Expression of Integrin Fraction and Adhesion Molecules on Human Granulosa Cells and Its Relation with Oocyte Maturity and Follicular Steroidogenesis

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Purpose: To study the correlation between the expression of integrin fractions and adhesion molecules on granulosa cells (GC) and follicular development.

Methods: GC and follicular fluid (FF) were obtained at oocyte retrieval for ICSI. Expression of adhesion molecules on GC was studied by flow cytometry. Statistics were evaluated using the Student *t* test and simple linear regression analysis.

Results: $\alpha 5$ integrin fraction was significantly ($p < 0.01$) higher, while $\alpha 6$ fraction and CD9 were significantly ($p < 0.01$ and $p < 0.001$, respectively) lower in GC from FF with metaphase II oocytes. A direct significant correlation was observed between FF progesterone and the $\alpha 5$ expression on GC ($r = 0.54$). In contrast, an inverse significant correlation was observed between FF progesterone level and the expression of $\alpha 6$ and CD9 ($r = -0.40$ and -0.41 , respectively).

Conclusions: The expression pattern for integrin fractions and adhesion molecules may be of predictive value in assessing the state of differentiation of the human follicle.

KEY WORDS: Adhesion; flow cytometry; granulosa; integrin; steroidogenesis.

INTRODUCTION

Ovarian granulosa cells (GC) are unique endocrine cells. In growing follicles, GC proliferate and achieve functional maturation, secreting estrogens and functioning as regulators of oocyte maturation. After ovulation, GC differentiate morphologically and functionally into luteal cells, which mainly secrete progesterone. If implantation occurs, they differentiate further into the corpus luteum of pregnancy, but if not, they fall into luteolysis. Follicular differentiation is mainly regulated by pituitary and chorionic gonadotropins under the control of an endocrine

mechanism. Several growth factors and sex steroid hormones are considered to have various effects on individual follicular development by autocrine and/or paracrine manners. However, the precise local mechanisms of follicular differentiation remain to be clarified (1).

Integrins appear to be the major receptors by which cells attach to extracellular matrices, and they also have a role in signal transduction (2). It has been reported that membrane proteins are differentially and stage-specifically expressed on human GC, and therefore their involvement in folliculogenesis and GC function is very possible (3–6).

Studies have found the expression of integrins and adhesion molecules in GC (3, 5–7) and these molecules have been shown to regulate cellular function in GC and other cellular types (8). There are some integrins with specific functions in GC, like the integrin fraction $\alpha 5$ which is involved in corpus luteum

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formation via interaction with its ligand fibronectin (7) or the integrin $\alpha 6 \beta 1$ which regulates the luteinization of human GC (9).

This accumulating evidence for a functional significance of adhesion molecules in GC and follicular function led us to study the correlation between the expression of integrin fractions ($\alpha 2$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$) and adhesion molecules (CD9, CD47, CD56) in GC by flow cytometry and oocyte maturity, fertilizability, and follicular steroidogenesis.

MATERIAL AND METHODS

Granulosa cells were obtained from a total of 69 follicles from 57 couples with male infertility. Repeated semen analyses for the husband were determined as pathological according to World Health Organization recommendations (World Health Organization, 1999). All women had regular menstrual cycles and day 3 FSH and PRL and day 21 progesterone were normal. Tubal and uterus normality was based on hysterosalpingography and ultrasonography studies. The patients were aged from 24 to 35 years.

The women were downregulated with gonadotrophin releasing hormone agonist triptorelin (Decapeptyl 0.1, Lasa, Barcelona, Spain) for 3–5 weeks, starting on cycle day 21 until the administration of FSHr, when the doses were reduced by half. After 10–14 days they received FSHr (Gonal F., Serono, Madrid, Spain) until the follicles reached maturity. When more than three follicles had a diameter of >18 mm, ovulation was stimulated by the administration of 5000 IU of human chorionic gonadotrophin (hCG) (Profasi HP, Serono, Madrid, Spain). Oocyte retrieval was performed by transvaginal needle-guided ultrasound aspiration 36 h after hCG injection. Each follicle was systematically washed in order to avoid any spill to the next follicle. Oocytes were enzymatically denuded of cumulus cells with a solution containing 80 IU hyaluronidase/mL (HYASE, IVF Science Scandinavia, Gothenburg, Sweden). Corona radiata cells were removed mechanically with the fire-polished tip of a Pasteur pipette. Oocyte maturity was judged by morphological criteria. The oocytes were always kept individually. Only follicles with metaphase I or II oocytes were analyzed. Germinal vesicle stage oocytes were excluded from the study. The ICSI procedure was carried out only in morphologically normal metaphase II oocytes that had extruded the first polar body. Motile spermatozoa were selected by the Swim-up procedure, which consists of washing the

