Localization of Glutamate Cysteine Ligase Subunit mRNA Within the Rat Ovary and Relationship to Follicular Apoptosis

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ABSTRACT Ovarian levels of the antioxidant tripeptide glutathione (GSH) increase following gonadotropin administration, suggesting that GSH synthesis in the ovary may be associated with follicular growth. In situ hybridization with ^{35}S-labeled riboprobes was used to localize ovarian mRNA expression of the catalytic and modulatory subunits of glutamate cysteine ligase (*Gclc* and *Gclm*), the rate-limiting enzyme in GSH synthesis, during each stage of the rat estrous cycle. *Gclm* was highly expressed in the granulosa cells and oocytes of healthy, growing follicles, not in atretic follicles. *Gclc* was also highly expressed in follicles; however, unlike *Gclm*, *Gclc* was also expressed in corpora lutea and interstitial cells. In a subsequent experiment, the hypothesis that GSH synthesis occurs in healthy, but not in apoptotic, follicles was tested. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to detect apoptotic cells in the ovaries, and in situ hybridization for *Gclm* and *Gclc* was performed in adjacent sections of the same ovaries. TUNEL staining was found to be significantly associated with absence of *Gclm* hybridization in granulosa cells and oocytes and with lack of strong *Gclc* hybridization in granulosa cells. These results suggest that follicular apoptosis may be associated with down-regulation of *Gclm* and *Gclc* transcription in granulosa cells and oocytes. *Mol. Reprod. Dev. 65: 254–261, 2003.*

Key Words: follicle; glutathione; gamma-glutamylcysteine synthetase; atresia; estrous cycle

INTRODUCTION

The tripeptide glutathione (γ-glutamylcysteinylglycine, GSH) plays critical roles in protecting cells from oxidant injury by both endogenous and exogenous agents (Deneke and Fanburg, 1989). GSH also acts as a cofactor for enzymes, regulates protein and DNA synthesis by altering redox status, and participates in microtubule assembly (Deneke and Fanburg, 1989). GSH synthesis occurs by a two-step process (Griffith and Mulcahy, 1999; Soltaninassab et al., 2000). The first and rate-limiting reaction, forming γ-glutamylcysteine, is catalyzed by glutamate cysteine ligase (GCL, also known as γ-glutamylcysteine synthetase). The second reaction, forming GSH, is catalyzed by glutathione synthetase. GCL is a heterodimer, composed of a catalytic subunit (GCLc) and a modulatory subunit (GCLm) (Griffith and Mulcahy, 1999; Soltaninassab et al., 2000). GCLc possesses all of the catalytic activity of the enzyme. However, binding of GCLm to GCLc considerably increases the catalytic activity (Griffith and Mulcahy, 1999; Soltaninassab et al., 2000).

GSH is present at moderately high concentrations in the ovary (Mattison et al., 1983; Clague et al., 1992; Luderer et al., 2001). GCL subunit protein and mRNA are expressed in the rat ovary (Luderer et al., 2001), indicating that the ovary possesses the machinery for GSH synthesis. Uptake of circulating GSH is probably not a significant source of GSH for the ovary, as neither follicles nor oocytes have been found to possess significant γ-glutamyltranspeptidase activity (Hanigan et al., 1994). As it does in other tissues, ovarian GSH likely plays important roles in detoxifying reactive oxygen species (ROS) and in conjugating electrophilic toxicants via glutathione-*S*-transferase catalyzed reactions (Deneke and Fanburg, 1989).

Reactive oxygen species (ROS), such as superoxide, are produced in the ovary, as in other tissues, as a result of normal cellular metabolism (Laloraya et al., 1988). Steroid synthesis by the steroidogenic cytochrome P450 enzymes has been shown to be a significant source of ROS in the testis and the adrenal, and likely is also in ovarian follicles (Hornsby and Crivello, 1983; Hall,

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1994). ROS disrupt normal granulosa cell function (Riley and Behrman, 1991). ROS may also promote the apoptotic process of ovarian follicular degeneration known as atresia (Tilly and Tilly, 1995). Follicular atresia can be blocked in vitro by administration of *N*-acetylcysteine, a precursor of GSH (Tilly and Tilly, 1995). The observation that stimulation of follicular development by the administration of exogenous gonadotropin increases ovarian GSH concentrations and GCL subunit protein levels (Luderer et al., 2001) further supports a protective role of GSH in ovarian follicular development.

To determine whether GSH is synthesized in ovarian follicles, the first experiment described herein used in situ hybridization to localize expression of *Gclc* and *Gclm* mRNA in rat ovaries during each stage of the estrous cycle. The second experiment tested the hypothesis that GCL subunit mRNA is not expressed in apoptotic follicles, using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) to label cells committed to apoptosis within follicles combined with in situ hybridization for *Gclc* and *Gclm*.

