Trichostatin A, lead compound for development of antifibrogenic drugs

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Abstract

Eukaryotic gene expression has mainly been studied in the context of trans-acting transcription factors and their interaction with regulatory cis-elements. Evidence is accumulating, that the higher order structure of chromatin also plays an essential role in eukaryotic gene expression.

Hepatic stellate cells are the major cellular source of extracellular matrix synthesis in chronic liver diseases leading to fibrosis. We explored the antifibrogenic effect of the histone deacetylase inhibitor trichostatin A (TSA) on hepatic stellate cells *in vitro*. Primary hepatic stellate cells as well as activated, subcultured stellate cells were exposed to 10^{-7} M - 10^{-9} M TSA. Collagens type I and III, and smooth muscle α-actin (α-SMA), a marker for transdifferentiation, were investigated at the protein and mRNA level by performing Northern hybridisation and quantitative immunoprecipitation. The antiproliferative effect was examined by ^{3}H-thymidine incorporation and cell counting. Hyperacetylation of histone H4 was demonstrated by acid urea Triton-X-100 (AUT) polyacrylamide gel electrophoresis.

TSA at 10^{-7} M retarded the morphological changes characteristic for activation of primary stellate cells. Synthesis of collagens type I and III, and α-SMA was strongly inhibited at both protein and mRNA level. The proliferation rate of primary hepatic stellate cells was strongly suppressed by 10^{-7} M TSA. Hyperacetylation of histone H4 showed to be maximal at 10^{-7} M TSA. Primary hepatic stellate cells were more affected by TSA than subcultured stellate cells. (Acta gastroenterol. belg., **2001**, 64, **239-246**).

Key words : hepatic stellate cells, liver fibrosis, trichostatin A, extracellular matrix, proliferation.

Introduction

In the last decade much effort has been made to find therapeutic agents to prevent or treat liver fibrosis and cirrhosis (1,2). The ultimate goal of fibrosis research is the development of a rational basis for effective antifibrotic therapy. Previous research has focused on several cellular and molecular mechanisms which lead to fibrosis, whereby different signal transduction pathways have been partially elucidated (1,3).

In liver, the major fibrogenic cell type is the hepatic stellate cell (previously called lipocyte, Ito-cell, fat-storing cell, or perisinusoidal cell). Stellate cells are resident nonparenchymal (non-hepatocyte) cells found in the subendothelial space between hepatocytes and sinusoidal endothelial cells. These cells undergo activation during liver injury. This process is associated with induction of key genes which can be influenced at the transcriptional, translational or post-translational level (4). Stellate cell activation refers to the transition from a quiescent vitamin A-rich cell to one that is proliferative, fibrogenic, and contractile with reduced vitamin A content. This transition is gradual and occurs through an intermediate stage defined as "transdifferentiation" (Fig. 1). Activation of stellate cells can be considered to occur in two phases : initiation and perpetuation. The first phase, initiation, refers to the earliest changes in phenotype that render the hepatic stellate cells more responsive to paracrine and juxtacrine growth factors. This process of initiation is followed by a phase of perpetuation which leads to prolonged proliferation, production of extracellular matrix, and synthesis of a number of factors that maintain transdifferentiation (1). Synthesis of extracellular matrix is increased. Because of enhanced synthesis of tissue inhibitors of metalloproteinases (TIMP) the cells have also a reduced ability to degrade and remodel extracellular matrix. Several groups have worked at identifying transcription factors such as c-myb (5), GC receptor (6), Stat1 (7), Zf9 (8), Ets-1 (9), PPAR-γ (10) and others involved in transdifferentiation of stellate cells (4,11).

