Hyaluronic Acid Inhibits Apoptosis in Granulosa Cells via CD44[1]

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Purpose: *This study was designed to examine whether hyaluronic acid (HA) inhibits apoptosis in cumulus and mural granulosa cells and to examine whether this effect of HA was mediated through CD44.*
Methods: *Mural and cumulus granulosa cells were obtained from in vitro fertilization patients. The cells were cultured with various concentrations of HA or HA plus various concentrations of anti-CD44 antibody without serum supplement. After 24 hr of culture, the cells were fixed and stained with Hoechst 33258. One thousand granulosa cells of each conditions were observed by fluorescence microscopy.*
Results: *HA inhibited apoptosis in both kinds of granulosa cells, and anti-CD44 antibody prevented this effect of HA.*
Conclusions: *The incidence of apoptotic granulosa cells with fragmented condensed nuclei was reduced by HA via CD44.*

KEY WORDS: Apoptosis; CD44; human, hyaluronic acid; granulosa cells.

INTRODUCTION

Hyaluronic acid (HA) is an extracellular glycosaminoglycan found in almost all types of extracellular matrix in mammalian systems. HA has been found to cause cell aggregation in a number of different types of cells and has been implicated in the stimulation of cell proliferation, cell migration, and cell adhesion. The presence of HA in granulosa cell layers during the period of ovulation has been confirmed by many investigators. Antral follicles have been shown to express HA in the cumulus oophorus and mural granulosa layers after induction of ovulation by luteinizing hormone (1). The concentration of HA in cumulus granulosa cell masses is higher than that in mural granulosa cell masses, and this gradient may be due to a soluble factor produced by fully grown oocytes that stimulate the production of HA in cumulus and mural granulosa cells. Mature oocyte–cumulus complexes are more expanded, larger, and contain more HA than the immature complexes. HA in cumulus or mural granulosa cell layers may be involved in cell locomotion (2), the prevention of fragmentation or segmentation of oocytes in vitro (3). HA decreases the frequency of degenerated oocytes (4).

Apoptosis has been investigated in terms of folliculogenesis and has been found to be responsible for the perinatal attrition of oogonia and oocytes, the postnatal loss of follicles through atresia, and regression of the corpus luteum in diverse species. Follicular atresia was found to be associated with apoptosis in chickens, pigs (5), gonadotropin-hyperstimulated rats, and humans (6). Formation of apoptotic bodies is one of the morphological hallmarks of apoptosis. The incidence of apoptotic bodies has been used as a morphological marker for physiological cell renewal (5) and for prognosis of patients with neoplasms (7,8). Recently, we reported the presence of apoptotic bodies in the granulosa cells in the stimulated cycles during in vitro fertilization (IVF) treatment, and membrana granulosa cells in the follicles from which oocytes were subsequently fertilized showed a significantly lower incidence of apoptotic bodies than those in follicles from which the oocytes were not fertilized (9). The incidence of apoptotic granulosa cells is a very sensitive indicator with which to estimate the quality of follicles and oocytes in an IVF program. We also found that the incidence of apoptotic bodies in cumulus cells was always lower than that in mural granulosa cells in

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ovarian follicles in any condition (9). This gradient in the incidence of apoptosis is the reverse of that in the concentration of HA between mural and cumulus granulosa cell masses.

CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule involved in cell-to-matrix interactions. CD44 is known to be a receptor for hyaluronic acid (10–12). Recently, CD44 was discovered on the surface of granulosa cells and on the surface of the cells of early stage embryos (13). The level of expression of CD44 in the cumulus granulosa cell masses is higher than that in the mural granulosa cell masses, and mature oocyte–cumulus complexes show higher expression of CD44 than immature oocyte–cumulus complexes (14). These observations suggested that CD44 may contribute to the quality of oocytes. However, the precise functions of the complex of HA and CD44 on granulosa cells remain unknown.

Therefore, the present study was performed to determine whether HA inhibits apoptosis in mural and cumulus granulosa cells and to determine whether this effect of HA is produced through CD44.

