Distributional Changes of Acridine Orange-Stained Lysosomes in Hamster Eggs and Embryos during the Folliculogenesis and Early Development

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Abstract: *Distributional changes in lysosomes in hamster eggs and embryos during the folliculogenesis and early development were observed using the acridine orange (AO) staining method. AO-stained lysosomes were densely observed all over the cytoplasm of eggs in secondary follicles and of eggs in antral follicles until 13 hrs before ovulation, but were sparsely observed in the cytoplasm of eggs in primordial follicles. The lysosomes were distributed throughout the cytoplasm in about a half of eggs in antral follicles at 12 and 10 hrs before ovulation, while they gathered around nuclei in the remaining half of the eggs. The lysosomes were distributed all over the cytoplasmic region of all eggs from 8 to 3 hrs before ovulation and of unfertilized eggs 2 hrs after ovulation. Such a distributional pattern remained unchanged until uncompacted 8-cell embryos. In compacted 8-cell embryos and blastocysts, the lysosomes were densely observed all over the cytoplasm in round blastomeres or inner-cell-mass cells, whereas they were sparsely observed in flattened blastomeres or trophoblast cells, mainly around nuclei. From the results on 8-cell embryos and blastocysts, it was suggested that there is the close relation between distributional changes of lysosomes and differentiation of blastomeres.*

Key words: *Hamster, Egg, Embryo, Lysosome, Acridine orange.*

It is generally considered that the lysosome is a cytoplasmic organelle surrounded by the limited membrane, and involves intracellular digestion of foreign bodies of intra- and extra-cellular origins. Lysosomes can be conventionally observed under electron microscopy, however, fluorescent microscopic observation of them has been performed using acridine orange (AO) as a vital staining agent [1–8] since lysosomes were found to appear as orange-yellow fluorescent particles in the cytoplasm of viable cells stained with AO in 1963 [1]. Since the AO staining method allows a rapid and accurate visualization of lysosome distribution in the cytoplasm, this method is considered to have many advantages over the electron microscopic method [1, 6].

In mammalian eggs and embryos, the distribution of lysosomes has been investigated using the AO staining method in rats [5–7] and mice [8]. It has been determined *in vitro* that lysosomes are distributed all over the cytoplasm in rat eggs with germinal vesicles (GVs), but they temporarily move to the perinuclear region prior to the breakdown of GVs, and then rapidly spread again over the entire cytoplasmic region after the GV breakdown [5–7]. It has also been reported that lysosomes are distributed throughout the cytoplasm of blastomeres in mouse embryos from the 2-cell to the uncompacted 8-cell stages, however, they are not only distributed in the peripheral cytoplasm in compacted 8-cell embryos and morulae, but also distributed all over the cytoplasm in inner-cell-mass cells of blastocysts and localized in trophoblast cells, mainly around nuclei [8].

However, distributional changes of lysosomes in eggs or embryos during the meiotic maturation and early development have been studied in rats and mice, but not in other species. In the present study, we observed distributional changes of lysosomes in hamster eggs from primordial follicles to antral follicles, and also from unfertilized eggs to blastocysts, according to the folliculogenesis, resumption of meiotic maturation and early development.

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Materials and Methods

Adult female hamsters (*Mesocricetus auratus*) were used. They were kept and fed normally in a room at 24 °C and lit 14 hrs a day, 4 a.m. through 6 p.m. Their estrous cycles were checked by examination of the post-estrous discharge [9], and only animals which became estrous at about 6 p.m. were used. Ovaries were removed from 22 females, and eggs were obtained from the follicles of different sizes as follows: small primordial follicles of 30 to 40 μm in diameter, large primordial follicles of 50 to 90 μm, small secondary follicles of 100 to 240 μm and large secondary follicles of 250 to 600 μm. As it is known that ovulation occurs 8 hrs after the onset of estrus in hamsters [10], the recovery hours of eggs and embryos were shown as the hours estimated from ovulation time. For the recovery of eggs from antral follicles, ovaries were removed from 46 animals at 88, 64, 40, 16, 14, 13, 12, 10, 8, 6 and 3 hrs before ovulation, and the eggs were recovered by tearing the follicles. Twenty-three females mated with fertile males were killed 6 hrs after ovulation for eggs just penetrated with sperm, 12 hrs for pronuclear eggs, 32 hrs for 2-cell embryos, 52 hrs for 4-cell embryos, 65 hrs for 8-cell embryos, 78 hrs for blastocysts, and the eggs and embryos were recovered by flushing the oviducts or uteri with Earle's balanced salt solution (EBSS, pH 7.3) [11]. Unfertilized eggs were also collected from unmated 4 females at 2 hrs after ovulation.

