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Basal lamina of avian ovarian follicle: influence on morphology of granulosa cells in-vitro

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Abstract

Experiments were conducted to determine the influence of basal lamina on the morphology of ovarian granulosa cells in vitro. Pure and intact basal lamina was isolated from the large preovulatory follicles of the chicken ovary and designated basal lamina of avian ovarian follicle (BLAOF). Examination of the isolated basal lamina with electron microscope revealed an ultrastructure that is similar to that of basal lamina in the intact ovarian follicle. Pieces of the intact basal lamina were attached to the bottom of 32 mm culture dishes (BLAOF-coated dishes) in which differentiated granulosa cells isolated from the largest preovulatory follicle or undifferentiated granulosa cells isolated from immature small yellow chicken ovarian follicles were cultured; uncoated dishes served as controls. Granulosa cells incubated on intact basal lamina assumed spherical shape, whereas granulosa cells incubated directly on plastic in control dishes became highly flattened. Interestingly, granulosa cells that attached to plastic close to BLAOF (in BLAOF-containing dishes) became rounded. The storage of BLAOF-coated culture dishes at 4°C for 2 years had no apparent effect on its ability of the matrix material to induce changes in granulosa cell shape. Some components of the basal lamina could be solubilized with guanidine–HCl alone (fraction 1; 90–95% of total protein in BLAOF) with the remaining components solubilized with β-mercaptoethanol containing guanidine-HCl (fraction 2; 5–10% of total protein in BLAOF). Differentiated and undifferentiated chicken granulosa cells became rounded when incubated in fraction 1 -pre-coated wells; whereas those incubated directly on plastic in control wells were flattened. Similarly, when fraction 1 of solubilized basal lamina was added as liquid to incubation mixture, it caused both differentiated and undifferentiated granulosa cells to assume spherical shapes. The storage of fraction l-coated culture dishes at 4°C for 12 or more months had no apparent effect on its ability to influence granulosa cell shape. Fraction l-induced changes in granulosa cell shape were similar to those observed for complete and intact basal lamina (BLAOF). These findings demonstrate that intact homologous basal lamina (BLAOF) or its solubilized (fluidized) form can induce normal (in vivo) morphology in granulosa cells. It is suggested that BLAOF or its solubilized form can be used to culture cells in experiments designed to examine the influence of the natural basal lamina microenvironment on cellular behavior and function.

Keywords: Basal lamina; Basement membrane; Extracellular matrix proteins; Cell morphology; Granulosa cell; Ovary; Follicular development; Chicken

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1. Introduction

Basement membranes are extracellular matrix sheets that compartmentalize tissues and act as physical barriers in separating different types of cells such as endothelia, epithelia and muscle fibers. The thickness of basement membranes vary in different organs and tissues (Inoue and Leblond, 1988). Two, and sometimes three layers have been identified in basement membranes (Kefalides et al., 1979; Inoue and Leblond, 1988). The layer closest to the basolateral plasmalemma of associated cells is pale and is referred to as lamina lucida (lamina rara). The next layer known as lamina densa is a dark continuous sheet made up of fairly compact material. The third layer, often absent, is in continuity with the connective tissue and is known as lamina fibroreticularis (Kefalides et al., 1979; Laurie and Leblond, 1985; Inoue and Leblond, 1988). In some cases the lamina fibroreticularis contains collagen fibrils (anchoring fibers) (Yurchenco and Schittny, 1990). The lamina lucida (rara) and lamina densa are collectively known as basal lamina (Laurie and Leblond, 1985). The basement membrane (basal lamina) of the rat ovarian follicle appears to have all three layers of basement membranes Bagavandoss et al., 1983, however, the lamina lucida is absent at points where the anchoring fibrils are attached to the lamina densa (Bagavandoss et al., 1983). The basal lamina of the chicken ovarian follicle is a 1 μm thick continuous sheet and appears homogenous at low magnifications and lacks lamina fibroreticularis (Perry et al., 1978). At high magnifications however, indistinct laminations and focal densities can be seen (Perry et al., 1978).

In situ, cells are either associated or surrounded with extracellular matrix which may regulate their structure and function; granulosa cells in the avian ovarian follicle exist in association with basal lamina. The influence of homologous basal lamina on the behavior of granulosa cells is unknown. Therefore, the goal of the present study was to isolate pure and intact basal lamina from the chicken ovarian follicle and assess its effect on the shape of granulosa cells.

The unique anatomical structure of the avian ovarian follicle made possible the isolation of pure intact basal lamina. In the mature avian ovarian follicle, a single layer of granulosa cells is located between basal lamina and perivitelline layer to form the granulosal layer (membrana granulosa) (e.g. see Wyburn et al., 1965; Perry et al., 1978; Bakst, 1979; Callebaut et al., 1991). The membrana granulosa (basal lamina-granulosa cell layer-perivitelline layer complex) can be easily separated from the theca layer (Gilbert et al., 1977) and basal lamina isolated in hypotonic solution.

2. Materials and methods

2.1. Chemicals

N-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), collagenase type IV, soybean trypsin inhibitor, bovine serum albumin (BSA, Fraction V), penicillin G, streptomycin, fungizone and Trizma-base were purchased from Sigma, St. Louis, MO. Medium 199 (M199) containing Hank's salts was from Gibco-BRL, Grand Island, NY.

2.2. Animals

Single Comb White Leghorn hens obtained from Purdue University Poultry Research Farms, West Lafayette, IN, in their first year of reproductive activity, were caged individually in a windowless, air-conditioned room with a 14 h light:10 h darkness cycle. They had free access to a layer ration and tap water. The time of egg lay of each bird in the colony was noted to the nearest 30 min (daily). The animals were killed by cervical dislocation about 10–12 h before the expected time of ovulation of the largest preovulatory follicle. The first, second, third, fifth to seventh largest (F_1, F_2, F_3, F_{5-7},) preovulatory follicles and a pool of small yellow follicles (SYF) were removed. The follicles were classified according to the criteria of Robinson and Etches (1986). The follicles were placed in ice-cold Hank's salt solution containing NaCl 140 mM, KC1 5 mM, $MgCl_2$ 1.1 mM, $CaCl_2$ 2.5 mM, Hepes 10 mM, Glucose 5.6 mM, pH 7.4. The thecal and granulosal layers (membrana granulosa) were separated by the method described by Gilbert et al. (1977). The granulosa cells were dissociated in M199 containing $NaHCO_3$ (350 mg/l), HEPES (10 mmol/l), pH 7.4, penicillin G (100 000 U/l), streptomycin (100 mg/l), fungizone (250 μg/l), collagenase (500 000 U/l) and trypsin inhibitor (200 mg/l) (Novero and Asem, 1993). Cell viability, determined by the

trypan blue exclusion method, was routinely greater than 90%.

