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Trichostatin A—histone deacetylase inhibitor with clinical therapeutic potential—is also a selective and potent inhibitor of gelatinase A expression

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Abstract

Modulation of histone acetylation is currently being explored as a therapeutic strategy in treatment of cancer. Specifically, inhibition of histone deacetylase by trichostatin A (TSA) has been shown to prevent tumorigenesis and metastasis. In the present paper we demonstrate that increased histone acetylation by TSA-treated 3T3 cells decreases mRNA as well as zymographic activity of gelatinase A, a matrix metalloproteinase, which is itself, implicated in tumorigenesis and metastasis. Furthermore, TSA inhibits cytochalasin D-induced activation of gelatinase A, but TSA does not affect other members of the gelatinase A activation complex, MT1-MMP and TIMP-2. Thus, TSA is a selective and potent inhibitor of expression and activation of gelatinase A. This finding not only strengthens the rationale for continuing to investigate the therapeutic utility of TSA in cancer, but also, provides evidence that TSA inhibition of gelatinase A expression and activation can be used as a biological marker to monitor and determine end-points of clinical trials involving TSA.

Keywords: Cancer; Gelatinase A; Histone deacetylase; Trichostatin A; Chromatin; Transcription

The geometry of DNA organization in chromatin affects such processes as transcription, replication, repair, recombination, and segregation [1]. Thus, the degree and extent of chromatin compaction can exert a crucial influence on gene expression by controlling access of transcription factors to the genome [2]. Ultimately, it is the spatial and temporal expression of genes that are pivotal to tissue differentiation, organogenesis, organism development, and disease [2]. In general, transcriptional activators target two types of enzymatic complexes which affect chromatin remodeling in the promoter region: (i) ATP-dependent SW1/SNF-like complexes and (ii) covalent modifiers of nuclear histones, most notably histone acetylases and deacetylases [1–9]. In particular, the attachment of an acetyl group to lysine in the histone tail by histone acetylases (HATs) causes unraveling of DNA through repulsion of the negatively charged phosphates on the DNA backbone [10,11]. The resulting decompaction of chromatin is believed to allow access of transcriptional complexes and thereby promote initiation of transcription. Conversely, removal of the acetyl group from histones by histone deacetylases (HDACs) causes chromatin compaction and inhibition of gene expression. Although chromatin decompaction generally leads to enhanced gene expression, surprisingly, inhibition of histone deacetylation does not result in a wholesale increase in gene expression, but rather produces remarkably specific activation of genes [11,12].

Histone acetylation has been implicated in the development of cancer [13]. For example, it has been shown that fermented fibers in the colon release butyric acid which enhances histone acetylation through inhibition of HDAC. This is believed to be the mechanism that underlies the protection against colon cancer afforded by a high fiber diet [14,15]. In addition, HDAC synthetic inhibitors induce differentiation or apoptosis of trans-

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formed cells and therefore are of interest in cancer therapeutics [11]. One of these inhibitors, trichostatin A specifically inhibits HDAC in the nanomolar range and causes a selective change in only about 2% of gene expression [11,16,17].

Matrix metalloproteinases (MMPs), most notably gelatinases A and B, are secretory enzymes involved in matrix remodeling and have been implicated in tumorigenesis and metastasis [18–20]. Many cancer cells secrete MMPs. However, other cancer cells that do not secrete these enzymes recruit the surrounding stroma to secrete MMPs in a paracrine fashion [21,22]. Although MMPs have been heralded as promising targets for cancer therapy, so far, the results of clinical trials using MMP inhibitors have not lived up to expectation [23]. One possible explanation for such clinical test failures may be the substantial side effects experienced in the in vivo setting, because the dosages required for drug inhibition of secreted MMP activity may be relatively high compared to what might be required to affect MMP gene expression.

In the present communication we report that TSA acts as a selective, potent inhibitor of gelatinase A expression and activation. Following histone acetylation after treatment with TSA in 3T3 cells, gelatinase A mRNA and gelatinolytic activity of latent gelatinase A is reduced in a dose-related manner, while mRNA levels of other members of gelatinase A activating complex, MT1-MMP, and TIMP-2 remain unchanged. TSA also inhibits cytochalasin-D-induced activation of gelatinase A. This study, for the first time, identifies gelatinase A as a selective target for histone acetylation-induced gene expression in 3T3 fibroblast cells. There are two important implications of this finding: (i) since gelatinase A is believed to contribute to metastatic potential of cancer cells, our results provide additional rationale for exploring the utility of TSA in anti-tumor trials with gelatinase A gene expression as the target; and (ii) monitoring of gelatinase A activity in clinical trials involving TSA may provide a useful end-point measure for such trials.