Collected eggs and embryos were washed in EBSS, and cumulus cells surrounding eggs were dispersed beforehand by immersing in EBSS containing 0.1% hyaluronidase (Sigma Chemical Co., St.Louis, U.S.A.). The eggs and embryos were washed 3 times in EBSS containing 25 mM HEPES (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.3% bovine serum albumin (Sigma Chemical Co.) (mEBSS), and then immersed in a staining solution composed of 500 μg AO (Sigma Chemical Co.) and 100 ml mEBSS for 5 min at room temperature. After staining, eggs and embryos were immersed in mEBSS containing 0.2% pronase (Sigma Chemical Co.) to dissolve the zona pellucida, and then they were washed 3 times in mEBSS and placed on glass slides to be photographed under a reflected-light fluorescing microscope (Nikon Corporation, Tokyo, Japan). The same procedures for the demonstration of lysosomes were applied 3 times to 20 to 30 eggs or embryos from each developmental stage.

Results and Discussion

The distribution of lysosomes has been investigated in rat eggs [5–7] and early mouse embryos [8]. However, there have been no reports on distributional changes of lysosomes during the entire course of folliculogenesis, or in eggs or early embryos of mammals except rats and mice.

In the present experiments, we observed distributional changes of lysosomes in hamster eggs derived from a series of primordial to antral follicles immediately before ovulation. As shown in Figs. 1 and 2, AO-stained lysosomes were sparsely distributed throughout the cytoplasm of eggs derived from small and large primordial follicles. The lysosomes increased in eggs of small secondary follicles, spreading uniformly all over the cytoplasm (Fig. 1-a). This distributional pattern remained unchanged in eggs of antral follicles until 13 hrs before ovulation. In eggs of antral follicles at 12 and 10 hrs before ovulation, the lysosomes were uniformly distributed all over the cytoplasm in about half of those, while they gathered around nuclei of the remaining half of the eggs (Fig. 1-b). In addition, all of eggs during these stages had GVs. The lysosomes were uniformly observed all over the cytoplasm in eggs from 8 to 3 hrs before ovulation, and the GV breakdown occurred in all eggs.

Lysosomes were observed around nuclei of eggs at 12 and 10 hrs before ovulation in the present experiment. Since the gathering of lysosomes around nuclei prior to the breakdown of GVs has also been noted in rat eggs [5–7], this change is thought to occur in association with the resumption of meiotic maturation of rodent eggs. The appearance of such eggs corresponded with the time of starting LH surge in hamsters [12], therefore stimulation with LH was thought to be involved in the movement of lysosomes to the perinuclear region prior to the GV breakdown. The involvement of LH in the movement of lysosomes to the perinuclear region prior to the breakdown of GVs has also been determined in rat eggs [5]. When rat eggs at the GV stage were incubated in a medium containing LH, movement of lysosomes to the perinuclear region was facilitated, and the GV breakdown was also enhanced, whereas such enhancement of the lysosome movement or the GV breakdown was not observed in incubation in a medium containing FSH or prolactin.

