Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture

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Abstract

The receptor tyrosine c-Kit and its cognate ligand, c-Kit ligand (KL, stem cell factor, SCF), are involved in ovarian follicular development in several animal species. We studied the expression of KL and c-Kit using *in situ* hybridization and immunohistochemistry in donated human ovarian cortical tissue. The KL transcripts were expressed in granulosa cells of primary follicles, whereas the expression of c-Kit was confined to the oocyte and granulosa cells in primary and secondary follicles. We employed an ovarian organ culture using firstly serum-containing and then serum-free medium to study the effects of KL and an anti-c-Kit antibody, ACK2, on the development and survival of ovarian follicles *in vitro*. Culture of ovarian cortical slices for 7 days resulted in a 37% increase in the number of primary follicles and a 6% increase in secondary follicles. The proportion of viable follicles decreased in all cultures. The addition of KL (1, 10 and 100 ng/ml) into the culture media did not affect the developmental stages of the follicles or the proportion of atretic follicles. Inclusion of ACK2 (800 ng/ml) in the culture medium significantly increased the proportion of atretic follicles on days 7 (49 vs 28% in control cultures) and 14 (62 vs 38%) of culture. In conclusion, c-Kit and KL are expressed in human ovaries during follicular development. Blocking the c-Kit receptor induces follicular atresia. The KL/c-Kit signaling system is likely to control the survival of human ovarian follicles during early follicular development.

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Introduction

During a woman's reproductive life less than 1% of the follicles present in the human ovary at birth reach the stage of ovulation, while the rest undergo atresia and are lost. Some of the follicles present at birth start to grow during infancy and childhood, but most of them remain in the resting stage until they either degenerate or enter the growth phase during adult life (Adashi 1991, Gougeon 1996). It is likely that the mechanism behind the loss of germ cells is apoptotic cell death (Tilly 1996). Follicle-stimulating hormone (FSH) has been considered to be a key survival factor for follicles from the antral follicular stage onwards (McGee *et al.* 1998). However, the regulators of early follicular survival are still poorly characterized.

The c-Kit ligand (KL, also called stem cell factor, SCF), a ligand for the c-Kit proto-oncogene receptor tyrosine kinase, is a pluripotent growth factor involved in the differentiation and growth of certain stem cell lineages including hematopoietic stem cells, neuroblasts, melanoblasts and primordial germ cells (PGCs) (Galli *et al.* 1994). The expression of KL has been detected in granulosa cells in mouse ovaries, while c-Kit receptor is confined to the oocytes and theca interna cells (Manova *et al.* 1990, 1993, Horie *et al.* 1991, Keshet *et al.* 1991, Motro *et al.* 1991, Motro & Bernstein 1993). In contrast to mice, c-Kit protein and mRNA have been detected in both human oocytes and granulosa cells (Horie *et al.* 1993, Tanikawa *et al.* 1998). KL promotes the survival of mouse PGCs in culture independently and in combination with leukemia inhibitory factor (LIF) (Dolci *et al.* 1991, Godin *et al.* 1991, Matsui

et al. 1991, Pesce *et al.* 1993). Morita *et al.* (1999) further showed that KL alone has no inhibitory effect on germ cell apoptosis in cultured prenatal mouse oogonia/oocytes, whereas it was able to promote germ cell survival when administered in combination with LIF. In cultures of fetal mouse ovarian tissue, KL has been found to initiate the growth of oocytes (Klinger & De Felici 2002). KL and c-Kit are also active in fetal mouse and sheep follicular formation (Tisdall *et al.* 1997, McNatty *et al.* 2000).