In this study we have shown that TSA is a potent antifibrogenic molecule (12). *In vitro* experiments showed that TSA exert its antifibrogenic effects in submicromolar concentration range (10^{-7} M), while other drugs under investigation with putative antifibrogenic properties exert their effects in a concentration range between 10^{-3} M and 10^{-6} M (13,14). TSA is a potent inhibitor of histone deacetylases which are the counterparts of histone acetyltransferases (15). One major process by which chromatin structure can be modulated is the histone acetylation level of which is determined by an equilibrium between histone acetyltransferases and deacetylases (table I) (16).

Acetylation of N-terminal lysine residues of histones lowers the positive charge of these N-terminal domains thereby weakening the interaction with the negatively charged DNA in the nucleosomes and decreasing the stability of the 30 nm chromatin fiber (17,18). Experimentally, one can interfere with this mechanism of transcriptional regulation by using specific histone deacetylase inhibitors. Specific pharmacological inhibitors of histone deacetylases can be used to further investigate the possible working mechanism of histone

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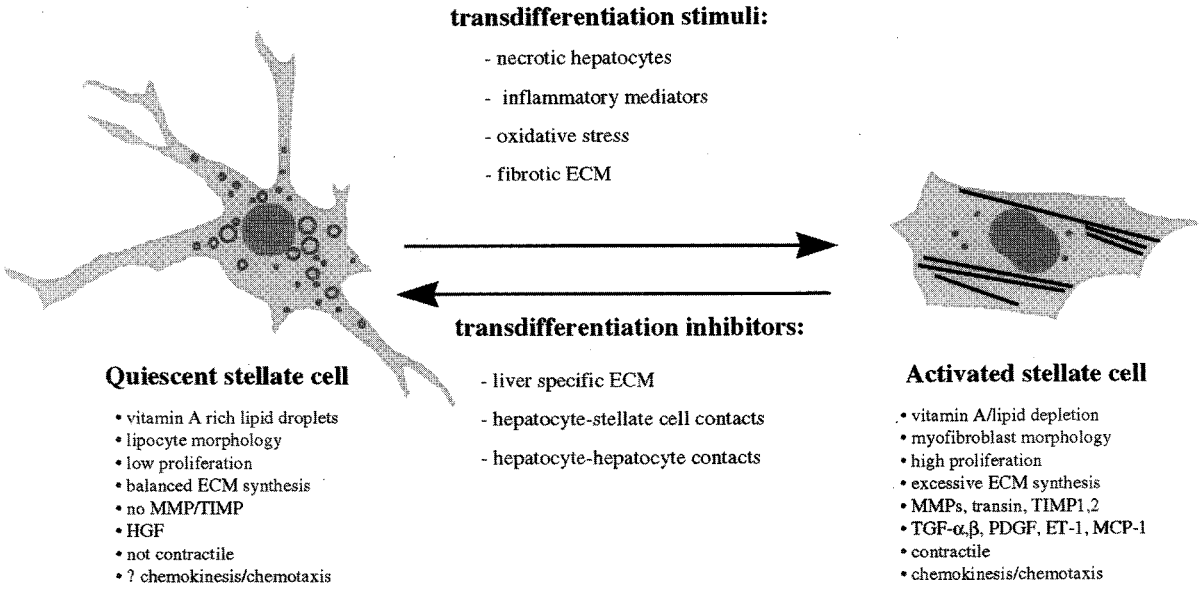


Fig. 1. — Transdifferentiation of hepatic stellate cells is regulated by three types of local control mechanisms : cell-cell contacts, cell-matrix contacts and soluble factors secreted in the micro-environment.

Table I. — **Overview of different histone acetyltransferases (HATs), histone deacetylases (HDACs) and HDAC inhibitors**

Histone acetyltransferases	Histone deacetylases	Histone deacetylase inhibitors
GCN5 (49)	* Class I :	Trichostatin A (60)
p300 (50)	HDAC1 (54),	Butyrate and derivatives (16,61)
CBP (51)	HDAC2 (55),	cyclic tetrapeptides (62,63)
TAF II 250 (52)	HDAC3 (56),	trichostatin / hybrid polar compounds (64)
SRC-1 (53)	* Class II :	depudecin (65)
ACTR (49)	HDAC4 (57),	bicyclic peptide (66)
	HDAC5 (58),	DMBA (67)
	HDAC6 (58),	benzamides (68,69)
	HDAC7 (59)	diallyl disulfides (61)

acetyltransferases and histone deacetylases on influencing transcriptional regulation of genes. Recently, several histone deacetylase inhibitors were synthesised (table I).

