Reconstituted Basement Membrane Reduces Proliferation and Increases Prolactin Expression of GH₃ Cells

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Abstract. This study investigated the effects of reconstituted basement membrane Matrigel on the proliferation and prolactin expression of GH₃ cells in culture for 6 days. When cells were cultured on Matrigel, the initial attachment was increased but the cell number was not changed with time whereas rapid increase in cell number was observed in cultures on plastic. Bromodeoxyuridine (BrdU)-labeling showed that BrdU incorporation ratio of GH₃ cells cultured on Matrigel was about one half of that observed with cells cultured on plastic (9.7±0.7% vs. 18.7±1.2%). Immunocytochemistry revealed that the ratio of the prolactin-immunoreactive GH₃ cells was about 3.6 times (58.4±2.9% on Matrigel vs. 16.2±1.4% on plastic), which was compatible with the results of Western blot analysis. In situ hybridization demonstrated that prolactin mRNA-positive cells were identified more frequently when cells were cultured on Matrigel compared to cultures on plastic. These findings indicate that Matrigel is a proper culture substrate for the long-term culture of GH₃ pituitary cells due to the inhibition of overgrowth and promotion of prolactin expression.

Key words: GH₃ cells, Matrigel, Cell proliferation, Prolactin expression

GH₃ cells have been widely used for the investigation of in vitro regulation of prolactin (PRL) expression [1]. The advantage of using GH₃ cells compared to normal pituitary cells is that a single type of hormone-containing cell can be studied in a fibroblast-free environment. In addition, GH₃ cells lack dopamine receptors [2] and provide a useful experimental model for the study of regulation of PRL in dopamine-resistant pituitary tumors. Thus, it seems to be worth finding a culture substrate that enables GH₃ cells to maintain PRL expression without causing a confluent condition.

Since extracellular matrix (ECM) can affect the cell growth and differentiation in vitro [3, 4], the effects of ECM on GH₃ cells have been studied. ECM produced by bovine corneal endothelial cells (BCEC) increased the synthesis and secretion of PRL up to 2 times; however, high rate of cell proliferation, which causes the confluent condition in long-term culture, was maintained [5]. Thus, another culture substrate that can inhibit the overgrowth of GH₃ cells as well as increase the PRL expression is in need.

Matrigel, a reconstituted basement membrane, has
been shown to provide a better environment for cultures of various epithelial cells than other substrate [6, 7]. Although it has been reported that laminin, the major component of Matrigel, increased the secretion of PRL of GH3 cells up to 1.5 times [8], the effect on the proliferation has not been studied. Furthermore, Matrigel contains several other components that may influence the proliferation and differentiation of GH3 cells.

Therefore, the present study investigated the effects of Matrigel on GH3 cells with respect to the proliferation, PRL synthesis and storage in order to observe whether it can provide the proper substrate for the long-term culture of GH3 cells. The effects of epidermal growth factor (EGF), which have been reported to increase the PRL mRNA and PRL secretion of GH3 cells [9], and laminin on GH3 cells were observed for comparison.

We found that Matrigel as a culture substrate increases the initial attachment (2-fold) and PRL expression (3.6-fold) of GH3 cells, but decreases the proliferation rate (50% of those on plastic) and provide a proper environment for the long-term culture of GH3 cells.

