Human granulosa cells in vitro: Characteristics of growth, morphology and influence of some cytokines on steroidogenesis*

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Granulosa cells (GCs) were characterised morphologically by light and electron microscopy. The steroidogenic capability of GCs in vitro was estimated by radioimmunoassay (RIA): oestradiol (E₂), progesterone (P) and androstenedione (A) secreted into the culture medium were measured. The influence of several culture media and anchorage of the cell either to plastic vessels (monolayer) or to collagen fibrils (in gel) were studied. As the various culture media were assayed with regard to their suitability for IVF, it was found that Ham's F10 is quite satisfactory (in agreement with other observations on embryo cultures). A chemically defined medium BM 86 was found to be inadequate. In addition to the two cell types which are known, a third cell type which can perform efficient aromatisation (E₂ production) in vitro is characterised here. The influence of cytokines/growth factors (GF) like insulin-like GF (IGF-I), epidermal GF (EGF), platelet-derived GF (PDGF) and fibroblast GF (FGF) on steroidogenesis was tested either alone or with human chorionic gonadotrophin (hCG). Except for oestradiol (E₂) from early GCs, hCG generally stimulated progesterone (P) and E₂ secretion. EGF by itself enhanced the secretion of P but not of E₂. EGF did not affect hCG stimulation of P, but reduced that of E₂. In contrast, in pre-ovulatory GCs IGF-I reduced the stimulatory effect of hCG on both E₂ and P. In early GCs IGF-I potentiated hCG stimulation of P. In early GCs, neither hCG nor IGF-I nor a combination of IGF-I with hCG had any effect on E₂ production.

The development of ovarian follicles (folliculogenesis) is a highly complex physiological process which involves not only several hormones of the endocrine system but also locally acting autocrine/paracrine growth factors which are known as cytokines. The influences of some of these on the growth and steroid producing behaviour of granulosa cells (GCs) of a few animal species have variously been reported. With the practice of in vitro fertilisation (IVF), human GCs have become available for experimental studies and current interests also focus on the role of cytokines as modulators of ovarian function. Despite a large number of reports, a clearcut picture has yet to emerge on the regulatory mechanisms involved in the triggering of the primordial oocyte to folliculogenesis, selection of the dominant follicle from the cohort of antral follicles and its development until ovulation, followed by luteinisation. GCs utilised in most experimental work have been obtained from Graafian Follicles and in the case of human GCs they are generally pre-ovulatory GCs. Thus, findings from such experimental studies represent temporal specificities in a much broader spectrum of events.

Steroidogenesis is regulated by gonadothropins. Morphologically GCs are peculiar because they demonstrate histological ambivalence. Until luteinisation the GCs are true epithelial cells delimited by a basement membrane. On luteinisation, the basement membrane is destroyed. Vascularisation of the granulosa follows and the tissue trans-differentiates. The resulting granulosa lutein cells acquire an altered phenotype and a new steroidogenic profile.

GCs can grow in culture without any need of special growth factors. Pre-luteal GCs in culture have been reported to show a limited proliferative potential of about one population doubling (PD). Pre-luteal GCs may, thus, represent another peculiarity since somatic euploid cells, excepting cardiomyocytes, generally have a large proliferative potential (50-70 PDs) in culture. The proliferative activity to which the granulosa cells are subjected during their growth from a primordial to a pre-ovulatory Graafian follicle could plausibly be the cause of proliferative exhaustion.

In vitro studies usually employ monolayers, which is the growth of cells on a two-dimensional growth surface. Under monolayer conditions, two populations of distinct morphological types have been described. The reported cell types are essentially a stellate fibroblastoid type and a polygonal epithelioid type. In collagen gels - which permit growth in a

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of the University. The patients follicular aspirates for oocyte retrieval from about were hyperstimulated with human menopausal fibroblast growth factor (FGF) was suggested to either as monolayers or in collagen gels are presented. Different culture conditions is discussed in connection induce intraovarian oocyte maturation and to FGF and PDGF on the biosynthesis of steroids in pre-ovulatory human GCs cultured as monolayers are with their steroidogenic behaviour. Materials and Methods

Granulosa cells (GCs)

Pre-ovulatory granulosa cells were obtained from follicular aspirates for oocyte retrieval from about 40 patients who opted for assisted reproduction at the Gynaecological Clinics of the University. The patients were hyperstimulated with human menopausal gonadotrophin (hMG). Ovulation was induced by injection of 10000 IU of hCG. GCs from Graafian follicles from natural cycles were obtained from 13 patients with regular cycles who underwent laparoscopy because of benign gynaecological ailments between day 7 to 13 of cycle. These patients gave informed consent to follicular aspiration.