We have shown that hyperacetylation of core histones in primary stellate cells inhibited strongly three main features of myofibroblast transdifferentiation of cultured hepatic stellate cells : (1) synthesis of collagens type I and III, the predominant collagens in fibrosis of liver and other organs, (2) cellular proliferation, and (3) expression of α-SMA, a marker for differentiated myofibroblasts (19,20). These results demonstrated that chromatin structure plays an important role in determining the phenotype of hepatic stellate cells. The chromatin structure is therefore a novel target in the search for agents that can prevent transdifferentiation of hepatic stellate cells.

Materials and Methods

Reagents

(R)-Trichostatin A, 7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide, was prepared from the culture broth of *Streptomyces platensis No. 145* as described previously (21). Stock solutions of TSA were prepared in ethanol (2 mg/ml), stored at -20°C, and diluted as required for each experiment. The final concentration of ethanol in the medium was 0.0016%.

Isolation, culturing and characterisation of hepatic stellate cells

Hepatic stellate cells were isolated from male Wistar rats (400-550 g) by collagenase / pronase digestion followed by density gradient centrifugation as described previously (22). All rats were fed *ad libitum*, and received humane care in compliance with the institution's guidelines for the care and use of laboratory animals in research. Cells were finally collected and diluted appropriately in Dulbecco's modified Eagle's medium with 10% foetal calf serum, at a density corresponding to the experiments (12). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Purity of cultures was evaluated by examining the characteristic stellate shape and fat-droplets under phase

contrast microscopy, and was at least 95%. Immunocytochemistry was performed using specific antibodies for α-SMA, Glial Fibrillary Acidic Protein (GFAP), desmin and vimentin, all known markers for hepatic stellate cells (23,24).

Immunoprecipitation

Cells at day 3 after isolation were exposed to 10^{-7} M - 10^{-9} M TSA for 24 hours. Subsequently, cells were metabolically labelled for 24 hours using 25 μCi/ml of Trans ^{35}S-label (specific activity of ^{35}S-methionine > 1,000Ci/mmol, ICN Biomedicals, Costa Mesa, CA, USA) while exposure to the test compound continued. Media and cell layers were harvested separately as described previously (25). Total incorporation of Trans ^{35}S-label into protein was determined by the hot trichloroacetic acid precipitation method (25,26). Total incorporation in media and cell layers was expressed per 9.6 cm^2 culture dish. The immunoprecipitation procedure was performed according to the method described by Niki *et al.* (25). The results were quantified by Phosphor Imaging (BioRad).

Northern Hybridisation Analysis

At day 3 cells were exposed to 10^{-7} M TSA for 24 hours. Total RNA was extracted by the method of Chomczynski and Sacchi (27). For Northern hybridisation, 10 μg total RNA of each sample was loaded on 1% agarose gels containing 3% formaldehyde. Electrophoresis and blotting were performed as described (25), using ^{32}P-labelled cDNA probes for rat procollagen α_1(I) (1.6 kb Pst I fragment) (28), rat procollagen α_1(III) (0.5 kb Hind III/EcoRI fragment) (29), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (0.5 kb XbaI/HindIII fragment (30). For α-SMA, a cRNA probe corresponding to the 5'-untranslated region of mouse α-SMA mRNA was used as described previously (31). The results were quantified by Phosphor Imaging and corrected for GAPDH.

