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Effect of the histone deacetylase inhibitor trichostatin A on spontaneous apoptosis in various types of adult rat hepatocyte cultures

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Abstract

Acetylation and deacetylation of histones, catalysed by histone acetyl transferases and histone deacetylases (HDAC), respectively, are known to be involved in gene expression regulation. Here, the effect on the activity and expression of several apoptosis-related proteins of trichostatin A (TSA), a well-known HDAC inhibitor, were studied in short-term (conventional monolayer) and long-term cultured (collagen I gel sandwich cultures and co-cultures) adult rat hepatocytes. No significant effects of TSA on the caspase-3-like activity were seen in rat hepatocytes cultured in a sandwich configuration or in a co-culture with rat liver epithelial cells of primitive biliary origin. In both culture models, the basal level of apoptosis was found to be much lower than in control monolayer cultures. In the latter system, it was found that, after 4 days of culture, TSA decreased the levels of caspase-3 (both proform and p17 fragment) and of the pro-apoptotic protein Bid. No effect of TSA was found on the expression of Bax. As expected, a TSA-mediated increase of acetylated histones H3 and H4 was observed in all culture systems examined. In addition, in the presence of TSA, increased albumin secretion and cytochrome P450 1A1/2 and 2B1-dependent enzyme activities were found in conventional cultures after 7 days. In conclusion, TSA delayed the occurrence of apoptosis and loss of liver specific functions in conventional hepatocyte monolayers. In contrast, in hepatocyte culture models in which spontaneous apoptosis is already minimised through the addition of either extracellular matrix components (sandwich cultures) or non-parenchymal liver cells (co-cultures), TSA did not have any additional anti-apoptotic effect.

Keywords: TSA; Apoptosis; Rat hepatocytes; Monolayer; Co-culture; Sandwich culture

1. Introduction

TSA, a fungistatic antibiotic purified from *Streptomyces platensis*, is currently tested as a potential drug against liver fibrosis [1–3]. In various cancer cell lines, low concentrations of TSA have been shown to affect a variety of biological processes, including the induction of cell cycle arrest, differentiation and apoptosis [1,4–9]. It is well documented that changes in gene expression upon TSA treatment are attributable to its strong and specific reversible inhibitory effect on HDACs [1,2,10]. Together with the histone acetyltransferases, HDACs are known to form one of the key regulatory mechanisms of chromatin configuration [11]. When deacetylation, catalysed by HDAC, occurs at the N-terminal tail of core histones H3 and H4, the positive charge to the lysines that bind more tightly to the negatively charged phosphate backbone of the DNA are restored, thereby condensing the structure of the nucleosomes. On the other hand, by acetylation the positive charge of lysine residues is neutralized, thereby decreasing their affinity for DNA and generating a more open DNA conformation. Consequently, transcription factors, regulatory complexes and RNA polymerase regain a better access to the DNA and the expression of corresponding genes is promoted. In general, although not exclusively, histone hyperacetylation is associated with gene transcription, whereas deacetylation of histones is associated

Abbreviations: Ac-DEVD-AFC, acetyl–Asp–Glu–Val–Asp–7-amino-4-trifluoromethylcoumarin; CYP, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; EROD, ethoxyresorufin *O*-deethylase; HDAC, histone deacetylase; PROD, pentoxyresorufin *O*-deethylase; TSA, trichostatin A

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with gene silencing [7,11,12]. Therefore, HDAC inhibitors such as TSA, provide useful tools to investigate the role of histone acetylation/deacetylation in the regulation of transcription and chromatin function.

Despite the wealth of knowledge regarding the effects of TSA on proliferation, differentiation and apoptosis in cancer cell lines at present, little data are available with respect to the effect of TSA on non-transformed cells and particularly on hepatic biotransformation and hepatocyte functioning. After all, hepatocytes are the main site of biotransformation and being a drug candidate, the effects of TSA should be investigated in hepatocytes. Previous studies from our laboratory have identified a number of TSA-altered cell cycle genes in primary cultures of adult rat hepatocytes [13]. In the present work, we have further analysed the role of histone hyperacetylation in regard to apoptosis and differentiation in both, short-term and long-term primary adult rat hepatocyte cultures.