Materials and methods

All experiments were repeated at least three times. Means were compared using Student's *t* test. All reagents were from Sigma (Oakville, ONT) unless otherwise indicated. NIH 3T3 cells were obtained from American Type Cell Culture (ATCC, Rockville, MD).

Western immunoblot. 3T3 cells were plated in 100 mm dishes (6×10^6/dish) in serum-containing medium. The following day, cells were washed and treated in serum-free medium with TSA (50 ng/ml) or vehicle alone for 24 h, after which cells were extracted with boiling gel loading buffer. Forty μg protein aliquots were separated using SDS–PAGE and transferred to nitrocellulose membrane. Histone 4 acetyl bands were visualized with specific antibody [anti-acetyl-histone H4 (lys 16); Upstate Biotechnology, Lake Placid, NY]. This antibody recognizes 10 kDa histone H4 acetylated at lysine in position 16. Acetylation of histones is associated with gene activation [24]. Signal was developed using HRP-second antibody as recommended by the manufacturer. Film was exposed for varying times (5–60 s) to ensure comparison in the linear range of the signal. After documentation, nitrocellulose membrane was stripped with 0.5 N NaOH and re-probed with anti-histone H4 (Upstate Biotechnology). This antibody recognizes 10 kDa total H4 histone with broad species cross-reactivity.

Northern blot analysis. Twenty-four hours after treatments with TSA or Cyto D, total RNA was extracted using RNeasy columns (Qiagen, Mississauga, ON). Five μg total RNA was separated by agarose–formaldehyde gel electrophoresis and transferred to positively charged nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Baie d'Urfe, QC) as described [25]. Probes (200–400 bp size range) were generated with gene-specific PCR primers, using RT-PCR of 3T3 cells. Probes were verified by restriction analysis and sequencing. Probes were labeled by random priming (Rediprime II; Amersham Pharmacia Biotech). On certain occasions, membrane was re-probed after stripping with Tris–HCl, pH 8.0, containing 2 mM EDTA and 0.1% Dendhardt's solution for 2 h at 68 °C.

SDS–PAGE zymography. Cells were plated in serum-containing medium for 24 h following which cells were washed 3× with HBSS and treated in serum-free media for 24 h. The next day, conditioned media were collected, spun to remove cell debris, and subjected to SDS–PAGE zymography as previously described [26,27]. Latent and active gelatinase A migrate on SDS zymography as 68 and 57 kDa species, respectively. This was confirmed by treatment of latent gelatinase A with APMA or treatment of cells with Cyto D followed by zymography and Western immunoblotting [26]. Thus, appearance of a lower molecular species following treatment with Cyto D in the present study represents active gelatinase A.

Protein assay. Protein levels were assessed using the Lowry assay (Bio-Rad, Mississauga, Ont.)

Results

Treatment of 3T3 cells with the HDAC inhibitor TSA causes increased acetylation of histone 4 (Fig. 1). This is accompanied by a dose-related inhibition of gelatinase A mRNA with no change in mRNA levels of other genes involved in the gelatinase A activation/activity complex, TIMP-2 and MT1-MMP (Fig. 2). The inhibition observed was not a result of reduced cell viability, as confirmed by the MTT assay (data not shown). In the

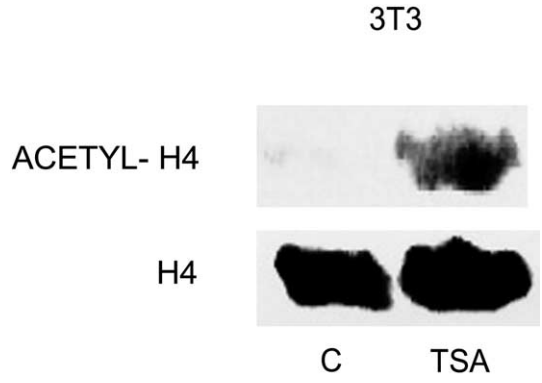

Fig. 1. Effect of TSA on acetyl histone H4 in 3T3 cells. Cells were treated with 50 ng/ml TSA for 24 h. and processed as described in Materials and methods. Note enhanced acetylation of histone H4 after treatment with TSA (upper panel). Equal loading was verified by stripping and reprobing with anti-histone H4 antibody (lower panel).

