Estrogen agonists, 17 β -estradiol, bisphenol A, and diethylstilbestrol, decrease cortactin expression in the mouse testis*

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Summary. Previous reports have revealed that estrogen agonists or anti-androgenic chemicals induce abnormal spermiogenesis in rodents. In the seminiferous epithelium, the apical ectoplasmic specialization (ES) is an actin-based (cell-cell) junctional structure developing between the Sertoli cells and spermatids as is the basal ES also — although it is located between adjoining Sertoli cells. In the apical and basal ES are several adhesion complex proteins that control the spermatid developing process. Cortactin, an actin-binding protein, is one of the ES adhesion proteins, combining with several cell-cell adhesions associating proteins. In the present study, 17 β -estradiol (E2, 1.2 μ g/kg), bisphenol A (BPA, 2.4 μ g/kg), and diethylstilbestrol (DES, 2.5 μ g/kg) were subcutaneously injected in ICR 12-week-old male mice. Mice testes

were observed for the expression of cortactin protein after E2, BPA, and DES treatments by Western blot analysis, immunohistochemical analysis, and immunoelectron microscopic analysis. Observations showed that the immunoreactivity of the treated testes was significantly decreased. The immunohistochemical reactivity of cortactin in the apical ES was decreased in the treated testis. In immunoelectron microscopic observations, ultrastructural immunolocalizations of cortactin protein in the apical ES by both E2 and BPA were decreased, and the immuno-gold particles of apical and basal ES by DES were much less than the control. In the toxicological field, cortactin may be considered to be one of the indicator proteins of abnormal spermiogenesis which is affected by exogenous chemicals, such as endocrine disrupting chemicals. In summary, this study helps toward understanding the cortactin protein expression underlying the histological abnormalities of spermatogenesis induced by exogenous hormonal chemical treatment.

Received January 30, 2006

* This work was supported by funds for research on endocrine disrupters from the Ministry of the Environment, and by a Grant-in-Aid for Scientific Research on a Priority Area (A) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Introduction

Exogenous chemicals which act as sex hormone agonists or antagonists may disrupt spermiogenesis. Previous reports have revealed that 17 β -estradiol (E2) (Toyama *et al.*, 2004), β -estradiol 3-benzoate (E2B) (Toyama *et al.*, 2001a, 2004), bisphenol A (BPA) (Toyama *et al.*, 2004; Toyama and Yuasa, 2004), and flutamide (FLUT) (Anahara *et al.*, 2004) induce abnormal spermiogenesis

in rodents. E2 and E2B are estrogens, BPA is a weak estrogen-like endocrine disrupting chemical (Sohoni and Sumpter, 1998), and FLUT is an anti-androgen compound used for the treatment and prevention of prostatic cancer (Neri *et al.*, 1979; Trump *et al.*, 2001). From a histological viewpoint, the effects of E2, E2B, BPA, and FLUT induce the deletion of the apical ectoplasmic specialization (ES) between the Sertoli cells and the spermatids, deforming the acrosomes and the nuclei of the spermatids (Toyama *et al.*, 2003; Anahara *et al.*, 2004). In addition, diethylstilbestrol (DES), an estrogen-like chemical, affects newborn rodent testes and causes an inhibition of Sertoli cell proliferation (Sharpe *et al.*, 1998) and retardation of meiosis (Toyama *et al.*, 2001b). Further, newborn DES-treated mice have a delayed formation of the basal ES construction (Toyama *et al.*, 2001b), but electron microscopy revealed no abnormal histological changes in adult DES-treated mice (unpublished data).

Spermatogenesis, a specific process of the proliferation and differentiation of the germ cells, is controlled by endocrine and local intercellular communications (Weinbauer and Wesseles, 1999). The apical ES is an actin-based (cell-cell) junctional structure developing in the Sertoli cells that faces the plasma membrane of the elongated spermatids covering the acrosomal cap (Toyama, 1976). The basal ES is also an actin-based junctional structure although it is located between adjoining Sertoli cells. The plasma membrane of the Sertoli cell, a subsurface cistern of the endoplasmic reticulum, and a layer of actin filaments are observed in both of the ES systems. The role of the apical ES has been hypothesized as: 1) shaping of the head of the spermatids (Fawcett *et al.*, 1971), 2) holding the younger spermatids (Brokelmann, 1963) orientating the elongating spermatids (Russell *et al.*, 1988), or 3) ejecting the mature spermatids into the lumen (spermiation) (Vitale-Calpe and Burgos, 1970). The biological signification of the basal ES as the blood-testis barrier has been established (Fawcett *et al.*, 1970).