2. Materials and methods

2.1. Chemicals

Crude collagenase type I, bovine serum albumin fraction V, insulin, glucagon, pepstatin A, leupeptin, aprotinin, TSA, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), dithiotreitol (DDT), EDTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma–Aldrich. Dulbecco's modified Eagle's medium (DMEM) came from BioWhittacker and fetal bovine serum (FBS) was from Invitrogen. Hydrocortisone hemisuccinate came from Upjohn s.a. 3,3′,5,5′-tetramethylbenzidine-hydrochloride (TMB) was from Organon Teknika. 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefablock) was obtained from Roche Diagnostics GmbH and Ac-DEVD-AFC came from Calbiochem. All other chemicals were readily available commercial products of analytical grade and were used without further purification.

2.2. Cell isolation and culture

Rat hepatocytes were isolated from outbred adult male Sprague-Dawley rats (200–250 g; Iffa Credo), with free access to food and water, as described previously [14]. Cell integrity was tested by trypan blue exclusion and cells were cultured either as a monolayer [15], as co-cultures with rat liver epithelial cells [15] or in a collagen gel sandwich configuration [16], either in the absence or presence of 1 μM TSA. The medium used was DMEM containing 0.5 U/ml insulin, 7 ng/ml glucagon, 1% (v/v) antibiotics (sodium ampicillin (10 μg/ml), kanamycin monosulfate (50 μg/ml), benzyl penicillin (7.3 IU/ml) and streptomycin sulfate (50 μg/ml)) and 10% (v/v) FBS. After 4 h of incubation at 37 °C in an atmosphere of 95% air and 5% CO_2 at 100% relative humidity, the medium was renewed with the same medium as described above but supplemented with 7.5 μg/ml hydrocortisone hemisuccinate. After 24 h of culture, the medium was daily renewed with serum-free medium.

2.3. Albumin secretion assay

Secretion of albumin into the medium was analysed at different culture times using an ELISA technique [17]. The content of antibody bound was detected by oxidation of the substrate TMB by the conjugated peroxidase. Absorbance was measured at 450 nm using a Titertek® Multiskan (Flow Laboratories, SA).

2.4. Preparation of cell lysates

Cells were harvested from the culture plates either by scraping off (monolayer culture and co-culture) or by collagenase digestion (sandwich culture) [18] and washed twice with ice-cold PBS. They were lysed with 100 μl lysis buffer pH 7.0 (40 mM sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 1 mM Pefablock, 1 μg/ml pepstatin A, 1 μg/ml leupeptin and 1 μg/ml aprotinin) by 5 freeze-thawing cycles. The lysates obtained were centrifuged at 16,060 × *g* for 30 min at 4 °C and protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.5. Caspase-3-like activity assay

The volume of cell lysate, containing 100 μg of total protein, was adjusted to 950 μl with pure water and 40 μl reaction buffer pH 7.4 (final concentrations: 10 mM PIPES, 2 mM EDTA, 1.6 mM CHAPS, 5 mM DDT) was added to each sample. After 2 min pre-incubation at 37 °C, 10 μl of 5.5 mM Ac-DEVD-AFC substrate was added. After 1 h incubation at 37 °C, the fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The background readings from cell lysates and buffer were subtracted from the readings of the samples and the enzyme activity was expressed as pmol/minute mg total protein.

2.6. Western blot analysis

Fifty micrograms of total protein was loaded onto 12% SDS-PAGE and transferred onto nitrocellulose membranes. Equal protein loading was controlled by reversible 0.1% Ponceau Red staining of the membranes. After blocking the membranes for 1 h in 0.1% Tween-20 containing 5% (w/v) non-fat milk, the blots were

probed overnight at 4 °C with primary antibody, followed by a one hour incubation at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized by enhanced chemiluminescence (Super Signal West Pico®). The primary antibodies used in this study were reactive to caspase-3 (rabbit; Calbiochem), Bax (mouse; Santa Cruz Biotechnology) and Bid (rabbit; R&D Systems). The goat–anti-mouse and goat–anti-rabbit secondary antibodies came from Dako Diagnostics.