Separation and culture of GCs

Follicular aspirates were centrifuged 10 min at 175 g. The cell pellets were resuspended in cold phosphate buffered saline (PBS), pooled and layered over 45% Percoll (Pharmacia, Sweden) in PBS and recentrifuged 20 min at about 700 g. When present, red blood corpuscles (RBC) pelleted at the tip of the conical tubes while the intact GCs settled at the interphase of the supernatant and Percoll. The GCs were picked with Pasteur pipettes, washed in PBS, spun down and counted in Neubauer Cytometers after resuspension in a small volume of PBS and staining with trypan blue (TB). Approximately 5x10^7/0.5 ml viable GCs (TB exclusion was 70 to 95%) in culture medium were plated into multiwell (growth surface 1.5 cm^2) plastic culture dishes (Nunc, Denmark).

Culture media

Several commercially available culture media were tested for their adequacy in supporting GC cultures. With the exception of one chemically defined medium BM 86 (Boehringer Mannheim, Germany) which was obtained ready for use, all other media were made by dissolving the respective powders in distilled water of injection quality. The maximal storage time under refrigeration was 4 weeks. The only supplements to the media (excepting BM 86) were 10% foetal calf serum (FCS) and antibiotics (100 IU/ml penicillin + 100 μg/ml streptomycin). The following media: MEM with Earle's salts, Ham's F10, RPMI 1640, Medium 199, McCoy's 5a and BM 86 (all from Boehringer Mannheim, Germany) were bicarbonate-buffered and were, therefore, kept in an atmosphere of 5% CO2 in air. Two other media CMRL 1415 and Leibowitz 15 (Seromed/Biochrom, Berlin,Germany) were amino acid buffered and were, therefore, kept in air. All cultures were incubated at 37° C with saturated humidity.

For collagen gel cultures, only Ham's F10 medium was employed, after it was established that this medium performed better than any other. Rat tail collagen solution 2 mg/ml in 0.1% acetic acid was obtained from Serva, Heidelberg, Germany. The cultures were set by mixing at ice-bath temperature 7
parts of collagen solution, 1 part of 10x concentrated Ham's F10 and 2 parts NaHCO₃ (11.76 mg/ml). GCs suspended in FCS were incorporated by replacing 1 part of the mixture with FCS containing the cells. Thus, the final gel cultures contained 10% FCS.

Stimulation of GC cultures
The influence of hCG on steroidogenesis was tested. A 100x concentrated hCG (Pregnesin, Serono, Germany) stock solution in PBS was made and kept at -20°C. After dilution in culture medium hCG was used at a concentration of 10 IU/ml.

Radioimmunoassay (RIA)
The steroidogenic activity of the GC cultures was monitored through daily estimations of oestradiol (E₂), progesterone (P) and androstenedione (A) secreted into the culture medium. The concentrations of these hormones in spent medium was measured by radioimmunoassay (RIA) using commercially available RIA kits (Biermann, Bad Nauheim, Germany). ¹²⁵I-activity was measured in a well-type crystal scintillation counter (LKB, Sweden).

Microscopy
Routine inspections of GC cultures were done with an inverted microscope equipped with phase contrast optics and a camera for microphotography. For transmission electron microscopy (TEM), cultures were fixed with 3% glutaraldehyde in cacodylate buffer at pH 7.4 containing 3% sucrose. Post-fixation in 2% osmic acid was followed by dehydration in ethyl alcohol. Specimens were embedded in araldite. Thin sections cut with a Reichert ultramicrotome using either glass knives or a diamond knife, were contrasted with uranyl acetate and lead citrate and examined in a Zeiss EM 9 electron microscope at 60 kV.

Results
General features of GCs in monolayers
After plating GCs attached within 24 hr. The GCs acquire stellate to elongated fibroblastoid shapes depending on density. Isolated single cells tend to be stellate while those in low density cultures displaying cell to cell contact appear elongated. Early cultures showed predominantly one or the other cell form, depending on dispersion. Fig. 1A shows such an early culture of elongated cells in loose contact. The inset demonstrates stellate cells. The predominantly stellate cells seen in Fig. 2A, despite cell to cell contact, are the result of hCG treatment.

