Effects of Trichostatin A, a Histone Deacetylase Inhibitor, on Mouse Gonadal Development In Vitro

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Abstract. Sry, Sox9 and M33 are thought to act as architectural transcription factors or as a chromatin regulator in gonadal development. However, the direct relationship between chromatin structure and sex determination has not yet been revealed. To clarify the effect of chromatin structural change on gonadal development, we examined the effects of trichostatin A, a histone deacetylase inhibitor, on mouse gonadal development in vitro. In the 0.1 µM treated testicular explants, the size of the gonad was significantly decreased, although the testicular cord formation occurred normally. In the 1.0 µM treated explants, the gonads revealed one or two large testicular cords. Sox9 and MIS expressions suggest that Sertoli cell differentiation is induced normally within the testicular cord, while Dnmt3b expression suggests that several immature Sertoli cells are located on the outside of the testicular cord. The 3β-hsd expression indicates that Leydig cell differentiation occurs normally. On the other hand, germ cell loss was observed in the treated testicular explants. In the treated ovarian explants, the number of premeiotic germ cells was reduced without gonadal size change. Thus, trichostatin A affects the development of germ cells, but does not affect sex determination.

Key words: Sex determination, Gonad, Germ cell, Trichostatin A, Histone acetylation

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elucidated.
Recently, it has become obvious that transcriptional regulation by histone acetylation and deacetylation play crucial roles in gene expression during the differentiation and development of various cell types and tissues in mammals. Changes in chromatin organization have been observed in many biological systems that exhibit developmental or tissue-specific controls in gene expression [6, 7], suppression [8], cell cycle progression [9], and cell differentiation [10]. The histone code theory proposes that the post-transcriptional modification of the histone amino terminal is an epigenetic chromatin marking system that can regulate “ON-OFF” switches of transcription [11].

Given these findings, we were very interested in the role of histone hyperacetylation in mammalian sex determination and gonadal development. Histone hyperacetylation is thought to be induced by incubation of the mouse gonad in the presence of histone deacetylase inhibitors (HDIs), such as trichostatin A (TSA) and sodium butyrate (NaB). TSA is generally regarded as a highly potent and highly specific inhibitor of histone deacetylase [12]. HDIs suppress the activities of multiple HDACs, leading to an increase in histone acetylation (hyperacetylation). This histone acetylation induces an enhancement of the expression of specific genes that elicit extensive cellular morphological and metabolic changes, such as growth arrest, differentiation, and apoptosis [13].

In order to clarify the possible involvement of histone acetylation in sex determination, we here examined the effects of trichostatin A, a histone deacetylase inhibitor, on gonadal development of mice in vitro.

**Materials and Methods**

**Organ culture of mouse genital ridges**

Embryos were obtained from pregnant mice (ICR strain; SLC Japan Inc., Shizuoka, Japan) at approximately 10.5–11.5 dpc. After counting the tail somite number, heads were separated from embryos for rapid sex determination [14]. The genital ridges (gonads and mesonephros) were also isolated under a dissecting microscope (Fig. 1 A, B).

Trichostatin A (TSA) (Wako Chemicals, JAPAN) was dissolved in vehicle (DMSO) and administered to the organ culture of the genital ridge at concentrations of 0.1 and 1 µM. The left genital ridge was incubated with TSA, while the right one was incubated with DMSO (final concentration, 0.05%) as a control explant. Each genital ridge was placed onto an ISOPORE membrane filter (pore size, 3.0 µm; Millipore), floated on Dulbecco’s Modified Eagle’s Medium (SIGMA, JAPAN) containing 10% horse serum (GIBCO, BRL) and penicillin/streptomycin (GIBCO,BRL) as described previously [15], and cultured at 37°C for 3 or 5 days. All explants were subjected to histological and PCR analyses as described below.

**Morphometric analysis of gonadal explants**

After 3 days culture, gonadal explants were
imaged, and the size of gonad and the number of germ cells on sections were analyzed by Image J (runs on Mac OS X: The author, Wayne Rasband, Research Services Branch, the National Institute of Mental Health, Bethesda, Maryland, USA.). Germ cells were counted on histological sections. All the germ cells in every twenty sections from one explant were counted, and the total number of germ cells counted per testis was designated the total count. Values are means of 5 explants. All data were statistically evaluated by one-way ANOVA. To obtain the significance of difference between treated and control explants, Student’s t-Test was performed at the level of P<0.05.

