Adhesion Proteins Increase Cellular Attachment, Follicle-Stimulating Hormone Receptors, and Progesterone Production in Cultured Porcine Granulosa Cells (43994)

C. K. SITES,*[1] B. KESSEL,*[2] AND A. R. LABARBERA†[3]

Department of Obstetrics and Gynecology, and Departments of Obstetrics and Gynecology, and Molecular and Cellular Physiology,† University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0526*

Abstract. **We sought to determine the influence of different constituents of the extracellular matrix on porcine granulosa cell function by assessing cellular attachment, cellular morphology, follicle-stimulating hormone (FSH) receptors, and progesterone production. Cells from immature porcine ovarian follicles were cultured for up to 6 days in serum-free medium containing porcine FSH (pFSH, 10 ng/ml) in culture dishes either uncoated or coated with one of the following adhesion proteins: gelatin (1 mg/cm^2), fibronectin (1 μg/cm^2), laminin (1 μg/cm^2), type I collagen (10 μg/cm^2), or type IV collagen (7.8 μg/cm^2). Fibronectin, laminin, type I collagen, and type IV collagen increased cellular attachment significantly ($P < 0.05$). All adhesion proteins except gelatin influenced cellular morphology. Cells cultured on laminin or type IV collagen formed dense clusters of rounded cells. Cells cultured in dishes coated with each adhesion protein except gelatin had higher ^{125}I-pFSH binding per cell than cells cultured in uncoated dishes, with increases of 7- to 12-fold over control ($P < 0.05$). All adhesion proteins increased progesterone production, ranging from 10- to 50-fold over control ($P < 0.05$). In summary, not only did adhesion proteins increase attachment to the dishes but they also increased FSH receptors and differentiated function (progesterone production) of granulosa cells from immature porcine ovarian follicles.**

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Adhesive interactions between cells and the surrounding extracellular matrix play a critical role in cellular physiology and gene expression. Many of the interactions between cells and the extracellular matrix are mediated by integrins, a family of cell-surface receptors (1). This receptor family is composed of 14 distinct α subunits and eight β subunits which associate in various combinations in many cell types to bind extracellular matrix proteins such as laminin, fibronectin, and collagen. Unlike the "single ligand–single receptor" concept believed important for hormone-receptor activation, an integrin receptor may recognize several extracellular matrix proteins, or several integrins may interact with a certain extracellular matrix protein (1). This flexibility in integrin receptor activation may allow for variability in gene expression by the cell.

Ovarian follicular granulosa cells as well as their male analog, testicular Sertoli cells, rest on a basement membrane, *in vivo*, which contains type IV collagen, laminin, and fibronectin (2, 3). Studies of rat follicles *in vivo* indicate that proximity to the extracellular matrix influences granulosa cell differentiation. The most differentiated cells, which contain the highest density of luteinizing hormone (LH) receptors, cytochrome

[1] Present address: Department of Obstetrics and Gynecology, University of Vermont, Given Building C254, Burlington, VT 05405.
[2] Present address: Department of Obstetrics, Gynecology, and Reproductive Biology, Harvard Medical School, Beth Israel Hospital, Dana 881, 330 Brookline Avenue, Boston, MA 02215.
[3] To whom requests for reprints should be addressed at University of Cincinnati College of Medicine, P.O. Box 670526, Cincinatti, OH 45267-0526.

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P-450 and 3β-hydroxysteroid dehydrogenase activity, are found closest to the basement membrane (4, 5).

Previous investigators have examined dependence on progesterone production, LH receptors, and human chorionic gonadotropin (hCG)-responsive adenylyl cyclase activity of rat and human granulosa cells on particular adhesion proteins (6–10). The goal of this study was to compare the abilities of five different adhesion proteins to promote cellular attachment to culture dishes and to increase follicle-stimulating hormone (FSH) receptors in immature granulosa cells of the pig. Progesterone production was assessed as an index of differentiated function.

