactions among the other hormones, as it neither increased nor decreased the amount of cumulus cell or MGC DNA synthesis in any of the treatment groups in which it was present.

Both IGF-I and FSH tended to increase the amount of progesterone secreted by granulosa cells of both GM –/– and GM +/+ mice compared with controls, although this trend was not significant (P = 0.08; Fig. 7). Culture of granulosa cells from both genotypes in a combination of both IGF-I and FSH resulted in progesterone secretion reaching a maximum, with values from these groups significantly higher than in all other treatments. The addition of GM-CSF in vitro did not affect granulosa cell progesterone secretion, whether alone or in various combinations of IGF-I and FSH. However, when the effect of genotype was examined over all treatment groups (ANOVA analysis), granulosa cells from GM –/– mice secreted significantly less (69%; P < 0.05) progesterone than did granulosa cells from GM +/+ mice.

**Discussion**

The present study was undertaken to determine whether GM-CSF can influence follicular functions by evaluating the effects of GM-CSF on isolated follicle cells in vitro and by comparing the responses of cells isolated from GM-CSF-deficient (GM –/–) and wild-type (GM +/+ ) mice either with or without recombinant murine granulocyte-macrophage colony-stimulating factor (rec-mGM-CSF) in vitro. Bars represent the mean c.p.m. expressed as a percentage of GM +/+ control ± SEM of values obtained from 11 replicates (n = 220 COCs per treatment).

**Fig. 3.** Effect of granulocyte–macrophage colony-stimulating factor (GM-CSF) genotype on denuded oocyte (DO)-stimulated mural granulosa cell (MGC) tritiated thymidine incorporation. GM –/– or GM +/+ DO (0.25 per ml) were co-cultured with MGC from either GM –/– (■) or GM +/+ (□) mice. Bars represent the mean c.p.m. per 1000 cells ± SEM of values obtained from five replicates. Bars with different superscripts are significantly different (P < 0.05).

**Fig. 4.** Tritiated thymidine incorporation in cumulus-oocyte complexes (COCs) from GM –/– (■) or GM +/+ (□) mice either with or without recombinant murine granulocyte-macrophage colony-stimulating factor (rec-mGM-CSF) in vitro. Bars represent the mean c.p.m. expressed as a percentage of GM +/+ control ± SEM of values obtained from 11 replicates (n = 220 COCs per treatment).
Effect of GM-CSF deficiency on follicular cells

Table 1. Dose response of granulocyte–macrophage colony-stimulating factor (GM-CSF) in vitro on tritiated thymidine incorporation in cumulus–oocyte complexes (COCs) from GM –/– and +/+ mice

<table>
<thead>
<tr>
<th>Dose of GM-CSF in vitro (ng ml⁻¹)</th>
<th>COC genotype</th>
<th>Relative [³H]thymidine incorporation in COC (mean c.p.m. ± SEM)ᵃ</th>
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<tbody>
<tr>
<td>0</td>
<td>+/+</td>
<td>100</td>
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<tr>
<td>0.1</td>
<td>–/–</td>
<td>188 ± 63</td>
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<tr>
<td>1.0</td>
<td>+/+</td>
<td>142 ± 80</td>
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<tr>
<td>10.0</td>
<td>–/–</td>
<td>166 ± 57</td>
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<tr>
<td>100</td>
<td>+/+</td>
<td>98 ± 15</td>
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<tr>
<td>1000</td>
<td>–/–</td>
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<td>93 ± 29</td>
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<tr>
<td>4000</td>
<td>–/–</td>
<td>253 ± 95</td>
</tr>
</tbody>
</table>

ᵃValues represent the mean ± SEM c.p.m. expressed as a percentage of data from GM +/+ mice. Data are obtained from six replicates (n = 80 COCs per treatment). There was no significant effect of treatment or genotype on [³H]thymidine incorporation.

to the number of cells, values were found to be comparable between genotypes (114 and 139 c.p.m. per 10⁴ cells for GM –/– and GM +/+ mice, respectively). It might be speculated that genotype differences in the numbers of cumulus cells per complex are a result of differences in the growth-promoting activity of oocytes, since oocytes are clearly able to promote proliferation and attenuate the differentiation of cumulus and MGCs (Vanderhyden et al., 1992; Li et al., 2000). However, the present results demonstrate that GM +/+ oocytes can promote MGC proliferation to the same extent as GM –/– oocytes, and MGCs from both genotypes respond equally well to factors secreted by the oocyte.

DNA synthesis by cumulus and granulosa cells can be contrasted by expressing DNA synthesis per 10⁴ cells. The amount of DNA synthesis in cumulus cells was considerably greater than it was in granulosa cells of either genotype, with c.p.m. per 10⁴ cells approximately sixfold greater in cumulus cells than in granulosa cells. This finding indicates that mouse cumulus cells exhibit a greater proliferative capacity than do granulosa cells, as has been reported in bovine follicular cells (Armstrong et al., 1996; Li et al., 2000).

Both IGF-I and FSH independently increased DNA synthesis in COCs from both genotypes in vitro, with the stimulatory effect of FSH being considerably greater than that of IGF-I. Culture of COCs in the presence of IGF-I and FSH combined resulted in a reduction in the FSH response, indicating that IGF-I has an antagonistic effect on the ability of FSH to stimulate cumulus cell proliferation in the mouse. A similar antagonistic effect of combined treatments with IGF-I and FSH has been observed for bovine COCs, except that in cattle, IGF-I has a greater mitogenic ability than FSH (Armstrong et al., 1996). This finding indicates subtle differences in the regulation of proliferation between mouse and bovine cumulus cells, although the mechanisms by which this IGF-I × FSH interaction affects the ability of FSH to induce cumulus cell proliferation remains unknown. This interaction may be related to FSH × IGF-I interactions in the production of IGF-binding proteins within the extracellular matrix (Ingman et al., 2000). In MGCs, IGF-I and FSH each stimulated DNA synthesis and therefore cell proliferation to a similar extent and, when administered together, the effects of the two factors were additive, similar to the responses reported previously in bovine MGCs (Armstrong et al., 1996).

