Subpopulations of physiological and ovarian follicular fluid peptide induced apoptotic cells

M S Rajadhyaksha
Department of Life Sciences, Sophia College, B. Desai Road, Mumbai 400 026, India
and
T D Nandedkar
Department of Biology, Institute for Research in Reproduction (ICMR), Parel, Mumbai 400 012, India

Received 20 April 1999; revised 26 July 1999

Ovarian follicular fluid peptide (OFFP) purified from sheep ovaries enhances apoptotic changes in ovarian granulosa cells of mice. To get an insight into the cell subpopulations responding to OFFP, the heterogeneity of granulosa cells was resolved. Subpopulations of granulosa cells were obtained from ovaries of immature mice treated with PMSG alone and autopsied 48 hr (control) and 72 hr after injection (atretic) and from animals injected OFFP 24 hr after PMSG injection and autopsied 24 hr later (OFFP treated) by separation on discontinuous Percoll gradient. Four fractions were collected and studied for their relative distributions and percent apoptotic cells measured by acridine orange staining. FSH binding to granulosa cell (sedimenting as a major) fraction was studied by radio receptor assay. There is a difference in densities in subpopulations of apoptotic cells induced by OFFP and those generated during the physiological process of atresia. This difference may be a reflection of different granulosa cell subpopulations involved in peptide response or differences in phases as the cells transit from normal to apoptotic phenotype. FSH binding to granulosa cells from OFFP treated animals was significantly less than those from control and atretic group.

Intra ovarian nonsteroidal factors are known to play a role in follicular maturation and atresia. Nandedkar et al. have shown that ovarian follicular fluid (OFFP) purified from sheep ovaries when injected into cycling mice induce degeneration of large follicles. In immature mice, PMSG induces follicular growth and granulosa cell proliferation and differentiation and at 48 hr preovulatory follicles are formed. Injection of HCG at this time is known to induce ovulation. If HCG is not given follicular atresia takes place by 72 hr. Apoptosis may be the mechanism underlying cell death in follicular atresia. OFFP prepones apoptosis in granulosa cells.

Granulosa cells are known to be heterogeneous with respect to morphology, biophysical characters like size and density, and responsiveness to hormones. It was of interest to examine granulosa cell heterogeneity involved in cells undergoing apoptosis due to atresia and due to OFFP induced degeneration. Density gradient techniques have been used to study subpopulations of granulosa cells in culture and in rat developing follicles. Structural and functional differences have been demonstrated in cell subpopulations separated on continuous density gradient of Percoll. Density gradient separation was, therefore, used to resolve the granulosa cell heterogeneity from ovaries of control, atretic and OFFP treated animals. The results indicated that a fraction of apoptotic cells sediment at a lower density as compared to fractions separated from the OFFP treated group. The FSH binding to cells in the major fraction separated in OFFP treated was less than those from control and atretic ovaries.

Materials and Methods

Purification of active fraction of OFFP—Follicular fluid was separated from sheep ovaries obtained from local abattoir. Pooled follicular fluid was centrifuged at 3000 rpm for 15 min to remove granulosa cells which were used for radio-receptor assay. The supernatant, about 150 ml, was purified by ultrafiltration using PM 10 amicon filter (cut off 10,000 Da). The filtrate but not the retentate showed FSH binding inhibitory activity in radio receptor assay (RRA). The filtrate was gel chromatographed on sephadex G-25 column and the active fraction was employed in the present study.

Treatment of animals—Immature, 21-23 days old female Swiss mice bred in our animal colony were used in the study. A group of animals were injected with 20 iu PMSG on day 0 and autopsied on day 2 (N) or on day 3 (A). The other group of animals was injected 20 iu PMSG on day 0 followed by 20 µg of
oGF5 on day 1 and was autopsied on day 2 (P). All the injections were given subcutaneously. At autopsy the ovaries were removed, freed of fat and connective tissue and pressed lightly in a homogenizer to harvest granulosa cells.

Separation of cells—Polyvinylpyrrolidone coated colloidal silica particles, (Percoll; Pharmacia Fine Chemicals AB, Uppsala, Sweden) were diluted to form isotonic solutions of densities 1.025, 1.035 and 1.045 and layered to form the discontinuous gradient. About 5x10^7 ovarian cells were loaded on the gradient, centrifuged at 2000 rpm for 20 min and four fractions of cells with density 1.045 (Fraction I), 1.045-1.035 (Fraction II), 1.035-1.025 (Fraction III) and <1.025 (Fraction IV) were collected. The percent distribution of cells in each fraction and the fraction of apoptotic cells in each was studied by fluorescence microscopy of acridine orange stained cells.

Staining with acridine orange and fluorescence microscopy—To 20 μl of 10^6 granulosa cells in medium, 20 μl of 1 mg/ml acridine orange (Sigma) was added on a glass slide. After mixing thoroughly, a coverslip was placed on the mixture. The cells were observed under the Leitz green fluorescence microscope using 500-525 nm filters. Normal and apoptotic cells were identified on the basis of morphological criteria reported by Evans et al. Normal cells were identified by their intact cell membrane, round nucleus with scanty chromatin. Cells with bright green condensed nuclei (intact or fragmented) were interpreted as apoptotic. Apoptotic cells were counted and represented as percent of the total cells scanned.

FSH binding studies—lodination of oFSH was carried out using the Chloramine T method. The specific activity of the labelled hormone used was 20 μCi/ng. Unseparated cells were incubated with radiolabelled hormone (20,000 cpm) for 2 hr prior to separation. Fraction III, which contained the major proportion of the Percoll separated cells from ovaries of control, PMSG alone treated and PMSG+OFFP treated mice were washed and the radioactivity in the pellets thus obtained were counted in Kontron gamma spectrometer. The specific binding was then calculated as reported previously.