2.3. Isolation of basal lamina

The granulosal layer (membrana granulosa) was placed in a hypotonic solution containing Tris-HCl 10 mM (pH 7.4), leupeptin 0.5 mg/l, EDTA–Na_2 1 mM, pepstatin 0.7 mg/l, and phenylmethylsulfonyl fluoride (PMSF) 0.2 mM in a petri dish. The granulosa cells, sandwiched between the basal lamina and perivitelline layer were lysed hypoosmotically and the basal lamina and perivitelline layer were separated. The duration of time required for complete separation of the two layers (basal lamina and perivitelline layer) was dependent on the hypotonicity of the solution. The separation was much faster (l-3 min) in the absence of Tris-HCl than in Tris–HCl-containing solution (4-8 min). This basal lamina of avian ovarian follicle (BLAOF) preparation is intact and complete basal lamina. The side of basal lamina in contact with granulosa cells in situ is designated as 'granulosa-side' and the side in contact with thecal tissue was assigned the name 'theta-side'.

2.4. Transmission electron microscopy

Granulosal layer or isolated basal lamina was fixed in 3% glutaraldehyde in 0.15 M Millonig's phosphate buffer pH 7.2 for 24 h. The fixed tissue was rinsed in phosphate buffer and postfixed in 1 % osmium tetroxide- 1.5% potassium ferrocyanide at 4°C for 1.5 h. Tissue was then rinsed in buffer, dehydrated through a graded ethanol series, rinsed 2 x in propylene oxide and infiltrated with epoxy resin (Poly/Bed 8 12, Polysciences, Warrington, PA). After resin polymerization at 60°C for 48 h, thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined in a JEOL JEM-100CX transmission electron microscope.

2.5. Scanning electron microscopy

Granulosal layer (granulosa cells, basal lamina, perivitelline membrane complex) or isolated basal lamina or perivitelline layer was fixed in 3% glutaraldehyde in 0.15 M Millonig's phosphate buffer pH 7.2 for 24 h. The fixed tissue was rinsed in phosphate buffer and postfixed in 1% osmium tetroxide for 1.0 h. Tissue was then rinsed in buffer, dehydrated through a graded ethanol series, and then a graded Freon 113 series. The granulosa cell layer or the individual components were then picked up from a petri dish containing Freon 113 with a glass microscope cover-slip and air dried. The cover-slips were glued to aluminum stubs with silver paint, sputter coated with gold, and examined in an IS1 100A scanning electron microscope.

2.6. Light microscopy

Photomicrographs of granulosa cells were obtained with a 40 x objective on a Nikon Diaphot microscope equipped with Hoffman Modulation Contrast Optics.

2.7. Differential interference contrast microscopy

The images of some cells were obtained with differential interference contrast optics (20 x objective) on a laser scanning confocal microscope, Bio-Rad MRC 1024 UV/vis (Bio-Rad, Hercules, CA). The images were visualized using 488 nm light from a Krypton-Argon laser and a frequency matched detector. The images were electronically magnified with Lasers lamp 1024 software.

2.8. Preparation of intact basal lamina-containing dishes for cell culture

Pieces of intact basal lamina (BLAOF) isolated from the largest (32--35 mm in diameter) or second (27-30 mm in diameter) largest preovulatory follicles (F, and F_2) were spread in the bottom of a 35 mm Falcon or Corning culture dishes (a piece per dish) and allowed to dry for 2 h in a laminar flow hood at room temperature (23°C). The bottom of the culture dish was not covered completely by BLAOF. The BLAOF-containing dishes were either used following the attachment procedure or were wrapped in aluminium foil for storage at 4°C. Tissue isolation and preparation of culture dishes were carried out under sterile conditions. The basal lamina was spread with 'theta-side' down (in contact with the bottom of the dish); in a few cases it was spread with 'granulosa-side' in contact with the bottom of the dish. Unless stated otherwise, the cells were incubated on the 'granulosa-side' of intact basal lamina in the present studies.

2.9. Soluhilization of basal lamina

2.9.1. Fraction 1

Basal laminae were placed in a microfuge tube and solubilization buffer containing 6 M guanidine–HCl, 50 mM Tris-HCl pH 7.4 was added (100 μl buffer per basal lamina per follicle). After shaking at 4°C overnight, some membrane fragments remained. The mixture was centrifuged at 1000-2500 x g for 10 min. The supernatant designated fraction 1 was placed in a 3 kDa cutoff dialysis membrane and dialyzed against 150 mM NaCl, 50 mM Tris-HCl pH 7.4 overnight at 4°C. After dialysis, fraction 1 became cloudy, presumably due to the precipitation of some proteins. The dialyzed fraction 1 was aliquoted and stored at 70°C in the same buffer. Protein content of solubilized basal lamina was determined by the method of Bradford (1976) using BSA as standard. Fraction 1 contained 90–95% of total protein in the basal lamina.

2.9.2. Fraction 2

The basal lamina fragments collected by centrifugation during the preparation of fraction 1 were solubilized with 6 M guanidine–HCl, 50 mM Tris-HCl pH 7.4 containing 5% β-mercaptoethanol with shaking for 60 min at 4°C and designated fraction 2. Similar results were obtained when 8 M urea was substituted for guanidine–HCl. The fraction 2 solution was placed in a 3 kDa cutoff dialysis tube and dialyzed against 150 mM NaCl, 50 mM Tris-HCl pH 7.4 overnight at 4°C. The dialysate was aliquoted and stored at − 70°C in the same buffer. The dialysate of fraction 2 did not turn cloudy. The exclusion of β-mercaptoethanol from the buffer led to incomplete solubilization of the basal lamina (fragments remained). Fraction 2 contains 5–10% of total protein in the basal lamina.

