Changes in expression of cell cycle regulators after G1 progression upon repetitive thioacetamide treatment in rat liver

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Abstract

Repetitive low dose thioacetamide (TA) treatment of hepatocytes was found to induce cells in G2 arrest. In the present study, an attempt was made to investigate alterations in expression of cell cycle regulators after G1 progression in the same repetitive low dose TA treated hepatocytes system and to define the determinators involved in G2 arrest. TA was daily administered intraperitoneally, with a dose of 50 mg/kg for 7 days. Expression levels of cyclin E and CDK2 were similar, increased at day 1 and reached a peak at day 2. And they recycled from day 3 reaching a second peak at day 5. Expression level of cyclin A was similar to p27^{Kip1} and p57^{Kip2} but not to CDK2 and increased to a peak level at day 2. Expression levels of cyclin B1 and cdc2 were similar although the cyclin B1 level was generally low, decreased from day 1 to basal levels at day 3 and persisted at a low level till day 7. The expression level of cyclin G1 was similar to p53 that peaked at day 3 and again at day 6 elevated over basal level. BrdU-labeled hepatocytic nuclei increased from 12 h, reached a peak at day 2, then decreased, and were not detectable from day 6. The number of PCNA-labeled nuclei increased immediately, peaked at day 2, and maintained till day 7. These results suggest that G2 arrest induced by repeated TA treatment might be p53-dependent, via activation of cyclin G1, rather than inhibition of cyclin B1-cdc2 complex, and inhibitors holding S phase progression might be p27^{Kip1} and p57^{Kip2}.

Introduction

Thioacetamide (TA) is a well known hepatotoxin (Fitzhugh et al., 1948). Earlier literature suggests that an obligate intermediate of TA metabolism by cytochrome P450 binds to proteins and forms acetylimidolysine derivatives which are responsible for hepatotoxic effect (Vadi et al., 1981). According to dose-response relationship, TA causes several types of liver damage and ultimately produces malignant transformation. TA has been reported to endorse chemically induced cell death via both apoptosis or mild necrosis in lower dose (Ledda-Columbano et al., 1991) and massive necrosis in higher dose. Administration of low dose of TA is known to open cell cycle, and stimulate DNA synthesis and mitosis in the rat liver for hepatocyte regeneration. In rats treated with a single low dose of 50 mg/kg of TA, peak S phase stimulation occurs at 36 h and subsides by 72 h (Reddy et al., 1969: Ramaiah et al., 1998). Hepatocellular regeneration and tissue repair in response to the pretreated low dose of TA are confirmed to be a pivotal priming role in mechanism of autoprotection toward lethal doses of TA (Mangipudy et al., 1995a, b). The mitotically dormant state of the hepatocytes of the adult liver belies the tremendous proliferative potential of the mature cells of the hepatocyte lineage in response to injury (Sell et al., 2001).

The eukaryotic cell cycle is regulated by signal transduction pathways mediated by complexes of cyclin dependent kinases (CDKs), and their partner, cyclins (Van et al., 1993; Pines et al., 1994). The catalytic activity of CDK is dependent on tyrosine and threonine phosphorylation by CDK activating kinases, binding to cyclins and interaction with CDK inhibitors (CKIs) (Draetta et al., 1994; Heichman et al., 1994; Hunter et al., 1994; King et al., 1994; Nurse et al., 1994; Pines et al., 1994; Sherr et al., 1994). Cell cycle regulators play an important role in physiologic

cellular regeneration, but dysregulated forms are closely related with pathologic cellular proliferation, including malignant transformation (Grana *et al.*, 1995; Shin *et al.*, 2000).

