Glutathione S-transferase theta 1 expressed in granulosa cells as a biomarker for oocyte quality in age-related infertility

Megumu Ito, M.D.,[a] *Miho Muraki, B.S.,*[a,f] *Yuji Takahashi, Ph.D.,*[a] *Misa Imai, M.S.,*[a] *Tohru Tsukui, Ph.D.,*[b] *Naomi Yamakawa, Ph.D.,*[c] *Koji Nakagawa, M.D.,*[a] *Shirei Ohgi, M.D.,*[a] *Takashi Horikawa, M.D.,*[a] *Wakako Iwasaki, M.D.,*[a] *Ayumi Iida, B.S.,*[a] *Yoshihiro Nishi, M.D.,*[d] *Toshihiko Yanase, M.D.,*[e] *Hajime Nawata, M.D.,*[e] *Kenji Miyado, Ph.D.,*[a] *Tomohiro Kono, Ph.D.,*[f] *Yoshihiko Hosoi, Ph.D.,*[g] *and Hidekazu Saito, M.D.*[a]

[a] Division of Reproductive Medicine, Department of Perinatal Medicine and Maternal Care, National Center for Child Health and Development, Tokyo, Japan; [b] Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan; [c] Research Team for Geriatric Diseases, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; [d] Department of Physiology, Kurume University, Kurume, Japan; [e] Department of Medicine and Bioregulatory, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan; [f] Department of BioScience, Tokyo University of Agriculture, Tokyo, Japan; and [g] Division of Biological Science, Graduate School of Biology-Oriented Science and Technology, Kinki University, Wakayama, Japan

Objective: The goal of this study was to identify a reliable biomarker for age-related infertility.
Design: Laboratory study.
Setting: ART laboratory.
Patient(s): Patients undergoing intracytoplasmic sperm injection or IVF cycles.
Intervention(s): Expression of Glutathione S-transferase (GST) mRNA and protein in mural and cumulus granulosa cells obtained from infertile patients were examined by reverse transcriptase-polymerase chain reaction and immunofluorescence.
Main Outcome Measure(s): Correlation between the expression of GST theta 1 (GSTT1) in granulosa cells and oocyte quality was a main outcome measure.
Result(s): Expression of GSTT1 in granulosa cells from male factor patients was positively correlated with age and negatively with cumulus-oocyte complex maturity. When samples with high and low GSTT1 in granulosa cells were extracted from the other infertility factors, cumulus-oocyte complex maturity in the high GSTT1 group was significantly lower than that in the low GSTT1 group (high: 27.2% vs. low: 51.3%). The developmental capacity of oocytes in the high GSTT1 group was likely to be lower (high: 26.4% vs. low: 43.9%). Up-regulation of GSTT1 during aging may be promoted by FSH and H_2O_2, determined by an in vitro model.
Conclusion(s): GSTT1 is a good indicator for age-related infertility. (Fertil Steril® 2008;90:1026–35. ©2008 by American Society for Reproductive Medicine.)

Key Words: Glutathione S-transferase theta 1 (GSTT1), aging, granulosa cell, biomarker, oocyte quality

Maternal age is a risk factor for infertility. The decline in fecundity becomes clinically evident when women reach their mid-30s (1). Despite the disadvantages, women in Western industrialized nations now tend to delay the birth of their first child until a later age than before. This tendency leads to declining birth rates, resulting in aging populations, which is a serious social issue. Although the exact mechanism by which aging causes female reproductive disorders is unclear, age-related changes in the ovary—including hormonal imbalance (2), decrease of the ovarian follicle pool (3), increase of oocyte aneuploidy (4), and mitochondrial dysfunction in oocytes (5)—account for the loss of reproductive function.

Oxidative stress is a major source of aging; it damages genomic and mitochondrial DNA, causing tumors and/or apoptosis in many cell types. Oocytes and somatic cells stored in ovaries are thought to be exposed to reactive oxygen species (ROS) during both ovulation and aging (6). In mice, repeated ovarian stimulation with gonadotropins increased the incidence of oxidative DNA damage and mitochondrial DNA mutation (7). In humans, accumulation of 8-hydroxy-2'-deoxyguanine, a byproduct of oxidative stress-induced digestion of DNA, in mural and cumulus granulosa cells from infertile patients correlated negatively with the oocyte quality (8). Moreover, the amount of ROS in human follicular fluid

Received April 3, 2007; revised July 6, 2007; accepted July 30, 2007.
M. Ito, M. Muraki, and Y. Takahashi contributed equally to this work.
Partly supported by a grant of Research on Child Health and Development (2005–2008) from the Ministry of Health, Labour and Welfare, and partly by grants (2005–2008: #17791147, #17791148; 2006–2009: #18591818) from the Ministry of Education, Culture, Sports, Science and Technology.
Reprint requests to: Yuji Takahashi, Ph.D., Division of Reproductive Medicine, Department of Perinatal Medicine and Maternal Care, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan (FAX: +81-3-3416-2222; E-mail: takahashi-yj@ncchd.go.jp).