2.10. Preparation of solubilized basal lamina -con taining dishes for cell culture

Fraction 1 of the solubilized basal lamina was diluted with either deionized water or modified Hank's salt solution or M199. Aliquots of 100–200 μl containing 5-50 μg of protein were transferred into 96-well, 24-well Falcon culture dishes (Fisher Scientific) or Lab-Tek 8-well chambered coverglass (Nunc, Naperville, IL) and allowed to dry under a tissue culture hood. Some wells received vehicle only and served as controls. The plates were then used after the drying procedure or were wrapped in aluminium foil for storage at 4°C. Culture wells that received Hank's salt solution or Ml99 were rinsed twice with deionized water prior to the incubation of cells. Tissue isolation, solubilization, dialysis and preparation of culture dishes were carried out under sterile conditions.

2.11. Incubation of cells

2.11.1. Incubation of cells in intact basal lamina -con taining dishes

Chicken granulosa cells were isolated by collagenase dispersion as described by Novero and Asem (1993). Isolated granulosa cells were designated as differentiated (from mature F, follicle), differentiating (from F_3 or F_{5-7} follicles) and undifferentiated (from SYF). The granulosa cells were plated at a density of 0.1-2 x 10^5 live cells/ml and incubated at 37°C in serum-free M 199 containing 0.1% (wt./vol.) BSA as described by Novero and Asem (1993). The cells were cultured in Ml99 containing Hank's salts, penicillin G 100 000 U/l, streptomycin 100 mg/l, fungizone 250 μg/l, HEPES 10 mM (pH 7.4), and BSA 0.1%. The granulosa cells were placed in the culture dishes in a 2 ml suspension. In BLAOF-containing dishes, granulosa cells attached to both the basal lamina and plastic. Unless indicated otherwise, all cells were cultured in serum-free media without any other additive.

2.12. Morphometric analysis of cells

Light microscopic images of granulosa cells were collected from at least five identical locations of each incubation well or coverslip on an inverted Nikon microscope (20 x objective) and stored. The outlines of individual cells were traced, and the following parameters: mean surface area covered by each cell, cell perimeter and circularity were determined with Optimas 6.0 Software (Bothell, WA). Higher estimates of circularity (which is independent of size) were associated with greater irregularity of cell profile. A perfect circle has a circularity of 12. The data presented in Figs. 5 and 6, are the image parameters for granulosa cells incubated in control dishes (CON), on intact basal lamina (BL), on plastic

3-5 mm distance from the intact basal lamina (designated close to basal lamina; PL-BL-C) and on plastic more than 7 mm distance from intact basal lamina (designated far from basal lamina; PL-BL-F).

2.13. Statistical analyses

A t-test (two-tailed) was performed to compare the differences among treatment means and control values. Differences at $P < 0.05$ were considered significant.

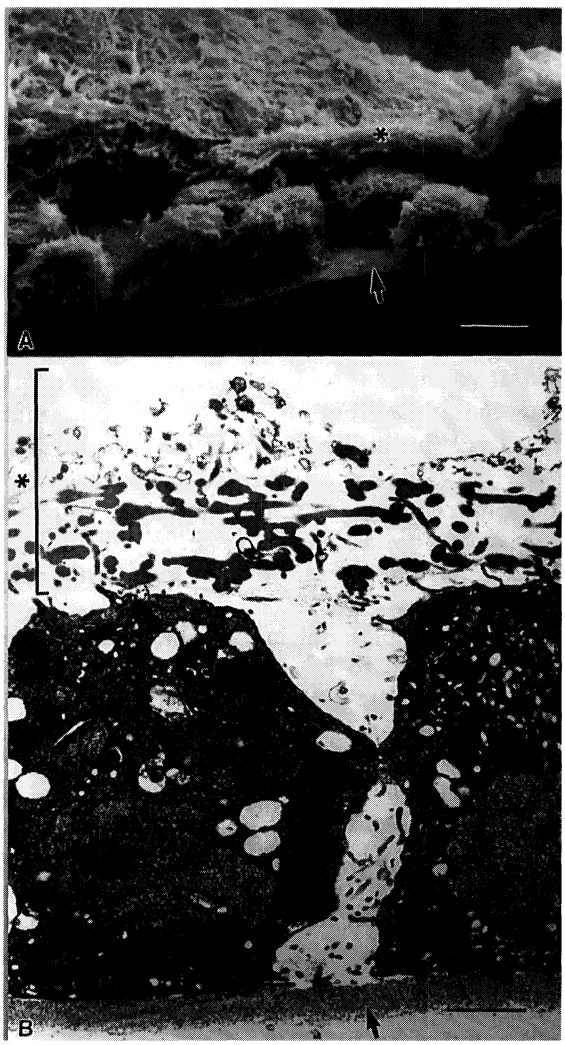

Fig. 1. Scanning (SEM) and transmission (TEM) electron micrographs of granulosal layer (membrana granulosa) of a mature follicle (F,) of chicken ovary showing granulosa cells located between the basal lamina (denoted by arrow) and perivitelline layer (denoted by *). Panel A, SEM of membrana granulosa; panel B, TEM of membrana granulosa. Scale bar: 5 μm (panel A); 1 μm (panel B).

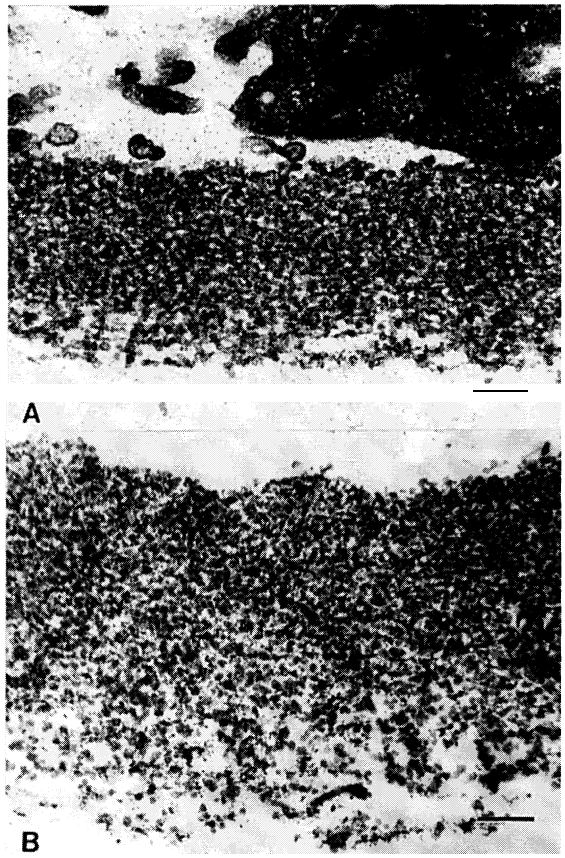

Fig. 2. Transmission electron micrographs of intact basal lamina of chicken ovarian follicle before (panel A) and after (panel B) isolation in hypotonic sol ution. Scale bar: 0.25 μm in both panels.