Literature on the effect of repeated exposure to TA inflicting liver injury is limitted. It has been reported that one of the most characteristic toxicities in rat liver is observed with repeated exposure of low dose TA (50 mg/kg per day) which induces cellular, nuclear and nucleolar enlargement, much different from those in regenerating liver (Jeong *et al.*, 1994). And these cellular organellomegaly was considered as a status of G2 arrest reflected toward continuous compensatory stimulation (Jeong *et al.*, 2001). Another study concerning about tissue repair after repeated exposure of TA with intervals of 96 h represented that S-phase stimulation did not occur following 4th dose and suggested that tissue repair was not operational (Mangipudy *et al.*, 1998). In the previous report on repeated low dose TA-treated rat liver, entry of G1 phase and G1 progression have been permitted through the action of cyclin D1-CDK4/CDK6 complex, $p21^{Waf1}$ as their nuclear transporter, and functionless $p16^{Ink4a}$ (unpublished data).

In the present study, an attempt was made to investigate changes in expressions of cyclins, CDKs, and CDKIs after G1 progression, to the response of repeated low dose administration of TA, ultimately inflicted on G2 arrest and eventually to search possible determinators of G2 arrest.

Materials and Methods

Experimental animals

Male Sprague Dawley rats weighing approximately 200 g were fed with laboratory chow and had free access to water *ad libitum.* The facility was conditioned with an alternating 12 h-light and 12 h-darkness cycle. A $21 \pm 1°C$ temperature and 50% relative humidity were preserved all times. All animal experiments were conducted in accordance with the institutional guidance and approved by the Animal Experimentation Committee of the Dong-A University School of Medicine.

Primary antibodies

Primary antibodies toward cyclin E, cyclin A, cyclin B1, cyclin G1, $p27^{Kip1}$, $p57^{Kip2}$ and cdc2 were purchased from Santa-Cruz Biotechnology Inc. (Santa Cruz, CA), CDK2 from NeoMarkers (Union City, CA), p53 from Oncogene Research Products (Boston, MA), proliferating cell nuclear antigen (PCNA) from DAKO-PATTS (Glostrup, Denmark), and BrdU from Novocastra Laboratories LTD (Newcastle, UK).

Carcinogen treatment and liver sample preparations

TA (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% saline to make 1% and was intraperitoneally administered daily, with a dose of 50 mg/kg for 7 days. During administration of TA for 7 days, TA-treated rats were sacrificed everyday.

Under anesthesia with ether, rat liver was perfused with 0.9% saline through the portal vein to remove red blood cells. Removed livers were directly used or fresh-frozen in liquid nitrogen and stored at -80°C. For getting better condition for preservation of nuclear antigen during immunohistochemistry, the liver was continuously perfused with 10% neutral buffered formalin for 1 h through the portal vein at 4°C. After removal, liver slices were fixed in 10% neutral buffered formalin for 24 h, at 4°C, again. These slices were embedded in paraffin after processing. Five μm thick liver sections were stained with hematoxylin and eosin for routine histology.

BrdU injection

At 2 h before sacrifice of TA-treated rat, 2 mg of 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co. St. Louis, MO) dissolved in 0.2 ml of normal saline solution was injected, intraperitoneally.

Whole tissue protein extraction

Using Teflon-glass homogenizer, 0.5 gm of liver tissue was homogenized in 1 ml of homogenization buffer (Tris-HCl, pH 7.4, 20 mM; NaCl, 100 mM; NP-40, 1%; sodium deoxycholate, 0.5%; $MgCl_2$, 5 mM; pepstatin, 0.1 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.2 mM; aprotinin, 0.4 mM; soybean trypsin inhibitor, 0.5 mg/ml). Tissue homogenates were centrifuged at 15,000 *g* for 5 min and the supernatant fraction was collected. After measuring the protein concentration, aliquots of samples were stored at -80°C. All procedures were done at 4°C.

SDS polyacrylamide gel electrophoresis and immunoblotting

Proteins (20 μg/μl) were fractionated on 12% SDS-polyacrylamide slab gels, according to Laemmli (Laemmli *et al.*, 1970). All samples were treated at the same concentration level and gels were checked with Coomassie blue staining. The gels were transferred into nitrocellulose membrane for 2 h at 60 volt. And rechecked the nitrocellulose membrane with Ponceau S staining for evaluation of blotting. The membranes were preincubated in TBS including 10% defatted milk powder for 1 h at room temperature. Then, adequately diluted antibodies were added, and incubated for

1 h. After washing with Tris Buffered Saline (TBS)-0.1% Tween 20, the membranes were incubated with alkaline phosphatase conjugated secondary antibodies for 30 min. After washing with TBS-0.1% Tween 20, the reaction was visualized with nitro-blue tetrazolium/S-bromo-4-chloro-3-indoyl-1-phosphate.

