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Original article

Clinical utility of decorin in follicular fluid as a biomarker of oocyte potential

Yuki Sawada, Takeshi Sato*, Chieko Saito, Fumiko Ozawa, Yasuhiko Ozaki, Mayumi Sugiura-Ogasawara

Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

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ABSTRACT

This study investigated the concentration of decorin (DCN) in mature follicular fluid and the existence in the granulosa cells. It also investigated whether DCN is useful as a biomarker for outcomes of assisted reproductive technology (ART). A retrospective cohort study was performed involving 130 oocytes of 88 patients treated with ART because of unexplained infertility. The concentration of DCN in the follicular fluid (F-DCN) was 39.26 ng/ml (median value); it was higher than that in serum. F-DCN of the oocytes fertilized by intracytoplasmic sperm injection (ICSI) was significantly lower than that of oocytes that were not fertilized (33.24 ng/ml vs 40.18 ng/ml; $P = 0.043$). When a cut-off level of 34.5 ng/ml was set according to the receiver-operating characteristic curve, the fertilization rate of the oocytes from the follicles in which F-DCN was lower than the cut-off level tended to be good compared to that of the oocytes with F-DCN higher than the cut-off level ($P = 0.052$). DCN is less likely to be produced by the granulosa cells (GCs), because it was not detected in GCs by immunostaining and Western blot analysis. F-DCN has a possibility to be a biomarker indicating the quality of oocytes collected from the corresponding follicle.

1. Introduction

Various bioactive substances such as hormones and cytokines exist in the pre-ovulatory follicular fluid (FF). They are related to the development and maturity of oocytes. Oocyte development and maturity are important steps in the process of reproduction and infertility treatments. It is thought that treatment outcomes can be improved by applying clinical findings after analyzing the associated bioactive substances. Examples of these bioactive substances are granulocyte colony-stimulating factor (G-CSF) and macrophage inflammatory protein (MIP)-3α. It has been reported that the concentration of G-CSF in FF is correlated with the number of the fertilized oocytes, and that it can be a useful biomarker of the quality of a corresponding oocyte [1,2]. On the other hand, it has been reported that MIP-3α in FF is related to the maturity of an oocyte because its concentration in FF containing mature oocytes is significantly higher than that in FF containing immature oocytes [3].

Decorin (DCN), another bioactive substance, belongs to the small leucine-rich proteoglycan family and exists in some organs such as the bone, tendon, skin, aorta, and cornea. It has been reported that DCN constructs the extracellular matrix and has an effect on the formation of collagen fiber by combining with collagen type I and type II [4]. In addition, it regulates cell proliferation by combining with epidermal growth factor receptor (EGFR) or insulin-like growth factor 1 receptor (IGF-1R). In normal cells such as endothelial and renal cells, it combines with EGFR, thereby inactivating EGFR and inhibiting cell proliferation [5,6]. However, by combining with IGF-1R, it promotes protein synthesis and controls apoptosis by activating the phosphatidylinositol 3-kinase/protein kinase B (PI3 K/Akt) pathway [5,7,8]. DCN has also been reported to halt tumor growth by antagonizing oncogenic tyrosine kinase receptors and restraining angiogenesis [9,10]. In addition, DCN actions at the fetal–maternal interface include restrained trophoblast migration, invasion, and uterine angiogenesis, and they might have a causal role in preeclampsia by compromising endovascular differentiation of the trophoblast and uterine angiogenesis, resulting in poor arterial remodeling [11,12]. However, it has also been reported that ectopic DCN overexpression in mouse cerebral endothelial cells upregulated vascular endothelial growth factor A to promote angiogenesis [13].

In the ovary, previous studies have shown that DCN presents in the connective tissue, follicular thecal compartments, FF of ovulatory follicles, and corpus luteum [14]. In addition, it has been reported that

* Corresponding author at: Takeshi Sato Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 4678601, Japan.
E-mail address: og.sato@med.nagoya-cu.ac.jp (T. Sato).

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DCN does not exist in the granulosa cells (GCs) of pre-antral and antral follicles [14]. However, the origin of DCN is unclear and the expression of DCN in GCs of mature follicles was not analyzed.

Regarding the function of DCN, it has been reported that it plays roles in some important processes such as follicle growth, ovulation, and retaining the corpus luteum by regulating growth factors [14], but its detailed functions are unknown. The association between DCN and the outcomes of infertility treatments has not been examined.

In the present study, we examined the concentrations of DCN in FF (F-DCN) and in serum (S-DCN), the localization of DCN in GCs, and the relationships with outcomes of assisted reproductive technology (ART) and, as a result, whether DCN is useful as a biomarker for ART.

2. Materials and methods

2.1. Patients

A retrospective cohort study involving 88 patients treated with ART because of unexplained infertility was performed at Nagoya City University Hospital between April 2010 and March 2016.

FF, serum, and GCs were collected from the patients. F-DCN, S-DCN, and the concentration of IGF-1 in FF were examined. We investigated the relationships between F-DCN and patient age, pulsatility index (PI) of the blood flow around the follicle at ovum pick-up (OPU), S-DCN, the concentration of IGF-1 in FF, fertilization, and quality of the embryo. We tried to identify the existence and localization of DCN in GCs.