3. Results

3.1. Electron microscopy

Scanning and transmi ssion electron micrographs of cross-section of granulosal layer (membrana granulosa) of preovulatory chicken follicle (35 mm in diameter) are shown in Fig. 1. The single layer of compressed cuboidal granulosa cells located between the perivitelline layer and basal lamina are separated by intercellular spaces, however, the cells are connected by cellular projections or cytoplasmic extensions. Microvilli-like extensions could also be observed at the apical portions of some of the granulosa cells (Fig. 1A,B). Fig. 2A shows the structure of the intact basal lamina of a mature chicken ovarian follicle in vivo. The basal lamina appears to consist of a network of granular material with irregular spaces (Fig. 2A). Similar network of granular material

with irregular spaces has been reported for basement membranes in other tissues (e.g. see Inoue and Leblond (1988)). Fig. 2B shows the transmission electron micrograph of an intact basal lamina of a mature chicken ovarian follicle following its isolation in hypotonic solution. The structure of basal lamina appeared to be unaltered following isolation in hypotonic solution (compare Fig. 2A with B).

3.2. *Effect of intact basal lamina on the morphology of granulosa cells*

The effect of intact basal lamina (BLAOF) on cell morphology was assessed. Within 60 min of incubation in serum-free medium, freshly isolated chicken granulosa cells (differentiated and undifferentiated) became flat on plastic. In contrast, granulosa cells incubated on BLAOF were less flattened (not shown). The effect of BLAOF on granulosa cell morphology became more dramatic when they were incubated for longer periods of time (see Fig. 3 for an example from a 24-h culture). Under these conditions, the granulosa cells incubated on intact basal lamina (prepared a few hours earlier) had a morphology that approximated the shape of in situ granulosa cells in intact membrana granulosa (compare Fig. 3B with Fig. 1A).

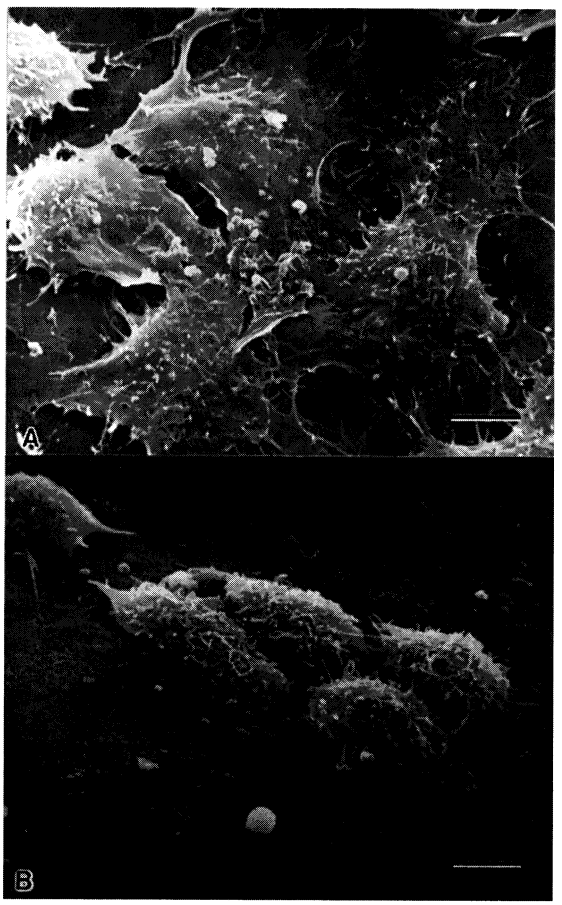

Fig. 3. Scanning electron micrographs of granulosa cells incubated on intact basal lamina in vitro. Differentiated granulosa cells isolated from the largest (F_1) preovulatory chicken ovarian follicle were incubated in serum-free medium 199 (M199) for 24 h on plastic (panel A) or on intact basal lamina that was isolated a few hours earlier (panel B). Scale bar: 5 μm in both panels.

In order to determine the effect of storage on the ability of BLAOF to influence cell shape, BLAOF-containing dishes were stored at 4°C for various periods of time. When granulosa cells were incubated in BLAOF-coated dishes stored for 12-24 months, the basal lamina caused the cells to assume spherical shapes similar to observations made for granulosa cells which were incubated on freshly prepared BLAOF (Fig. 4). Intact basal lamina prepared three months earlier caused granulosa cells to become rounded (Fig. 4B,C). It is noteworthy that both 'granulosa-side' (Fig. 4B) and 'theta-side' (Fig. 4C) of basal lamina influenced cell shapes. This observation indicated that both sides of the isolated basal lamina could influence cell shape in vitro. Photomicrographs of cells cultured on dehydrated BLAOF stored at 4°C for 18 months are shown in Fig. 4D (see also Fig. 4H).

The effects of basal lamina on granulosa cell shape appeared to be influenced by cell density. The effect of BLAOF on cell shape under low-density condition is illustrated in Fig. 4A–D. At high densities, the granulosa cells tended to form clusters on intact basal lamina (compare Fig. 4E with F). It is interesting to note that the granulosa cells which attached to plastic a few millimeters (3-5 mm) away from BLAOF (in BLAOF-containing dishes) were much less flat than cells in the control dishes (compare Fig. 4G,H with Fig. 4A,E).

