Inhibition of Histone Deacetylase Activity by Trichostatin A Modulates Gene Expression during Mouse Embryogenesis without Apparent Toxicity

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Abstract

Remodeling of the chromatin template by inhibition of histone deacetylase (HDAC) activities represents a major goal for transcriptional therapy in neoplastic diseases. Recently, a number of specific and potent HDAC-inhibitors that modulate in vitro cell growth and differentiation have been developed. In this study we analyzed the effect of trichostatin A (TSA), a specific and potent HDAC-inhibitor, on mouse embryos developing in vivo. When administered i.p. to pregnant mice (at a concentration of 0.5–1 mg/kg) at postimplantation stages (embryonic day 8 to embryonic day 10), TSA was not toxic for the mother and did not cause any obvious malformation or somitogenesis or at later stages of development. Treated embryos were born at similar frequency and were indistinguishable from control animals, developing normally, and were fertile. Interestingly, embryos from TSA-treated mice killed during somitogenesis were modestly but consistently larger than control embryos and presented an increased (+2 to +6) number of somites. This correlated with an increased acetylation of histone H4, the number of somites expressing the myogenic factor Myf-5, and the expression of Notch, RARα2, and RARβ2 mRNAs. These data indicate that the effects of TSA on transcription: (a) are not toxic for the mother; (b) transiently accelerated growth in mouse embryos without perturbing embryogenesis; and (c) do not result in teratogenesis, at least in rodents. Thus, TSA might represent a nontoxic and effective agent for the transcriptional therapy of neoplasia.

Introduction

A common multiprotein complex containing catalytically active HDACs mediates the transcriptional repression of transcription factors such as Mad/MAX, E2F, YY1, or members of the steroid/thyroid receptor superfamilies (1–3). Deacetylation of nucleosomal core histone tails by the HDACs leads to a chromatin conformation that inhibits transcription, whereas histone acetylation results in transcriptional activation after local chromatin remodeling and recruitment of the RNA polymerase II complexes (2, 3).

Drugs modulating the acetylation status of histones have been proposed recently as a novel approach for the molecular treatment of cancer (4, 5). In AMLs, different chromosomal translocation-generating fusion gene products, such as PML/RARα, PLZF/RARα, and AML1/ETO, induce an abnormal regulation of HDAC activities, an event that plays a crucial role in the pathogenesis and maintenance of the transformed phenotype (6–10). In agreement with these biological findings, clinical efficacy has been reported for the HDAC-inhibitor butyrate and its analogue phenylbutyrate in the treatment of AML and in a RA-resistant acute promyelocytic leukemia patient (11, 12). A potential use of HDAC-inhibitors is also foreseen for cancer, with inactivation of tumor suppressor genes attributable to the hypermethylation of their promoters. HDAC, indeed, is involved in the transcriptional silencing of methylated promoters (3, 13).

However, the availability of HDAC-inhibitors for clinical use is limited. Butyrates and their derivatives have limited toxicity, but they act as nonspecific, transient, and reversible HDAC-inhibitors. Recently, a number of highly specific, stable, and more potent inhibitors of HDAC activities have been developed (14). Among these, TSA is one of the most active and studied. TSA has been found effective in modulating cell growth and differentiation in a number of in vitro systems, including carcinoma cell lines and myeloid leukemias, suggesting its potential use in transcriptional therapy of neoplasia (4, 6, 15–17).

A major issue concerning transcriptional therapy with HDAC-inhibitors is the expected toxicity of these compounds. Indeed, the general involvement of HDACs in a variety of fundamental cellular processes suggests a wide range of biological effects for HDAC-inhibitors when used as drugs. In support of this view, a recent publication reported malformations in embryos treated with TSA in vitro (18). However, a systematic analysis of the toxicity of HDAC-inhibitors during embryo development and in the adult life is lacking. In this study, we investigated the effect of TSA treatment in adult mice and during embryonic development and report that, despite its wide effects on histone acetylation and gene expression, TSA is not toxic and does not perturb mouse embryonic or postnatal development.

