Effects of Various Types of Extracellular Matrices on Adhesion, Proliferation, Differentiation, and c-fos Protein Expression of Porcine Thyroid Follicle Cells

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ABSTRACT. Thyroid follicles in vivo are embedded in extracellular matrix (ECM). The composing epithelial cells are in close contact with ECM at the basal side. To examine cell-to-ECM interactions, we studied adhesion, proliferation and differentiation of porcine follicle cells monolayer-cultured on type I and IV collagen, fibronectin or laminin. At 3 h in culture, laminin had the lowest rate of cell adhesion. In proliferation, type IV collagen induced the highest level of nuclear bromodeoxyuridine intake. In a functional differentiation, laminin had about 3 times as much triiodothyronine production as the other ECM molecules. In confluent culture cells, we also examined an expression of c-fos protein, a transcription factor that plays crucial roles in signal transduction. Immunocytochemistry detected the protein mainly in the nuclei. Western blot showed that laminin induced the highest level of its expression. Thyrotropin (TSH, 10 mU/ml) did not affect adhesion of the cells on any of the substrata or proliferation of the cells on fibronectin; nor did TSH affect c-fos protein expression of the cells on the substrata except for fibronectin. Our results suggest that type IV collagen and laminin, major components of basement membrane, play positive roles in proliferation and differentiation of follicle cells, respectively, while laminin has no positive effect on adhesion of the cells at early culture; that the cells express c-fos protein even in contact inhibition of growth and its expression is regulated in part by ECM; and that ECM controls some behaviors of the cells in a TSH-dependent or TSH-independent manner.

Extracellular matrix (ECM) plays vital roles in both embryonic morphogenesis and regulation of cell behaviors in adult tissues, involving adhesion, migration, proliferation and differentiation of many cell types (22, 18, 19). Recently, many studies and ours have shown that gel of type I collagen, a major component of ECM, induces structural and/or functional differentiation of multiple cell types, including thyroid follicle cells (8, 11, 13, 16, 20, 21, 25, 30–33, 38–40, 45). In thyroid gland, individual follicles, its structural and functional unit, are embedded in interfollicular ECM (3). At the basal side, composing epithelial cells are in close contact with the intricate network of ECM containing mainly type I collagen and fibronectin through basement membrane, which consists chiefly of type IV collagen, laminin, and

heparan sulfate proteoglycans (24). Each of the ECM components is thus expected to play crucial roles in regulating the behaviors of follicle cells. To ultimately clarify pathogenesis of human thyroid disorders, it is, therefore, essential to study follicle cell-to-ECM interactions. However, it has not yet been examined what effects on the cells various ECM components induce. To estimate the roles of ECM, we have studied the effects of type I and IV collagen, fibronectin and laminin on the adhesion, proliferation and differentiation of porcine thyroid follicle cells in monolayer cultures with plastic substratum (polycation-coated dish) as a reference.

Protein c-fos is encoded by the c-fos oncogene which is the cellular cognate of v-fos, the transforming gene of the FBJ-murine osteosarcoma virus (10, 14). This oncoprotein is a transcription factor that regulates proliferation and differentiation of many cell types (1, 9, 29). In thyroid follicle cells, recent studies indicate that c-fos mRNA expression is transiently enhanced in proliferation of the culture cells without contact inhibition of growth (6, 17, 35, 41). However, it has hardly been examined whether the level of c-fos protein is actually in-

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Abbreviations: ECM, Extracellular matrix; PBS, Phosphate buffered saline; FCS, Fetal calf serum; TSH, Thyrotropin; BrdU, Bromodeoxyuridine; T₃, Triiodothyronine; RIA, Radio-immunoassay; ABC, Avidin-biotin complex; PAG, Polyacrylamide gel; KD, Kilodalton; VLA₃, Very late antigen 3.

creased in these cells. The effects of ECM components on c-fos protein expression of follicle cells are also unknown. In addition, follicle cells in normal thyroid essentially have low mitotic ability (5, 12). To clarify the roles of c-fos protein other than proliferation, it is essential to study the protein expression in the cells under such a situation as would be simulated by contact inhibition of growth *in vitro*. To estimate an expression of c-fos protein in cell-to-ECM interaction, we performed immunocytochemical and Western blot analyses for the protein in follicle cells confluent cultured on different types of ECM components.