0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.07.1389

was negatively correlated with oocyte development potential (9). The apoptotic status of rat follicular cells was also in parallel with ROS production (10). In agreement with this report, apoptosis possibly induced by ROS in granulosa cells from infertile patients was closely related to the oocyte quality (11, 12). Thus, repeated exposure of oocytes and granulosa cells to oxidative stress must be associated with reproductive failure.

Organisms have many adaptive devices to oxidative stress and genotoxins. Glutathione S-transferases (GSTs) are well known to detoxify the metabolites of genotoxic molecules to more water-soluble and readily excretable forms. In addition, they are known to protect cells from ROS-induced membrane lipid peroxidation (13). Because of their roles in self-defense, mutations of GSTs are often linked to certain diseases. Several studies have indicated that GSTs may play a role in predisposition to cancer, with the GSTM1 and GSTT1 null phenotypes (14–16). A number of common polymorphisms affect enzyme activity; these include gene deletions in the GSTM1 and GSTT1 genes, which result in lack of the corresponding enzyme activity (17). The mutated products modulate chemical binding to DNA, and are associated with myocardial infarction as well as the tobacco-related cancers in smokers (18, 19). In addition, polymorphisms of GSTM1 and GSTT1 may increase the risk of recurrent pregnancy loss (20), and susceptibility to polycystic ovaries (21).

Because of the limited availability of molecular information and biomarkers for age-related infertility, effective diagnosis and therapy have not yet been established. The aim of our present study, therefore, was to explore the possibility that the expression of GSTs in granulosa cells obtained from infertility patients is associated with age-related changes of fecundity. We screened for the expression of GSTs in young or older (stressed) granulosa cells by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, to select GSTs as potential biomarkers for age-related infertility; we then compared the expression levels of each potential molecule in various patients with oocyte quality. Finally, we examined the cause of aberrant expression of GSTs in in vitro model systems.

MATERIALS AND METHODS

Reagents

Hoechst 33342, human FSH, human LH, and hCG were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against GSTT1 and BAX were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Donkey anti-rabbit IgG-Cy3 was purchased from Chemicon International Co. Ltd. (Temecula, CA).

Patients and Samples

Mural granulosa cells and cumulus granulosa cells were obtained from infertile patients undergoing IVF/intracytoplasmic sperm injection cycles between September 2004 and June 2007 at the National Center for Child Health and Development, Japan. One hundred seventy-six infertile patients (male factor: 50; tubal factor: 32; unknown factor: 94) participated in the study. The institutional review board approved the experiments on these samples, and individual patients provided prior informed consent.

Mural granulosa cells were isolated from follicular aspirates and washed three to four times in phosphate-buffered saline (PBS) containing 1 mg/mL bovine serum albumin (BSA) (PBS + BSA). In addition, cumulus-oocyte complexes (COCs) were isolated and washed twice in IVF medium (human tubal fluid medium purchased from Irvine Scientific Co., Santa Ana, CA). Cumulus cells were then detached from the COCs physically using 27-G fine needles in IVF medium and washed three times in PBS + BSA. Just before detachment of the cumulus cells, the maturity of the COCs was evaluated based on the following morphologic criteria: [1] complete expansion of cumulus with visible halo (mature), [2] incomplete expansion of cumulus without halo (immature), and [3] incomplete or complete expansion and dissociation of cumulus with dark spots (dysmature).

A portion of the mural and cumulus granulosa cells was transferred into 1.5-mL microtubes, centrifuged for 10 minutes at 15,000 rpm at 4°C to remove excess buffer, and stored at −80°C until use. The remaining samples were fixed in 4% formaldehyde in PBS + BSA for 30 minutes at room temperature, washed three times in PBS + BSA, put on glass slides, air-dried, and stored at 4°C until use.

Preparation of Mouse Ovaries

Experiments were approved by the Animal Ethics Committee at the National Center for Child Health and Development. ICR female mice (8 weeks old) were purchased from Sankyo Labo Service Co. Ltd (Tokyo, Japan). They were injected with 6 IU of pregnant mare serum gonodotropin (PMSG), and ovaries were collected at 72 hours post-injection to induce the atretic follicles. In addition, some mice were injected with 6 IU hCG following PMSG administration, and ovaries were collected at 8 hours post-hCG injection to obtain ovaries with preovulatory follicles. Those ovaries were then fixed in 4% paraformaldehyde in PBS overnight at 4°C. They were then sectioned serially by Cryostat (8 μm interval, Leica Microsystems Japan Co. Ltd, Tokyo, Japan), and subjected to immunofluorescence studies. The sections were kept at 4°C until use.

Cell Culture and Treatment

A human granulosa-like tumor cell line, KGN, was established previously (22). The cells were maintained in Dulbecco's minimum essential medium/Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum, 100 μg/mL penicillin, and 100 IU/mL streptomycin at 37°C in a CO_2 incubator. For the immunofluorescence study, the cells were seeded onto eight-well culture slides (BD Japan Co. Ltd, Tokyo, Japan) and stimulated with either gonadotropins (FSH, LH, hCG) or oxidative stress (H_2O_2, 10 μM) for 6 to 24 hours at the indicated concentration. The cells