Materials and Methods

Cell Culture. Ovaries of prepubertal pigs were obtained at a local abbatoir within 20 min of slaughter. Granulosa cells were aspirated aseptically from immature (1- to 3-mm diameter) follicles and washed twice in medium containing Ham's F10:Dulbecco's modified Eagle's medium (1:1; Gibco, Grand Island, NY) containing penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (250 ng/ml), mycostatin (100 U/ml), and gentamicin (50 μg/ml, all from Gibco). Cells were plated at a density of 15×10^6 cells (75 μg DNA) per 35-mm diameter wells in 6-well plates (Nunclon, Scientific Supply Company, Schiller Park, IL) and cultured for up to 6 days in medium containing the above antibiotics plus bovine serum albumin (BSA, 0.4% [w/v], Sigma Chemical Co., St. Louis, MO), low-density lipoprotein cholesterol (10 μg/ml, Calbiochem, LaJolla, CA), thrombin (1 U/ml, Sigma), bovine insulin (1 μg/ml, Gibco), and purified porcine FSH (10 ng/ml), prepared and characterized previously (11). This serum-free medium was similar to that described by Buck and Schomberg (12) and has been described previously (13). Medium was changed every 2 days and stored at −20°C for progesterone radioimmunoassay. Cells were scraped from wells in phosphate-buffered saline with a rubber policeman and stored at −70°C for radioreceptor assay and DNA assay.

Preparation of Adhesion Proteins. Wells were coated with one of the following adhesion proteins: fibronectin (1 μg/cm²) (14, 15), laminin (1 μg/cm²) (14, 16), type I collagen (10 μg/cm²) (14), or type IV collagen (7.8 μg/cm², all from Sigma) (16), or gelatin (1 mg/cm²) (17). Each adhesion protein was diluted in medium and added to wells in 1 ml. Fibronectin-coated wells were placed in a humidified 5% CO_2 incubator at 37°C for 2 hr. Wells containing gelatin, laminin, type I collagen, or type IV collagen were placed in a tissue culture hood for 4 hr. All solutions were aspirated before cells and culture media were added.

Radioreceptor Assay. All cells, including freshly harvested cells and cells cultured for increasing lengths of time, were treated with mild acid prior to radioreceptor assay to remove any surface-bound FSH (13). Briefly, cells were incubated for 45 sec in 500 μl minimal essential medium (MEM, Gibco) without $NaHCO_3$ adjusted to pH 3.5 with acetic acid and containing 0.1% bovine serum albumin (BSA). The reaction was terminated by addition of 2 ml of phosphate-buffered saline (PBS) (0.14 *M* NaCl, 0.01 *M* sodium phosphate buffer, pH 7.4). Cells were centrifuged, and the pellet was saved for binding assay. This treatment removed 86% of bound FSH with retention of 89% of the original binding capacity.

Purified pFSH (11) was iodinated with ^{125}I by a modified chloramine-T method (18). Specific radioactivity, calculated according to the method of Greenwood *et al.* (19), was 12.2 μCi/μg. Cells were incubated in 500 μl assay buffer (0.15 *M* NaCl, 10 m*M* Tris, pH 7.4, 0.1% BSA) for 14–16 hr at 22°C with ^{125}I-pFSH (5 ng/ml) in triplicate. Pregnant mare's serum gonadotropin (PMSG, Sigma) was added to parallel tubes in duplicate at a 2000-fold excess by mass to estimate nonspecific binding. Incubations were terminated with the addition of 2.5 ml ice-cold assay buffer followed by centrifugation (1500*g* for 15 min) to pellet the cells. Bound ^{125}I-pFSH was measured by gamma scintillation spectrometry. Nonspecific binding was subtracted from total binding to yield specific binding. We demonstrated previously that changes in binding assessed in this manner reflect changes in receptor number (13).

Radioimmunoassay. Progesterone in culture medium was measured by radioimmunoassay using a specific progesterone antibody provided by Dr. Robert Chatterton (20). Interassay and intraassay coefficients of variation were less than 10%.

DNA Quantitation. A fluorometric assay using calf thymus DNA (Sigma) as standard was used to quantitate cellular DNA (21).

Data Analysis. Treatment time courses were compared by multivariate analysis (22). Values for progesterone were logarithmically transformed prior to analysis. Means at individual time points were then compared by univariate analysis using Duncan's multiple range test. An α level of $P < 0.05$ was accepted as significant.

