NOTE

Reconstituted Basement Membrane Induces Glandular-like Morphogenesis but no Difference in ACTH Synthesis of Anterior Pituitary Cells

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Abstract. Basement membrane, a thin extracellular matrix, promotes tissue integrity and differentiated phenotype. This study was performed in order to investigate the effect of basement membrane components on adrenocorticotropin (ACTH) synthesis and to observe its relationship with cell morphology. Rat anterior pituitary cells were cultured on plastic culture plate coated with either Matrigel or poly-D-lysine. Phase-contrast microscopy and scanning electron microscopy showed that cells cultured on Matrigel appeared as a three-dimensional glandular-like cell aggregate, while those cultured on plastic showed a flat, confluent monolayer. Reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analysis revealed that ACTH synthesis in the Matrigel culture was not significantly different from that in the plastic culture. Our results suggest that the relationship between the morphological changes caused by cell-substrate interaction and pituitary hormone synthesis does not exist in all pituitary cell types and that other factors associated with cell-substrate interaction influence the hormone synthesis of some pituitary cells.

Key words: Anterior pituitary, Matrigel, ACTH, Morphology

(Endocrine Journal 47: 771–776, 2000)

IT has been shown that monolayer cell culture of anterior pituitary cells causes loss of characteristics which are expressed *in vivo*. For this reason, optimization of culture environment has been investigated in an attempt to maintain the differentiated phenotype of pituitary cells. The basement mem-

brane is a kind of extracellular matrix (ECM) which contains the essential elements of the epithelial cell environment and promotes the differentiation of a variety of cell types. In normal pituitaries, the basement membrane exists around the endocrine cell cords and plays an important role in initiating pituitary cytodifferentiation, in addition to its structural role [1]. Previous studies used components of ECM as the culture substrate of anterior pituitary cells and demonstrated an increase in prolactin secretion in cultures on collagen [2], and on ECM produced by bovine corneal endothelial cells [3]. Matrigel, which is a reconstituted basement membrane and known to

Received: February 21, 2000

Accepted: August 7, 2000

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be a better culture substrate for various epithelial cells, increased the secretion of PRL, LH and FSH from anterior pituitary cells [4]. However, the effect of ECM on the ACTH synthesis of anterior pituitary cells has not been reported.

This study used Matrigel as a culture substrate and compared the ACTH synthesis of anterior pituitary cells to that in plastic culture plate using reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. For Northern blot, mRNA levels of pro-opiomelanocortin (POMC), the common precursor to ACTH, β -endorphin, and β -lipotropin, were used as an index of the biosynthesis of ACTH. In addition, morphology was observed by phase-contrast and scanning electron microscopy, in order to understand the relationship between cell shape and pituitary hormone synthesis. The results of our study showed that the culture of anterior pituitary cells on Matrigel does not cause differences in ACTH synthesis in spite of the remarkable morphological changes.

Materials and Methods

Preparation of culture surface

Matrigel (10 mg/ml media) was prepared from the Engelbreth-Holm-Swarm (EHS) tumor as described previously [5], and 250 μ l of Matrigel was used to coat each well of a 24-well plate and then incubated overnight at 4°C to polymerize and form a gel. Half of the wells were coated with 500 μ l poly-D-lysine (50 μ g/ml sterilized water) (Sigma, St. Louis, MO) for 30 min.

Cell culture

Anterior pituitary cells were obtained from male Sprague-Dawley rats (150–200 g) after decapitation as described previously [6]. Briefly, anterior pituitary lobes were chopped into small pieces and dispersed by trypsin treatment. Forty thousand cells in 500 μ l of Dulbecco's Modified Eagles Medium (DMEM), pH 7.5 (Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco BRL) and ascorbic acid (1.9 mg/100 ml) (Sigma) were plated on each well of the 24-well culture plate coated with poly-D-lysine or Matrigel. Cultured cells were

maintained for 6 days and the medium was changed every 3 days. More than three pituitaries were pooled for each experiment and four experiments were performed.

