The extracellular matrix component laminin promotes gap junction formation in the rat anterior pituitary gland

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Abstract

Folliculo-stellate (FS) cells in the anterior pituitary gland are believed to have multifunctional properties. FS cells connect to each other not only by mechanical means, but also by gap junctional cell-to-cell communication. Using transgenic rats that express green fluorescent protein (GFP) specifically in FS cells in the anterior pituitary gland (S100b-GFP rats), we recently revealed that FS cells in primary culture markedly change their shape, and form numerous interconnections with neighboring FS cells in the presence of laminin, an extracellular matrix (ECM) component of the basement membrane. Morphological and functional changes in cells are believed to be partly modified by matricrine signaling, by which ECM components function as cellular signals. In the present study, we examined whether gap junction formation between FS cells is affected by matricrine cues. A cell sorter was used to isolate FS cells from male S100b-GFP rat anterior pituitary for primary culture. We observed that mRNA and protein levels of connexin 43 in gap junction channels were clearly higher in the presence of laminin. In addition, we confirmed the formation of gap junctions between FS cells in primary culture by electron microscopy. Interestingly, we also observed that FS cells in the presence of laminin displayed well-developed rough endoplasmic reticulum and Golgi apparatus. Our findings suggest that, in anterior pituitary gland, FS cells may facilitate functional roles such as gap junctional cell-to-cell communication by matricrine signaling.

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Introduction

The anterior pituitary gland comprises five types of hormone-producing cells plus folliculo-stellate (FS) cells, which do not produce classical anterior pituitary hormones. FS cells have a star-like appearance (Farquhar 1957, Vila-Porcile 1972), and pseudolumina form in the center of FS cell clusters (Soji & Herbert 1989). It has been suggested that FS cells are stem cells, phagocytes, or cells that regulate hormone release (Inoue et al. 1999, Allaerts & Vankelecom 2005). In addition, gap junctions between FS-cells form intercellular channels that facilitate transmission of ions and small molecules between adjacent cells and allow direct cell–cell communication within the anterior pituitary gland (Fauquier et al. 2001, Sato et al. 2005). It is also known that connexin 43, which is the protein subunit that forms gap junctions, is mainly expressed in the anterior pituitary (Meda et al. 1993).

It has been shown that the lobular structures that are surrounded by basement membrane are responsible for organizing the functional unit of the anterior pituitary gland (Soji & Herbert 1989, Shirasawa et al. 2004). FS cells are located in the core of these lobular structures, and, interestingly, the cytoplasmic processes of FS cells attach to the basement membrane (Inoue et al. 1999, Shirasawa et al. 2004). Generally, FS cells can receive extracellular matrices (ECM) as signals that can exert functional changes, a process called matricrine signaling (Koide & Ito 2000, Miyamoto et al. 2007). Based on the histological features of FS cells, we hypothesized that FS cells require matricrine signaling for their functional roles.

Recently, Itakura et al. (2007) succeeded in producing an S100b-GFP transgenic rat that expresses green fluorescent protein (GFP) specifically in FS cells in the anterior pituitary. Using S100b-GFP rat anterior pituitary cells, we found that gap junctional cell-to-cell communication was immunohistochemically reconstructed between FS cells in vitro (Horiguchi et al. 2008). Furthermore, using living-cell imaging, we revealed that FS cells under the influence of ECM components may play important roles in determining and/or maintaining local cellular arrangement (Horiguchi et al. 2010). The above-mentioned morphological and functional characteristics of FS cells lend weight to the supposition that matricrine action influences cell-to-cell communication within the anterior pituitary gland. In order to confirm this hypothesis, we attempted to determine whether laminin, which is an ECM component of the basement membrane, affects gap junction formation between
FS cells. Using the S100b-GFP transgenic rat, we were able to observe the localization of connexin 43 in gap junction channels by immunohistochemistry, investigate the assembly of gap junctions by electron microscopy, and analyze the expression of connexin 43 by real-time PCR and western blotting.

