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MORPHOLOGICAL TRAITS, CELL PROLIFERATION AND APOPTOSIS IN ADRENOCORTICAL X-ZONE IN PREGNANT MICE

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Adrenocortical X-zone, a transitory cortical region in virgin female and immature male, exists exclusively in mice, and its detailed nature, regression and turnover remain unclear. In this study, the X-zone was investigated in pregnant Jcl:ICR mice, in aspects of morphological traits, cell proliferation by bromodeoxyuridine (BrdU) immunohistochemistry and apoptosis by *in situ* nick end-labeling of genomic DNA (TUNEL). In the early pregnancy, the intact X-zone, which took more than 30 % of the total adrenocortex, was composed exclusively of non-vacuolated cells, with smaller size and a darker nuclei than other permanent cortical cells. From day 8 of gestation, the X-zone involuted gradually and this degeneration process lasted about one week. There are similarities and discrepancies between Jcl:ICR mice and reported other strains in cellular composition and period of the involution, suggesting strain-difference due to genetic background. BrdU immunohistochemistry demonstrated an apparently more active cell proliferation in the intact X-zone than in other permanent cortical zones. Apoptotic cells were not observed during the X-zone involution by either hematoxylin-eosin staining or TUNEL.

Key words: adrenocortical X-zone / pregnant mice / cell proliferation / involution / apoptosis

A transitory adrenocortical region, termed X-zone, exists exclusively in mice (1, 2). The X-zone is localized in the innermost cortical area surrounding the medulla and has been considered to be distinct from the permanent cortex (1-4).

The X-zone is present both in virgin female and immature male mice (2, 3). In both sexes, the X-zone becomes evident around 2 weeks after birth (2-4). In males, it entirely disappears before maturity (2-4). In females, however, it continues to grow and disappears during pregnancy or, in the absence of pregnancy, during advanced mature life (2-4).

Because of these events, the X-zone provides a unique *in vivo* model for studying cell degeneration, regression and the regulatory mechanism. Recent investigations demonstrate that the X-zone not only shows a sex difference but a strain difference in morphological traits and the cell turnover mechanisms, suggesting that the X-zone may primarily controlled by genetic mechanisms and modified by the endocrine system (5-16).

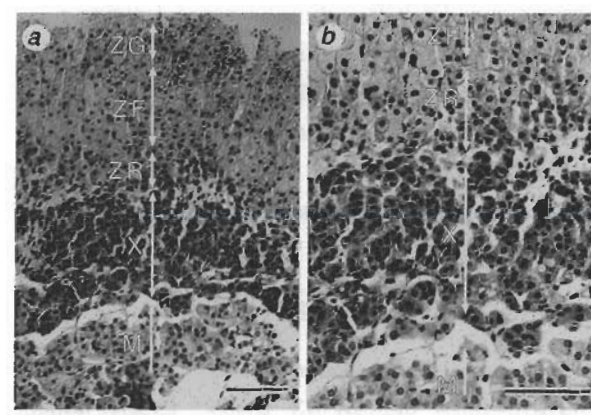

Fig. 1. Representative view of the intact X-zone at early gestation (day 6 of gestation). Between permanent cortex and medulla, the X-zone is visible and its width is more than 30% of total cortex (a). Compared with permanent cortex, X-zone cells are smaller and staining more darkly (a and b). The X-zone is composed exclusively of non-vacuolated cells (b). ZG: zona glomerulosa; ZF: zona fasciculata; ZR: zona reticularis; X: X-zone; M: medulla. Bars＝100 μm.

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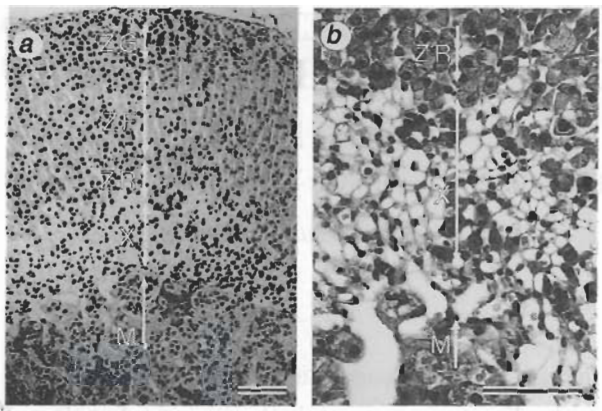

Fig. 2. Representative view of the involuting (degenerating) X-zone at mid-gestation (day 10 of gestation) (a). X-zone cells become highly vacuolated and nuclei are in the one side of cells, showing a fatty degeneration-like changes (b). ZG: zona glomerulosa; ZF: zona fasciculata; ZR: zona reticularis; X: X-zone; M: medulla. Bars =100 μm.

