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Altered Remodeling of Nucleolar Machineries in Cultured Hepatocytes Treated with Thioacetamide

Thioacetamide (TA) is converted into a hyperacetylating agent which causes hepatic necrosis, regeneration, cirrhosis and cancerous transformation. One of the most characteristic toxicities of TA in rat is observed with a 50 mg/kg per day which induces nucleolar enlargement different from that in regenerating liver. From TA-treated liver, the nucleoli were isolated and characterized for an altered nucleolar signal transduction system. Immunochemistry revealed that the poisoned nucleoli had increased levels of both nucleolus specific proteins (nucleophosmin and nucleolin) and various signal molecules (CK2, Erk1/2, p38, protein kinases A and C, and cyclin A). Using flow cytometry, the nucleoli were found to be in G2-arrested nuclei. Manifestation of the nucleolar enlargement could be readily observed using an ex vivo hepatocyte culture. There were two types of nucleolar enlargement. One was observed in normal hepatocytes with light density of enlarged nucleoli. The other was in TA-treated hepatocytes with dense and compact density of enlarged nucleoli, which contained a 3 to 5-fold higher nucleophosmin content than the control. In vitro induction of nucleolar enlargement with TA was possible. As soon as the hepatocytes anchored on a collagen coat, exogeneous TA (higher than 1 μg/mL) could induce dense and compact nucleoli. However, when an exogeneous drug was added after monolayer formation (1 day), no drug-induced nucleolar enlargement was observed.

Key Words: *Hepatocyte; Thioacetamide; Nucleolus Organizer Region; G2-Arrest; Regeneration*

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INTRODUCTION

Thioacetamide (TA) is a hepatocarcinogen which causes several types of liver damage in a dose-dependent manner and ultimately produces malignant transformation. In the rat liver system, a lethal dose (higher than 600 mg/kg) produces severe necrosis and fulminant hepatic encephalopathy leading to death (1, 2). A moderately high dose (200-400 mg/kg) causes hepatic zonal necrosis, followed by cirrhosis which ultimately leads to malignant transformation (4, 5). A low dose (40-100 mg/kg) induces mild apoptosis or necrosis accompanied by a cell cycle opening for regeneration (1, 3). These dose response relationships have been utilized for the genesis of liver disease models involved in acute hepatic failure, regeneration, cirrhosis and cancerous transformation. Among the above relationships, the low dose response pattern is of great interest because a novel TA toxicity is recognized in induction of the opening of the cell cycle and the remodeling of subnuclear structures, especially those associated with conversion of a multinucleolar structure into a dense and compact megalonucleolus (TA-induced nucleolar enlargement) (6). This toxic organelle remodeling is a unique characteristic sign of early TA-induced tumor promotion. Electron microscopic studies demonstrated that the nucleoli of TA-treated liver are clearly different from the nucleoli of normal rat liver, regenerating liver or hepatoma (7). Kinetic studies of ribosome synthesis indicated that the processing rate of 45S pre-rRNA all differs among these systems (8).

Rat liver is an excellent model system to study the nucleolus. Since rat liver provides various bulk tissue and cell sources in the forms of normal, regenerating, preneoplastic and neoplastic cells, biochemical analysis of the nucleolus became feasible to characterize differences among the above different cell nucleoli. Based on these studies, a number of important experiments were carried out on features of nucleolar composition, various kinetic

characters of synthesis and processing of nucleolar pre-rRNA, structural uniqueness of chromatin and matrix, and post-translational modifications. It is of great significance to indicate that the above studies yielded discovery of histone modification by ubiquitination-deubiquitination cycle, and protein modifications by short RNA adduct formation, snoRNAs and many others (9-13).

In the present study, an attempt was made to investigate nucleolar dynamics related to the cell cycle and nucleolar cycle and the nature of TA toxicity inflicted on nucleolar metabolism, especially that associated with the remodeling of nucleolar structures. Accordingly, the present work was to focus on the process of nucleolar enlargement and subdivide it into different approach systems to describe nucleolar morphology, intranucleolar signal transduction, cell and nucleolar cycle, and ex vivo manipulation of TA-treated hepatocytes. The present study of TA toxicity presents the following important findings. TA induced nucleolar enlargement is not a mere increase in size by the physical aggregation of pre-existing multinucleolar structures, but TA toxicity produces a cell cycle opener and G2 delayer which together generate anomalous nucleolar enlargement through intranucleolar signal networking and stimulation of genomic networking for genesis of the nucleolus. A minimum dose of 2 daily TA administrations is required for the production of dense nucleolar compaction, enhanced G2 delayer, and de-accelerator of nucleolar processing.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats were fed with laboratory chow and water ad libitum. The body weights were approximately 200 g.