The morphometric parameters of granulosa cells cultured on BLAOF, on plastic in BLAOF-containing dishes and on plastic of control wells are shown in Fig. 5. The cells were cultured on either the 'granulosa-side' (Fig. 5A,C,E) or on 'theta-side' of the basal lamina (Fig. 5B,D,F). As noted above, this was an investigation of whether both sides of the isolated basal lamina could

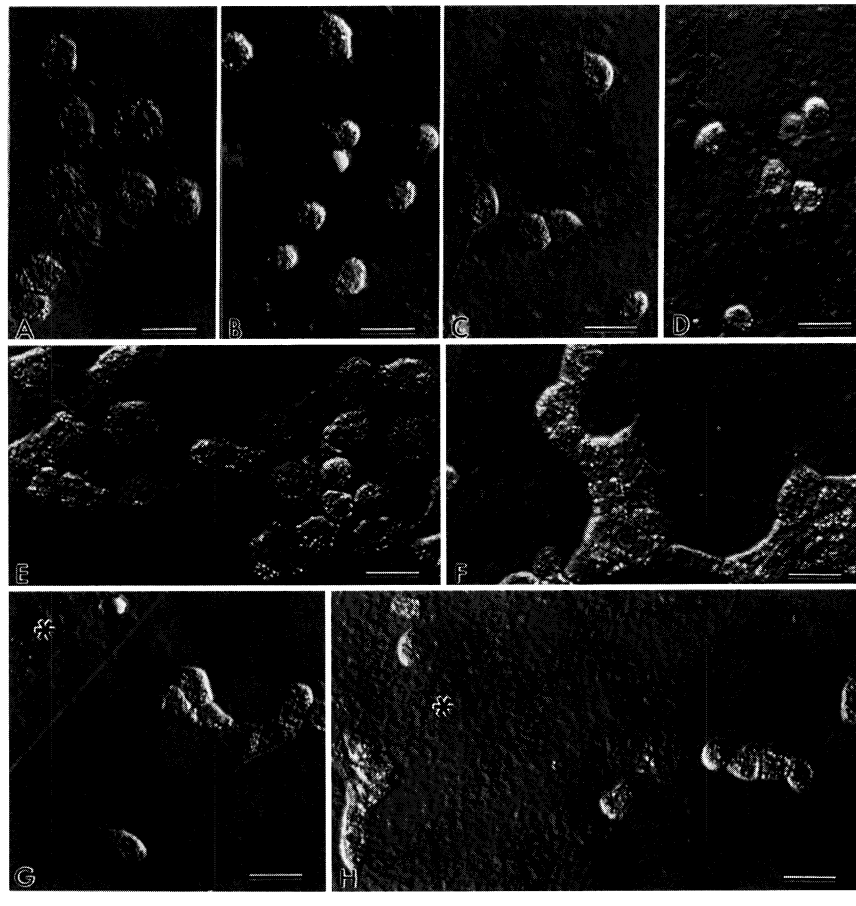

Fig. 4. Hoffman modulation contrast photomicrographs of granulosa cells incubated on intact basal lamina of avian ovarian follicle (BLAOF) at low and high densities. Differentiated granulosa cells isolated from the largest (F,) preovulatory chicken ovarian follicle were incubated in serum-free medium 199 (M 199) for 15 h at low density, 0.2 x 10^5 cells/ml (panels A-D). Panel A, cells incubated on plastic (control). Panel B, cells incubated on 'granulosa-side' of basal lamina prepared 3 months earlier. Panel C, cells incubated on 'theta-side' of basal lamina prepared 3 months earlier. Panel D, cells incubated on basal lamina prepared eighteen months earlier. Granulosa cells incubated at high density, 0.75 x 10^5 cells/ml on plastic (control, panel E) and on intact basal lamina prepared three months earlier (panel F). Granulosa cells incubated on plastic close to intact basal lamina prepared three months earlier (panel G) or on plastic close to basal lamina prepared 18 months earlier (panel H). Basal lamina in panels G and F are labeled with star (*). Scale bar: 20 μm.

influence granulosa cell shape. Independent of the side of BLAOF, the mean area occupied by cells incubated directly on the basal lamina or close to it was less than that of cells incubated in control dishes (Fig. 5A,B). Similarly, the morphometric parameters of perimeter (Fig. 5C,D) and circularity (Fig. 5E,F) were less for cells that were incubated directly on BLAOF or close to BLAOF. Fig. 6 shows morphologic parameters of granulosa cells incubated in BLAOF-containing dishes that had been stored for 18 months. The influence of 18-month-old BLAOF preparation on cell shape was not significantly different from that observed for BLAOF preparations stored for shorter duration of time (compare Fig. 6A–C with Fig. 5A,C,E). These data lend credence to the observation that the storage of dehydrated BLAOF had no apparent effect on its ability to influence granulosa cell shape.

3.3. Effect of solubilized basal lamina on the morphology of granulosa cells

The effect of solubilized basal lamina on granulosa cell morphology was assessed. Different amounts of fraction 1 of solubilized basal lamina were dried in the bottom of culture wells prior (pre-coated wells; solid-form) to the incubation of granulosa cells or were added as liquid (liquid-form) to the incubation mixture. Culture wells

that did not contain solubilized basal lamina served as controls. Granulosa cells were flat in control wells (Fig. 7A). In contrast, granulosa cells incubated in wells in which fraction 1 had been dried (30 $\mu g/cm^2$) were less flattened (Fig. 7B).

The morphometric parameters of differentiated (F_1), differentiating (F_3) and undifferentiated (SYF) granulosa cells incubated for 6 hr in wells containing dried fraction 1 are shown in Fig. 8. The solid-form of fraction 1 reduced the mean area occupied by granulosa cells obtained at all