Materials and Methods

Animals
Transgenic S100b-GFP rats express GFP under control of the promoter of the S100β protein gene, a marker of FS cells. The rats were donated by Prof. K Inoue of Saitama University and were bred in our laboratory (Itakura et al. 2007). Eight- to ten-week-old male rats weighing 250 to 300 g were given ad libitum access to food and water and housed under conditions of 12 h light:12 h darkness. The rats were killed by exsanguination from the right atrium under deep Nembutal anesthesia, and were then perfused with Ca\(^{2+}\) - and Mg\(^{2+}\)-free Hanks’ solution for primary culture. All animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University, which are based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell culture
Anterior pituitary cells of male S100b-GFP rats were dispersed as described previously (Horiguchi et al. 2008). Dispersed cells were separated into GFP-positive and GFP-negative cells by a cell sorter (MoFlo XDP: Beckman Coulter, Inc., Fullerton, CA, USA). GFP-positive cells were plated onto 8-well glass chamber slides (1 cm\(^2\)/well; Nalge Nunc International, Rochester, NY, USA), with or without a coating of 10 μg/cm\(^2\) of laminin (Millipore, Bedford, MA, USA), at a density of 1×10\(^5\) cells/cm\(^2\) in 400 μl of Medium 199 with Earle’s salts (Invitrogen) supplemented with 10% fetal bovine serum (Serum–Aldrich Corp.), 0.5 U/ml penicillin, and 0.5 μg/ml streptomycin (Invitrogen). In the other experiment group, laminin-coated slides were treated in medium containing 5 μg/ml monoclonal anti-integrin β1 antibody (clone Ha2/5, BD Biosciences, San Jose, CA, USA), which was shown to block signaling mediated by integrin β1 (Schultz & Arnett 1995); hamster IgM served as control. Cells were then cultured for 72 h at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air.

Time-lapse observation of primary culture
The GFP-positive cells were cultured in a CO\(_2\) gas culture chamber (Sankei Corp., Tokyo, Japan) with a thermostat (Kokensha Engineering Corp., Tokyo, Japan) on a fluorescence-inverted microscope (IX71; Olympus Corp., Tokyo, Japan). Culture conditions were the same as those described above. We time-lapse recorded the cells by using a digital camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan) and MetaMorph software (Molecular Devices Corp., Downingtown, PA, USA). Each observation was performed in triplicate.

Immunocytochemistry
Cultured cells fixed with 4% paraformaldehyde in 50 mM cacodylate buffer for 20 min at room temperature were first immersed in PBS containing 2% normal goat serum for 20 min at 30 °C, then incubated with anti-rat connexin 43 mouse monoclonal antibody (0.5 μg/ml, Zymed Lab, South San Francisco, CA, USA) overnight at room temperature. After washing with PBS, cells were incubated in PBS with Alexa Fluor 568-conjugated goat anti-mouse IgG diluted to 1:200. Absence of an observable nonspecific reaction was confirmed using normal mouse serum.

Electron microscopic observation
For observation of gap junctions between FS cells, FS cells in primary culture on the uncoated and laminin-coated surfaces were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 °C. Then, FS cells were postfixed with 1% OsO\(_4\) in 0.1 M phosphate buffer for 1 h at 4 °C, dehydrated in a series of graded alcohols, and embedded in epoxy resin (Quetol 812; Nissin EM Co., Tokyo, Japan). FS cells were sectioned into ultrathin slices with a Reichert–Nissei Ultracut S (Leica Microsystems, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and then observed under a Hitachi H-7600 electron microscope (Hitachi).

Quantification of connexin 43 mRNA levels by real-time reverse transcription-PCR
Total RNA fractions were prepared with Trizol reagent (Invitrogen) from cultured cells and incubated with RNase-free DNase I (1 U/tube; Promega Corp.). After inactivation of DNase I by heating for 10 min at 65 °C, cDNA was synthesized using the Superscript III reverse transcription (RT) kit with oligo-(dT)\(_{20}\) primer (Invitrogen). Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems, Carlsbad, CA, USA) was performed by using gene-specific primers and SYBR Premix Ex Taq (Takara, Tokyo, Japan). PCR cycling was performed in triplicate.
**Immunoblot analysis**