However, up to now, the detailed natures of the X-zone, where the X-zone is originated from; how cells turnover; how the X-zone involutes; what the fate of X-zone is and what function the X-zone plays remain unclear.

We therefore investigated the X-zone in aspects of cellular dynamics, its fate and possible steroidogenic functions. In this study, we report some data on the X-zone in pregnant females of a mouse strain Jcl:ICR. We addressed following issues, 1) morphological traits of the X-zone in this mouse strain; 2) the profile of the proliferation in the intact X-zone in pregnant female mice and 3) whether the involution of the X-zone during the pregnancy involves apoptosis.

MATERIALS AND METHODS

Animals and tissue preparation

Jcl:ICR mice (12 to 13 weeks, CLEA Japan, Tokyo) were used. The presence of a vaginal plug on the morning after mating indicated day 0 of gestation (G0). The gestation period was 18-19 days. The adrenal glands were harvested from mice at the interval of every two days: G2, G4, G6, G8, G10, G12, G14, G16 and G18. Two to four mice for each gestational day were used. The dissected adrenal glands were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) overnight, and then embedded in paraffin. The transverse sections (5 μm) through the central medullary tissue were stained with hematoxylin and eosin (HE) or immunohistochemistry as described below.

Light microscopic examination

Sections stained with HE were observed under a light microscope for examining morphological traits and apoptosis.

Immunocytochemical analysis of cell proliferation

Dams were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA) 50 mg/kg BW 1 h before sacrifice. The adrenal glands were fixed in 4% PFA in 0.1 M PB (pH 7.4) overnight. The sections were stained with a monoclonal anti-BrdU antibody (DAKO, Denmark) using avidin-biotin-peroxidase complex method as described (17). To estimate the labeling index (the percentage of labeled cortical cells), three to four sections of each gland containing the

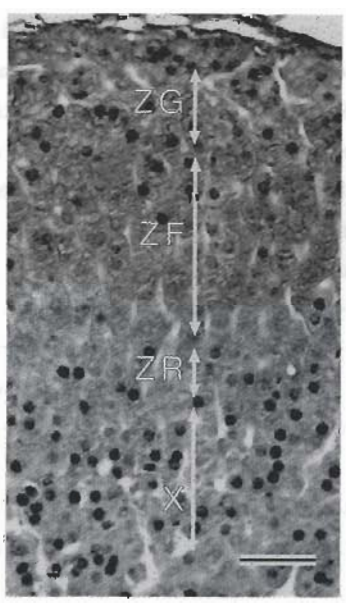

Fig. 3. Cell proliferation examined by BrdU immunohistochemistry in adrenal when the X-zone is intact (day 4 of gestation). Substantially more labeled nuclei are observed in the X-zone than permanent cortical zones. ZG: zona glomerulosa; ZF: zona fasciculata; ZR: zona reticularis; X: X-zone. Bar =50 μm.

central medulla were selected. The nuclei were counted in the cortex areas and medullary areas from the photomicrographs (X 100). The labeling index was calculated from the ratio of BrdU-labeled nuclei to the total cell number.

In situ nick end-labeling of genomic DNA (TUNEL) for analysis of apoptosis

DNA fragmentation associated with apoptosis was detected with TUNEL method (18). The staining was performed using ApopTag™ In Situ Apoptosis Detection Kit (ONCOR, Graithersburg, MD, USA), according to the protocol of the kit.

Statistical analysis

All data are shown as the mean ± SEM. Data were statistically analyzed with Mann-Whitney U-test.

RESULTS

1. Morphological views of the X-zone during pregnancy

The X-zone, localized between the zona reticularis of the cortex and medulla, was very evident during the early pregnancy (Fig. 1a). It took more than 30% of total cortex (Fig. 1a). Intact X-zone was composed exclusively of non-vacuolated cells, characterized by smaller cell size and darker-staining nuclei (Fig. 1b). From G8, degeneration of the X-zone was observed (Fig. 2). With progress of pregnancy, the X-zone gradually involuted. The width of the zone became smaller and the cells degenerated (Fig. 2a and b). Fig. 2 shows a representative view of degenerating X-zone on G10. Cells became highly vacuolated and nuclei localized in one side, showing a fatty degeneration-like change (Fig. 2b). On G14, the X-zone was not visible completely in the gland (data not shown).

2. Cell proliferation in the intact X-zone

Substantially more BrdU-labeled nuclei were observed in the intact X-zone than those in the other permanent cortical zones in the G4 adrenal (Fig. 3). Quantitative analysis revealed that BrdU-labeled index in the X-zone was significantly higher than that in the other permanent cortical zones (Fig. 4).

