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In Vitro Regeneration of Carcinogen Thioacetamide Treated Rat Hepatocytes

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ABSTRACT

Thioacetamide is a non-genotoxic carcinogen, a protein modifying agent. It causes nucleolar hypertrophy in short term treatment. In the present work, thioacetamide treated hepatocytes were observed *in vivo* and *in vitro* conditions. After 7 day treatment of rat liver with thioacetamide, the hepatocyte nucleoli were enlarged and their signalling molecules such as B23 and p38 MAPK were increased. When these hepatocytes were released by collagenases and were grown under the conditions of gene therapy grade tissue culture system, the enlarged nucleoli were further enlarged. The B23 content was again increased under *in vitro* conditions. From these experiments, it is clear that the hepatocytes possess approximately 100 fold flexibility of nucleolar capacity. It is suggested that thioacetamide enhances the ribosome genesis and exaggerates the nucleologenesis ability.

Key Words: Thioacetamide treated rat hepatocytes, Primary culture of hepatocytes, B23

INTRODUCTION

Thioacetamide is a weak carcinogen specific for the liver carcinogenesis (Kleinfeld, 1957). Although this simple molecule attacks the subcellular components of liver cells, it has not been clarified that nature of the chemical carcinogen is genotoxic. It requires a long term exposure of thioacetamide to cause neoplastic transformation and the short term exposure gives reversible premalignant states and does not involve carcinogenesis (Dyroff and Neal, 1983; Sharma, 1978). However, it has been clearly demonstrated that a short term administration of thioacetamide generates two major types of cytopathology. One is cytopathology of resting hepatocytes stimulated to perform apoptosis/compensatory regeneration and characterized by enlargement of nuclei and nucleoli. The other is cytopathology of oval cells stimulated to perform oval cell proliferation (Mironescu *et al.*, 1968; Olason and Smuckler, 1976). These two premalignant events show some common initial pathway in a form of stimulation of liver cell proliferation (Farber, 1991).

The detailed molecular events in the premalignant hepatocytes have not been elucidated. However, it is readily recognized that all the cytopathology occured could be translated into molecular terms. Therefore, it is very clear that the stimulatory effects can be described in terms of death /growth related signal pathways for apoptosis/ compensatory regeneration and selective gene activations for nucleolar enlargements which require net increase of nucleolar components involved in the genesis of ribosome.

Earlier biochemical studies by a number of investigators (Busch, 1967) indicated that the nucleolar enlargements are characterized by 1) increased activity of pol I, 2) increased synthesis of 45S pre-rRNA, 3) decreased processing of 45S pre-rRNA to smaller precursors, 4) increased A24 content, 5) increased nucleolus specific phosphoprotein (unpublished). However, the nature of nucleolar hypertrophy is not clarified.

In the present report, attempts were made to characterize pharmacological responses of the carcinogen injured hepatocytes under *in vivo* and *in vitro* conditions. The normal *in vivo* hepatocytes forms orderly structure in the hexagonal framework by extracellular matrix. Under the normal conditions, the hepatocytes are in the state of cell cycle arrest either at G0 or G2. Upon partial hepatectomy, the liver undergoes opening cell cycle leading to cell division (Steer, 1995). However, when the hepatocytes are separated from liver extracellular matrix, they divide *in vitro* culture system. Recently, the tissue culture technology has advanced to be cell replacement gene therapy grade (Grossman and Wilson, 1993; Wilson, 1995; Kozarsky and Wilson, 1993; Wilson *et al.*, 1992). Because the improved technology, it is readily feasible to obtain clinically acceptable grade of free dividing hepatocytes.

Based on these two different hepatocyte systems (*in vivo* and *in vitro*), an effort was made to examine the nature of the carcinogen caused injury. It was found that the thioacetamide treated hepatocyte are viable and grow *in vitro* conditions and moreover open the arrested cell cycle. It was particularly novel that the enlarged nuclei and nucleoli are further-more enlarged immediately after the cell culture. By immunochemical analysis, the most abundant nucleolar specific phosphoprotein B23 shows marked increase which supports the idea of nucleolar enlargement.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals were obtained from commercial sources. Antibodies were obtained from Dr. P.K. Chan for B23 from Baylor College of Medicine, U.S.A. and from UBI (New York, U.S.A.) for MAP kinases.