Scanning electron microscopy

Cells for scanning electron microscopy were washed with serum-free media and fixed in 5% glutaraldehyde in 0.125 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) for 2 hrs at room temperature, stored at 4°C overnight and postfixed in 2% osmium tetroxide in 0.125 M HEPES buffer overnight. Cells were processed in ascending concentrations of ethanol (50–100%) and Freon 12, adhered to an aluminum stub, critical point dried (Critical Point Drier, Hitachi, Japan), sputter-coated with Au-Pd, and observed using a scanning electron microscope (JEOL, Tokyo, Japan).

RT-PCR procedure

Total RNA from anterior pituitary cells was extracted using Trizol reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instructions and RNA integrity was confirmed by electrophoresis followed by ethidium bromide staining. In order to remove the Matrigel, cells were washed twice with serum-free media and then 500 μ l of Dispase II containing 2.4 U/ml of Puck's solution A (Boehringer Mannheim) was added to each well followed by incubation at 37°C for 0.5 to 1 h. The RT reaction was performed in a final volume of 20 μ l containing 0.5 μ g of total RNA, 4 μ l of 5 $\times$ RT reaction buffer, 1.6 μ g of oligo (dT)₁₅, 1 mM of each of deoxynucleotide triphosphate (dNTP) mix. The samples were heated at 65°C for 10 min, cooled on ice and briefly spun down. Then, 1 μ l of RNase inhibitor and 5 unit AMV reverse transcriptase (Promega, Madison, WI) were added. The mixture was heated at 37°C for 1 h, 75°C for 10 min and then immediately placed on and stored at –20°C. PCR amplification was performed for 35 cycles in 50 μ l final reaction volumes containing 1 μ l of RT reaction product as template cDNA, 1 $\times$ PCR buffer, 0.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 μ M of each sense and antisense primer, and 2.5 U of *Taq* polymerase (Promega). A 260-bp region was identified in the rat

POMC gene coding for ACTH (GenBank accession number J00759) provided by the NCBI (National Center for Biotechnology Information). The sequence of sense and antisense primers were 5'-GCA-GAG-GCG-TGC-GGA-GGA-AG-3' (nucleotides 253 to 271) and 5'-CGG-CCT-TCT-CGG-TAT-CCG-GC-3' (nucleotides 493 to 512), respectively. A 182-bp region in rat GAPDH cDNA was used as an internal control (GenBank accession number X02231). The primers used were 5'-TCA-CTG-CCA-CTC-AGA-AGA-CTG-3' (sense: nucleotides 607 to 627) and 5'-GGA-TAC-ATT-GGG-GGT-AGG-AAC-AC-3' (antisense: nucleotides 766 to 788), respectively. Programmable temperature cycling was performed using a thermal cycler (Perkin Elmer/Cetus 480, Norwalk, CT) with the following cycle profile: initial degeneration at 94°C for 4 minutes, followed by 35 cycles of 94°C for 45 seconds (denaturation), 70°C for 1 minute (annealing), and 72°C for 1 min (elongation). After each 5 cycles, 5 µl of PCR product was collected. The PCR products were separated by 2% agarose gel in a TAE buffer system (40 mM Tris acetate and 1 mM EDTA, pH 8.0) with a molecular weight marker (100-bp DNA ladder, New England Biolabs, Beverly, MA), stained with ethidium bromide and visualized under a UV illuminator.