After primary culture, cells were washed in PBS and lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1% w/v SDS, 1% v/v Triton X-100, pH 7.5), and total protein was estimated by the Bradford assay (Sigma). Twenty micrograms of protein from each sample was applied to 12% SDS-PAGE. Proteins were then transferred electrophoretically to Immobilon-P transfer membrane (Millipore). The membrane was blocked with 2% normal goat serum in TBST (50 mM Tris, 100 mM NaCl, 0-1% v/v Tween 20, pH 7.4) for 30 min. After washing with TBST, the membrane was incubated overnight with mouse monoclonal anti-connexin 43 antibody (0.05 µg/ml; Zymed Lab) or mouse anti-β-actin antibody (0.1 µg/ml, BioVision, Mountain View, CA, USA), diluted in Can Get Signal solution (Toyobo, Osaka, Japan), followed by TBST washes, and incubated for 1 h with HRP-labeled secondary antibodies (Envision + System-HRP, anti-rabbit, Dako, Glostrup, Denmark). After washing with TBST, bands were visualized by 3,3’-diaminobenzidine. The membrane was scanned, and densitometric analysis was performed with ImageJ software (NIH, Bethesda, MD, USA). The results were normalized with β-actin. Each analysis was performed in triplicate.

**Statistical analysis**

All results were presented as the mean±S.E.M. Statistical analysis was performed using ANOVA with Fisher’s protected least significant difference test (StatView 5.0 software, SAS Institute, Inc., Cary, NC, USA). Differences between groups were considered to be statistically significant at a P value of <0.05.

**Results**

**Time-lapse imaging of FS cells in primary culture**

A cell sorter was used to isolate FS cells from male S100b-GFP rat anterior pituitary for primary culture. At 2 and 72 h of primary culture, the percentage of FS cells among all cells was 96.0±0.4 and 96.4±0.2% (mean±S.E.M., n=3) respectively. We observed the behavior of FS cells in primary culture on the uncoated and laminin-coated surfaces (Fig. 1A–D, Supplementary Movies 1–3, see section on supplementary data given at the end of this article). In culture for 2–24 h, we noted that FS cells on the uncoated surface were remarkably motile as compared with cells on laminin-coated surface. FS cells aggregated and formed clusters of approximately 12 cells on the uncoated surface at 24 h (Fig. 1A and Supplementary Movie 1, see section on supplementary data given at the end of this article). Although FS cells on the laminin-coated surface were solitary, paired, or formed small clusters of only a few FS cells at 2 h (Fig. 1B), they flattened and markedly extended their cytoplasmic processes at 24 h (Fig. 1B and Supplementary Movie 2, see section on supplementary data given at the end of this article). Later, the cytoplasmic processes of a large number of FS cells interconnected (Fig. 1B). In contrast, only a few FS cells extended their cytoplasmic processes on the uncoated surface at 24 h (Fig. 1A and Supplementary Movie 1, see section on supplementary data given at the end of this article). At 72 h, almost all FS cells on laminin were flattened, with interconnected cell bodies (Fig. 1B). However, FS cells on the uncoated surface formed clusters of approximately ten cells (Fig. 1A). FS cells on the laminin-coated surface with integrin β1 antibody aggregated and formed clusters of approximately 12 cells at 2–24 h (Fig. 1D and Supplementary Movie 3, see section on supplementary data given at the end of this article). They remained clustered until 72 h of primary culture (Fig. 1D). The motility of FS cells on laminin-coated surface with hamster IgM and those on laminin-coated surface was similar (Fig. 1C).

**Localization of connexin 43 on FS cells**

We determined the localization of connexin 43 by immunohistochemistry after 72 h in primary culture on both uncoated and laminin-coated surfaces. Immunoreactive connexin 43 was located mainly on cell bodies and partly in the cytoplasmic processes at the interconnection of FS cells on both uncoated and laminin-coated surfaces (Fig. 2B, D, H, and J). Connexin 43 immunoreactivity of FS cells on the laminin-coated surface was clearly greater than that observed on the uncoated surface (Fig. 2B and D). In contrast, treatment with anti-integrin β1 antibody completely blocked the increase in connexin 43 immunoreactivity between FS cells on laminin-coated surface with hamster IgM (Fig. 2H and J).