In female mice carrying a distinct mutant allele, *Sl^{pan}*, that leads to decreased production of KL protein, an almost normal number of germ cells are generated but ovarian follicular growth is arrested at the one-layered granulosa cell stage of the primary follicle (Huang *et al.* 1993). A similar ovarian phenotype was observed in mice after administering ACK2, an anti-c-Kit antibody that blocks c-Kit/KL receptor–ligand interaction (Yoshida *et al.* 1997). Moreover, heterozygous mice carrying the mutation Kit (W-lacZ) show alterations in granulosa cell proliferation and oocyte growth in preantral follicles (Reynaud *et al.* 2001). In cultures of preantral mouse follicles, cytoplasmic maturation of the oocyte and testosterone production by the follicle were improved by addition of KL (50 ng/ml), and blocking the c-Kit receptor by use of an antibody decreased survival of the oocytes (Reynaud *et al.* 2000). Two oocyte-derived transforming growth factor beta family members, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15, also known as growth differentiation factor 9B) appear to regulate KL expression in a specific manner. GDF-9 suppresses the KL expression in mouse preantral granulosa cells, whereas in bovine antral granulosa cell culture GDF-9 increases KL transcript levels (Joyce *et al.* 2000, Nilsson & Skinner 2002). BMP-15 stimulates KL expression in rat antral granulosa cells while KL is able to negatively regulate BMP-15 transcripts in a paracrine manner (Otsuka & Shimasaki 2002).

There are differences in rodent and human follicular function, and little is known about the role of KL/c-Kit in early human follicular development. We have previously developed an organ culture method for human primordial, primary and secondary follicles (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999, Hreinsson *et al.* 2002). In this system, follicles are cultured within small slices of ovarian cortical tissue, allowing the maintenance of normal follicular structures and epithelial–stromal interactions. In this study we localized the expression of KL and c-Kit in human ovarian tissue and tested the effects of exogenous KL protein and an antibody against the *c-kit* receptor, ACK2, on the development and survival of early human ovarian follicles in organ culture.

Materials and Methods

Human ovarian tissue

Ovarian cortical tissue was obtained during gynecological laparotomies or laparoscopies from 55 subjects. All the

women, aged 19–44 (mean 34) years, donated tissue after informed consent. The study was accepted by the Ethics Committees of Karolinska Institutet, Sweden, Imperial College School of Medicine, Hammersmith Hospital, UK, Department of Obstetrics and Gynecology, Helsinki University Central Hospital and the Family Federation of Finland, Finland. The biopsy specimens were placed in Hepes-buffered culture medium (MEM; Gibco) and transferred to organ culture dishes (see below).

For immunohistochemistry and *in situ* hybridization analyses, ovarian tissue from six consenting subjects, aged 25–38 years (mean 32), was fixed in Bouin's solution and embedded in paraffin or directly embedded in OCT cryopreservation solution (TissueTek, Miles, Inc., Elkhart, IN, USA).

Human granulosa-luteal (GL) cell culture

Human GL cells were obtained by follicular aspiration from regularly menstruating women undergoing oocyte retrieval for IVF because of either tubal obstruction or infertility of the spouse. Ovarian stimulation was induced by combining a gonadotropin-releasing hormone analog (Suprecur; Hoechst, Frankfurt am Main, Germany) and human menopausal gonadotropin (Pergonal; Serono Nordic, Vantaa, Finland; or Humegon; Organon, Oss, The Netherlands). Oocyte retrieval was carried out 36–37 h after human chorionic gonadotropin (Profasi; Serono; or Pregnyl; Organon) administration at a total dose of 10 000 IU. The cells obtained the same morning from two to four patients were pooled, enzymatically dispersed, and separated from red blood cells by centrifugation through Ficoll-Paque (Pharmacia, Uppsala, Sweden), as previously described (Eramaa *et al.* 1993). Thereafter the cells were directly recovered for RNA extraction or plated at a density of $2\text{--}5 \times 10^5$ cells/well on 35 mm six-well dishes (Costar, Cambridge, MA, USA) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (Gibco) at 37°C in 95% air–5% CO₂ humidified environment. Cell culture media were changed every other day. The cells were cultured 6 days before RNA extraction.