Reagents and materials

Collagenase type I and IV, and most of chemicals were obtained from the Sigma Chemical Company. Selenium dioxide was from the Aldrich Chem. Co. Fetal bovine serum (FBS), RPMI 1640, and all antibiotics were from GIBCO/BRL and Williams' Medium E was from Sigma. Antibodies were obtained from Dr. P. K. Chan for nucleophosmin and Dr. H. Busch for nucleolin from the Baylor College of Medicine, U.S.A. and for MAP kinases and others from UBI (New York, NY, U.S.A.). Tissue culture flask was from Corning and Primaria dish from Falcon. The perfusion pump was from Manostat., U.S.A.

Carcinogen treatment

TA was dissolved in 0.9% saline to give 1% and injected daily, intraperitoneally in a dose of 50 mg/kg (6).

Partial hepatectomy

Approximately 70% of liver was excised and the regenerating liver was removed after 24 hr (14). For control liver, a sham operation was performed.

Histological examination

As soon as liver was removed from the rat, it was fixed with B5 fixative. Routine staining was made with a standard hematoxylin-eosin staining for observation of liver cell types and their subcellular organelles.

Immunohistochemistry

Frozen tissue slices were fixed on slides and reacted with mouse monoclonal antibody (anti-nucleophosmin), followed by the secondary reaction with fluorescein-labeled goat anti-mouse IgG.

Flow cytometry

Flow cytometry was performed on fresh nuclear preparation. After portal perfusion by physiological saline, liver was removed, teased, filtered and trypsinized for isolation of nuclei. They were stained with propidium iodide and analyzed on a FACSort flow cytometer (Becton Dickinson).

Isolation of hepatocytes and tissue culture

Rat liver was perfused using a Ca^{2+}-free buffer and 0.01% collagenase type IV solution. Free hepatocytes were isolated from the digested liver. Appropriately diluted hepatocytes (5×10^5 cells/mL) were plated to give 5×10^4 cells/cm^2 on the culture flasks. After anchoring for 1-2 hr, the culture medium was changed to the Williams' Medium E containing hormonally defined medium. The cells were maintained at a 37℃ and 5% CO_2 atmosphere (15, 16).

Isolation of nuclei

Minced liver (in vivo) or trypsinized cultured cells (in vitro) were homogenized with 20 volumes of 0.5% citric acid using an IKA homogenizer. They were centrifuged at 2,000 rpm for 10 min at 4℃ through a 0.88 M sucrose cushion. The resulting pellets were nuclear fraction. Alternatively, minced liver was homogenized in 2 M sucrose containing 3.3 mM calcium acetate, pH 5.5.

The nuclei were pelleted by centrifugation for 60 min at 15,000 rpm in a Beckman 20 centrifuge (17).

Isolation of nucleoli

Nuclei were isolated by the above 2 M sucrose method. Subsequently, they were suspended in 0.34 M sucrose and subjected to sonication by a Branson sonicator. The mechanically resistant nucleoli were collected on an overlayer of 0.88 M sucrose by centrifugation at 2,400 rpm for 20 min in a RC3 centrifuge (18).

SDS-polyacrylamide gel electrophoresis and western blotting

Proteins (20 μg/10 μL) were fractionated on 10% SDS-polyacrylamide slab gels. After electrophoresis, proteins were transferred to nitrocellulose membrane using Transphor™ Electro-Transfer (Pharmacia) apparatus. Western blotting was performed according to a commercial manual. All samples were loaded at the same concentration, checking gels with Coomassie brilliant blue staining and nitrocellulose membrane with Ponceau S.