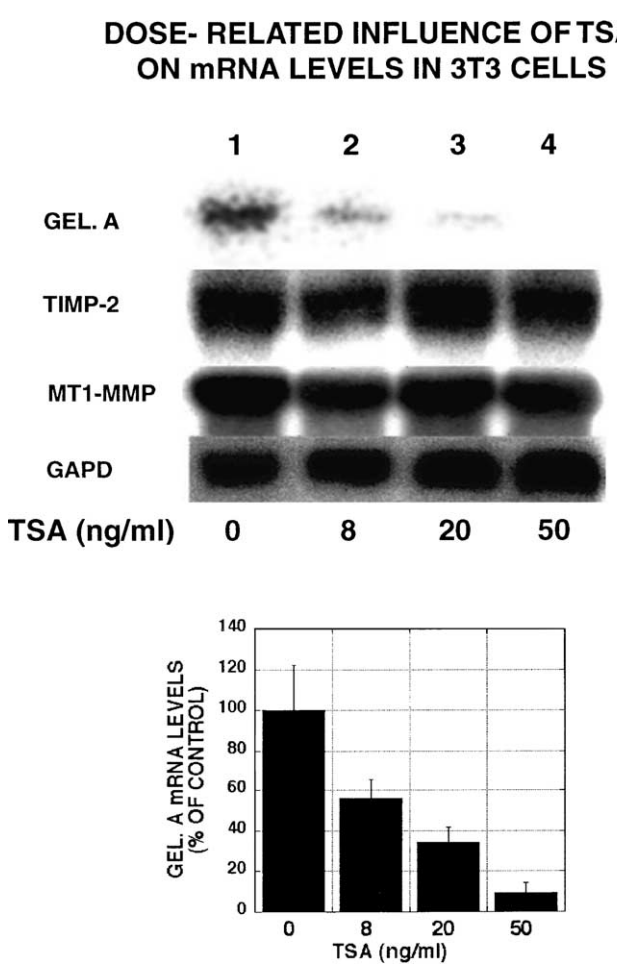

Fig. 2. Dose-dependent, selective inhibition of gelatinase A mRNA by TSA. 3T3 cells were treated with various concentrations of TSA for 24 h and 5 μg aliquots of total RNA were subjected to Northern blot analysis. After visualization, the membrane was stripped and re-probed with GAPD to confirm equal loading. Upper panel is a representative Northern blot showing dose-dependent inhibition of gelatinase A mRNA by TSA, with no apparent change in mRNA of the other genes tested. Lower panel: scan of gelatinase A blots. Bars: represent means + SE, All treatments are statistically different ($p < 0.001$, $n = 4$) from control.

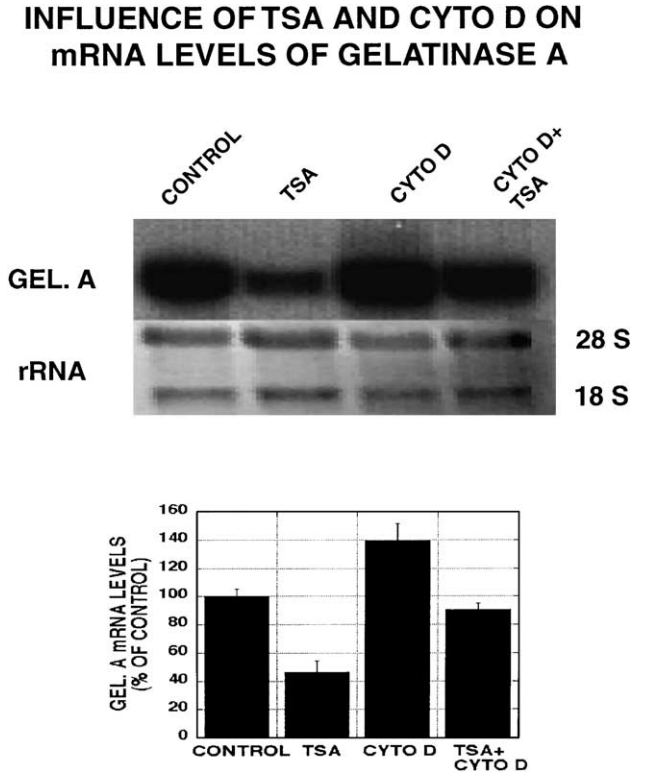

Fig. 3. Effect of TSA on gelatinase A mRNA in control (basal) and Cyto D-treated 3T3 cells. Cells were treated with 25 ng/ml TSA (lanes 2 and 4) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 1 μg/ml Cyto D, for 24 h. Five μg total RNA aliquots were subjected to Northern blot analysis. In parallel, 5 μg of each sample was run and stained with ethidium bromide to verify loading (rRNA). Upper panel-representative experiment. Lower panel: scan of gelatinase A blots. Bars represent means + SE. TSA and Cyto D treatments are statistically different ($p < 0.005$, $p < 0.01$, respectively) from control. TSA + Cyto D treatment is statistically different ($p < 0.01$) from TSA treatment alone.

