BASIC PROTEINS IN THE NUAGE OF GROWING CHINESE HAMSTER OOCYTES

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Two types of nuage aggregates, the mitochondria-associating nuage and the freely-existing nuage, appeared in the cytoplasm of the growing Chinese hamster oocytes in a growing stage-specific manner. Enzyme digestion studies on the ultrathin sections obtained from the glycol methacrylate-embedded materials showed that both types of nuage contain RNA and proteins. The ethanol-phosphotungstic acid staining and also the cobalt thiocyanate staining used to examine the localization of basic proteins at the ultrastructural level revealed that the mitochondria-associating nuage contains a considerable amount of basic proteins, whereas only a few may be present in the freely-existing nuage.

It was considered that the mitochondria-associating nuage may lose its basic proteins and transform into the freely-existing nuage, along with the oocyte's growth.

Germ line cells such as primordial germ cells, young oocytes and young spermatocytes of nearly all animal species contain discrete cytoplasmic bodies which lack surrounding membranes and consist of densely accumulated fibrillar and/or granular materials. A variety of names have been used to refer such intracytoplasmic bodies in the past (for review, cf. 10). In this study, the term "nuage," the French word for "cloud," is used according to the recommendation of Eddy (9, 10). Many cytochemical and autoradiographic studies have shown that the nuage may consist of RNA and proteins (2, 5, 7, 13, 23, 26, 27, 29, 30-33, 38). Much speculation has been devoted to the functional role of the nuage, but none has been confirmed by conclusive evidence (for review, 3, 10).

A number of authors have reported that two types of nuage are identified in germ line cells of some animal species; one is associated with numerous mitochondria and forms the mitochondrial cloud, and another exists freely in the cytoplasm except for the direct attachment of ribosomes on its surface (1-4, 8-10, 12, 13, 16-21, 24, 26, 34, 36-38). The interrelationship between these two types of nuage remains unknown.

Previously, we showed that the growing oocytes of the Chinese hamster ovaries contain the above-mentioned two types of nuage, and that neither type of the nuage is stained with the Ag-NOR staining method which preferentially reacts with

nucleolar acidic proteins (34). In this study, the ultrastructural localization of basic proteins is examined cytochemically in these two types of nuage.

MATERIALS AND METHODS

Ovaries were taken from 20-day-old females of the CHS/Idr Chinese hamster strain. For standard electron microscopic observation, they were fixed with Karnovsky's paraformaldehyde-glutaraldehyde fixative (cacodylate-buffered at pH 7.4) for 1 hr at room temperature, post-fixed with Dalton's chrome-osmium fixative (pH 7.2) for 1 hr at 4°C, and embedded into Quetol 812. Ultrathin sections were double-stained with a routine method using uranyl acetate and lead nitrate before observation with a JEOL JEM 100B electron microscope.

To test the presence of proteins and RNA in the nuage, ovaries were fixed with Karnovsky's fixative alone, and embedded in glycol methacrylate (GMA) according to the method described by Leduc and Bernhard (25). Ultrathin sections were mounted on carbon-coated nickel grids, and treated with pronase solution (Kaken Kagaku, 5 mg/ml in distilled water) for 3 or 15 hr at 37°C, or with pronase solution for 15 hr, followed by RNase solution (Sigma Type I-A, 1 mg/ml in distilled water) for additional 15 hr. After the completion of enzyme digestion, the grids were washed in distilled water, and double-stained with uranyl acetate and lead nitrate. Control grids were treated with distilled water under the same condition.

Two electron-staining methods, ethanol-phosphotungstic acid (E-PTA) staining (15) and cobalt thiocyanate (Co-Tc) staining, were employed for the ultrastructural localization of basic proteins in the nuage. For the E-PTA staining, ovaries were fixed with Karnovsky's fixative, dehydrated with an ethanol series and incubated for 15 hr at room temperature in 2% phosphotungstic acid in absolute ethanol; then, they were washed in absolute ethanol and embedded in Quetol 812. Acetylation for the blockade of amino groups was performed by incubating the fixed and dehydrated ovaries in 10% acetic anhydride in dry pyridine for 90 min at 37°C before the E-PTA staining. Control ovaries were incubated under the same condition in pyridine only (28). To examine whether the condensed state of the nuage materials blocks the access of the stain, the fixed ovaries were incubated for 2 hr at room temperature in 1% sodium dodecyl sulphate (SDS) in the presence of 0.002 M dithiothreitol (DTT) in 0.05 M sodium borate buffer at pH 9.0, before the dehydration and staining with the E-PTA staining (22). Ultrathin sections obtained from the E-PTA-treated materials were observed by an electron microscope without any further electron-staining.