Animals

Male Sprague Dawley rats were fed with labchow and water adlibitum. The body weights were approximately 200 g.

Thioacetamide administration

Thioacetamide was dissolved in 0.9% saline to give 1% and injected daily for 7 days intraperitoneally in a dose of 50 mg/kg a day (Mironescu *et al.*, 1968).

Hepatocyte isolation and primary cultures

Rat hepatocytes were harvested by sequential perfusion using Ca^{2+}-free buffer (118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 and 25 mM $NaHCO_3$, pH 7.4 with EGTA) and collagenase type IV solution (Berry and Freind, 1969; Selglen, 1972; Selglen, 1976). The perfused liver was transfered to a wide petri dish containing RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. It was teased apart with a dog's comb and filtered through one layer cheese cloth. The filtered hepatocytes were gently washed by centrifugation three times at 400 rpm (50 g) for 4 min. The cell pellet was resuspended and viable cells were quantified by exclusion of Trypan blue using hemocytometer. Appropriately diluted hepatocytes (5×10^5 cells/ml) were plated on the culture flasks. After anchoring for 1-2 h, the culture medium was changed to hormonally defind medium. Cells were maintained at 37℃, 5% CO_2 atmosphere (Wilson *et al.*, 1992; Ryan *et al.*, 1993; Guillouzo *et al.*, 1981; Yano *et al.*, 1996).

Isolation of nuclei

Rat liver was perfused with saline in a rate of 30 ml/min. Liver was removed and processed through Harvard press to obtain crude homogenate. Crude homogenate (*in vivo*) or trypsinized cultured cells (*in vitro*) were homogenized with 20 volumes of 0.5% citric acid using an IKA homogenizer. Removal of cytoplasm was examined by light microscope. They were centrifuged 2,000 rpm for 10 min at 4℃. The pellets were resuspended with 0.34 M sucrose, underlaid with 0.88 M sucrose and centrifuged 2,400 rpm for 15 min at 4℃. The resulting pellets were nuclear fraction (Busch, 1967). In some cases, the crude homogenate was

homogenized in 2 M sucrose containing 3.3 mM sucrose. The nuclei were pelleted by centrifugation for 60 min at 15,000 rpm in Beckman 20 centrifuge.

Isolation of nucleoli

The nuclei isolated by the 2 M sucrose method were washed in 0.88 M sucrose and pelleted by centrifugation for 10 min at 3,000 rpm. The washed nuclei were suspended in 0.34 M sucrose and subjected to sonication by Bronson sonicator. The mechanically resistant nucleoli were collected on overlayer of 0.88 M sucrose by centrifugation at 2,400 rpm for 20 min (Busch, 1967).

Electrophoresis and immunoblotting

Pellets were dissolved in SDS-sample buffer (0.125 M Tris-HCl, pH 6.8 / 4% SDS / 10% sucrose / 5% 2-mercaptoethanol) and boiled for 10 min. Protein content was determined by the method of Bradford *et al.* (1976). Proteins were fractionated on 10% SDS-polyacylamide slab gels (Lammli, 1970). After electrophoresis, proteins were transferred to nitrocellulose membrane using Transphor™ Electro-Transfer (Pharmacia) apparatus. The nitrocellulose membrane was incubated for 1 h at room temperature with monoclonal antibodies to B23 or MAP kinase R1 (both at 1: 5,000 dilution), washed, and then incubated for 1 h with goat anti-mouse IgG or goat anti-rabbit IgG conjugated with alkaline phosphatase respectively. After 5-10 min of color development, the nitrocellulose membranes were washed and photographed (Ausubel *et al.*, 1987).

