Soluble CD44 in Human Ovarian Follicular Fluid

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Purpose: *In the present study, we investigated the existence of soluble CD44 (sCD44) in human follicular fluid, the relationship between the concentration of sCD44 and that of other hormonal parameters, and the prognostic value of sCD44 in follicular fluid in in vitro fertilization (IVF) programs.*

Methods: *A total of 63 follicular fluid specimens from patients (n = 30) participating in our IVF programs was analyzed by RIA and enzyme-linked immunosorbent assay (ELISA).*

Results: *The mean concentration (±SE) of sCD44 in follicular fluid was 265.4 ± 7.8 ng/ml. The variation of the follicular fluid concentration of sCD44 was strictly associated with that of human chorionic gonadotropin (hCG) ($r = 0.572$, $P < 0.0001$). The mean concentration of sCD44 in follicular fluid was significantly higher in follicles containing subsequently unfertilized oocytes than that in those containing oocytes that had undergone fertilization ($P = 0.0428$). In the analysis of each follicle that contained an oocyte subsequently fertilized, the mean concentration of sCD44 was significantly higher in follicular fluid with the subsequently good-quality embryos than in that with the subsequently poor-quality embryos ($P < 0.05$).*

Conclusions: *These results indicated that the concentration of sCD44 in follicular fluid reflects the development of embryos derived from the same follicle, so the sCD44 in human follicular fluid may be useful in the assessment of the prognostic value of IVF programs.*

KEY WORDS: Follicular fluid; human chorionic gonadotropin; in vitro fertilization; soluble CD44.

INTRODUCTION

There is accumulating evidence of involvement of the structurally integrated immune system of the ovary and of inflammation-associated phenomena within ovulating follicles as potential physiological stimuli during follicular maturation and ovulation. Indeed, the ovulatory process has been interpreted recently as a kind of physiological inflammatory reaction characterized by infiltration of leukocytes and macrophages into ovulating follicles; ovarian release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, with their actions on granulosa and theca/interstitial cells; ultrastructural transformation of granulosa cells into macrophage-like phagocytic cells; and an increase in the prostaglandin level at the time of follicle rupture. In this context, recent findings have emphasized the specific role of adhesion-promoting receptors in the regulation of lymphoid–ovarian cell contact and interaction and granulosa/luteal cell differentiation (1–3).

CD44 is a cell surface transmembrane glycoprotein widely expressed in many types of cells. The wide distribution of CD44 expression, in addition to the highly regulated patterns of expression observed during functional maturation and cell differentiation, especially among lymphoid cells, suggests that CD44 may play a fundamental role in cell–cell and/or cell–matrix interactions that are important for cell differentiation and function (4). We demonstrated previously that the standard type of CD44 is expressed on luteinized granulosa cells, especially on cumulus cells in the follicle at the time of ovulation, and may play an important role in oocyte maturation by regulating the metabolic coupling of cumulus cells, mural granulosa cells, and oocytes (5).

CD44 on the cell surface, as well as some variant sequences generated by alternative splicing, are vulnerable to proteolytic cleavage. Therefore, CD44 not

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only exists as membrane-integrated molecules, but also is shed from the cell surface and thus occurs as soluble CD44 (sCD44) in body fluids. Measurement of sCD44 has been evaluated as a marker of disease activity in inflammatory diseases, such as rheumatoid arthritis and acute hepatitis (6, 7), and of tumor burden and metastasis in patients with cancer, such as lymphoma, ovarian cancer, and colon cancer (8–10). Elevated serum CD44 levels in these patients may be due to active shedding of CD44 molecules by the inflammatory cells and/or tumor cells.

Due to the similarities between the ovulatory and oocyte maturation processes and inflammation- or metastasis-associated reactions, we performed this study to investigate the prognostic value of sCD44, which may be shed mainly from granulosa cells into follicular fluid during these processes, in follicles of infertility patients.

MATERIALS AND METHODS

Patients and Stimulation Protocol

We studied follicular fluids obtained from a series of 30 patients who participated in in vitro fertilization (IVF) programs at Yamagata University Hospital, Yamagata, Japan. Patients with severe male factor were excluded because of the effects of this disease on fertilization. The patients gave their informed consent to participate in this study. Each patient was administered 600 μg of buserelin acetate/day (Suprecur nasal; Hoechst Marion Roussel, Tokyo) for pituitary desensitization beginning in the midluteal phase of the preceding menstrual cycle. Human menopausal gonadotropin (150 to 300 IU/day; Humegon; Sankyo, Tokyo) and/or pure follicle-stimulating hormone (FSH; Fertinom P; Serono, Tokyo) was administered starting on day 3 of the menstrual cycle. Human chorionic gonadotropin (hCG; 10,000 IU; Mochida, Tokyo) was administered when the leading follicle enlarged to a mean diameter of 15.5 mm or more. Thirty-five hours after administration of hCG, follicles were aspirated by transvaginal ultrasound retrieval (6.5 MHz; Mochida).