The Co-Tc staining was done according to the method described by Courtoy *et al.* (6).

RESULTS

The ovaries of 20-day-old Chinese hamster contained numerous oocytes in a variety of growing stages. These oocytes were divided into early-stage (oocytes enveloped by a single-layer of flattened follicle cells), middle-stage (follicle cells being cuboidal in shape and partially bi-layered), late-stage (oocytes entirely

FIGS. 1-5. Standard electron micrographs of the growing Chinese hamster oocytes, prepared by double-fixation and double-staining methods.

FIG. 1. Early-stage growing oocyte. The mitochondria-associating nuage is conspicuous (mNg). $\times 12,100$

FIG. 2. Middle-stage growing oocyte. Both types of nuage aggregates, the mitochondria-associating nuage (mNg) and the freely-existing nuage (fNg), are seen. In the freely-existing nuage, ribosomal particles are intermingled with fibrillar materials (an arrow), and a cytoplasmic core containing a few ribosomal particles is encountered (a crossed arrow). $\times 20,800$

FIG. 3. Middle-stage growing oocyte. Nuage materials appear to be eliminated from the mitochondrial cloud into the cytoplasm (an arrow). $\times 28,800$

FIG. 4. Late-stage growing oocyte. The mitochondria-associating nuage has disappeared, and each mitochondria exists freely in the cytoplasm. $\times 20,500$

FIG. 5. Freely-existing nuage in the late-stage growing oocyte. Note change in shape. $\times 15,700$

enveloped by bi-layered follicle cells), and antral-stage (antrum formation seen in follicles) growing oocytes, according to Takeuchi *et al.* (35).

In the cytoplasm of the early-stage growing oocytes, the nuage was observed as a dense accumulation of fine fibrillar materials, usually associated with a number of mitochondria and forming a mitochondrial cloud (Fig. 1). The size of nuage was 0.5–1.5 μm in diameter. The number of mitochondria associating with one

FIGS. 6–8. Electron micrographs taken from the glycol methacrylate-embedded sections. $\times 22,400$ in all these figures.

FIGS. 6a, b. Mitochondria-associating nuage (a) and freely-existing nuage (b) in the untreated section.

FIGS. 7a, b. Mitochondria-associating nuage (a) and freely-existing nuage (b) after pronase treatment. A large part of both types of nuage aggregates has been extracted, but a considerable amount of reticulated fibrillar materials remains undigested.

FIGS. 8a, b. Mitochondria-associating nuage (a) and freely-existing nuage (b) after pronase plus RNase treatment. Both types of nuage aggregates have been almost completely extracted.

nuage was from three up to ten in a cross section. This type of nuage was called a mitochondria-associating nuage.

In the middle-stage growing oocytes, the number of mitochondrial clouds increased considerably, but the size of the nuage in each mitochondrial cloud decreased to about 0.3–1.0 μm in diameter (Fig. 2). The number of mitochondria associating with one nuage was also decreased, mostly to three to five in a cross section (Fig. 2). In some mitochondrial clouds, an image suggestive of the elimination of nuage materials from the mitochondrial cloud into the cytoplasm was occasionally encountered (Fig. 3). On the other hand, another type of nuage aggregate was found in the vicinity of the mitochondrial cloud. It was a large round body measuring about 1.0–1.7 μm in diameter, and characterized by the absence of specific contiguity with any other cytoplasmic organelles except for the direct attachment of ribosomes on its surface (Fig. 2). This type of nuage aggregate was called a freely-existing nuage. The density of fibrillar materials in the freely-existing nuage was less than in the mitochondria-associating nuage, and ribosomal particles frequently intermingled with the fibrillar materials of the freely-existing nuage (Fig. 2). A cytoplasmic core containing a few ribosomal particles was sometimes present in the freely-existing nuage (Fig. 2). The number of freely-existing nuages in the middle-stage growing oocyte was far less than that of the mitochondria-associating nuage; in rare cases more than two were found in a cross section of one middle-stage oocyte.

In the late-stage growing oocytes, most of the mitochondria-associating nuages had almost entirely disappeared, and each mitochondria existed freely in the cytoplasm (Fig. 4). The freely-existing nuage was still occasionally encountered, but their shape had been collapsed (Fig. 5).

In the antral-stage oocytes, no types of nuage aggregate were found in the cytoplasm.

Cytochemical studies

After treatment of ultrathin sections obtained from GMA-embedded materials with pronase solution, a large part of both types of nuage was extracted, but a considerable amount of reticulated fibrillar materials remained undigested (Figs. 6a, 6b; 7a, 7b). After treatment of sections with pronase plus RNase, both types of nuage were mostly extracted (Figs. 8a, 8b). The pronase-resistant fibrillar materials remained undigested after treatment with distilled water containing no RNase.