Northern blot analysis

Northern blot hybridization was performed using the PCR-generated cDNA probe labeled with the nonradioactive steroid hapten digoxigenin (DIG), and signals were detected by means of chemiluminescent reaction. DIG-11-dUTP was incorporated into the DNA antisense probe by PCR using 1 µl of RT product (template cDNA), 0.2 mM dATP, dGTP, dCTP, 0.13 mM dTTP, 0.07 mM DIG-11-dUTP (Boehringer Mannheim), *Taq* polymerase in a final volume of 50 µl followed by elution of the probe band and 2.5 µg of total RNA extracted from anterior pituitary cells cultured for 6 days was separated by electrophoresis in a 1.2% agarose-formaldehyde gel. The RNA was transferred onto nylon membrane (Hybond-N) (Amersham, Buckinghamshire, UK) using capillary transfer followed by baking at 80°C for 2 h. Membranes were prehybridized for 2 h with 0.06 ml/cm² of 5 × SSC, 50% formamide, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate, 0.02% blocking reagent (Boehringer

Mannheim) at 42°C. The DIG-labeled 260-base cDNA probe (10 ng/ml) including the region complementary to the rat ACTH mRNA was denatured at 98°C, added to the prehybridization solution, and hybridized to a membrane-bound nucleic acid for 18 h at 50°C. The membrane was washed with 1 ml/cm² of 2 × SSC, 0.1% SDS twice for 15 min at room temperature and 68°C in 0.1% × SSC, 0.1% SDS. After incubation for 2 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5) at room temperature for 30 min, membranes were incubated with 10% Genius blocking reagent in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 for 30 min, with anti-DIG-alkaline phosphatase (1 : 10,000 final dilution) for 30 min at room temperature, followed by washing twice for 15 min each to remove unbound conjugates. Membranes were equilibrated in 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5 and incubated with chemiluminescent substrate solution (CSPD diluted 1 : 100 in detection buffer), semi-dried at 37°C for 10 min, and then exposed to Kodak X-ray film with an intensifying screen.

Statistical analysis

Quantitative analysis of the autoradiograms was performed with the aid of the software package 'Image' running on a Macintosh II. The 'image' software is a public domain package written by Wayne Rasband of NIH in the USA. Values were adjusted by GAPDH to correct for variations in starting cDNA concentrations. Data were expressed as means ± SE. For statistical analysis of difference between the groups, unpaired *t*-test was used and a *P* value of 0.05 or less was considered statistically significant.

Results

Morphological observation

Fig. 1 shows the results of the phase-contrast microscopy (A, B) and scanning electron microscopy (C) of the anterior pituitary cells at Day 6 of culture. Pituitary cells cultured on plastic culture plate coated with poly-D-lysine were flat and confluent. Fibroblasts with larger nuclei were predominant and occupied a large part of the culture surface. In contrast,

Fig. 1. Morphology of rat anterior pituitary cells cultured on plastic (A) or on Matrigel (B, C) for 6 days. Phase-contrast micrographs (A, B) show that cells cultured on Matrigel form three-dimensional, lobule-like cell aggregates with a connecting cell (arrow) while those cultured on poly-D-lysine form almost confluent cell monolayer with a high percentage of fibroblast-like cells (arrowheads). Cell masses in various sizes are shown in a scanning electron micrograph of a Matrigel culture (C). Bars = 20 μ m.

cells cultured on Matrigel showed three-dimensional lobule-like cell aggregates surrounded by Matrigel.

ACTH synthesis

We used oligo (dT)₁₅ as a primer to generate cDNA which was then amplified by PCR. Ethidium bromide staining of agarose gel electrophoresis of the amplified products produced detectable bands of

Fig. 2. RT was performed using total RNA (0.5 μ g) obtained from anterior pituitary cells cultured on Matrigel (lanes 1–5) or on plastic (lanes 6–10) for 6 days. Ethidium bromide staining after PCR amplification for 15 (lanes 1, 6), 20 (lanes 2, 7), 25 (lanes 3, 8), 30 (lanes 4, 9), and 35 (lanes 5, 10) cycles showing 260-bp bands for POMC and 182-bp bands for GAPDH (upper panel). Densitometric analysis demonstrated no difference between the cultures on Matrigel (MG) and those on plastic (PL). Results are the means $\pm$ SE from four different experiments (lower panel).