Materials and Methods

CD1 pregnant mice (E 8, 9, and 10) received i.p. injections of 15 mg of TSA (WAKO Chemicals, Neuss, Germany) in 0.2 ml of DMSO (Sigma, Milano, Italy) or 0.2 ml of vehicle alone. In some experiments, the same dose was repeated after 24 h. At the time indicated, mice were killed, and the embryos were observed under a dissecting stereomicroscope to evaluate their viability, the presence of external malformations, and the number of somites. Embryos were then fixed in 4% paraformaldehyde for histology, X-Gal staining, and whole-mount in situ hybridization using Notch and RARα1, -α2, and -β2 antisense probes (19–22). Immunoblot analysis was performed on total embryonic homogenates (60 μg) using anti-histone H4 and anti-acetylated histone H4 antibodies (Upstate Biotechnology). Densitometric analysis was performed using the Advanced Image Data Analyzer (Raytest, Milan, Italy).

Results and Discussion

The administration of a single TSA i.p. injection (15 μg) to pregnant CD1 outbred mice at E 8.9, or 10 did not result in any apparent toxicity for the mother and did not cause embryo malformations or defects at birth or during the animals’ postnatal growth. Animals treated in utero developed to normal adult size and were fertile.
Observation under a dissecting microscope revealed that 6- to 24-h TSA treatment of the mother constantly resulted in a slight, but statistically significant, increase in the number of somites in E 8.5-, 9-, or 9.5-treated embryos. Treatment with the vehicle (DMSO) induced no variations in any of the parameters analyzed (control; Fig. 1A). From E 8 to E 11, the number of somites represents the simplest and most faithful indicator of the embryonic stage of development.

We investigated whether the effect of TSA on embryos correlated with modifications of histone acetylation. Fig. 1B shows an immunoblotting analysis of extracts prepared from three control and three TSA-treated embryos using an antibody that recognizes the acetylated form of histone H4. Although these forms were already detectable in control whole embryos, they appeared more abundant in embryos from TSA-treated mice; this did not reflect an increased accumulation of the H4 protein as shown by an antibody recognizing the histone H4 protein in the same samples (Fig. 1, B and C). It appeared therefore that in vivo treatment with TSA affects mammalian embryonic development but is not embryotoxic. This is at variance with a previous study (18) that showed malformations in early postimplantation embryos exposed in vitro to TSA. In this case, direct exposure to high TSA concentrations of embryos, which in culture develop under critical conditions, may have caused effects that are not seen after exposure in vivo. Indeed, even repeating TSA administration after 24 h did not result in apparent abnormalities in treated embryos. This discrepancy might be attributable to the actual concentrations of TSA, which may not be directly comparable between the two studies.

During vertebrate embryogenesis, somites are formed in a strict cranio-caudal order by the successive segmentation of the paraxial mesoderm flanking the neural tube (23). Caudal to the most recently formed somite, the paraxial mesoderm appears morphologically unsegmented. Subsequently, somites become compartmentalized into a dorsal epithelial dermomyotome (future muscle and dermis) and a ventral mesenchymal sclerotome (future vertebral column and ribs).

We therefore evaluated whether i.p. injection of TSA could modulate in vivo the expression of Myf-5 and Notch, two genes involved in somitogenesis (24). Myf-5 is a basic-helix-loop-helix transcriptional regulator of muscle determination and is a useful marker of somites that contain cells already committed to skeletal myogenesis (25). Once formed, the somites follow a normal process of maturation, and Myf-5 is activated at the proper time, i.e., in the second- to third-last-formed somite. Myf-5 expression was evaluated by staining for X-Gal Myf-5/nlacZ (Myf-5/+ ) embryos, where the nLacZ reporter gene has been targeted into the Myf-5 locus (26). Fig. 2 (A and B) shows that both the total number of somites and the number of Myf-5-expressing somites (revealed in blue by X-Gal staining in A) were consistently increased in TSA-treated embryos. By whole-mount in situ hybridization we found that Notch expression was increased in the presomitic paraxial mesoderm of TSA-treated embryos (revealed in purple by the antisense probe; Fig. 2A; a higher magnification of the presomitic mesoderm is shown in C).