MATERIALS AND METHODS

Patients

A total of 71 patients in IVF cycles were analyzed between June 1997 and June 1998 at Yamagata University Hospital, Yamagata, Japan. Patients with endometriosis were excluded due to the effect of this condition on apoptosis in granulosa cells (15). This study was approved by Yamagata University Hospital Committee for Research on Human Subjects. Written informed consent was obtained from all patients, and their clinical information was concealed.

Stimulation Protocol and Follicle Aspiration

The ovulation induction protocol used a gonadotropin-releasing hormone (GnRH) analogue, buserelin acetate (Suprecur nasal; Hoechst, Tokyo) in a long suppression protocol starting from midluteal phase in the previous cycle. Administration of human menopausal gonadotropin (150 to 300 IU/day, Humegon; Sankyo, Tokyo) with or without follicle-stimulating hormone (FSH) (Fertinom P; Serono, Tokyo) was started on day 3 of the menstrual cycle. Human chorionic gonadotropin (hCG) (10,000 IU; Mochida, Tokyo) was administered when one follicle achieved a mean diameter of 16 mm. Thirty-five hours after administration of hCG, follicles were aspirated by transvaginal ultrasound retrieval (6.5 MHz; Mochida). All follicles with a mean diameter of $\geq$ 11 mm were aspirated using a 20-ml syringe.

Isolation of Granulosa Cells

Aspirated follicular fluid (FF) was transferred into tissue culture dishes (Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ). Cumulus oocyte complexes were isolated under a dissecting microscope (SZH-ILLB; Olympus, Tokyo) and were put into organ tissue culture dishes (Falcon 3037; Becton Dickinson and Company) with culture medium. Ham's F-10 medium was used for all experiments. Cumulus cell masses were separated mechanically from oocytes using 26-gauge needles and transferred into 15 ml centrifuge tubes (Iwaki Glass, Tokyo). After collecting oocyte–cumulus cells complexes, aspirated FF from individual patients was gathered into tubes and left 10 min to allow sedimentation of mural granulosa cells. Precipitated mural granulosa cells were transferred to another tube containing 5 ml of medium and then gently resuspended. One milliliter of 80% Percoll was carefully layered at the bottom of the tube followed by centrifugation at 600g for 10 min. The mural granulosa cells formed a layer on the Percoll solution, while the red blood cells sedimented at the bottom of the tube. The mural granulosa cells were transferred to another tube and washed again with 5 ml of medium. The tube was centrifuged at 300g for 5 min and the supernatant was decanted. Hyaluronidase solution (0.1% wt/vol in culture medium) (Sigma, St. Louis, MO) was added in the tubes containing cumulus cell masses or mural granulosa cell masses (final concentration of 0.05% wt/vol). Both kinds of cells were mechanically dispersed by pipetting for 2 min. The cells were washed twice with culture medium containing 10% of human plasma protein fraction (Plasmanate Cutter; Bayer, Osaka), then cultured under various conditions.

Cell Culture, Cell Fixation, and Quantification of Apoptotic Bodies

Twenty thousand of cells were seeded in a 35-mm dish and cultured in Ham's F-10 medium at 37°C in a humidified atmosphere of trimixture gas (5% CO_2: 5% O_2: 90% N_2). This culture medium was not supplemented with serum to clarify the effects of HA and anti CD44 antibody. We performed experiments under three different culture conditions: (i) The effects of culture time (0, 6, 12, 24, and 48 hr) on the incidence

apoptotic mural granulosa cells were examined. Further experiments were performed after 24 hr in culture. (ii) The effects of HA (Sigma, St. Louis, MO) in culture for 24 hr at the concentration of 50 ng/ml to 5μg/ml on the incidence of apoptotic mural and cumulus granulosa cells were examined. (iii) The effects of antihuman CD44 antibody (clone OS/37, Seikagaku Co., Tokyo) at concentrations of 2.5 ng/ml to 250 ng/ml on the preventive effect of HA on apoptosis of mural granulosa cells were examined. This human CD44 antibody has been reported to recognize the epitope of hyaluronan binding region of CD44. In this experiment, we added HA into culture medium at a concentration of 500 ng/ml. At the end of each culture period, the cells were scraped from the bottom of each dish and put into tubes. These tubes were centrifuged at 300g for 5 min and the supernatant was decanted. The precipitated cells were fixed with 4% wt/vol of neutral buffered formalin and placed on a slides.