presence of Cyto D, a drug that induces activation of gelatinase A [26,27], TSA also causes inhibition of basal and Cyto D-induced activation of gelatinase A mRNA levels (Fig. 3). Similarly, using zymography, we demonstrate that TSA inhibits the activity of latent, as well as Cyto D-induced active gelatinase A (Fig. 4). No detectable changes in secreted protein levels were observed in the conditioned media using the Lowry assay, following treatment with both both Cyto D and TSA, suggesting that no general effect on protein secretion was elicited by the drugs (data not shown). To ascertain whether the inhibitory effect of TSA on gelatinolytic activity of gelatinase A was a consequence of direct inhibition (e.g., irreversible binding of TSA to the zinc in the catalytic site of gelatinase A), various concentrations of TSA were added to conditioned media derived from 3T3 cells (containing both latent and active gelatinase A; Fig. 4 lane 6) and incubated for 24 h at 37 °C followed by 48 h at 4 °C. Inspection of Fig. 5 shows that there is no effect of TSA on gelatinase A gelatinolytic activity. Furthermore, when zymogram gels with 3T3 conditioned media were developed in the presence of added 100 ng/ml TSA, no influence was noted on gelatinolytic activity (data not shown).Taken together, these data suggest that TSA is a selective and potent inhibitor of both gelatinase A expression and activation.

Discussion

Histone deacetylase inhibitors are known to inhibit about 2% of cellular genes [11,16]. Most of the genes so affected exhibit enhanced expression, although some are inhibited. For example, treatment of cancer cells with the HDAC inhibitor oxamflatin causes stimulation of PAI-2 and inhibition of u-PA gene expression [28]. In

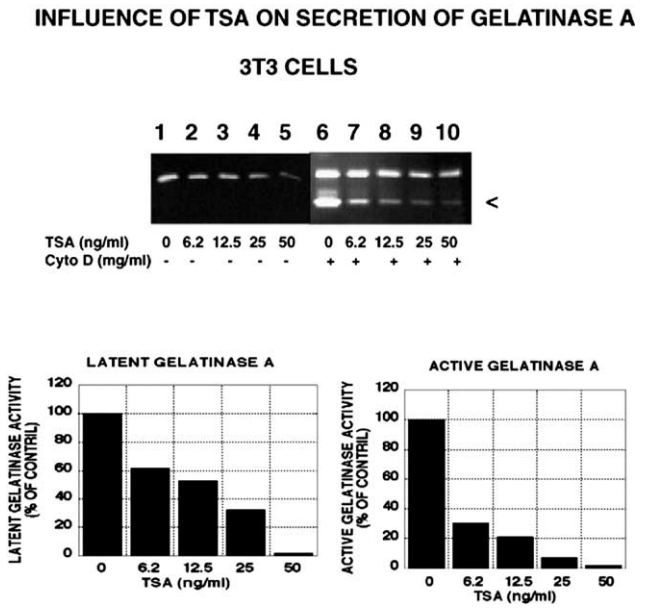

Fig. 4. Influence of TSA on gelatinolytic activity in conditioned media of 3T3 cells under basal and Cyto D-induced activation. Cells were treated with TSA alone (lanes 1–5) or TSA plus Cyto D (lanes 6–10) for 24 h. Lower panel depicts scanning of latent gelatinase A in lanes 1–5 and active gelatinase A (arrowhead) in lanes 6–10. Note dose-related inhibition of TSA on gelatinolytic activity of both latent and active gelatinase A.

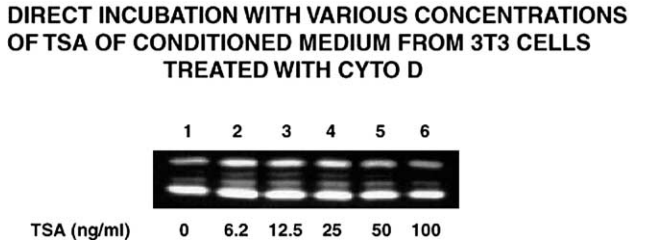

Fig. 5. Lack of effect of TSA on gelatinolytic activity of conditioned medium from Cyto D-treated 3T3 cells. Upper band shows latent gelatinase A, lower band-activated gelatinase A. There is no effect of TSA on gelatinolytic activity at any concentration tested. For duration of treatment, consult Materials and methods.

the present communication, we show that treatment of NIH 3T3 cells with TSA causes inhibition of gelatinase A expression. (Similar results were also obtained with STO cells—mice embryonic fibroblasts; Ailenberg and Silverman, unpublished data.) There are several possible explanations for our results. TSA could exert a direct inhibition on gelatinase A gene expression. Alternatively, TSA could stimulate other genes known to inhibit gelatinase A expression. In this regard, it has been reported that ATF3 represses gelatinase A expression by antagonizing p53-dependent trans-activation of the gelatinase A promoter [29]. It is possible that TSA inhibition of gelatinase A expression could be modulated through enhancement of ATF3 gene expression.

