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Granulocyte–macrophage colony-stimulating factor: presence in human follicular fluid, protein secretion and mRNA expression by ovarian cells

Melinda J.Jasper[1,6], Mats Brännström[2], Jan I.Olofsson[3], Ossie M.Petrucco[1], Helen Mason[4], Sarah A.Robertson[5] and Robert J.Norman[1]

[1]Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, The University of Adelaide, The Queen Elizabeth Hospital, Woodville, 5011, South Australia, Australia, [2]Department of Obstetrics and Gynaecology, The University of Göteborg, Sahlgrenska Sjukhuset, S-41345 Göteborg, [3]Department of Physiology and Department of Obstetrics and Gynaecology, Umeå University, S-901 87 Umeå, Sweden, [4]Department of Obstetrics and Gynaecology, Saint Mary's Hospital Medical School, Paddington, London W2 IPG, UK, and [5]Department of Obstetrics and Gynaecology, The University of Adelaide, Adelaide, 5005, South Australia, Australia

[6]To whom correspondence should be addressed

In recent years it has become evident that a leukocyte–cytokine network contributes to the paracrine regulation of ovarian function. The objectives of this study were to examine the presence of a potent lympho–haemopoietic cytokine, granulocyte-macrophage colony-stimulating factor (GM–CSF), in tissues and fluids from human ovaries. In a prospective study, follicular fluid and plasma were collected from naturally cycling women and women undergoing hyperstimulation for in-vitro fertilization (IVF). Granulosa–lutein cells were collected at the time of oocyte recovery for IVF and corpora lutea were collected at the time of hysterectomy for non-ovarian reasons. Culture supernatants from ovarian cell and tissue cultures were harvested on completion of a 48 h incubation. Immunoactive GM–CSF was measured by enzyme-linked immunosorbent assay, and was found to be present at statistically significantly higher levels in follicular fluid (8.9 ± 0.7 pg/ml) and plasma (11.3 ± 0.8 pg/ml) of women undergoing hyperstimulation compared to follicular fluid (5.3 ± 0.3 pg/ml) and plasma (7.1 ± 0.5 pg/ml) from naturally cycling women. Immunoactive GM–CSF was also detected in culture supernatants of granulosa-lutein cells (47.6 pg/10^5 cells), early luteal phase corpora lutea (0.52 pg/μg DNA) and mid-luteal phase corpora lutea (0.98 pg/μg DNA). Furthermore, transcripts for GM−CSF, and both the α and β subunits of the GM–CSF receptor, were detected by reverse transcription polymerase chain reaction (RT-PCR) in granulosa-lutein cell culture preparations and corpora lutea collected during the early, mid- and late luteal phase of the menstrual cycle. These results show that GM–CSF is expressed and secreted by cells within the human ovary, and, together with the finding of expression of mRNA for GM–CSF receptor, suggest a role for GM–CSF in the local regulation of ovarian events.
Key words: corpus luteum/cytokine/GM–CSFR/granulosa-lutein/ovary

Introduction

The classical model for the control of ovarian function pivots on the interaction between the glycoprotein hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the locally produced steroid hormones, oestradiol and progesterone. In recent years it has become evident that lympho–haemopoietic cells and the cytokine networks in which they participate have an important role in many reproductive events. In particular, there is now abundant evidence to show that cytokines, including interleukin-1β (IL-1β), tumour necrosis factor α (TNFα) and interferons (IFN), interact with gonadotrophins and growth factors to mediate the local regulation of ovarian function (Adashi, 1989; Stern and Coulam, 1992). The cellular origins of these mediators are not known with certainty in the ovary, but are presumed to include thecal and granulosa cells, luteal cells, and migrating and resident leukocytes (Brännström and Norman, 1993).

Granulocyte-macrophage colony-stimulating factor (GM−CSF), an 18–22 kDa glycoprotein, initiates biological activity through binding to the GM–CSF receptor (GM–CSFR), which is a heterodimeric complex comprised of a low affinity α-chain (GM–CSFRα) and affinity converting β-chain (KH97 or GM−CSFRβ). Whereas many cell lineages express the α-chain, only myeloid leukocytes have been found to express the β-chain. In peripheral tissues GM–CSF is thus thought to act primarily as a regulator of the activation and function of granulocytes, mononuclear phagocytes and dendritic cells, influencing their trafficking into and out of tissues, and promoting activities including cytotoxicity, phagocytosis, antigen presentation, and cytokine secretion (Crosier *et al.*, 1994).