**Quantification of connexin 43 mRNA and protein levels**

To determine whether laminin affects connexin 43 gene expression in FS cells, we performed quantitative real-time RT-PCR. As compared with the uncoated surface, connexin 43 expression was higher in cells on the laminin-coated surface at 72 h (Fig. 2E). Using western blot analysis, connexin 43 immunoreactivity was also detected in FS cells as an ~43-kDa band, which conforms to the expected size of rat connexin 43 (Fig. 2F). When normalized with β-actin, the expression of connexin 43 protein was greater in FS cells on the laminin–coated surface (Fig. 2F). In contrast, at 72 h, the connexin 43 expression level on the laminin-coated surface with anti-integrin β1 antibody was not lower than that on the laminin-coated surface with hamster IgM (Fig. 2K and L).

**Electron microscopic observation of FS cells**

To verify the presence of gap junctions between FS cells, we used an electron microscope to observe FS cells in primary culture on the uncoated and laminin-coated surface. At gap
junctions, the intercellular space is 2–4 nm, and the membranes of cells are lined up in parallel. On the uncoated surface, there were few gap junctions between FS cells (Fig. 3). In contrast, we confirmed the presence of gap junctions between FS cells on the laminin-coated surface (Fig. 4A and B). We also observed that FS cells displayed developed rough endoplasmic reticulum and well-developed Golgi apparatus in the presence of laminin, but not in its absence (Figs 3 and 4).

**Discussion**

In the present study, we isolated pure FS cells from S100b-GFP rat anterior pituitary to eliminate the physical and functional effects of the hormone-producing cells and examined the direct involvement of laminin on gap junction formation between FS cells in primary culture. We showed that laminin promoted gap junction formation between FS cells.

The S100b-GFP rat (Itakura et al. 2007) model is a powerful tool for studying cell-to-cell communication between FS cells, and between FS cells and hormone-producing cells. In a recent study using this rat, we observed that FS cells in primary culture used their cytoplasmic processes to interconnect and form cell clusters (i.e. FS cells aggregated homophilically), and reported that these histological features *in vitro* provided a suitable environment for cell-to-cell communication in the anterior pituitary (Horiguchi et al. 2008). In the present study, we observed how the histological features between FS cells were altered in primary culture without hormone-producing cells. We were able to obtain pure GFP-positive FS cells with a cell sorter, and to use them in primary culture. The histological features of FS cells were the same in the presence or absence of GFP-negative cells, which are primarily hormone-producing cells. These findings suggest that primary culture of isolated FS cells was appropriate for analyzing FS cell-to-FS cell communication in the absence of the effects of hormone-producing cells.
Figure 2  Connexin 43 expression levels in primary culture of FS cells after 72 h of incubation. (A, B, C, D, G, H, I, and J) Immunocytochemistry for connexin 43 in primary culture of FS cells; (A and B) 72 h culture of FS cells on an uncoated surface; (C and D) 72 h culture of FS cells on the laminin-coated surface; (G and H) 72 h culture of FS cells with hamster IgM on the laminin-coated surface; (I and J) 72 h culture of FS cells with anti-integrin β1 antibody on the laminin-coated surface. (A, C, G, and I) Phase contrast images. (B, D, H, and J) Fluorescent images. Connexin 43 (red) immunoreactivity was observed between FS cells (green). Scale bar, 10 μm. (E and K) The expression of connexin 43 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) after 72 h incubation. (E) Connexin 43 mRNA expression was higher on the laminin-coated surface (laminin) than on the uncoated surface (uncoated). (K) Connexin 43 mRNA expression was not lower on the laminin-coated surface with anti-integrin β1 antibody (laminin Itgb-1 AB) than on the laminin-coated surface with hamster IgM (laminin Ham IgM). Connexin 43 mRNA levels were calculated as ratios of uncoated (E) and laminin Ham IgM (K) values respectively. (F and L) The amount of connexin 43 protein was determined by western blotting. The upper panel shows connexin 43 and the lower panel shows β-actin, the loading control. Western blotting data from three experiments (mean ± S.E.M., n=3) were quantified by densitometry and normalized with β-actin. (F) Expression of connexin 43 in FS cells on the laminin-coated surface (laminin) was significantly higher than that in FS cells on the uncoated surface (uncoated) (*P<0.05). (L) Expression of connexin 43 in FS cells on the laminin-coated surface with anti-integrin β1 antibody (laminin Itgb-1 AB) was not significantly lower than that in FS cells on laminin-coated surface with hamster IgM (laminin Ham IgM). Connexin 43 protein levels were calculated as ratios of uncoated (F) and laminin Ham IgM (L) values respectively.
In a previous study using living-cell imaging of S100b-GFP rat anterior pituitary cells, we first examined whether FS cells respond to ECM components and observed that FS cells in primary culture formed numerous interconnections with neighboring FS cells (Horiguchi et al. 2010). In the present study, we observed the behavior of isolated FS cells in the presence of laminin without hormone-producing cells. As shown in Fig. 1B, it is clear that laminin affects morphological changes in FS cells and increases interconnections between them.

