Purification of porcine granulosa cells by continuous Percoll gradient[1]

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Summary. Granulosa cells purified on a continuous Percoll gradient have considerably increased responsiveness to FSH in formation of cAMP, secretion of progesterone, and ^{125}I [hCG] binding to cells incubated for 4 days in culture.

Granulosa cells were found to be a suitable model system for examination of hormone-mediated differentiation of ovarian cells. Granulosa cells are a homogenous cell population; they are easy to isolate, but the cells are contaminated to a great extent by cellular debris. The present paper describes the isolation of purified pig granulosa cells using a Percoll gradient. Continuous Percoll gradients have been applied to the separation of many types of cells and particles, including that of viable rat Leydig cells[2,3]. A percoll density gradient can be formed spontaneously by centrifugation; it has very low osmolarity and viscosity, so that cells are not damaged by the purification procedure.

Material and methods. A percoll density gradient (30 ml) was prepared by mixing 13.5 ml of Percoll (Pharmacia) with 14.7 ml of Medium 199, 1.5 ml of 10× concentrated Medium 199 and 0.3 ml of pig serum and centrifuging at 25,000× g for 60 min in Janetzki VAC 602 centrifuge with angle-head rotor. 2 ml of granulosa cells (approximately 10^8 cells) harvested from small (1-2 mm) porcine ovarian follicles[4] were layered on top of the gradient and centrifuged at 600× g for 20 min. The visible bands of granulosa cells were aspirated and the Percoll was removed from the cells by washing with 15 ml of Medium 199 followed by centrifugation at 500× g for 10 min.

Granulosa cells were cultured for 4 days at 37 °C in Falcon multiwells containing Medium 199 with Earle's salts and pig serum[4]. The cells were inoculated at a density of 3×10^5 viable cells per well. The culture medium was supplemented with glutamine (1 mmole l^{-1}), hFSH (0.1 μg ml^{-1}), thyroxine (0.1 mmole l^{-1}), insulin (1 mU ml^{-1}) and cortisol (0.01 μg ml^{-1}). At the end of incubation the progesterone content of the culture medium was assayed by RIA[5]. The granulosa cells from multiwells were harvested by scraping with a rubber policeman and cells were incubated with 2 ng ^{125}I [hCG] (sp.act. 28 Ci g^{-1}) for 1 h at 37 °C with or without excess of unlabeled hormone[6].

The concentration of cAMP in the granulosa cells was estimated by a protein binding assay[7]. The cells were incubated for 60 min at 37 °C in Medium 199 with 20 mmoles l^{-1} HEPES buffer, 0.2 mmoles l^{-1} 3-isobutyl 1-methylxanthine and 10 mg ml^{-1} bovine serum albumin fraction V in the presence or absence of 10 μg FSH (NIH-FSH-P-1).

Granulosa cell identification was confirmed by light and electron microscopic examination[8].

Results and discussion. Continuous Percoll gradient separated porcine granulosa cells into 5 visible bands (fig. 1). Band I and to some extent band II were composed mainly of damaged granulosa cells and cellular debris. Band V consisted practically of red blood cells. Morphological integrity of granulosa cells purified by the Percoll procedure was confirmed by electron microscopy (fig. 2). The purified granulosa cells were found to exibit characteristics of steroid synthesizing cells[8], i.e. lipid droplets, smooth endoplasmic reticulum and mitochondria. When granulosa cells with bound ^{125}I [hCG] were fractionated on a Percoll gradient, the greater part of the radioactivity was found in

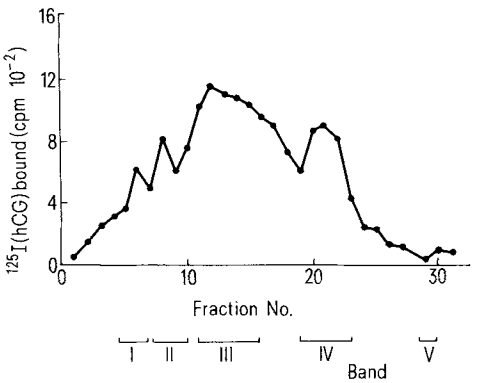

Figure 1. Distribution of ^{125}I [hCG] bound to granulosa cells fractionated on a continuous Percoll gradient. 10^8 cells were incubated with ^{125}I [hCG] before fractionation. The gradient was fractionated with a Beckman Fraction Recovery System and radioactivity of each 1 ml fraction was measured. The location of the visible bands of cells is shown on the abscissa.