semen with the Gamete medium (IVF Science Scandinavia, Gothenburg, Sweden) by centrifugation at 300 g. The supernatant was discharged and in the same tube 1 mL of the medium was added and left standing for 30 min to allow the sperm to swim up from the pellet. Just before injection 2 μ L of the prepared sperm suspension was added to 5 μ L of a polyvinylpyrrolidone solution (PVP) (ICSI, IVF Science Scandinavia, Gothenburg, Sweden) to slow down the spermatozoa. Only one spermatozoon was aspirated, tail first, from the PVP solution and was injected into the cytoplasm of the oocyte through the zona pellucida. The injection pipette was withdrawn slowly from the oolema and the oocyte was placed in 0.7 mL of IVF medium (IVF Science Scandinavia, Gothenburg, Sweden) on a 4-well dish, one oocyte per well. Between 16 and 20 h after the ICSI procedure, oocytes were observed under a stereomicroscope (Eclipse TE-200, Nikon, Tokyo, Japan) for the presence of pronuclei and polar bodies.

After removal of the oocyte from the aspirated FF, it was centrifuged for 5 min at 400 g, and the supernatant was collected and stored at -80°C until its analysis. The pelleted cells were resuspended in phosphate-buffered saline (PBS), and were carefully overlaid on Ficoll-Hypaque (Sigma Diagnostics, St Louis, USA) and centrifuged at 400 g for 30 min. Granulosa cells were collected from the interphase and washed twice with PBS.

Flow Cytometric Analysis of Adhesion Molecule Expression

Expression of adhesion molecules on the GC surface was analyzed by direct immunofluorescence. The isolated GC were incubated with 5 μ L of fluorescein (FITC) marked specific mAbs (anti-integrin $\alpha 2$, anti-integrin $\alpha 5$, anti-integrin $\alpha 6$, anti-integrin $\beta 1$, anti-integrin $\beta 3$, anti-CD9, anti-CD47, and anti-CD56) and 5 μ L of ficolitrin (PE) marked mAb anti-CD45 (Cytognos, Salamanca, Spain) for 30 min at 4°C in the dark. After washing in PBS, the cells were resuspended in the same solution and viable cells were analyzed by flow cytometry (Cytoron Absolute, Ortho, Madrid, Spain) (Fig. 1). The Ortho Control reactive (Ortho, Madrid, Spain), which consists of an isotype mouse mAb that is not reactive to any human component, was used as negative control. It constitutes a quality control the negativity of which guarantees the absence of autofluorescence of the cells. As positive control we used human lymphocytes stained according to the technique described. The cursor used in

Fig. 1. Expression of integrin $\alpha 6$ (FITC fluorescence) in granulosa cells (CD45 –) measured by flow cytometry.

flow cytometry separates the cells that present the expression of the molecule studied and the cells that do not present this molecule on their surface. This cursor was placed so that <2% of the events in the isotype negative control were considered positive.

Assay of Steroid Hormones in FF

The FF concentrations of estradiol and progesterone were determined by enzyme linked fluorescent assay (Biomerieux, Marcy-l'Etoile, France). Intra- and interassay coefficients of variation for both assays were <10%. Levels of testosterone in FF were measured by commercial radioimmunoassay kits (Orion Diagnostica, Espoo, Finland). Intra- and interassay variations were 7.4 and 9.2%, respectively.

Statistics

Shapiro-Wilk's test was used to test whether data were normally distributed. Statistics were evaluated using the Levenne test to check the variance homogeneity. If this test was significant the Welch test was used to compare the means, and if not, the student *t* test was used. Simple linear regression analysis (Pearson coefficient) was used to show the degree of linear association between the expression of adhesion molecules in GC and the hormones studied.

RESULTS

Expression of Integrin Fractions and Adhesion Molecules in GC

Flow cytometry showed that the $\alpha 6$ and $\beta 1$ integrin fractions, CD9 and CD47 are expressed on 70–80% of the studied GC. This high expression contrasts with that of the $\alpha 2$, $\alpha 5$, and $\beta 3$ integrin fractions and CD56, which are expressed in a lower percentage of GC.

