Visualization of the Three-Dimensional Distribution of Collagen Fibrils Over Preovulatory Follicles in the Hamster

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ABSTRACT  Before an oocyte can escape from a preovulatory follicle, the apical wall must thin to the point of rupture. Although numerous layers of cells are present, it is the collagen fibrils in the theca externa that provide most of the strength to the developing follicle. The three-dimensional distribution and integrity of these fibrils over a follicle cannot be appreciated with standard used methods such as examination of thin sections by transmission electron microscopy. In this paper we describe a technique that removes cells superficial to the collagen fibrils so that their distribution may be examined by scanning electron microscopy. On the third day of the hamster's 4-day estrous cycle, bundles of fibrils pass from intrafollicular areas and ascend follicles. Approximately halfway up the follicle wall, the bundles fan out and form a meshwork of fibrils which covers the apex. As the time of ovulation approaches, the number of layers of fibrils decreases over the apex until a tear forms in the weakened matrix. Experimental results demonstrating that the meshwork is composed of collagen fibrils are presented. The usefulness of this technique in visualizing the collagen content in preovulatory follicles is discussed as well as factors that may aid in weakening this layer so that follicle rupture may occur. Key words collagen, ovarian follicle, hamster, ovulation

Collagen fibrils are the main supportive structures in the wall of mature ovarian follicles (Espey, '78). For ovulation to occur, the follicle wall, and in particular its collagenous layers, must be weakened to the point of rupture. Numerous histological and electron microscopical studies have described the thinning of the apical follicle wall (Byskov, '69; Parr, '74; Motta and Van Blerkom, '75; Mori and Uchida, '81) and the loss of collagen fibrils as ovulation approaches (Espey '67; Bjersing and Cajander, '74; Okamura et al., '80). The mechanisms by which collagen is removed from the follicle wall are not known. Although numerous studies have implicated hydrolytic enzymes in this function (Espey, '74; Bjersing and Cajander, '75; Cajander and Bjersing, '76; Espey and Coons, '76; Strickland and Beers, '79; Yajima et al., '80), it is not clear if they degrade the collagen fibrils or the cross links that bind them together into a matrix (Espey, '67; Bjersing and Cajander, '74). It would be helpful in evaluating degradation of collagen fibrils in preovulatory follicles to have a technique that permits three-dimensional visualization of the collagen organization. Unfortunately such information is not easily obtained in thick-or thin-sectioned material. In this paper, we describe a technique for removing cells superfi-

cial to the collagen fibrils so that the distribution of these fibrils over developing follicles may be examined by scanning electron microscopy. The hamster is an excellent animal for this study because the collagen fibrils are restricted to the relatively thin theca externa of the follicle wall. Changes in the distribution and organization of collagen fibrils during the estrous cycle are described, and the role of these fibrils in ovulation is discussed.

MATERIALS AND METHODS

Female golden hamsters (Mesocricetus auratus), 8–16 wk old were used throughout this study. Hamsters were maintained on a 12.5:11.5-hr light:dark cycle and allowed free access to Purina laboratory chow and water. Animals on day 1 of their 4-day estrous cycle (as determined by the presence of a vaginal discharge in the morning) were given an intraperitoneal injection of pregnant mare's serum gonadotropin (25 IU, Organon) to induce superfollicularization. On the evening of day 3, hamsters were given an intraperitoneal injection of human chorionic gonadotropin (25 IU, Sigma) to induce ovulation approximately 13 hr later.

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At specific times during the estrous cycle, hamsters were sacrificed and the ovaries were removed. The ovaries were rinsed in 8.5% saline and placed in a 5% solution of sodium dodecyl sulfate (SDS) in distilled water. The ovaries were observed with a dissecting microscope until a clear layer lifted off the surface of the ovary. This normally took 2–4 min. Ovaries were then fixed in 3% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.4) for 3 hr and rinsed in the same buffer. The clear layer could be removed by treatment with a 0.02% solution of DNase (1 hr at 20°C). It was also lost during routine dehydration of the tissue through a graded series of acetone. The tissue was critical-point dried (Denton DCP-1), coated with gold-palladium, and viewed in a JOEL JSM 35c scanning electron microscope (SEM).