Materials and Methods

Cell culture

GH3 cells obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained in Ham's F-10 medium (Gibco, Grand Island, NY, USA) supplemented with 12.5% horse serum (Gibco), 2.5% fetal bovine serum (FBS) (Gibco), 0.25 μg/ml fungizone (Gibco), 80 μg/ml gentamycin (Sigma, St Louis, MO, USA) and 2 mM L-glutamine (Gibco). They were subcultured twice a week using Trypsin-EDTA (0.05%-0.53 mM) (Gibco) for detachment of cells and found free of mycoplasma by the DNA fluorescent staining method using 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim, Indianapolis, IN, USA) before seeded onto either culture plastic or surface coated with Matrigel (10 mg/ml). Cells were cultured in the 24-well tissue culture plates (Nunc, Inc., Naperville, IL, USA) at a density of 30,000 cells in 1 ml Ham's F-10 medium containing 10% FBS, 80 μg/ml gentamycin, 0.25 μg/ml fungizone for up to 6 days. Some cells were treated with 10 nM EGF (Biofluids Inc., Rockville, MD, USA). Culture medium was replaced every 3 days. To determine the rate of cell division, bromodeoxyuridine (BrdU) incorporation was used as an indirect measure of cell division. Cells were incubated in the CO2 incubator for 30 min after BrdU (Sigma) was added to the culture medium to give a final concentration of 100 μM. The experiments were performed in triplicate and repeated at least three times.

Extracellular matrix preparation

Matrigel was prepared from the Engelbreth-Holm-Swarm (EHS) tumor as described previously [10], diluted to a concentration of about 10 mg/ml and kept frozen until use. Matrigel was thawed, and 250 μl of Matrigel was used to coat each well of the 24-well plate and incubated overnight at 4°C to polymerize to form a gel. Laminin was prepared as described previously by Tougard et al. [11]. Ten microgram in 200 μl Milli-Q water was added to each well, and incubated at 37°C until dried.

Cell number

Cells were washed twice with serum-free media and then 500 μl of dispase II containing 2.4 U/ml of Puck's solution A (Boehringer Mannheim) was added into each well followed by incubation at 37°C for 0.5 to 1 h. Cells in each well were monodispersed, transferred to a hemocytometer and counted. This procedure was performed at Day 0 (90 min after plating), 3 and 6.

Immunocytochemistry

Single cells were obtained by dispase treatment and attached onto poly-D-lysine (Sigma) coated slides using a cytospin (Cytospin 2; Shandon Inc., Pittsburg, PA, USA). After fixation with modified Carnoy's fixatives (methanol: acetic acid = 3:1 v/v) overnight at 4°C, fixed cells were washed three times with phosphate-buffered saline (PBS), pH 7.5, containing 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and 0.14 M NaCl.

For the detection of BrdU incorporation, monoclonal antibody was used by the previously described procedures [12]. Briefly, cells were rinsed
with PBS, treated with 1 N HCl, 2 N HCl, and 0.1 M borax solution (Sigma) and were incubated with 1:100 mouse monoclonal anti-BrdU antiserum (Sanbio, Uden, Netherlands) for 1 h. After incubation with 1:100 biotinylated horse anti-mouse IgG (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1 h and with avidin-biotin-peroxidase complex (ABC; Vector Laboratories) for 30 min, 0.025% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris buffer (pH 7.2) containing 0.03% (w/v) hydrogen peroxide was used for detection. Controls for the specificity of the BrdU-immunostaining reaction included omission of either the BrdU incubation, the primary or secondary antisera or ABC.

The percentage of PRL-immunoreactive cells were detected again, as we have previously described [12]. Briefly, cells were incubated with 1:40,000 rabbit anti-rat PRL (NIDDK Hormone Distribution Program, Bethesda, MD, USA) for 2 h at room temperature and then with biotinylated goat anti-rabbit IgG (Vectastain Elite ABC kit) for 1 h. The rest of the procedures were the same as those for BrdU detection. Cells were dehydrated in an ethanol series and mounted with permount. The results were expressed as the percentage of PRL-positive cells among the counted cells. The specificity of anti-PRL antisera was confirmed by absorption of the antibodies with PRL (100 pg/ml) for 2 h at room temperature which abolished the immunoreaction.