Table I shows that the expressions of the $\alpha 5$ and $\alpha 6$ integrin fractions and CD9 are significantly different between follicles with metaphase I oocytes and those with metaphase II oocytes. While $\alpha 5$ integrin fraction expression was significantly ($p < 0.01$) higher in GC from FF with metaphase II oocytes, the $\alpha 6$ fraction and CD9 were significantly ($p < 0.01$ and $p < 0.001$, respectively) lower in these follicular types. Flow cytometry histograms of integrin fraction expression in GC are shown in Fig. 2, while histograms of adhesion molecules CD9, CD47, and CD56 are shown in Fig. 3. There was no difference in the expressions of the $\alpha 2$, $\beta 1$, $\beta 3$ integrin fractions and CD56 in GC from FF with metaphase I oocytes and those which oocytes were in the metaphase II stage. There was no difference in the expressions of the adhesion molecules

Table I. Relationship Between Expression of Integrin Fractions and Adhesion Molecules in GC and Oocyte Maturity

	Metaphase I <i>n</i> = 18	Metaphase II <i>n</i> = 48	<i>t</i> _{exp}	<i>p</i>
$\alpha 2$	48.7 $\pm$ 3.4 (32.4–66.9) ^a	55.3 $\pm$ 3.1 (27.9–93.0)	0.93	ns
$\alpha 5$	39.6 $\pm$ 3.6 (12.0–61.1)	54.4 $\pm$ 3.1 (19.5–84.0)	2.76	$p < 0.01$
$\alpha 6$	83.8 $\pm$ 2.2 (70.6–92.7)	73.0 $\pm$ 3.0 (33.3–96.0)	2.14	$p < 0.01$
$\beta 1$	84.4 $\pm$ 3.1 (59.6–65.0)	83.5 $\pm$ 1.8 (49.6–97.0)	0.37	ns
$\beta 3$	24.8 $\pm$ 3.3 (10.6–46.2)	32.3 $\pm$ 2.1 (7.3–58.0)	1.92	ns
CD9	83.6 $\pm$ 2.4 (63.1–99.0)	72.8 $\pm$ 1.6 (57.8–96.0)	3.58	$p < 0.001$
CD47	77.4 $\pm$ 3.2 (54.3–92.7)	73.1 $\pm$ 1.9 (51.3–88.0)	1.15	ns
CD56	1.7 $\pm$ 0.4 (0.5–4.3)	2.2 $\pm$ 0.5 (0.1–7.0)	0.66	ns

^a Values are mean $\pm$ SEM. Numbers in parentheses are minimum and maximum.

Fig. 2. Comparative flow cytometry histograms showing the expression in GC of some integrin fractions according to the maturity of the associated oocyte. The cursor was set on the isotype control.

Fig. 3. Comparative flow cytometry histograms of the expression in GC of the adhesion molecules CD9, CD47, and CD56 according to the maturity of the associated oocyte. The cursor was set on the isotype control.

Table II. Relationship Between Expression of Integrin Fractions and Adhesion Molecules in GC and Oocyte Fecundability

	Unfertilized $n = 13$	Fertilized $n = 33$	t_{exp}	p
$\alpha 2$	55.9 ± 4.6 (33.2–82.4) ^a	51.4 ± 3.7 (27.9–93.0)	0.75	ns
$\alpha 5$	53.6 ± 4.9 (20.7–80.6)	54.0 ± 4.1 (19.5–84.0)	0.05	ns
$\alpha 6$	67.1 ± 5.4 (41.6–86.5)	75.7 ± 3.7 (33.3–96.0)	1.33	ns
$\beta 1$	81.6 ± 3.8 (49.6–96.5)	83.0 ± 1.9 (68.9–95.0)	0.36	ns
$\beta 3$	36.8 ± 3.0 (21.7–51.2)	30.5 ± 2.8 (7.3–58.0)	1.3	ns
CD9	72.3 ± 2.6 (65.2–86.7)	72.9 ± 2.1 (57.8–96.0)	0.17	ns
CD47	73.4 ± 4.3 (52.0–87.0)	73.9 ± 2.2 (51.3–88.0)	0.1	ns
CD56	1.4 ± 0.3 (0.5–2.9)	1.9 ± 0.6 (0.1–5.0)	0.61	ns

^a Values are mean $\pm$ SEM. Numbers in parentheses are minimum and maximum.

studied in GC from FF with fertilized oocytes and those with oocytes that remained unfertilized after ICSI (Table II).