Immunohistochemistry

Processed and paraffin embedded tissue blocks were cut into 4-6 μm thickness onto Snowcoat X-tra™ MICRO SLIDES (Surgipath, Richmond, IL). They were deparaffinized with xylene and hydrated with decreasing concentrations of ethanol and washed with TBS-0.1% Tween 20 (LABVISION Co. Fremont, CA). For the retrieval of nuclear antigen, they were placed in Coplin jars, immersed in citrate buffer and put in microwave for 10 min. HISTOSTAIN-PLUS BULK KITS (Zymed, SF, CA) was used for immunostaining as follows: pretreated slides were incubated in normal goat serum for 30 min to block nonspecific bindings. The primary antibodies were incubated overnight at 4°C. Twice washing with TBS for 10 min followed by incubation with avidin biotin complex-linked peroxidase solution, and rinsed twice with PBS for 10 min. For colorization, 3-amino-9-ethylcarbazole in 0.01% hydrogen peroxide, Tris buffer 50 mmol/L, pH 7.2 was applied until positive controls were detected. After washing with TBS, the slides were mounted and microscopic examination followed. Negative control slides were processed in the same manners except primary antibody treatment.

For BrdU antibody, the paraffin sections required pretreatment. Deparaffinized and rehydrated slides were treated with trypsin at 37°C for 30 min, rinsed and preheated in distilled water at 37°C for 5 min. Then, slides were incubated with 2 N HCl at 37°C for 30 min, and incubated with 0.1 M di-sodium tetraborate for 10 min. After rinse in tap water, the slides were rinsed in TBS several times, and next procedures were same as above.

The labeling index of hepatocytes for BrdU and PCNA was determined by counting more than 2,000 nuclei in photographs of three randomly selected fields under a light microscope ($\times$100).

Results

Histopathologic findings

Inflammatory reaction and apoptosis of hepatocytes started at 6 h after TA administration, peaked at 18 h and day 1, subsided for next several days, and minimized at day 7. Inflammatory activity was more prominent at centrilobular areas. From day 1, enlarged nuclei and nucleoli were observed at enlarged

hepatocytes around the central vein, which gradually propagated toward portal areas.

Changes in expression of cyclin E and CDK2

Cyclin E and CDK2 represented similar expression patterns during TA treatment (Figure 1). They were constitutively expressed in control liver. From day 1, their expressions increased and peaked at day 2. Although slight decreases were observed at day 3, day 4 and day 6, increases were noted till day 6.

Changes in expression of cyclin A, $p27^{Kip1}$ and $p57^{Kip2}$

Cyclin A, $p27^{Kip1}$, and $p57^{Kip2}$ showed similar expression patterns during TA treatment (Figure 1). In control liver, cyclin A, $p27^{Kip1}$ and $p57^{Kip2}$ were lowly expressed. With time progression during TA treatment, their expressions increased and reached a peak at day 2 or day 3.

Changes in expression of cyclin B1 and cdc2

Cyclin B1 and cdc2 showed similar expression patterns during TA treatment (Figure 1). They were expressed in control liver and their expressions decreased during TA treatment, although at day 3 they reached control level. Cyclin B1 expression pattern generally showed low expression signal.
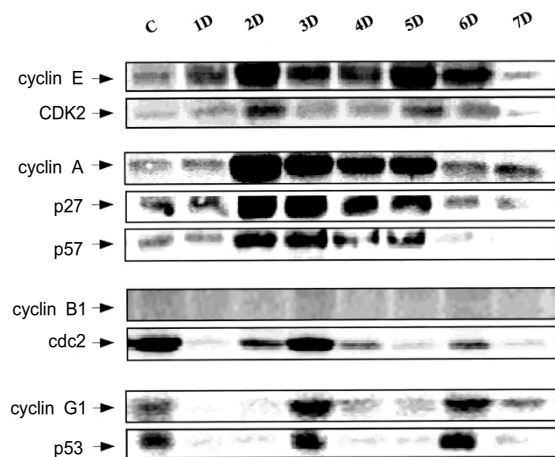