Organ cultures

We used an organ culture method that we have described earlier (Hovatta *et al.* 1997, 1999). Organ cultures were set up in 24-well plates (Nunc, Roskilde, Denmark) fitted with Millicell CM inserts (12 mm diameter, 0.4 µm pore size; Millipore, Bedford, MA, USA) previously coated with 100 µl of extracellular matrix (Matrigel; Becton Dickinson, MA, USA). Prior to coating, Matrigel was diluted 1:3 with Earle's balanced salt solution (EBSS; Gibco). Ovarian tissue samples were cut in slices, 0.5–1 mm thick, using a needle and scalpel. The tissue pieces were then either directly fixed for histological analyses (control at 0 days) or placed in the inserts and cultured for 7 or 14 days. Culture medium was EBSS supplemented with either

10% human serum albumin (HSA; Pharmacia Upjohn, Sweden) or inactivated human serum (5%) obtained from women undergoing pituitary desensitization for IVF treatment. Further supplements were FSH (0.11 U/ml; Metrodin or Gonal-F; Serono), and 0.5% antibiotic/antimycotic solution (50 IU/ml penicillin, 50 µg/ml streptomycin sulfate, 0.125 µg/ml amphotericin B; Gibco) at 37°C in a 95% air–5% CO₂ humidified environment. Culture medium (500 µl) was added to each well; three drops (150 µl) were pipetted into the insert with the remainder into the well outside the insert. Every second day, 150 µl culture medium were removed and three drops of fresh medium added to the inserts. Treatment groups in the serum-containing medium were culture medium without (controls) or with recombinant human (rh) SCF at 1, 10 or 100 ng/ml (Sigma) or monoclonal anti-c-kit antibody at 800 ng/ml (ACK2; Sigma), an antagonistic blocker of c-kit function. The diluent of ACK2 was PBS and we diluted it further into culture medium. HSA-containing medium was tested without or with rhSCF (KL) at 10 ng/ml. The cultured pieces were collected for histology and immunohistochemistry.

Histology and follicle counts

The freshly isolated ovarian biopsy material (day 0) or cultured specimens were fixed in Bouin's solution for 1–2 h, dehydrated in ethanol, embedded in paraffin and serially sectioned at 5 µm thickness. The sections were stained with hematoxylin and eosin. Cells possessing round nuclei along with maintenance of cytoplasmic volume and spherical plasma membranes were considered viable, whereas cells showing eosinophilia of the ooplasm, contraction and clumping of nuclear material as well as wrinkling of the nuclear membrane were considered atretic (Gougeon 1986). From the serial sections, the follicles were counted, their developmental stages were recorded and their diameters were measured at the level of the nuclei of the oocyte by using an ocular micrometer. Care was taken to count each follicle only once as we have also done in our earlier studies (Hreinsson *et al.* 2002).

Immunohistochemistry

The paraffin sections were incubated at 60°C for 15 min, serially rehydrated and placed in a water bath. They were incubated for 5 min with peroxidase to block endogenous peroxidase activity and for 30 min with ACK2 (4 µg/ml) diluted in PBS. The sections were then incubated for 30 min with peroxidase-conjugated goat anti-mouse secondary antibody (DAKO EnVision + System; Dako Corporation, Carpinteria, CA, USA). Washing twice with PBS followed each incubation. Finally, the antigenic sites were visualized by using 3,3'-diaminobenzidine (Dako) as a chromogen. Counterstaining was carried out with Erlich's hematoxylin for 1 min. Immunostaining with PBS + 0.1% BSA (Sigma) and the peroxidase-labeled

secondary antibody was used for negative controls. Also antibody against myelin basic protein was used as a negative control. All of the incubations were performed at room temperature unless otherwise specified.