Results

Granulosa cells attached to wells within 24 hr after plating in the absence or presence of adhesion proteins. The degree of attachment was dependent upon the adhesion protein used to coat the culture dishes prior to plating (Fig. 1). By Day 2, the number of cells attached, as reflected by DNA, declined in culture by approximately 60%–70% with all adhesion proteins except gelatin, where cell attachment declined by 93%,

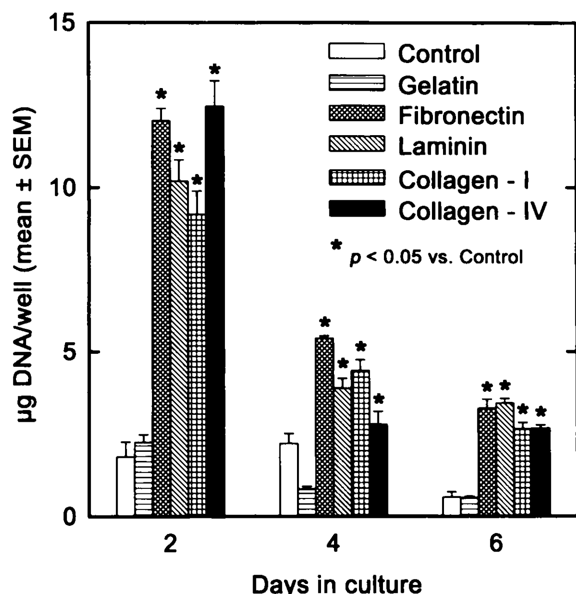

Figure 1. Effect of adhesion proteins on cellular attachment. Cells were cultured for up to 6 days with pFSH (10 ng/ml) in the absence or presence of one of the five adhesion proteins (see text for concentrations). The medium was changed every 2 days. Data are expressed as μg DNA/well and are graphed versus days in culture. Each vertical bar represents the mean and the vertical line the SEM of quintuplicate determinations in a representative of three experiments. *Significant differences from control values ($P < 0.05$).

similarly to control. The adhesion proteins, fibronectin, laminin, type I collagen, and type IV collagen, maintained significantly greater cell attachment at Day 2, 4, and 6 of culture. Gelatin did not significantly enhance cellular attachment to culture dishes compared with control. At Day 2, type IV collagen and fibronectin increased attachment 6.9- and 6.7-fold, respectively, compared with control. By Day 6, attachment was greatest on laminin- and fibronectin-coated dishes, 6.1- and 5.8-fold of control, respectively.

Adhesion proteins caused pronounced differences in morphologic appearance as observed by light microscopy (Fig. 2). These differences were most evident at Day 4 and 6 of culture. Control cells and cells on gelatin typically appeared as isolated, single cells, spread sparsely on the dishes (Fig. 2 A and B). Cells cultured on either fibronectin (Fig. 2C) or type I collagen (Fig. 2E) spread on the dishes and had a flattened, spindle-shaped appearance; some small clusters of rounded cells were evident. In contrast, cells cultured on laminin (Fig. 2D) or type IV collagen (Fig. 2F) formed numerous dense clusters of rounded cells on areas of flattened cells.

All adhesion proteins tested significantly increased FSH binding, expressed on a per microgram DNA basis (Fig. 3). The decrease in FSH binding with fibronectin, laminin, type I collagen and type IV collagen observed on Day 2 of culture was not significant. By Day 4, the increases in FSH binding compared with control ranged from 7.3-fold for gelatin to 12.9-fold for type IV collagen. FSH binding declined between Day 4 and 6 of culture. However, at Day 6 FSH binding in dishes coated with fibronectin, laminin, type I collagen, and type IV collagen remained greater than control; the difference between gelatin and control was not significant. The difference between type I collagen and control increased to 20.1-fold because FSH binding in control cultures declined to a greater extent than in the type I collagen cultures.

Progesterone production, expressed on a per cell basis, in cultures with gelatin, fibronectin, laminin, type I collagen, and type IV collagen was significantly lower compared with control cultures at Day 2 (Fig. 4) However, subsequent progesterone production was enhanced by each of the adhesion proteins. At Day 4 of culture, enhancement of progesterone production ranged from 3.0-fold for fibronectin to 7.0-fold for laminin. At Day 6 of culture, enhancement of progesterone production ranged from 6.7-fold for gelatin to 57.8-fold for type I collagen; gelatin was significantly less effective than the other adhesion proteins tested.