Figure 1. Expression of cyclins, CDKs, CKIs, and p53. Rat livers obtained during TA treatment were homogenated, extracted and subjected to 12% SDS-polyacrylamide gels, as mentioned in Materials and Methods. The gels were subsequently analyzed for immunoblot, using individual antibodies to cyclin E, CDK2, cyclin A, p27, p57, cyclin B1, cdc2, cyclin G1 and p53 as mentioned in Materials and Methods. C, control liver; 1D - 7D, 1st through 7th day during TA treatment.
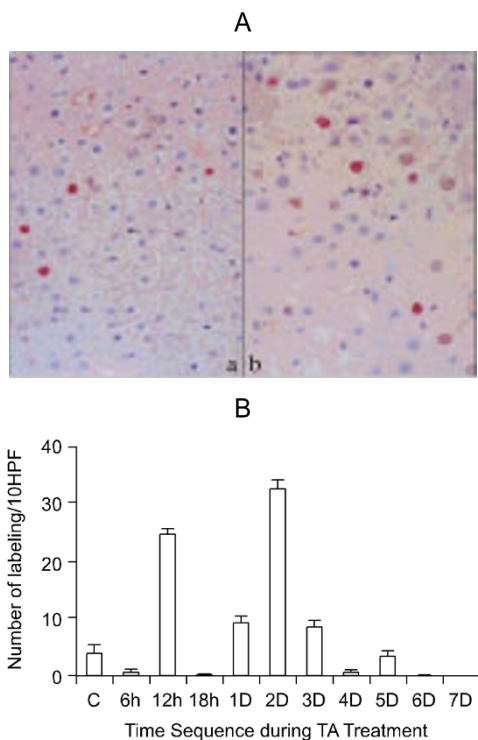
A

B

Figure 2. BrdU labeling incorporated into hepatocytes. 2 h before sacrifice, BrdU solution was injected, intraperitoneally, and then removed liver was adequately processed. Anti-BrdU was detected by LSAB method as mentioned in Materials and Methods. (A) Labeled hepatocyte nuclei are identified in red color. a, 12 h after TA treatment; b, 2nd day during TA treatment (B) Numbers of BrdU-labeling in time sequence during TA treatment were compared. The values expressed as means ± S.E (n=5).
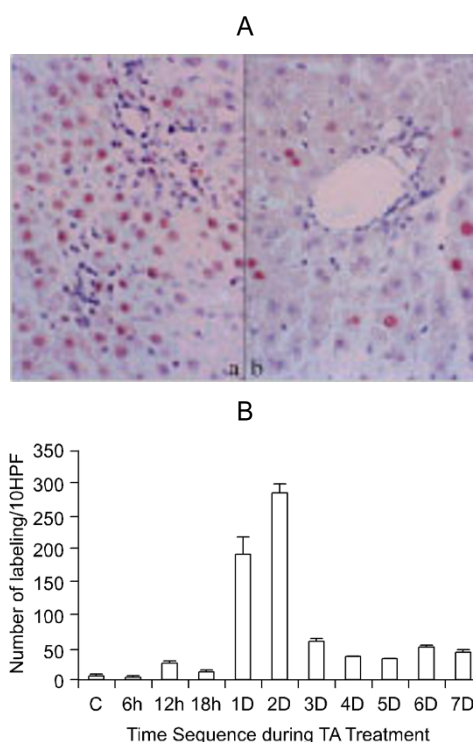
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Figure 3. PCNA labeling of rat hepatocytes. TA-treated rat liver tissue was adequately processed, paraffin-embedded, and anti-PCNA was detected by LSAB method as mentioned in Materials and Methods. (A) Labeled hepatocyte nuclei are identified in red color. a, 2nd day after TA treatment; b, 3rd day during TA treatment (B) Numbers of PCNA-labeling in time sequence during TA treatment were compared. The values expressed as means ± S.E (n=5).