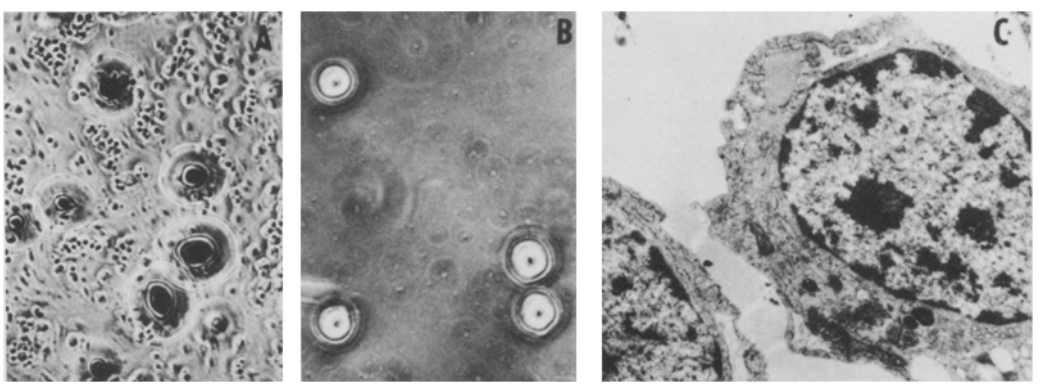

Figure 2. Photomicrographs of unfractionated (A) and Percoll purified (B) granulosa cells prepared under phase contrast microscopy (magnification ×200). Electron micrograph (magnification ×9600 of purified granulosa cell fraction III (C).

bands III and IV. Also, the fractionation illustrated in the table shows a large increase in responsiveness of granulosa cells to FSH in cAMP synthesis in bands III and IV. The FSH was used since granulosa cells harvested from small porcine follicles are more responsive to FSH then LH in terms of cAMP accumulation[9].

Functional integrity of the granulosa cells obtained by the Percoll procedure was demonstrated by secretion of progesterone and binding of ^{125}I [hCG] to the cells incubated for 4 days in culture medium. The increase of about 7.4-fold in LH/hCG receptors was accompanied by a 3.5-fold increase in progesterone secretion by purified granulosa cells in band III in comparison with unfractionated cells. Granulosa cells used for a long-term incubation in culture need to be 3 times washed with culture medium[4]. Purification of granulosa cells on continuous Percoll gradients possesses several advantages. The Percoll gradient separates cells from cellular debris quickly and effectively (fig. 2). Percoll did not significantly change the osmolarity of the culture medium (310 mOs/kg H_2O in our experiment) and it can be prepared in sterile conditions prior to use. Even though the granulosa cells were found in all bands tested, band III contained the highest activity of cells with only small contamination by cellular fragments. This method of purification has considerable value for the investigation of many aspects of intracellular mechanisms of the maturation and differentiation of granulosa cells.

Production of cAMP (in 60-min incubation), progesterone secretion and ^{125}I[hCG] binding (in 4-day culture) to unfractionated and Percoll fractionated pig granulosa cells

	cAMP content (pmoles 10^{-6} cells) n=4		Progesterone secretion (ng 10^{-6} cells)	^{125}I[hCG] bound (cpm 10^6 cells)
	−FSH	+FSH		
Unfractionated cells	0.79±0.05	10.90±0.99	89.7± 7.5	956±137
Band I	2.25±0.20	2.65±0.10	87.2± 8.7	341±109
Band II	0.78±0.12	2.87±0.26	159.2±12.8	735±122
Band III	2.21±0.25	41.50±0.63	317.0±23.2*	7096±980*
Band IV	2.61±0.21	59.20±7.50	181.5±18.6	2680±187

The mean values ± SEM are expressed per number of viable cells.
*$p < 0.001$: compared to band II and Band IV.

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A new strain of rat with an inherited cataract

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Summary. A new rat strain has been developed, in which a spontaneous cataract occurs without exception at 3–4 months after birth and matures completely at 4–6 months of age, indicating that this rare strain possesses a maturity-onset cataract. In the present report, histological data are presented and discussed.

Although the occurrence of hereditary cataracts in mice has been reported[2–12], the application of these cataracts to the study of the etiology of human cataract has been restricted, because hereditary mouse cataracts occur in the embryonic or neonatal period and the available samples are very small. If hereditary cataracts occurred after birth in larger animals, this would be a more useful tool in studying the etiology of human cataracts.

In rats, hereditary cataracts have also been developed[13–19]. Among them are the cataracts that occur only after birth in Bourne-Grüneberg[14] and the Léonard-Maisin[16] strains. However, the cataract in the former strain is complicated by retinal degeneration and remnants of the hyaloid artery, while in the latter strain, the anterior polar cataract occurs spontaneously in the neonatal period, associated with epithelial hyperplasia and lens fiber degeneration in this area, perhaps through a mechanical stimulus of the cornea. In contrast to these strains, the strain now developed is characterized by the fact that cataract formation occurs definitely at 3–4 months after birth in every animal without any significant complication in the eye. The strain, which is tentatively termed the ICR cataractous rat, was developed by successive mating and selection.

Methods. The occurrence of cataract was examined biomicroscopically by using a slit lamp (Kowa), and the excised lenses were examined macroscopically. For histological

Results of back-cross experiment

Sex		♂		♀	
Cataract		+	−	+	−
P-generation*	Number	8(ICR)			8(JCL)
F_1(ICR×JCL)	Number	0	46	0	50
F_2(P×F_1)	Number	15	46	17	42
	% Ratio	24.6%		34.8%	

*8 pairs of ICR(♂)×JCL(♀) were mated, resulting in 8 litters of F_1 offspring. Though crosses were made between ICR(♀) and JCL(♂), no cataractous offsprings (♂: 48, ♀: 51) were obtained.