The number of clonal precursors of the follicular epithelium in the mouse ovary

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Summary. The number of clonal precursors of granulosa cells in mouse ovarian follicles has been estimated using a technique based on the phenomenon of random X-chromosome inactivation of somatic cells and the use of an X-linked alloenzyme variant of the glycolytic enzyme PGK-1. The granulosa cells of follicles were oligo-clonal in origin and founded by a small number of cells (about 5) which was consistent with histological observations. When the analysis was extended to two sub-compartments of the follicle, the mural and cumulus granulosa cells, the results indicated that the cumulus and mural granulosa cells had a common origin.

Keywords: granulosa cells; primordial follicles; phosphoglycerate kinase; ovary

Introduction

The oocyte develops within an envelope of granulosa cells which multiply to form a solid follicle with several cell layers. The entire granulosa cell population, which numbers about 50 000 in mature follicles of mice, is presumed to be derived from a single (and sometimes incomplete) layer of squamous cells associated with the small oocyte in primordial follicles. It is not known whether the pre-granulosa cells of individual follicles are derived from one or several ancestral cells, nor is it certain whether they all contribute to the mature epithelium. Distinct populations of granulosa cells have been identified by their antigenic differences (Erickson *et al.*, 1985) and a gradient of biosynthetic activity exists across the epithelium according to the distribution of several enzymes (Zoller & Weisz, 1978, 1979; Zoller & Enelow, 1983). Whether these sub-populations of granulosa cells have distinct developmental lineages is unknown.

Once a follicle has accumulated 2000–3000 cells to form a solid multilaminar structure it subsequently undergoes a morphogenetic transition in which the extracellular space expands to form the antrum of the Graafian follicle (Gosden & Telfer, 1987). The epithelium of the latter is uniform in thickness except at the pole where granulosa cells support the oocyte. Thus, two morphologically distinct sub-populations exist: those forming an epithelial lining of the follicle (mural granulosa cells) and those in which the oocyte is embedded (cumulus granulosa cells). Differences in secretory products have been found in cumulus and mural granulosa cells (Gilula *et al.*, 1978) but it is not known whether these differences are related to differences in developmental lineage.

The present study was designed to estimate the numbers of clones and their relationships in differentiating granulosa cell populations of mouse follicles using two alloenzyme variants of an X-linked enzyme, phosphoglycerate kinase-1 (PGK-1; EC 2.7.2.3), to detect tissue mosaicism. The numbers of clonal precursors have been estimated from the proportions of the two phenotypes present which are distinguishable by their differences in electrophoretic mobility. These results have been compared with the numbers of somatic cells which, on morphological grounds, are intimately associated with oocytes in primordial follicles.

Materials and Methods

Analysis of granulosa cell heterogeneity using PGK alloenzymes. Granulosa cells were obtained from a colony of CBA/Ca $\times$ CBA/Ca-PgK-1a mice maintained at the Department of Zoology, University of Edinburgh. These animals were heterozygous for the two electrophoretic variants (A and B) of the X-linked glycolytic enzyme PGK-1 and, as a result of X chromosome inactivation (Lyon, 1974), each cell and its descendants express only one or other form of the enzyme.

Mice which were 21–28 days old (i.e. pre-pubertal) were injected with 10 i.u. PMSG (Organon Laboratories, Surrey, U.K.) i.p. to stimulate antral follicle development. Animals were killed by cervical dislocation 24 h later, and their ovaries were excised and placed in a dish containing Hepes-buffered Medium 199 (Flow Laboratories, Irvine, U.K.). The large follicles were dissected using watchmakers' forceps and transferred to 50 μ l droplets of Medium 199 under paraffin oil. The granulosa cells were expressed by gentle teasing and the follicle wall (mainly theca-stroma cells) and the oocyte were discarded.

In the first series of experiments granulosa cells, which included both mural and cumulus cells, from 75 follicles were transferred by pipette to separate plastic tubes. In the second series mural and cumulus granulosa cells from 34 follicles were isolated separately by rupturing the follicle wall and transferring the oocyte in its associated cumulus mass to another droplet of medium where the oocyte was removed by pipetting. The mural and cumulus granulosa cell components from individual follicles were transferred to separate tubes for processing. In both series the cells were centrifuged at 18 000 *g* for 40 sec at room temperature in an Eppendorf centrifuge. Excess medium was removed and the cells were resuspended in 7 μ l of a lytic buffer containing 50 mM-triethanolamine hydrochloride (Sigma), 0.3 mg dithioerythritol (Sigma)/ml, 0.5 mg bovine serum albumin/ml, and 2 mg digitonin (Sigma)/ml (Ansell & Micklem, 1986). Each sample was snap-frozen on solid CO₂ and stored at -70°C until enzyme activities were measured. PGK-1 enzyme activity is stable at -70°C for up to 3 months (J. D. Ansell, unpublished).