Changes in expression of cyclin G1 and p53

Cyclin G1 and p53 showed similar expression patterns during TA treatment (Figure 1). They were expressed in control liver. Their expression persisted at a low level till day 7, but showed two peaks at day 3 and day 6 which were stronger than basal level.

Attempts for immunohistochemical visualization of all cell cycle regulators were not successful.

Assessment of BrdU and PCNA labeling

BrdU-labeled nuclei were noted in control liver. According to time progression during TA treatment, the number of BrdU-labeled nuclei increased and peaked at day 2 (Figure 2). Then, the number remarkably decreased from day 3, and during following days, rarely recognized. PCNA-labeled nuclei were detected in control liver, as well. From 12 h of TA treatment, the number of PCNA-labeled nuclei increased and peaked at day 2 (Figure 3). In contrast to BrdU, from day 3 to day 7, the increased number of PCNA labeling persisted.

Discussion

TA causes various liver damages in a dose-dependent manner, including hepatic necrosis, regeneration, cirrhosis and malignant transformation. A single low dose of TA induces apoptosis or mild necrosis of rat hepatocytes accompanied by a cell cycle opening for regeneration (Ledda-Columbano et al., 1991; Mangipudy et al., 1995). These changes are associated with nucleolar enlargement, increase in polyamine synthesis, alterations in the relative proportion of histone, and increase of nuclear RNA into the cytosol. The rise in DNA synthesis induced by TA follows a time sequence. Rats treated with a single low dose of 50 mg/kg of TA undergo hepatocellular proliferation as suggested by ^{3}H-thymidine incorporation, which peaks at 36 h after treatment. It is confirmed by cell cycle proliferation and cell division by PCNA assessment (Reddy et al., 1969; Ramaiah et al., 1998). In a single low dose of TA 50 mg/kg, hepatocytic necrosis was evident at 12 h, peaked at 36 h, persisted up to 72 h and resolved by 96 h (Reddy et al., 1969).

And peak S-phase stimulation occurred at 36 h and subsided by 72 h.

Repeated daily exposures of low dose TA in this study showed that inflammatory activity with apoptosis of hepatocytes started at 6 h, peaked at 18 h and day 1, gradually diminished and minimized at day 7. These findings are different from cyclic response of necrosis and repair in other pattern of repeated TA treatment with intervals of 96 h (Ohtsubo *et al.*, 1995). Throughout histologic screening during TA treatment, mitoses were not observed in hepatocytes. These histologic findings coincided with BrdU incorporation into hepatocyte nuclear DNA (Figure 2). At day 2, S-phase stimulation reached a peak but decreased, and during last 4 days, BrdU incorporation was under the control level or not seen. On the contrary, PCNA assay revealed constantly increased labeling during TA administration, and shows a peak at day 2, corresponding to BrdU incorporation. The PCNA study represented corresponding stimulation to urge cell cycle progression for compensatory tissue repair. A unique feature was nuclear and nucleolar enlargement of hepatocytes, especially around the centrilobular area. Above results were coincided with the recent study of flow cytometric analysis in which hepatocytes treated with repeated low dose of TA were arrested at G2 (Jeong *et al.*, 2001). Till now, determinators concerned about G2 arrest induced by repeated low dose of TA treatment have not been reported.

In our previous study, it has been noticed that repeated daily administration of TA endorses rat hepatocytes to enter cell cycle and progress G1 phase, under the possible activation of cyclin D1-CDK4/CDK6 complex (unpublished data). The expression pattern of p21^{Waf1} was similar to cyclin D1 not p16^{Ink4a}, supporting a role of transporter protein (Juamot *et al.*, 1999) rather than inhibitory regulator (Oh *et al.*, 2002). Also the expression of p16^{Ink4a} increased but localized at cytoplasm, suggesting functionless status. The present study was undertaken to determine alterations of cell cycle regulators involved in G1/S transition, S phase and G2 checkpoint.