Because Notch and members of its signal transduction pathway con-

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Fig. 1. A, number of somites in control (open symbols) and TSA-treated (closed symbols) embryos. The number of somites was counted, including the two occipital somitomers. Time of killing is indicated in the upper abscissa, and the length of previous TSA treatment is indicated in the lower abscissa. B, immunoblot analysis of the acetylation status of histone H4 was performed in total embryo extracts using the antiacetylated histone H4 antibody. Representative samples from three controls and TSA-treated embryos are shown. Expression of histone H4 was used to control the histone H4 acetylation levels. C, ratio Ac-H4/H4 histone in control (open bars) and in TSA-treated embryos (dashed bars).

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Fig. 2. Gene expression in TSA-treated embryos. Note than in all panels, TSA-treated embryos are slightly larger than control embryos. A, whole mount in situ hybridization for Notch on a control and a TSA-treated embryo, also stained for Myf-5 expression by X-Gal (28). Note that the TSA-treated embryo has two additional somites expressing Myf-5. This is quantified in B, where the total number of somites (closed bars) and the number of X-Gal somites expressing Myf-5 (closed bars) are shown. Somites were scored as Myf5+ when they contained in excess of five X-Gal + nuclei. Treated embryos had a higher level of Notch expression in the presomitic mesoderm; this is more evident in C, where only the dissected caudal portion of embryos hybridized with the Notch probe are shown. D, whole-mount in situ hybridization for RAR a1 in control and TSA-treated E-9.5 embryos. Note comparable levels of expression throughout the mesoderm and in the branchial arches. E, whole-mount in situ hybridization for RAR a2 in control and TSA-treated E-9.5 embryos. TSA-treated embryos expressed higher levels of RAR a2 in the cranial spinal cord and in the hindbrain. F, whole-mount in situ hybridization for RAR b2 in two controls (above) and in two TSA-treated (below) E-9.5 embryos. Note that TSA-treated embryos express higher levels of RAR b2 in the presomitic mesoderm. C, control.
trol numerous cell-fate decisions during development and are involved in the patterning of somites (24), its increased expression might accelerate this process, resulting in the additional somites formed in response to TSA treatment. Together these results show that TSA treatment during the postimplantation stage, when critical morphogenetic events occur, results in the increased expression of Notch, accelerated formation of somites, and normal commitment to myogenesis.

Recently, a number of studies showed that unliganded nuclear receptors, such as the RARs (RARα, -β, and -γ), recruit the nuclear corepressors N-CoR and SMRT that associate with adapter proteins like Sin3 and histone deacetylases (HDAC1 and HDAC2) to form a transcriptional repressor complex at the response element of RA target genes (RAREs). (1, 2). Ligand binding releases corepressors and HDACs and recruits transcriptional adapters and coactivators that include histone acetyltransferase activities such as p300/CBP, ACTR, and TIF2 (1, 2). Interestingly, cells derived from mice lacking a functional p300 gene proliferate poorly and display specific transcriptional defects of RA signaling (27). Each RAR gene generates distinct mRNA isoforms by the use of different promoters and alternative splicing. The RAR isoforms RARα2 and RARβ2 posses a RARE in their promoters that is responsible for their RA inducibility occurring through a HDAC-dependent signaling pathway (1, 20, 21). The RARE is not present in the promoter of the RARα1 isoform, which, at variance with RARα2, is expressed constitutively in mouse adult tissues as a housekeeping gene and is widely distributed during mouse embryogenesis (20–22). We therefore examined by whole-mount in situ hybridization the expression levels of RARα1, RARα2, and RARβ2 in control and in TSA-treated E-9.5 mouse embryos. Fig. 2D shows similar levels of RARα1 transcript in the expected sites of expression, such as the branchial arches, somite mesoderm, and limb buds of embryos from control and TSA-treated mice. In contrast, significantly higher levels of expression of RARα2 in the spinal cord and hindbrain, as well as of RAR-β2 in the presomitic mesoderm, could be detected in TSA-treated embryos when compared with control embryos. Thus, the HDAC-inhibitor TSA relieved a repressive activity associated with the HDAC-complex at the RARE of RA target genes.

In summary, the results presented in this study indicate that in vivo modulation of gene transcription by inhibition of HDAC activity by TSA treatment is neither toxic nor teratogenic, at least in rodents, suggesting that TSA might represent a novel and safe transcriptional therapeutic agent.

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References

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