2.7. Histone extraction

Histones were acid-extracted from cell pellets as previously described [19]. The cell pellets were resuspended in 1 ml ice-cold lysis buffer pH 6.5 (10 mM Tris–HCl, 50 mM sodium bisulfite, 1% (v/v) Triton X-100, 10 mM $MgCl_2$, 8.6% (w/v) sucrose) and Dounce-homogenized. The nuclei were collected by centrifugation (4 °C) at 380 g for 10 min and washed trice with lysis buffer and once with ice-cold 10 mM Tris–HCl, 15 mM EDTA, pH 7.4. The pellets were resuspended in 100 μl ice-cold pure water and 25 μl 2N H_2SO_4 was added. After incubation for 1 h on ice, the suspension was centrifuged for 5 min at 1,606 × *g* and 4 °C, and the supernatant was mixed with 1 ml acetone. After overnight incubation at −20 °C, the coagulated pellets were collected by centrifugation (5 min at 1,606 × *g* and 4 °C) and resuspended in 25 μl pure water. The protein amount was determined using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Thirty microgram of isolated histones were used for Western blot analysis. Hyperacetylated histones were detected using antibodies that specifically recognize the hyperacetylated forms of histones H3 and H4 (rabbit; Upstate Biotechnology).

2.8. EROD and PROD enzyme assays

Microsomes were prepared from freshly isolated and hepatocytes cultured for 7 days according to Hales and Neims [20]. Microsomal protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as a standard. The microsomes were incubated with either 5 μM ethoxy- or penthoxyresorufin. The resorufin formed was measured fluorimetrically according to a modified procedure of Burke and Mayer [21].

2.9. Statistical analysis

Data are presented as mean ± S.D. of three independent experiments. Results were evaluated by a two-sided paired Student's *t*-test for statistical difference between treatments. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of 1 μM TSA on apoptosis in short-term and long-term hepatocyte cultures

The caspases are the effector enzymes of apoptosis. They are synthesized as inactive procaspases (30–60 kDa) that contain three domains: an N-terminal prodomain, a large subunit (∼20 kDa) and a small subunit (∼10 kDa). Upon proteolytic cleavage, two large and two small subunits reassemble in order to form an active heterotetramer [22,23]. Here, the involvement of caspase-3, the main executioner of apoptosis, was investigated in rat hepatocyte cultures. The effect of TSA on caspase-3-like activity was measured towards Ac-DEVD-AFC as a function of culture time in the various types of adult rat hepatocyte cultures (Fig. 1). It was found that monolayer cultures under control conditions, show a significant ($P <$