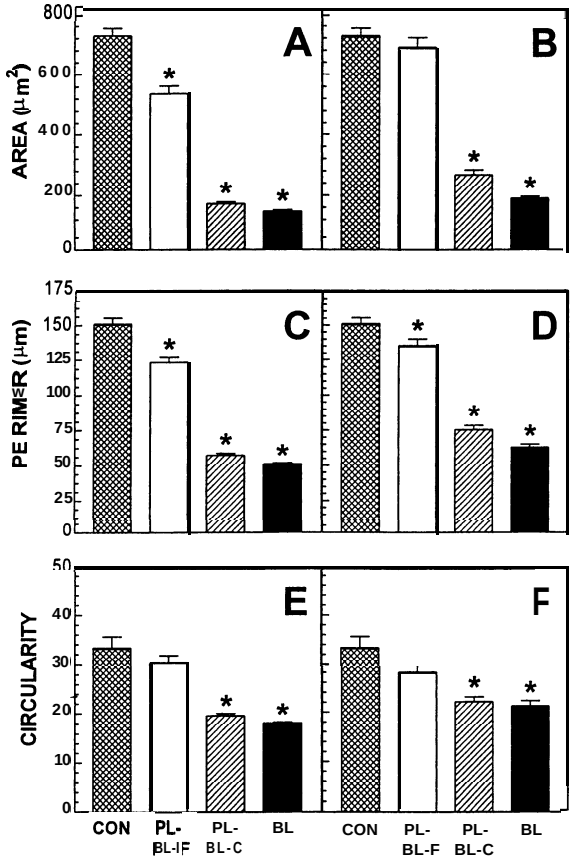

Fig. 5. Morphometric parameters of granulosa cells incubated on intact basal lamina of avian ovarian follicle (BLAOF) stored for 3 months. The cells were incubated on the 'granulosa side' (left panels) or 'theca side' (right panels) of intact basal lamina. The morphometric parameters were area occupied (panels A and B), perimeter (panels C and D) and circularity (panels E and F). Differentiated granulosa cells isolated from the largest (F,) preovulatory chicken ovarian follicle were incubated in serum free medium 199 (M 199) for 15 h on plastic in control dishes (CON), on intact basal lamina (BL), on plastic close to basal lamina (PL-BL-C) or on plastic far from basal lamina (PL-BL-F). Data are mean ± S.E.M. of 50 or more cells. * $P < 0.05$ vs. control (CON).

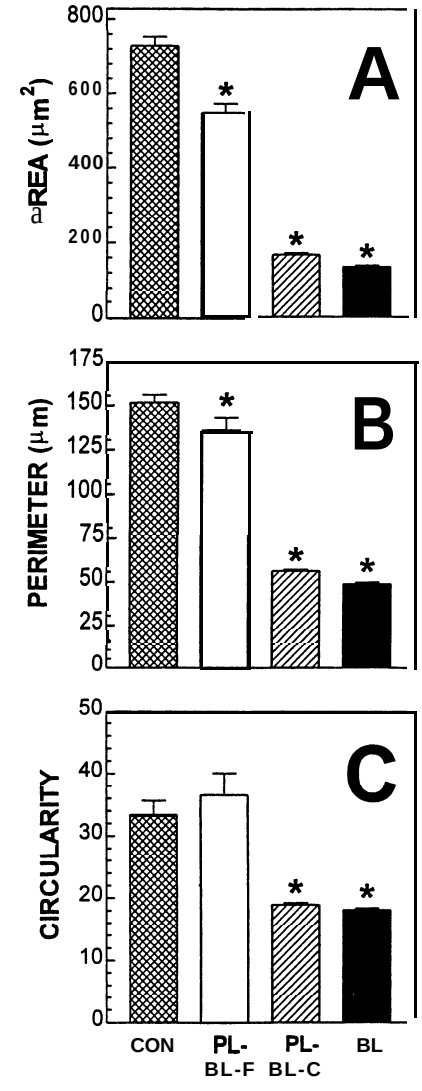

Fig. 6. Morphometric parameters of granulosa cells incubated on intact basal lamina of avian ovarian follicle (BLAOF) stored for 18 months. The morphometric parameters were area occupied (panel A), perimeter (panel B) and circularity (panel C). Differentiated granulosa cells isolated from the largest (F,) preovulatory chicken ovarian follicle were incubated in serum free medium 199 (Ml 99) for 15 h on plastic in control dishes (CON), on intact basal lamina (BL), on plastic close to basal lamina (PL-BL-C) or on plastic far from basal lamina (PL-BL-F). Data are mean ± S.E.M. of 50 or more cells. * $P < 0.05$ vs. control (CON).

stages of differentiation (Fig. 8A–C). Similarly, the perimeter of cells incubated in fraction 1 pre-coated wells was less than that of cells incubated on plastic (Fig. 8D–F). Moreover, the cells incubated on plastic were more irregular than those incubated on fraction 1 (Fig. 8G–I). As such, in wells pre-coated with fraction 1 of solubi-

lized basal lamina, granulosa cells assumed a morphology that approximated the shape of chicken granulosa cells in vivo (in intact membrana granulosa). Granulosa cells incubated on solid-form of fraction 1 formed clusters (data not shown) similar to what has been observed for intact basal lamina (BLAOF). To assess the influence of storage on the effect of solubilized basal lamina on granulosa cell shape, fraction 1 was dried in wells and kept at 4°C. Granulosa cells incubated in fraction l-containing wells that had been stored at 4°C for 12 or more months became rounded similar to observations made for granulosa cells incubated in freshly prepared fraction 1 -containing dishes (results not shown).

The observation that granulosa cells that attached to plastic in close proximity to intact basal lamina (BLAOF) were less flattened than cells in control wells (Figs. 5 and 6) suggested that BLAOF is the source of soluble morphogenic factor(s). Therefore, additional experiments were conducted to examine the influence of fraction 1 added as liquid on cell shape. When added as liquid to the incubation mixture, the effect of fraction 1 fluidized basal lamina on granulosa cell shape was similar to that observed for cells incubated in wells that were pre-coated with the matrix material (results not shown). Fig. 9 shows the morphometric parameters of granulosa cells incubated in wells to which fraction 1 was added as liquid. The mean area occupied by differentiated (F_1), differentiating (F_3) or undifferentiated (SYF), cells was less in wells to which fraction 1 was added (Fig. 9A–C). Likewise, the perimeter (Fig. 9D–F) and circularity (Fig. 9G–I) of granulosa cells incubated in wells to which fraction 1 was added were less than those of cells in control wells. It was noted that the cells were covered by fluidized basal lamina especially in the wells that contained 50 μg/ml or greater matrix material.

In experiments in with liquid-form of basal lamina, 50 μg/ml of protein caused a significant change in granulosa cell shape. The addition of 50 μg/ml of solubilized basal lamina represents only 5% increase in the concentration of total protein in the incubation medium. When experiments were conducted in 0.2% BSA containing medium (representing 100% increase in total protein) the cells remained flattened (data not shown).