A diagram of the distributional changes of fluorescent lysosomes in hamster eggs and embryos during the early development is shown in Fig. 3. In unfertilized

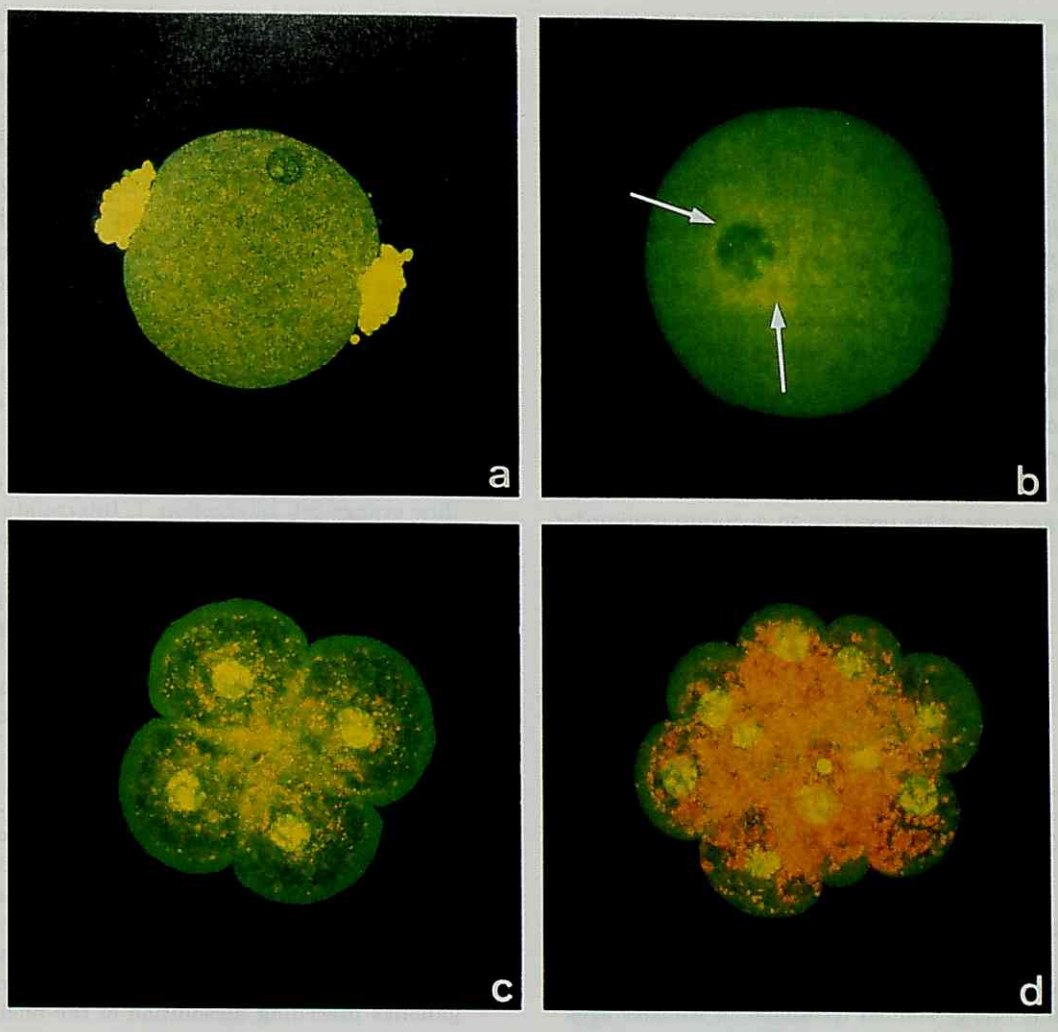

Fig. 1. Whole mount preparations of hamster eggs (a and b) and embryos (c and d) with a magnification of × 450 under an epifluorescence microscope. All eggs and embryos were photographed after staining with acridine orange.

a. An egg collected from a small secondary follicle. Fluorescent lysosomes are densely distributed throughout the cytoplasm.
b. An egg collected from an antral follicle 12 hrs before ovulation. Fluorescent lysosomes (arrows) are mainly distributed in the perinuclear cytoplasm.
c. A 4-cell embryo. Fluorescent lysosomes are densely distributed throughout the cytoplasm of each blastomere.
d. A compacted 8-cell embryo. Fluorescent lysosomes are densely distributed throughout the cytoplasm of round blastomeres while they are sparsely and mainly distributed in the perinuclear cytoplasm of flattened blastomeres.

eggs, a larger number of the lysosomes were uniformly observed throughout the cytoplasm. Such a distributional pattern remained unchanged until the uncompacted 8-cell stage (Fig. 1-c). In compacted 8-cell embryos and blastocysts, the lysosomes were only sparsely observed in flattened blastomeres or trophoblast cells, mainly around nuclei, but they were densely observed in round blastomeres or inner-cell-mass cells, spreading all over the cytoplasm (Fig. 1-d).