Analysis of Apoptosis by Fluorescence Microscopy

After drying, the slides were washed with phosphate-buffered saline (PBS) three times and the nuclei of cells were stained with 0.5 μg/ml of Hoechst 33258 (fluorescent dye; Waco, Osaka) with 5% wt/vol of DABCO (1,4-diazabicyclo-2,2,2-octate; Sigma Tokyo) in 90% glycerol: 10% 0.2M Na_2HPO_3 to block the bleaching effect of fluorecence.

Apoptotic cells were defined as those cells that contained condensed or fragmented condensed chromatin, or with fragmented cytoplasm containing condensed chromatin. We counted the apoptotic cells with fragmented condensed chromatin or fragmented cytoplasm containing condensed chromatin among 1000 granulosa cells.

Flow Cytometric Analysis of DNA in Granulosa Cells

The nuclear DNA condition also was determined by flow cytometry using a modification of the method described by Guthrie *et al.* (16). Cumulus granulosa cells were not numerous enough for this analysis, We then used mural granulosa cells. To fix the individual mural granulosa cells, they were left in 80% ethanol overnight at 4°C to remove the DNA fragment produced by apoptosis from each cell. Then, the ethanol was decanted after centrifugation at 300g for 5 min. The granulosa cells were stained in 1 ml of propidium iodide (PI) solution [50 μg/ml PI; 0.1% Triton X100; 0.1 mM EDTA $(Na)_2$ and 50μg/ml RNase A in modified PBS(−)]. The stained cells were stored at 4°C for 24hr until flow cytometric analysis. Before flow cytometry, the cell suspensions were put on 100 μm nylon mesh filters to remove aggregated cells. The nuclei of granulosa cells were analyzed with a FACS Calibur (Becton Dickinson, Mountain View, CA). An argon laser was used to excite the PI nuclear stain at a wavelength of 488 nm. DNA histograms were gated out by pulse processing using peaks and integrated DNA fluorescence. The software ModFit LT TM (Verty Software House, Inc., Topsham, ME) was used to analyze the percentage of granulosa cells containing hypodiploid levels of DNA (apoptotic cells).

Statistical Analysis

The data are presented as mean ± SEM. For statistical comparisons between groups, Friedman's test, Wilcoxon's signed rank test, and analysis of variance (ANOVA) were applied. P values < 0.05 were considered significant.

RESULTS

The incidences of apoptotic cells in mural granulosa cells cultured for various periods are shown in Fig. 1. The incidence of apoptotic mural granulosa cells increased significantly with time (0 hr: 4 ± 1; 6 hr: 23 ± 4; 12 hr: 28 ± 2; 24 hr: 43 ± 4; 48 hr: 40 ± 4). Consequently, we used a culture period of 24 hr in further examinations.

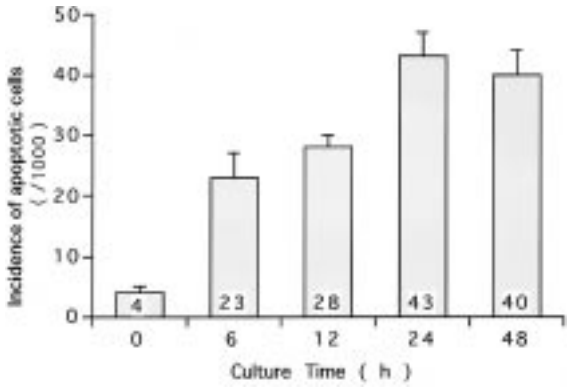

Fig. 1. Effects of different culture periods on the incidence of apoptosis in mural granulosa cells. Values are mean ± SEM of six trials. The incidence of apoptotic granulosa cells increased with the length of culture ($P < 0.01$, one way ANOVA).