Proliferation assay

Cells were cultured in triplicate or quadruplicate in 24 well plates (Costar, Cambridge, USA). At day 4, cells were exposed to 10^{-7} M-10^{-9} M TSA for 24 hours. Subsequently, medium was changed and cells were further incubated for 20 hours with the same concentrations of TSA in the presence of 10 μCi/ml ^{3}H-thymidine (specific activity 25 Ci/mmol, 10 μCi/ml) (Amersham, Gent, Belgium). Radioactivity incorporated into the 2% perchloric acid/95% ethanol-insoluble fraction was measured by scintillation counting. Parallel cultures incubated with ^{3}H-thymidine in the presence of 10 mmol/L hydroxyurea provided the baseline value, which was subtracted from each measurement (32). Final data were normalised for cell number which was determined by trypsinization of parallel wells.

Cell counting

Cells were cultured in triplicate or quadruplicate in 24 well plates (Costar, Cambridge, USA). Cells at day 2 were exposed to 10^{-7} M - 10^{-9} M TSA for the 4 subsequent days. Culture medium and test compounds were replaced every day. At day 6 cells were trypsinized and counted using a hemocytometer.

Acid Urea/Triton-X-100 PAA Gel Electrophoresis

Histones were extracted from primary stellate cells, seeded in 177 cm^2 dishes at a cell density of 2×10^6 cells/dish, cultured with or without 10^{-7} M TSA for 12 hours. The histone extraction was performed according to Cousens *et al.* (33). The level of acetylation was analysed by gel electrophoresis using acid urea / Triton-X-100 (AUT) gels (33). Gels were stained using Silver Stain kit (BioRad).

Statistics

With regard to immunoprecipitations and Northern experiments, the ratios of protein or mRNA level of treated versus control cultures were calculated for each concentration of TSA and expressed as mean ± SD (n > 3). An effect was considered statistically significant when 1.0 did not belong to the 95% confidence interval of the treated / control ratio (25,34). Incorporation of ^{3}H-thymidine into DNA and cell-counting experiments, were performed on stellate cells cultures of at least three animals. Data were expressed as mean ± SD.

Results

Characterisation and morphology of primary hepatic stellate cells

The morphology of hepatic stellate cells in culture was studied by light microscopy. When freshly isolated cells were placed in culture, they initially reproduced their characteristic shape observed *in vivo* with abundant cytoplasmic lipid-droplets. With time in culture, however, cells lost their fat-droplets and gained myofibroblast-like morphology.

Stellate cell origin was established by immunocytochemistry for α-SMA (35), GFAP (25), desmin (25,36) and vimentin (24) (Fig. 2). Immunocytochemistry was performed three days after isolating hepatic stellate cells. α-SMA was present in bundles of filaments but also more diffusely in the perinuclear and subcortical cytoplasm (Fig. 2a). α-SMA showed a diffuse perinuclear staining. GFAP was present in bundles of intermediate filaments mainly around the nucleus (Fig. 2b). Desmin and vimentin were present in filamentous structures both in the perinuclear and more distant paracortical cytoplasm (Fig. 2c, 2d).

Primary hepatic stellate cells transdifferentiate into myofibroblast-like cells after several days in culture. When primary hepatic stellate cells (three day old) were

