Effect of Histone Deacetylase Inhibitors on Early Preimplantation Development in Mouse Embryo

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Abstract: *Reversible acetylation of core histone plays an important role in the regulation of gene expression and DNA replication. We examined the effect of histone deacetylase inhibitors, trichostatin A and trapoxin, on the early preimplantation development of mouse embryos. When the embryos were cultured with trapoxin and trichostatin A at the concentration at which hyperacetylation of histone is induced in the 1- and 2-cell embryos, almost all the embryos cleaved to the 2-cell stage but not to the 4-cell stage. To determine the phase in which the embryos were arrested in the second cell cycle by trapoxin, analyses of DNA synthesis and DNA stain were conducted. DNA synthesis was detected in the embryos treated with trapoxin 23 h after insemination, and chromosome condensation was not observed in the embryos arrested at the 2-cell stage 48 h after insemination. These results suggest that the embryos were arrested at the G2 phase. Immunoblotting with anti p34^{cdc2} antibody showed that phosphorylated forms of p34^{cdc2} were detected in the 2-cell arrested embryos, indicating that p34^{cdc2} remained in an inactive form. These results suggest that hyperacetylation of histones prevent the embryos from expressing the genes involved in the activation of p34^{cdc2} to inhibit p34^{cdc2} activation.*

Key words: *Histone acetylation, Mouse, Embryo, Preimplantation development*

Acetylation of histone is involved in the various aspects of cellular functions, cell growth, differentiation and development [1–3]. Several biochemical analyses revealed that hyperacetylated histones play pivotal roles in the change in chromatin structure and the progression of the cell cycle [4–6], but little is known about the role of histone acetylation in the early developmental processes, such as cell cycle control and transcriptional regulation, in which chromatin functions differ from those of somatic cells [7].

Inhibitors of histone deacetylases can experimentally change the levels of acetylated histones, since the acetylation status of chromatin is determined by the equilibrium between activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [5]. Trichostatin A (TSA) and trapoxin are known as potent inhibitors of histone deacetylases [8]. TSA reversibly inhibits the mammalian histone deacetylase [9], whereas trapoxin causes inhibition by binding to the enzyme irreversibly [10]. Both of them are useful in analyzing the role of histone acetylation in chromatin structure and function as well as in determining the genes whose activities are regulated by histone acetylation.

Histone deacetylases has been shown to be involved in the regulation of embryonic development in several species [11, 12]. In the starfish embryo, TSA inhibits development before the formation of mesenchyme cells during the early gastrula stage. The TSA-sensitive period is limited to the midblastula stage, in which the rate of RNA synthesis increases [13]. In *Xenopus* embryos, HDAC protein and acetylated non-chromosomal histones are accumulated in the developing oocyte [14]. Hyperacetylated histones first accumulate early in gastrulation [7, 15]. Concentrations of TSA sufficient to induce histone hyperacetylation in Xenopus embryos delay gastrulation and cause diminished midtrunk and posterior formation, suggesting defects in mesoderm formation. Although the constitutive hyperacetylation of the histones induced by TSA does not affect either the cell division or differentiation before gastrulation, it prevents midblastula transition [7, 16].

In preimplantation mouse embryos, acetylation of hi-

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stones has been shown to play an important role in the regulation of gene expression [17, 18]. Culture of 1-cell embryos with TSA and trapoxin results in an increase in the amount of acetylated histone H4, indicating that histone deacetylase and acetyltransferase are already active at this time [19, 20]. Transcriptional activity is increased in the 2-cell embryo by the treatment with trapoxin [21]. Histone deacetylase prevents the repression of genes that are transiently expressed during the 2-cell stage [22, 23]. Activation of the embryonic genome is accompanied by a transient enrichment of acetylated histone H4 [20]. Much information is therefore accumulated about the involvement of histone acetylation in the regulation of gene expression, but no information about that in the regulation of development in early preimplantation embryos.

In order to obtain information on the role and significance of histone acetylation in driving the progression of the development and cell cycle in the mouse embryos, we examined the effects of two specific inhibitors of histone deacetylase, trapoxin and TSA, on early preimplantation development. We show that histone deacetylases inhibit progression from the 2-cell to the 4-cell stage. Moreover, The experiments on chromosome condensation and the BrdU incorporation suggested that embryos were arrested in the G2 phase of the 2-cell stage, in which $p34^{cdc2}$ protein kinase is accumulated in the phosphorylated form.

