A New Approach to the Study of Ovarian Follicles by Scanning Electron Microscopy and ODO Maceration

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Summary. The ultrastructure of the follicle-oocyte complex in rodents and humans was revealed by high resolution scanning electron microscopy (SEM) following the Osmium-DMSO-Osmium maceration method (TANAKA and NAGURO, 1981).

In primary follicles, the majority of oocyte organelles such as mitochondria and Golgi complex components are concentrated in a juxtannuclear area. In particular, many spherical mitochondria are oriented all around the nucleus. After maceration of the ooplasm matrix, most of these mitochondria appear intermingled with numerous microtubules (MT) and associated with many Golgi vesicles. Such a nuclear polarization of organelles, essential to the oocyte metabolism, might depend upon a MT activity. MT might guide mitochondria to gather in the perinuclear region and further maintain their close association to the nuclear envelope. A similar relationship among microtubules, vesicular Golgi complex and mitochondria has been also observed when, in maturing oocytes, these organelles migrate and gather in other areas of the ooplasm.

A pattern common only to human developing follicles appears in the occurrence of long microvilli projected from follicle cells deep into the oocyte. These unusual microvilli running within the ooplasm are surrounded by several vesicles of the Golgi complex and endoplasmic reticulum, and often end close to the nucleus.

In the antral follicle, the microvilli of corona cells, directed toward the oocyte (after their full exposure through the chemical dissolution of the zona pellucida matrix) are extremely numerous (up to 70/cell), long (up to 7/10 μm) and tortuous. They resemble epididymal stereocilia, may be ramified and possess bulbous tips. In contrast, oocyte microvilli are thin and short. The "curly hair-like microvilli" of corona follicle cells and especially those terminating deep in the oocyte cytoplasm, besides being the expression of active exchange of nutrients, may serve to finely modulate oocyte maturation.

The study of intracellular organelles by scanning electron microscopy (SEM) began about 20 years ago with the use of various cracking methods, including cryofracture and frozen resins (TANAKA, 1972; HAGGIS and PHIPPS-TODD, 1977). At that time, the utility of these techniques was quite limited in that the low resolution power of SEM rendered it impossible to remove the cell matrix embedding and masking the cellular structures.

Today, the Osmium-DMSO-Osmium (ODO) method, developed by TANAKA and NAGURO (1981) and considered to be the most effective technique for visualizing cell organelles, is used extensively. Using this method, the present SEM study offers original illustrations of the three-dimensional (3-D) submicroscopic details of intracellular structures of oocytes. Microvilli embedded in the zona pellucida of oocytes and corona radiata cells are similarly studied.

MATERIALS AND METHODS

Ovaries taken from 14 to 22-week-old human fetuses, and from adult humans and rats were used in the present study.

Human ovaries were fixed *in toto* by immersion with 2.5% glutaraldehyde.

Following Nembutal anesthesia, the rats were perfused via the abdominal aorta first with Ringer's solution, and then with 0.5% glutaraldehyde plus 0.5% paraformaldehyde in a M/15 phosphate buffer. After dissection, the rat ovaries were post-fixed in 1% osmium tetroxide in M/15 phosphate buffer for 1-2 h.

After fixation, the specimens were rinsed with the buffer solution and were successively immersed in 15%, 30% and 50% aqueous solutions of dimethyl

Fig. 1. Rat ovary. Fractured surface of a developing follicle. $\times 800$

Fig. 2. Part of an oocyte of a developing follicle in the adult rat ovary. Mitochondria (*M*), small Golgi complexes (*GC*), and membranes of smooth and rough endoplasmic reticulum are seen in the cortical zone of the ooplasm. *ZP* Zona pellucida. $\times 12,000$

Fig. 3. Adult human ovary showing a primary follicle. *F* follicular cells. The matrix of the oocyte cytoplasm is partially dissolved and the nucleus is removed. The assemblage of vesicles corresponds to mitochondria. Golgi complex and endoplasmic reticulum (*GC*) are seen. $\times 990$

Figs. 4-6. Legends on the opposite page.

sulfoxide (DMSO) for 30 min each in order to prevent ice-crystal damage resulting from freezing occurring during the next step.

Specimens were frozen on metal plates chilled with liquid nitrogen, cracked into two parts with a razor blade and hammer, and were immediately placed in a 50% DMSO aqueous solution.

After rinsing with the buffer solution, the specimens were immersed in 0.1% osmium tetroxide in a M/15 phosphate buffer at 20°C for 1–3 days and then fixed again for 1 h in 1% osmium tetroxide in a M/15 phosphate buffer.