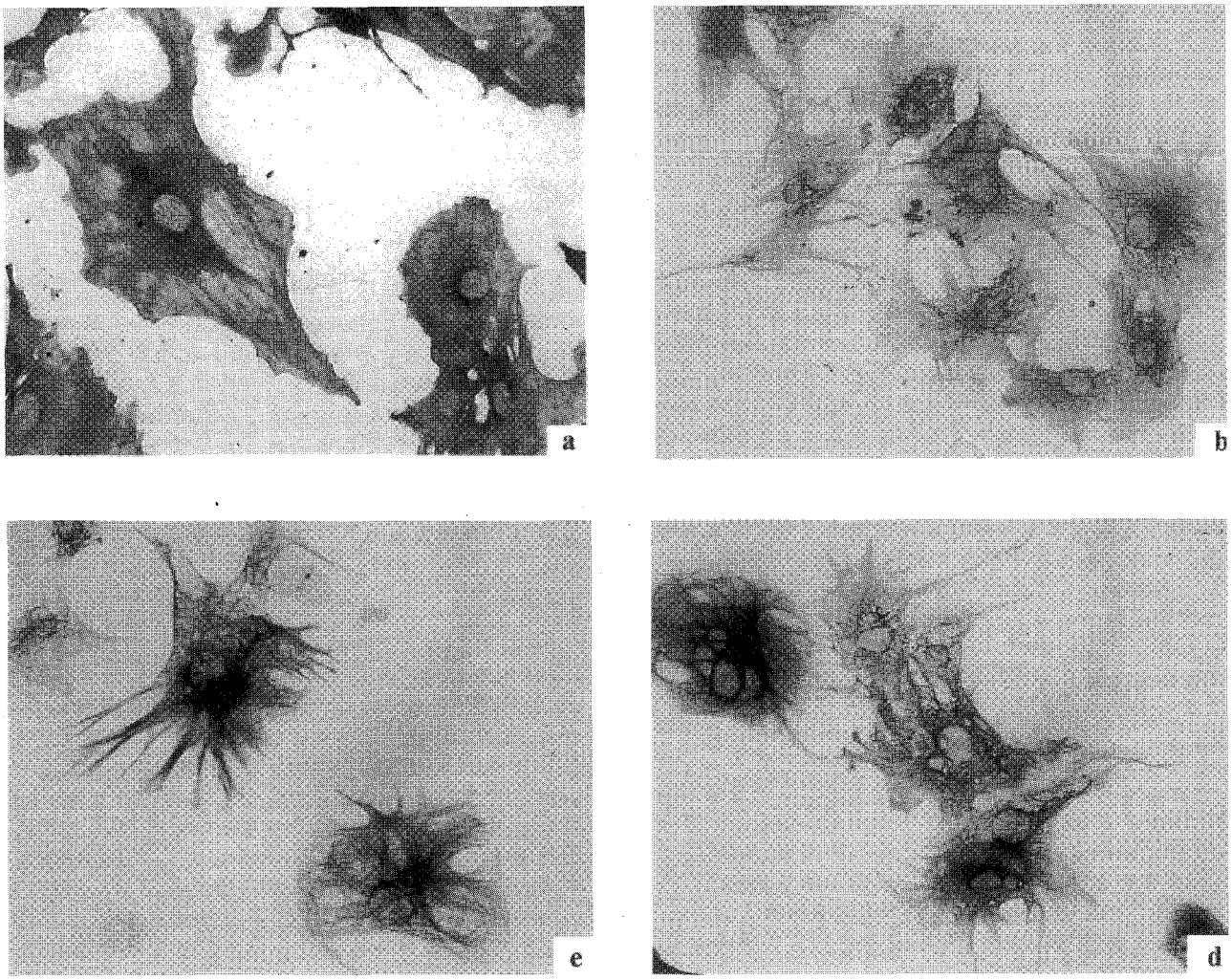


Fig. 2. — Immunocytochemistry of primary hepatic stellate cells in culture. The stellate cell origin of primary cells was established by immunocytochemistry for (a) α-SMA, (b) GFAP (c) desmin and (d) vimentin. Original magnification : 110 ×.

treated with 10^{-7} M TSA for 24 hours these morphological alterations are inhibited without affecting the viability of the cells. Primary hepatic stellate cells treated with 10^{-7} M TSA retained their slender shape and long cytoplasmic extensions (Fig. 3).