Often (in about 85% of cases), beginning about the third to fourth day of culture (or later), isolated clusters of actively proliferating oval or polygonal epithelioid cells appeared (Fig. 1B) thus representing a second population. Active proliferation could be documented through the rapid increase in numbers as well as the presence of mitotic cells. The origin and hormonal relevance of these cells is unclear and will be discussed. Only rarely, a third cell type could be identified. These cells were in morphology and growth pattern indistinguishable from diploid human fibroblasts (HDF) in culture (Fig. 1C). Owing to possible follicular mural ruptures during flushing (for oocyte retrieval), the presence of fibroblasts in the aspirates may be expected. However, the steroidogenic activity of these cells excludes this possibility.

Longer culture duration led to hypertrophy and loss of filopodia. Excepting the larger size, such GC cultures (Fig. 1D) resembled a senescing phase III HDF culture. When the second epitheloid cell type (Fig. 1B) appeared, they overgrew the fibroblastoid type.

The fine structure of monolayer GCs seen under TEM usually showed cytopathological features. The presence of condensed mitochondria, vacuoles and areas of rarified cytoplasm were typical. The vacuoles probably contained lipids which got lost during processing for TEM (see Fig. 2B and C). Completely degenerated cells were frequently observed. The presence of smooth endoplasmic reticulum (SER) was difficult to detect in monolayer GCs. The nuclei were predominantly euchromatic with only scarce peripheral heterochromatin. The presence of stress fibres (Fig. 2D) is typical for monolayer GCs.

Influence of culture media on steroidogenesis
The synthesis of androstenedione (A), progesterone (P) and oestradiol (E₂) was detected through the presence of these hormones in the culture medium. The kinetics of A and P synthesis were closely correlated. Fig. 4 shows daily concentrations of P in the different culture media which were tested. The curves for A production are not shown since the kinetic pattern is identical to that for P. Both A and P production increased during the first three days of culture, reaching peak values about the third day, and declining to base-line values by the end of the first week. Noteworthy, was the difference between the performances of two culture media: Ham’s F10
Fig. 3—GCs in collagen gel culture. (A) Phase contrast microphotograph showing a typical GC collagen culture. The inset shows a single cell with arborescent filopodia. (B) TEM of a gel GC. The relative absence of cytopathological features is conspicuous. The arrows indicate intercellular contacts (x2500). (C) TEM of a GC in collagen gel showing smooth endoplasmic reticulum (arrow heads) and tubular mitochondria. L = lipid droplet, N = nucleus and arrow points to filopodial contact (x8000). (D) TEM of a native GC obtained from the follicular aspirate. The condensed mitochondria is attributable to unavoidable time lags before cells from the aspirate can be processed for TEM (x8000).

[Upper panel] Fig. 1—Phase contrast microphotographs of monolayer cell types grown from follicular aspirates. Arrow heads point to mitotic cells. (A) A typical early GC culture 24 to 48 hr after plating. The cells are in loose contact and represent the primary GC population. The inset shows isolated stellate cells in early culture. (B) Oval or polygonal cells which appear later. (C) HDF-like cells which also appear later. (D) Typical late cultures (about 2 weeks). The cell type here belongs to the primary population shown in A. The inset depicts isolated late cells which have hypertrophied. Microscope magnification was 20 X 10.

[Lower panel] Fig. 2—Primary GC type in monolayers. (A) Altered morphology upon hCG treatment: lamellipodia are retracted and replaced by filopodia. The inset shows the altered morphology after treatment with hCG either in combination with IGF-I or EGF. Microscope magnification was 20 X 10 (B) TEM overview of monolayer GC (x1800). (C) TEM of monolayer GC showing cytoplasmic details. Mitochondria (M) are condensed; lipid droplets are typically lost leaving vacuoles (L) (x8000). (D) Late monolayer GCs hypertrophy and often reveal extensive stress fibres (arrow head) (x1800).

showed high P and A levels with rather broad peaks while BM 86, which is a chemically defined medium (i.e., no serum supplementation) specially designed for the culture of mammalian cells (particularly hybridomas), performed exceedingly poorly owing to cell death. Cell growth and hormone biosynthesis could, however, be restored (not shown), by supplementing BM 86 with 10% FCS.