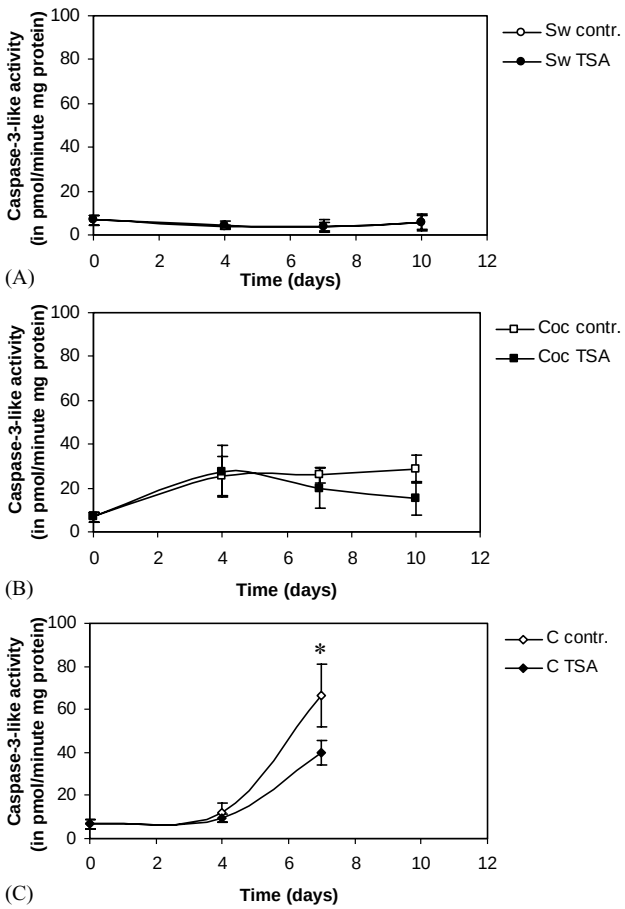

Fig. 1. Caspase-3-like activity measured in rat hepatocytes either cultured in the absence or presence of 1 μM TSA in a collagen I gel sandwich configuration (A), as a co-culture with rat liver epithelial cells (B) or as a conventional monolayer culture (C). Rat hepatocytes were cultured, harvested at the indicated time points and the DEVDase activity measured as described under Section 2. The activities are expressed as mean ± S.D. of three separated experiments. Statistical significance between control and TSA-treated hepatocyte cultures was tested by a paired Student's *t*-test (*$P < 0.05$). C, Conventional monolayer; Coc, co-culture; contr., control; Sw, collagen I gel sandwich culture.

0.01) 6-fold increase of caspase-3-like activity from day 4 (12.1 ± 4.3 pmol/min mg total protein) to day 7 (66.4 ± 14.4 pmol/min mg total protein) (Fig. 1C). In contrast, in the presence of TSA, only a four-fold increase is seen. This demonstrates that significantly ($P < 0.05$) higher caspase-3-like activity is reached on day 7 in control monolayers as compared to TSA-treated cultures. In contrast to monolayer cultures, a low and constant caspase-3-like activity is observed in control and TSA-treated sandwich cultures during the whole culture period (Fig. 1A). In addition, sandwich cultures (control and TSA-treated), show a significantly ($P < 0.05$) lower caspase-3-like activity than their co-culture counterparts (Fig. 1B). However, co-cultures contain two different cell populations and the contribution of rat hepatocytes versus rat liver epithelial cells to the DEVDase activity is not known.

In order to confirm our results obtained for the caspase-3-like activity assay, we further analysed the processing of caspase-3 in both short-term and long-term cultures by Western blotting. As shown in Fig. 2, and in agreement with the activity assay, no major differences between control and TSA-treated sandwich cultures (Fig. 2A) and co-cultures (Fig. 2B) were found regarding caspase-3 processing. Although the 17 kDa large subunit (p17) was found in freshly isolated hepatocytes, both control and TSA-treated long-term cultures showed lower levels of this fragment on day 4 with further apparent decreases on days 7 and 10. No large subunit was detected in pure rat liver epithelial cells (Fig. 2B last lane).

In the case of control monolayer cultures (Fig. 2C), an up-regulation of procaspase-3 (p32) was seen by day 4. Unlike sandwich and co-cultures, control monolayer cultures showed a continuous presence of the 17 kDa fragment, suggesting a process of ongoing caspase-3 activation in line with the observed increase in DEVDase activity. In TSA-treated cultures, the expression of procaspase-3 remained approximately at the same level during the whole culture period. However, a tendency towards a decrease in the level of the 17 kDa large fragment was seen by day 5 in TSA-treated monolayers, while its expression in control cultures stayed constant or was slightly increased which is in agreement with the lower caspase-3-like activity observed in TSA-treated monolayer cultures at day 7.

Caspase activation is controlled by members of the Bcl-2 protein family. Both pro- (e.g. Bax and Bid) and anti-apoptotic (e.g. Bcl-2 and Bcl_{xL}) members have been identified and proven to play a pivotal role in mitochondria-dependent apoptosis [24–26]. We, therefore, investigated whether changes in the expression of pro-apoptotic Bid and Bax could be involved in the activation of caspases and induction of spontaneous apoptosis. As shown in Fig. 3A, the expression of Bid remained constant in control monolayer cultures. In contrast, in TSA-treated monolayer cultures, the expression of Bid showed a marked decrease in Bid levels by day 5. Except for day 7, no major