RESULTS

Time course study of alterations in subnuclear structures

After the first administration of TA, various liver tissue responses occurred rapidly, including centrilobular necrosis and inflammation, and apoptosis. In addition, it was recognized that the process involved in the enlargement of nuclear and nucleolar sizes predominantly localized at the central and mid-zone of liver. It appeared that small multinucleolated structures assembled and aggregated mostly into one large, dense and compact nucleolus as the administration continued. As TA administration continued, the hepatocyte population with a remodeled nucleolar structure peaked at the 7th to 9th day and gradually reversed into a multinucleolated subnuclear structure after the 14th day. Fig. 1A shows a representative example of the time-dependent progression of nucleolar remodeling. Fig. 1B shows a representative graphic description of time-dependent alterations of nuclear and nucleolar sizes.

Nucleolar signal transduction molecules

For the further visualization of the altered nucleolar status, nucleolus-specific proteins were selected for metabolic characterization. They are a granular component phosphoprotein, nucleophosmin (~10^5 copies/cell) and a fibrillar component phosphoprotein, nucleolin (~10^4 copies/cell) which are highly abundant and essential in nucleologenesis (19, 20). In contrast to these high abundance proteins, low abundance proteins were included to specify

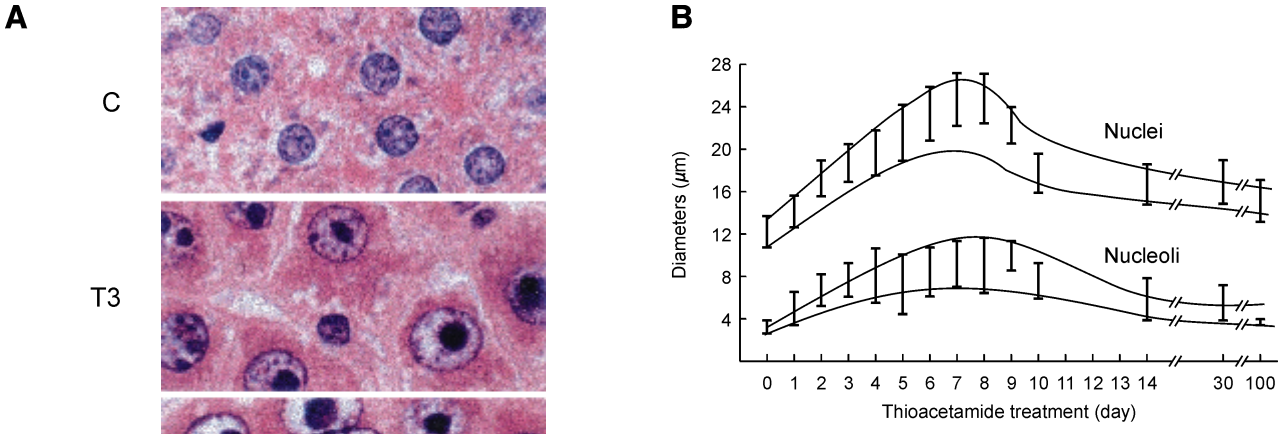

Fig. 1. Time course study of TA-induced alterations of nuclear substructures. **A:** Hepatocytes showed time-dependent variations of nuclear and nucleolar sizes (H&E, ×1,000). Nuclear and nucleolar sizes of 3-day (T3) and 7-day (T7) TA-treated hepatocytes were bigger than control (C). 100-day TA-treated hepatocytes (T100) showed decreased nuclear and nucleolar sizes similar to control. **B:** Time course variations of nuclear and nucleolar diameters are graphed.

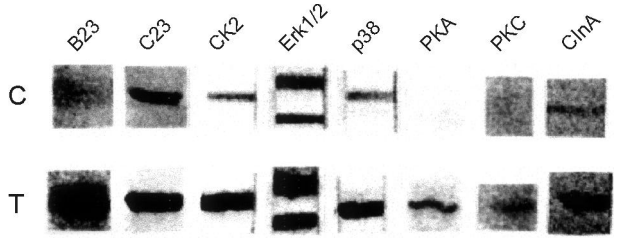

Fig. 2. Increased expressions of nucleolar specific phosphoproteins and signal transduction molecules [C, control; TA, 7-day TA-treated liver; B23, nucleophosmin (36-38 kDa); C23, nucleolin (110 kDa); CK2, casein kinase 2 (42 kDa); Erk 1/2, Erk 1/2 MAP kinase (44 kDa/42 kDa); p38, p38 MAP kinase (38 kDa); PKA, protein kinase A (41 kDa); PKC, protein kinase C (82 kDa); Cln A, Cyclin A (58 kDa)]. Control nucleoli and 7-day TA-treated nucleoli were isolated and their proteins were analyzed by immunoblotting. All their expression were higher in TA-treated than control.

