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Quantification of insulin-like growth factor I receptors on granulosa cells with flow cytometry after follicular stimulation

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The presence of insulin-like growth factor I (IGF-I) receptors on granulosa cells was investigated by flow cytometric analysis. Granulosa cells were retrieved from follicular fluid after oocyte retrieval during assisted reproduction technology procedures. Whole samples of follicular fluid were pooled and the cellular fraction analysed. In order to analyse granulosa cells only we developed a dual labelling technique whereby granulosa cells were identified as CD45 negative cells, distinguishing them from leukocytes which are CD45 positive. Analysis of the IGF-I receptor was done by staining the sample with a monoclonal anti-IGF-I receptor antibody (αIR3 clone) and goat anti-mouse phycoerythrin labelled antibody. After identification of the presence of IGF-I receptors, receptor expression was quantified by calibration of the fluorescence signals. We analysed 10 patients' samples and showed 559–1774 binding sites per granulosa cell with a mean value of 1125 ± 382 (SD).

Key words: flow cytometry/granulosa cells/IGF-I receptors

Introduction

There is increasing evidence that the insulin-like growth factor (IGF) system has a role to play in follicular dynamics. Much of the attention has been focused on IGF-I since initial experimental data from the rat showed it to promote granulosa cell differentiation and proliferation. IGF-I has the ability of synergizing with pituitary gonadotrophins and is capable of augmenting the effect of follicle-stimulating hormone (FSH) on oestradiol, progesterone, inhibin biosynthesis and on acquisition of luteinizing hormone (LH) receptors (Adashi *et al.*, 1985). However, the human granulosa cell apparently does not contain detectable IGF-I mRNA (El Roeiy *et al.*, 1993), hence the suggestion of a role for systemic IGF-I in human follicular development. IGF-II mRNA and protein were detectable in all antral follicles and IGF-II synthesis has been shown in granulosa cells from dominant follicles in the human (El Roeiy *et al.*, 1993).

The IGF-I receptor (IGF-Ir) comprises a heterotetrameric structure of two α and two β subunits. The two α subunits are entirely extracellular and mainly involved in ligand binding; the two transmembrane β subunits include a tyrosine kinase domain in their cytoplasmic position (Werner *et al.*, 1994). The presence of IGF-I receptors on granulosa cells was shown using binding studies in the animal and human (Baranao and Hammond, 1984; Davoren *et al.*, 1986; Gates *et al.*, 1987; Adashi *et al.*, 1988, 1989; Spicer *et al.*, 1994) and immunohistochemistry (Balboni *et al.*, 1987; Samoto *et al.*, 1993). Expression of IGF-Ir mRNA and localization of the protein was detected only in granulosa cells (El Roeiy *et al.*, 1993). It is suggested that IGF-II plays a critical role in follicular maturation by means of an autocrine action on granulosa cells via IGF-I receptor (Adashi *et al.*, 1989).

A relationship between poor response in ovarian stimulation and abnormalities in the IGF system was suggested but could not be confirmed. No differences were noted in follicular fluid concentrations of IGF-I, IGF-II, IGF binding protein (BP)1 and IGFBP3 when poor and normal response to ovarian stimulation was compared (Hamori *et al.*, 1991). Serum concentrations of IGF-I are higher than those of follicular fluid (Hamori *et al.*, 1991; Pellegrini *et al.*, 1995), suggesting a systemic origin of IGF-I, whereas concentrations of IGF-II in follicular fluid and serum were comparable (Hamori *et al.*, 1991). In order to elucidate further the response to ovarian stimulation we decided to investigate the IGF-I receptor and to quantify IGF-I receptors on granulosa cells.

Aspirates of follicular fluid from oocyte retrievals in assisted reproduction technology procedures contain granulosa cells, large vaginal epithelial cells and may contain an important fraction of blood cells. Blood contamination is inherent to the procedure of oocyte retrieval. Large vaginal epithelial cells can be gated during flow cytometric analysis and red blood cells are lysed during the staining procedure. Leukocytes are much more difficult to discriminate as they show an important overlap for size range with the granulosa cells. We established a method whereby granulosa cells could be analysed with flow cytometry without physically separating them, allowing the quantification of IGF-Ir expression by calibrated flow cytometric measurement (De Neubourg *et al.*, 1996).