**Western blot analysis for PRL**

At culture Day 6, equivalent number of cells cultured on different substrates were obtained by cell counting after dispase treatment, washed with cold PBS, and lysed with lysis buffer [0.05 M PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate (SDS), and 2 mM phenylmethylsulfonyl fluoride] for 30 min on ice. Protein extract was separated by electrophoresis at 125 V for 90 min on 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) at 130 mA for 100 min. Molecular weights were determined using standard proteins (Amer sham, Arlington Heights, IL, USA). The membrane was blocked for 1 h at room temperature with 3% nonfat dried milk in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Tween-20 (TTBS) and incubated overnight at 4°C with the rabbit anti-rat PRL polyclonal antiserum (NIDDK) diluted 1:1,000 in TTBS containing 0.5% nonfat dried milk. The membrane was washed 5 times (10 min each) with the same buffer and the HRP-conjugated anti-rabbit IgG antibody (Amersham) binding was detected by enhanced chemiluminescence (Amersham).

**In situ hybridization for PRL mRNA**

Probes were prepared as follows: The plasmid containing the rat PRL cDNA cloned in the EcoRI site of pSP64 was generously provided by Dr. R.A. Maurer (University of Iowa, USA). Linearized DNA was transcribed to obtain single stranded antisense and sense RNA probes labeled with digoxigenin-UTP using SP6 RNA polymerase and T3 RNA polymerase, respectively, using an RNA labeling kit (Boehringer Mannheim). RNA *in situ* hybridization was performed by the method described previously [13]. Cells were fixed in 4% paraformaldehyde in PBS, pH 7.0, washed in PBST (1% Tween 20 in PBS) three times and treated with 10 mM Tris-HCl, pH 7.5, containing 1 μg/ml proteinase K in 5 mM EDTA at room temperature for 5 min. After endogenous alkaline phosphatase was inactivated with 0.2 M HCl (10 min), cells were treated with 0.1 M triethanolamine-HCl (TEA), pH 8.0 for 1 min, and 0.25% acetic anhydride in 0.1 M TEA for 10 min. Prehybridization solution (50% formamide, 5 x SSC, pH 5.0, 50 μg/ml yeast t-RNA, 1% SDS) was added to the cells at 70°C for 20 min.

Cells were covered with 100 μl of hybridization mixture (1 mM EDTA, 50% formamide, 10 mM Tris-HCl, 200 μg/ml tRNA, 1 x Denhardt’s solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS) containing RNA probe (1 μg/ml) at 50°C for 16–18 h in an humidified chamber. Cells were washed in 2 x SSC (1 x SSC = 15 mM sodium citrate, 150 mM NaCl, pH 7.0) containing 50% formamide at 50°C for 15 min, and at 37°C for 15 min and at room temperature for 30 min, and followed by RNase A treatment (10 μg/ml) for 15 min at 37°C. Color detection was carried out using the Genius-detection system (Boehringer Mannheim), in which the specific transcripts were detected by an anti-digoxigenin antibody conjugated with alkaline phosphatase. After washing with the solution containing 100 mM Tris-
HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂, cells were immersed in the color-development solution of HistoMark Red Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and in the 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. Cells were dehydrated with ethanol and mounted in permount.

Statistical analysis

The percentages of BrdU-incorporated nuclei, apoptotic cells, mitotic cells and PRL-positive cells were calculated. More than 1,000 GH₃ cells were counted on each of the triplicate specimens under a light microscope (Olympus, Tokyo, Japan) at a magnification of ×200, ×400 or ×1,000 and each experiment was repeated three times. Data were evaluated with the Student’s t-test. Differences were considered to be statistically significant when the P value was <0.01.

Results

Morphological observations

Phase contrast microscopy shows the morphology
of GH3 cells at Day 6 of culture (Fig. 1). Cells attached on plastic culture surfaces grew in the monolayer of polygonal cells while cells plated on Matrigel remained rounded or formed three-dimensional cell aggregates.

**Cell number**

The number of attached GH3 cells was assessed at culture Day 0 (90 min after plating), 3 and 6 (Fig. 2). Cell attachment at 90 min after plating was higher when GH3 cells were plated on Matrigel (85.5 ± 2.2%) compared to those plated on either plastic or laminin (39.3 ± 2.3% and 38.3 ± 2.2%, respectively). For the 6-day time period, GH3 cells cultured on either plastic or laminin showed rapid increase in cell number (cell doubling time was about 1.2 days) whereas cells on Matrigel did not show significant changes in cell number. Addition of EGF to the cells plated on plastic also decreased the cell number.