Relationship Between Expression of Integrin Fractions and Adhesion Molecules in GC and Levels of Steroid Hormone in FF

We analyzed the correlation between expression of integrin fractions and adhesion molecules in GC and levels of steroid hormones in FF of stimulated cycles, finding that progesterone levels correlate significantly ($p < 0.001$) and in a direct way with the expression of the $\alpha 5$ integrin fraction in GC ($r = 0.54$). In contrast, progesterone levels correlate significantly ($p < 0.005$) but in an indirect way with the expression of the $\alpha 6$ integrin fraction and CD9 ($r = -0.40$ and -0.41 , respectively). No correlations were found between the expression of the remaining adhesion molecules and integrin fractions studied and steroid levels in FF (Table III; Fig. 4).

DISCUSSION

Integrins and adhesion molecules mediate cell contacts to the extracellular matrix and initiate intracellular signalling cascades in response to a variety of factors. These molecules play a role in controlling development, structural integrity, and tissue function. Despite their importance, little is known about the regulation of adhesion molecules in human ovaries and how they function in folliculogenesis. To determine their relevance to ovarian physiology, the present study was intended to investigate the correlation between the expression of integrin fractions and adhesion molecules in GC and follicular steroidogenesis, oocyte maturity, and fertilizability.

We found that the expression of the $\alpha 5$ integrin fraction is significantly higher in GC obtained from follicles in which the oocytes were at the metaphase II stage. Honda *et al.* (7) reported a higher expression of this integrin fraction in GC just before ovulation, when the oocyte is mature. Therefore the $\alpha 5$ integrin can be considered a differentiation marker of GC, due to its induced expression in these cells during ovulation. Honda *et al.* (7) showed that $\alpha 5$ integrin expression in GC cultivated with hCG was higher, which suggests that LH increases the expression of this molecule in GC. This hypothesis is supported by our finding of a direct relationship between progesterone levels in FF and expression of $\alpha 5$ in GC. Moreover, increased progesterone synthesis in cultivated GC stimulated with fibronectin, the ligand of the $\alpha 5$ integrin, has been reported (10). Therefore, we propose that the $\alpha 5$ integrin fraction is involved in GC differentiation during corpus luteum formation through its union with fibronectin causing an increase in GC progesterone production.

We examined the relationship between the $\alpha 6$ integrin fraction and oocyte maturity and observed that the expression of this molecule was significantly ($p < 0.01$) lower in follicles with metaphase II oocytes. These findings are not in agreement with those of

Table III. Relationship Between Expression of Integrin Fractions and Adhesion Molecules in GC and Steroid Hormones in FF

	E_2	Progesterone	Testosterona
$\alpha 2$	0.06	0.24	0.11
$\alpha 5$	0.34	0.54*	-0.13
$\alpha 6$	-0.19	-0.40**	0.09
$\beta 1$	0.13	0.11	-0.05
$\beta 3$	0.16	0.04	-0.15
CD9	-0.27	-0.41**	0.20
CD47	0.16	0.01	0.29
CD56	-0.29	-0.06	-0.04

* $p < 0.001$; ** $p < 0.005$.

Fig. 4. Relationship between expression of $\alpha 5$ and $\alpha 6$ integrin fractions and CD9 in GC and progesterone levels in FF.

some authors, who have observed an increase in the expression just before ovulation in women without stimulation of ovulation by immunohistochemistry (11,12). These discrepancies could be due to

the different mechanisms of intraovarian regulation that take place in women with or without stimulation of ovulation (13,14). Therefore $\alpha 6$ integrin can be considered a differentiation marker of GC,

due to its different expression at different follicular stages.

Granulosa cell luteinization is a process in which the capacity of progesterone production is augmented. The serum level of progesterone gradually increases until it reaches a peak 5–6 days after the LH surge. In this study, we found an indirect correlation between progesterone levels in FF and expression of $\alpha 6$ on GC ($p < 0.005$), which coincides with the increase in progesterone production by human GC when the interaction between the $\alpha 6\beta 1$ integrin and its ligand laminin is blocked (9). Therefore, it seems that the $\alpha 6\beta 1$ integrin expressed on GC interacts with laminin and suppresses the luteinization of GC, which may regulate the timing of the onset of the LH surge, thus preventing the premature luteinization of GC (15,16). These findings support the idea that the $\alpha 6$ integrin fraction is a local regulator of GC luteinization during the periovulatory phase.