Materials and methods

Patients

Follicular fluid was collected during oocyte retrieval after informed consent had been obtained from couples attending the Nottingham University Hospital (NURTURE) for male and female infertility treatment. Pituitary desensitization was obtained with gonadotrophin-releasing hormone (GnRH) analogues (Suprefact®; Hoechst, Hounslow, UK), which were started during the midluteal phase of

the pretreatment cycle for a minimum of 2–3 weeks. Daily i.m. injections of human menopausal gonadotrophin (HMG) (Pergonal®; Serono SA, Aubonne, Switzerland; Humegon®; Organon, Cambridge, UK) were used as follicular stimulation. Ovulation was induced with human chorionic gonadotrophin (HCG) 10 000 IU i.m. (Profasi®; Serono SA). Oocytes were retrieved 36 h later under transvaginal ultrasound guidance. All the follicular fluid and flushes (with Hartman's solution containing 2 IU of heparin/ml) collected from one patient were pooled. All 10 patients were considered as normal responders to ovarian stimulation. Their stimulation characteristics were (mean ± SD): oestradiol concentration on day of HCG administration was 8841 ± 3165 pmol/l; 32.4 ± 5.5 ampoules (2430 ± 412.5 IU) of HMG were given over 11 ± 2.7 days; 10.7 ± 4.7 oocytes were retrieved.

Sample preparation

Follicular fluid was centrifuged at 300 *g* for 5 min and cell pellets were transferred into one tube and resuspended in RPMI 1640 (Sigma UK, Poole, UK) + 1% fetal calf serum (FCS). Cell concentration varied between 2.5 and 25×10^6 cell/ml. This preparation was then adjusted to 1×10^6 cell/ml with RPMI 1640 + 1% FCS. Viability was tested using trypan blue and revealed a minimum of 98% viable granulosa cells. Addition of fetal calf serum did not alter physiological conditions of granulosa cells since concentration of IGF-I and IGF-II in serum and follicular fluid are comparable (Hamori *et al.*, 1991).

Staining procedure

Ten µl of IGF-I receptor antibody (Oncogene Science, Cambridge, UK; αIR3 clone) was pipetted into Falcon tubes and 100 µl of the sample of 1×10^6 cell/ml was added and gently mixed. This sample was incubated for 30 min at room temperature, then 1 ml of flow cytometric analysis (FACS) lysing solution (Becton Dickinson UK, Cowley, UK) was added and incubated for 10 min. The samples were centrifuged at 300 *g* for 5 min and the supernatant discarded. The sample was resuspended in 1 ml of RPMI 1640 + 1% FCS, centrifuged and the supernatant discarded. Ten µl of a 1:10 dilution of goat anti-mouse (GAM)-phycoerythrin (PE) conjugated antibody (Dako AS, Glostrup, Denmark) was then added and incubated for 15 min at room temperature in the dark. The sample was washed three times with RPMI 1640 + 1% FCS. To block residual anti-mouse binding sites, 10 µl of mouse whole serum (Dako) was added and incubated for 10 min in the dark at room temperature. Without washing the serum away, 5 µl of CD45-fluorescein isothiocyanate (FITC) (Sigma, UK) was added and incubated for 15 min in the dark at room temperature. The sample was washed twice and the resuspended cells fixed with 300 µl of 0.5% of formaldehyde and stored in the dark until analysis.

Autofluorescence was assessed using an unstained sample, and mouse IgG1 (Dako) was used as an isotype control.

Flow cytometric analysis

Flow cytometric analysis was performed on FACScan® (Becton Dickinson, UK) using standard settings: fluorescence 1 (FL1), 4 decades (logarithmic), detector 648V, log amplifier, compensation 1.1%; fluorescence 2 (FL2), 4 decades (logarithmic), detector 496V, log amplifier, compensation 22.8%. Data analysis was performed using Lysis® software (Becton Dickinson, UK). A total of 10 000 events per sample were analysed.

Quantification of IGF-I receptors

In order to assess how many binding sites were represented by the fluorescence signals, 50 µl of Quantum Simply Cellular Microbeads® (Sigma, UK) were incubated with 10 µl IGF-Ir antibody for 30 min at room temperature. The bead standards consisted of four populations of simply cellular microbeads coated with goat anti-mouse antibody which bind a different number of mouse IgG monoclonal antibody molecules (4063, 14 354, 54 401, 203 303 molecule binding capacity for the batch used) and a blank population. The beads were then washed with 1 ml RPMI 1640 + 1% FCS for 5 min at 300 *g*. The IGF-Ir stained beads were incubated with 10 µl GAM-PE diluted 1:10 with RPMI 1640 + 1% FCS for 15 min in the dark at room temperature. The beads were then washed three times in RPMI 1640 + 1% FCS and fixed with 300 µl of 0.5% formaldehyde. Flow cytometric analysis was done on a FACScan® flow cytometer (Becton Dickinson, UK) using the same settings as for cell analysis. A histogram of red fluorescence (FL2) was produced for the beads and each peak of fluorescence corresponding to a bead type was identified by histogram markers. The mean fluorescence channel number for each peak was taken. A best fit curve was drawn to relate linear channel number to logarithmic binding capacity (molecules) from which values for the IGF-Ir samples and GAM-PE control samples could be read. These values were subtracted in order to account for autofluorescence and non-specific binding (QuickCal, QSC calibration software, Sigma, UK).