the signal status of nucleolar dynamics. These are membrane linked signal proteins such as casein kinase 2 (CK2), MAP kinases, protein kinase A (PKA) and protein kinase C (PKC) and cyclin A. From Fig. 2, which shows results from a comparative analyses by immunoblotting, it became possible to visualize up-regulation of nucleolar metabolism characterized by 5-fold nucleophosmin (B23), 3-fold nucleolin (C23), 5-fold CK2, 2 to 3-fold Erk1/2 MAP kinase, 5-fold p38 MAP kinase, 5-fold protein kinases A and C, and 5-fold cyclin A.

Time course cell cycle fractions by flow cytometry

To strengthen the above evidence of proliferative signal molecules (Erk1/2 and p38 MAP kinases, and cyclin A) in the remodeled nucleoli, the hepatocyte status for the cell cycle was analyzed by flow cytometric technique (Fig. 3). The results of analysis assigned mostly resting hepatocytes (61%) to the G0/G1 phase and TA-treated hepatocytes (40-55%) to the G2/M phase arrest, suggesting a good correlation to the above signal status. When the flow cytometric profiles were compared among the control, 3-day TA-treated, 7-day TA-treated and 10-day TA-treated livers, there was an increase of delayed G2/M up to 7 days, followed by a subsequent decrease.

Two modes of nucleolar enlargement by in vitro manipulation of hepatocytes

In addition to the above manipulation of in vivo hepatocytes which yielded a number of important findings, ex vivo hepatocytes were utilized for observations under a tissue culture system. Isolated normal hepatocytes could be maintained on a collagen coated layer for four days with a well defined survival program, consisting of

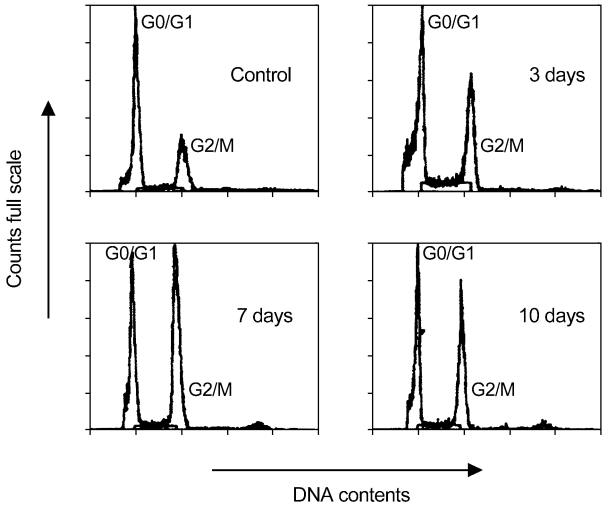

Fig. 3. Flow cytometric analysis of isolated nuclei of TA-treated liver (3- day, 7-day and 10-day; all the duration of TA treatment). The fraction of G2/M increases in 3-day TA treatment and reached the peak in 7-day TA treatment and decreases in 10-day.

anchoring stage (2 hr), monolayer formation (first day), cell division (2nd-3rd days), stability (4th day), and sudden death (5th day). To characterize three nucleolar types (resting, regenerating and TA-induced), normal control livers subjected to sham operation, livers 24 hr after partial hepatectomy, and three daily TA-treated livers were used for in vivo and ex vivo analyses. Nucleoli of in vivo hepatocytes were stained with nucleophosmin monoclonal antibody (Fig. 4), whereas those of ex vivo hepatocytes were not stained under a phase microscope (Fig. 5). By fluorescent immunostaining for in vivo hepatocytes (Fig. 4), distinct differences in size and density were observed among the three liver groups. The biggest in vivo nucleolar size was noted in TA-treated, followed by in regenerating livers and smallest in normal livers. Fig. 5 demonstrates ex vivo hepatocytes showing three different nucleolar morphologies, that is, resting, regenerating and TA-induced nucleolar configurations. When in vivo hepatocytes were placed in ex vivo tissue culture environments, they showed the opening of the cell division cycle after the stage of monolayer formation. Consequently, there were no marked differences between sham operated liver and partially hepatectomized liver in nucleolar morphology. Also, it was interesting to observe that, unlike the in vivo system, the hepatocytes from both sham operated and partially hepatectomized livers showed a similar pattern of nucleolar size increase as large as that of TA-treated hepatocytes. A most striking difference was found in nucleolar density: normal hepatocytes (sham operated and 24 hr post-hepatectomy) showed light nucleolar density, while TA-treated hepatocytes re-