The study was conducted with the approval of the Research Ethics Committee of Nagoya City University. Written informed consent was obtained from all patients.

2.2. Ovarian stimulation

Controlled ovarian stimulation was performed with a long protocol of gonadotropin-releasing hormone (GnRH) agonist, a short protocol of GnRH agonist, a GnRH antagonist protocol, or a clomiphene citrate (CC) protocol. The protocol was selected according to patient age and the ovarian reserve predicted by serum anti-Mullerian hormone. The details of each protocol are described here.

2.2.1. Long protocol of GnRH agonist

Downregulation of the pituitary gland was performed by administering buserelin acetate in a nasal spray (0.9 mg/day) (Suprecur; Mochida Pharmaceutical, Tokyo, Japan) starting from the mid-luteal phase of the previous cycle. When the serum estradiol levels were reduced to less than 30 pg/ml during the early phase of the menstrual cycle, intramuscular injection of gonadotropin (Gonpure; ASKA Pharmaceutical, Tokyo, Japan) was started (150–450 IU/day).

2.2.2. Short protocol of GnRH agonist

Administration of buserelin acetate in a nasal spray was started during the early phase of the menstrual cycle. Then, intramuscular injection of gonadotropin was started 2 days later.

2.2.3. GnRH antagonist protocol

Intramuscular injection of gonadotropin was started on day 3 to day 5 of the menstrual cycle. Since the leading follicle reached 16–18 mm in diameter when measured by transvaginal ultrasonography, daily subcutaneous injections of 0.25 mg Cetrotide (Merck Serono Co., Ltd., Tokyo, Japan) were administered until stimulation was completed.

2.2.4. CC protocol

On day 5 of the menstrual cycle, 100–150 mg/day of CC (Fuji Pharmaceutical, Toyama, Japan) was started orally. When the leading follicle reached 10–12 mm, intramuscular injection of gonadotropin was started.

2.3. OPU, fertilization, and embryo evaluation

During all protocols, ovulation was induced by intramuscular injection of 10,000 IU human chorionic gonadotropin (Gonatropin; ASKA Pharmaceutical, Tokyo, Japan) when the leading follicle reached a diameter of more than 20 mm as measured by transvaginal ultrasonography. Thirty-six hours later, transvaginal ultrasonography–assisted OPU was performed after PI of the blood flow around the follicle was calculated by transvaginal ultrasonography. The oocytes collected from the first punctured follicles that were selected according to accessibility and size at least 18 mm in diameter were cultured individually in separate dishes. In vitro fertilization (IVF) was performed; however, intracytoplasmic sperm injection (ICSI) was performed for patients with low fertilization rates during previous IVF. In addition, when more than six oocytes were collected, split ICSI was performed if requested by the patient. Embryo quality was evaluated 2 days after OPU according to the Veeck classification. Grade 1 or grade 2 embryos were considered good, whereas the others were determined to be poor embryos.

2.4. Samples (serum, FF, and GC)

On the day of OPU, serum and FF samples were collected. Blood was sampled just before OPU. Blood samples were centrifuged at 250 × *g* for 5 min to separate and collect serum. At OPU, FF was aspirated and collected from the first punctured follicle of each ovary to avoid contamination with blood. These samples were centrifuged at 250 × *g* for 5 min to separate the supernatant as FF and the precipitate as the cellular components. The serum and FF samples were cryopreserved at −20 °C until analysis.

Some FF samples were layered over a Ficoll-Paque gradient at 250 × g for 30 min at 4 °C. The cell samples in the mononuclear cell layer were cryopreserved as GCs at 4 °C for use during immunocytochemistry and SDS-PAGE and Western blot analyses.

2.5. Enzyme-linked immunosorbent assay

Commercial enzyme immunoassay kits were used to examine the concentration of DCN (RayBiotech, Norcross, GA, USA) in serum and FF and the concentration of IGF-1 (R&D Systems, Minneapolis, MN, USA) in FF. Because the kits were validated for serum samples but not for FF, before using FF samples, the recovery rate of at least three FF samples was checked. All samples were measured in duplicate according to the manufacturer's instructions. In cases with very high or low concentrations, the measurements were repeated with the appropriate dilution.

2.6. Immunostaining

2.6.1. Immunocytochemistry

Immunocytochemistry was performed for three GC samples to examine the localization of DCN in GCs.

GC samples were smeared on MAS-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan) after fixation using Mildholm (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After washing in phosphate-buffered saline (PBS) (Dulbecco's phosphate-buffered saline; Invitrogen, Inc., Carlsbad, CA, USA) for 5 min three times, treatment with 0.1% TritonX (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS was performed for 5 min. Endogenous peroxidase activity was blocked by incubating GC samples with 0.3% H_2O_2 in methanol (Sigma-Aldrich Co.) for 30 min. GC samples were blocked with Histofine SAB-PO (M) (Nichirei Bioscience Inc., Tokyo, Japan) for 1 h at room temperature. Then, GC samples were incubated with primary antibodies diluted in PBS overnight at 4 °C. Anti-decorin antibody sc-73896 (mouse, 1/100 and 1/500 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used. Negative controls were created by omitting the primary antibodies and incubating with PBS only. GC samples were incubated with