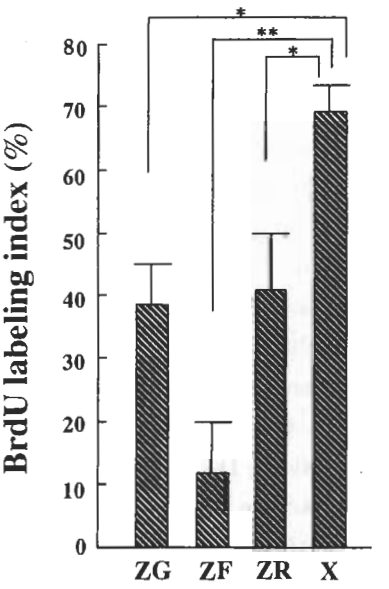

Fig. 4. BrdU labeling index in the X-zone significantly higher than that in other permanent cortexes of the adrenal (day 4 of gestation). * $P<0.05$; * * $P<0.01$. ZG: zona glomerulosa; ZF: zona fasciculata; ZR: zona reticularis; X: X-zone.

3. Apoptosis in the X-zone during pregnancy

Apoptosis in the intact or degenerating X-zone was checked by HE staining and TUNEL. On HE stained sections, there were not cells with characteristic appearances of apoptosis: membrane blebbing, cytoplasmic condensation, fragmentation and/or condensation of cell nuclei (19, 20).

In either intact or degenerating X-zone, apparent apoptotic cells were not observed by TUNEL (data not shown).

DISCUSSION

Our first major finding is that the intact X-zone is constituted exclusively of non-vacuolated cells in Jcl:ICR strain, a common laboratory mouse strain. Regarding the cell composition of the X-zone, three types of data have been reported using different mouse strains (9-12). According to the previous investigations in various natural strains or recombinant inbreds, X-zones were composed exclusively of vacuolated cells in A/J or A^w/A^w mice (10, 11) and were constituted only of non-vacuolated cells in SM/J, A^w/a or a/a mice (10, 11), while they contained both in KK or NC mice (9). It has been reported that agouti (a), a well-known locus controlling the coat color, on chromosome 2, in mouse

strain SM/J also control the adrenocortical X-zone cells (10). Accordingly, it has been proposed that the X-zone morphological traits is under the genetic control (8-12). In this study, the morphological traits of the X-zone in mouse strain Jcl: ICR may also be interpreted due to the genetic background of mouse strain.

The reports on degeneration process of the X-zone in female mice during the first pregnancy, were also not uniform. Jones using Wistar albino mice, reported that the X-zone gradually disappeared (4), while Deacon et al. suggested an abrupt disappearance in Swiss albino strain (7). We found that the X-zone in mouse strain Jcl:ICR disappeared gradually and its degeneration process lasted about one week. The mechanism responsible for the discrepancy in the above issues may also be the genetic differences in various strains.

The another striking finding in this study is an apparently active cell proliferation as deduced by BrdU immunohistochemistry in the intact X-zone compared to other cortical zones. At present, it is difficult to interpret what such abundant proliferating cells imply. Following up the migration of such BrdU-labeled cells may offer some information for whether or not and how the X-zone is involved in the adrenal zone formation, adrenal-cell replication and turnover.

The mechanism of the involution in the X-zone is still uncurtained. Early literatures of morphological description have suggested that the X-zone disappeared by "fatty degeneration" and vacuolization usually in females or by collapse of the cells and pyknosis of the nuclei in both sexes (1, 2, 13). Our HE staining did show the "fatty changes" or"fatty degeneration" of the X-zone during the involution. Since involution of some other transitory adrenocortical zones, such as the fetal zone in primates and part of the inner zone in rats, has been suggested associated with apoptosis (19-21), we examined apoptosis in the involuting X-zone in pregnant female mice. But our HE staining or TUNEL results did not show apparent apoptotic cells during this involution. These preliminary findings imply that apoptosis may not be associated with the involution of the X-zone in pregnant Jcl:ICR mice. Further confirmation using other methods for checking apoptosis like DNA ladder and transmission electron microscopy has yet to be performed. Because of the sex and strain differences, the relevant changes in male mice and other mouse strains might be diverse.

In this study, we used the adrenal in pregnant Jcl:ICR mice. Further comparison of the X-zone between the different mouse strains and between male and female during different life stage or in different endocrine conditions should promote understanding of how the X-zone is controlled by genetic background and by endocrine hormones.

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