Discussion

We have demonstrated that the adhesion proteins, gelatin, fibronectin, laminin, type I collagen, and type IV collagen all increase FSH receptors *in vitro*. Binding is maximal by Day 4 of culture and declines slightly by Day 6, perhaps reflecting FSH receptor downregulation (13). The decrease in FSH binding in porcine granulosa cells cultured with FSH is consistent with the progressive decrease in FSH receptors in growing porcine follicles (23). Thus, under these *in vitro* conditions, the response to FSH parallels the *in vivo* response to FSH in porcine granulosa cells.

Others utilizing culture systems of rat or human granulosa cells pretreated with hormones *in vivo* have shown that the extracellular matrix influences LH/hCG receptors. Granulosa cell LH receptors, also critical for development and coordination of oogenesis, increase in culture when either bovine corneal extracellular matrix (6, 8) or fibronectin (12) is employed as the attachment substrate. The increase in LH receptors corresponds to increased responsiveness to cAMP (6).

We have shown that fibronectin, laminin, type I collagen, and type IV collagen significantly enhance cell attachment compared with either no adhesion protein or gelatin for culture up to 6 days. The initial decline in cell number despite progressive differentiation and viability of the cell population may be caused by the selection by the adhesion protein of a subpopulation of granulosa cells that is most steroidogenically active. To our knowledge no prior study has examined granulosa cell number as measured by cellular DNA as a function of various adhesion proteins. Buck and

