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Note

Histochemical Analysis of Laminin α Chains in Diethylstilbestrol-Induced Prolactinoma in Rats

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Laminin, a major basement membrane component, is important in structural support and cell proliferation and differentiation. Its 19 isoforms are assemblies of α, β, and γ chains, and the α chains (α1-5) determine the isoform characteristics. Although our previous studies showed alterations in α chain expressions during anterior pituitary development, their expressions in pituitary tumors yet to be determined. The present study used a rat model of diethylstilbestrol (DES)-induced prolactinoma to examine α chain expressions during prolactinoma tumorigenesis (0–12 weeks of DES treatment) by *in situ* hybridization and immunohistochemistry. mRNA of α1, α3, and α4 chains was detected in control and after 4 weeks of DES treatment. These expressions were undetectable after 8 weeks of DES treatment and in prolactinoma (12 weeks of DES treatment). Immunohistochemistry showed that the α1 chain was localized in some anterior pituitary cells in control and after 4 weeks of treatment and in endothelial cells after 8 weeks of treatment. The α3 and α4 chains were expressed in endothelial cells, and immunoreactivity and the number of immunopositive cells decreased during DES treatment. These findings suggest that alteration of laminin α chains is related to abnormal cell proliferation and neovascularization during development of DES-induced prolactinoma.

Key words: anterior pituitary, laminin, prolactinoma, diethylstilbestrol, rat

I. Introduction

The anterior pituitary gland comprises 5 types of hormone-producing cells plus non-endocrine cells. Soji and Herbert [14] observed that these cells form microlobules enclosed by extracellular matrix including basement membrane. The basement membrane is a thin sheet-like extracellular matrix and is composed of several components, the most important of which is laminin. Laminin is essential for basement membrane assembly [11] and also plays a role as a ligand in affecting cellular activities through cell surface receptors [1, 2]. The laminin molecule is a heterotrimer of the α, β, and γ chains [5, 17]. Durbeej reported that the five α, three β, and three γ chains can form 19 laminin isoforms [3].

Among the three chains, the α chain is the most important, as it determines the characteristics of the laminin isoform and is required in laminin assembly and secretion [10, 18, 19]. Our previous study showed that expression of α chains varied during pituitary development [9]. Since expression of the α chains differs in relation to the stage of pituitary development, alteration of α chains might be important in cell growth and proliferation in the gland. Abnormal cell proliferation is a characteristic of tumorigenesis, as seen in pituitary adenoma. It is thus important to determine whether α chain expression is altered during tumorigenesis. In the present study, we used *in situ* hybridization and immunohistochemistry to identify expression and localization of α chains during prolactinoma tumorigenesis. Diethylstilbestrol (DES)-treated rats were used as the animal model of prolactinoma.

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II. Materials and Methods

Animals

This study used LEXF recombinant inbred (RI) rats [4, 15], which were established by Shisa *et al.* [12] to investigate genes involved in tumorigenesis. Compared with other spontaneous prolactinoma rat models, these rats develop prominent prolactinoma more rapidly after DES treatment, because of their high sensitivity to estrogen [15]. The animals were maintained on a 12-hr light/dark cycle and given access to conventional food and water *ad libitum*. Room temperature was maintained at around 22°C. The animals were used after receiving approval from the Institutional Animal Experiment Committee of Jichi Medical University. All experiments were conducted in accordance with the Institutional Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the Jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

DES administration

Sixty-day-old male LEXF RI rats were subcutaneously implanted with a silastic tube (inner diameter: 2 mm, outer diameter: 3 mm, length: 20 mm; Kaneka Medix, Osaka, Japan) containing DES (Sigma-Aldrich, St. Louis, MO, USA). The rats were sacrificed at 4, 8, or 12 weeks after implantation. LEXF RI rats not exposed to DES were used as control.