The samples were conductively stained by immersion in a 2% tannic acid aqueous solution for 1 h and in 1% osmium tetroxide in a M/15 phosphate buffer for 1 h.

After dehydration in a graded ethanol series and treatment with isoamyl acetate, the specimens were critical-point dried and coated with about 3 nm of platinum in an ion coater equipped with a rotary tilt state (VX-10R, EIKO Engineering Co., Ltd., Japan) and observed with a field emission SEM (HFS-2ST, Hitachi Co., Ltd., Japan).

RESULTS

A number of organelles within the oocyte cytoplasm were revealed by this method, and their 3-D surface features and spatial arrangements were easily demonstrated under high-resolution field emission SEM.

The present report describes some particular and original data on the arrangement and fine structure of the Golgi complex, mitochondria and microtubules in the oocyte. The features of the follicular corona cells and oocytes as well as the interrelationship existing between these cells and microvilli are also illustrated for both rat and human ovaries.

Golgi complex

After 24 h maceration, the fractured surface of the developing follicle showed a centrally located oocyte

surrounded by numerous follicular cells. The zona pellucida was easily observed but, as a consequence of the fracture, the oocyte nucleus sometimes appeared to have fallen off the fractured surface during the preparation process (Fig. 1). If areas such as these were viewed at higher magnifications, typical intracellular organelles such as Golgi complexes and mitochondria (Fig. 2) could be readily observed in detail. Since the matrix of the zona pellucida could not be removed within 24 h of maceration, the zona material surrounding the oocyte appeared as an amorphous structural layer not unlike that normally observed in thin sections under transmission electron microscopy (TEM), thereby preventing visualization of the features of the developing microvilli of oocytes and follicular cells in the zona.

The Golgi apparatus within the ooplasm was clearly revealed; it was disposed throughout the cell to form many separate dictyosomes in a fashion similar to that of higher plants. Golgi membranes were distributed mainly in the cortex of the ooplasm, adjacent to the zona pellucida (Fig. 2).

In growing follicles, the Golgi complex of the oocyte was often located more deeply in the cytoplasm, and consisted of five to six flattened cisternae piled one upon the other in close parallel array, forming a typical Golgi stack (Figs. 2, 3). In these growing oocytes, rounded mitochondria and small vesicular types of endoplasmic reticulum membranes were also frequently detected in areas where the Golgi complex was present (Figs. 2, 3).

Primary follicles consisting of a single layer of cuboidal follicular cells were easily found in the cortex of human adult ovaries. When these follicles were fractured following the ODO method, in some instances the nucleus of the oocyte was able to be removed from the preparation, thus offering a favorable and complete view of the oocyte's cytoplasm and organelles (Fig. 3). Small intercellular spaces were also noted between the oocyte and the surrounding follicle cells at this stage, but the zona material was poorly developed and unremarkable (Fig. 3). At this time, the relationship between oocyte and follicular

Fig. 4. Human developing follicle. A long microvillus (*arrow*) projected from a follicle cell runs deep within the ooplasm and is surrounded by vesicles of the Golgi complex and endoplasmic reticulum. $\times 21,000$

Fig. 5. Higher magnification of part of Figure 3, showing long and tortuous microvilli (*arrows*) from adjacent follicle cells deeply projected into the oocyte. Numerous membranous structures belonging to the endoplasmic reticulum and Golgi complex surround and even mask the microvilli. The filamentous network in these areas corresponds to microtubules (*MT*). $\times 21,000$

Fig. 6. One of the multiple Golgi complexes dispersed in the ooplasm (primary follicle in adult human ovary). Outermost cisterna of the Golgi stack (*GC*). Vesicles of about 80 nm in diameter and vacuoles of various sizes are found. Many microtubules (*MT*) and intracytoplasmic microvilli (*arrows*) from a follicle cell can be noted. $\times 25,000$

Figs. 7-10. Legends on the opposite page.

cells was so strict that, even following maceration, the oocyte rarely detached itself from the fractured surface.