Relative proportions of PGK-1A and PGK-1B activity were measured using an assay based on that described by Bucher *et al.* (1980) and modified by Ansell & Micklem (1986). PGK-1A and 1B alloenzymes were separated on Titan III cellulose acetate membranes (Helena Labs, Beaumont, TX, U.S.A.) by electrophoresis for 40 min at 15 mA (constant current) at 4°C . The electrophoresis buffer was a barbitol citrate buffer pH 8.8 containing 2 mM-EDTA, 10 mM-sodium citrate, 5 mM-magnesium sulphate, 20 mM-sodium barbital and 0.25 mg adenosine monophosphate (AMP: Sigma)/ml. Ten samples were applied per gel, approximately 0.5 μ l per application. The substrate for PGK-1 (1,3-diphosphoglycerate) is unstable and is prepared *in situ* after electrophoresis by a series of linked enzyme reactions (aldolase, glycerol-3-phosphate dehydrogenase, and glyceraldehyde phosphate dehydrogenase) catalysing the conversion of fructose 1,6-diphosphate to the substrate for PGK-1 in the presence of nicotinamide adenine dinucleotide (NAD) and inorganic phosphate. ATP produced by the action of PGK-1 on its substrate generates 6-phosphoglucono-lactone from glucose through a second series of linked reactions catalysed by hexokinase and glucose 6-phosphate dehydrogenase. The principle of the autoradiographic assay is that a proportion of the glucose substrate is universally labelled with ^{14}C and that the phosphate-containing products of [^{14}C]glucose metabolism will covalently bind to a polyethylene imine thin-layer chromatographic (t.l.c.) membrane. Unconverted [^{14}C]glucose can be washed off. A solution of the enzymes and substrates described above was spread evenly over the t.l.c. membrane. The Titan III membrane was then applied making a sandwich of t.l.c. membrane, substrate solution and Titan III membrane. This was incubated for 45 min at 37°C . When a PGK band was present on the Titan III membrane, [^{14}C]gluconolactone was formed and bound to the t.l.c. membrane, forming an image of the PGK band. After washing and drying the t.l.c. membrane, this band was visualized by the application of Kodak XS X-ray film to the membrane. The autoradiographs were scanned with an automated microdensitometer (Chromoscan 3, Joyce Loebel, Gatheshead, U.K.) and the areas under the peaks were integrated to estimate the proportions of the products.

Cytological studies of primordial follicles. The numbers of somatic cells in primordial follicles were counted in serial histological sections from a similar group of animals. The ovaries were fixed in aqueous Bouin's fluid for 24 h, embedded in epoxy resin and sectioned at 3 μ m with a glass knife. The sections were stained with haematoxylin and eosin and examined at $\times 1000$ using oil immersion optics. Through a camera lucida attachment the outlines of oocytes and their surrounding pre-granulosa cells in primordial follicles were drawn. The numbers of pre-granulosa cells were then determined by tracing cells through successive drawings.

Results

Numbers of granulosa cell clones

The results of measuring PGK activity in the granulosa cells (cumulus and mural cells combined) of mouse follicles are shown in Fig. 1: 9 of 75 follicles (12%) contained one of the two phenotypes, A or B, and the other follicles contained various proportions of the two, demonstrating that each population had been founded by more than one cell. These results are shown with those obtained for the frequency of PGK-1A activity in peripheral blood (107 samples) during the course of another study. The distribution is comparable in both tissue types.

Fig. 1. Frequency distribution of the percentage of phosphoglycerate kinase activity (PGK) which is of the A phenotype in granulosa cell (cumulus and mural cells combined) from 75 individual mouse follicles (unhatched) and 107 samples of peripheral blood (hatched area). The frequency is expressed as a proportion of the total in each sample.