After E-PTA staining of the ovaries, the mitochondria-associating nuage was well contrasted (Fig. 9a), whereas the freely-existing nuage remained only faintly

Figs. 9–12. $\times 22,400$ in all these figures.

Figs. 9a, b. After ethanol-phosphotungstic acid (E-PTA) staining. The mitochondria-associating nuage is well contrasted (a), whereas the freely-existing nuage is only faintly contrasted (b).

Figs. 10a, b. Mitochondria-associating nuage treated by E-PTA staining after acetylation with acetic anhydride in pyridine (a), or after incubation in pyridine only (b). The stainability is greatly reduced by acetylation.

Figs. 11a, b. E-PTA staining of mitochondria-associating nuage (a) and freely-existing nuage (b) after incubation in sodium dodecyl sulphate plus dithiothreitol. The stainabilities of both types of nuage aggregates remain unchanged.

Figs. 12a, b. After cobalt-thiocyanate staining. The mitochondria-associating nuage is densely stained (a), whereas the freely-existing nuage is only faintly stained (b).

contrasted (Fig. 9b). Exposure of the ovaries to 10% acetic anhydride in dry pyridine greatly reduced the stainability of the mitochondria-associating nuage (Fig. 10a). Pyridine alone did not suppress the staining (Fig. 10b). The treatment of the ovaries with SDS plus DTT before the E-PTA staining did not change the stainability of both types of nuage (Figs. 11a, 11b).

After Co-Tc staining of ultrathin sections, the mitochondria-associating nuage was densely stained (Fig. 12a), whereas the freely-existing nuage remained only faintly stained (Fig. 12b).

DISCUSSION

Although a number of cytochemical and autoradiographic studies have shown that the nuage materials consist of proteins and RNA (2, 5, 7, 23, 26, 27, 29, 30–33, 38), only a limited number of studies have been published concerning the nature of proteins in the nuage. In the spermatogenesis of the rat, Sud (31, 32) examined the composition of chromatoid body (one kind of nuage) by light microscopic histochemistry, and reported that this body contains basic proteins rich in arginine. In the ovarian oocytes of the golden hamster, Weakley (38) revealed by electron-microscopic autoradiography that the freely-existing granular-fibrillar bodies and the intermitochondrial substances are heavily labeled after the injection of tritiated lysine into the animal.

The present cytochemical studies using E-PTA staining and Co-Tc staining showed that the mitochondria-associating nuage in the growing Chinese hamster oocytes contains a considerable amount of basic proteins, whereas only a few may be present in the freely-existing nuage. This result appears to disagree with Weakley's autoradiographic study indicating that the freely-existing granular-fibrillar cytoplasmic bodies in the golden hamster oocytes are heavily labeled with tritiated lysine, as are the intermitochondrial substances (38). In the present study, it is unlikely that the basic protein sites reactive to the E-PTA staining may be masked in the freely-existing nuage, since the pre-treatment with SDS plus DTT did not increase the stainability of the freely-existing nuage. It may be probable that, though the amount of basic proteins is less in the freely-existing nuage than in the mitochondria-associating nuage, their turnover rate is faster in the former type of nuage.

Two possibilities may be considered for the interrelationship between the mitochondria-associating nuage and the freely-existing nuage; either they have no relation to each other and are formed independently in the cytoplasm of the growing Chinese hamster oocytes, or one of the two may be a precursor of the other. The early-stage growing oocytes were found to contain only the mitochondria-associating nuage, and the freely-existing nuage appeared in the oocyte's cytoplasm from the middle-stage growing oocytes onward; these findings, together with the fact that an electron microscopic image suggestive of the elimination of nuage materials from the mitochondrial cloud into the cytoplasm was occasionally encountered in the middle-stage growing oocytes, make it very plausible that the mitochondria-associating nuage may be the precursor of the freely-existing nuage. In oogenesis of a viviparous teleost, Azevedo (2) also reported that the mitochondria-associating nuage must be transformed into the freely-existing nuage during the oocyte's growth.

There may be also two possibilities concerning the functional role of basic proteins in the nuage: 1) The basic proteins might inactivate the nuage materials (especially their RNA components) in the mitochondria-associating nuage, the nuage materials becoming able to function by virtue of their elimination from the mitochondrial cloud and simultaneous disappearance of basic proteins. 2) The basic proteins might play function in conjunction with other components of the nuage in the mitochondria-associating nuage and, after completion of this function, the nuage materials would leave from the mitochondrial cloud and the basic proteins would rapidly disappear from the nuage aggregate. This problem awaits further elucidation in forthcoming studies.

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