Statistical analysis—Student's t test was used to analyze the data. Difference of p value < 0.05 was considered significant.

Results
Analysis of unseparated cells—A majority of granulosa cells were normal in appearance (Fig. 1) while only about 7% were apoptotic (Fig. 1) from animals autopsied on day 2 after PMSG injection. However, a large fraction of cells were apoptotic from animals treated with OFFP (about 36%) or autopsied on day 3 (35%).

Analysis of subpopulations of percoll separated cells
Fraction I: This fraction of cells from both control and atretic animals had a small percentage of total cells. (Fig. 2a) Significant increase in number of this fraction was seen in cells from peptide treated group. Majority of these cells were apoptotic (Fig. 2b).

Fraction II: A significantly greater number of cells sedimented as this fraction from OFFP treated animals in contrast to cells from control and atretic group. (Fig. 2a). About 80% of cells in this fraction from OFFP treated group were apoptotic (Fig. 2b).

Fraction III: A majority of cells sedimented as this fraction from all three groups. However, there were significantly less number of cells in the fraction from OFFP treated group (Fig. 2a). About 50% of the cells in this fraction were apoptotic from the OFFP treated group while about 21% are apoptotic from atretic group (Fig. 2b).

Fraction IV: This fraction had a small percentage of total cells from OFFP as well as control group (Fig. 2a). On the other hand, this fraction of atretic group had significantly large number of cells (Fig. 2b). This fraction also had fragmented cells or the cell debris.

Fig. 3 depicts the oFSH binding to cells sedimenting in fraction III. Binding of oFSH to granulosa cells is significantly less in oFSH to granulosa cells stained with Acridine orange. [N=Normal cell, A=Cell undergoing apoptosis]
Fig. 2—Distribution of total and apoptotic granulosa cells in fractions separated by Percoll density gradient (Fraction I, Fraction II, Fraction III, Fraction IV. C=Control, A=Atretic, P = OFFP treated. *significant difference (p<0.05) compared to control (C))

Discussion

In the present study, ovarian granulosa cell heterogeneity with respect to density in normal, atretic and OFFP treated groups has been resolved. The majority of cells from PMSG induced preovulatory follicles (N) sedimented at density between 1.025-1.035. A small fraction of these cells was apoptotic. The oFSH binding features of this fraction suggests that this fraction contained majority of the normal granulosa cells.

There were relatively few cells in the fractions at lower and higher densities in cells from control animals. This was in contrast to cell distribution characters seen in cells from atretic and OFFP treated animals.

A change in the density distribution profile of cells was observed in case of cells from ovaries from the atretic group. A significantly larger number of cells sedimented at a lower density as fraction IV. In addition oFSH binding of the cells in the major fraction was significantly less than those of the normal cells, suggesting less number of normal cell population in this fraction. Change in specific gravity of cells possibly has occurred as cells transit from normal to atretic phenotype. Change in specific gravity of porcine granulosa cells has been reported to be associated with luteinization in culture and change in FSH binding. A similar increase of cell density has been reported from porcine follicles cultured for 72 hr in PMSG. Of the atretic sample, a majority of cells sedimenting at lower density were apoptotic. Changes in cell density due to apoptosis have been reported in other systems. However in the present study, there seems to be a fraction of cells that did undergo morphological changes associated with apoptosis without concomitant changes in cell density.

On the other hand, cell distribution profile of granulosa cells from OFFP treated group indicated increase in cell number in fraction at higher densities. Unlike cells from atretic group, small fraction of cells sedimented at lower density (<1.025) in case of OFFP treated group. Significant increase in number of apoptotic cells was seen at higher densities (>1.045 and 1.045 - 1.035) in cells from OFFP treated group as compared to control and atretic group. In addition, the normal fraction of cells was lower in OFFP treated group as indicated by oFSH binding.

It therefore appears that there is a difference in the changes in the cells that take place in apoptosis associated with atresia and associated with OFFP induced degeneration. Though the oFSH binding profile of the major fraction of cells that contain normal cells is very similar the change in density that...
the cells undergo as they degenerate are contrasting in nature. Changes in density of granulosa cells reflect dynamic changes in their biology.

A decrease in density is seen in cells undergoing the physiological process of atresia while an increase is seen in cells at higher densities when apoptosis is induced by OFFP. Differences in physiological responses and induced responses of granulosa cells have been reported in relation to apoptosis. Protooncogene involvement has been suggested in inducing apoptotic changes in granulosa cells.

Further it has been proposed that protooncogene function may be determined by availability of growth factors. Such modulation would lead to differences in the kinetics of apoptotic process and would reflect as variation in biophysical feature such as density. The possibility that a cell subpopulation different from atretic cells responds to OFFP can not be ruled out. Earlier studies had shown that OFFP prepones apoptosis associated with atresia. The present study suggests that OFFP modulates the process of apoptosis associated with atresia in granulosa cells, either by changing the dynamics of the process or by inducing different subpopulations of cells to degenerate.

Acknowledgement:
The authors are thankful to Dr. H.S. Juneja, Director, IRR for his encouragement throughout the study. Thanks to Messrs S T Ghanekar and S M Rewdekar for technical assistance. Supply of FSH from Dr. L E Reichert Jr. Alba, USA PMSG from the hormone bank of IRR is gratefully acknowledged.

References