In the apical and basal ES are several adhesion complex proteins that control the spermatid developing process (Lee and Cheng, 2004). Cortactin, an actin-binding protein, is one of the ES adhesion proteins, combining with several cell-cell adhesions associating proteins such as β 1-integrin, cadherin, or ILK (Chapin *et al.*, 2001). Kai *et al.* (2004) reported that the dominant spermiogenesis defect (Dspd) mutation mouse showed morphological deformations in the testes, and that their histological characterization was very similar to those found in E2, E2B, BPA, and FLUT-treated mice (Toyama *et al.*, 2003; Anahara *et al.*, 2004). The expression of

cortactin is significantly decreased in the *Dspd* mouse (Kai *et al.*, 2004); the same hold true for the testes of FLUT-treated mice is (Anahara *et al.*, 2006b). We therefore investigated the dynamics of the expression pattern of cortactin protein after E2, BPA, and DES treatments. In the toxicological field, cortactin may be considered to be one of the indicator proteins of abnormal spermiogenesis that is affected by exogenous chemicals, such as endocrine disrupting ones. This study helps toward understanding the cortactin protein expression underlying the histological abnormalities of spermatogenesis induced by exogenous hormonal chemical treatment.

Materials and Methods

Animal

ICR mice (12-week-old) were purchased from CLEA Japan Co. (Hamamatsu) and housed under a 12:12 h light: dark cycle, in a controlled temperature (24–26°C) room. Water and food were available *ad libitum*. All animals were treated according to the Chiba University Guide for the Care and Use of Laboratory Animals.

Experimental design

E2 (Sigma, St. Louis, MO, USA) 1.2 μ g/kg body weight/day, BPA (Wako Pure Chemical Industries, Osaka) 2.4 μ g/kg body weight/day, or DES (ICN Biomedicals Inc, OH, USA) 2.5 μ g/kg body weight/day were subcutaneously injected for five sequential days and the animals ($n = 5 - 7$) were sacrificed on the 6th day after the sequential five-day treatment. Control animals received the vehicle (corn oil). The injection protocol was based on that of Anahara *et al.* (2004).

Western blot analysis

The decapsulated testes ($n = 7$) from treated and control mice ($n = 7$) were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA 2Na, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin. The lysates were then homogenized on ice and centrifuged at 15,000 $\times$ g for 20 min at 4°C and homogenized. The resulting supernatants were used for SDS-polyacrylamide gel electrophoresis. Equal amounts of the proteins (2 μ g/lane) from the treated or control mice were applied on 10% SDS-polyacrylamide gels. The gels were then transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA,

USA) for semi dry electrophoresis (ATTO Corporation, Osaka). The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 10% skimmed milk and then incubated at 4°C for 24 h in a 1:4000 dilution of a mouse anti-cortactin monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA). Following incubation, the membranes were washed and incubated for 1 h at room temperature in a 1:20000 dilution of an HRP-labeled goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA, USA). The ECL Western-blotting detection system (Amersham, Piscataway, NJ, USA) was used to visualize the results. Analysis of the protein bands was performed by densitometry by using a software package from Scion Image (Frederick, MD, USA). Densitometry data from the treated groups and the control were expressed as means $\pm$ the standard error (S.D.) and compared by an independent *t*-test. The significance level for all the statistical tests was set at $P < 0.01$ or 0.05 .

Immunohistochemistry

The treated ($n = 5$) and control ($n = 5$) mice testes were perfusion-fixed with 10% formalin in PBS and embedded in paraffin wax. The nonspecific sites were blocked by incubation in 5% normal goat serum (NGS; Gibco BRL, Grand Island, NY, USA) for 1 h, and the sections were reacted with an anti-cortactin mouse monoclonal antibody (Upstate) at a 1:80 dilution at 4°C for 24 h. After rinsing, the sections were first incubated with a biotinylated anti-mouse IgG antibody (Funakoshi, Tokyo) at a 1:100

dilution for 1 h at room temperature and then with an avidin-biotinylated peroxidase complex (Streptavidin Biotin Complex Peroxidase Kit, Nacalai Tesque, Kyoto) for 30 min at room temperature. Immunohistochemical reactions were visualized using 3,3-diaminobenzidine (Peroxidase Stain DAB Kit (Dark gray Stain), Nacalai Tesque). The sections were then counterstained with Mayer's hematoxylin (Wako).