MATERIALS AND METHODS

Experimental Animals

Adult, 9–10-week-old female Sprague–Dawley rats (Crl:CD(SD)IGS BR) were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, the animals were housed in an AAALAC-accredited facility, 3 to a cage, with free access to deionized water and standard laboratory chow, on a 12-hr/12-hr (Experiment 1) or a 14-hr/10-hr (Experiment 2) light/dark cycle. After a 7-day acclimatization period, daily vaginal cytology was performed for at least two 4-day estrous cycles prior to initiation of the experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington (Experiment 1) or at the University of California at Irvine (Experiment 2), where the experiments were performed.

Experiment 1: Localization of GCL Subunit mRNA Within the Rat Ovary During the Estrous Cycle

Rats weighing 249–284 g were rapidly killed by decapitation between 09:00 hr and 11:00 hr on each day of the estrous cycle as determined by vaginal cytology ($n = 4$ for each cycle stage except proestrous on which $n = 3$). Trunk blood was collected for estradiol and progesterone assays to verify estrous cycle stage (data not shown). Estrous cycle stage was further verified by examination of the uterus for ballooning on proestrus (Armstrong, 1968). Ovaries were rapidly dissected out, trimmed of fat, and one ovary from each animal was prefixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 1 hr, then immersed for 3 hr in 15% sucrose in PBS at 4°C. The ovary was then embedded in Tissue Tek OCT (Sakura Finetek USA, Torrance, CA) and stored at −70°C until sectioning. The other ovary was used for a separate study. Embedded, frozen ovaries were serially sectioned (11 μm) using a cryostat onto charged microscope slides (Fisher Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and stored at −70°C until in situ hybridization. Subsequently, every third section was subjected to in situ hybridization with antisense *Gclc* probe, and adjacent sections were subjected to hybridization with antisense *Gclm* probe or with sense *Gclc* or *Gclm* probes.

Experiment 2: Detection of Apoptotic Follicles by TUNEL and Co-Localization With GCL Subunit mRNA Expression

Rats weighing 230–285 g were rapidly killed by decapitation on either estrus ($n = 4$) or metestrus ($n = 4$) between 07:00 hr and 08:30 hr. The ovaries were removed and dissected as for Experiment 1. One ovary was then prefixed, embedded, and stored as for Experiment 1. Embedded, frozen ovaries were serially sectioned (10 μm) using a cryostat onto charged microscope slides. Slides were then stored at −70°C until further processing. Every fourth section was subjected to TUNEL, and adjacent sections were used for in situ hybridization with antisense *Gclc* probe, antisense *Gclm* probe, or sense *Gclc* or *Gclm* probes.

In Situ Hybridization

The hybridization procedure was adapted from Wilcox (Wilcox et al., 1986) utilizing ^{35}S-labeled antisense riboprobes transcribed from full-length 0.82 kb mouse GCLm cDNA in pCR II (Reid et al., 1997) or a 0.6 kb mouse GCLc cDNA fragment in pBluescript II (Kang et al., 1997) using the appropriate RNA polymerase. Sense probes were also transcribed for use as negative controls. These cDNAs have 100% homology with the rat and human cDNAs. After removal from storage, sections were fixed in 4% paraformaldehyde in PBS. Sections were then pretreated with Proteinase K, washed, prehybridized in hybridization buffer (10 mM DTT, 0.3 M NaCl, 20 mM Tris, 5 mM EDTA, 1× Denhardt's solution, 10% Dextran sulfate, 50% formamide) at 42°C for 1 hr, hybridized overnight at 55°C with the appropriate riboprobe (3×10^6 cpm/slide) and transfer RNA (150 μg/slide) in hybridization buffer, washed, and dried. Slides were exposed to Kodak Biomax MS film at 4°C for 3 days. After the film was developed, slides were dipped in Kodak NTB2 emulsion diluted 1 to 2 with deionized water at 42°C, exposed at 4°C for 14 days, developed, and counterstained with toluidine blue or hematoxylin. Silver grains were visualized by dark field microscopy. Sections of kidney were used as "positive controls" because of the known high levels of GCL subunit mRNA in this organ (Li et al., 1996). Two kinds of negative controls were also run to test the specificity of the antisense riboprobes: (1) some slides were incubated with sense, rather than antisense, riboprobes; (2) some slides were pre-incubated with RNAse A (Qiagen, Valencia, CA) at a concentration of 20 μg/ml for 25 min prior to incubation with the antisense riboprobes.

Serial sections of ovaries were examined under light microscopy and follicles were classified as primary

(Pederson stages 1–3), secondary (Pederson stages 4–5), or antral (Pederson stages 6–8) (Plowchalk et al., 1993; Pedersen and Peters, 1968). The follicle diameter was measured as the average of two perpendicular diameters in the section in which the oocyte nucleolus was visible. From Experiment 1, this was found to eliminate most follicles in late stages of atresia, as these follicles often do not exhibit nucleoli (Hirshfield, 1988). Therefore, for Experiment 2, the diameter of highly atretic follicles was measured in the section in which the oocyte was visible, if there was no section with a nucleolus. The follicle granulosa cells and oocytes were scored for *Gclm* hybridization and for *Gclc* hybridization (absent, weak, or strong signal) using dark field microscopy.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