Tissue preparation

For hematoxylin and eosin (H&E) staining, deep anesthesia was induced by intraperitoneal administration of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). The pituitary glands were excised and immersed for 6 hr in sublimate-formalin at 4°C. The fixed tissues were then processed routinely and embedded in Pathoprep embedding media (Wako Pure Chemical Industries, Osaka, Japan), after which sagittal sections (2–3 µm) of the lateral wing of the gland were made. For *in situ* hybridization and immunohistochemistry, a solution with 4% paraformaldehyde in 0.05 M phosphate buffer (PB), pH 7.4, was perfused for 5 min through the left ventricle after anesthesia. The pituitary glands were excised and immersed overnight in the same fixative at 4°C. The tissues were transferred into 30% sucrose in 0.05 M PB buffer, pH 7.2, for 2 days at 4°C and then embedded in Tissue-Tek compound (Sakura Finetechnical, Tokyo, Japan). Cryosections (8 µm) were obtained using a cryostat (CM3000; Leica Microsystems, Wetzlar, Germany).

In situ hybridization

In situ hybridization for each α chain was performed as described previously (for protocol and primer information, see Ramadhani *et al.* [8]). mRNA expression was visualized with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Penzberg, Germany) by using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). DIG-labeled sense RNA probes and no probe were used as negative controls. No signal was detected in either control. Sections were observed using a BX-63 microscope (Olympus, Tokyo, Japan).

Immunofluorescence

Immunofluorescence was performed as described previously [8], with some modifications. Briefly, sections were boiled using a microwave device (MI-77; Azumaya, Tokyo, Japan) in 0.01 M citrate buffer, pH 6.0, for 10 min at 90°C for antigenic retrieval. The sections were then incubated in primary antibodies (for antibody information, see Ramadhani *et al.* [8]) for 90 min at 30°C. The endothelial cell marker biotinylated isolectin B4 (B-1205; Vector Laboratories, Burlingame, CA, USA) was added to the primary antibodies to detect endothelial cells. The sections were then incubated in secondary antibodies, namely, Alexa Fluor 488-conjugated goat anti rabbit IgG (1:200) and Alexa Fluor 568-conjugated goat anti rabbit IgG (1:200) (Life Technologies, Carlsbad, CA, USA). Biotinylated isolectin B4 was detected by Alexa Fluor 633-conjugated streptavidin (1:400). Sections were scanned using a confocal laser microscope (FV-1000; Olympus).

III. Results and Discussion

In control tissue, the anterior pituitary gland was composed mainly of chromophil cells (acidophils and basophils) and chromophobe cells, as well as some sinusoidal capillaries (Fig. 1a). Cell types remained mixed after 4 weeks of DES treatment (Fig. 1b), but monomorphic cells were predominant after 8 weeks of treatment (Fig. 1c). Capillaries were tortuous after 4 and 8 weeks of DES treatment (Fig. 1b, c). At 12 weeks monomorphic cells were present throughout the gland, and capillaries were branched (Fig. 1d). In addition to these morphological changes, previous studies using DES-treated LEXF RI rats found increased pituitary gland weight, high serum prolactin levels [15], an increase in prolactin cells (as determined by immunohistochemistry), and fine structural characteristics of high hormone synthesis and release in prolactin cells [4]. Prolactinoma was induced through hyperplasia after 12 weeks of DES treatment.

In situ hybridization was performed to determine α chain expression in the anterior pituitary during DES treatment. Expression of α1, α3, and α4 chain mRNAs was detected in control (Fig. 1e, m, q), but expression of α2 and α5 chain mRNAs was not (Fig. 1i, u). *In situ* hybridization signals for α1 and α3 chain mRNAs were detected in the cytoplasm of some anterior pituitary cells (Fig. 1e, m), while α4 chain mRNA was detected in cells around capillaries (Fig. 1q, asterisk). These results are consistent with our previous observation of α chain expression in adult