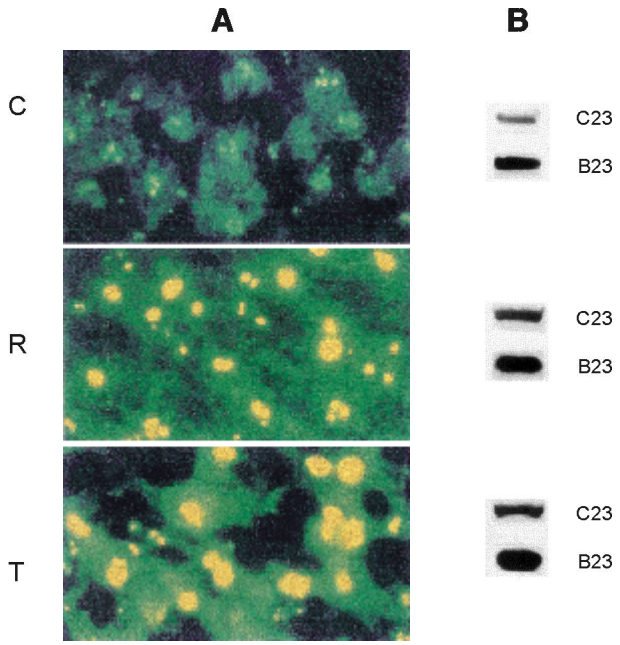

Fig. 4. Immunohistochemical identification of nucleolar proteins. A: immunofluorescent identification of nucleolar sizes using nucleophosmin antibody (×400) (C, control; R, regenerating liver; T, 7-day TA-treated liver). B: immunoblotting of nucleolar specific phosphoproteins (B23, nucleophosmin; C23, nucleolin).

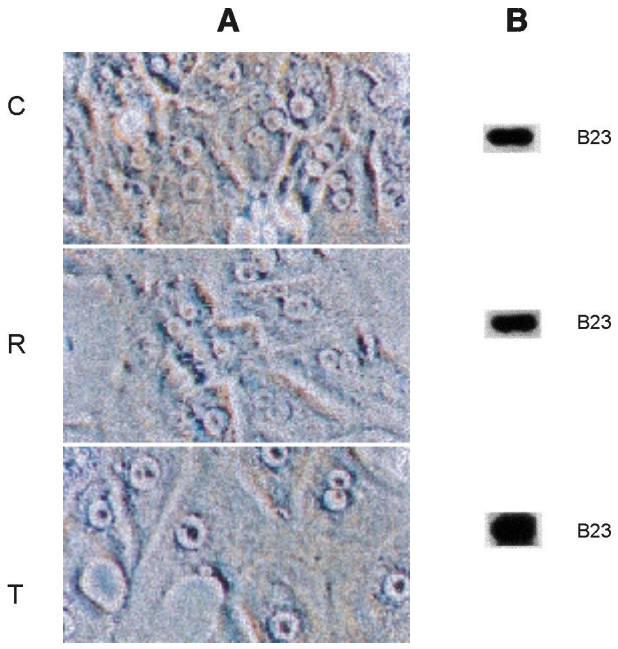

Fig. 5. Nucleolar differences among ex vivo grown hepatocytes (×300) (C, hepatocytes from normal liver; R, hepatocytes from regenerating liver; T, hepatocytes from 7-day TA-treated hepatocytes). A: comparison of nucleolar morphology. B: immunoblotting comparison among above samples with B23 (nucleophosmin).

vealed a much more dense and compact nucleolar architecture. Furthermore, immunoblottings showed that nucleophosmin in TA-treated hepatocytes contained 3-fold as much nucleophosmin as in normal or regenerating hepatocytes. When the hepatocytes were maintained longer, the enlarged nucleoli in normal hepatocytes quickly disappeared due to the normal nucleolar cycle, while the TA-induced enlargements of the nucleoli persisted.