TUNEL was accomplished using the In Situ Cell Death Detection Kit, POD (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, paraformaldehyde-prefixed ovaries were sectioned and stored as for in situ hybridization. On the day of TUNEL, slides representing every fourth serial section from each ovary were thawed at room temperature, fixed in 4% paraformaldehyde for 10 min, washed, treated with 3% hydrogen peroxide in methanol for 10 min, washed in PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, washed, blocked for 30 min with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS), washed, incubated at 37°C for 1 hr with terminal deoxynucleotidyl transferase/label solution (containing fluorescein-labeled dUTP), and washed. Slides were then observed under fluorescence microscopy and photographs were taken. Subsequently, slides were blocked with 3% BSA in PBS, washed, incubated for 30 min at 37°C with peroxidase converter solution (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase) diluted 1:2 in TUNEL dilution buffer, washed, and incubated for 5 min with diaminobenzidine substrate solution (Roche Molecular Biochemicals), washed, and counterstained with hematoxylin. All washes and incubations were at room temperature unless otherwise indicated.

For Experiment 2, follicles were scored as atretic according to the following histological criteria, which are based on Hirshfield (Hirshfield and Midgley, 1978; Hirshfield, 1988): (1) more than 3 pyknotic granulosa cell nuclei in any one cross-section, except for follicles of less than 50 cells in cross section, in which case one pyknotic nucleus; (2) separation of the oocyte from the surrounding granulosa cells; (3) thinning of the granulosa cell layer in antral follicles; (4) loss of distinct boundary between granulosa and theca cell layers. Follicles were judged definitely atretic if they met the pyknosis criteria, and were judged as advanced atresia if they met at least two of the remaining criteria. Follicles were judged possibly atretic if they met one of the other criteria, but not the first criterion. Finally, follicles were scored in terms of TUNEL staining: (1) no TUNEL positive granulosa cells; (2) lightly TUNEL positive granulosa cells or fewer than three strongly positive granulosa cells; or (3) three or more strongly positive granulosa cells.

Statistical Analysis

The Pearson chi-square statistic was used to determine whether the distribution of GCL subunit antisense riboprobe hybridization to follicles varied with estrous cycle stage and to determine whether the distribution of TUNEL staining of follicles varied with GCL subunit

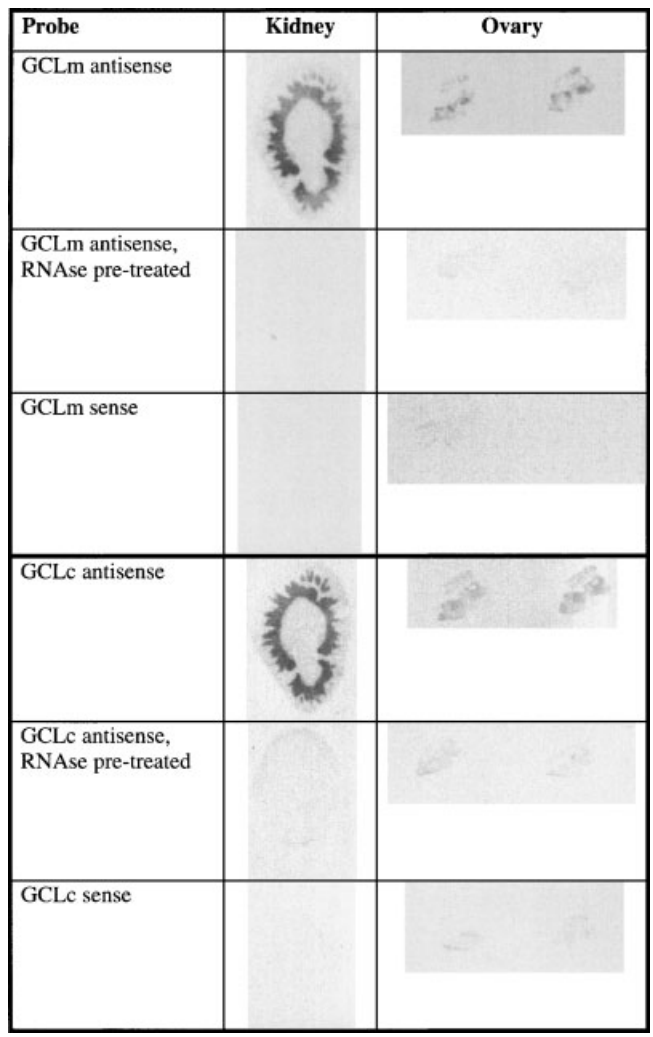

Fig. 1. Demonstration of the specificity of *Gclm* and *Gclc* antisense riboprobes. Paraformaldehyde-fixed sections of rat kidney ("positive control" tissue) or ovary were hybridized overnight at 55°C with the indicated ^{35}S-labeled riboprobe, with or without 25 min pretreatment with 20 μg/ml RNAse A, as indicated. After serial washes and dehydration, the slides were exposed to Kodak Biomax MS film for 3 days at 4°C. The film was then developed and the autoradiographs were scanned. All sections shown here were from a single experiment and were scanned from a single autoradiograph; all the ovary sections are from the same ovary; all the kidney sections are from the same kidney. *Gclm* and *Gclc*: glutamate cysteine ligase modulatory and catalytic subunits.

