Effect of glycodelin on the production of vascular endothelial growth factor in cumulus cells

Glycodelin modulates vascular endothelial growth factor (VEGF) production in cumulus cells in vitro. Patients with normal gonadotropin responses who were undergoing IVF demonstrated increased VEGF production to glycodelin, whereas poor responders had a decreased response to glycodelin. (Fertil Steril® 2006;85:1553–6. ©2006 by American Society for Reproductive Medicine.)

The physiology of the preovulatory follicle is undergoing extensive investigation to gain insight into the development of oocytes and subsequent embryos. One specific area of investigation involves the role of angiogenic factors and cyclic neovascularization in the ovarian follicle and its effect on the developing oocyte. Understanding the factors that control follicular blood supply may eventually lead to improved oocyte quality and treatments for ovarian factor infertility.

Glycodelin, previously referred to as placental protein-14 and progesterone-associated endometrial protein, is a 28 KD glycoprotein produced in the female reproductive tract (1). More extensively studied in the endometrium, glycodelin levels are known to increase in the late secretory phase of the menstrual cycle and peak at approximately 10–12 weeks gestation when pregnancy occurs (2). Glycodelin is under the regulation of progesterone in the endometrium, where it is known to produce both immunosuppressive and contraceptive effects (1, 3, 4). It is thought that its ability to inhibit natural killer lymphocytes by impairing cytotoxicity of these cells in the endometrium is crucial for functioning of the fetomaternal defense mechanisms that allow embryo implantation and growth (5). Glycodelin also inhibits the binding of sperm to the oocyte zona pellucida at concentrations found in the midluteal endometrium. The absence of glycodelin in the periovulatory period allows fertilization to occur (6).

In 1992, Critchley et al. (7) found that the ovary is a significant source of serum glycodelin, and that glycodelin levels were significantly decreased in subjects with nonfunctioning ovaries due to premature ovarian failure. Kamarainen et al., (8) in 1996, used immunohistochemical staining to provide evidence for the presence of glycodelin within the granulosa, theca, and stromal cells of the ovary during all phases of the ovarian cycle.

In 2001, Song et al. (9) published findings that provided evidence for an angiogenic role of glycodelin mediated through vascular endothelial growth factor (VEGF) in multiple cell lines in vitro, including human ovarian adenocarcinoma cells. This was followed by elucidation of the ontogeny of glycodelin in the ovarian follicle by Tse et al., (10) who determined that although glycodelin immunoreactivity was noted in both the luteinized granulosa and cumulus cells of late secondary follicles, glycodelin mRNA was present only in granulosa cells, not cumulus cells. They concluded that granulosa cells had the ability to synthesize glycodelin and release it into the follicular fluid where it was taken up by cumulus cells; however, the function of glycodelin in the ovary is unknown. We have therefore elected to study cumulus cells instead of granulosa cells because this cell type and its secretory products are likely to have the greatest effect on the developing oocyte.

Vascular endothelial growth factor is a potent angiogenic factor that facilitates neovascularization (11). Vascular endothelial growth factor is stimulated by hypoxia, cytokines, and hormones, such as estrogen, and is expressed in the ovary throughout most of the ovarian cycle (12, 13). Within each follicle, granulosa cells become positive for VEGF staining at the preantral stage and remain so throughout folliculogenesis, thus emphasizing the association of this factor with oocyte development (11).

Friedman et al. (14, 15) demonstrated an inverse relationship between VEGF concentration in follicular fluid and pregnancy rate, number of oocytes retrieved, and peak E_2 in patients undergoing in vitro fertilization. They also found a positive correlation between VEGF concentration, patient age, and number of ampoules of gonadotropins required for ovarian stimulation. Barrosa et al. (16) found a negative correlation between follicular fluid VEGF concentration and day 3 embryo quality. Similarly, Neulen et al. (17) noted that in patients undergoing in vitro fertilization, levels of VEGF in follicular fluid were highest in the patients with poor ovarian response to gonadotropin stim-

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Reprint requests: Ana A. Murphy, M.D., Department of Gynecology and Obstetrics, Emory University, 1639 Pierce Drive, Suite 4301, Atlanta, Georgia 30322 (FAX: 404-727-8615; E-mail: amurphy@emory.edu).

ulation. He also found that poor responders have the lowest concentration of bioavailable VEGF, and concluded that a high concentration of follicular fluid VEGF is due to diminished bioavailability of VEGF, which leads to follicular hypoxia and poor oocyte quality. These studies suggest that VEGF may play an important role in the developing ovarian follicle.

The preceding data suggest that ovarian follicular angiogenesis is at least partially dependent on VEGF production, which may be mediated by granulosa cell secreted glycodelin. We hypothesize that cultured cumulus cells will produce VEGF in response to the addition of glycodelin peptide, and that the concentration of VEGF produced by cultured cumulus cells will differ based on the subject's level of ovarian response to gonadotropin stimulation during an in vitro fertilization cycle.