Materials and Methods

In vitro fertilization and culture of embryos

Female ddY mice, 21–23 days of age, and mature male ICR mice were purchased from SLC Japan (Shizuoka, Japan). The Female mice were superovulated with 5 IU of pregnant mare serum gonadotropin (SANKYO CO., LTD. Tokyo, Japan) and 5 IU of human chorionic gonadotropin (SANKYO CO., LTD. Tokyo, Japan) 48 h later. The ovulated oocytes were collected in Whitten's medium (WM medium) [24] from the ampullae of the oviduct 15–16 h after hCG injection. Sperm were obtained in WM medium from the cauda epididymis. The ovulated oocytes were inseminated with capacitated sperm that had first been incubated for 2 h at 37°C. One and a half hours after insemination, the embryos were washed with glucose-free CZB medium [25] and cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. The embryos that had formed no pronucleus (PN) 6 h after insemination were regarded as unfertilized and were removed. To examine the effect of histone deacetylase inhibitors on early development, the embryos were transferred to CZB medium containing 10 ng/ml trapoxin or 30 ng/ml TSA 1.5 h after insemination, and were observed 24 h and 48 h after insemination. These concentrations of the inhibitors are enough to induce hyperacetylation of histones in the 1- and 2-cell embryos [19, 20].

Detection of DNA synthesis

Embryos were incubated with 10 μM bromodeoxyuridine (BrdU) for 1 h from 22 h after insemination. After incubation with BrdU, the embryos were washed with phosphate buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) (PBS/BSA) and then fixed with 3.7% paraformaldehyde. The DNA was denatured by incubating the embryos with 2 N HCl at 37°C for 1 h and the sample was then neutralised by incubating with 0.1 M borate buffer, pH 8.5, for 15 min. The incorporated BrdU was detected as previously described [22]. Briefly, washed with PBS/BSA, the embryos were incubated with anti-BrdU monoclonal antibody (Boehiringer-Mannheim; dilution 1: 50) for 45 min, washed, and then incubated with an anti-mouse IgG antibody conjugated with Texas Red (Jakson ImmunoResearch Laboratories, Burlingame, CA). The embryos were mounted on a glass slide with a VectaShield (Vector Laboratories, Burlingame, CA), and observed under a Leica TCS 4D laser-scanning confocal microscope.

Observation of chromatin status

Embryos treated with trapoxin were collected 48 h after insemination in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (PBS/BSA) and the zona pellucida was removed with acid memco [26]. The zona pellucida free embryos were briefly washed in PBS containing 4 mg/ml polyvinylpyrrolidone and fixed in 3.7% paraformaldehyde. The embryos were washed in two drops of PBS/BSA, and transferred to RNase reaction buffer (40 mM Tris-Cl pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$) containing 0.5 mg/ml RNase for 1 h. The embryos were washed with three drops of RNase stop solution (PBS containing 15 mM EDTA) and then incubated with PBS containing 0.5 mg/ml propidium iodide for 10 min. The embryos were washed with PBS/BSA and mounted on a glass slide with VectaShield (Vector Laboratories, Burlingame, CA), and observed under a Leica TCS 4D laser-scanning confocal microscope.

Separation of phosphorylated forms of $p34^{cdc2}$ on SDS-PAGE

To obtain whole cell extracts, 50 embryos were ly-

sed in 15 μl of extraction buffer (pH 7.4) composed of 50 mM Tris-HCl, 1% Nonidet P-40, 5 mM EGTA, 15 mM $MgCl_2$, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 20 μg/ml phenylmethylsulfonyl fluoride. To the samples was added an equal volume of 2 × SDS sample buffer [27] and the mixture was boiled for 3 min. The proteins were separated on 10% polyacrylamide gel and the fractionated proteins were electrotransferred onto Immobilon-NC (Millipore, Bedford, MA, USA). The membrane was first blocked with Blocking Ace (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) for 30 min, and then incubated with anti PSTAIR antibody [28] in Tris-buffered saline containing 0.01% Tween-20 (TBST). The membrane was washed 3 times with TBST for 30 min each time, followed by overnight incubation at 4°C with anti-mouse IgG $F(ab')_2$ fragment conjugated with horseradish peroxidase (Life Science, Amersham, UK) at a 1:1000 dilution. Immunoreactive proteins were then detected with chemiluminescence reagent plus (NEN Life Science Product, Inc.,Boston, MA, USA).

Results

The embryos were incubated with trapoxin from 1.5 h after insemination, and its effects on development were examined. The results showed that the reagent did not affect PN formation (Table 1). Most of the embryos incubated with trapoxin formed PN until 6 h post insemination as well as the control embryos incubated without trapoxin. The first cleavage also was not affected by the treatment with trapoxin. Almost all the embryos cleaved to the 2-cell stage (Table 1). The second cleavage, however, was noticeably inhibited. Only 4% of the embryos incubated with trapoxin cleaved to the 3- or 4-cell stage, whereas more than 60% of the control embryos cleaved (Table 1). TSA, another inhibitor of histone deacetylase whose inhibitory mechanism is different from trapoxin, also arrested the embryos at the 2-cell stage (Table 1).