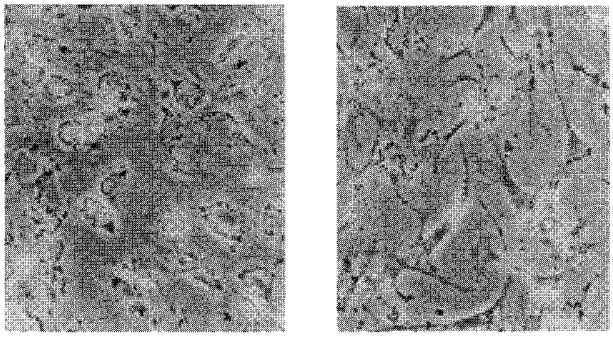
Influence of TSA on de novo protein synthesis of primary hepatic stellate cells

We first examined the effect of TSA on the synthesis of collagens type I and III, the major fibril-forming collagens that predominate in fibrotic livers (37). TSA at 10^{-7} M strongly suppressed the synthesis of collagens type I and III by 62% and 70%, respectively (table II).

Inhibition of histone deacetylase also suppressed the synthesis of α-SMA, an established marker for myofibroblast differentiation (19). As shown, synthesis of this molecule was inhibited by 84%, 46%, and 12% of the control levels by 10^{-7} M, 10^{-8} M and 10^{-9} M TSA (table II). Suppression of α-SMA expression suggested that TSA blocked synthesis of collagens type I and III at least partially by preventing the differentiation of stellate cells into myofibroblasts. This assumption was supported by the observation that TSA was less effective when tested on cells at day 14, when transition into myofibroblasts had already occurred (data not shown) (24,38,39).

Influence of TSA on mRNA synthesis of primary hepatic stellate cells

To explore at which level of collagen synthesis TSA exerted its effects, we performed Northern hybridization analysis on RNA extracted from primary stellate cell cultures. TSA (10^{-7} M) suppressed collagen α_1(III) mRNA levels by 61% and α-SMA mRNA level by 75%, which was in keeping with the extent of suppression at the protein level (- 70%) (table II). In contrast, collagen α_1(I) mRNA levels were modestly altered by TSA (- 21.5%). This suggested that the suppressive effect of TSA on collagen type I synthesis occurred mainly at the posttranscriptional level. The inhibitory action of TSA was selective for collagens type I, III and α-SMA, since mRNA levels of collagen α_1(IV) and the house-keeping gene GAPDH were not altered (data not shown).



day 4 day 4 + 10^{-7}M TSA

Fig. 3. — Morphology of hepatic stellate cells *in vitro*. Untreated control cells at day 4 showed intermediate morphology between quiescent and activated stellate cells. Cells treated with trichostatin A (10^{-7} M) at day 4 were slender-shaped and retained cytoplasmic extensions. Magnification : 125 ×.

Effect of TSA on proliferation of primary hepatic stellate cells

We examined also the effect of TSA on proliferation of stellate cells. 10^{-7} M TSA strongly inhibited proliferation by 89% ; lower concentrations were not inhibitory (table II). The suppressive effect of 10^{-7} M TSA on cell proliferation observed by ^{3}H-thymidine incorporation was confirmed by counting cells (Table II).

Effect of TSA on hyperacetylation of histone H4 in primary stellate cells

In order to gain insight into the molecular mechanism behind the observed effects, we examined whether the observed morphological and biochemical changes brought about by TSA, were preceded by hyperacetylation of histones, in particular histone H4 (Fig. 4). Stellate cells in primary culture were incubated with 10^{-7} M TSA for 12 hours. Next, cells were harvested for extraction of histones. Histones were subjected to acid urea / Triton-X-100 PAA electrophoresis which allowed to separate the different acetylation forms of histone molecules. Incubation with 10^{-7} M TSA caused strong hyperacetylation of histone H4 (Fig. 4).