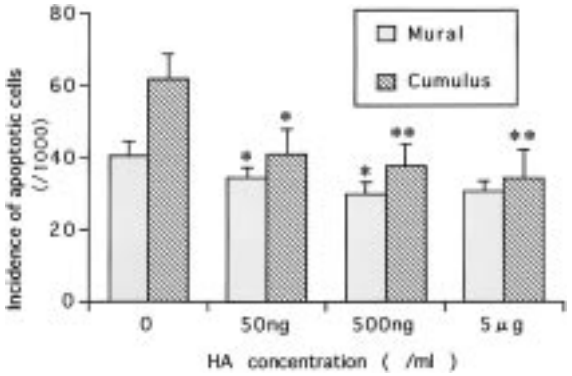

Fig. 2. Effects of culture with different concentrations of HA on the incidence of apoptosis in the mural and cumulus granulosa cells. Values are mean ± SEM of 24 trials. HA inhibited the apoptosis of mural granulosa cells at concentrations of 50 ng/ml and 500 ng/ml (*$P < 0.05$) compared with that at concentration of 0 ng/ml. HA also inhibited apoptosis of cumulus granulosa cells at concentrations of 50 ng/ml (*$P < 0.05$), 500 ng/ml, and 5 μg/ml (**$P < 0.01$) compared with that at 0 ng/ml.

In mural granulosa cells, HA inhibited apoptosis at concentrations of 50 ng/ml and 500 ng/ml (control: 40.5 ± 4; 50 ng/ml: 34.3 ± 3; 500 ng/ml: 30 ± 3; 5 μg/ml: 30.5 ± 3) (Fig. 2). HA also inhibited apoptosis in cumulus granulosa cells at concentrations from 50 ng/ml to 5 μg/ml (control: 61.7 ± 7; 50 ng/ml: 40.9 ± 7; 500 ng/ml: 37.7 ± 6; 5 μg/ml: 34.3 ± 8) (Fig. 2). The incidence of apoptotic cells was always higher among cumulus granulosa cells than mural granulosa cells.

Apoptotic changes of granulosa cells also were examined by flow cytometry. The incidence of apoptotic cells in cultures with HA was lower than that in cultures without HA (Fig. 3).

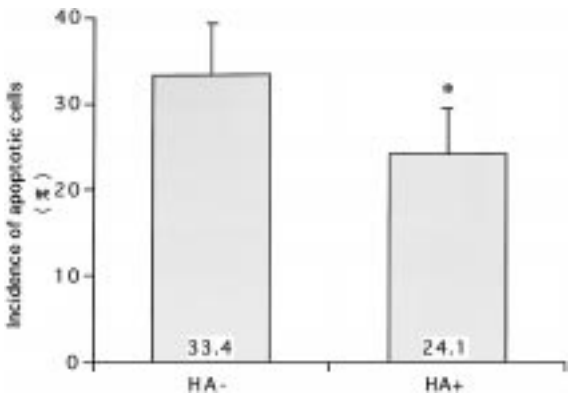

Fig. 3. Effects of culture with HA on the incidence of apoptosis in mural granulosa cells as determined by flow cytometry. Values are mean ± SEM of seven trials. The incidence of apoptotic granulosa cells cultured with HA (500 ng/ml) was significantly lower than that without HA (*$P < 0.05$).

The results described above implied that HA inhibits apoptosis of both kinds of granulosa cells. Thus we further investigated whether this inhibition of apoptosis by HA is mediated through an interaction with CD44. Anti-CD44 antibody prevented HA from inhibiting apoptotis in granulosa cells at concentrations of 25 ng/ml and 250 ng/ml (Fig. 4). As the concentration of anti-CD44 was higher, the incidence of apoptotic cells was larger. The effect of anti-CD44 antibody that prevent HA-inhibiting apoptosis in granulosa cells was in a concentration-dependent manner.

DISCUSSION

The functions and roles of HA have been investigated in various kinds of cells. HA is responsible for promoting cell locomotion and regulating cell contact behavior (2,17), expressing CD44 in endothelial cells, and correlating with metastatic behavior (18). In tumor cells, HA promoted tissue neovascularization (19), increasing blood flow and graft vessel growth (20), and suppressing cell-mediated antitumor responses in inflammatory cells (21).