4. Discussion

The present results demonstrate that pure and intact basal lamina of avian ovarian follicle (BLAOF) has a dramatic effect on the morphology of granulosa cells. The influence of BLAOF on cell shape was evident within l-2 h after plating. Granulosa cells that attached to plastic in control dishes were more flattened than cells that attached to plastic in BLAOF-containing culture dishes. Although the reason(s) for this observation is unknown, BLAOF may have released certain substances that influenced cell shape. This intriguing result was observed for all BLAOF preparations irrespective of duration of their storage. Interestingly, the storage of BLAOF-containing dishes at 4°C for 2 years or longer did not

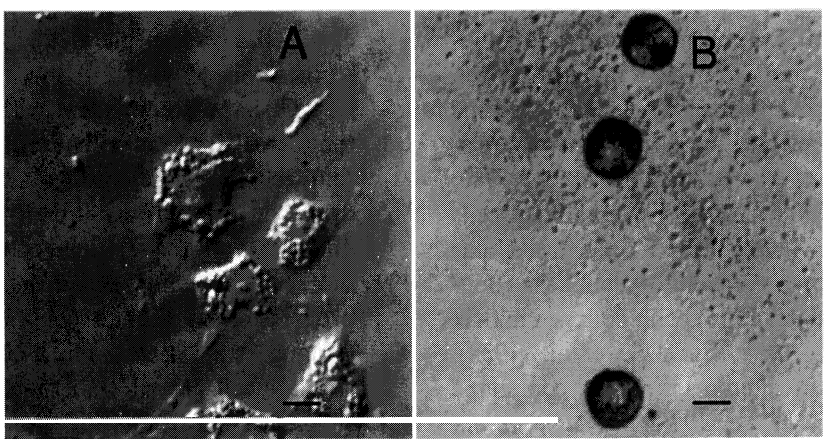

Fig. 7. Differential interference contrast photomicrographs of granulosa cells incubated on uncoated plastic (panel A) and in wells precoated with fraction 1 (30 μg/cm^2) of solubilized basal lamina (panel B) in serum-free medium 199 (M 199) for 15 h. Scale bar: 10 μm.

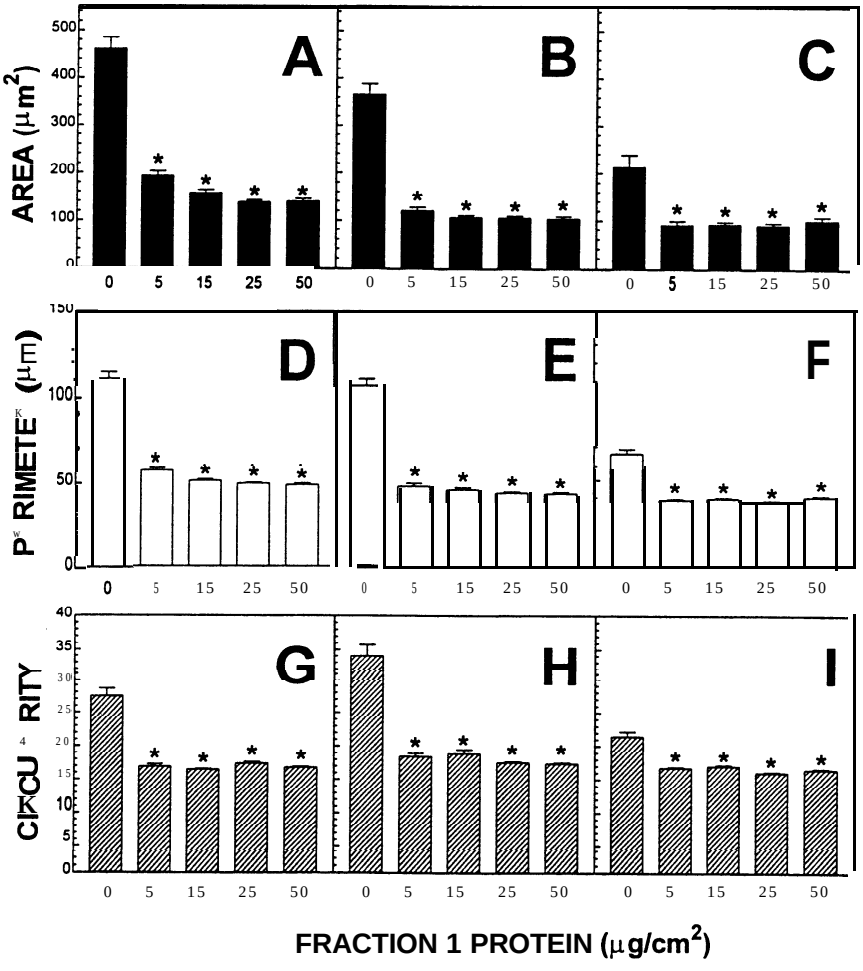

Fig. 8. Morphometric parameters of granulosa cells incubated in wells pre-coated with fraction 1 of solubilized basal lamina. The morphometric parameters were area occupied (top panels), perimeter (middle panels) and circularity (bottom panels). Granulosa cells isolated from mature (F, ; panels A, D, G), developing (F,; panels B, E, H), immature (SYF; panels C, F, I) chicken ovarian follicles were incubated in serum-free medium 199 (M199) for 6 h on plastic or in wells pre-coated with different quantities of fraction 1 protein (5-50 μg/cm^2). Each point is mean +_ S.E.M. of 40 or more cells. * $P < 0.05$ vs. no fraction 1 protein (0 μg/cm^2).

affect the ability of the matrix material to influence the shape of granulosa cells in vitro. When grown on plastic, granulosa cells spread, became flat and were connected with cytoplasmic processes (Lawrence et al., 1979; Soto et al., 1986; Carnegie et al., 1988). However, granulosa cells became rounded when cultured on extracellular matrix produced by bovine endothelial cells (Ben-Ze'ev and Amsterdam, 1986) or in collagen matrix (Ben-Rafael et al., 1988; Carnegie et al., 1988). Gonadotropins also altered granulosa cell shape in vitro (Lawrence et al., 1979; Carnegie et al., 1988). Both gonadotropin- and collagen matrix-induced alteration in granulosa cell shape were accompanied by disruption of cytoskeleton (Soto et al., 1986; Carnegie et al., 1987, 1988). Future studies will determine if the influence of BLAOF on cell shape is coincident with the dismantling of the cytoskeleton. Recent studies have revealed that cell shape determines whether capillary endothelial cells from human and bovine origin undergo apoptosis (Chen et al., 1997). In addition, cell shape modulated the control of cell cycle progression in human capillary endothelial cells (Huang et al., 1998). Thus, cell shape per se can regulate cell growth and function.