Immunoelectron microscopy

Treated ($n = 5$) and control ($n = 5$) mice were perfused transcardially with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4). The testes were cut into small pieces, immersed in the same fixative for an additional 1 h, rinsed with PBS, embedded in LR White (London Resin Company Limited, Reading, England), and sectioned at a thickness of 75 nm using an ultramicrotome (Leica No.702503, Tokyo).

After ultra-thin sections were placed on the nickel grid, the sections were treated with a solution of 5% NGS for 1 h at room temperature. After rinsing with PBS, the sections were incubated with an anti-cortactin antibody (Upstate) at a 1:80 dilution at 4°C for 24 h. The samples were then incubated with the rat anti-mouse IgG antibody conjugated 5nm gold particles (Funakoshi, Tokyo) at a 1:200 dilution at room temperature for 4 h. The sections were then rinsed with PBS, and stained with lead citrate. Observation was made using an electron microscope (JEM 1200EX; JEOL, Tokyo).

Fig. 1. Western blot analysis of cortactin in control and E2, BPA, and DES treated mouse testes. Compared with the control, the immunoreactive band from all the treated testes is faint. The relative level of cortactin in the control and treated testes is shown in the bar charts. Quantitative ratios of cortactin immunoreactivity are inserted in the bar charts. Values are expressed as ratio $\pm$ S.D. *: Significantly different ($p =$ or < 0.05) from the control, **: Significantly different ($P < 0.01$) from the control.

Fig. 2. Immunohistochemical localization of cortactin in control (A), E2-treated (B), BPA-treated (C), and DES-treated (D) mouse testis, counterstained with hematoxylin (blue). **A:** Cortactin immunoreactivity (brown) is localized at the apical ES (arrows). The basal ES (arrowheads) is also immunostained. **B–D:** Cortactin immunoreactivity (brown) is decreased in the apical ES (arrows). **D:** Cortactin immunoreactivity (brown) tends to be weak in the basal ES (arrowheads). Bar = 50 μ m

Results

Western blot analysis

Cortactin in the E2, BPA, and DES-treated testes was analyzed by Western blotting. The results showed that the treated testes showed weaker reaction bands than those of the control testes, although an immunoreactive band at around 83–85kDa was detected in both the control and treated mouse testes (Fig.1). A quantitative analysis of cortactin in the immunoreactivity of the control and treated testes (E2, BPA and DES) is also shown in Figure 1. The immunoreactivity of the treated testes was significantly decreased.

Immunohistochemical localization

The immunohistochemical localization of cortactin in the testis was examined in the control and treated mice (Fig. 2). In the controls, immunoreactivity was found in the apical and basal ES (Fig. 2A). Immunostaining of cortactin in the apical ES was observed around the head of the spermatids older than step 7. In contrast, the immunoreactivity in the apical ES had conspicuously decreased in the treated testes (Fig. 2B–D arrows).

Especially in the seminiferous tubules at stages VI to IX did cortactin immunoreactivity tend to be depressed. However, the immunoreactivity in the basal ES was not obviously affected in E2- and BPA-treated animals (Fig. 2B, C arrowheads). On the other hand, the DES-treated seminiferous tubules showed a weak immunoreactivity in the basal ES compared with the control (Fig. 2D arrowheads).

Immunoelectron microscopic observation

The ultrastructural immunolocalization of cortactin protein in the apical and the basal ES is shown in Figure 3. In the apical ES of the control testis, the most abundant cortactin expression appeared at the Sertoli cell sites, i.e. in the layer of actin filaments (Fig. 3A). In the basal ES of the control testis, a cortactin expression also appeared in the actin layer (Fig. 3E). In the E2- and BPA-treated testis — where the structure of the apical ES is deficient, immuno-gold particles were not seen around the heads of the spermatids, and deformation of the nuclei and acrosomes was also found (Fig. 3B, C). In the DES-treated testis, the immuno-gold particles of the apical and basal ES were much less than in the control; however, there was no deletion of the ES or deformation of the spermatids with the DES-treatment (Fig. 3D–F).