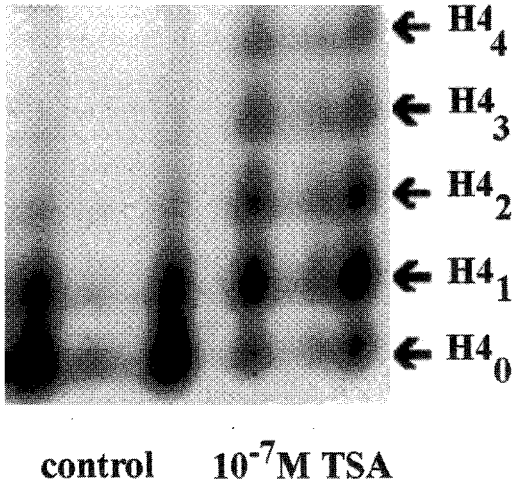
Discussion

We have shown that hyperacetylation of core histone H4 has a potent antifibrogenic effect on hepatic stellate cells. These cells are the major cellular sources of extracellular matrix proteins in fibrotic livers (1,3). To our knowledge, this is the first evidence that hyperacetylation of histones has a suppressive action on collagen synthesis and other cellular features of fibrogenesis.

In view of the generally assumed role of histone acetylation in gene regulation, how selectivity is achieved needs clarification. In the present study, we found that TSA affects strongly expression of collagens type I and III, and α-SMA but does not alter gene

Table II. — **Inhibitory effect of TSA on ECM *de novo* protein synthesis and collagens type I, III and α-SMA mRNA levels in primary hepatic stellate cells. The ratio of treated versus control was calculated for different concentrations of TSA. Cell counting and anti-proliferative effect of TSA**

	10^{-7} M TSA		10^{-8} M TSA	10^{-9} M TSA
	mRNA	protein	protein	protein
coll. α1(I)	25%	62%	31%	4%
coll. α1(III)	61%	70%	25%	24%
αSMA	75%	84%	46%	12%
proliferation	89%		1%	2%
cell counting	72%		4 %	2%



control 10^{-7}M TSA

Fig. 4. — Analysis of hyperacetylation of histone H4. Stellate cells in primary culture were incubated with vehicle only (control) or 10^{-7} M trichostatin A for 12 hours. Next, histones were extracted and subjected to acid urea/Triton-X-100 PAA electrophoresis which allowed to separate the different acetylation forms of histone molecules. Incubation with trichostatin A caused strong hyperacetylation of histone H4.

expression of collagen type IV and GAPDH. A recent study using differential mRNA display analysis has shown that in lymphoid cell lines, TSA affects the expression of only 2% of genes analysed (42). When applying differential mRNA display to stellate cells exposed to TSA, comparable low numbers of genes were affected (K. Rombouts *et al.*, unpublished observations). The selectivity of TSA is brought about by different mechanisms. First, this may be explained by the presence of several histone deacetylases that show different sensitivity to TSA (43). Preliminary experiments in our laboratory showed the presence of histone deacetylases class I and II in hepatic stellate cells (M. Chavez *et al.*, unpublished data). Neither histone acetyltransferases or histone deacetylases appear to work alone, as both exist in multiprotein complexes. Recruitment of these complexes to specific regions of the genome plays a major role in creating the spectrum of expressed and silenced genes, that characterises a cell type. Second, promoter regions of different genes are organised in different ways in nucleosomes. Third,

acetylation of nucleosomes varies topographically within the nucleus and with time. In general, approximately 10% of nucleosomes are acetylated ; 90% are not. The latter nucleosomes are located in transcriptionally silenced heterochromatin. The degree of acetylation of each individual nucleosome is the result of local action of histone acetyltransferases that increase the level of acetylation and of histone deacetylases that decrease the level. Fifth, selectivity in regulation of transcription is brought about by the composition of the protein complexes in which histone deacetylases are incorporated (44). Recent co-immunoprecipitation experiments have shown that these enzymes are present in large transcriptional complexes, the composition of which is depending on the cell type and state of differentiation (44-47). Besides the well known mSin3 / histone deacetylase 1 complex more evidence is growing for the existence of other alternative complexes without Sin3 whereby the repression domain of the corepressor directly binds to histone deacetylases of class II. Huang et al. have demonstrated the existence of multiple histone deacetylase complexes in one cell type functioning in different protein complexes with different results (48).