RESULTS

The ultimate goal in the present work was to observe the features of thioacetamide treated hepatocytes under actively dividing conditions, which can't be attained by partial hepatectomy. In order to design such an experiment, it was necessary to obtain fundamental technology available in the laboratory. The basic experiments were performed to isolate *in vivo* nucleoli from thioacetamide treated liver and determine molecular features of the isolated *in vivo* nucleoli. To grow the thioacetamide treated hepatocytes, it was mandatory to make available the tissue culture system of gene therapy grade. From these experiments, it was possible to observe the *in vitro* hepatocyte nucleoli and compare with the *in vivo* nucleoli to achieve the above ultimate goal. These investigations are described in a series of the experiments as below.

Effect of thioacetamide on liver histology

Thioacetamide (50 mg/kg) was administered intraperitoneally daily into rat and the liver is found to be the most susceptible organ to the administration. There is no apparent manifestation of liver to thioacetamide exposure within 3~ 4 days. However, in seven days of thioacetamide administration, an induction of oval cell proliferation took place in the area of portal triad. The oval cells were smaller than hepatocytes and the H-E staining shows very light density in the nuclei. Fig. 1 shows a comparison of normal liver architecture with thioacetamide treated liver architecture. The oval cells shown in Fig. 1b exhibits their location resided in the periphery of the lobule and close proximately to biliary system. When the period of thioacetamide administration exceeds two weeks, the population of oval cells increase dramatically that the liver nuclear preparation reveals considerable heterogeneity mixed with smaller oval cell nuclei in the range of 20%.

Effect of thioacetamide on hepatocyte subcellular structures

In addition to the oval cell proliferation induced, the hepatocytes shows distinct subcellular responses to the carcinogen. It was observed that the nuclear size increased and was accompanied by nucleolar enlargement. Fig. 2 shows a comparison of normal hepatocytes and 7 day thioacetamide treated hepatocytes stained with H-E. There was a distinct difference in the detailed structures of nuclei between the two system. Normal hepatocytes contained smaller but more numerous number of nucleoli/nucleus whereas thioacetamide treated hepatocytes were characterized by fewer but larger nucleoli/nucleus. The size is larger than red blood cells. In some cases the nucleolus becomes so gigantic that the entire nuclear compartment is occupied by an enlarged single nucleolus. Fig. 3 demonstrates much clearer features of nucleoli which were iso-

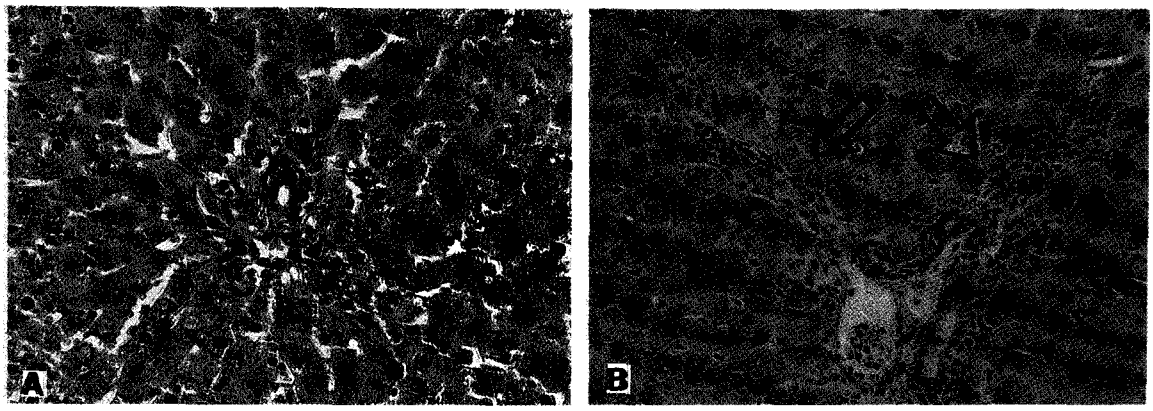

Fig. 1. Induction of oval cell proliferation at portal area of rat liver. A: Normal, B: Oval cell proliferation after 1 week thioacetamide administration.