For the first time, we show herein that each type of the ECM components has crucial effects on the biological behaviors of thyroid follicle cells.

MATERIALS AND METHODS

Preparation of ECM-coated culture dishes. Type I and IV collagen (Nitta Gelatin Co., Ltd., Osaka, Japan) were diluted by $\times 30$ with 1 mM HCl to the final concentration of 100 $\mu\text{g}/\text{ml}$. Fibronectin and laminin (Nitta Gelatin Co., Ltd.) were diluted by $\times 40$ with water to the final concentration of 12.5 $\mu\text{g}/\text{ml}$. Using these matrix solutions, ECM-coated dishes were prepared by the following standard method. After 6, 1 and 0.036 ml of the matrix solutions were placed on 100 mm- and 35 mm-diameter culture dishes, and 96 well dishes, respectively, the culture dishes were kept at 25°C for 1 h. The solutions were removed and dried overnight at 25°C. Type I and IV collagen-coated dishes were washed twice with phosphate-buffered saline (PBS) immediately before use. In addition to these ECM substrata, plastic substratum (polycation-coated culture dish, Corning Costar Corporation, Cambridge, MA) was used as a reference substratum in this study.

Cell culture. Isolated thyroid follicle cells from 6-month-old porcine were prepared as described elsewhere (38). These cells had no contamination with smooth muscle cells or fibroblasts. The cells were cultured in ECM-coated or plastic dishes with Ham's F-12 medium supplemented with 5% fetal calf serum (FCS) and 50 $\mu\text{g}/\text{ml}$ gentamicin. Some cultures were stimulated with 10 mU/ml thyrotropin (TSH, Sigma, St. Louis, MO).

Assay of cell adhesion. To examine the effects of different substrata on adhesion of follicle cells, 1.5×10^5 cells were seeded and cultured in ECM-coated or plastic dishes (35 mm-diameter) for 3 h with or without 10 mU/ml TSH. The culture dishes were aspirated and washed three times with Ham's F-12 medium. The adherent cells were trypsinized and processed by the standard method, and were counted with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL), as previously described (38). The rate of cell adhesion was calculated as follows: the number of adherent cells/ $1.5 \times 10^5 \times 100$ (%). In addition, at 6, 24 or 48 h in culture, we calculated the rate of adherent cells on each substratum by the same method. These results in part also involved those of proliferation of the cells

cultured on each substratum.

Assay of cell proliferation. To estimate the effects of different substrata on proliferation of follicle cells, we performed immunocytochemical assay in microtiter plate wells, using monoclonal anti-bromodeoxyuridine (BrdU) antibody to detect BrdU incorporated in cellular DNA (cell proliferation assay kit, Amersham, Arlington Heights, IL). This kit has high sensitivity in detection of cell proliferation with wide range of cell number (1×10^3 to 1×10^5) (4). The proliferation assay was carried out after the rates of cell adhesion reached to about 90% (see Fig. 2). After 100 μl cell suspension containing 5,000 cells were seeded in each of 96 wells, the cells were cultured for 24 h in type I collagen-, type IV collagen-, fibronectin-coated, or plastic wells, and cultured for 48 h in laminin-coated wells. After 100 μl labeling medium (30 mg/ml BrdU) was added to each well, the cells were cultured for 2 h with or without 10 mU/ml TSH. The culture cells were fixed with 30% acetic ethanol and processed according to the protocol of the kit. The absorbance of each well was measured at 410 nm.