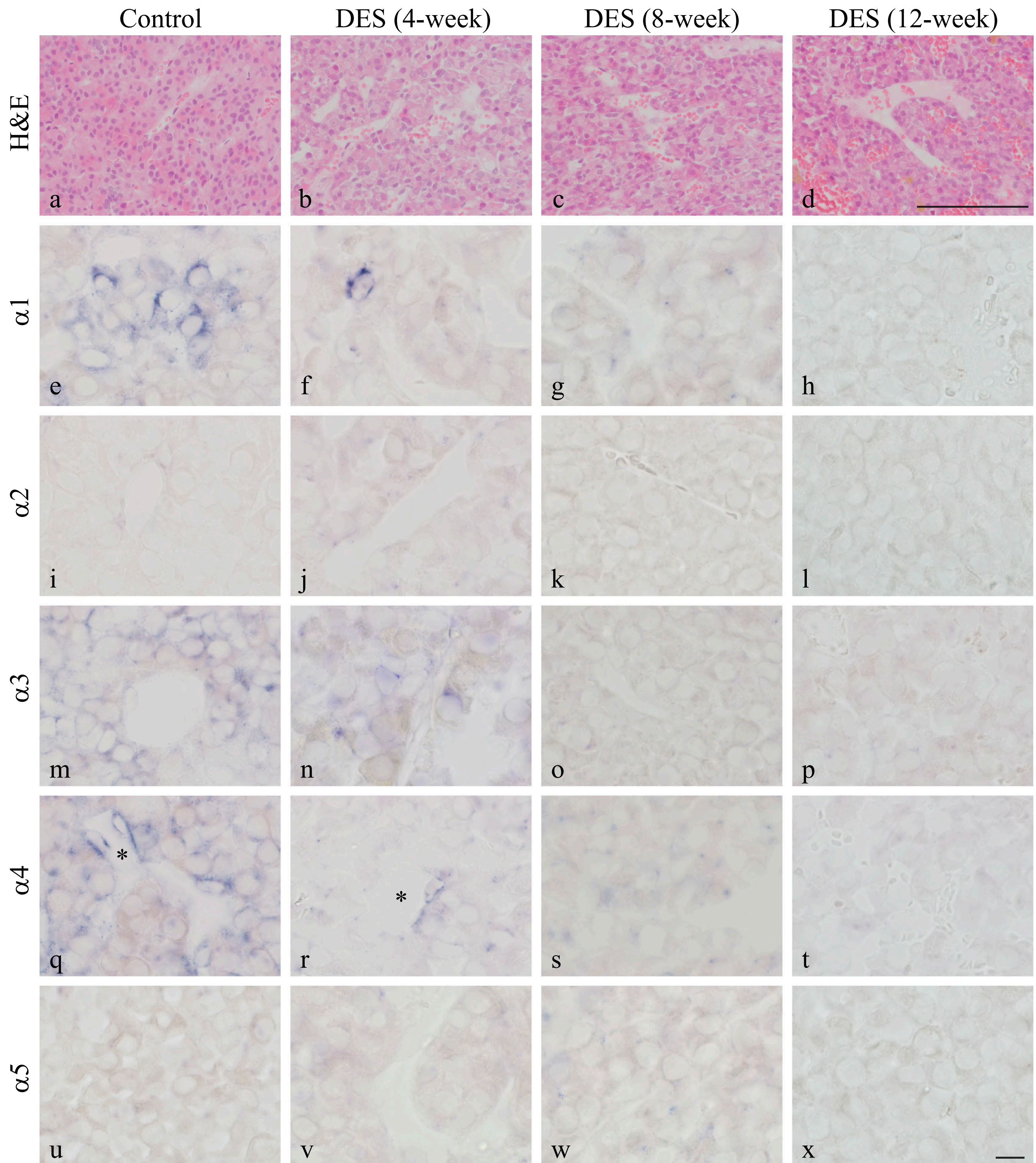

Fig. 1. Expression and localization of α chains in rat anterior pituitary during diethylstilbestrol (DES) treatment (normal: **a, e, i, m, q, u**; 4-week DES: **b, f, j, n, r, v**; 8-week DES: **c, g, k, o, s, w**; 12-week DES: **d, h, l, p, t, x**). The top panels show hematoxylin and eosin staining (H&E) in control and after 4, 8, and 12 weeks of DES treatment (**a–d**): (**a**: control) A sagittal section of the lateral wing of the gland shows capillaries lined by flat endothelial cells with spindle nuclei; (**b**: 4-week DES) Capillaries are tortuous, and the lumens are larger than in control; (**c**: 8-week DES) Capillaries are tortuous, and the lumens are larger than in 4-week DES; (**d**: 12-week DES) Capillaries are branched, and there is an extensive area of hemorrhage and hemosiderin deposition. *In situ* hybridization of the α1, α2, α3, α4, and α5 chains is shown in (**e–h**), (**i–l**), (**m–p**), (**q–t**), and (**u–x**), respectively. Positive signals for α chains are seen in cell cytoplasm (purple). Asterisk: capillary. Bars=100 µm (**a–d**) and 10 µm (**e–x**).