The present results also show that the structural integrity of the basal lamina isolated from the chicken ovarian follicle is similar to the structure of this matrix material in situ. The current obser-

vations confirm an earlier report of Perry et al. (1978) that the structure of avian ovarian basal lamina is granular in nature. The structure of the avian ovarian basal lamina is similar to that described for the lamina densa layer of basement membranes of rat seminiferous tubule, vas deferens, epidermis, trachea, jejunum; monkey seminiferous tubule, mouse lens capsule, rat Reichert's membrane (Inoue and Leblond, 1988) and rat ovarian basal lamina (Bagavandoss et al., 1983).

BLAOF is easy and quick to isolate and can be used in biomedical research especially in experiments designed to assess the effects of basement membranes exactly in the form in which they exist in situ. The present observations support the view that BLAOF can cause cells to adopt morphology that is distinctly different from that expressed when cultured on plastic. Importantly, BLAOF can induce normal (in situ) morphological appearance in certain cell types; therefore, cells maintained on BLAOF can serve as a model system for in vitro studies.

The ability of the basal lamina to influence cell shape was not affected by solubilization as fraction 1 also caused granulosa cells to become rounded. It is noteworthy that, the storage of dried solubilized basal lamina (fraction 1) containing dishes at 4°C for longer that 12 months

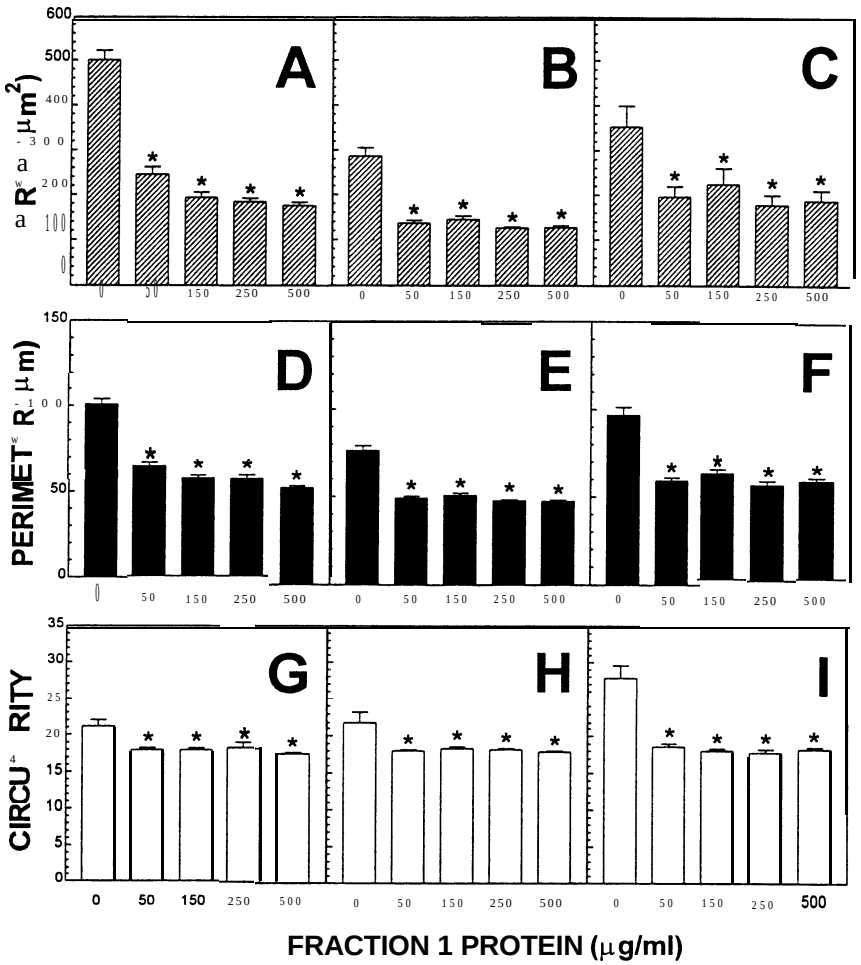

Fig. 9. Morphometric parameters of granulosa cells incubated in wells to which fraction 1 of solubilized basal lamina was added as liquid. The morphometric parameters were area occupied (top panels), perimeter (middle panels) and circularity (bottom panels). Granulosa cells isolated from mature (F_1; panels A, D, G), developing (F_3; panels B, E, H), immature (SYF; panels C, F, I) chicken ovarian follicles were incubated in serum free medium 199 (M 199) for 15 h in the absence or presence different quantities of fraction 1 protein (50-500 μg/ml) which was added as liquid. Each point is mean ± S.E.M. of 29 or more cells. * $P < 0.05$ vs. no fraction 1 protein (0 μg/ml).

had no apparent effect on its ability to cause shape changes in granulosa cells. In addition to its influence on cell shape, solubilized basal lamina regulated the function of granulosa cells as well; fraction 1 stimulated progesterone production by chicken granulosa cells dose dependently (Asem et al., 2000). The effect of fraction 1 on progesterone synthesis was influenced by both the state of granulosa cell differentiation and the form of the matrix material, whether solid or liquid (Asem et al., 2000). These results indicate that the effect of basal lamina on granulosa cell shape is associated with the regulation of the function of these cells.

In summary, the structure of pure and intact basal lamina isolated from hen ovarian follicle is similar to that observed for basal lamina in vivo. The homologous basal lamina influenced the shape of cultured granulosa cells; the cell type with which it is associated in situ. Both the intact basal lamina and its solubilized form can be stored, dehydrated, for l-2 years without losing their ability to influence cell shape. The effects of fraction 1 of fluidized basal lamina on the morphology of granulosa cells were neither influenced by the state of cell differentiation nor by its form (solid or liquid). One advantage of the utility of intact basal lamina such as BLAOF is that it could provide data pertinent to the in situ behavior (responses). Moreover, it can be used for the culture of cells in experiments designed to examine the influence of the basement membrane microenvironment on cell structure and function.

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