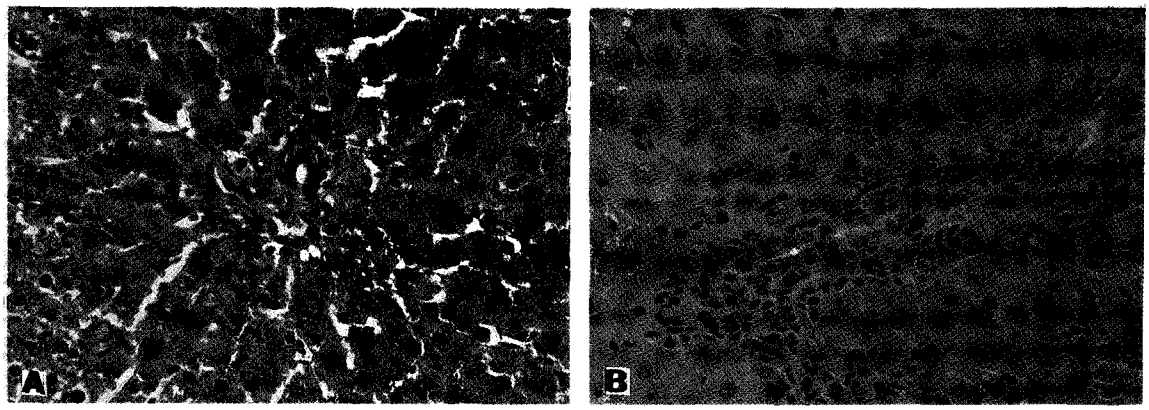

Fig. 2. H-E staining of rat liver (×400). Nucleolar hypertropy is induced by thioacetamide administration for 7 days. A: Normal, B: Thioacetamide administration

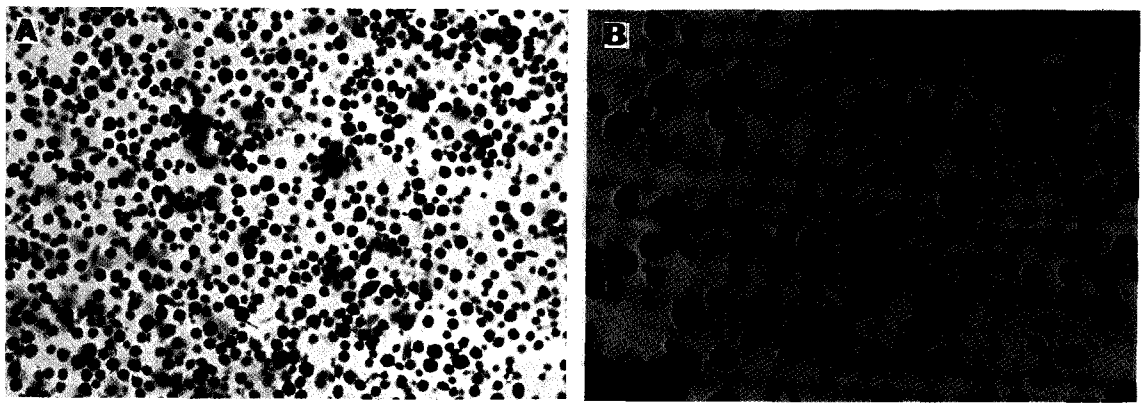

Fig. 3. Liver nucleolar isolation obtained from 7 days thioacetamide treated liver. A: Normal, B: Thioacetamide administration