The CD9 molecule is a cell surface glycoprotein which is reported to be involved in cell adhesion and migration (17). It is shown to be associated with other surface proteins and to influence their functions. Takao *et al.* reported that CD9 is associated with the $\alpha 6\beta 1$ integrin in GC (4), suggesting that CD9 is concerned with $\alpha 6\beta 1$ integrin functions, which may include the regulation of GC luteinization found in this study. By flow cytometry we have demonstrated that CD9 is present on the cell surface of 75.8% of GC, which coincides with the expression found by Takao *et al.* by immunohistochemistry (4). The expression of CD9 on GC was significantly ($p < 0.001$) lower in follicles with metaphase II oocytes. This expression of CD9 resembled that obtained in this study of the $\alpha 6$ integrin fraction. Although the relationships found do not coincide with those of the study carried out by Takao *et al.* (4), this can be explained as resulting from the utilization of immunohistochemistry, a technique that is less objective and precise than flow cytometry (18), and to the fact that the above authors studied GC from women without stimulation of ovulation.

The indirect relationship observed between the expression of CD9 on GC and the levels of progesterone in FF can be related to the association between $\alpha 6\beta 1$ integrin and CD9. Thus, we can conclude that CD9 and $\alpha 6$ integrin may be involved in the regulation of follicular luteinization, both molecules producing an inhibition of the luteinization of GC and preventing premature follicular luteinization.

We did not find any relationship between the $\alpha 2$ integrin fraction and oocyte maturity and follicular steroidogenesis. These results are in agreement with

those obtained by Yamada *et al.* who did not find any differences in the expression of the $\alpha 2$ fraction during follicular growth (3). However, the expression of this molecule changes during formation of the corpus luteum, which shows that $\alpha 2$ integrin can be considered a differentiation marker for luteinized GC (3). These authors also found an interaction between $\alpha 2\beta 1$ integrin and collagen IV, which is produced by GC. It has been postulated that $\alpha 2\beta 1$ integrin interacts with collagen IV in an autocrine way, modulating the differentiation of GC during corpus luteum formation. Therefore, although our results show that $\alpha 2$ integrin does not influence follicular physiology after hCG stimulation, we cannot discard the possibility that this molecule may play a role at other moments of ovarian physiology.

In relation to the $\beta 1$ integrin fraction, we did not find any differences between the expression of this integrin fraction and oocyte stages and steroid levels, which is in accordance with the results obtained by Honda *et al.* (11). It is possible that the $\beta 1$ integrin fraction has no specific function due to the union of this molecule to different α fractions.

We did not find any relationship between $\beta 3$ integrin expression on GC and oocyte stage and GC steroidogenesis. However, Makrigiannakis *et al.* showed that GC adhesion can be inhibited by the addition of anti-integrin $\beta 3$ monoclonal antibodies (6), which indicates that although there is no function of this molecule in follicular physiology after hCG stimulation, it can be of importance in the process of follicular remodelling.

In relation to the expression of CD47 on GC, we found no relationship with oocyte maturity. Higuchi *et al.* showed that CD47 expression does not change on the GC of growing and preovulatory follicles (5), which coincides with the results observed in our study. The low expression of CD56 on GC found in our study can be explained by the evidence that GC isolated from FF does not express CD56, while after cultivating these cells, increased expression is observed in GC, forming cellular aggregates (19). Therefore it has been postulated that the antrum formation requires a diminution in the adhesion properties of the adjacent GC (20), which might explain the low expression of CD56 on GC from FF found in the present study. Therefore, we conclude that the $\alpha 2$, $\beta 1$, and $\beta 3$ integrin fractions and the CD47 and CD56 adhesion molecules have no involvement in GC physiology after hCG administration, although we cannot rule out their importance at other follicular stages.

The absence of any correlation between integrin fractions and adhesion molecules expressed in GC and estradiol and testosterone levels in FF, and the significant correlation observed between these expressions and progesterone levels suggests that after LH surge progesterone production may have a more important role in ovarian physiology than estradiol or testosterone production. The discovery that the midcycle gonadotrophin surge stimulated the progesterone receptor in the luteinizing granulosa cells of the preovulatory follicle led to the hypothesis that periovulatory events previously attributed to LH are mediated or at least modulated by the intrafollicular action of progesterone (21).

Our results show that there is no relationship between the molecules included in the study and oocyte fertilizability. This can be explained because of the use of the ICSI technique, which omits some oocyte functions, such as the union to the spermatozoa, that could be altered by inadequate oocyte maturation.

In conclusion, the expression pattern for integrin fractions and adhesion molecules may be of predictive value in assessing the state of differentiation of the human follicle.

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