Northern and in situ hybridization

Total RNA from freshly isolated GL cells and cytoplasmic RNA from cultured GL cells were extracted with the guanidine isothiocyanate–cesium chloride method (Chirgwin *et al.* 1979) and by the modified NP-40 lysis procedure (Ritvos & Eramaa 1991) respectively. Northern blotting was performed as previously described (Laitinen *et al.* 1997). For filter hybridization reactions we used [α -³²P]deoxy-CTP-labeled single- or double-stranded cDNAs. Human c-Kit cDNA was purchased from ATCC (clone no. 59492; ATCC, Manassas, VA, USA). To prepare the cDNA probe, the c-Kit cDNA was first subcloned into pGEM7 vector (Promega, Madison, WI, USA) and cut with EcoRI. The 546 bp (nt 704–1249) single-stranded c-Kit cDNA probe was then synthesized by PCR amplification for 40 cycles using the primer 5'-TAAATCCACTGTGATATCTTA (complementary to the SP6 promoter sequence) (Laitinen *et al.* 1997). Double-stranded rat glyceraldehyde-6-phosphate dehydrogenase (GAPDH) cDNA (Laitinen *et al.* 1997) was used as a control for even loading in filter hybridization. GAPDH cDNA was labeled with [α -³²P]deoxy-CTP and a Prime-a-gene kit (Promega). For *in situ* hybridization analyses, the [α -³³P]UTP-labeled antisense cRNA probes were transcribed *in vitro* from EcoRI-linearized plasmids containing a 905 bp human KL cDNA (Laitinen *et al.* 1995). *In situ* RNA analyses were carried out on 9 µm cryostat sections as previously described (Heikinheimo *et al.* 1997). The slides were dipped in NTB-2 emulsion (Eastman Kodak, New Haven, CT, USA) and exposed for up to 57 days.

Statistical analyses

The data were analyzed using Chi-square and Mann–Whitney U-tests. $P < 0.05$ was considered significant.

Results

Expression of KL and c-Kit in human ovarian follicles

As no earlier data have been available about the role of KL/c-Kit in early human folliculogenesis, we conducted *in situ* hybridization and immunohistochemical studies to localize the expression of KL and c-Kit in human ovarian sections obtained as biopsy samples. *In situ* hybridization revealed that KL is expressed in the granulosa cells of primary follicles (Fig. 1). To control the technique of hybridization, we also used a probe for an oocyte-specific gene, GDF-9, whose expression is confined to oocytes (Aaltonen *et al.* 1999).

To investigate the distribution of c-Kit protein in human ovaries, we performed immunohistochemistry on ovarian

Figure 1 *In situ* hybridization analysis of KL mRNAs in human ovarian biopsy samples (bright-field (A) and dark-field (B) micrographs). The bar represents 25 μ m.

tissue samples using an anti-*c-kit* antibody, ACK2. Immunoreactivity to *c-kit* was readily detectable in oocytes and granulosa cells of both primary and secondary follicles and granulosa cells of preantral follicles, whereas no staining was seen in thecal cells of preantral and antral follicles (Fig. 2A and C). No staining was observed when sections were incubated with only the secondary antibody (Fig. 2B and D).

We further confirmed the expression of *c-kit* mRNAs in granulosa cells by studying their expression in freshly isolated human GL cells and in cultured human GL cells by Northern analysis. We detected a faint but visible, approximately 5.1 kb, transcript and a shorter hardly detectable, approximately 4.8 kb, transcript for *c-kit* in freshly isolated preovulatory GL cells (Fig. 3, lanes 1 and 2). In cultured GL cells we detected only the shorter transcript (Fig. 3, lane 3). The 5.1 kb transcript represents most likely incompletely processed nuclear transcript, whereas the 4.8 kb transcript is completely processed cytoplasmic RNA and therefore shorter. The absence of longer transcript in cultured GL cells is probably due to the different RNA source (total RNA in freshly isolated GL cells vs cytoplasmic RNA in cultured cells as described in the Materials and Methods section).

Exogenous KL supplementation does not affect human follicular development in ovarian biopsy cultures

During organ culture, the follicles initiated their growth within 1 week, as had been shown in our earlier reports (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999,

Figure 2 (A) Localization of *c-kit* protein in the granulosa cells and oocytes of primordial-intermediary (pr) and secondary (s) follicles. (B) The same section processed with only the secondary antibody. (C) Positive *c-kit* immunoreactivity in the mural granulosa cells of a recently ruptured antral follicle. (D) Counterstained section of the same follicle, without the primary antibody. All the theca cells (th) were negative. Original magnification $\times 400$.