![Photomicrographs of GH3 cells after cultured for 6 days. Immunocytochemistry for BrdU (A, B) or for PRL (C-F) was performed after dispase treatment and cytopinning. BrdU-labeld nuclei (arrows) are seen more frequently in GH3 cells cultured on plastic (A) compared to cells cultured on Matrigel (B) while PRL-immunoreactive cells are seen more frequently when cells were cultured on Matrigel (D). Note that cytopsin cells cultured on Matrigel (D–F) remained rounder and smaller than those cultured on plastic (C). Nuclei of apoptotic cells (arrows) with peripheral chromatin condensation (E) or nuclear fragmentation (F) are seen. Counterstained with hematoxylin (A–F). In situ hybridization for PRL mRNA (G, H) demonstrates that red signals (arrowheads) are weak in GH3 cells cultured on plastic (G) while it is intense when cells were cultured on Matrigel (H). Bars: A–D, and G = 20 μm; E, F and H = 10 μm.](image-url)
cytochemistry for BrdU (Figs. 3 and 4A, B) revealed that the relative ratio of BrdU-incorporation into the cells cultured on Matrigel to that into the cells cultured on plastic was 51.9% (9.7±0.7% vs. 18.7±1.2%) at culture Day 6. The presence of EGF also decreased the BrdU-labeling ratio. In addition, mitotic cells were observed less frequently when GH3 cells were cultured on Matrigel (1.0±0.4% vs. 2.7±0.2% on plastic). On the contrary, apoptotic cells with peripheral nuclear condensation or fragmented were observed under oil immersion lens and it was shown that they occupied 2.1±0.6% of GH3 cells cultured on Matrigel (Figs. 4E, F) while they were seen rarely when cultured on plastic surface (0.2% or lower).

**Effects on PRL**

In in situ hybridization (Figs. 4G and 4H), stronger and more frequent red signals were observed in cells cultured on Matrigel compared to cells cultured on plastic. The results of immunocytochemical staining for PRL (Figs. 4C, D and 5) revealed that cultures on Matrigel increased the percentage of PRL-positive GH3 cells by 3.6-fold (58.4±2.9% vs. 16.2±1.4% on plastic) after culture for 6 days. EGF also increased the proportion of PRL-positive cells to 48.3±4.1%. Western blot analysis (Fig. 6) also showed similar results to those of immunocytochemistry in which both Matrigel and EGF increased the PRL content of GH3 cells.

**Discussion**

The results of the present study show direct evidences for effects of Matrigel on the proliferation and PRL expression of GH3 cells. When GH3 cells are plated on Matrigel, they form cell aggregates and surrounded by Matrigel which contains high protein components. Thus, it is not possible to compare the proportion of immunostained cells or PRL content with those of cells cultured on plastic. We prepared single cells free from Matrigel by dispase treatment, which enabled us to quantify the ratios of BrdU-labeled cells, of PRL-immunoreactive cells and PRL content. To the best of our knowledge, no study has quantified the effect of Matrigel on pituitary tumor cells.

The present study showed rapid cell growth of GH3 cells cultured on plastic. Growth rate of cells cultured on laminin was similar to that of cells cultured on plastic whereas cells cultured on Matrigel did not show significant increase in cell number. This result indicated that Matrigel is a proper culture substrate to prevent the overgrowth of GH3 cells in the long-term culture. We performed BrdU-labeling assays at Day 6 of culture to observe if the cells cultured on Matrigel have stopped or decreased cell division. Our data showed that the cells on Matrigel were still replicating but with significantly lower BrdU-incorporation ratios compared to those cultured on plastic (9.7% on Matrigel vs. 18.7% on...
plastic), which was compatible with the difference in the mitotic ratios. Since cell growth is determined by the relative ratio between cell division and cell death, it is possible that cell proliferation on Matrigel is masked by the increase in apoptosis (2.1% on Matrigel vs. less than 0.2% on plastic). Since pituitary tumors in vivo are not surrounded by basement membrane [14], this apoptotic effect of Matrigel on GH3 cells may be affected by the difference in the surrounding condition; however, further studies on the apoptotic effect of Matrigel on the transformed cells are needed.