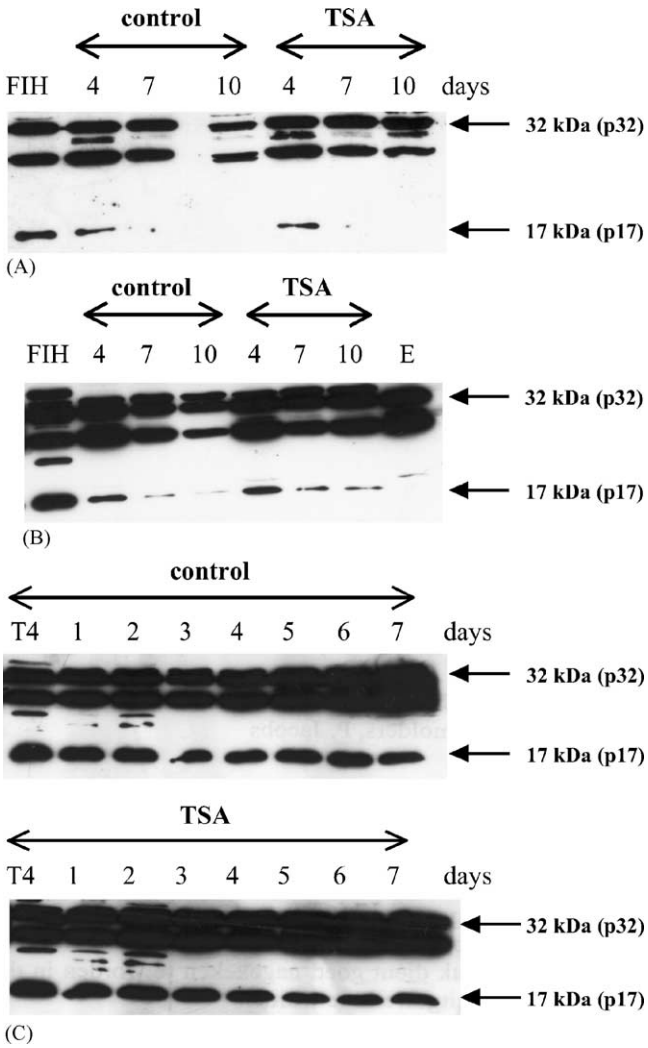

Fig. 2. Expression of (pro)caspase-3 in rat hepatocytes either cultured in the absence or presence of 1 μM TSA in a collagen I gel sandwich configuration (A), co-culture with rat liver epithelial cells (B) or a conventional monolayer culture (C). Rat hepatocytes were cultured, harvested at the indicated time points and cellular extracts were probed for caspase-3 by Western blot analysis as described under Section 2. Equal loading has been confirmed with Ponceau Red staining (FIH, freshly isolated rat hepatocytes; E, pure rat liver epithelial cells; T4, rat hepatocytes cultured for 4 h after the isolation).

difference could be observed in the levels of Bax between control and TSA-treated cultures (Fig. 3B).

3.2. Effect of 1 μM TSA on histones H3 and H4 acetylation in short-term and long-term hepatocyte cultures

To determine whether the acetylated state of core histones was involved in decreasing the levels of caspase-3 and Bid, we analysed the amount of acetylated histones H3 and H4 in the various adult rat hepatocyte cultures in response to 1 μM TSA. Using antibodies that specifically recognize the acetylated state of the histones H3 and H4, we found a higher expression of acetylated H3 and H4 in TSA-treated monolayer cultures in comparison with

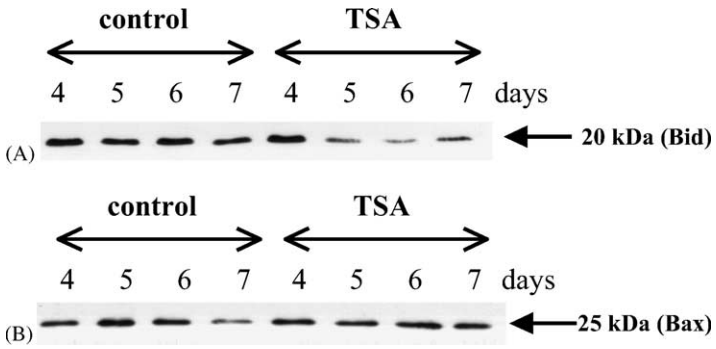

Fig. 3. Expression of Bid (A) and Bax (B) in conventionally cultured adult rat hepatocytes either in the absence or presence of 1 μM TSA. Rat hepatocytes were cultured, harvested at the indicated time points and cellular extracts were probed for Bid and Bax by Western blot analysis as described under Section 2. Equal loading has been confirmed with Ponceau Red staining.