Fig. 3. Regulation of POMC mRNA expression by culture substrate. Northern blot of total RNA (2.5 μ g) extracted from anterior pituitary cells after culture on Matrigel (left lane) or on plastic (right lane) for 6 days. Hybridization to DIG-labeled cDNA probe showed only one band in each lane (upper panel). Densitometric analysis demonstrated no difference between the cultures on Matrigel (MG) and those on plastic (PL). Results are the means $\pm$ SE from four different experiments (lower panel).

260-bp for POMC and 182-bp for GAPDH, respectively (Fig. 2). The results obtained with RT-PCR were confirmed by Northern blot hybridization using a DIG-labeled cDNA probe and chemiluminescent detection, which showed a single band (Fig. 3). Both sets of data showed that the amount of POMC mRNA, which has been used as an index of the biosynthesis of the POMC-derived peptide including ACTH, was not different in either culture.

Discussion

The anterior pituitary gland consists of several types of hormone secretory cells and fibroblasts. Since hormone secretory cells in long-term culture lose the characteristics they express *in vivo*, optimal culture conditions are needed to overcome this problem. However, not all the pituitary hormone cells respond in the same way to the same environmental changes. For example, estradiol treatment caused an increase in PRL cell numbers and a decrease in GH cell numbers [7]. Furthermore, hypothyroidism increased TSH cell numbers while GH cell numbers were decreased [6]. Thus, the optimal culture conditions for each type of pituitary hormone cells need to be ascertained in pituitary culture.

Among the ECM components which have been reported to affect cell differentiation, a reconstituted basement membrane, Matrigel, has been shown to provide a better environment for cultures of various epithelial cells than other substrates [8]. With respect to the effects of Matrigel on pituitary hormones, few studies have been performed using normal pituitary cells [4]. Since no study to our knowledge has reported the effects of ECM on ACTH synthesis in the anterior pituitary cells, we investigated whether Matrigel as a culture substrate affects the expression of POMC mRNA, which has been used as an index of the biosynthesis of the POMC-derived peptides including ACTH. Our results showed that there was no difference between cultures on Matrigel and those on plastic.

Cell-matrix interactions that occur on Matrigel are different from those that occur on glass or laminin and a more spherical shape and enhanced differentiation of epithelial cells have been demonstrated. It has been suggested that there is an association be-

tween cell shape and differentiation [9] and that Matrigel-induced cell differentiation is caused by the inhibition of cell spreading [10]. With respect to the effects of Matrigel on pituitary hormones, an increase in the secretion of PRL, LH, and FSH was observed [4]. In comparison, laminin, which is the most abundant component of Matrigel, did not increase pituitary hormone secretion. Thus, it was suggested that Matrigel influences the functional status of anterior pituitary cells by preserving a more spherical morphology. However, another study showed that an ECM component, which was produced by bovine corneal endothelial cells, stimulated the PRL secretion of pituitary cells in spite of increased cell spreading [3]. Thus, factors other than cell shape may also contribute to effects of culture substrate on pituitary hormone expression. In the present study, anterior pituitary cells cultured on Matrigel formed three-dimensional, glandular lobule-like cell aggregates, but ACTH synthesis which was investigated by RT-PCR and Northern blot analysis for POMC mRNA was not increased. Thus, ACTH increase may not be correlated with the three-dimensional cell morphology.

Although immunocytochemical methods have been used in most of the studies on pituitary hormone production, suitable antibodies are not always commercially available. We were not able to find any commercial antibody against rat ACTH which is reliable. Furthermore, it is not possible to distinguish between *de novo* synthesis and uptake by tissues with hormone receptors by immunocytochemistry. The development of molecular biology techniques has allowed investigators to distinguish between uptake and *de novo* synthesis, since the increase of mRNA usually indicates synthesis by the cells. Northern hybridization can be used for the detection of mRNA, but RT and amplification by PCR have provided us with more sensitive methods to detect specific products and to distinguish between cell uptake and *de novo* synthesis.