Meanwhile, in ovarian follicles Chen *et al.* (22) reported that optimal expansion of cultured oocyte cumulus cell complexes requires the presence of substrates of HA synthesis, and an expanded cumulus mass may positively influence oocyte viability and fertilizability. HA produced naturally by granulosa cells prevents fragmentation or segmentation of oocytes in vitro (3). However, the precise roles and

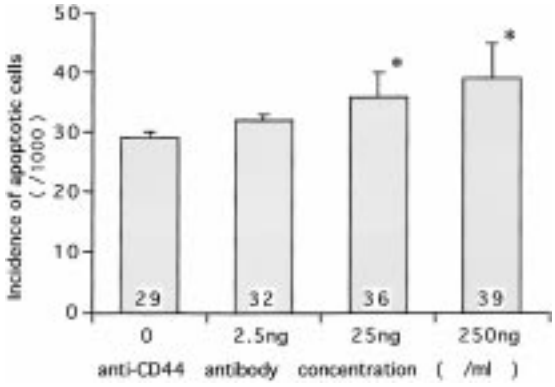

Fig. 4. Effects of culture with different concentrations of anti-CD44 antibody on the incidence of apoptosis in mural granulosa cells. HA was added to all culture dishes at a concentration of 500 ng/ml. Values are mean ± SEM of seven trials. The anti-CD44 antibody prevented HA from inhibiting apoptosis in granulosa cells at concentrations of 25 ng/ml and 250 ng/ml (*$P < 0.05$) as compared with that at 0 ng/ml.

functions of HA during the period of ovulation remain to be resolved.

In this study, we found that HA inhibited apoptosis in cumulus and mural granulosa cells. A similar phenomenon was observed in immature thymocytes and peripheral T lymphocytes exposed to anti-CD3 monoclonal antibodies (23). And at all concentrations of HA examined, the incidence of apoptotic cumulus granulosa cells was higher than that in mural granulosa cells due to the ready dispersion of cumulus granulosa cells into single cells. In contrast, mural granulosa cells are relatively difficult to disperse by hyaluronidase. Peluso *et al.* (24) used granulosa cells from immature rats to determine the relationship between the rate of apoptosis and cell aggregation. They found that a single cell contact was sufficient to suppress apoptosis, with a small nonsteroidogenic granulosa cell being as effective as a large steroidogenic granulosa cell; that is, cell contact blocks apoptosis in a progesterone-independent manner. The cumulus granulosa cells showed a high incidence of apoptosis, but the variation of the incidence of apoptosis in cumulus granulosa cells was larger than that in mural granulosa cells. CD44, which is one of the receptors of HA, is present on the surface of human cumulus and mural granulosa cells and the level of CD44 expression by cumulus granulosa cells is much higher than that by mural granulosa cells (14). The effects of HA expressed as the suppression of apoptosis were stronger in the cumulus granulosa cells than in the mural granulosa cells.

The anti-CD44 antibody used in this study recognizes the epitope to which HA binds. The anti-CD44 antibody significantly prevented HA from inhibiting the production of apoptosis in granulosa cells at concentrations of 25 ng/ml and 250 ng/ml. In the murine CD4 and CD8 single positive T lymphocytes, the same phenomenon was observed, and anti-CD44 antibody enhanced the apoptosis and decreased the proliferation of T cells. Then, as the concentration of anti-CD44 antibody became higher, the incidence of apoptosis became higher.

Our previous study (25) showed that co-culture of early stage embryos with their own cumulus mass maintains embryo quality over prolonged culture periods. Considering that preimplantation embryos also express CD44 on the surface of blastomeres (13), HA in the cumulus mass may maintain embryo quality through suppressing detrimental effects, like apoptotic change, on embryos via the direct interaction between CD44 expressed in embryos and HA in the cumulus mass.

CONCLUSIONS

HA reduced the incidence of apoptotic granulosa cells via CD44 receptor on the granulosa cells.

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