Histological analysis
Each explant was fixed in Bouin’s solution, dehydrated in a graded series of ethanol, cleaned in xylene, and embedded in paraffin wax; then sectioned at 4 µm, stained with periodic acid Schiff (PAS)-hematoxylin [16], and observed by light microscopy. Some sections were subjected to immunohistochemistry as described below. For immunohistochemistry, anti-hsp86 (a germ cell marker; Santa Cruz Biotechnology, Santa Cruz, CA), anti-laminin (a basal membrane marker; Cappel, ICN Pharmaceuticals, Inc), anti-MIS (a differentiated Sertoli cell marker; Santa Cruz Biotechnology), and anti-Dnmt3b (DNA methyltransferase marker; Santa Cruz Biotechnology), were used in this study. Deparaffinized sections were blocked with 1% normal goat serum/PBS at room temperature for 30 min and incubated at 4 C for 12 h with anti-hsp86 (1:500 dilution), anti-laminin (1:500 dilution), anti-MIS (1:250 dilution), or anti-Dnmt3b (1:50 dilution) antibody. Before incubation with antibody, 0.4% pepsin (DAKO S300) digestion was required only for laminin staining. Immunoreactivities were visualized by successive incubation with biotinylated anti-rabbit IgG amplified with ABC kit (Vector Laboratory, Burlingame, CA, USA). After washing with PBS, they were placed in a reactive mixture with diaminobenzidine and H2O2.

Isolation of RNA and RT-PCR
Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s manual. Two micrograms of total RNA were used for synthesis of cDNA using Superscript reverse transcriptase (Gibco BRL, Gaithersburg, Maryland). The 1 µl of cDNA was amplified in a thermal cycler under a PCR condition with primers for M33 [17], Sox9 [18], MIS [19], 3β-hds and sf-1 [20], and Dnmt1 [21], described previously. The PCR products were run on 2% agarose gel in EDTA buffer. G3pdh primer was used as an internal standard control.

Results
The effects of TSA on gonadal development were assessed using 11.5 dpc mouse gonad organ cultures. The left genital ridge was treated with TSA, while the right one was treated with DMSO as a control explant. Through a preliminary experiment, the optimal concentrations of TSA were determined to be 0.1 µM and 1 µM.

Decrease in the size of the TSA treated testicular explant
At 0.1 µM and 1 µM TSA treatment, testicular development was inhibited and the size of the explant was remarkably decreased compared to that of control testicular explant (Fig. 1C, E, G, and Fig. 2). While, in the ovarian development, the size of the ovarian explant showed no change at 0.1 µM TSA treatment, although in the 1 µM treatment of TSA, the size of ovarian explants was significantly decreased (Fig. 1D, F, H, and Fig. 2). Thus, the size reduction of the gonad appears in a sex specific manner after TSA treatment.

Reduction of the testis size without affecting testicular cord formation
To determine the cause of the size reduction in the 0.1 µM TSA treated testicular explants, histological analysis and immunohistochemistry of laminin were performed. In the presence of 0.1 µM TSA, testicular cord formation was observed to be normal(Fig. 3B, H) as in the case of control explants (Fig. 3A, G), though the size was remarkably decreased. In the 1 µM treated testicular explants (Fig. 3C, I), the number of testicular cords was decreased and the diameter of each testicular cord was increased (data not shown). Inhibition of testicular cord formation occurred mainly at the surface area of the gonad. These data suggest that the reduction of gonadal size doesn’t affect testicular cord formation and laminin production in vitro in the 0.1 µM TSA treated testicular explants.
Germ cell loss in TSA treated gonadal explants both sexes

To determine the effects of TSA on germ cell development, the germ cell number was counted and compared with that of control explants. To identify the germ cells in the gonad, immunohistochemistry of hsp86 was performed. Hsp86 was detected in germ cells [22] of the control testis (Fig. 3D) and ovary (Fig. 4A). In the ovary, hsp86 was localized from primordial germ cells to premeiotic oocytes. Thus, hsp86 is a useful marker of germ cells in the developing gonad.