To determine the phase in which the embryos were arrested in the second cell cycle by trapoxin, analysis of DNA synthesis and DNA stain were conducted. First, DNA synthesis was detected in the embryos treated with trapoxin 23 h after insemination when DNA synthesis was detected in the control embryos (Fig. 1A). This indicates that embryos had entered the S phase. Second, DNA was stained with propidium iodide in the embryos arrested at the 2-cell stage 48 h after insemination. DNA was uniformly distributed in the nucleus, and chromosome condensation, a sign of the M phase in morphology, was not observed (Fig. 1B).

Nevertheless, cell cycle regulators to induce the M phase may be activated in these cells, since acetylation of histone changes the chromatin structure and can prevent chromosome condensation even if M phase regulator proteins are activated. To address this possibility, the phosphorylation state of $p34^{cdc2}$ was examined in the embryos treated with trapoxin 48 h after insemination (Fig. 2). $p34^{cdc2}$ is bound to cyclin B1 and is phosphorylated during the interphase and dephosphorylated in the M phase [29]. We previously reported that phosphorylated forms of $p34^{cdc2}$ were detected as the two upper bands of three different migrating bands on SDS-PAGE followed by immunoblotting with anti-PSTAIR antibody [30, 31]. In the embryos arrested at the 2-cell stage by the incubation with trapoxin, phosphorylated forms of $p34^{cdc2}$ were detected at a similar level to those in the embryos in the G2 phase 32 h after insemination, indicating that the cell cycle regulator to control G2/M phase transition was not activated. These results strongly suggest that the embryos were arrested in the G2 phase in the second cell cycle by trapoxin.

Table 1. Effect of histone deacetylase inhibitor on the development of mouse embryos*

Treatments [a]	No. (%) of embryos which formed pronuclei [b]	No. (%) of embryos developed to:	
		2 cells [c] (24 h)	>2 cell [c] (48 h)
None	152/195 (77.9)	145/152 (94.5)	99/152 (65.1)
Trapoxin	154/196 (78.6)	150/154 (97.4)	6/154 (3.9)
Trichostatin A	160/212 (75.5)	156/160 (97.5)	2/160 (1.3)

*Experiments were repeated three times, and data were accumulated. [a] Fertilized eggs were transferred to the medium containing 10 ng/ml trapoxin or 30 ng/ml trichostatin A 1.5 h after insemination. [b] Embryos which formed pronuclei were scored 6 h after insemination. [c] Embryos that had formed pronuclei 6 h after insemination were collected, further incubated and observed 24 h and 48 h.

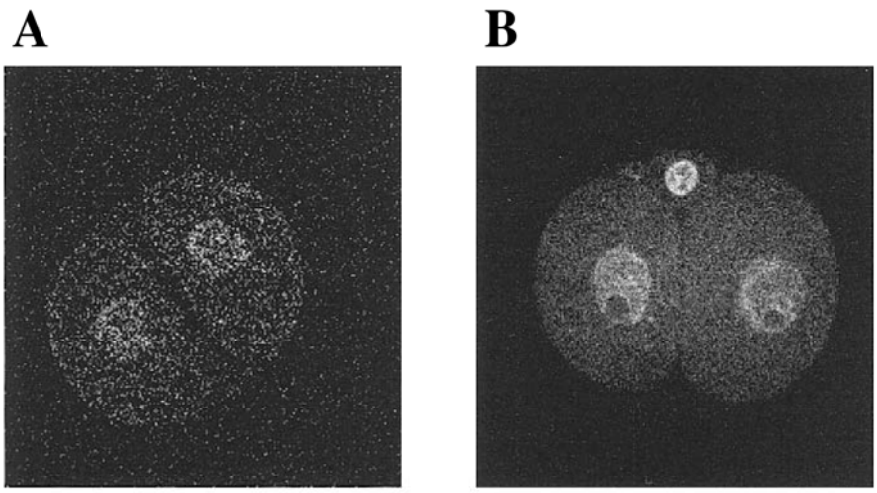

Fig. 1. Determination of the phase in the cell cycle in which the 2-cell embryos were arrested by trapoxin. Embryos were incubated with BrdU for 1 h at 22 h after insemination, and then immunostained with anti-BrdU antibody (A). DNA was stained with propidium iodide in the embryos arrested at the 2-cell stage 48 h after insemination (B). These embryos were observed by laser-scanning confocal microscopy.