Overall, the granulosa cells of GM –/– mice showed lower progesterone secretion than the granulosa cells of GM +/+ mice. Progesterone secretion by granulosa cells is an indicator of their differentiation status, with higher values observed from terminally differentiated granulosa cells or luteal cells. Ovaries of GM –/– mice perfused in vitro also produced less progesterone per oocyte ovulated compared with GM +/+ mice (Jasper et al., 2000). Taken together, these data indicate that the granulosa cells of GM –/– mice are less differentiated toward luteinization than the granulosa cells of GM +/+ mice. Both IGF-I and FSH also tended to increase progesterone secretion when added alone to MGCs and, when added in combination, progesterone synthesis was considerably enhanced in cells of both genotypes. This response appeared to be additive in both genotypes, similar to results observed for granulosa cell proliferation in this and previous studies (Armstrong et al., 1996).

Despite the presence of GM-CSFRα and β transcripts in follicular somatic cells, supplementation of culture media...
with GM-CSF did not affect the amount of DNA synthesis in COCs or MGCs of either genotype, nor did it affect the characteristic proliferative responses of these cells to FSH or IGF-I. Similarly, addition of GM-CSF in vitro had no effect on either basal or hormone-elicited progesterone secretion. GM-CSF is known to stimulate the proliferation of other cell types such as monocytes, macrophages, granulocytes and non-haemopoietic cells, for example, some carcinoma cell lines (for review, see Gasson, 1991). The GM-CSF receptor transcripts detected in follicular cells using RT–PCR are probably expressed at physiologically meaningful concentrations, since studies in haemopoietic cells demonstrate that only extremely low receptor expression is required to elicit a biological response mediated through the receptor dimer (Walker and Burgess, 1985). Therefore, these results indicate that GM-CSF does not directly act on follicular somatic cells to influence their function, but instead may act indirectly via some alternative mechanism in vivo. Such a mechanism may involve some other GM-CSF responsive cell lineage present in the follicle, for example, macrophages, which are known to secrete a number of mitogenic cytokines (Brännström and Norman, 1993).

Macrophages are a potent source of GM-CSF and other cytokines, and are found within the stromal and thecal regions of the ovary before and after ovulation (Brännström et al., 1993; Jasper et al., 2000). The number of macrophages increases approximately fivefold within the thecal region before ovulation (Brännström et al., 1994b), providing a potential source of secreted factors. Co-incubation of peritoneal macrophages with mouse granulosa cells results in a dose-dependent increase in progesterone secretion (Kirsch et al., 1981), most likely mediated through the action of cytokines, including tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) (Roby and Terranova, 1988, 1990). Steroidogenic events occurring within thecal cells can affect the functions of both granulosa cell subtypes. Granulosa cells are reliant on androgens produced via the conversion of cholesterol to androstenedione within thecal cells; this androstenedione is the substrate that granulosa cells convert to the oestrogens crucial for follicular development. These data might be explained by macrophage-derived factors influencing steroidogenesis within the ovarian thecal cells and, therefore, the differentiation of these and other follicular cells. GM−/− mice exhibit a reduced number of activated macrophages within the ovarian stroma and thecal cell layer (Jasper et al., 2000), which may correspond to a reduction in the secretion of various factors that act directly or indirectly on the follicular environment. This may explain the difference in the number of cumulus cells per oocyte between GM−/− and GM+/+ mice, as well as the difference in progesterone secretion by granulosa cells between the two genotypes.

If factors secreted by thecal macrophages are involved in the differentiation of follicular cells in vivo, this may help to explain why the mass of ovaries from GM−/− mice on day 4 of pregnancy is significantly lower than the mass of GM+/+ mouse ovaries, even though numbers of corpora lutea are not different (Jasper et al., 2000). The results of the present study suggest that GM-CSF does not directly affect follicular cells in vitro, although it may affect thecal macrophages in vivo.
study support this interpretation, since the greater proportion of cumulus cells:granulosa cells within GM –/– mouse follicles, compared with that within GM +/+ mouse follicles, may result in the expulsion of a greater number of follicular cells in the form of cumulus cells during ovulation in GM –/– mice, leaving a smaller population of MGCs which, upon luteinization, form corpora lutea. Comparison of the size, number and cellular composition of follicles of the two genotypes is required to support or refute this speculation.

Lack of GM-CSF does not appear to impair follicular cell function or development, since no differences are observed between GM-CSF-deficient and wild-type mice in the numbers of oocytes ovulated (Jasper et al., 2000) or rates of fertilization (Robertson et al., in press). However, observations in this and other studies indicate that GM-CSF deficiency is associated with subtle alterations in follicular cell composition and function. These differences may be the result of attenuated differentiation in follicles in the absence of GM-CSF, possibly occurring as a consequence of a perturbation in the behaviour of the local macrophages that regulate steroidogenesis.

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Fig. 7. Progesterone secretion from granulosa cells of granulocyte–macrophage colony-stimulating factor (GM-CSF) –/– (○) or GM-CSF +/+ (●) mice with or without insulin-like growth factor I (IGF-I; 50 ng ml −1), FSH (10 ng ml −1), GM-CSF, and combinations of the three in vitro. Bars represent mean progesterone pmol per 10⁶ cells ± SEM of values obtained from eight replicates.
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