In the uterus, GM–CSF secreted by the uterine epithelium is implicated as a principal mediator of the steroid regulated recruitment and activation of neutrophils and macrophages during the oestrous cycle and early pregnancy (Roberston *et al.*, 1994). Studies in ovariectomized animals have shown that oestradiol promotes, whereas progesterone inhibits, GM–CSF mRNA expression and protein secretion. In an in-vitro perfusion model, we have shown that bioactive GM–CSF is

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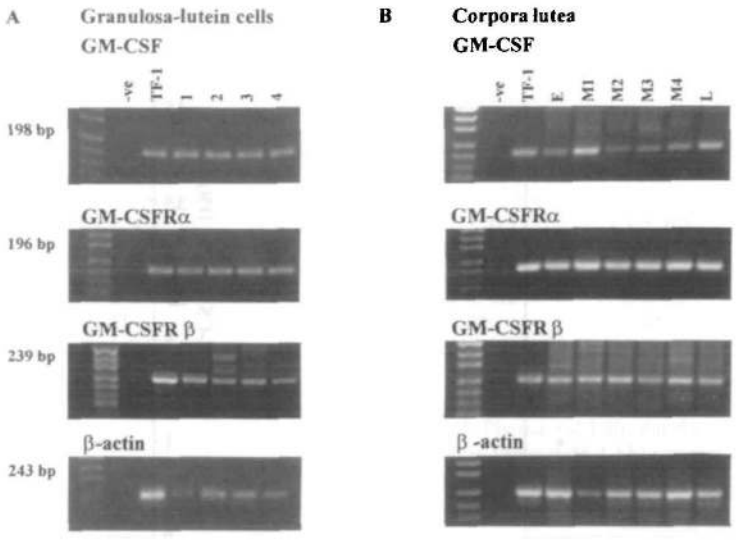

Figure 5. Electrophoretic analysis of the reverse transcription-polymerase chain reaction (RT-PCR) amplification products for granulocyte-macrophage colony-stimulating factor (GM–CSF), GM–CSF receptor (GM–CSFR)α, GM–CSFRβ and β-actin. (**A**) Human granulosa–lutein cell preparations (n = 4; lanes 1–4) and (**B**) early (n = 1; E), mid- (n = 4; M1–M4) and late luteal phase corpora lutea (n = 1; L). Negative (no cDNA) and positive controls (TF-1 cells) were also analysed, and the molecular weight ladder (Hpa-digested pUC-19) was included for estimation of RT-PCR product size.

the activity of various biochemical mediators involved in the ovulatory pathway, including progesterone, prostaglandins (Wang *et al.*, 1991; Brännström *et al.*, 1993a), gelatinase (Hurwitz *et al.*, 1993), plasminogen activator (Bonello *et al.*, 1995) and nitric oxide synthase enzymes (Ellman *et al.*, 1993; Bonello *et al.*, 1996). TNFα and IL-6 have also been shown to be present in the ovaries of mice and rats (Sancho-Tello *et al.*, 1992; Chen *et al.*, 1993; Brännström *et al.*, 1994).

A potential role for GM–CSF in the ovulatory process was first suggested by the finding that bioactive GM–CSF is secreted from the rat ovary with maximal release occurring at the time of ovulation (Brännström *et al.*, 1994). In the current study, GM–CSF was found to be present in follicular fluid of both naturally cycling and hyperstimulated women prior to ovulation at values ~2-fold less than that of plasma, similar to findings for IL-1 and IL-2 (Wang and Norman, 1992). Granulosa–lutein cells and corpora lutea were shown to secrete immunoactive GM–CSF *in vitro* and to express GM–CSF and GM–CSFRα and β mRNAs. Together these data demonstrate the occurrence of GM–CSF and its receptor in the human ovary.

Immunoactive GM–CSF was detected in human plasma at similar values throughout the menstrual cycle in both normal and ovariectomized women, suggesting that plasma GM–CSF is primarily derived from tissues other than the ovary. However, gonadotrophin or steroid hormone-sensitive cells or tissues other than the ovary may contribute to the circulating concentrations of this cytokine since plasma of hyperstimulated women contained significantly more GM–CSF than those undergoing a natural LH surge. Although the mean plasma GM–CSF concentration did not show menstrual variation, some individuals did exhibit a pre-ovulatory increase with a secondary luteal rise, concomitant with oestradiol fluctuations.

Interestingly, hyperstimulation also significantly increased the concentration of GM–CSF in follicular fluid. This increase suggests that the synthesis of GM–CSF by ovarian cells may be at least partially regulated by gonadotrophins or steroid hormones, as is the case for uterine epithelial cell GM–CSF synthesis (Robertson *et al.*, 1996). Alternatively, the effect of gonadotrophins may be mediated through the enhanced recruitment of trafficking cells, such as macrophages, which can produce GM–CSF when activated. In contrast, the disease state of PCO did not appear to alter values of GM–CSF in follicular fluid, similar to findings for TNFα (Jasper and Norman, 1995).