The Emory University Institutional Review Board approved this prospective case-control study. From October 2002 to September 2003, we enrolled 37 women who were undergoing IVF at the Emory Center for Reproductive Medicine and Fertility. The need for IVF was based on accepted indications for this treatment. The subjects enrolled were between 25 and 42 years of age at the time of oocyte retrieval. All patients undergoing oocyte retrieval during this time were eligible to participate. Subjects were asked to participate on the day of oocyte retrieval and gave written informed consent before administration of analgesia.

Each subject was categorized as either a normal or poor responder to exogenous gonadotropin stimulation. Poor responders were defined as subjects with [1] an E_2 of <100 pg/mL on stimulation day 5, [2] fewer than six follicles at the time of oocyte retrieval, or [3] a previous poor response to an IVF cycle. All other subjects were classified as normal responders. The majority of normal responders underwent a lupron down-regulation protocol, whereas the majority of poor responders underwent a lupron flare protocol. A minority of subjects in each group were stimulated with other individualized protocols.

Following oocyte retrieval, the cumulus cells were dissected from each oocyte. The cumulus cells from all follicles were pooled for each subject, treated with hyaluronidase (Sage BioPharma, Inc., Bedminster, NJ) to create a single cell suspension, and counted on a hemocytometer. A concentration of 20,000 cumulus cells was placed into each of 12 wells of a 48-well culture plate (Corning, Inc., Corning, NY) to achieve 80% confluence.

The cells were incubated for 24 hours at 37°C with 5% CO_2 in air in 0.5 mL of M199 medium (Sigma, St. Louis, MO) with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO) and 1% penicillin-streptomycin (PS) (Sigma, St. Louis, MO). A second 24-hour incubation was performed with concentrations of glycodelin peptide (synthesized by the Microchemical Facility of Emory University) of 50 ng/mL, 100 ng/mL, and 200 ng/mL contained in 0.5 mL of M199 medium with 0.1% FCS and 1% PS. Cells cultured in M199 with 0.1% FCS and 1% PS without glycodelin were used as controls. Each of the three glycodelin concentrations and control medium was added to wells in triplicate. At the end of this 24-hour incubation, cell culture medium was removed from each well and centrifuged for 5 minutes at $240 \times g$. The supernatant from each sample was obtained and stored at −80°C. Cells were released from each culture well using 0.5 mL of Trypsin-EDTA (Sigma, St. Louis, MO) and then centrifuged for 5 minutes at $240 \times g$. Each remaining cell pellet was stored at −80°C.

An enzyme-linked immunosorbent assay (ELISA) was performed in duplicate for each cell culture supernatant sample using Quantikine human VEGF immunoassay (R&D Systems, Minneapolis, MN) and quantified using an ELISA plate reader (Lab-Tek, Rochester, NY). The optical density of each well was read at 405 nm. These data were converted to picograms (pg) of VEGF/mL using a standard curve. The intraassay and interassay variability was $<6.8\%$ and 8.9% respectively. The sensitivity of the assay was 15.6 pg/mL.

Each cell pellet was then analyzed for total protein content. Briefly, each pellet was thawed and sonicated with a pellet pestle and placed in a 96-well plate. After addition of Bradford reagent to each sample, the optical density was read at 570 nm using an ELISA plate reader. The VEGF levels were then standardized for each sample by total protein content, allowing results to be expressed as pg VEGF/mL per mg protein.

Embryo grading was performed daily starting on postretrieval day 1, according to the morphological criteria of Tesarik and Greco (18). The day 1 morphology was weighted more heavily in final selection of embryos for transfer.

Results are expressed as mean ± SEM. Patient characteristics were analyzed using an analysis of variance (ANOVA) and Fischer's exact test. The difference in VEGF between the groups was tested for significance using ANOVA and Tukey's post hoc test. A P value of $<.05$ was considered significant for all comparisons.

Thirty-seven patients were recruited for this study, including 27 subjects classified as normal responders and 10 as poor responders. The normal responders were significantly younger than the poor responders (31.4 vs. 36.0 years, $P=.0376$). The normal responders had a significantly higher peak E_2 level during the stimulation cycle (3,130 pg/mL vs. 1,214.8 pg/mL, $P=.002$) as well as more oocytes obtained at oocyte retrieval (20.7 vs. 7.8, $P=.002$). The number of embryos surviving until day 3 postretrieval was higher (10.8 vs. 2.8, $P=.0007$). The grade of those embryos was significantly better in the normal responders than in the poor responders (1.0 vs. 1.7, $P=.0037$), result-