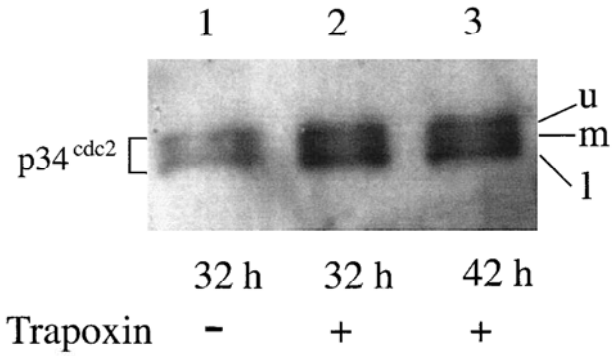

Fig. 2. Phosphorylation state of $p34^{cdc2}$ in the embryos arrested by trapoxin. The embryos were incubated with no addition (lane 1) or with 10 ng /ml trapoxin from 32 h (lane 2) and from 42 h (lane 3) after insemination. The lysates of the embryos were subjected to immunoblotting with the anti-PSTAIR antibody after SDS-PAGE. The three bands, referred to as upper, middle and lower bands, are indicated as u, m and l, respectively, on the right side.

Discussion

Our results support the idea that histone acetylation is involved in early preimplantation development [20]. We report here that trapoxin and TSA, inhibitors of histone deacetylase, did not affect PN formation or the first cleavage, but arrested embryo at the G2 phase of the 2-cell stage. Hyperacetylation of histones, induced by these inhibitors, may disrupt the proper pattern of expression of stage specific genes, since the status of histone acetylation affects the structure of chromatin and regulation of transcription [8, 32]. A previous report showed that mouse embryos could progress to the first cleavage, but that did not develop beyond the G2 phase of the 2-cell stage, when the transcription was inhibited by α-amanitin [33]. It is therefore possible that the abnormality in transcriptional regulation by histone hyperacetylation, induced by histone deacetylase inhibitor, did not affect the first cleavage, but prevented the second cleavage.

Trapoxin and TSA may decrease the level of expression of the genes required for the second cleavage. These inhibitors may increase the expression of repressed genes to decrease that of the genes that had been actively expressed, since the amount of transcription factors seems to be limited to the 1- and 2-cell embryos [22]. Increased histone acetylation relieved the chromatin from the repressive state and facilitated the random binding of transcription factors to DNA in chromatin, resulting in a lack of transcription factors to bind the genes required for the second cell progression. This hypothesis is consistent with the results showing that oxamflatin, an inhibitor of histone deacetylase, increased the expression of some genes, but decreased

that of others in NIH3T3-derived transformed cells [34], so that the inhibitor of histone deacetylase does not always increase the level of expression of the genes.

It is known that the stage specific expressions of genes are important in early development [7, 35]. In the starfish embryo [13], the treatment with TSA induced hyperacetylation of histones in chromatin during the blastula stages, but cell cycle progression proceeded normally with slower rates than that of the control. Development of the treated embryos was arrested during the early gastrula stage, thereby preventing subsequent formation of mesenchyme cells. The TSA sensitive period was limited to the midblastula stage, when no obvious morphological manifestation occurs, suggesting that the progression of development through the gastrula stage required some events occurring in the midblastula stage which are dependent on the normal cycle of histone acetylation and deacetylation. In the early mouse embryo, the temporally restricted spatial localization of acetylated histone controlled the zygotic gene activation [20, 36], suggesting that proper acetylation levels are required for the stage specific expression of genes.

It has been proposed that histone acetylation might be involved as a mechanism for maintaining cell memory through the S and M phases [37]. Patterns of gene expression need to be reinstated after passage of the DNA replication fork in the S phase and after general transcriptional repression during mitosis. Existing nucleosomal acetylation states can be transmitted to newly assembled chromatin after replication [38], and hyperacetylated regions of chromosomes in the metaphase appear in the same chromatin domains in the daughter cells [39]. Inhibitors of histone deacetylase would change the pattern of histone acetylation in the 1- and 2-cell embryos and disrupt the proper reinstatement of the patterns of gene expression after the S and M phases, resulting in the arrest in the G2 phase of the 2-cell stage. In the mouse embryo, proper modulation of acetylation levels is therefore very important for development during the early preimplantation stage. Specific inhibitors of histone acetyltransferases would also aid in more clearly defining the role of acetylated core histone in the processes of chromatin remodeling and preparation for Zygotic Gene Activation (ZGA) in preimplantation embryos.

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