Granulosa–lutein cells from hyperstimulated individuals were found to secrete GM–CSF *in vitro*. Considerable variation between individuals was apparent for both basal GM–CSF secretion and responsiveness to co-incubation with gonadotrophins and cytokines, which may reflect differences in the degree of external hyperstimulation, or may be an indication of the functional state of granulosa–lutein cells in each case. Notably, co-incubation of granulosa–lutein cells with HCG appeared to up-regulate, while IL-1 diminished, GM–CSF secretion in one of two individuals, but conclusive data on the effect of these factors on GM–CSF secretion awaits the study of a larger number of samples. Messenger RNA for GM–CSF was detected by RT-PCR in all granulosa–lutein cell preparations, confirming the production of protein by cells constituting the culture preparation.

Corpora lutea from early and mid-luteal phase ovaries were found to secrete GM–CSF *in vitro*. Production was found to be stimulated by both IL-1β and TNFα, in accordance with other cell systems where these cytokines are known to promote GM–CSF production (Munker *et al.*, 1986; Churchill *et al.*, 1992). RT-PCR demonstrated clearly the presence of mRNA transcripts for GM–CSF in early, mid- and late luteal phase corpora lutea indicating local production of the cytokine within the corpus luteum. These data are consistent with the findings of Zhao *et al.* (1995) of GM–CSF protein and mRNA expression in the small and large luteal cells of early, mid- and late luteal phase corpora lutea.

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In addition, detection of both α and β GM–CSF receptor subunit mRNAs by RT-PCR in granulosa–lutein cell preparations and early, mid- and late luteal phase corpora lutea suggests the presence of GM–CSF responsive cells within the ovary. Both GM–CSF receptor subunit mRNAs have been localized to luteal cells of corpora lutea, whereas protein has been localized to theca, granulosa cells and luteal cells of the corpus luteum (Zhao *et al.*, 1995).

Until recently, epithelial cells had been the only ovarian cell reported to secrete bioactive GM–CSF *in vitro* (Ziltner *et al.*, 1993). We have demonstrated protein secretion and mRNA expression in various crude ovarian cell preparations, but due to the multi-lineage constitution of granulosa–lutein cell culture preparations and the corpus luteum, the cellular origin of GM–CSF and target cells remains uncertain. Evidence from Zhao *et al.* (1995) suggests that GM–CSF is not synthesized by granulosa cells but rather that cells of the theca may produce GM–CSF which targets granulosa cells, while luteal cells appear to be both a source and a target for GM–CSF. Leukocytes, which comprise up to 20% of granulosa–lutein cell preparations (Wang *et al.*, 1995), and connective tissue and endothelial cells as well as leukocytes which comprise up to 80% of the cells in the corpus luteum (Lei *et al.*, 1991), are also potential sources of this cytokine in the culture preparations.

The biological significance of GM–CSF in the ovary remains unclear. The concentration of GM–CSF present in culture supernatants was comparable to the physiological values required to stimulate survival, proliferation and activation of myeloid cells *in vitro* (Lopez *et al.*, 1992), with local values at the site of synthesis likely to be even higher. Given the role of GM–CSF in the regulation of myeloid cell recruitment and activation during inflammatory processes (Wang *et al.*, 1987) and in the reproductive tract (Robertson *et al.*, 1994), it is possible that this cytokine may act to recruit leukocytes into the ovary at the time of ovulation and corpus luteum development, and subsequently to regulate their behavioural and secretory profile. Indeed, GM–CSF may at least partially account for the chemotactic activity observed in follicular fluid (Herriot *et al.*, 1986). GM–CSF may also target ovarian endothelium, inducing neutrophil adherence (Gamble *et al.*, 1990) and the proliferation and migration of endothelial cells during angiogenesis (Bussolino *et al.*, 1991). Indeed, we have recently shown migration of leukocytes into rat and human ovaries, particularly into the area surrounding the ovulatory follicle and in the developing corpus luteum (Wang *et al.*, 1992; Brännström *et al.*, 1993b). These leukocytes may mediate tissue remodelling of ovarian structures, while cytokines secreted from activated macrophages and neutrophils, especially IL-1 and TNF, may contribute to the regulation of steroidogenesis, local vascular permeability and biochemical responses to gonadotrophins.

It is now apparent that cytokines also play a role in the pathophysiology of disease states. Interestingly, GM–CSF receptors have been identified on the surface of various types of non-haematopoietic tumour cells (Baldwin *et al.*, 1991) and more recently GM–CSF has been shown to stimulate the growth of human ovarian cancer cells (Cimoli *et al.*, 1991). Together with the finding that GM–CSF mRNA is expressed in biopsies from epithelial ovarian carcinoma (Pisa *et al.*, 1992), this has led us to postulate that GM–CSF may have an autocrine activity in ovarian cancer. Future therapies for ovarian diseases will need to confront the role of cytokines in ovarian physiology.

In conclusion, we have demonstrated that another member of the cytokine family, GM–CSF, is secreted by the cells of the human ovary and may, through regulating the recruitment and function of myeloid leukocytes in the ovary, be important in the local regulation of ovarian events.

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