Although the distributional changes of lysosomes in hamster embryos in the present experiments were consistent with those of mouse embryos observed by Batten *et al.* [8] until uncompacted 8-cell embryos and in blastocysts, the distribution of lysosomes in compacted 8-cell embryos considerably differed between mouse and hamster embryos. This discrepancy in the distribution of

lysosomes in compacted embryos may have been caused by the fact that the authors used hamsters while Batten *et al.* [8] used mice. On the other hand, Ishida [13] noted that the activity of acid phosphatase (Acp), known as a marker enzyme of lysosomes, did not differ among blastomeres in uncompacted 8-cell hamster embryos, equally at a low level, whereas the activity was remarkably higher in round blastomeres than in flattened blastomeres in compacted 8-cell embryos. Because the results of Acp activity examined by Ishida [13] agreed with distributional changes of lysosomes in the present experiments, the distribution of lysosomes in compacted hamster embryos observed in the present experiments is thought to be plausible.

Batten *et al.* [8] assumed that a distributional change of lysosomes cannot be used as an accurate marker for the transformation of flattened blastomeres to trophoblast cells because the distribution of lysosomes in flattened and round blastomeres in compacted embryos differed from that in trophoblast cells and inner-cell-mass cells in blastocysts. In the present experiments, it was, however, determined that localization of lysosomes in trophoblast cells and inner-cell-mass cells in blastocysts was comparable to that seen in flattened and round blastomeres in compacted 8-cell embryos, indicating the close relation between distributional changes of lysosomes and differentiation of blastomeres.

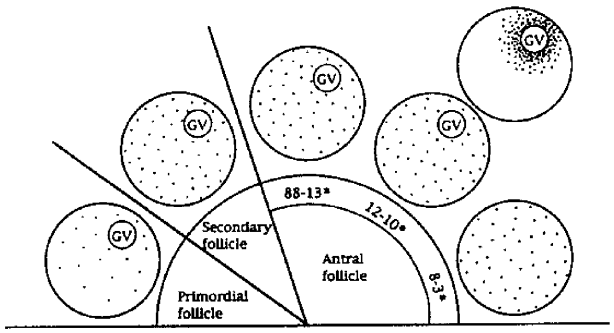

Fig. 2. A diagram of the distributional patterns of acridine orange-stained lysosomes in hamster eggs during the folliculogenesis. GV: Germinal vesicle. * Hours before ovulation.

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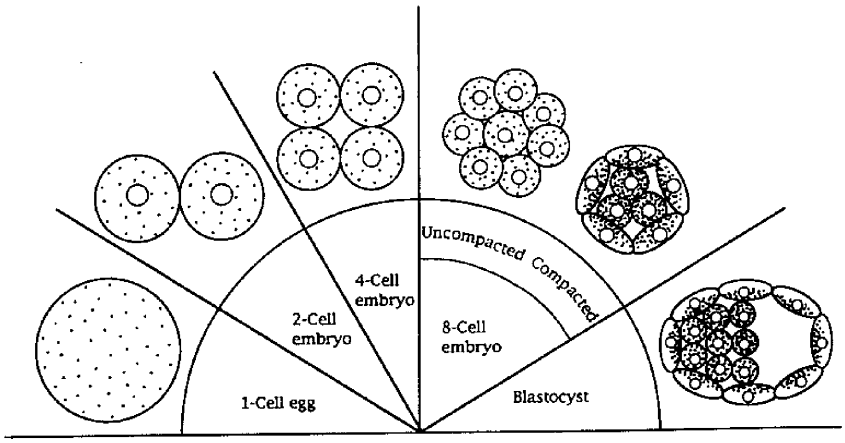

Fig. 3. A diagram of the distributional patterns of acridine orange-stained lysosomes in hamster eggs and embryos during the early development.

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