control cultures on days 5 and 6 (Fig. 4C). In sandwich cultures (Fig. 4A) and co-cultures (Fig. 4B), a higher acetylation state of histones H3 and H4 could also be observed after 4 and 7 days of culture.

3.3. Effect of 1 μM TSA on the differentiated state of short-term and long-term hepatocyte cultures

Since TSA is known to have the ability to induce differentiation in several cell lines [1,4,6–8], we also investigated whether TSA had an effect on the maintenance of liver-specific functions as assessed by the amount of albumin secreted into the culture medium and the phase I CYP1A1/2 (EROD) and CYP2B1 (PROD)-dependent activities.

In control monolayer cultures, a significant ($P < 0.05$) decrease of albumin secretion from day 2 to day 7 was found, indicating a continuous dedifferentiation of the cultured rat hepatocytes (Fig. 5C). TSA-treated cultures also showed an initial significant ($P < 0.05$) decrease in albumin secretion till day 4, remaining constant thereafter and resulting on day 7 in a higher albumin secretion ($P < 0.05$) in TSA-treated monolayers in comparison to control monolayers. For sandwich cultures and co-cultures, no significant differences in albumin secretion between TSA-treated and non-treated cultures could be observed Figs 5A and B).

In order to evaluate the effect of TSA on the maintenance of the biotransformation capacity in cultured hepatocytes, we investigated the effect of 1 μM TSA on phase I EROD and PROD activities after 7 days of culture (Table 1). In the presence of TSA, EROD and PROD activity values showed increases of 55% ($P < 0.05$) and 20%, respectively, as compared to control cultures. Similar to the observations made for albumin secretion, no effects of TSA on EROD and PROD activities could be found after treating sandwich cultures and co-cultures with TSA (not shown).

Table 1
EROD and PROD activity in 7-day conventionally cultured adult rat hepatocytes in the absence and presence of 1 μM TSA

Culture medium	Activity (pmol resorufin/min mg microsomal protein)	
	EROD	PROD
Control	2.8 ± 0.5	3.4 ± 0.6
TSA	4.3 ± 0.6*	4.1 ± 0.7

Note: Mean values ± S.D. of three separate experiments ($n = 3$) are shown. Statistical significance between control and TSA-treated hepatocyte cultures was tested by a paired Student's *t*-test (*$P < 0.05$).