The large variance in the follicle data implies that the actual number of progenitors is small, and this has been confirmed with the use of the binomial equation. The binomial statistic relates variance to mean as follows:

$$n = p(1 - p)/\sigma^2$$

where p = the mean percentage value obtained for PGK-1A activity, $1 - p$ = the mean value of the B allele, σ^2 = the sample variance, and n = the mean number of clonal precursors. The estimate of the variance between samples indicates the number of clones of cells contributing to the tissue (Nesbitt, 1971; Stone, 1984). If there is only one founder cell the enzymic phenotype of the resultant tissue will be either A or B. If many cells found the tissue, the ratio of A:B is expected to be similar and close to 1:1 in all samples. Intermediate situations can be analysed by application of binomial statistics to the variance of phenotypes between follicles. From the application of the binomial equation to data obtained from granulosa cells it is predicted that on average 5 precursor cells give rise to this population in a mouse follicle. Examination of alloenzyme phenotypes may be used to determine whether or not the clonal descendants of these precursors are distributed randomly between mural and cumulus sub-populations of the granulosa membrane. The binomial statistic for 34 follicles in which mural and cumulus granulosa cells were examined separately predicted 5.2 precursor cells *in toto* with 10% monoclonal follicles, confirming the previous findings. The proportions of total enzyme activity which was due to the single phenotype PGK-A were compared for the mural and cumulus cell sub-groups by linear regression analysis (Fig. 2). The numbers and phenotypes of the two sub-groups were linearly related, proportions being highly correlated ($r = 0.89$, $P < 0.01$) and indicating that both cell types were derived from the same precursor pool.

Numbers of morphologically identified pregranulosa cells

A frequency distribution for the numbers of (presumptive) pregranulosa cells in 40 primordial follicles is shown in Fig. 3. Most follicles were associated with 3–6 squamous cells, the average being 4.8 (s.d. 2.05). Follicles with 1 and > 11 somatic cells were not observed. Occasionally, naked oocytes were encountered.

1987). This is the first report for ovarian cells. The results have verified the assumption which was made *a priori* that granulosa cells express only one X chromosome for otherwise monoclonal types could not have occurred and, moreover, the proportion of total PGK activity which is due to the A phenotype was 0.69 which compares with the number found in other polyclonal tissues. A mean value greater than 0.50 is expected because of the presence of alleles of an inactivating locus (Xce) which affects the probability that a particular X chromosome will be inactivated (Cattanach & Papworth, 1981; Forrester & Ansell, 1985).

The technique for measuring enzyme activity has been demonstrated to be sufficiently sensitive for use even with the small numbers of cells obtained from a single cumulus oophorus (<2000). Reliable estimates of the number of clones in follicles rest on the assumptions that differential cell multiplication and death are insignificant as appears to be the case for the following reasons. Firstly, very few apoptotic bodies are present in normal healthy follicles of the type obtained during PMSG treatment (Braw & Tsafirri, 1980). And, secondly, on the basis of tritiated thymidine autoradiography, it appears that the granulosa cell population in solid follicles of adult mice is multiplying uniformly but asynchronously, although at late stages in Graafian follicle development cell activity is restricted to the cumulus region (Gosden *et al.*, 1983). The pattern of cellular activity in immature mice is not known but in rats there is no differential growth at these ages (Hirshfield, 1986). Polyploidy is another potential source of error when estimating the relative amounts of PGK activity and, hence, the estimated number of clones. It has been claimed that some pig and cow granulosa cells are polyploid (Coulson, 1979) but microdensitometric measurements of Feulgen-stained nuclei plainly show that murine cells are diploid (E. Telfer & R. G. Gosden, unpublished results).

The presence of the two alloenzymic phenotypes in widely varying proportions provides unequivocal evidence that the populations are oligoclonal. The binomial statistic suggests that the average number of clones is 5, but this figure should be used as a guide rather than carrying the weight of numerical precision. Follicles with a single alloenzyme phenotype are uncommon. These may indicate monoclonality or the chance coincidence of cells with the same phenotype in the primordial follicle. The average number of clones corresponds closely to the numbers of observed pre-granulosa cells, supporting the hypothesis that they are the progenitors of the mature follicle. The founding of the granulosa cell population of most follicles by more than one cell is reminiscent of other organ structures and its significance may be that plurality confers developmental flexibility.

Whilst detailed analysis of the spatial distribution of clones will require different methods, measurements of PGK activity in the two major cellular subpopulations gives some indication of the clonal relationships between them. The results show that the cumulus cells, which represent approximately 20% of the total population, were founded by a number of precursors similar to that for the granulosa cells. The high degree of correlation between the proportions of alloenzymes in cumulus and granulosa cells indicates that these precursors were probably the same. If the separate layers forming the follicle were founded by separate clones these proportions would not be expected to be highly correlated. Further, the correlations indicate that before the differentiation of cumulus cells there was extensive proliferation and cell mixing of granulosa cells by the time the antrum had formed and that a large number of these (probably >200) generated the cumulus mass. These results do not suggest what mechanisms are driving the differentiation but might indicate that a critical number of granulosa cells must be present before the cumulus can differentiate.