The synthesis of E2 dropped drastically within 48 hr of culture (see Fig. 5). The inverse levels of P and A imply the inability of the cells to synthesise aromatic precursors of E2, also shown by the fact that the initial E2 levels for the individual media corresponded approximately to those of final P and A. The steroidogenic activity of monolayer GCs is easily stimulated by the addition of hCG (10 IU/ml) to the culture medium (see Fig. 6 for P and Fig. 7 for E2). When on rare occasions the third cell type (vide supra), whose morphology was indistinguishable from typical human diploid fibroblasts (HDF), appeared in cultures, a resurgence of E2 levels was measured. These cells were initially seen at about the seventh or
eight day (later than the oval/polygonal ones). The steep rise in E2 seen at the beginning of the second week (see Fig. 8) could be explained on the basis of the exponential increase in cell number. P and A levels were less dramatically affected. This reactivation of aromatisation was observed only in medium 199, MEM and McCoy, not in the other media that were tested. The co-culture of GCs with a strain of HDF (IMR-90) did not mimic this effect.

**GC morphology in collagen gels**

In collagen gels, GCs expressed a completely different phenotype. As shown in Fig. 3A the cells showed ramified filopodia. The perinuclear cytoplasmic volume became greatly reduced presumably due to the extensive formation of filopodia. No cellular hypertrophy was noticeable with increasing age of culture. Although collagen gel cultures always appeared scanty in cell numbers when compared to monolayers for the same initial plating, cell counts performed after enzymatic resuspension of the GCs revealed the same numbers for both gels and monolayers. Thus, the apparent density was the result of altered spatial distribution.

TEM fine structure (Fig. 3B & C) was free of cytopathological alterations, indicating healthy cells. The mitochondria were not condensed and lipid droplets which are typical for pre-luteinised GCs remained intact (cf. within Fig. 3 panels B & C with D which shows a native GC from an aspirate). Rough endoplasmic reticulum (RER) and SER were clearly identifiable, and SER predominated both here and in native GCs. Further, the mitochondria seen in Fig. 3C are of the tubular type characteristic of steroid producing cells. In short, it can be said that the fine structure of gel culture GCs was closely akin to that of native GCs. Frequent cell to cell contacts and the presence of gap junctions were observed.

**GC steroidogenesis in collagen gels**

Figures 6 and 7 show the comparative hormone production of P and E2 for GCs as monolayers or in gel cultures, respectively. Ham's F10 medium was used for all gel cultures. As in the case of monolayers, the kinetics of A and P were identical and are, therefore, not shown.

The addition of hCG to monolayer cultures stimulated hormone production significantly. Although a similar trend was observed also for GCs in collagen gels, the increase was statistically not significant. The major difference between the steroidogenic activities in monolayers and collagen
gels was the significantly higher baseline hormone production levels of the gel cultures.

**Influence of some cytokines on steroidogenesis**

In monolayer cultures, at a concentration of 25 ng/ml culture medium, IGF-1 alone did not affect the steroid biosynthetic activity of pre-ovulatory GCs. As shown in Figs. 9 and 10 the secretion of P and E2, respectively, were both unaffected by IGF-1 alone. When IGF-1 was applied together with hCG, the stimulatory effect of hCG was reduced. In gel cultures, neither hCG alone (data not shown) nor in combination with IGF-1 (Figs. 9 and 10) affected hormone production significantly.

The temporal secretory pattern for both P and E2 from early GCs which were obtained from young follicles from natural cycles, was essentially the same as that of pre-ovulatory GCs, as shown in Figs. 11 and 12. hCG could only slightly stimulate P secretion but not that of E2. Neither P nor E2 secretion was influenced by IGF-1 alone. In combination with hCG, IGF-1 provoked a significant elevation of P secretion (Fig. 11). This was accompanied by morphological change to a predominantly fibroblastoid phenotype with bipolar cells, similar to the change observed for hCG and EGF acting together as shown in the inset in panel A, Fig. 2. EGF stimulated P secretion (Fig. 11). The level of EGF stimulation was lower than that of hCG stimulation and EGF did not interact with hCG.

The level of EGF stimulation was significant from day 5 to 10. EGF alone or in combination with hCG inhibited E2 secretion (Fig. 12).

FGF and PDGF were tested at concentrations of 1 and 5 ng/ml culture medium (data not shown). Both of these growth factors did not affect P and E2 secretion, respectively, in pre-ovulatory GCs. Morphological effects were also absent.