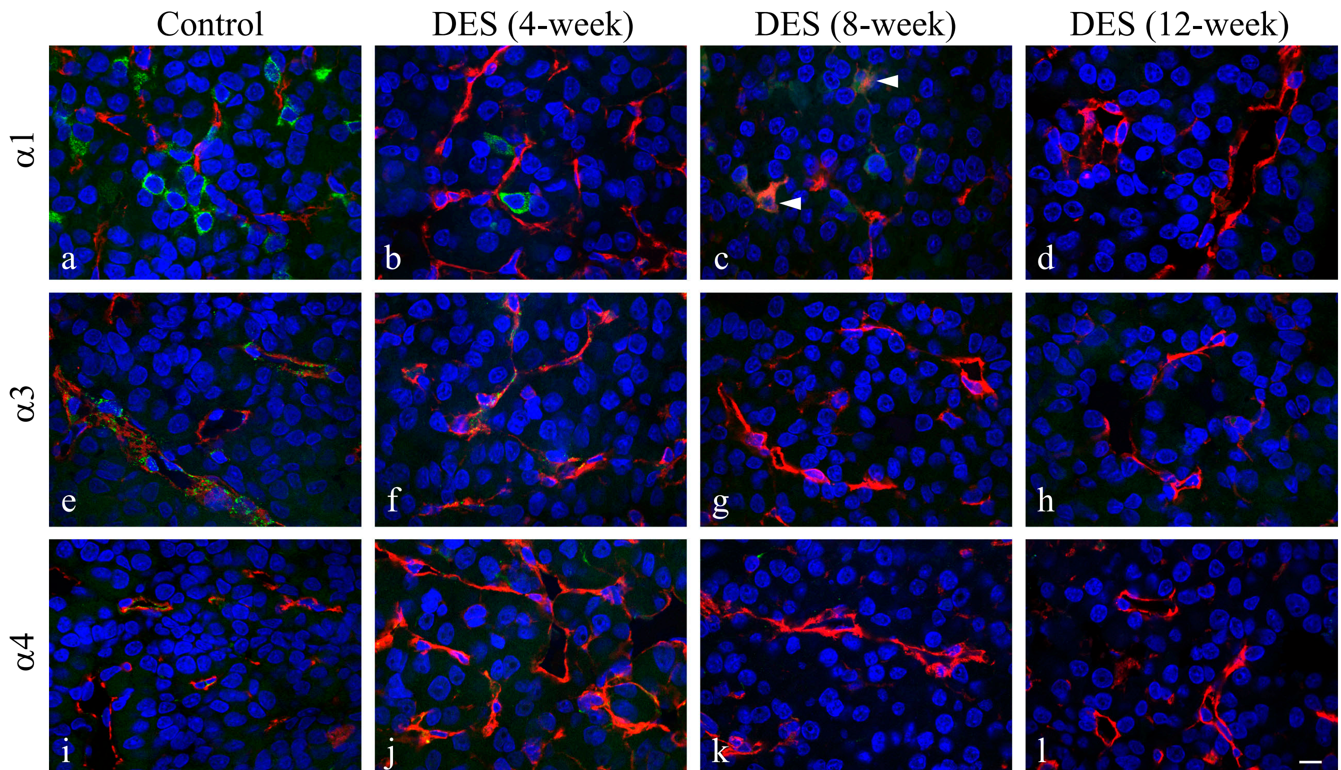

Fig. 2. Immunohistochemistry of α chains in rat anterior pituitary during diethylstilbestrol (DES) treatment (normal: **a, e, i**; 4-week DES: **b, f, j**; 8-week DES: **c, g, k**; 12-week DES: **d, h, l**). Laminin immunofluorescence using anti-α1 chain (**a–d**), anti-α3 chain (**e–h**), and anti-α4 chain (**i–l**) antibodies is shown in green. Isolectin B4 was used as an endothelial cell marker (red) and DAPI was used a nuclear marker (blue). Arrowhead: α1 chain-immunopositive endothelial cells. Bar=10 µm.