There is abundant evidence that in the mature follicle the granulosa cells differentiate to serve different functions. For example, the cumulus cells produce glycosaminoglycans in response to FSH (Eppig, 1981). Evidently, these differences have not emerged purely from the cell lineages but will have to be understood on the basis of other factors, such as cell position and interactions between granulosa cells with the stroma-theca and oocyte.

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References

- Ansell, J.D. & Micklem, S. (1986) Genetic markers for following cell populations. In *Handbook of Experimental Immunology*, 4th edn, vol. 2, ch. 56. Eds D. M. Weir, L. A. Herzenberg & C. C. Blackwell. Blackwell, Oxford.
- Braw, R.H. & Tsafiri, A. (1980) Effect of PMSG on follicular atresia in the immature rat ovary. *J. Reprod. Fert.* **59**, 267–272.
- Bucher, T., Bender, W., Hofner, H. & Linke, I. (1980) Quantitative evaluation of electrophoretic allo- and isozyme patterns. *FEBS Lett.* **115**, 319–324.
- Cattanach, B.M. & Papworth, D. (1981) Controlling elements in the mouse. V. Linkage tests with X linked genes. *Genet. Res.* **38**, 57–70.
- Coulson, P.B. (1979) Characterization of polyploidy in ovarian granulosa cells. In *Ovarian Follicular Development and Function*, pp. 385–394. Eds A. R. Midgley & W. A. Sadler. Raven Press, New York.
- Eppig, J.J. (1981) Ovarian glycosaminoglycans: evidence for a role in regulating the response of oocyte-cumulus cell complex to FSH. *Endocrinology* **108**, 1992–1994.
- Erickson, G.F., Hofeditz, C., Unger, M., Allen, W.R. & Dulbecco, R. (1985) A monoclonal antibody to a mammary cell line recognises distinct sub types of ovarian granulosa cells. *Endocrinology* **117**, 1490–1499.
- Forrester, L.M. & Ansell, J.D. (1985) Parental influences on X chromosome expression. *Genet. Res.* **45**, 95–100.
- Gilula, N.B., Epstein, M.L. & Beers, W.H. (1978) Cell-cell communication and ovulation: A study of the cumulus-oocyte complex. *J. Cell Biol.* **78**, 58–75.
- Gosden, R.G. & Telfer, E. (1987) Scaling of follicular sizes in mammalian ovaries. *J. Zool., Lond.* **211**, 157–168.
- Gosden, R.G., Laing, S.C., Flurkey, K. & Finch, C.E. (1983) Graafian follicle growth and replacement in anovulatory ovaries of ageing C57BL/6J mice. *J. Reprod. Fert.* **69**, 453–462.
- Hirshfield, A.N. (1986) Patterns of [³H]thymidine incorporation differ in immature rats and mature, cycling rats. *Biol. Reprod.* **34**, 229–235.
- Lyon, M.F. (1974) Mechanisms and evolutionary origins of variable X-chromosome activity in mammals. *Proc. R. Soc. B* **187**, 243–268.
- Micklem, H.S., Lennon, J.E., Ansell, J.D. & Gray, R.A. (1987) Numbers and dispersion of repopulating hematopoietic cell clones in radiation chimeras as functions of injected cell dose. *Exptl Hematol.* **15**, 251–257.
- Nesbitt, M.E. (1971) X chromosome inactivation mosaicism in the mouse. *Devl Biol.* **26**, 252–263.
- Stone, M. (1984) Variance-covariance modelling with chromosome markers. *J. theor. Biol.* **107**, 275–286.
- Woodruff, M.F.A., Ansell, J.D., Hodson, B.A. & Potts, R.B. (1986) Oligoclonal tumours. *Int. J. Cancer* **38**, 747–751.
- Zoller, L.C. & Enelow, R. (1983) A quantitative histochemical study of lactate dehydrogenase and succinate dehydrogenase activities in the membrana granulosa of the ovulatory follicle of the rat. *Histochem. J.* **15**, 1055–1064.
- Zoller, L.C. & Weisz, J. (1978) Identification of cytochrome P-450, and its distribution in the membrana granulosa of the preovulatory follicle, using quantitative cytochemistry. *Endocrinology* **103**, 310–313.
- Zoller, L.C. & Weisz, J. (1979) A quantitative cytochemical study of glucose-6-phosphate dehydrogenase and Δ^5 - 3β -hydroxysteroid dehydrogenase activity in the membrana granulosa of the ovulable type of follicle in the rat. *Histochemistry* **62**, 125–135.

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