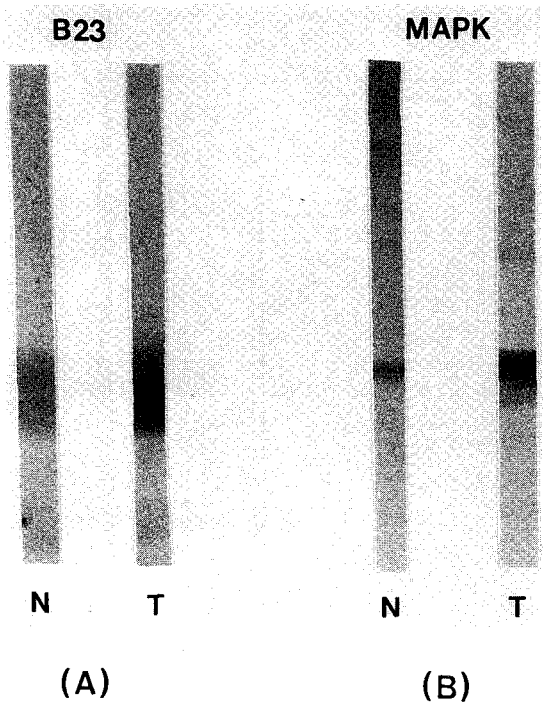

Fig. 4. Immunoblots of nuclear proteins of rat hepatocytes after thioacetamide treatment. (A) anti-B23 antibody, (B) anti-MAP kinase antibody, N; normal, T; 7 days thioacetamide treatment

lated by a sonication method (Busch, 1967). It is very clear that the isolated nucleoli, from the 7 day thioacetamide treated liver, show their size 2 ~5 time as large as the normal liver nucleoli.

Analysis of nucleolus specific signal molecule

Nucleolus is defined functionally the site of ribosome genesis (Busch and Smetana, 1970). It contains nucleolus specific pol I dependent transcription, RNP assembly and post-transcriptional processing systems. It also contains nucleolus specific signalling molecules. Two specific molecules were assayed in the study. It was revealed that p38 B23 phosphoprotein and p38 MAP kinase were involved in the nucleolar enlargement. Fig. 4 shows a comparative immunoblotting findings. It is very clear that the thioacetamide treated nucleoli contain more nucleolus specific B23 and MAP kinase. It was further revealed that other signal molecules such as CKII, PKA and C23 were increased (unpublished).

***In vitro* behaviors of normal hepatocyte and thioacetamide treated hepatocyte**

To further characterize the *in vivo* hepatocytes described above, *in vitro* characterization of the hepatocyte were performed. The hepatocytes culture system selected was the cell replacement therapy grade where the hormonally defined media were used in addition to collagen coated

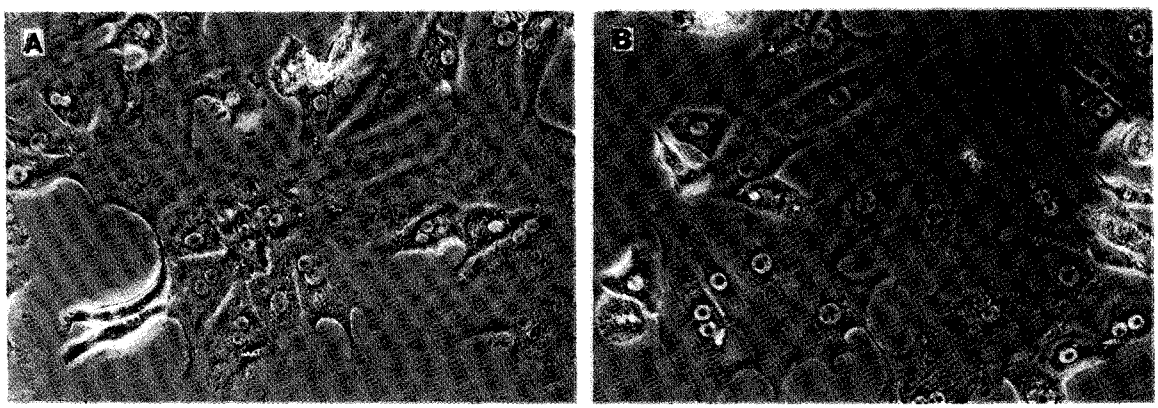

Fig. 5. Rat hepatocytes primary cultures *in vitro* (×200). A: Normal hepatocytes, B: Thioacetamide treated hepatocytes