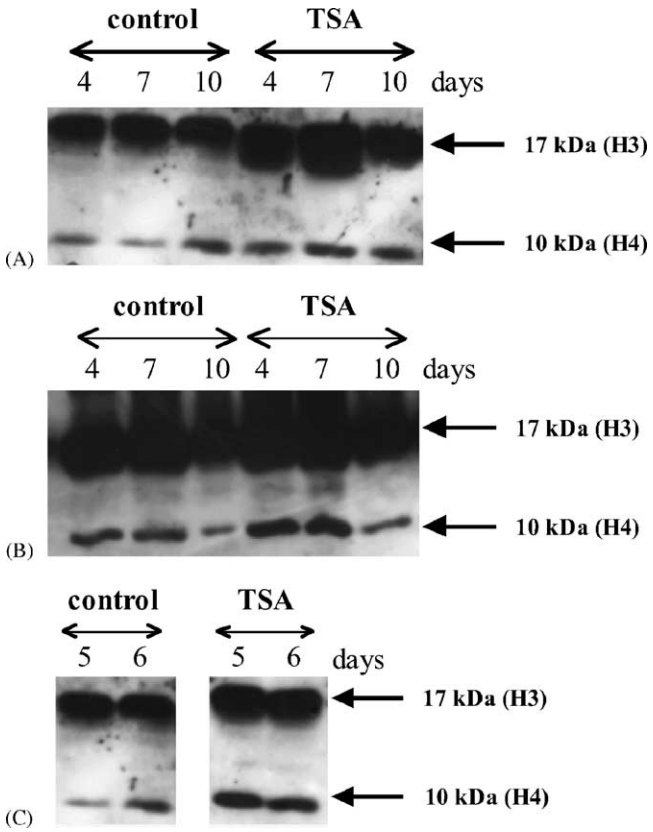

Fig. 4. Expression of acetylated histones H3 and H4 in collagen I gel sandwich cultures (A), co-cultures with rat liver epithelial cells (B) and conventionally cultured adult rat hepatocytes (C) in the absence and presence of 1 μM TSA. Rat hepatocytes were cultured, harvested at the indicated time points and cellular extracts were probed for acetylated H3 and H4 by Western blot analysis as described under Section 2. Equal loading has been confirmed with Ponceau Red staining.

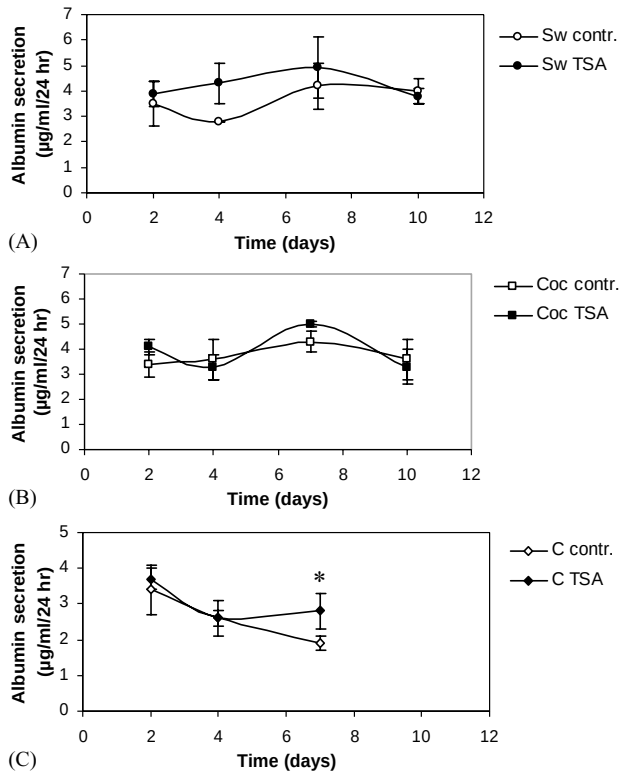

Fig. 5. Amount of albumin secreted into the culture medium of a collagen I gel sandwich configuration (A), co-culture with rat liver epithelial cells (B) or a conventional monolayer culture (C) in the absence and presence of 1 μM TSA. Results are expressed in μg albumin secreted per ml medium in 24 h and are mean values ± S.D. of three separate experiments ($n = 3$). Statistical significance between control and TSA-treated hepatocyte cultures was tested by a paired Student's t-test ($^{*}P < 0.05$). C, Conventional monolayer; Coc, co-culture; contr., control; Sw, collagen I gel sandwich culture.

4. Discussion

Primary adult rat hepatocyte cultures are important in vitro models to study the pharmacological and toxicological properties of newly developed drugs [27]. As such, these models are frequently used to investigate the occurrence of chemically induced cell death. Cytotoxicity, as measured for instance by lactate dehydrogenase leakage or trypan blue exclusion, often has been taken as the end point for toxicological cell damage. However, it has become clear that many cytotoxic agents kill cells predominantly by triggering apoptosis (programmed cell death) rather than necrosis, unless excessive concentrations of the drug are involved [28,29]. Here, the effects of 1 μM TSA (non-cytotoxic concentration), a drug candidate for the treatment of liver fibrosis [3], have been investigated on the cell death programme in short-term (conventional monolayer) and long-term (collagen I gel sandwich and co-cultures) primary adult rat hepatocyte cultures. Therefore, the expression of downstream caspase-3 as well as Bid and Bax have been investigated.