Assay of triiodothyronine (T_3) in culture medium. In this study, we selected T_3 production as an indicator of differentiation of follicle cells, because porcine follicle cells stably produce T_3 in culture. To estimate the effects of different substrata on T_3 production of follicle cells, 6×10^5 cells were first cultured in ECM-coated or plastic dish (35 mm-diameter) until they formed a confluent monolayer within 1–2 days. After the medium was exchanged for fresh medium and the cells were cultured for 48 h with or without a 12 h-stimulation of 10 mU/ml TSH, T_3 secretion from follicle cells into medium was assessed in culture supernatants by the standard radioimmunoassay (RIA) method (T_3 assay kit, Dinabot Co., Ltd., Tokyo, Japan).

Immunocytochemical and Western blot analyses for c-fos protein. On 4 days in culture, we observed the effects of different substrata on c-fos protein expression in confluent culture cells under contact inhibition of growth, a condition simulating low mitotic ability of follicle cells in normal thyroid (5, 12). At first, 6×10^5 (for immunocytochemistry) and 42×10^5 (for Western blotting) cells were cultured on different substrata in 35 mm- and 100 mm-diameter dishes, respectively until they formed a confluent monolayer within 1–2 days. After 2 days in culture, the medium was exchanged for fresh medium and the cells were cultured for 48 h with or without a 12 h-stimulation of 10 mU/ml TSH. Before the detection of c-fos protein, we also confirmed by phase contrast microscopy that the culture cells were completely confluent. Using the culture cells, we performed immunocytochemistry and Western blotting with anti-c-fos polyclonal antibody (Ab-2, Oncogene Science, Inc., Uniondale, NY). In immunocytochemistry, after culture cells were fixed for 5 min by 3.7% paraformaldehyde/PBS, air-dried and processed by the standard methods, the cells were incubated with anti-c-fos antibody for 1 h at 25°C. The protein was detected by the avidin-biotin complex immunoperoxidase (ABC) method. We also examined immu-

Fig. 1. Phase contrast micrograph of follicle cells cultured on different substrata at 3 h in culture. Type I (I, b) and IV collagen (IV, c), and fibronectin (F, d) induce good outcomes of cell adhesion and spreading, while plastic substratum (P, a) has poorer results of those. Laminin (L, e) shows the poorer cell adhesion and has cell spreading similar to plastic substratum.

nohistochemically the distribution of c-fos protein in normal thyroid on sections of 3.7% paraformaldehyde/PBS-fixed tissues. To examine whether this immunostaining was specific for c-fos protein, we performed an absorption test as a negative control. The c-fos antibody (1 μg) neutralized with c-fos antigen (10 μg, peptide-2, Oncogene Science, Inc.) was used in-

stead of a primary antibody and processed by the same methods above. For Western blotting, after the culture cells were washed twice with cold PBS, in the dishes the cells were suspended in 0.6 ml of 0.1 M Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The samples were boiled for 20 min and then centrifuged for

Fig. 2. Rates of cell adhesion in follicle cells cultured on different substrata. At 3 h in culture, rates of cell adhesion to type I (I) and IV collagen (IV), and fibronectin (F) are about 60%, respectively, while those of adhesion to laminin (L) and plastic substratum (P) are about 8 and 30%, respectively ($P < 0.001$ between I, IV or F and L or P; $P < 0.001$ between L and P). In continuous culture, the rate of cell adhesion to each substratum gradually increases. The rates of adhesion to the substrata except for laminin (L) reach to about 90% at 24 h in culture, while the rate of adhesion to laminin (L) reached to almost the same range at 48 h. TSH (10 mU/ml) does not affect the rates of adhesion of cells cultured on any of the substrata. The values represent the mean $\pm$ S.D. of five independent experiments.

Fig. 2.

Fig. 3. Nuclear BrdU intake of follicle cells cultured on various substrata. Type IV collagen (IV), plastic substratum (P) and type I collagen (I) induces the higher intake of BrdU in that order, as compared with fibronectin (F) and laminin (L), although there is no statistical significance between type IV collagen (IV) and plastic substratum (P) or type I collagen (I). There is statistical significance only between type IV collagen (IV) and fibronectin (F) or laminin (L) ($P < 0.02$ between IV and F; $P < 0.05$ between IV and L). TSH (10 mU/ml) clearly inhibits the BrdU intake of cells cultured on any of the substrata except for fibronectin (F). TSH does not suppress the BrdU intake of the cells cultured on fibronectin (F). The values represent the mean $\pm$ S.D. of five independent experiments.