Evidence for a link between hyperacetylation and carcinogenesis comes from studies indicating that histone deacetylase inhibitors such as butyric acid and TSA are able to induce growth arrest and differentiation in a variety of cancer cells [13]. In the present communication, we demonstrate that TSA is a potent and selective inhibitor of gelatinase A expression and activation. Gelatinase A itself has been implicated in carcinogenesis [18–22]. The active enzyme binds to integrin $\alpha V\beta 3$ and promotes angiogenesis as well as tumor invasion [30]. It has also been shown that gelatinase A affects cell proliferation and produces anti-apoptotic signals either directly or through release of growth factors associated with the extracellular matrix [31–34]. Hence, inhibition of gelatinase A could be one of the reasons for the anti-tumorigenic effect of TSA. Furthermore, monitoring gelatinase A levels could serve as an early stage biological parameter for monitoring efficacy studies of the effect of TSA in clinical trials.

In the present study we have used fibroblast NIH 3T3 cells for our investigation of gelatinase A inhibition by TSA. In contrast to our results with normal cells, it is interesting that treatment of SW620 colon carcinoma cells with TSA did not affect the expression of gelatinase A [35]. Recently, we have also noted that treatment of HT-1080 fibrosarcoma cells with TSA does not affect gelatinase A expression (Ailenberg and Silverman, unpublished data). Moreover, unlike 3T3 cells, we and others have also found that treatment of cancer cells with TSA results in induction of apoptosis ([36]; Ailenberg and Silverman, unpublished data). It has been reported that tumor invasion of metastatic cells is facilitated by release of gelatinase A either by the invading cells themselves or indirectly by recruiting the surrounding stroma to secrete gelatinase A [18–22]. Thus, the differential effect that TSA seems to exert on cancer and stromal cells could be of relevance in the use of this compound as anti-tumor agent. On the one hand, TSA could induce apoptosis in cancer cells without affecting stromal cell survival, e.g., by influencing genes involved in cancer cell cycle control; on the other hand, although TSA does not seem to alter expression of gelatinase A by cancer cells, it may influence tumorigenesis and cancer cell invasion by inhibiting gelatinase A in stromal cells. From another perspective, it is the general experience that cancer cells often develop resistance to drug treatment by undergoing mutations, while normal cells are genetically more stable [37]. Since TSA seems to selectively target mesenchymal rather than cancer cells and inhibit gelatinase A expression in the former but not the latter, it would appear that TSA offers some potential added advantage for cancer treatment.

Crystallographic studies have revealed that HDAC is a metalloprotein that contains zinc in its catalytic site [38]. Hydroxamates like trichostatin A bind to the zinc thus inhibiting the catalytic activity. Interestingly, other

hydroxamates are also potent inhibitors of metalloproteinases such as gelatinase A (see below). However, it is unlikely, that TSA exerts its action on gelatinase A by irreversibly inhibiting the activity of gelatinase A because: (i) TSA inhibits expression of gelatinase A mRNA; (ii) zymography of active or latent gelatinase A in the presence of TSA fails to demonstrate inhibition of its activity (Fig. 5).

Clinical trials using gelatinase A inhibitors, most notably hydroxamates, are in more advanced stages than HDAC inhibitors. However, evidence for the therapeutic efficacy of gelatinase A inhibition is modest at best [23]. Some of the problems arising in such clinical trials are a consequence of lack of appropriate endpoints that can be utilized to monitor drug efficacy, which in many instances has meant administering increased drug dosages. However, making use of the selective effect of TSA on gelatinase A might provide a useful biological marker that could assist with early evaluation of the trial rather than waiting for the actual effect on tumor sizes and/or degree of metastasis. Another advantage in using TSA as an anti-gelatinase inhibitor rather than other specific hydroxamates is that TSA inhibits gelatinase A expression whereas the other inhibitors inhibit gelatinase A activity, after synthesis and secretion—at a stage that is too late in the ontogenesis of the disease.

Acknowledgment

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