TSA inhibited synthesis of collagen type I presumably at the posttranscriptional level. This may seem paradoxical in view of the presumed role of acetylation of histones in transcriptional regulation. We hypothesise that genes affected by TSA may have a role in translational or post-translational processing and / or secretion of collagen type I.

At present we use the following hypothetical working model to explain how hyperacetylation of histones could bring about the observed antifibrogenic effects (Fig. 5). In healthy liver, the quiescent hepatic stellate cells express a fairly constant array of genes. When homeostasis is disrupted, either by liver injury or by isolating and subculturing cells, paracrine and autocrine factors (cytokines, growth factors, reactive oxygen species, eicosanoids) induce a new type of repressor complex that contains one or more histone deacetylase subtypes. This repressor complex binds to promoters of genes that are characteristic for the quiescent phenotype. Incubation of the cells with a selective inhibitor of histone deacetylases will lead to alleviating gene repression and to reverting activated into quiescent cells. This model is consistent with the available experimental data and is also the leading working hypothesis to explain the differentiating effect of TSA on promyelocytic leukemia cells (40).

Whether TSA has other effects besides inhibition of histone deacetylation cannot be entirely excluded but is unlikely. TSA is a potent and specific histone deacetylase inhibitor. Tumour cell lines with mutant histone deacetylase are resistant to the antiproliferative effect of TSA ; if TSA would have other effects, they should show up in these mutant cells which is not the case (41).

In summary, we found that submicromolar concentrations of TSA inhibited strongly (1) synthesis of collagens type I and III, (2) cellular proliferation, and (3) expression of α-SMA in primary culture of rat hepatic stellate cells. These biochemical changes were paralleled by inhibition of the morphological changes characteristic for activation of the cells. Thus, the histone deacetylase inhibitor TSA may be a promising lead compound in development of novel agents to treat fibroproliferative diseases.

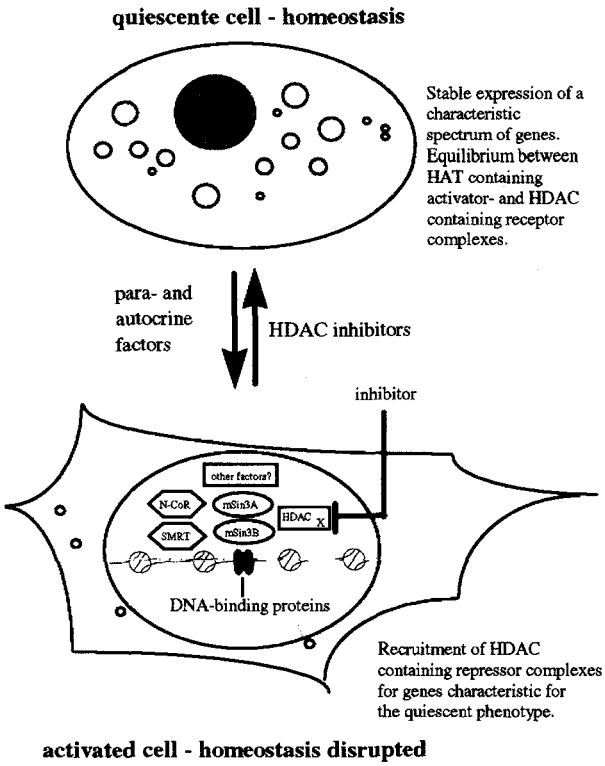


Fig. 5. — Hypothetical working model to explain why inhibition of histone deacetylases, in primary hepatic stellate cells, keeps the cells in a quiescent phenotype.

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