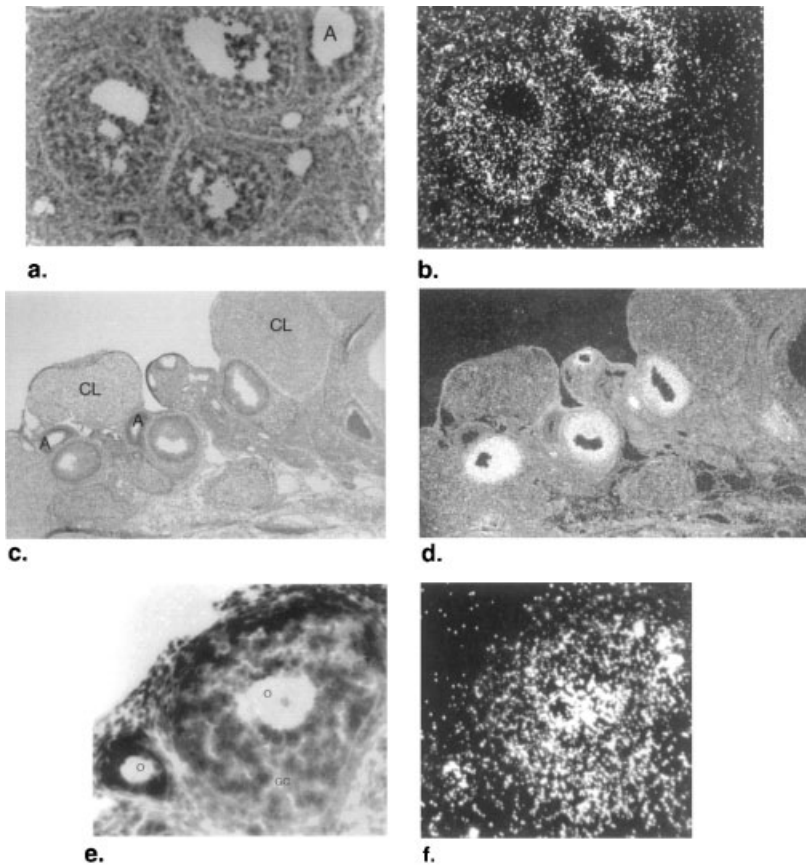

Fig. 2. *Gclm* antisense riboprobe hybridization to rat ovary during the estrous cycle. Paraformaldehyde-fixed 11 μm sections of rat ovaries from each stage of the estrous cycle were hybridized with ^{35}S-labeled antisense riboprobe to mouse *Gclm*. Under bright field microscopy (**a**, **c**, **e**), follicles were scored as primary, secondary, or antral and as healthy or atretic (indicated by pyknotic nuclei, oocyte separated from cumulus cells, and loss of distinct boundary between theca and granulosa cells). *Gclm* hybridization was observed under dark field (**b**, **d**, **f**). *Gclm* antisense riboprobe hybridized strongly to granulosa cells and oocytes of healthy secondary and antral follicles, but hybridized minimally to interstitial cells or to corpora lutea. There was no significant cycle stage dependent variation in *Gclm* hybridization. a, b: Estrous ovary. *Gclm* probe hybridized strongly to the granulosa cells of healthy antral follicles, but not to an atretic follicle (A). Original magnification ×66. c, d: Metestrous ovary. *Gclm* probe hybridized strongly to the granulosa cells of healthy secondary and antral follicles, but not to atretic follicles (A). *Gclm* probe also showed minimal hybridization to corpora lutea (CL). Original magnification ×13.2. e,f: Metestrous ovary. *Gclm* probe hybridized strongly to granulosa cells (GC) and oocytes (O) of healthy secondary follicles. Original magnification ×132. *Gclm* and *Gclc*: glutamate cysteine ligase modulatory and catalytic subunits.

mRNA hybridization to follicles or with follicle class. Analyses were performed using SPSS 10 for the MacIntosh or Systat 5.2.1 (SPSS, Chicago, IL).

RESULTS

Demonstration of Riboprobe Specificity

Figure 1 shows representative kidney ("positive control") and ovary sections treated with antisense GCL riboprobes, without and with RNAse A pretreatment, or treated with sense GCL riboprobes. Antisense riboprobes to both *Gclc* and *Gclm* showed the characteristic paracortical hybridization in the kidney that has been previously described (Diaz et al., 2001), as well as hybridization to ovarian structures. Sense riboprobes for both subunits showed minimal hybridization to any ovarian structure or to kidney (Fig. 1). Preincubation with RNAse A prior to incubation with the antisense riboprobes also abolished the hybridization signals (Fig. 1).