Follicles from some ovaries were prepared for transmission electron microscopy (TEM) so that the effect of the SDS treatment on normal follicle structure could be studied. Examination of sectioned material also allowed us to determine the optimal treatment time so that the collagen matrix was exposed while deeper layers of the wall were not affected. SDS-treated follicles to be sectioned were fixed and dehydrated as described above. They were then embedded in Spurr’s (69) plastic and sectioned on a Porter-Blum MT-2B ultramicrotome. Thin sections were stained with uranyl acetate (1 hr) and lead citrate (5 min) and viewed in a Hitachi H500 TEM.

To test for the presence of collagen in the matrix of fibrils revealed by the SDS technique, ovaries observed by SEM were soaked in a 0.02% solution of collagenase (Sigma type II) for 3 hr, dehydrated, and reexamined by SEM. To determine if the matrix was produced or contaminated by debris from the lysed cells,

**Abbreviations**

| A. Apex     | I. Intrafollicular area |
| C. Cables  | S. “Skirt” of collagenous fibrils |
| F. Follicle |                         |

Fig. 1. Photomicrograph of a follicle 2 hr before expected ovulation. × 35.

Fig. 2. Photomicrograph of the follicle in Figure 1, 1 min after being placed in a 5% solution of SDS. Note the layer of cells lifting off the surface of the follicle. × 35.
they were soaked in trypsin (10 μg/ml pH 7.6; Sigma type IX) or urea (8 M). After 3 hr the tissue was dehydrated and reexamined by SEM.

RESULTS

Observation on ovaries in SDS

When ovaries from selected stages of the estrous cycle were placed in a 5% SDS solution, a thin, transparent layer lifted from the ovarian surface in 2–4 min (Figs. 1, 2). After 4 min, there was no further change in this layer. Additional material extended about 20 mm from the ovary in all directions. This material could not be seen with a dissecting microscope but the ovary could be moved by forces placed in this zone. When ovaries were placed in a 0.02% DNase solution both layers disappeared, suggesting these layers are composed of DNA from lysed cells.

Thick sections through follicles treated with SDS for varying lengths of time were examined to determine the optimal treatment time. The penetration of the detergent was easily observed by the presence of lysed cells. When follicles were treated for 1 min or less, the transparent layer was not fully formed and in sectioned material some surface epithelial cells could still be distinguished. When follicles were treated for 5–10 min, theca interna and granulosa cells of the apical follicle wall were lysed. In follicles treated for 30–60 min, all cells of the follicle wall and many cells of the ovarian stroma were lysed and the structure of the follicle was difficult to interpret. Treatment for 2–4 min was optimal for exposing collagen fibrils of the theca externa layer and caused minimal disruption of the deeper cell layers.

SEM observations of collagenous matrices

Figures 3 and 4 show a normal and an SDS-treated ovary on the second day of the cycle. The surface of untreated ovaries is smooth and covered by epithelial cells (Fig. 3, inset). The SDS-treated ovary has the same general shape but is distinguished by numerous "straps" that extend from intrafollicular areas over developing follicles (Fig. 4). At higher magnification, each follicle is seen to be covered by several layers of fibrils (400–700 Å diameter) that are oriented randomly (Fig. 5). The pattern of fibrils over developing follicles is indistinguishable from that over intrafollicular areas (Fig. 6).

By day 3, follicles protrude further above the ovarian surface and the fibrils are organized into two zones. Beginning near the base of the follicles, wide bands or straps ascend the sides of the follicle (Figs. 7, 8). There are several layers of fibrils in these bands. They are oriented parallel to one another and run toward the apex of the follicle (Fig. 9). At the apical end of these straps, the fibrils fan out into a meshwork that covers the apex of the follicle (Fig. 10).