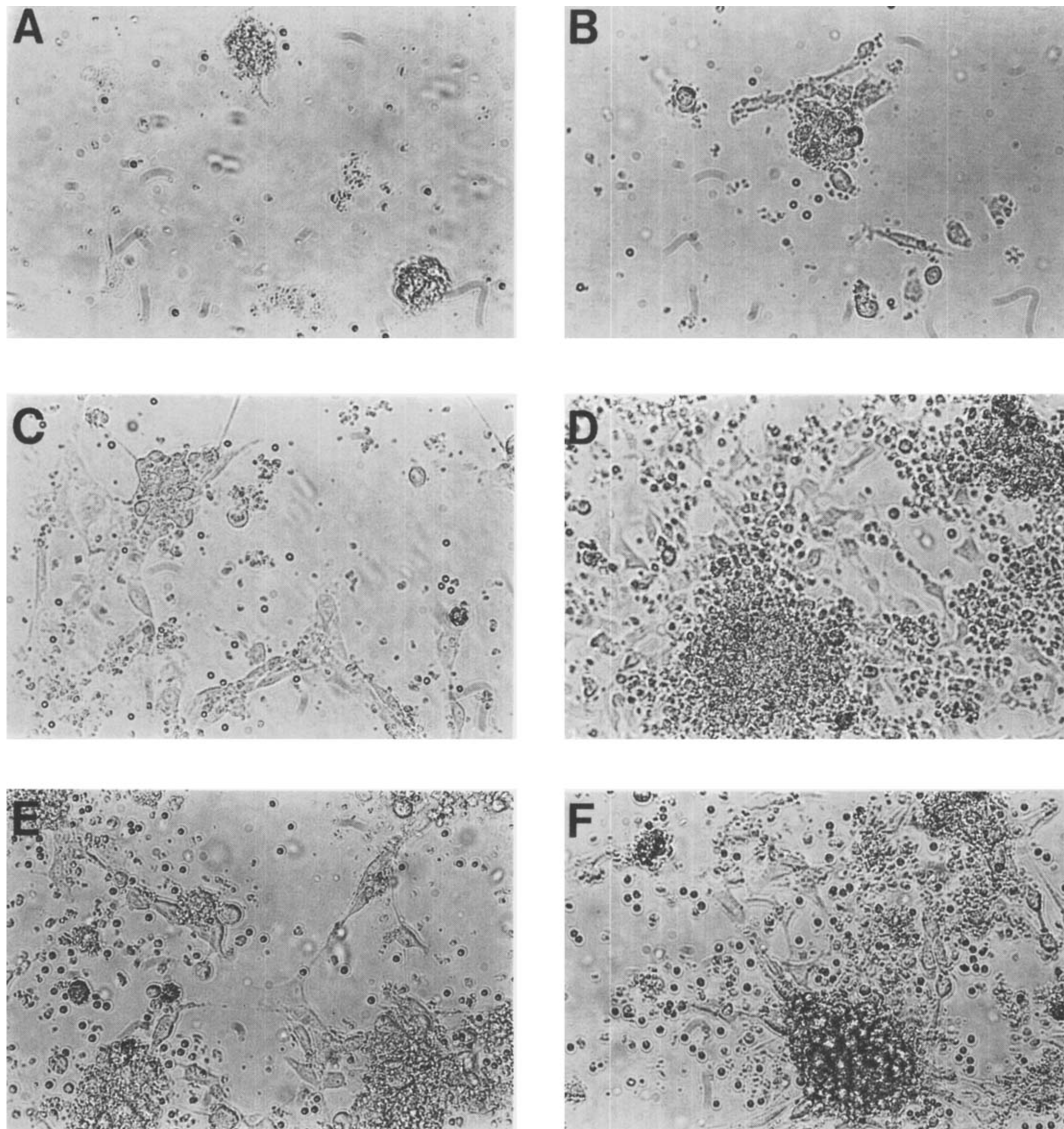

Figure 2. Phase contrast microscopy of cells cultured on either uncoated dishes or dishes coated with one of the following adhesion proteins: control (uncoated plastic) (A); gelatin (B); fibronectin (C); laminin (D), type I collagen (E), type IV collagen (F). Photomicrographs represent typical morphology observed on day 6 of culture in a representative of three experiments. Magnification: ×600.

Schomberg demonstrated an increase in cellular protein in porcine granulosa cells cultured on fibronectin (12), although it was not clear whether the quantitation of cellular protein was influenced by the fibronectin, a glycoprotein, used to coat the plates. Aten *et al.* (24) recently demonstrated that laminin and Matrigel enhance progestin production in mature rat granulosa cells from preovulatory follicles. They further demonstrated that addition of integrin receptor antibody to the culture medium inhibited progestin production (24).

It is not known how granulosa cells attach to adhesion proteins. Other cell types, including fibrosarcomas, breast carcinomas, muscle cells, melanomas, neuroblastomas, and Chinese hamster ovary cell lines, possess integrins, receptors for laminin (25). Also, Sertoli cells in the testis, which possess FSH receptors, laminin receptors that have been proposed to promote formation of testis cords and subsequent differentiation into seminiferous tubules during embryogenesis (3, 26). Testicular Leydig cells similarly attach to type IV collagen, fibronectin, or laminin, and form spherical shapes (27). An antibody directed against a peptide which competes with laminin for laminin receptors (YIGSR) on mouse testicular cells blocks attachment and aggregation of those cells, further supporting the importance of integrins for cellular attachment and physiologic cell clustering in gonadal tissue (28).

Adhesion proteins have been shown to influence hormone production in several circumstances. Our studies with fibronectin, laminin, gelatin, type I collagen, and type IV collagen agree with previous studies in which an increase in progesterone production by granulosa cells cultured on collagen or bovine corneal