Oocyte Preparation, Culture, Insemination, and Embryo Transfer

Aspirated follicular fluids were transferred into tissue culture dishes (Falcon 3002; Becton Dickinson, Lincoln Park, NJ). Cumulus–oocyte complexes were isolated under a dissecting microscope (SZH-ILLB; Olympus, Tokyo) at 10–20× magnification and transferred into an organ culture dish (Falcon 3037; Becton Dickinson) with human tubal fluid (HTF) medium prepared at our institution (11). Oocytes were isolated mechanically from cumulus cell masses using 26-gauge needles. The semen was washed twice in HTF medium by centrifugation at 300*g*. The centrifugation tube was then left standing for 15 min to allow the sperm to swim up from the pellet. Then individual oocytes were inseminated with the washed sperm at concentrations of between 50,000 and 100,000 motile sperm per ml 3 hr after oocyte recovery. The individual gametes were cultured in 150 μl of HTF medium at 37°C in trimixture gas (5% CO_2, 5% O_2, and 90% N_2). Twenty hours after IVF, the individual oocytes were observed under a dissecting microscope, and the number of pronuclei was determined. Oocytes that had two pronuclei were defined as fertilized oocytes and cultured for an additional 24 hr. At the end of the culture period, the embryos were assessed morphologically as described previously (12). The good-quality embryos had equal blastomeres and no fragmentation. The fair-quality embryos showed $<$30% fragmentation of the embryo mass. The poor-quality embryos showed $\geq$30% fragmentation of the embryo mass. Embryos were transferred into the patients' uterine cavities approximately 42 to 48 hr after ovum capture.

Follicular Fluid Assay

Aspirated follicular fluid samples were stored at −20°C until assay. The levels of soluble CD44 were measured by sCD44std enzyme-linked immunosorbent assay (ELISA; Bender Med Systems, Bender, Vienna, Austria). This test is based on the immunological recognition of human standard sCD44 by murine monoclonal antibodies immobilized on microwell strips. The standard sCD44 assay has a limit of detection of 0.07 ng/ml, intraassay coefficients of variation ranging from 1.7 to 9.7% (average, 4.7%), and interassay coefficients of variation ranging from 2.6 to 5.1% (average, 3.8%).

Quantification of other hormonal concentrations was performed using commercially available immunoassay kits: E_2 by time-resolved fluoroimmunoassay (DELFIA Estradiol; Pharmacia, Tokyo, Japan), progesterone and free testosterone by RIA (DPC Progesterone Kit and DPC Free Testosterone Kit; DPC Corporation, Tokyo), and hCG by chemiluminescent

Table I. Univariate Linear Regression Analysis Between sCD44 and a Panel of Selected Variables[a]

	r	*P*
f-volume	−0.162	NS
E_2	−0.276	0.0129
Progesterone	0.272	0.0142
f-testosterone	−0.118	NS
hCG	0.572	<0.0001

[a] hCG, human chorionic gonadotropin; NS, not significant; *r*, Spearman's correlation coefficient by rank.

enzyme immunoassay (IMMULYSE; Diagnostic Products Corporation, Los Angeles, CA).

Statistical Analysis

The data are presented as means ± SE. Statistical methods included Fisher's protected least significant difference (PLSD) method, Pearson's correlation coefficient test, and Mann–Whitney's *U* test; $P < 0.05$ was defined as statistically significant.

RESULTS

Soluble CD44 existed in all follicular fluid specimens. The mean concentration (±SE) of sCD44 in follicular fluid was 265.4 ± 7.8 ng/ml ($n = 63$). Table I shows the results of univariate regression analysis, choosing sCD44 as a dependent variable and a selection of various indicated parameters as predictor variables. The variations in follicular fluid concentration of sCD44 were strictly associated with those of hCG ($r = 0.572$, $P < 0.0001$). Table II shows selected follicular fluid variables in relation to oocyte fertilization. The mean follicular fluid concentration of sCD44 was significantly higher in follicles that contained subsequently unfertilized oocytes than in those with oocytes that were subsequently fertilized ($P = 0.0428$), whereas the levels of other selected variables did not differ significantly between follicles with fertilized oocytes and those with unfertilized oocytes. Table III shows selected follicular fluid variables in relation to embryo quality. The mean follicular fluid concentration of sCD44 was significantly higher in the group containing oocytes that subsequently developed into good embryos than in the group with poor embryos ($P < 0.05$).