**Discussion**

The presence of two cell types in cultured GCs from pre-luteinised human follicles is known from the literature. We have, in addition, observed a third cell type whose unique E2 biosynthetic potential was maximally expressed in Medium 199. Whether follicular GCs in vivo consists of a homogenous or a mixed population of cells is not definitely known. The phenotype of the major population of these cells in culture (stellate and elongate cells) is not typical of epithelial cells. No attempt has been made to demonstrate the secretion of a basal lamina or laminin in GCs cultured from pre-luteinised follicles, although Schmidt et al demonstrated in 1984 the presence of Call-Exner Bodies. If the features described here for the major cell population is representative of luteinised cells, then the oval or polygonal subpopulation may be regarded as the non-luteinised fraction or, in other words, the true epithelial GC.
component observed in culture. As luteinisation progresses, this fraction vanishes. Despite active steroidogenesis, the fine structure of the oval cells does not demonstrate the features regarded as typical for luteinisation i.e. the accumulation of lipids and the abundance of SER. Basing on ultrastructural features, these cells would not be considered to be steroid producing cells.

Despite its spectacular ability to carry out aromatisation in medium 199, the third cell type described here is also devoid of lipid droplets and SER. Obviously, GCs are able to realise their steroidogenic potential through an alternative pathway. The mitochondria, instead of the SER, might serves as the site of synthesis. It is also worthy of mention that mitochondrial pleomorphism was frequently observed in the GCs. It may be said that both the oval and HDF-like cells display an abundance of structurally intact mitochondria. The origin of the HDF-like cells may be disputable and it remains to be shown whether these cells are GCs or cells of thecal origin.

Despite interest in the sustained production of E2 in chemically defined culture medium, no other study has compared the influence of culture media on the steroidogenic activity of GCs. The interesting finding on the restoration of aromatisation, i.e. E2 production in the presence of a third population of cells, in certain culture media, should be critically appraised with regard to composition. A perusal of the components of medium 199, MEM and McCoy draws attention to the higher concentrations of Ca^{2+} in these media. The importance of calcium in steroidogenesis has been discussed by others. Interestingly the relative levels of Ca^{2+} correlates to E2 production. As molecular O_2 is known to be essential for the process of aromatisation, the availability of O_2 ought to be investigated in view of the high levels of P and A.

Fig. 11—Comparison of the influences of EGF and IGF-1 either alone or in combination with hCG on the secretion of progesterone in early GCs in monolayer cultures. [(a) HAM’s F 10, (b) HAM’s F 10 + hCG (10 IU/ml), (c) HAM’s F 10 + EGF (2 ng/ml), (d) HAM’s F 10 + hCG (10 IU/ml) + EGF (2 ng/ml), (e) HAM’s F 10 + IGF (25 ng/ml), (f) HAM’s F 10 + hCG (10 IU/ml) + IGF (25 ng/ml)]

Fig. 12—Comparison of the influences of EGF and IGF-1 either alone or in combination with hCG on the secretion of oestradiol in early GCs in monolayers. [(a) HAM’s F 10, (b) HAM’s F 10 + hCG (10 IU/ml), (c) HAM’s F 10 + IGF (25 ng/ml), (d) HAM’s F 10 + hCG (10 IU/ml) + IGF (25 ng/ml), (e) HAM’s F 10 + EGF (2 ng/ml), (f) HAM’s F 10 + hCG (10 IU/ml) + EGF (2 ng/ml)]
Follicular growth is the nett result of a concerted action of several concomitant factors in addition to gonadotrophins.

Of the four frequently studied cytokines that have been tested here, two of them viz. FGF and PDGF showed absolutely no effects with regard to steroidogenesis and morphology. Owing to the already mentioned complexity of granulosa development during folliculogenesis, a possible involvement of these two growth factors at some other developmental stage cannot be ruled out, even if unlikely, because of the epithelial nature of the granulosa until luteinisation commences, which is at or about the time of ovulation. Thereupon, the granulosa transdifferentiates into a non-epithelial tissue and sensitivity to FGF and PDGF could develop.

Conversely, GC reaction to EGF is only to be expected. The nature of this reaction is, nonetheless, interesting. It is the only growth factor of the four frequently studied cytokines that have numbered from 1 to 5, the data here on IGF-1 shows that IGF-1 by itself does not influence steroidogenesis. It also by itself did not provoke morphological change. This was the case for both pre-ovulatory as well as early GCs from developing follicles. However, IGF-1 consistently interacted with hCG in that it altered hCG effects. The nature of this interaction depended on the age of the GCs. In mature GCs from the pre-ovulatory follicles IGF-1 generally inhibited hormone production, but in young GCs, the reverse was the case. This enhancing effect was, however, selective and restricted only to P secretion.

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References