10 min. Twenty μ l of the solubilized proteins were subjected to electrophoresis on 10% polyacrylamide gel (PAG), and then transferred to a nitrocellulose membrane sheet (Bio-Rad, CA). The antigen on the membrane was visualized according to the manual supplied by Bio-Rad, using the ABC method.

Statistical analyses. We performed statistical analyses of the data obtained through four or five independent experiments, using student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Cell adhesion. The results of adhesion assays were as follows. At 3 h in culture, the rates of cell adhesion to type I and IV collagen, and fibronectin were all about 60%. In contrast, the rates of cell adhesion to laminin and plastic substratum were about 8 and 30%, respectively (Figs. 1 and 2). At this culture time, laminin and plastic substratum induced poor cell spreading (Fig. 1, a and e). In continued culture, the rate of cell adhesion to each substratum gradually increased. The rates of adhesion to the substrata except for laminin reached to

Fig. 4. T₃ production of follicle cells cultured on various substrata. Laminin (L) shows about 3 times as much T₃ production as type I (I) and IV collagen (IV), and fibronectin (F) ($P < 0.01$ between L and I, IV or F). Plastic substratum (P) has the highest level of T₃ production and shows about 1.6 times the production of laminin (L) ($P < 0.05$ between P and L). TSH (10 mU/ml) clearly increases T₃ production from cells cultured on each substratum. The values represent the mean $\pm$ S.D. of four independent experiments.

about 90% at 24 h in culture, while adhesion to laminin reached to almost the same range at 48 h (Fig. 2). The rates of cell adhesion to all substrata or cell spreading exhibited no differences with or without 10 mU/ml TSH (Fig. 2). Finally, cells cultured on type I and IV collagen, fibronectin or plastic substratum seemed to enter the proliferative phase after 24 h in culture, while adhesion of cells on laminin was almost completed at 48 h and thereafter the cells were expected to come into the proliferative phase (Fig. 2). In addition, none of the cells cultured on the substrata had distinctive differences in morphology except for cell spreading.

Cell proliferation. The proliferation assay was performed after the rate of cell adhesion to each substratum reached to about 90% (Fig. 2). In nuclear BrdU intake of culture cells, type IV collagen was the highest, and then came plastic substratum, type I collagen, laminin and fibronectin in that order (Fig. 3). In fact, the number of the cells cultured on type IV collagen was highest after 48 h (see Fig. 2). In these values, there was statistical significance only between type IV collagen or plastic substratum and fibronectin or laminin. TSH (10 mU/ml) clearly inhibited nuclear BrdU intake of the cells cultured on each substratum except for fibronectin.

Fig. 5. Immunocytochemistry for c-fos protein. Immunocytochemistry shows that c-fos protein is localized in the nuclei of cells cultured on laminin (a). An absorption test results in negative staining for this protein (b). In the porcine thyroid (c and d), c-fos product is also positive immunostained in the nuclei (arrowhead) of some follicle cells and is also weakly stained in some cytoplasm (arrow). Most smooth muscle cells of blood vessels are positive-stained in both nuclei (arrowhead) and cytoplasm (arrow). The staining intensity is stronger in smooth muscle cells than in follicle cells.

Fig. 5.

tin. Interestingly, the BrdU intake of cells cultured on fibronectin was not suppressed by TSH (Fig. 3).