Experiment 1

Gclm antisense riboprobe hybridized to granulosa cells and oocytes of healthy, growing secondary and antral follicles in ovaries from all estrous cycle stages (Fig. 2). *Gclc* antisense riboprobe also hybridized to granulosa cells of secondary and antral follicles, with stronger *Gclc* hybridization observed to granulosa cells of some larger secondary and antral follicles than to smaller follicles (Fig. 3). *Gclm* antisense riboprobe hybridized minimally to primordial follicles, but hybridized to some primary follicles. There was also minimal *Gclm* hybridization to corpora lutea or interstitial cells. Unlike *Gclm*, *Gclc* riboprobe hybridized strongly to corpora lutea and hybridized to interstitial structures and primordial follicles (Fig. 3). *Gclm* antisense probe appeared not to hybridize to atretic follicles as judged by histological criteria (Fig. 2). Although *Gclc* riboprobe hybridized to all ovarian structures, strong hybridization with *Gclc* riboprobe was rarely observed

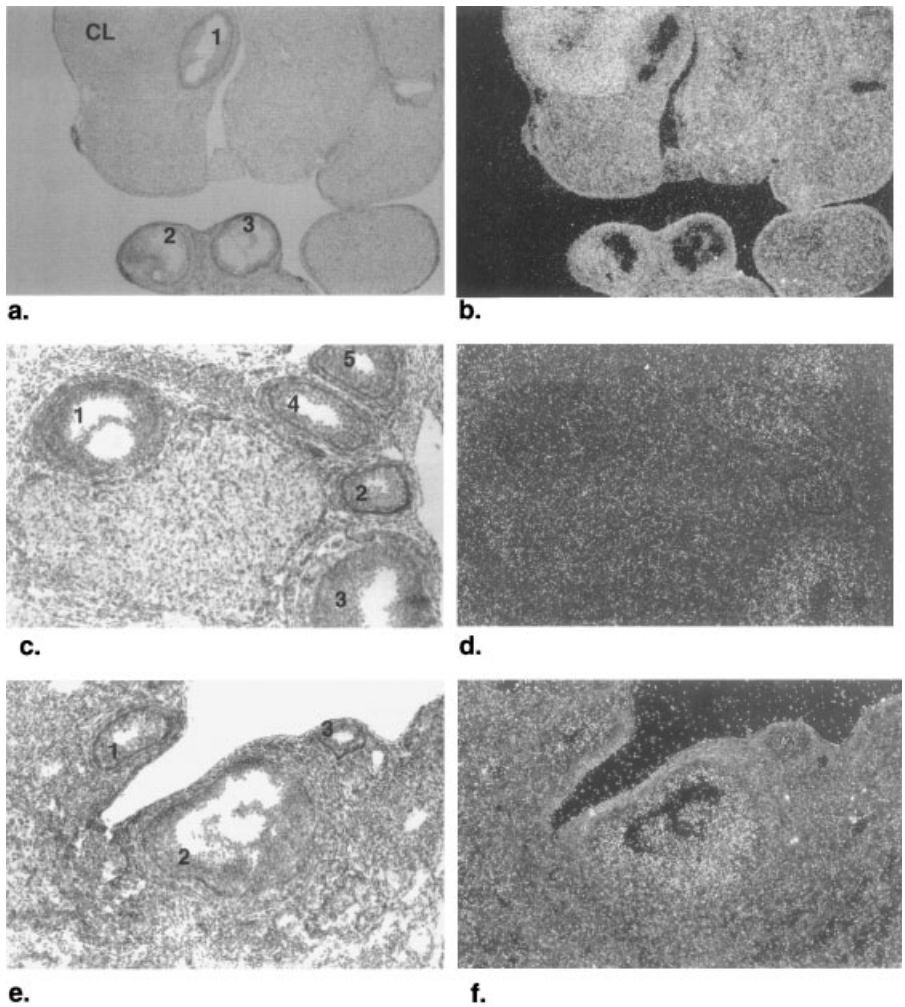

Fig. 3. *Gclc* antisense riboprobe hybridization to rat ovary during the estrous cycle. Ovaries were treated as for Figure 2 except that *Gclc* antisense riboprobes were used. Bright field (**a**, **c**, **e**) and dark field (**b**, **d**, **f**) images of the same fields are shown. There was no significant cycle stage dependent variation in *Gclc* probe hybridization. a, b: Metestrous ovary. *Gclc* probe hybridized to most structures within the ovary, with strongest hybridization to some corpora lutea (CL) and granulosa cells of some follicles (2). The weakest hybridization was observed in granulosa cells of obviously atretic follicles (1,3). Original magnification ×13.2. c, d: Estrous ovary. *Gclc* probe hybridized strongly to granulosa cells of follicles 3, 4, and 5, but less strongly to granulosa cells of atretic follicle 1 and secondary follicle 2. Original magnification ×33. e, f: Estrous ovary. *Gclc* probe hybridized to granulosa cells of antral follicle (2) and secondary follicle (1), but less to granulosa cells of small secondary follicle (3). Original magnification ×33. *Gclm* and *Gclc*: glutamate cysteine ligase modulatory and catalytic subunits.

in atretic follicles (Fig. 3). However, the difference in hybridization between healthy and atretic follicles was much more pronounced for *Gclm* than for *Gclc* (Fig. 4).