Wistar rats [8]. mRNA expression of the three α chains was sustained after 4 weeks of DES treatment (Fig. 1f, n, r), while the α chain-expressing cells were not detected after 8 weeks of DES treatment (Fig. 1g, h, o, p, s, t).

To identify the immunohistochemical localization of each α chain in DES-treated LEXF RI rats, the pituitaries were stained using α chain-specific antibodies and isolectin B4 to visualize endothelial cells. These α chain-specific antibodies recognize unassembled α chains in the cytoplasm but not assembled laminin in the extracellular space [8]. In the control and after 4 weeks of DES treatment, the α3 and α4 chains were stained in capillary endothelial cells detected by isolectin B4 staining (Fig. 2e, i, f, j), and the α1 chain was stained diffusely in some pituitary cells, but not in endothelial cells (Fig. 2a, b). LH cells produce the α1 chain in adult Wistar rats [8]. Therefore, we performed double-immunostaining for the α1 chain and LHβ to identify α1 chain-immunopositive cells. LH cells were α1 chain-immunopositive in the control and after 4 weeks of DES treatment. The α1 chain-immunopositive LH cells were undetectable after 8 weeks of DES treatment (data not shown). Several studies reported loss of cytoplasmic laminin staining in LH cells [6] and a decrease in the population of LH cells after estrogen treatment [13]. Taken together, these past observations and the present results suggest that α1 chain expression in LH cells and the number of LH cells change in response to DES.

Interestingly, after 8 weeks of DES treatment, the α1 chain was expressed in a few endothelial cells (Fig. 2c, arrowheads) but not in LH cells. We previously observed transient α1 chain expression in endothelial cells, along with vascular development in the gland during the late prenatal stage (embryonic day 19.5 [9]). These findings suggest a relationship between temporal expression of α1 chain in endothelial cells and new vascular formation in the developing pituitary. It is likely that α1 chain expression after 8 weeks of DES treatment is associated with neovascularization in prolactinoma tumorigenesis. In addition, the morphology of α1 chain-immunopositive endothelial cells (Fig. 2c, arrowheads) differed from that of α3 chain- and α4 chain-immunopositive endothelial cells (Fig. 2e, i). The α1 chain-immunopositive endothelial cells were large, polygonal, had large round nuclei in the center of the cells, and sometimes displayed cytoplasmic processes (Fig. 2c). The α1 chain was diffusely stained in cytoplasm. In contrast, α3 chain- and α4 chain-immunopositive endothelial cells were flat, had long cytoplasmic projections, and contained spindle nuclei (Fig. 2e, i). The α3 chain staining showed a punctate pattern in cytoplasm, while the α4 chain was diffusely stained in cytoplasm.

Immunoreactivity for the α3 chain was seen in the cytoplasm of endothelial cells in control and after 4 weeks of DES treatment (Fig. 2e, f), while the *in situ* hybridization signal for the α3 chain was seen only in some pituitary

cells (Fig. 1m, n). While this discrepancy requires further clarification in future studies, our previous study showed similar α3 chain expression in anterior pituitary cells during gland development (at postnatal day 30) [9]. Immunoreactivity for the α4 chain and the number of α4 chain-immunopositive cells were decreased after 8 weeks of DES treatment (Fig. 2k, l). Previously, we showed that α4 chain expression transiently decreased during normal development (postnatal day 5–10 [9]), and Jindatip *et al.* [7] observed a partial basement membrane around capillaries during the same period. Furthermore, Thyboll *et al.* [16] showed that α4 chain deletion impairs microvessel maturation. These findings suggest that the α4 chain is important in maturation of the vascular basement membrane.

In conclusion, the results of *in situ* hybridization and immunohistochemistry showed altered laminin expression and localization in rat anterior pituitary during tumorigenesis of DES-induced prolactinoma. These changes in expression and localization might be related to abnormal cell proliferation and neovascularization.

IV. Declaration of Interest

The authors have no conflict of interest that might prejudice the impartiality of this research.

V. Disclosure Statement

The authors have nothing to declare.

VI. Acknowledgments

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