DISCUSSION

These results provide the first piece of evidence that human follicular fluid contains sCD44 and that follicular sCD44 level may be a useful marker for monitoring fecundity in infertility patients.

CD44 in follicular fluid may be transferred from the serum and also may be shed from granulosa cells, at least in part, because granulosa cells express CD44, as we described previously (5). hCG appears in follicular fluid following intramuscular administration, and its concentrations in follicular fluid differ among individual follicles because the permeability of hCG is unique to the individual follicles, and the number of LH/hCG receptors available to bind hCG differs with each individual follicle (13). Our results suggested an association between high hCG and high sCD44 concentrations in follicular fluid, and therefore it was speculated that hCG may induce shedding of CD44 from granulosa cells.

The mechanisms responsible for shedding of cell surface protein are still unclear. Recent reports provided evidence for the existence of a single common mechanism leading to the release of a wide variety of surface proteins (14, 15), while other reports suggested that specific mechanisms may be involved in shedding in different molecules (16,17). There have been no reports indicating a relationship between

Table II. Relationship Between Oocyte Fertilization and the Mean Follicular Levels of sCD44 and of Other Factors[a]

	Fertilized ($n = 48$)	Unfertilized ($n = 15$)	Significance[b]
f-volume (ml)	3.2 ± 0.2	3.2 ± 0.3	NS
sCD44 (ng/ml)	260.6 ± 7.3	296.7 ± 21.1	$P = 0.0428$
E2 (ng/ml)	530.9 ± 29.5	499.4 ± 42.5	NS
Progesterone (μg/ml)	11098.3 ± 770.6	13539.3 ± 1781.8	NS
f-testosterone (pg/ml)	36.7 ± 2.9	33.1 ± 6.4	NS

[a] Data are means ± SE.
[b] Statistical analysis was performed by Mann–Whitney's *U* test. NS, not significant.

Table III. Relationship Between Morphological Embryo Quality and the Mean Follicular Levels of sCD44 and of Other Factors

	Good (n = 13)	Fair (n = 22)	Poor (n = 13)	Significance[b]
f-volume (ml)	3.5 ± 0.5	2.9 ± 0.4	3.6 ± 0.3	NS
sCD44 (ng/ml)	285.5 ± 15.6[a]	262.6 ± 10.9	244.4 ± 8.6[b]	$P < 0.05$[a,b]
E_2 (ng/ml)	538.4 ± 64.5	491.8 ± 39.7	524.9 ± 57.7	NS
Progesterone (μg/ml)	12073.9 ± 1190.5	10894.2 ± 1192.1	10829.1 ± 2013.7	NS
f-testosterone (pg/ml)	37.9 ± 6.3	33.3 ± 4.4	41.4 ± 6.1	NS

[a] Data are means ± SE.
[b] Statistical analysis was performed by Fisher's protected least significant difference (PLSD) test. NS, not significant.

hCG and sCD44. We speculated that follicular hCG influences shedding of CD44 through an as yet unknown specific mechanism.

In the present study, we found that the amount of sCD44 in follicles containing oocytes that were not fertilized was higher than that in follicles containing oocytes that were fertilized. We speculate that once CD44 on the granulosa cells is shed excessively into the follicular fluid during the maturation process toward ovulation, the protective function of the oocyte by CD44 on the granulosa cells may be sharply reduced and, in turn, this reduction decreases the oocyte quality, resulting in inhibition of fertilization. In the fertilized oocytes, shedding CD44 optimally resulted in the development of oocytes into good-quality embryos, while the oocytes in the follicles containing a small amount of sCD44 developed into poor-quality embryos. In line with this point of view, we have hypothesized that the balance of CD44 on the granulosa cells and sCD44 in the follicular fluids appears to influence the quality of the embryos. This suggests that assessment of sCD44 in follicular fluids may be used as a method of monitoring the prognosis of IVF therapy.

CD44 is known to be one of the receptors of hyaluronic acid (HA) (18). HA is an extracellular glycosaminoglycan found in almost all types of extracellular matrix in mammalian systems. HA in follicles may be involved in cell-to-cell adhesion, maintenance of quality of oocytes (19), and good development of embryos (20, 21). As these functions of HA may be mediated through cell surface CD44, sCD44 in follicular fluid shed from CD44 expressed on granulosa cells may indirectly reflect the function of HA.

In conclusion, the results presented here support the following new observations: (1) follicular fluid from women subjected to ovarian hyperstimulation contain sCD44; (2) levels of sCD44 in follicular fluid are correlated significantly with those of hCG in the same follicles; (3) levels of sCD44 in follicular fluid may be associated with oocyte prognosis and embryonal quality; and (4) the measurement of sCD44 in follicular fluid could be useful in assessing the prognostic value of IVF cycles.

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