T₃ production. To examine the effects of various substrata on the differentiation of follicle cells, we assayed T₃ in culture supernatants at 4 days in culture. Laminin had about 3 times as much T₃ production as type I and IV collagen, and fibronectin. However, plastic substratum induced the highest T₃ production and showed about 1.6 times the production of laminin (Fig. 4). TSH (10 mU/ml) clearly increased T₃ production from the cells cultured on each substratum (Fig. 4). In addition, after 8–10 days in culture, none of the cells cultured on each substratum had any significant differences in T₃ production. This suggested that at the late culture stage, the cells themselves may newly produce ECM molecules and fall into almost the same ECM-environment.

Expression of c-fos protein. To estimate c-fos protein expression of follicle cells in cell-to-ECM interaction under contact inhibition, we performed immuno-

cytochemistry and Western blotting for the protein. In immunocytochemistry, c-fos protein was detected mainly in nuclei of some cells cultured on each substratum even in contact inhibition (Fig. 5a), although weakly positive staining was observed in cytoplasm of some cells (data not shown). An absorption test resulted in negative staining (Fig. 5b). TSH (10 mU/ml) did not appear to affect the expression of c-fos protein in any of the cells cultured on the substrata when examined immunocytochemically. In thyroid tissue, c-fos protein was detected mainly in the nuclei of some follicle cells, and was occasionally and weakly seen in cytoplasm (Fig. 5c). Most smooth muscle cells of blood vessels were positively stained in nuclei and cytoplasm (Fig. 5d). The staining intensity was stronger in smooth muscle cells than in follicle cells (Fig. 5, c and d). Western blotting of the cell extracts showed that cells cultured on various substrata each contained slightly different levels of c-fos product of 62 kilodalton (KD) (Fig. 6). Cells cultured on laminin appeared to express the highest level of the

Fig. 6. Western blot analysis for c-fos protein in thyroid tissue, and follicle cells cultured on various substrata with or without 10 mU/ml TSH. Thyroid tissue (lanes b and c) and follicle cells cultured on plastic substratum (d and e), type I collagen (f and g), type IV collagen (h and i), fibronectin (j and k) or laminin (l and m) in the presence (e, g, i, k and m) or absence (d, f, h, j and l) of TSH were boiled with SDS, and the extracts were subjected to SDS-PAGE and immunoblot with an anti-c-fos antibody as described in Materials and Methods. Lanes a and b, Coomassie blue stain. lanes c–m, immunoblot. Lane a, molecular mass standards (MW) in kilodalton (KD). Arrowhead indicates 62 KD c-fos protein. Arrow indicates the faint expression of endogeneous biotin-binding protein of about 116 KD.

protein. TSH (10 mU/ml) did not affect the expression of the protein in the cells cultured on the substrata except for fibronectin, although TSH seemed to slightly enhance its expression in the cells on fibronectin (Fig. 6).

DISCUSSION

Our present study has shown that each of the ECM components tested (type I and IV collagen, fibronectin and laminin) induces differential effects on adhesion, proliferation, differentiation or c-fos protein expression of follicle cells. Interestingly, we have also demonstrated that TSH, a major effector for follicle cells, does not affect adhesion of the cells on any of the substrata or proliferation of the cells on fibronectin; nor does TSH affect c-fos protein expression of the cells on the substrata except for fibronectin under contact inhibition. Our results indicate that some behaviors of follicle cells are regulated not only by soluble factors, such as TSH, but also by insoluble ECM molecules.

Beta-1-integrin family plays a vital role in cell-to-ECM interaction. This group of cell surface heterodimers, cell adhesion molecules, is characterized by a common β_1 chain noncovalently associated with a distinctive α subunit (18). In the thyroid, Vitale *et al.* have shown that more than 97% of follicle cells express the β_1 chain, associated with a high level of α_3 and very low levels of α_1 , α_5 and α_6 (42). This indicates that follicle cells exhibit mainly VLA₃ (very late antigen₃, $\beta_1\alpha_3$, a receptor for collagen, fibronectin, entactin or epiriglin/kalinin) and express poorly a receptor ($\beta_1\alpha_6$) specific for laminin (36). These findings are consistent with our results that adhesion of follicle cells to laminin at least at early culture stages is very poor, as compared with adhesion to other ECM molecules. However, the rate of cell adhesion to each substratum gradually increased during continuous cultures. It is conceivable that in order for the cells to become adhesive to a substratum, the cells themselves may change expressions of integrin receptors (18, 27).