In the TSA treated gonadal explants, hsp86 was detected in the germ cell cytoplasm of both sexes. In the control and 0.1 µM treated testicular explants, most of hsp86 positive germ cells were located within the developing testicular cords (Fig. 3D, E), while in the 1 µM treated explants, about half of hsp86 positive germ cells were located on the outside of the testicular cords (Fig. 3F). On the outside of the cord, hsp86 positive germ cells did not enter meiosis. The number of the male germ cells was significantly decreased in the TSA treated explants (Fig. 5).

In the control ovarian explants, both hsp86 positive germ cells (Fig. 4B) and premeiotic germ cells (showing nuclear condensation) (Fig. 4A) were spread throughout the gonad, while, in the treated ovary, though hsp86 positive cells were located in the central portion of the gonad (Fig. 4E, F), premeiotic germ cells were present within the gonad (Fig. 4B, C). These results suggest that the germ cells at the surface area of the ovary may be affected by TSA treatment. The number of both hsp86 positive cells and premeiotic cells was significantly decreased in the TSA treated ovarian explants (Fig. 5), though the size of the ovary showed no change. Thus, germ cell loss independent of gonadal size reduction occurred in the 0.1 µM TSA treated ovarian explants.

Sertoli cell development in TSA treated testicular explants

Müllerian inhibiting substance (MIS) is a member of the TGF-β super family of cytokines and promotes regression of the male Mullerian duct. MIS was detected in the Sertoli cell of the testis from 12.5 dpc when cord formation is accomplished [19]. To examine the Sertoli cell differentiation in the TSA treated explants, immunohistochemistry of MIS was performed.

In the control explants, MIS was localized in the Sertoli cells of the developing testicular cords (Fig. 3J) suggesting that Sertoli cell differentiation was induced normally. In the TSA treated explants (0.1 µM-1 µM), MIS was detected in the Sertoli cells of the testicular cord (Fig. 3K, L). Thus, the Sertoli cells in the testicular cord developed normally in the TSA treated gonadal explants.

Dnmt3b expression in TSA treated gonadal explants

In mammals, silenced genes are associated with a defined chromatin state. The formation of an inactive chromatin structure associated with DNA methylation may be associated with histone deacetylase (HDAC), which interacts with methyltransferases. The Dnmt (DNA methyltransferase) family has a key role in cytosine methylation. Dnmt3b contains a PWWP domain and a plant homeodomain like the Zn finger domain, which binds to DNA and is thought to relate to chromatin remodeling [23]. Dnmt3b was detected in the Sertoli cells of the developing testicular cords (Fig. 3M). In the TSA treated gonad, Dnmt3b positive cells were detected both within and on the outside of the testicular cords (Fig. 3N, O). On the outside of the testicular cord, Dnmt3b positive cells were aggregated (Fig. 3O).
Expression of various cell type markers in TSA treated gonads (Fig. 6)

To examine the expression of sex determination related genes in the TSA treated gonad, RT-PCR was performed. The most severely affected gonadal explants (1 μM treated) were...
compared with control samples. No apparent changes were detected in the TSA treated testes (Fig. 6) and ovaries (data not shown). Sex9 and MIS were induced normally in the TSA treated testis, suggesting that Sertoli cell differentiation occurs normally [18]. Leydig cell marker (3β-Hsd) expression was induced normally in the TSA treated testis, suggesting that Leydig cell development also occurs normally in the TSA treated testis. M33 and Dnmt1, which react with HDAC and are thought to act as chromatin regulators during gonadal development, were induced normally in the TSA treated gonad.

Discussion

In this study, we revealed the effects of trichostatin A, a histone deacetylase inhibitor, on gonadal development in vitro. Histone hyperacetylation is induced by TSA and its effects appear in a sex specific manner.