The matrix over day 4 follicles is similar to that described for day 3. However, a larger area at the apex is covered by the meshwork. Unruptured follicles prepared at the time of expected ovulation are covered by a very thin meshwork of fibrils. In some follicles, a tear in the meshwork was fixed as it was apparently in the process of forming (Figs. 11, 12). Increased magnification of the torn area shows the thin mesh layer pulling away from the site of eventual rupture (Fig. 13).

Day 4 follicles that had been examined by SEM were infiltrated with Spurr's plastic and sectioned for examination by TEM. Collagen fibrils with a banding pattern of approximately 650 Å could be seen.

Effect of collagenase, trypsin, and urea on collagenous matrix

Ovaries that had been treated with SDS and examined by SEM to verify the presence of the matrix were used to assess the effect of three chemicals on the meshwork. Ovaries were treated with collagenase, trypsin, or urea and reexamined by SEM. Collagenase removed the fibrils from the ovaries, and the loss of the thick straps around the base of follicles could be followed using a dissecting microscope (Fig. 14). Trypsin and urea, however, had no apparent effect on the matrix.

DISCUSSION

We have described changes in the three-dimensional distribution of collagen fibrils over hamster ovarian follicles as the time of ovulation approaches. These observations were obtained by the use of the ionic detergent, SDS, to solubilize cells superficial to the collagen fibrils. This technique was originally developed to investigate the matrix produced by and surrounding smooth muscle cells from the rat heart grown in culture (Jones et al., '79). The cultures were treated with a 1% solution of SDS for up to 5 hr with no detectable extraction of collagen. The matrix was analyzed biochemically and consisted primarily of collagen, some elastin, and small amounts of a glycoprotein that has similarities to fibronectin.

We treated hamster ovaries with a 5% solution of SDS for 2–4 min. This time was sufficient to lift a transparent layer off mature follicles. This layer consists of lysed epithelial
Fig. 3. Scanning electron micrograph of a normal day 1 ovary. × 26. Inset: higher magnification (× 550) showing surface epithelial cells from the apex of a follicle.

Fig. 4. Scanning electron micrograph of a day 2 ovary that was treated for 1–2 min with SDS. Note the strands crossing intrafollicular areas (I) and ascending developing follicles (F). × 30.

Fig. 5. Scanning electron micrograph showing the meshwork of collagen fibrils over the apex of a day 2 follicle. × 1,700.

Fig. 6. Scanning electron micrograph showing the meshwork of collagen fibrils over the interfollicular areas of a day 2 ovary. × 1,450.
Fig. 7. Scanning electron micrograph showing a nearly continuous "skirt(s)" of dense collagen fibrils ascending the sides of a day 3 follicle. × 130.

Fig. 8. Scanning electron micrograph looking down at the apex (A) of a day 3 follicle. Note the cables (C) of collagen fibrils wrapping around the sides of the follicle. × 170.

Fig. 9. Scanning electron micrograph showing the dense and parallel packing of collagen fibrils found on the "skirt" or cables ascending a day 3 follicle. × 6,300.

Fig. 10. Scanning electron micrograph of the side of a follicle showing collagen fibrils fanning out from a cable (C) to form a meshwork. This meshwork covers the apex of day 3 follicles. × 4,000.
and thecal cells and their DNA. The transparent layer could be removed by DNase, although it was also lost in normal processing for SEM.

The matrices we examined by SEM were not analyzed biochemically but are thought to consist primarily of collagen for three reasons. First, the size and distribution of the fibrils coincide with those of collagen fibrils in thin sections of normal follicles examined by TEM. Second, the matrix is removed when SDS-treated ovaries are subsequently treated with collagenase. Third, DNase, urea, and trypsin had no effect on the matrix, suggesting that cellular debris was not abundant.