Fig. 3. Immuno-gold electron micrographs of elongated spermatids in control (A), E2- (B), BPA- (C), and DES-treated (D) mice stained with anti-cortactin antibody, and the basal ES in control (E) and DES-treated (F) animals. **A:** Immuno-gold is localized in the Sertoli cell (arrows) but not in the germ cell. The **inset** shows a higher magnification in the boxed area. **B** and **C:** Nuclei and acrosomes of spermatids are deformed (*). **Insets** show higher magnifications in the boxed areas. An immuno-gold particle is shown at the actin layer (arrows), but cortactin immunoreactivity is not found because of deletion of the apical ES (arrowheads). **D:** The apical ES is not deleted, but the cortactin immunoreactivity (arrows) with DES treatment is less than that of the control. The **inset** shows a higher magnification in the boxed areas. **E:** Immuno-golds are localized in the layer of actin adjoining the Sertoli cells (arrows). **F:** The basal ES is not deleted but the cortactin immunoreactivity with DES treatment is also less than that of the control. Ac: acrosome, N: nucleus, Se: Sertoli cell. Bars = 0.5 μ m

Discussion

The present study demonstrated that E2, BPA, and DES decreased the cortactin protein expression in the testes. The results suggest that estrogen and estrogen agonists affect the regulation of actin binding *via* cortactin expression in the mouse testis. From an immunohistological perspective, the cortactin

protein expression in E2 and BPA-treated testis was conspicuously decreased compared with the control. Immuno-electron microscopy revealed that cortactin protein was not seen at the deleted portions of the apical ES. This indicates that the expression of cortactin is related to the apical ES deletion. In the DES-treated mice, the decrease in cortactin protein was less than that with the other treatments. Immuno-electron microscopy showed that the number of immuno-gold particles was decreased

in the apical and basal ES in the DES-treated testis, though there was no abnormal ES with the DES-treatment. The results suggested that the target of cortactin protein in seminiferous tubules differed between the E2, BPA and DES. Toyama *et al.* (2001b) revealed that DES delayed the establishment of the basal ES for four weeks after neonatal DES treatment; however, our results showed that the basal ES was not changed in the adult animal with DES treatment. These results also show that DES affects protein regulation in the ES with treatment in adults but does not delete the ES. Different reactions of estrogen agonists in the ES were confirmed. Chapin *et al.* (2004) demonstrated that adherence junctional proteins, such as β 1-integrin, cadherin, and ILK, are related to the process of spermiation; however, there was little spermiation failure in our treated mice. Cortactin plays the role of shaping the acrosome and nucleus of the spermatids rather than the holding and preservation of spermatids in the seminiferous tubules. We speculate that the differences in the histological changes from E2, BPA, and DES proceed from variations in the chemical-targeting proteins, such as cortactin and other proteins. These results suggest that not only cortactin but also several cell-cell associating proteins may be related to the ES deletion.

According to previous studies, E2 (Toyama and Yuasa, 2004), BPA (Toyama and Yuasa, 2004; Toyama *et al.*, 2004), FLUT (Anahara *et al.*, 2004)-treated mice, and macrophage migration inhibitory factor (MIF) knockout mice (Anahara *et al.*, 2006a) show a similar abnormal histology in the testes, such as deletion of the apical ES and deformation of the acrosomes and spermatids. The study by Sharpe *et al.* (1998) demonstrated that the exposure to DES leads to permanent defects in Sertoli cell function rather than an effect on the germ cells themselves. The present study also reconfirmed that exogenous chemicals affect not only the germ cells, but also the intercellular junctions (the ES) of the Sertoli cells.

We speculate that the ES is sensitive and becomes a target of the seminiferous epithelium easily affected by exogenous chemicals, such as sex hormonal agonists and antagonists. It is hypothesized that several exogenous chemicals damage the ES junctional proteins — such as cortactin — and then have adverse effects on protein regulation in the Sertoli cells. For these reasons, further examination of the expression of junctional protein is needed to reveal the details of apical and basal ES function. In the future, the regulation of cortactin protein will be one of the indices of adverse effects on spermiogenesis by exogenous chemicals.

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