Figure 3 Expression of *c-kit* mRNAs in human GL cells. Twenty micrograms of total RNA from different cell pools (lanes 1 and 2) of freshly isolated GL cells or cytoplasmic RNA from GL cells cultured for 6 days (lane 3) were used for preparing Northern blots. The migration of 28S and 18S ribosomal RNAs are shown. GAPDH transcripts are shown as a control for even loading (below).

Hreinsson *et al.* 2002). There was no significant difference between the numbers of follicles in the cultures with and without KL or the developmental stages of the follicles in cultures without KL supplementation or with any of the three concentrations of added KL (the pooled results are shown in Fig. 4).

The sizes of the follicles were similar within all the supplemented groups and in control cultures. The mean diameter of the follicles in non-cultured tissue was 48.8 μm ; after 7 days it was 56.6 μm with KL and 45.0 μm without KL (data pooled from three concentrations; no significant differences separately). After 14 days in culture supplemented with KL the mean diameter

was 51.4 μm , and without KL, 49.0 μm . The proportion of atretic follicles increased with time in all cultures, but there was no significant difference between the control cultures and those with KL added (Fig. 5).

Because human serum might have masked the possible effect of KL in the serum-containing cultures we carried out another series of experiments using serum-free culture medium containing 10 ng KL. The results from 7 and 14 day cultures are shown in Fig. 6. Similarly to the serum-containing cultures, the proportions of the primary and secondary follicles increased in both KL-containing and control cultures, but there were no significant differences between the KL-containing and control cultures at the two time points (7 and 14 days) studied. There were no differences in the viability, either (data not shown).

Anti-c-Kit antibody increases atresia of follicles in organ culture

The distribution of the developmental stages of the follicles – primordial, primary, secondary (a follicle containing several layers of cuboidal granulosa cells) and tertiary (a follicle with antrum formation) – underwent a significant change during culture. However, there was no difference between control cultures and those in which ACK2 had been added (Fig. 7). There were no differences in the mean diameters of the follicles cultured with and without ACK2. The diameters of follicles cultured with ACK2 were 49 μm (79 total follicles) at 7 days and 45 μm (52 total follicles) at 14 days, while the follicles cultured without ACK2 were 47 μm (150 total follicles) at 7 days and 42 μm (71 total follicles) at 14 days of culture.

The proportion of atretic follicles was significantly increased in the cultures which contained ACK2 after both 7 (49 vs 28%) and 14 days (62 vs 38%), when compared with the control cultures (Fig. 8). Atresia was already seen in primordial follicles that were cultured in a

Figure 4 Numbers and developmental stages of follicles cultured for 7 or 14 days with and without KL. Numbers of follicles are shown in parenthesis above each column.

Figure 5 Proportions of viable (A) and atretic (B) follicles cultured for 7 or 14 days with and without KL. Numbers of follicles are shown in parenthesis above each column.

Figure 6 Proportion of the developmental stages of the follicles in serum-free cultures for 7 or 14 days. Numbers of follicles are shown in parenthesis above each column.

Figure 7 Numbers and developmental stages of follicles cultured for 7 or 14 days with and without ACK2. Numbers of follicles are shown in parenthesis above each column.

Figure 8 Percentage of viable (A) and atretic (B) follicles cultured for 7 or 14 days with and without ACK2. In (B), asterisks denote a significant difference between ACK2-treated culture vs control culture (* $P < 0.0005$; ** $P < 0.0001$).

medium containing ACK2, while it was uncommon in control cultures. The oocytes of primary follicles often had abnormalities in the nucleus after 7 days in cultures containing ACK2. It was observed that some of the granulosa cells were pyknotic or had disappeared leaving only a hole, whereas healthy viable primary follicles were most frequently seen in the control cultures (Fig. 9).

Discussion

Our results showed the expression of KL mRNA and c-Kit mRNA and protein in human ovarian tissue, from early to antral follicles, and the decreased survival of the follicles in long-term organ culture when an antibody blocking c-Kit receptor was added. This indicates that KL/c-Kit signaling is important in early human ovarian follicular development.