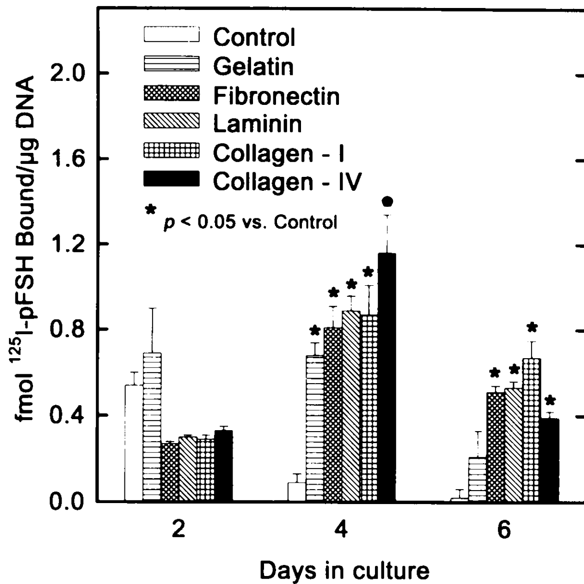

Figure 3. Effect of adhesion proteins on ^{125}I-pFSH binding. Cells were cultured for up to 6 days with pFSH (10 ng/ml) in the absence or presence of one of the five adhesion proteins. The medium was changed every 2 days. Surface-bound FSH was removed by treatment with mild acid and FSH binding was quantified by radioreceptor assay. Data are expressed as fmol ^{125}I-pFSH bound/μg DNA and are graphed versus days in culture. Each vertical bar represents the mean and the vertical line the SEM of triplicate determinations in a representative of three experiments. *Significant differences from control values ($P < 0.05$).

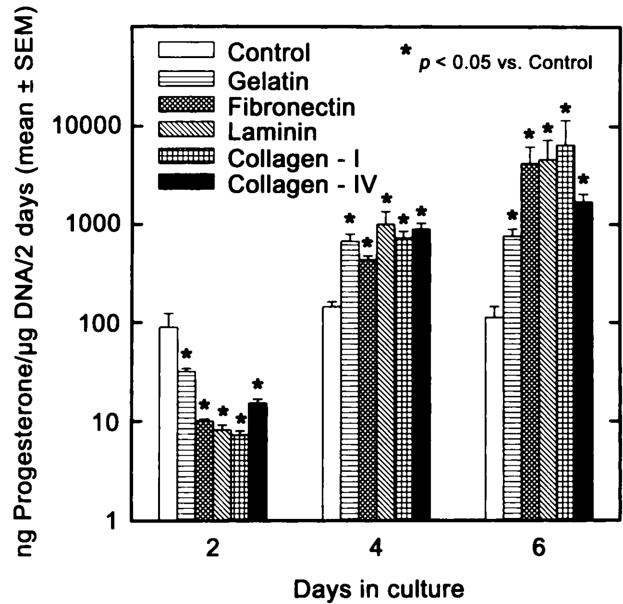

Figure 4. Effect of adhesion proteins on progesterone accumulation. Cells were cultured for up to 6 days with pFSH (10 ng/ml) in the absence or presence of one of the five adhesion proteins. The medium was changed every 2 days. Data are expressed as ng progesterone/μg DNA/2 days and are graphed on a logarithmic scale versus days in culture. Each vertical bar represents the mean and the vertical line the SEM of triplicate determinations in a representative of three experiments. *Significant differences from control values ($P < 0.05$).

extracellular matrix (6–10, 29) was observed. The substantially greater increase in progesterone per microgram DNA in the present studies compared with other studies (7–9) might be explained by the addition of LDL cholesterol as a substrate for progesterone biosynthesis, by a longer culture time in the present studies, or by both. Other glandular cells also are influenced by adhesion proteins. In cultured pituitary cells, FSH, LH, and prolactin secretion are increased by extracellular matrix (30). Mammary gland cells demonstrate increased milk protein secretion when maintained on extracellular matrix (31). Leydig cells in the testis maintain their ability to express 3β-hydroxysteroid dehydrogenase activity for longer periods of time on extracellular matrix (27). Thyroid follicles maintain their polarity when cultured on extracellular matrix (32).

Our observations of pronounced granulosa cell clustering and height with extended intercellular processes on laminin, fibronectin, type I collagen, or type IV collagen are similar to those of others who cultured with bovine corneal extracellular matrix (6–8, 33), collagen (9, 29) or fibronectin (12). In our experience, all attachment substrates are fairly easy to use and all except gelatin maintain clusters of cells which become fuller as steroidogenesis ensues. Laminin yielded optimal cell attachment and FSH binding along with its consistently small standard deviations between replicates. The microscopic appearance of granulosa cell clustering on adhesion proteins is similar to the description of Sertoli cell cord formation *in vitro* on laminin and entactin but not on plastic (26).

These experiments demonstrate that the structure and function of immature granulosa cells of the pig *in vitro* are influenced by the substrate on which they are cultured. Cell attachment, FSH receptor binding and steroidogenesis all are enhanced by the adhesion proteins fibronectin, laminin, type I collagen, and type IV collagen. These studies support the concept of the necessity for adhesion proteins for full expression of differentiated function in monolayer cultures.

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