Using the SDS technique, we found a thick meshwork of fibrils covering day 2 follicles. By day 3 the fibrils either form a continuous skirt around the sides of a follicle or form distinct cables that cross intrafollicular areas and continue up the sides of a follicle. Toward the apex, the skirts or the cables fan out to form a fine mesh of fibrils. On day 4, the general distribution of the meshwork is similar to that of day 3 follicles. The layer of fibrils at the apex appears very thin. Follicles prepared at the time of expected ovulation show tears in the collagenous matrix at the apex. Once the integrity of the meshwork has been broken, only theca interna cells remain in the follicle wall at the point of eventual rupture. Presumably, these cells stretch until their connections break and ovulation occurs.

The presence of a meshwork of collagen fibrils over preovulatory follicles helps to ex-
plain the spherical shape of the follicle. As long as follicle shape is determined by intrafollicular pressure, and the meshwork lacks focal weaknesses, the follicle will be spherical. Although intrafollicular pressure does not increase and cause follicle rupture (Blandau and Rumery, '63; Espey and Lipner, '63; Bronson et al., '79), it is important in inflating the follicle. In addition, as the follicle swells, the apical wall thins because it is not supported by tissue as are the sides and base of the follicle. Shortly before expected ovulation, the profile of the hamster follicle changes from a low to a high dome (Martin and Talbot, '81). This change has been correlated with the contraction of smooth muscle cells (SMC) in the theca externa at the base of follicles (Martin and Talbot, '81; Talbot, '82; Talbot and Chacon, '82; Talbot and Schroeder, '82). It is suggested that force generated by the contraction of these cells is transmitted in the collagen meshwork to the apex, where it aids in thinning of the wall and rupture. When the SMC shorten, the diameter of the follicle decreases, the walls straighten (Martin and Talbot, '81; Talbot, '82), and presumably tension increases on the apical wall.

In other mammals, such as the rat and rabbit, a secondary cone forms at the site of eventual rupture within minutes of ovulation (Walton and Hammond, '28; Hill et al., '35; Blandau, '55). In these species, a thicker layer of cells may remain in the wall following disruption of the collagen matrix. Intrafollicular pressure may be sufficient to cause this area of the follicle to balloon outward forming the secondary cone which thins until rupture occurs.

There has been considerable interest in the presence and role of hydrolytic enzymes in the ovulatory process (Espey, '74). Plasmin (Strickland and Beers, '79), collagenase (Espey and Coons, '76; Yajima et al., '80), and lysosomal enzymes (Cajander and Bjersing, '76) have been detected in the follicle. Only plasmin has been shown to increase in concentration within preovulatory follicles in response to the surge of luteinizing hormone. Espey ('80) has recently suggested that plasmin may activate procollagenase. At the morphological level, it is not clear whether the "ovulatory enzyme(s)" degrades the collagen fibrils or the intermolecular bonds that link them together. Our observations support the idea that it is the connections between fibrils that are weakened for the following reasons. First, fibrils at the site where the meshwork tears show no morphological changes at the SEM level. Second, TEM of the matrix near the tear shows no signs of disintegration and the fibrils show the banding pattern typical of collagen. Third, we have shown that as the follicle swells there is a change in the orientation of the fibrils. Collagen fibrils are not very distensible (Harkness, '68); therefore, it is either the weave of the fibrils or the presence of elastic elements that allows for the swelling of the follicle. We are not aware of any study reporting the presence of elastin in the follicle wall, although Espey and Coons ('76) found a decrease in the integrity of the follicle wall when strips were incubated in elastase. As the follicle enlarges, the meshwork becomes thinner at the apex where most of the stretching occurs. We suggest that the reorientation of these fibrils is caused by the follicle swelling and may involve the breaking and possible reforming of cross links between fibrils. Continued swelling of the follicle and the activity of hydrolytic enzymes results in a tear of the meshwork and a loss in the integrity of this layer. In this view, follicle swelling plays an essential role in altering the meshwork pattern and in stressing the cross links between fibrils that may be enzymatically weakened.

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