We showed that KL mRNA is expressed at the primary follicular stage in human ovaries. This is well in line with other studies showing KL expression in granulosa cells in mouse and rat ovaries during early folliculogenesis (Manova *et al.* 1993, Ismail *et al.* 1996). We also showed that c-Kit is expressed both in oocytes of early follicles and in granulosa cells of early and antral follicles in human ovaries. These results confirm earlier studies showing expression of c-Kit protein in oocytes and expression of its mRNA in oocytes and preovulatory granulosa cells (Horie *et al.* 1993, Tanikawa *et al.* 1998). Although several studies on mice have shown that c-Kit is confined to theca interna cells (Manova *et al.* 1990, 1993, Horie *et al.* 1991, Keshet *et al.* 1991, Motro *et al.* 1991, Motro & Bernstein 1993), we did not locate its expression in these cells in human ovarian biopsy samples. In this respect, our result is different from that reported by Ito *et al.* (2001), who used RT-PCR and showed the presence of c-Kit mRNA in human ovarian theca and stroma cells. Our results suggest a species-specific difference in the expression profile of c-Kit.

We have previously developed an extracellular matrix-based method to culture human ovarian follicles *in vitro* (Hovatta *et al.* 1997). In these cultures, the structural

Figure 9 Morphology of primary follicles cultured with (A) and without (B) the blocking antibody ACK2. In (A), the nuclei of the granulosa cells of follicles cultured in the presence of the antibody were pyknotic, and some granulosa cells had disappeared, leaving empty vacuoles. There was also abnormal condensation of chromatin in the nucleus of the oocyte. Two-micron paraffin sections, original magnification $\times 400$.

integrity of the ovarian tissue is maintained and this allows paracrine interactions, which appear necessary, because isolated follicles do not survive or grow as well (Hovatta *et al.* 1999). Under these culture conditions the follicles

grow in diameter and develop to further follicular stages. This is reflected by a 43% decrease in the proportion of primordial follicles during the first 7 days of culture. FSH acts as a survival factor in these cultures (Wright *et al.* 1999), and insulin and insulin-like growth factors promote the growth and survival of the follicles (Louhio *et al.* 2000). Furthermore, GDF-9, secreted by the oocyte, is an important factor that promotes the growth and survival of early human follicles in organ culture (Hreinsson *et al.* 2002). When we added KL to the cultures, there was no clear effect on the development or survival of the follicles. However, increased atresia of early follicles was seen after adding the c-kit blocking antibody to the cultures. The lack of effect of adding KL is probably due to the presence of endogenous KL in the cultures, but the Matrigel could also have been a source of the growth factor. We excluded the possible masking effect of the serum in the culture medium by carrying out also serum-free cultures, with similar results. In cultures of mouse preantral follicles using serum-containing medium, no clear effect was seen, but in serum-free medium KL was mitogenic for granulosa cells (Reynaud *et al.* 2000). The FSH in our culture medium might also have had a simultaneous effect in preventing apoptosis, masking the effect of ACK2, making further effects difficult to observe (Wright *et al.* 1999). Also the high rate of spontaneous maturation *in vitro* may have masked the effect of added KL.

In the rat, KL stimulates the promotion of the developmental stage from primordial to primary follicles (Parrott & Skinner 1999). This was not observed in our cultures, in which we saw an initiation of growth of the primordial follicles within 1 week. Species differences between rodent and human early follicular development may be reflected here, or perhaps the effect of endogenous KL in our cultures. When we added the c-kit blocking antibody to our cultures, we saw similar promotion of the developmental stages as we saw in our control cultures, before the follicles underwent atresia, which is different from the situation in mice and rats (Yoshida *et al.* 1997, Parrott & Skinner 1999). This indicates a real species difference. A blocking antibody has also been reported to cause a decrease in the survival of mouse oocytes in culture (Reynaud *et al.* 2000).

We can conclude that *c-kit* and KL are expressed in human ovaries during early follicular development. Exogenous KL does not improve the survival of follicles in organ culture, but blocking the *c-kit* receptor induces follicular atresia. The KL/*c-kit* signaling system is likely to control the survival of human ovarian follicles during early folliculogenesis.

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