Estrous cycle stage variation in GCL subunit hybridization was evaluated only for follicles in which a cross-section containing the oocyte nucleus was observed. This eliminated grossly atretic follicles. There was no significant estrous-cycle stage dependent variation in *Gclm* or *Gclc* hybridization.

Experiment 2

In situ hybridization using antisense riboprobe to *Gclm* or *Gclc* mRNA and TUNEL were performed on adjacent sections of estrous and metestrous ovaries. Pearson's chi-square test on the resulting cross-tabulated data (TUNEL by GCL subunit hybridization) revealed a strong association between absent *Gclm* hybridization and strong TUNEL staining ($P < 0.001$ for hybridization to granulosa cells or to oocytes; Fig. 5). Of the strongly TUNEL positive follicles, 67.4% showed no granulosa cell *Gclm* hybridization. Similarly, there was a strong association between meeting histological criteria for atresia and *Gclm* hybridization to granulosa cells ($P < 0.001$) or oocytes ($P < 0.001$), with 60.3% of atretic follicles showing no granulosa cell *Gclm* hybridization. *Gclc* hybridization to granulosa cells was also significantly negatively associated with TUNEL staining ($P = 0.022$) and histological criteria for atresia ($P = 0.005$). However, *Gclc* hybridization to oocytes was not significantly associated with TUNEL ($P = 0.244$) or histological criteria for atresia ($P = 0.459$).

Most (85.1%) of the strongly TUNEL positive follicles were antral follicles. Few secondary follicles and virtually no primordial or primary follicles exhibited TUNEL

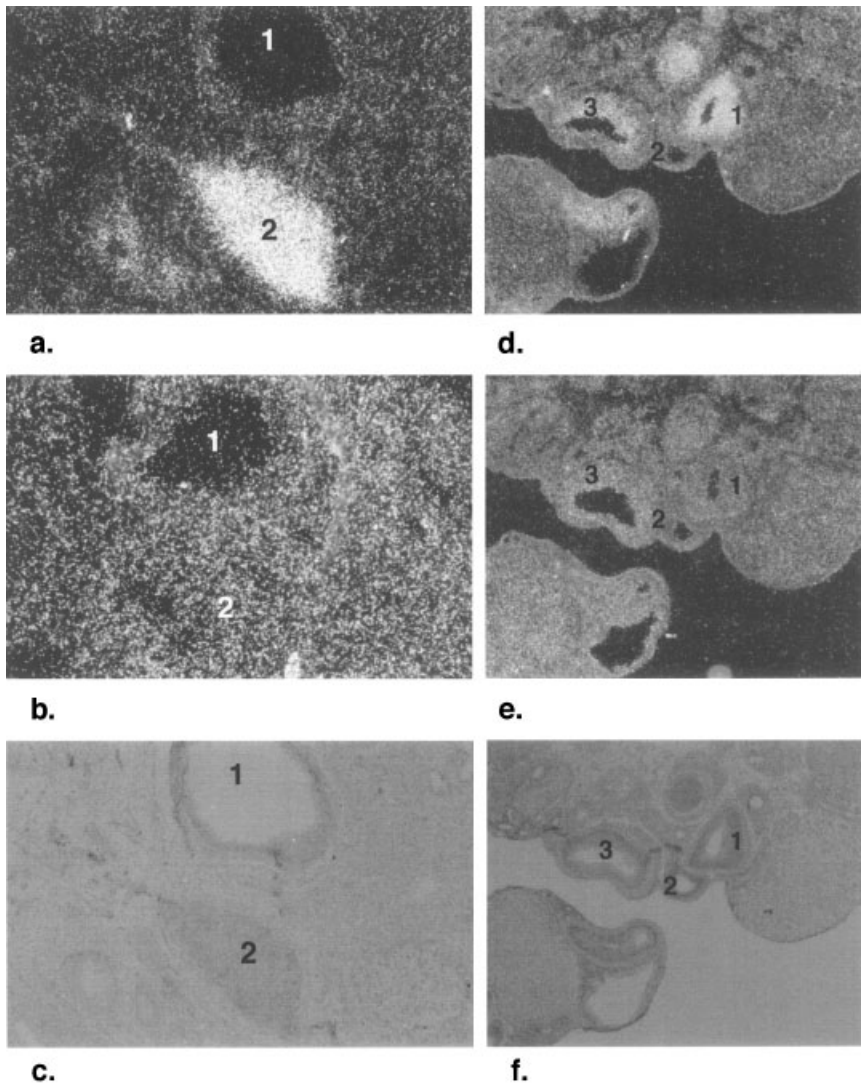

Fig. 4. Comparison of *Gclm* and *Gclc* antisense riboprobe hybridization in adjacent ovarian sections from metestrous ovaries. Dark field (**a**, **b**, **d**, **e**) and bright field (**c**, **f**) images of follicles from two different ovaries (a–c and d–f) are shown. a: *Gclm* riboprobe hybridized strongly to granulosa cells of a healthy follicle (2), but not to an atretic follicle (1). b: In contrast to *Gclm*, *Gclc* riboprobe did not hybridize differentially to the same follicles as in (a). d: *Gclm* riboprobe hybridized strongly to granulosa cells of two healthy follicles (1, 3), but not to an atretic follicle (2). e: *Gclc* riboprobe did not hybridize differentially to follicles (1) and (2), but hybridized more strongly to follicle (3). Original magnification ×33 (a, b, c) or ×13.2 (d, e, f).

positive cells. The association between follicle class and TUNEL was statistically significant ($P < 0.001$).