In control monolayer cultures, an increase in caspase-3-like activity is seen from day 4 to day 7. However, Martin et al. [30] reported an increase in caspase-3 activity in monolayer cultures already at day 2. This discrepancy is probably attributable to the use of different culture media, culture plate coatings and cell plating density. In TSA-treated monolayer cultures the caspase-3-like activity was also increased. However, this was significantly less than in controls. In accordance with the caspase-3-like activity measurements, it was further found at the protein level that, after 4 days, TSA led to a reduction in the processing of downstream caspase-3. Likewise, the presence of TSA caused a decrease in the levels of Bid. No effect of TSA on the expression level of Bax could be found. These effects were accompanied by a TSA-mediated increase of acetylated histones H3 and H4. In addition, a better maintenance of albumin secretion as well as CYP1A1/2 and CYP2B1-dependent enzyme activities were found in TSA-treated monolayers, indicating an improved differentiation state of these cells.

The effect of TSA on apoptosis has been previously investigated using different cell lines, including the human hepatoma cell line HepG2 [8,9]. In this cell line, 1 μM TSA rather induced than inhibited apoptosis, as evidenced by an increase in caspase-3 activity together with an up-regulation of Bax and down-regulation of Bcl2. TSA-induced apoptosis was even more pronounced in Hep1B compared to HepG2 cells, which can be attributed to the lower differentiation degree of the latter [9]. However, Yamashita et al. have been unable to detect apoptosis in HepG2 cells after exposure to 1 μM TSA, whereas 250 nM TSA seemed to be enough to induce apoptosis in Huh-7 hepatoma cells [31]. Thus in both, cancerous hepatocytes and primary cultured hepatocytes, apoptosis is regulated via acetylation/deacetylation of histone proteins but in exact opposite ways. Whereas in primary hepatocytes TSA is anti-apoptotic ([13] and this study), it usually is pro-apoptotic in liver cancer cells. However, different sensitivities towards TSA-induced apoptosis exist within individual hepatoma cell lines that might be related to various differentiation degrees of the cells and/or the culture conditions used.

Here, sandwich cultures and co-cultures, two long-term culture models known to better preserve hepatocyte specific functions including biotransformation [32–35] and albumin secretion [36,37], have also been tested for their response to TSA. However, it was found that in untreated as well as in TSA-treated co-cultures and sandwich cultures that showed already a higher expression of acetylated histones H3 and H4, the caspase-3-like activity remained low during culture time, indicating only a very modest spontaneous apoptosis in these long-term culture systems. A decreased processing of caspase-3 in co-cultures and sandwich cultures, as observed by Western blotting, confirmed this finding. No data are available with respect to caspase-3-like activity in co-cultured hepatocytes. However, we have previously shown that, compared to conventional monolayers, co-cultures are much less susceptible to oxidative stress [38,39], known to trigger

apoptosis in hepatocytes [40]. Thus, less apoptosis is indeed to be expected in co-cultures. Likewise in sandwich cultures, low levels of oxidative stress are observed [41] and are accompanied by a low caspase-3-like activity ([42] and this study), which is in agreement with the results obtained here. Taken together, our findings point to the fact that, probably due to reduced oxidative stress because of the presence of either an extracellular matrix or cell-cell interactions, sandwich cultures and co-cultures, respectively, are not as susceptible to spontaneous apoptosis compared to conventional monolayers. However, further studies are needed to fully elucidate a possible direct relationship between apoptosis and the maintenance of the differentiated state of hepatocytes in the various types of culture systems.

From our results, we conclude that, although 1 μM TSA is unable to completely block spontaneous apoptosis in primary monolayer cultures of adult rat hepatocytes, the onset of apoptosis and loss of liver specific functions occur later than observed in untreated control cultures. However, under culture conditions where spontaneous apoptosis is already minimised either through the addition of extracellular matrix components (sandwich cultures) or the presence of non-parenchymal liver cells (co-cultures), TSA does not exhibit an additional beneficial effect. Further experiments are currently underway to elucidate whether TSA also inhibits chemically-induced apoptosis in hepatocyte monolayer cultures. However, it is already clear that the level, onset and regulation of apoptosis in primary hepatocyte cultures largely depend on the culture conditions used.

Acknowledgments

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