It has been observed that GH3 cells show poor attachment when plated onto the plastic surfaces. In the present study, some cells were loosely attached onto the plastic surface; however, detached cells were rarely seen for the 6-day culture period once GH3 cells were attached. The results of the present study showed that Matrigel increased the initial attachment of GH3 cells about 2-fold (85.5% vs. 39% on plastic) 90 min after plating. This relationship was reversed by Day 3 of the culture, and more cells were attached onto the plastic than onto the Matrigel. Thus, we speculate that the difference in the mitotic ratio, rather than that in cell attachment, between the cultures is likely to account for the different cell number.

Although the total number of GH3 cells cultured on plastic is much greater than those cultured on Matrigel, the number of PRL-immunoreactive cells are almost the same in both groups because of high proportion of PRL-immunoreactive cells in the Matrigel culture. Thus, it is not the total number of PRL cells but the degree of PRL expression of each cell that determines the difference in PRL content.

Immunocytochemical staining for PRL demonstrated that the proportion of PRL-immunoreactive cells in cultures on Matrigel was remarkably higher (58.4%) by Day 6 compared to those on plastic (16.2%) or laminin (17.1%). This result was further confirmed by Western blot analysis and by in situ hybridization. Taken together, these results indicated that Matrigel decreases the rate of the proliferation of GH3 cells and increases the amount of PRL in the equivalent number of GH3 cells. Therefore, it was suggested that Matrigel is an effective culture substrate for maintaining the differentiated condition of GH3 cells.

It has been proposed that there is an association between cell shape, proliferation and differentiation. Previous studies that used normal epithelial cells such Sertoli cells [15] or endothelial cells [4] observed a greatly reduced proliferation rate in cultures on Matrigel and suggested that low rate of mitosis was induced by the morphological changes causing high cell density. It has been also suggested that preventing epithelial cells from spreading on Matrigel is responsible for the differentiation [16, 17]. This speculation, however, is not in agreement with findings of the study by Elias (1990). ECM component produced by BCEC increased spreading, attachment, proliferation but still stimulated PRL secretion (2-fold) of GH3 cells simultaneously [5]. Although our results indirectly showed that Matrigel is a more effective culture substrate for the culture of GH3 cells compared to laminin or ECM produced by BCEC, further studies are needed to elucidate the relationship between the effects of ECM on the attachment, proliferation and PRL expression of GH3 cells.

In conclusion, when GH3 cells were cultured on Matrigel, overgrowth of GH3 cells was inhibited and marked increase in the PRL content was observed compared to cells cultured on plastic. Therefore, Matrigel as a culture substrate appears to provide a promising experimental model for the study of GH3 cells, especially in the long-term culture. Although it was clearly shown that Matrigel decreases the mitosis of GH3 cells, contribution of the increased apoptosis to the inhibition of overgrowth was also suggested.

Acknowledgement

We express our gratitude to Dr. Richard A Maurer of the University of Iowa, for supplying PRL cDNA, to Drs. Raiti and Parlow at NIDDK for providing anti-rat PRL antisera, and to Dr. Yoshi Yamada at NIDR for letting us to use his laboratory facilities for in situ hybridization. We are also grateful to Mr. Sung-Jae Park, Mr. Jin-Young Kim and Mrs. Hee-Sun Park at Seoul National University for their excellent technical assistance.

This study was supported by the academic research fund of Ministry of Education, Republic of Korea (1995 and 1996; BM 95, 96-099)
References