Minimum requirement of TA for nucleolar remodeling

To further clarify the initial stage for formation of compact nucleolar enlargement, a minimum requirement of daily TA doses was examined. After examination of three types of hepatocyte samples (prepared from zero time, 1-day TA, and 2-day TA-treated livers) for identification of TA-induced large compact nucleoli, it became clear that a minimum of two daily doses was necessary to induce similar large compact nucleolar structures similar as the 3-day TA-treated nucleoli shown in Fig. 5.

Ex vivo study to reproduce in vivo TA-induced nucleolar enlargement

To induce nucleolar enlargement, all the above experiments required TA pre-treatment under in vivo conditions, followed by a manifestation expressed both under in vivo and ex vivo conditions. Thus, it remained an unanswered question as to whether or not ex vivo hepatocytes can produce a nucleolar enlargement like the in vivo system. To perform such an experiment, two parameters were determined. One was TA concentration in a tissue culture system. Since the in vivo system (whole rat) was administered with 10 mg of TA which circulated in 14 mL of blood (7% of 200 g rat) to bathe approximately 7 g of liver, it is calculated that an in vitro equivalent (1) corresponds to 10 μg TA/10^6 hepatocytes/100 cm^2 collagen coat/10 mL medium. Based on this approximation, various concentration ranges (0.1-1,000) were prepared. The second parameter was concerned with the duration of TA exposure. Since the minimum of initial 48 hr of TA exposure plus a subsequent 24 hr was required for in vivo hepatocytes to manifest compact nucleolar enlargement, ex vivo hepatocytes were exposed to TA at least for 48 hr at the various time points around the stage of monolayer formation. The results showed that ex vivo hepatocytes' behavior was much different from that of in vivo hepatocytes. Unlike the in vivo TA experiments, the in vitro TA effect was not consistently toxic, but rather was characterized by dual pharmacological actions. TA toxicity was pronecrogenic only at the pre-monolayer stage where the freshly anchored hepato-

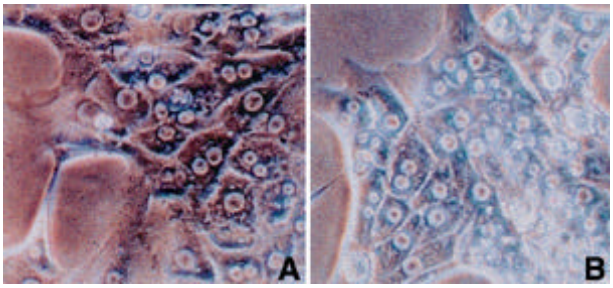

Fig. 6. Ex vivo induction of dense and compact nucleolar structure using exogenous TA (1 μg/mL) (×300) (**A:** normal hepatocytes, **B:** TA-treated hepatocytes). TA-treated hepatocytes contain slightly dense compact nucleoli.

cytes became quickly detached and positively stained with trypan blue at a concentration of 0.1 μg/mL. The higher the TA concentrations, the more dead cells there were. After the hepatocytes formed a monolayer, the ex vivo hepatocytes suddenly became resistant toward TA and were well maintained even in the presence of 100-1,000 μg/mL concentration of TA. When the hepatocytes were exposed to 1 μg TA/mL from the time of anchoring to the stage of cell division spanning over 3 days, two mixed populations of enlarged nucleoli were clearly observed. They consisted of hepatocytes containing light enlarged nucleoli and another hepatocytes containing somewhat dense and compact nucleoli. It was indicated that TA-induced dense and compact enlarged nucleoli were not produced as effectively as in vivo conditions (Fig. 6).

DISCUSSION

The present study was an effort to characterize molecular lesions involved in the mechanism of TA-induced megalonucleolus formation by remodeling in the rat liver. For this purpose, the work was manipulated into three TA-treated hepatocyte biological systems, composed of in vivo TA treatment and in vivo TA manifestation, in vivo TA treatment and ex vivo TA manifestation, and ex vivo TA treatment and ex vivo TA manifestation. In the work, a search was made to detect alterations in nucleolar signal networking.