Table 1. Change nucleolar size during *in vitro* culture

Day \ Type	Nuclear size (μm)		Nucleolar size (μm)	
	Normal	TA	Normal	TA
Day 0	7.33±2.08	10.07±2.57	1.88±0.75	3.75±0.53
Day 1	11.44±1.50	15.95±3.73	3.46±1.23	6.11±1.70
Day 3	13.18±3.28	23.28±5.57	5.24±1.38	6.65±2.46

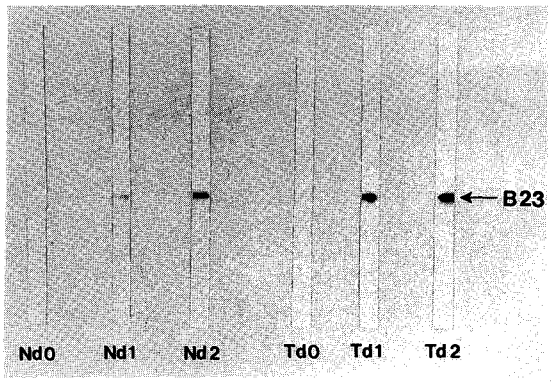

Fig. 6. Immunoblots of nuclear proteins of in vitro cultured rat hepatocytes detected by anti-B23 antibody. Nd0; normal hepatocytes, Nd1; cultured hepatocytes day 1, Nd2; cultured hepatocytes day 2, Td0; thioacetamide-treated hepatocytes, Td1; cultured thioacetamide-treated hepatocytes day 1, Td2; cultured thioacetamide-treated hepatocytes day 2.

matrix (Wilson, 1992). This culture system has been proven to be clinically suited. It supports the hepatocyte for active cell cycle and prevents from dedifferentiation.

Fig. 5 shows a comparison of the two hepatocyte systems. It is clear that the carcinogenic injury has not resulted in gross cell behavioral changes. They are as stable as normal hepatocytes engaged in active cell division cycle.

Table 1 shows a numerical comparison tabulated on the time course changes of nuclei and nucleoli in terms of size. It is very clear that the nucleolus becomes enlarged as entering cell cycle from resting state of *in vivo* hepatocyte. It is also clear that the enlarged nucleoli of the thioacetamide treated hepatocytes became more enlarged. It is suggested that the molecular injury caused by thioacetamide actions may be resided feedback pathway of ribosome biogenesis. It is possible that the metabolic demand is signalized from cytoplasm where ribosomes are functionally involved in translation.

Most thioacetamide sensitive signalling molecules

B23 is most abundant nucleolus specific phosphoprotein. Its molecular population is approximately 10^6 copies/cell and this copy number is similar to the abundance of ribosome in cytoplasm (Chan *et al.*, 1988). In order to examine the enlargement of nucleoli, it is natural to analyze the major nucleolar protein B23 by western blotting. Fig. 6 shows a representative time course change of B23 from *in vivo* states to *in vitro* states at cell cycle opening. It was demonstrated that B23 increase as the cells grow. It is noteworthy that B23 detection was possible with 10^6-10^7 cells. Since it is difficult to detect B23 using whole cell sample, the nuclei were isolated by a citric acid method using a microprobe (0.8 cm diameter) of IKA homogenizer (Busch, 1967).

DISCUSSION

The present study made an experimental effort to obtain new evidence for the carcinogenic nature of thioacetamide actions. The chemistry of thioacetamide is very simple and is similar to acetamide (Olason and Smuckler, 1976). However, both chemicals are hepatotoxic. Acetamide causes cirrhotic pathology. The only difference is replacement of oxygen of acetamide with sulfur which can form S-oxide. It is this atomic difference that makes carcinogenic. An isotopic experi-

ment with ^{3}H-thioacetamide suggested the mechanism of organic reaction which involves in attacking ε-amino moiety of lysine to form a N-acetylated product acetyl-lysine (Dyroff and Neal, 1983). Thus, it is clear that thioacetamide is a protein modification agent. However, it is not proved that it reacts with other biological molecules such as DNA, RNA, lipids and carbohydrates to form acetylated derivatives, although there are many naturally occuring acetylated products. Therefore, it is known as a non-genotoxic carcinogen.