Statistical analysis

Paired Student's *t*-test was used for differentiation between control and sample group. Spearman's rank test was used to analyse a possible correlation between IGF-Ir and stimulation characteristics.

Results

The dual labelling technique with IGF-Ir-PE and CD45-FITC was used to analyse 10 patients' samples (Figure 1). Live gating was performed during acquisition of the data to exclude large cells such as epithelial cells. On a forward scatter (FSC) versus side scatter (SSC) dot plot the population of interest was delineated as region 1 (R1). This population was then analysed on a fluorescence 1 (FL1) versus fluorescence 2 (FL2) plot and the granulosa cell population was identified as CD45 negative and gated as region R2. Histograms of FL2 were then calculated in the CD45 negative population for a PE unstained sample, IgG1-PE, GAM-PE and for the IGF-Ir-PE stained sample (Figure 2). Statistical characteristics of the histograms were used to describe the intensity of the signal and thus the presence of IGF-Ir. The mean channel number (± SD) for GAM-PE control was 101.8 ± 39.8 and for IGF-Ir-PE was 129.2 ± 7.2. The paired Student's *t*-test showed the populations to be significantly different ($P < 0.001$). From these data it was concluded that granulosa cells from follicular fluid collected from dominant follicles during oocyte retrieval for assisted reproduction techniques, ~36 h after HCG was given, contained a low concentration of IGF-Ir. To calculate the amount of IGF-Ir per granulosa cell, Quantum Simply Cellular Microbeads® were used to calibrate the indirect PE labelling, and the QuickCal® program used to calculate the equivalent numbers of antibody molecules bound (Figure 3). Binding capacities were calculated for the control and IGF-Ir sample and subtracted. Binding sites for IGF-I receptor antibody varied from 559 to 1774 per granulosa cell with a mean value of 1125 ± 382 (SD) binding sites per cell. There was no correlation between stimulation characteristics and number of IGF-Ir. This is to our knowledge the first report of quantification of IGF-I receptors in human lutein-granulosa cells.

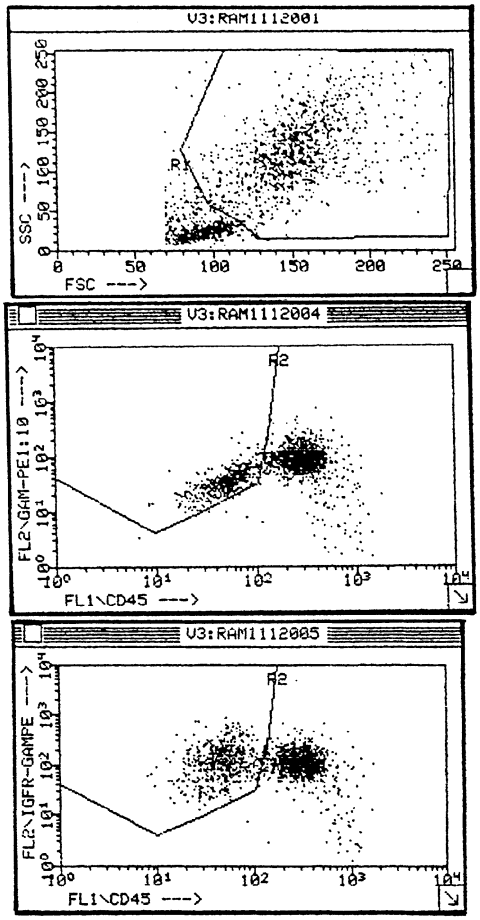

Figure 1. Setting the region for the CD45 negative population. **(top)** forward scatter (FSC) versus side scatter (SSC) dot plot of the sample; the population of interest is delineated as region 1 (R1); **(middle)** fluorescence 1 (FL1) (CD45) versus fluorescence 2 FL2 (GAM-PE) is measured in R1, region 2 is the CD45 negative population; **(bottom)** FL1 (CD45) versus FL2 (IGF-Ir-PE) in R1.