In proliferation, we have shown that nuclear BrdU intake clearly increases in the cells cultured on type IV collagen, as compared with those cultured on laminin or fibronectin, although there is no statistical significance between type IV collagen and type I collagen or plastic substratum. Woodley *et al.* also have demonstrated that type IV collagen promotes proliferation of skin keratinocytes (43). At least in type IV collagen and laminin, which are the main components of basement membrane, their report and ours suggest that type IV collagen plays a more positive role in proliferation of follicle cells than laminin. In addition, TSH inhibited proliferation of follicle cells cultured on the substrata except for fibronectin. In contrast, proliferation of the cells cul-

tured on fibronectin was not suppressed by TSH. The inhibition of proliferation by TSH is consistent with other reports in porcine follicle cells (15, 44). To our knowledge, our present study is the first demonstration that TSH does not affect proliferation of porcine follicle cells cultured on fibronectin, although at present the reason for that and its significance remain to be elucidated.

In a functional differentiation, we have shown that follicle cells cultured on laminin produce about 3 times as much T₃ as compared with those on other ECM molecules. Some investigators have reported that laminin clearly induces functional differentiation of mammary epithelial cells or hepatocytes (22, 7). Miettinen *et al.* indicate that laminin expression of follicle cells is associated with the differentiated state of normal or neoplastic thyroid tissues (23). These studies and ours suggest that laminin plays a more positive role in a functional differentiation than type I and IV collagen or fibronectin. However, plastic substratum induced the highest level of T₃ production of the cells among all of the substrata examined. This suggests that some differentiative properties of follicle cells may be regulated at an appropriate level through cell-to-ECM interaction.

For the first time, we have shown in this study that follicle cells express c-fos protein even in contact inhibition. In addition, TSH does not affect c-fos protein expression in any of the cells cultured on the substrata except for fibronectin, although TSH seems to slightly enhance its expression in the cells on fibronectin. Many studies indicate that ECM regulates gene expression and signal transduction (7, 18, 19, 28). Our present study supports these outcomes. In this study, we have shown that laminin induces the highest levels of both c-fos protein expression and T₃ production among the ECM molecules examined. Some studies indicate that c-fos protein expression is closely associated with the differentiated state of the epithelial cells in colon, salivary gland, kidney and thyroid (2, 34, 37). Furthermore, laminin has been shown to clearly induce functional differentiation of hepatocytes and mammary epithelial cells, and to be related to the differentiated state of follicle cells (7, 22, 23). These reports and ours suggest that c-fos protein expression of follicle cells under contact inhibition may be related to the laminin-induced cell differentiation.

In the cytologic localization of c-fos protein, we have shown that the protein is seen mainly in the nuclei of follicle cells, and occasionally and weakly in the cytoplasm. Roux *et al.* indicate that transport of c-fos protein from the cytoplasm, where it is synthesized, into the nucleus, depends on the stimulation of the cells by serum factors. In their study, c-fos protein is localized in the nucleus of fibroblasts fed with 10% FCS, while the protein is detected in the cytoplasm of the serum-

starved cells (26). This report and ours suggest that the cytologic localization of c-fos protein is transportable between the cytoplasm and the nucleus, which is controlled by extracellular signals. In addition, we have shown that c-fos protein expression in normal thyroid is stronger in smooth muscle cells of blood vessels than in follicle cells. Although the significance of this finding is unknown at present, the result will prompt further studies to clarify roles of c-fos protein in the thyroid gland in more detail.

In conclusion, ECM has been shown to affect some biological behaviors of thyroid follicle cells in a TSH-dependent or TSH-independent manner. To further understand behaviors of the cells or thyroid diseases, it is hoped that our study will prompt investigations of the cells, involving both cell-to-soluble factor and cell-to-ECM interactions.

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