From the present study where hepatotoxicity was manifested under in vivo and ex vivo conditions, TA produced pro-necrotic metabolites which were manifested immediately after its administration. An ex vivo study showed that the poisoned and anchored hepatocytes became immediately detachable, trypan blue-positive and nonviable, suggesting loss of cell membrane integrity to death. An in vivo and ex vivo study showed that the hepatocytes, which survived a pro-necrotic effect, manifested subtle metabolic alterations. These toxic effects revealed in the survived hepatocytes were shown in forms of the interference with the cell cycle as a G2 delayer, nucleolar remodeling to form a compact megalonucleolus, and alteration in an ex vivo growth program. Although the molecular mechanisms are not clarified, the above effects must be correlated to a hyperacetylating effect. It is noteworthy that G2 delay can be induced by inhibition of a cell cycle-related acetylation-deacetylation cycle. One of the most representative examples is the histone deacetylase inhibitors, trichostatin A and trapoxin, which disrupt the G2 checkpoint (21, 22). TA may not directly inhibit histone deacetylase. However, it is proposed that TA can inhibit the histone acetylation-deacetylation cycle, because continuous administration results in inappropriate acetylation, which interrupts the timing of the regulatory acetylation-deacetylation cycle. The mechanism of compact megalonucleoli formation is unclear. Also, it is not clear whether or not direct acetylation on nucleolar proteins is responsible.

Comparing 24 hr post-hepatectomy nucleoli and TA-induced nucleoli on thier metabolic alterations, there was a disproportionate discrepancy between the two systems. One was exaggerated increases in size (2-fold), phosphoprotein (2 to 3-fold), and polymerase I activity (2-fold). The other was an 8-fold decrease in the RNA processing rate. These paradoxical alterations suggested that the TA-induced megalonucleolus formation was caused by the anomalous acetylation-deacetylation cycle resulting from a damaged coordination of gene networking for genesis of nucleolar constituents. Also, it may be attributed to damaged synthesis of snoRNA necessary for efficient nucleolar processing (13). Whether or not this decrease is associated with a pre-mitotic shutoff of RNA metabolism is also not clear.

The present study established the presence of a nucleolar signal transduction system. Analyses of the signal molecules (Fig. 2) indicated that the nucleolus contains its own signal networking involved in the regulatory mechanism of nucleolar metabolism. It is suggested that TA-induced nucleolar enlargement does not result from simple physical aggregation of pre-existing nucleolar structures, as well. The presence of proliferative signal MAP kinases proved the close linking both to a proliferative membrane receptor pathway as a downstream portion and to a nucleolocytoplasmic trafficking mechanism (23). These findings are most significant in that they suggest the long distance interactions between the cell membrane and the nucleolus are regulated by the signalling molecules. The detection of cyclins for cell division cycle indicated the existence of signal cascades which signify the progression of the cell cycle status. Although cell cycle regulators involved in G2/M transition were not

investigated, it was obvious with the flow cytometric analysis (Fig. 3) that TA-treatment induces G2-arrest. Thus, it is indicated that TA-induced nucleolar remodeling requires at least two simultaneous metabolic conditions. One is a normal condition in which light nucleolar enlargement is produced by a ploidy-dependent manner through cell cycle progression. Since TA is a cell cycle opener, it could be regarded as a light nucleolar enlarger. On the other hand, TA could also be regarded as a nucleolar compactor through an intranucleolar overload of pre-rRNP. Since TA is an up-regulator of polymerase I activity (24) and a down-regulator of processing and export (8), the kinetic consequence of TA treatment is the organization of the nucleoli into large, dense and compact structures by the accumulation of processing intermediates.

In vitro reproduction of TA-induced large, dense and compact nucleoli was not fully successful. Although the ex vivo hepatocytes could be used for cell replacement therapy, they were much different from in vivo hepatocytes. The action of TA is singularly toxic to in vivo hepatocytes, but dually toxic to ex vivo hepatocytes. When the isolated hepatocytes were fresh or less than 10 hr, they became readily stained with trypan blue in the presence of TA. Cell death occurred at the pre-monolayer stage. At the post-monolayer stage, the ex vivo hepatocytes became TA-resistant and showed a normal growth pattern. In some cases, TA had a protective effect so that the ex vivo hepatocytes survived longer than the control hepatocytes. The reason is not clear. It may be attributed to the fact that the freshly prepared hepatocytes quickly lose 85% of P450-dependent enzyme activity associated with FMO-dependent oxidation (25). A more improved culture system is necessary for future work.

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