DISCUSSION

Ovarian follicular atresia is an apoptotic process, as demonstrated by the observation of various indicators of apoptosis in atretic follicles, including nuclear pyknosis, DNA laddering, TUNEL staining, and caspase activation (Zelesnik et al., 1989; Tilly, 1998; Tilly and Robles, 1999). However, the factors that cause some follicles to remain healthy and eventually ovulate and others to become atretic are only partially understood. Gonadotropins clearly play an important role, as evidenced by the ability of follicle stimulating hormone to rescue cultured follicles from apoptosis (Tilly and Tilly, 1995). The protective effect of gonadotropins may be mediated by upregulation of antioxidant defenses, including GSH (Tilly and Tilly, 1995). The experiments described herein further support a role for GSH in the protective effect of gonadotropin hormones against follicular atresia.

The onset of follicular *Gclm* mRNA expression appeared to be associated with the recruitment of follicles into the growing pool. *Gclm* mRNA was minimally expressed in primordial follicles, began to be expressed in primary follicles, and was strongly expressed in granulosa cells and oocytes of healthy secondary and antral follicles. *Gclc* mRNA was more ubiquitously expressed in the ovary, but the highest levels of expression were observed in granulosa cells of healthy antral follicles and in some corpora lutea. In a preliminary experiment using a polyclonal GCLc antibody, GCLc protein