When the cortical ooplasm was observed at high magnifications (Fig. 4), numerous, closely associated small vesicles were seen. Long, tubular extensions corresponding to microvilli were also found within the ooplasm. These microvilli projected from adjacent follicular cells and ran deep within the oocyte. Here they were in close relation to and surrounded by various vesicular elements which corresponded to Golgi complexes and endoplasmic reticulum membranes (Fig. 5). In some cases, these intracytoplasmic microvilli assumed a tortuous course, penetrating more deeply into the oocyte, and were always surrounded and masked by numerous small membranes of endoplasmic reticulum, even near the nuclear zone (Figs. 4, 5). At higher magnifications, an interior view of the oocytes of similar primary follicles showed multiple Golgi elements of various shapes and sizes in great detail. Further, cytoplasmic projections of associated follicular cells could be more easily detected in close proximity to the Golgi complex (Fig. 7). In addition, the outermost cisternae of the Golgi stack in these preparations showed fenestrations and vesicles measuring about 80 nm in diameter, and vacuoles of various sizes. Numerous fibers about 25 nm in diameter were noted around the Golgi complex. These structures likely corresponded to microtubules (Figs. 5, 6). Ordinarily, microtubules and all other cytoplasmic filamentous components are removed when the ODO maceration process (TANAKA and NAGURO, 1981) is employed. In the current study, the successful observation of microtubules was attributable to the prolonged pre-fixation of human specimens in 2.5% glutaraldehyde. As a result, it was easy to find a well preserved and developed microtubular network concentrated around the Golgi complex in many of the oocytes observed (Fig. 6).

Perinuclear mitochondria and microtubules

Another noteworthy finding obtained by the ODO

method was the spatial arrangement of mitochondria around the nucleus. These features were observed mostly in young oocytes still contained in the sexual cords of 14 to 22-week-old fetuses and in the primordial and primary follicles of both fetal and adult human ovaries. The most typical aspects are those represented in Figures 7 and 8, which illustrate the fractured surfaces of primary meiotic oocytes (pachytene) in 14-week-old human fetal ovaries. The large spherical nucleus containing chromosomes is clearly demarcated by a double nuclear envelope to which large numbers of spherical mitochondria adhere. Many microtubules were also seen in close association with these areas. Very rich clusters of mitochondria and networks of microtubule assemblages were more evident when the structures were found intact around the nuclear surface in unfractured cells (Figs. 9, 10).

Microvilli of cumulus corona cells and oocytes

After 3 days' maceration, the matrix of the zona pellucida was completely removed. In developed antral follicles, oocytes often fall away from the fractured surface. This suggests that, at this point, there no longer existed any strong connection between oocyte and corona radiata cells. Therefore, once the oocyte has fallen away, the study of the 3-D features of developing microvilli of follicular cells, which, in fresh specimens, are ordinarily masked by the presence of a sticky and abundant zona pellucida (Fig. 11), becomes easier. In these antral follicles, many long microvilli of follicular cells were seen spreading tortuously in various directions. At higher magnifications, corona cells appeared typically pear-shaped, and their fine microvilli were mostly concentrated in the form of a "plume" at the apex of the cell directed towards the oocyte (Fig. 12). Generally these microvilli were numerous (up to 70/cell), long (7/10 μ m) and curly. Moreover, many of these appeared entwined and ramified, possessing bulbous tips. As revealed by these methods, the microvilli of corona cells were generally more numerous than

Fig. 7. Primary meiotic oocyte (pachytene) in a sexual cord of a 14-week human fetal ovary. The large spherical nucleus (N), containing chromosomes in prophase, is surrounded by many mitochondria (M). $\times 7,500$

Fig. 8. Human fetal ovary at 14 weeks. Mitochondria (M) are closely related to the outer nuclear membrane in a primary meiotic oocyte. Nucleus with chromatin (N). $\times 25,000$

Fig. 9. Primary oocyte in a 14-week human fetal ovary. Mitochondria (M) assembled around the nucleus are associated with microtubules (arrows). The spherical mitochondria have sparse cristae. Outer surface of the nuclear envelope (N) is evident. $\times 15,000$

Fig. 10. Human fetal ovary at 22 weeks. Mitochondria (M) of meiotic oocyte are intermingled with numerous microtubules (arrows) and are present in a cytoplasmic area closely associated with the nucleus (N). $\times 12,000$

Figs. 11-14. Legends on the opposite page.

previously reported (MOTTA and VAN BLERKOM, 1974, 1975) (Fig. 14). In contrast, the microvilli of oocytes were thin and short when compared to those of follicular cells, and extended straight over the oocytes plasma membrane. Oocytes possessed many more microvilli than corona cells; indeed, these structures occupied almost the entire surface of the oocyte (Fig. 13). Complete dissolution of the ooplasm sometimes made it possible to observe the oolemma from the interior of the oocyte. From this unusual vantage point, the inner surface of the oolemma was fully and clearly visible, revealing a great number of tiny holes (Fig. 13). These orifices corresponded to the positions occupied by the microvilli on the surface of the oocyte and extended towards the zona pellucida. These holes were not seen wherever the microvilli of the follicular cells were in close contact with the oocyte's plasma membrane. Considering the very high number and regular distribution of these microvillous holes, they well parallel the number and distribution of oocyte microvilli ordinarily seen on the outer surface of the cell (compare Figs. 13, 14).