In testicular development, the size of the gonad was remarkably reduced (40–50%) in the TSA treated gonadal explants (Fig. 1, 2), though the decrease of the gonadal size didn’t affect testicular cord formation in the 0.1 µM TSA treated testicular explant (Fig. 3). TSA may affect Sertoli cell proliferation and mesonephric cell migration. In addition, the form of testicular cord changed
slightly in the 1 µM TSA treated gonad. One or two large testicular cords were induced in the 1 µM TSA treated gonad. Inhibition of cell proliferation is thought to induce such an effect in the testis. Cell proliferation has an important role in the development and pattern formation of the gonadal development, especially in the determination between male or female pathway [24]. Thus, the 1 µM TSA treated gonad had potency to form testicular cord, but not enough to form complete testicular cord.

Hsp86 immunohistochemistry showed that the number of male germ cells were significantly decreased in the TSA treated gonadal explants. Testicular germ cells affect MIS expression in Sertoli cells by TNF-α [25]. However, in this study, MIS and SF-1 expressions revealed no change. Although, germ cells are generally thought to be not required for testicular cord formation [26], recent reports have shown that meiotic germ cells antagonize mesonephric cell migration and testicular cord formation [27], and that germ cells enter meiosis at 13.5 dpc when they develop on the outside of the gonad [28]. In our results, germ cells on the outside of the testicular cord were observed in the TSA treated testis (Fig. 3), and they may have a regional effect on testicular cord formation. A further analysis is required to clarify the exact role of the germ cells on the outside of the gonad.

The expressions of Sox9, MIS, SF-1 by RT-PCR, and that of MIS and laminin by immunohistochemistry show that TSA did not affect Sertoli cell development. Expression of Dnmt3b [29] was detected in Sertoli cells of the testicular cord. In the 1 µM TSA treated testis, Dnmt3b positive cells were localized not only within the cord but also on the outside of the cord. MIS and laminin were not detected in Dnmt3b positive cells on the outside of the cord. This suggests that testicular cord is not formed completely in the TSA treated testis, because Sertoli cell differentiation is partially affected by TSA treatment. The 5 days culture of the treated testis showed the same pattern of Dnmt3b expression, suggesting that the expression of Dnmt3b in the Sertoli-like cells on the outside of the cord doesn’t cause the delay of Sertoli cell differentiation (data not shown). The 3β-Hsd expression suggests that Leydig cell differentiation was normally induced in TSA treated testicular explants. Thus, the effect of TSA on the testis is one of inhibition of the germ cell proliferation.

In the ovarian development, the same concentration of TSA for testicular explants showed no effect on the size of ovary, although the number of germ cells and premeiotic germ cells were significantly reduced. Recently, it has been shown that histone acetylation plays an important role in meiosis, but its role is less well known in meiosis. In oocyte meiosis, meiosis specific events such as successive M phase without intervening DNA replication, pairing of homologous chromosomes, and asymmetric cell division, occur. It has been reported that HDAC1 is detected in *Xenopus*...
oocytes [30] and plays a crucial role in deacetylation of histone during meiosis. Recently, it was reported that TSA treatment affects the acetylation pattern in mouse oocytes [31], suggesting that most of these meiosis specific events and functions probably involve meiosis specific changes in chromatin structure and histone acetylation. In this study, TSA may have induced the arrest of meiosis and inhibited germ cell proliferation in the ovary. The inhibition of HDAC by TSA is thought to induce cell cycle arrest and inhibit cell proliferation in various cancer cells [32], and HDAC inhibition by TSA arrested the cell cycle [33]. TSA may arrest the cell cycle and inhibit cell proliferation resulting in reduction of gonadal size and germ cell number in the gonadal development in vitro.

Recently, it has been shown that TSA, a histone deacetylase inhibitor, induces cell cycle arrest, differentiation and/or apoptosis of many tumors. Therefore, TSA or HDIs are useful for chemotherapy for cancer [33]. In this study, we have revealed that TSA affects germ cell proliferation, but shows no direct effect on sex determination. Thus, TSA has low reproductive toxicity. The organ culture system described in this study will be a useful tool for studying epigenetical regulation of sex determination and for examining the direct effect of toxic chemicals on gonadal development.

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References