Morand et al. (1996) showed that FS cells in vitro can transfer small molecules to neighboring FS cells through gap junction coupling in FS cell-to-FS cell communication. We observed that the immunoreactivity of connexin 43, which is the protein subunit forming the gap junction in the anterior pituitary gland (Meda et al. 1993) was greater on the laminin-coated surface than on the uncoated surface. Furthermore, fine structural formation of gap junctions between FS cells on the laminin-coated surface was confirmed by electron microscopy. These cells had developed rough endoplasmic reticulum and Golgi apparatus, as shown in Fig. 3. These morphological findings were expected due to the hyperfunctional status of FS cells in the presence of laminin, and they suggest that the increase in gap junction between FS cells under the influence of laminin is partly due to the up-regulation of connexin 43 mRNA and protein levels.

Laminin transduces signals mediated by integrins, which are the principal ECM receptors, and regulates gene expression and protein synthesis (Belkin & Stepp 2000, Hynes 2002). Studies indicate that there are two possible cascades mediated by integrins for connexin 43 up-regulation. One possibility is that the connexin 43 gene is a downstream target for an outside-in integrin signaling cascade (Spragg & Kass 2005); the other is that laminin, via the integrin signaling cascade, promotes the assembly of cell-to-cell adhesion mediated by E-cadherin before gap junction assembly, thereby up-regulating connexin 43 (Jongen et al. 1991, Frenzel & Johnson 1996). FS cells express integrin α3, α6, and β1, which are specific laminin receptors (Horiguchi et al. 2010), and E-cadherin (Kikuchi et al. 2006). As shown in Fig. 2, an antibody that inhibits the integrin β1 cascade did not block connexin 43 up-regulation. Connexin 43 up-regulation in the presence of laminin may be regulated by another integrin signaling cascade, such as integrin α6β4, and/or may depend on the mere increase of juxtaposed membranes that result from FS cell-to-FS cell adhesion.
mediated by E-cadherin. In addition, it is interesting that anti-integrin β1 antibody inhibited gap junction formation between juxtaposed membranes of FS cells (Fig. 2). Connexin 43 delivery to the cell surface has been shown to be facilitated by interactions with many connexin 43-binding proteins (Giepmans 2004). Their genes may be a downstream target for the integrin β1 signaling cascade. It remains to be confirmed whether connexin 43 up-regulation and gap junction formation in FS cells are caused by one or a combination of mechanisms. Nevertheless, to our knowledge, the present study is the first to demonstrate directly that laminin–integrin interaction triggers an increase in gap junction formation in FS cells.

Data from recent morphological and electrophysiological studies have led to speculation regarding the function of FS cells. One hypothesis maintains that FS cells interconnect via gap junctions to form a network in anterior pituitary gland that transmits signals regulating hormone release from hormone-producing cells, in addition to the established hypophysal portal vein system (Shirasawa et al. 2004, 2007, Sato et al. 2005). Matricrine signaling, which interacted with laminin as a signal mediated by integrin, may underlie the formation of this network of FS cells.

In conclusion, in a study using primary culture of FS cells isolated from S100b-GFP rat anterior pituitary, we revealed that gap junction assembly between FS cells was promoted by matricrine signaling. These findings suggest that FS cells in the presence of laminin may assume functional roles such as FS cell-to-FS cell communication in the anterior pituitary gland.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/JOE-10-0297.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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