The translation of the chemical protein modification into its biological consequence remains to be defined. It was most prominent that hepatocytopathology was the nucleolar enlargement (Jeong *et al.*, 1994). The present study indicated that its characteristic feature was the increased signalling content of B23, p38 MAP kinase and other nucleolar components including C23, PKA, CKII, pol I, RNP (unpublished). The observed net increase of nucleolar machinery suggests two underlying events. The first suggests the enhanced genesis of both ribosome and nucleolar components, and the second the increased demand of ribosome genesis. It has been known that ribosome genesis is regulated by cell size, ribosome turnover rate and cell division cycle. Based on the present observation, it is possible that thioacetamide produces above three effects. However, it is not certain that the acetylated proteins are directly responsible for the three effects. It is conceivable that the lysine containing ribosomal proteins are readily attacked by thioacetamide to become more acidic and hydrophobic and the injured ribosomes thus are responsible for the signal generation of increased demand of ribosome genesis.

Why then the protein acetylation modifications can lead to hepatocyte apoptosis / compensatory regeneration and oval cell proliferation? It is only to assume an idea that the acetylatable molecules belong to those of upstream signalling initiation pathway and downstream interface with gene activation in above complex biological phenomena.

The increased size of thioacetamide treated nucleoli are as large as many malignant cells which have characteristic pleiotropic nucleoli of 10~15 μm in diameter. In the present work, it was possible to observe the increasing nucleolar size from resting normal nucleoli (2~5 μm) → TA nucleoli (5~10 μm) → TA nucleoli from dividing cells (10 ~15 μm). When the increased nucleolar sizes were compared with the most abundant nucleolar specific B23, it was observed that the B23 content increased in relative term from resting normal nucleoli (1 X) → resting TA nucleoli (10 X) → TA nucleoli from dividing cells (100 X). Based on the these observation, it is possible to assume that 10^{6} B23 molecules/nucleolus could increase approximately 10^{7} B23 molecules/nucleolus. Although it is approximate in the computation, the immunoblotting findings support the idea of su per-increase of B23. Therefore, the nucleolar enlargement require not only nucleolar affair itself but gene involvement in nucleoplasm for the synthesis of all the nucleolar, ribosomal, signal and gene activating components.

From the *in vivo* and *in vitro* study, it is demonstrated that the nucleolar dynamics of hepatocyte manifests tremendous flexibility (100 fold) of functional reserve. In a sense of cytopathology, the reversible premalignant cells of thioacetamide treated liver can be regarded as manifestation of nucleolar RNP storage disease.

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=국문초록=

Thioacetamide처리한 백서간세포의 *in vitro* 상에서의 재분열

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Thioacetamide는 non-genotoxic 발암제로서 세포단백의 변형을 초래하는 것으로 알려져 있으며 이것을 단기간 처리하면 핵소체의 비대를 초래하게 된다. 본 연구에서는 thioacetamide를 처리한 간세포를 *in vivo*와 *in vitro* 상태에서 관찰하였다. *In vivo* 상태로서 thioacetamide를 쥐의 복강에 7일간 주사하면 (50 mg/kg), 핵소체 비대와 B23 및 MAP kinase와 같은 신호전달분자들이 증가하는 것을 관찰할 수 있었다. *In vitro*상태로서 쥐의 간장을 collagenase로 분리하여 유전자 치료에 사용될 수 있는 배양조건으로 간세포를 배양하여 핵소체를 관찰한 결과 핵소체 비대가 현저하였으며, B23의 양도 증가하였다. 본 실험의 결과로 미루어 볼 때, 간세포는 핵소체 비대 수용능력이 약 100배 이상이라고 할 수 있으며, thioacetamide 처리에 의하여 라이보좀 생성과 핵소체 증가 능력이 증폭되어 나타나는 것으로 사료된다.