It has been reported that some pituitary hormones are found in normal or abnormal extrapituitary tissues and have been designated as ectopic, although predominantly produced by the anterior pituitary gland [11]. ACTH has been shown to be produced in extrapituitary cells such as macrophages, lymphocytes [12] and various neoplasms [13]. Thus, a very sensitive technique such as PCR should be an excel-

lent method of examining the pituitary hormone production in normal and neoplastic tissues, especially when the amount of specific mRNA produced by the various tissues may be very low.

In conclusion, the effects of Matrigel on anterior pituitary cells are probably attributable to changes in cell morphology, and to the synthesis of some pituitary hormones but not to ACTH synthesis. It was indicated that Matrigel may be a useful culture substrate for synthesis of some pituitary hormones but not for ACTH and that the morphological changes of pituitary cells do not affect ACTH synthesis. Thus,

cell-substrate interaction depends on pituitary cell type and other factors may influence pituitary cell differentiation. Further studies are needed to find the optimal culture substrate for ACTH synthesis.

Acknowledgement

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the 1997–1999 program years.

References

1. Leardkamolkarn V, Heck LW, Abrahamson DR (1989) Gonadectomy induces laminin biosynthesis and basement membrane assembly in anterior pituitary glands of adult rats. *Cell Tissue Res* 257: 587–596.
2. Martinez-Compos A, Dannies PS (1986) A possible differentiation of anterior pituitary cells in collagen gels into neurons. *Cell Tissue Res* 244: 21–26.
3. Bethea CL, Romsdell JS, Jaffe RB, Wilson CB, Weiner RI (1982) Characterization of the dopaminergic regulation of human prolactin-secreting cells cultured on extracellular matrix. *J Clin Endocrinol Metab* 54: 893–902.
4. Horacek MJ, Dada MO, Terracio L (1992) Reconstituted basement membrane influences prolactin, LH and FSH secretion from adult and fetal adenohypophyseal cells in vitro. *J Cell Physiol* 151: 180–189.
5. Kibbey MC, Royce LS, Dym M, Baum BJ, Kleinman HK (1992) Glandular-like morphogenesis of the human submandibular cell line A253 on basement membrane components. *Exp Cell Res* 198: 343–351.
6. Childs GV, Taub K, Jones KE, Chin WW (1991) Triiodothyronine receptor beta-2 messenger ribonucleic acid expression by somatotropes and thyrotropes: effect of propylthiouracil-induced hypothyroidism in rats. *Endocrinology* 129: 2767–2773.
7. Boockfor F, Hoeler J, Frawley LS (1986) Estradiol induces a shift in cultured cells that release prolactin or growth hormone. *Am J Physiol* 250: E103–105.
8. Royce LS, Kibbey MC, Mertz P, Kleinman HK, Baum BJ (1993) Human neoplastic submandibular intercalated duct cells express an acinar phenotype when cultured on a basement membrane matrix. *Differentiation* 52: 247–255.
9. Ben-Ze'ev A (1986) The relationship between cytoplasmic organization, gene expression, and morphogenesis. *Trends Biochem Sci* 11: 478–481.
10. Opas M (1989) Expression of the differentiated phenotype by epithelial cells in vitro is regulated by both biochemistry and mechanics of the substratum. *Dev Biol* 131: 281–293.
11. Fields K, Kulig E, Lloyd RV (1993) Detection of prolactin messenger RNA in mammary and other normal and neoplastic tissues by polymerase chain reaction. *Lab Invest* 68: 354–360.
12. Lyons PD, Blalock JE (1995) The kinetics of ACTH expression in rat leukocyte subpopulations. *J Neuroimmunol* 63: 103–112.
13. Monig H, Oldfield EH, Schulte HM (1993) Structure of POMC promoter region in pituitary and extrapituitary ACTH producing tumors. *Exp Clin Endocrinol* 101: 36–38.