In mammalian cells, cyclin E expressed later than cyclin D1, and the formation of cyclin E-CDK2 for activation occurred just before S phase. The cyclin E-CDK2 complexes are another candidates for G1 phase pRb protein kinases (Ohtsubo *et al.*, 1995), which phosphorylates pRb and free E2F transcription factor goes on progression of cell cycle. In addition to pRb phosphorylation, the complex induces progression to S phase *via* phosphorylation of Cdc25. Figure 1 represents expressions of cyclin E and CDK2, showing increases from day 1 and 1st peak at day 2 which correspond with the peak of BrdU incorporation as shown in Figure 2. Between day 1 and day 2, G1/S transition might be successfully progressed.

BrdU incorporation abrogated after day 3, nevertheless expressions of cyclin E and CDK2 cycled once more, which represented corresponding stimulation to urge cell cycle progression, suggesting a highly stimulated compensatory response in tissue repair to chemical injury.

Cell cycle regulators related with progression of S phase were known to be consisted of cyclin A and CDK2 which were responsible for DNA synthesis (Nurse *et al.*, 1994). Expression and activation of cyclin A increased during late G1 and S phases, and decreased during mitosis. The accurate substrate of cyclin A-CDK2 complex was still not clearly identified. Figure 1 showed the expression of cyclin A reached a peak at day 2 and persisted till day 5 and returned to control level at day 6 and day 7. Cyclin A revealed similar expression patterns with p27^{Kip1} and p57^{Kip2} rather than CDK2, suggesting two possibilities; one, activation of cyclin A-CDK2 complex at day 2 and another, inhibition by p27^{Kip1} or p57^{Kip2}.

The expression of cyclin B1 increased at early G2 and peaked at late G2 and early M phases (King et al., 1994; Nurse *et al.*, 1994). For the success of G2/M transition, activation of cdc2 bound with cyclin B1 is essential. The activation of cdc2 is regulated by its phosphorylation at Tyr15 residue *via* wee1 and dephosphorylation at Tyr15 residue *via* Cdc25, as well. The expression patterns of cyclin B1 and cdc2 during treatment of low dose TA were similar, even though the expresssion level of cyclin B1 was generally low, and showed decrease from day 1 till day 7 although basal levels at day 3.

Since G1 arrest by the expression of p53 was confirmed, p53 was suggested to regulate cell cycle at G1 checkpoint and proved as a safeguard through the induction of p21^{Waf1} expression, resulting into DNA repair or apoptosis (Agarwal *et al.*, 1995). A numerous hypothesis of mechanisms about signal transduction pathway in G1 arrest have been investigated and a questionable role of p53 in concern with G2 arrest was arisen. Recently, it was reported that p53 inactivated Cdc2 through inhibition of transcriptional expression of cyclin B1 and Cdc2 (Stewart *et al.*, 1995). p53 was also known as an inducer of cyclin G1 gene that was recently discovered, contained two functional binding sites for p53 and linked to p53-dependent G2 arrest, although exact mechanism was not known (Jensen *et al.*, 1998). In this study, cyclin G1 expression showed two peaks at day 3 and day 6, coinciding with the expression of p53.

It is suggested that G2 arrest induced by repeated TA treatment might be p53-dependent, *via* activation of cyclin G1 rather than inhibition of cyclin B1-cdc2 complex, and inhibitors holding S phase progression might be p27^{Kip1} and p57^{Kip2}, even though immunoprecipitation and kinase activity studies were not

performed. Persisted PCNA labeling after peak S-phase stimulation and repeated increased expressions of positive regulators were considered as corresponding stimulation to urge cell cycle progression, suggesting a highly stimulated response for compensatory tissue repair.

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