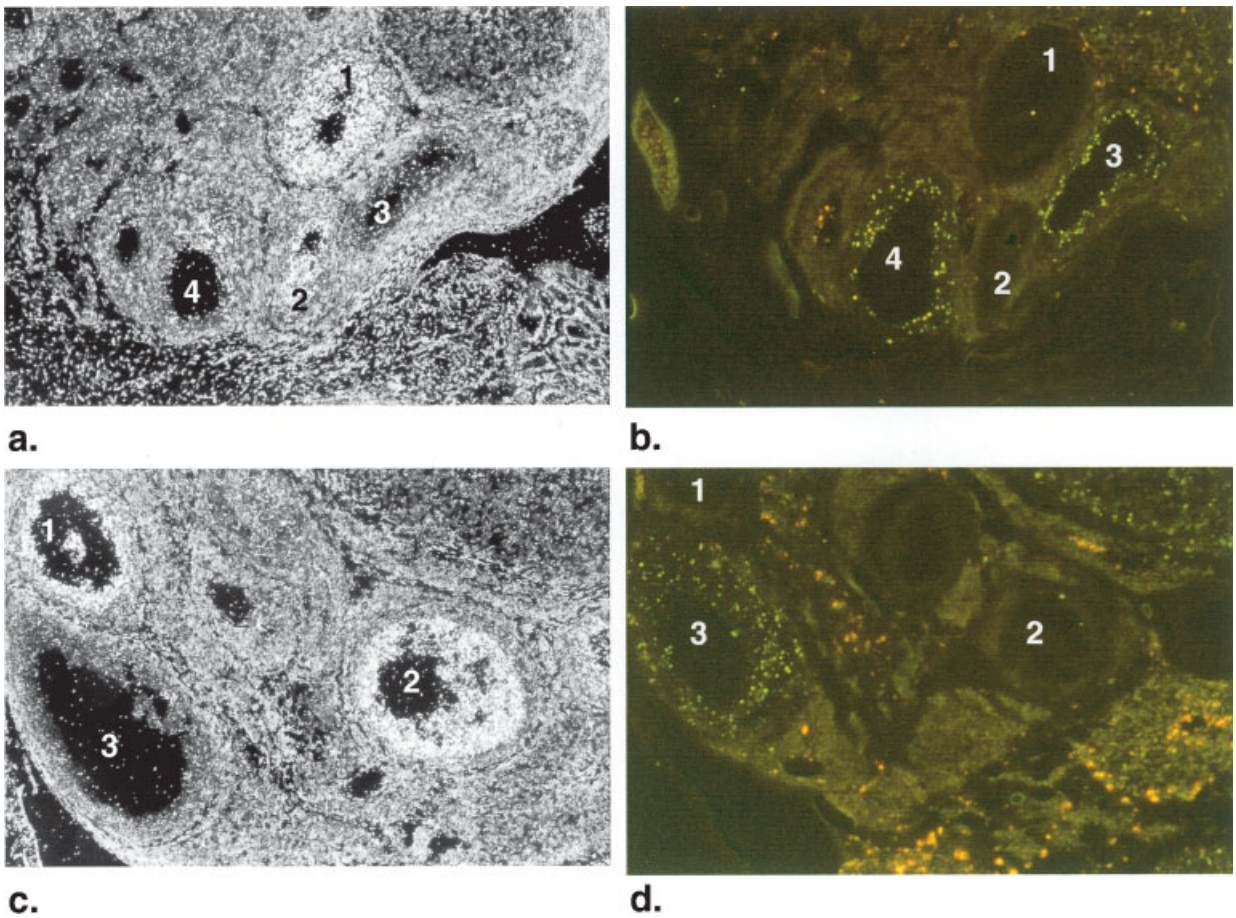

Fig. 5. Comparison of TUNEL and *Gclm* antisense riboprobe hybridization in adjacent ovarian sections. **a**: Dark-field view of estrous ovary shows *Gclm* riboprobe hybridization to granulosa cells of follicles 1 and 2, but not to follicles 3 and 4. Original magnification ×33. **b**: Fluorescence view of the same follicles as in (a) in an adjacent section shows numerous TUNEL-stained green fluorescent granulosa cells in two atretic follicles that lack *Gclm* hybridization (3 and 4), and no TUNEL-stained cells in healthy follicles that display strong *Gclm* hybridization (1 and 2). Original magnification ×33. **c**: Dark-field view of estrous ovary shows *Gclm* riboprobe hybridization to follicles 1 and 2, but not follicle 3. Original magnification ×33. **d**: Fluorescence view of same follicles as in (c) shows TUNEL-stained green granulosa cells in atretic follicle (3) that lacks *Gclm* hybridization, and no TUNEL-stained cells in healthy follicles (1 and 2) that display strong *Gclm* hybridization. Original magnification ×33. *Gclm* and *Gclc*: glutamate cysteine ligase modulatory and catalytic subunits. TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

expression in immature rat ovaries stimulated with pregnant mare's serum gonadotropin showed a similar pattern of localization as *Gclc* mRNA in the present study (unpublished observations). It has recently been reported that protein expression of another GSH-related enzyme, glutathione reductase, displays a pattern in the ovary that overlaps the pattern observed here for *Gclc* and *Gclm*. Glutathione reductase protein expression was the strongest in oocytes and was also strong in granulosa cells and corpora lutea (Kaneko et al., 2001). Taken together these findings suggest that follicles possess the ability to synthesize GSH de novo as well as the ability to regenerate reduced GSH from oxidized GSSG. The ability to detoxify ROS may be of particular importance to growing follicles because their granulosa and theca cells are actively engaged in steroid synthesis. Increased production of ROS has been shown to be associated with the action of the steroidogenic cytochrome P450 enzymes in the adrenal gland and testis (Hornsby and Crivello, 1983; Hall, 1994).

The results of the present experiments suggest that GSH protects against granulosa cell apoptosis and follicular atresia. Strong hybridization of *Gclm* riboprobe was observed in the granulosa cells of healthy secondary and antral follicles, while atresia was associated with absence of *Gclm* expression. This was confirmed by the significant negative relationship observed between strong TUNEL positivity and absent or low *Gclm* and *Gclc* hybridization in Experiment 2.

In contrast to *Gclm*, the association between *Gclc* hybridization and TUNEL or histological criteria for atresia was weaker. This is consistent with our previous observation that regulation of GSH levels by gonadotropin in the ovary seems to occur mainly via modulation of GCLm and not GCLc. Gonadotropin stimulation significantly increased GCLm protein levels, but not GCLc protein levels, in immature rat ovaries (Luderer et al., 2001). An isolated down-regulation of *Gclm* mRNA expression within granulosa cells would result in decreased catalytically active GCL enzyme (GCLc/

GCLm heterodimer) if GCLc protein is more abundant than GCLm within the cell. It is not known whether GCLc protein is more abundant than GCLm protein in ovarian granulosa cells.

The rates of atresia during various estrous cycle stages observed in the present study are in agreement with previous studies. Hirshfield and Midgley (Hirshfield and Midgley, 1978) examined serial sections of rat ovaries on each stage of the estrous cycle. They reported that 35–45% of all follicles greater than or equal to 170 μm in diameter were atretic by histological criteria (Hirshfield and Midgley, 1978). In Experiment 2, 57% and 42% of all follicles greater than or equal to 170 μm, were atretic on metestrus and estrus, respectively. The slightly higher percentages of atretic follicles in the current study are likely due to the fact that Hirshfield and Midgley (1978) counted only follicles in which the oocyte nucleolus was visible. This would have excluded many of the highly atretic follicles that were counted in the present study (Hirshfield, 1988). The observation in the present study that atresia was much more common in antral than in secondary follicles is also consistent with previously reported findings (Hirshfield and Midgley, 1978; Hirshfield, 1988).

CONCLUSIONS

The results of the present experiments suggest that follicular apoptosis may be associated with down-regulation of GSH synthesis. In situ hybridization localized mRNA expression of *Gclm*, the modulatory subunit of the rate-limiting enzyme in GSH synthesis, to granulosa cells and oocytes of healthy, growing follicles and not to atretic follicles. Follicular atresia, identified both by histological criteria and TUNEL, was significantly associated with absent *Gclm* and reduced *Gclc* hybridization.

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