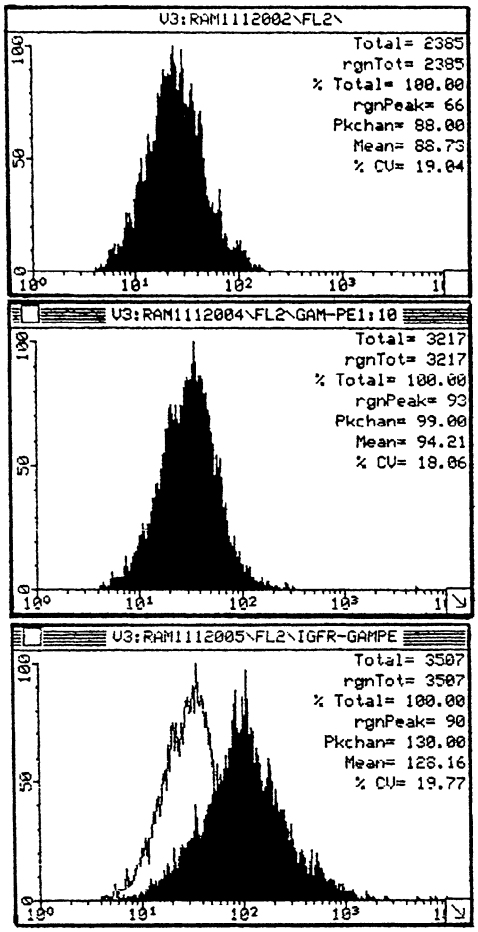

Figure 2. Analysis of insulin-like growth factor (IGF)-I receptor in the CD45 negative population. **(top)** histogram of fluorescence 2 (FL2) (autofluorescence) in region 2 (R2); **(middle)** histogram of FL2 (GAM-PE) in R2; **(bottom)** histogram of FL2 (IGF-Ir-PE) in R2 (shaded) versus histogram of FL2 (GAM-PE) (open).

Discussion

We developed a method whereby characteristics of granulosa cells could be analysed in a fresh and untreated patient's sample without physically separating them. With this method CD45 was used as a marker for leukocytes to distinguish them from granulosa cells for which there is an important size overlap (De Neubourg *et al.*, 1996). Red blood cells were lysed during the staining procedure and the few large vaginal epithelial cells could be excluded because of their high scatter. With this method we were able to detect IGF-Ir on granulosa cells from follicular fluid with flow cytometric analysis using an antibody directed against the α subunit of the IGF-I receptor. Using standard beads with fixed amounts of binding sites we were able to convert the fluorescence signal to number of binding sites per cell. This is to our knowledge the first report of quantification of IGF-I receptors in human lutein-granulosa cells.

Davoren *et al.* (1986) showed that in the rat there were 3259 ± 662 binding sites for ^{125}I-IGF-I per granulosa cell. These high affinity, low capacity binding sites for IGF-I had lower affinities for IGF-II and insulin. In the human, Gates *et al.* (1987) suggested the presence of IGF-I binding sites in

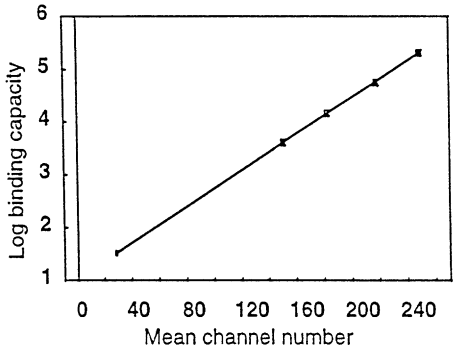

Figure 3. Binding capacity calibration curve, using simply cellular microbeads stained with anti IGF-I receptor antibody and GAM-PE. Mean fluorescence of each bead population plotted against the corresponding antibody binding capacity (r = 0.998). Logarithmic binding capacity α molecules.

granulosa cells from follicular fluid after follicular stimulation with ^{125}I-IGF-I, but these binding studies were conducted in the presence of isotonic sucrose solution and 0.1% of bovine serum albumin which do not resemble the in-vivo conditions. However, initially the same group of investigators (Poretsky *et al.*, 1985) were unable to prove ^{125}I-IGF-I binding to

granulosa cells in physiological assay conditions. Immunohistochemistry studies using IGF-I receptor antibody (αIR3 clone) in the human have shown its presence in antral follicles. Immunoreactive granulosa cells were scattered within the follicular wall with an apparent concentration in the cumulus in follicles >6 mm (Balboni *et al*., 1987). The number of immunoreactive cells in each follicle was variable. Samoto *et al*. (1993) described moderate immunostaining of the IGF-I receptor in antral and pre-ovulatory follicles. IGF-Ir mRNA and its protein are present in granulosa cells with increasing expression in the dominant follicle (El Roeiy *et al*., 1993).

All the follicular fluid from dominant follicles was pooled per patient, so the value for the number of binding sites per granulosa cell from dominant follicles is an overall assessment for the patient rather than per follicle. We could not detect a correlation between oestradiol concentrations on the day of HCG administration and number of IGF-I receptors; neither was there a correlation between number of ampoules of HMG, duration of HMG treatment or number of oocytes and IGF-Ir quantity.

The use of this dual labelling technique enables us to analyse a whole range of parameters on granulosa cells with minimal manipulation of the cell population. Only by comparison with IGF-I receptor content in granulosa cells from unstimulated cycles with the same timing or during follicular phase in stimulated and unstimulated cycles will it be possible to draw conclusions about the role and dynamics of the IGF-I receptor.

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