DISCUSSION

This study, employing the ODO maceration method as well as high-resolution SEM, showed some peculiar 3-D ultrastructural features occurring during the maturation of oocytes and follicles. The main points focused on in this report concern: a) the characteristic assemblage of oocyte organelles and microtubules and, b) the microvillous pattern of oocytes and follicle cells.

Assemblage of oocyte organelles and microtubules

This study demonstrated that, in sexual cords as well as in primordial and primary follicles of both fetal and adult human ovaries, the majority of primary oocyte organelles, such as mitochondria, endoplasmic reticulum and Golgi complex elements, were typically concentrated in certain areas of the cytoplasm

where a rich network of microtubules was also present. In particular, in some special developmental stages, such as in the primary meiotic human oocytes, especially in the pachytene stage, many spherical mitochondria with single, scarce cristae typically appeared oriented about the nucleus in a rosette fashion, in close contact with the nuclear membrane (POZO et al., 1990). Similar concentrations of mitochondria associated with elements of the endoplasmic reticulum, lysosomes and annulate lamellae were, as a rule, also observed in oocytes of primordial follicles in adult human ovaries, where they form the so-called "Balbiani body" (HERTIG and ADAMS, 1967; ZAMBONI, 1971; SATHANANTHAN et al., 1986). After careful removal of the ooplasm matrix by maceration, most of the mitochondria assembled around the nucleus appeared intermingled with numerous microtubules (MAKABE, NAGURO and MOTTA, 1990). It is likely that this nuclear polarization of organelles, essential for the oocyte metabolism at this early stage, depends upon microtubular activity. A microtubular network might help to guide mitochondria towards the perinuclear region, and—with the help of other (intermediate) microfilaments—to maintain them in close association with the nuclear membrane.

Similar relationships among microtubules, Golgi complexes and mitochondria have also been observed in maturing oocytes, in which these organelles migrated towards the cortex and assembled in other areas of the ooplasm (VAN BLERKOM and MOTTA, 1979; DVORAK and TESARIK, 1980; MOTTA and MAKABE, 1981; FAMILIARI et al., 1989; 1991).

Microvillous pattern of corona cells and oocytes

The application of the ODO maceration technique and SEM revealed a microvillous pattern commonly seen only in human developing follicles: the occurrence of long microvilli originating from follicle cells and projecting deep into the oocyte. These unusual microvilli running within the ooplasm were surrounded by several Golgi vesicles and endoplasmic

Fig. 11. Adult human ovary. Microvilli of corona radiata cells in the zona pellucida of antral follicle. The matrix of the zona pellucida has been dissolved in diluted osmium solution and disappeared. *FA* follicular antrum, *CO* cumulus oophorus *GL* granulosa layer $\times 6,000$

Fig. 12. Higher magnification of part of Figure 11. Numerous microvilli radiating from corona radiata cells are entangled. $\times 14,000$

Fig. 13. Rat ovary. Microvilli of the oocyte at very high magnification appear short and numerous, compared to the long and tortuous microvilli (*arrow*) of corona radiata cells. $\times 30,000$

Fig. 14. Rat oocyte. Inside view of the oolemma. The holes correspond to the areas from which microvilli arise (*arrows*). Long and short microvilli of corona radiata cells and the oocyte, respectively, are also evident. $\times 32,000$

reticulum, and often terminated close to the nucleus. Though the occurrence of a similar microvillous pattern was reported in TEM studies (BACA and ZAMBONI, 1967; ZAMBONI, 1971), a clear demonstration of their nature and exact distribution within the ooplasm is now possible by 3-D SEM. Moreover, the number of microvilli noted by SEM is much higher if compared to the occasional finding obtained solely by TEM observations.

In antral follicles, the microvilli of corona cells are directed towards the oocyte. After full exposure of the oocyte through chemical dissolution of the zonal matrix, these structures appeared very numerous and extremely long and tortuous. They greatly resembled the epididymal stereocilia as seen in SEM studies (MURAKAMI et al., 1975; MOTTA et al., 1977). In some cases these microvilli were ramified and possessed bulbous tips. On the contrary, oocyte microvilli were thin and short. Besides being the expression of the active exchange of nutrients, the "curly hair-like microvilli" of follicle cells, and especially those terminating deep within the ooplasm, may further serve to finely modulate or regulate, promote and/or inhibit the maturation of the oocyte.

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