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研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Onset of hepatocarcinogen-specific cell proliferation and cell cycle aberration during the early stage of repeated hepatocarcinogen administration in rats

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ABSTRACT: We have previously reported that a 28-day treatment of carcinogens evoking target cell proliferation activates G₁/S checkpoint function and apoptosis, as well as induction of aberrant ubiquitin D (Ubd) expression, suggesting disruptive spindle checkpoint function, in rats. The present study aimed to determine the onset time of rat liver cells to undergo carcinogen-specific cell cycle aberration and proliferation. Animals were treated orally with a hepatocarcinogenic dose of methyleugenol or thioacetamide for 3, 7 or 28 days. For comparison, some animals were subjected to partial hepatectomy or treated with noncarcinogenic hepatotoxicants (acetaminophen, α -naphthyl isothiocyanate or promethazine). Carcinogen-specific liver cell kinetics appeared at day 28 as evident by increases of cell proliferation, p21^{Cip1+} cells, phosphorylated-Mdm2⁺ cells and cleaved caspase 3⁺ cells, and upregulation of DNA damage-related genes. Hepatocarcinogens also downregulated *Rb12* and upregulated *Cdkn1a* and *Mdm2*, and decreased Ubd⁺ cells co-expressing phosphorylated-histone H3 (p-Histone H3) and p-Histone H3⁺ cell ratio within the Ki-67⁺ proliferating population. These results suggest that it takes 28 days to induce hepatocarcinogen-specific early withdrawal of proliferating cells from M phase due to disruptive spindle checkpoint function as evidenced by reduction of Ubd⁺ cells staying at M phase. Disruption of G₁/S checkpoint function reflected by downregulation of *Rb12* as well as upregulation of *Mdm2* suggestive of sequestration of retinoblastoma protein is responsible for the facilitation of carcinogen-induced cell proliferation at day 28. Accumulation of DNA damage probably in association with facilitation of p53 degradation by activation of *Mdm2* may be a prerequisite for aberrant p21^{Cip1} activation, which is responsible for apoptosis. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: cell proliferation; hepatocarcinogen; G₁/S checkpoint; spindle checkpoint; ubiquitin D; apoptosis

Introduction

Carcinogenicity assays using rodent animals are versatile for evaluating the carcinogenic potential of test compounds. However, they have several disadvantages, such as long duration, high costs and use of a large number of animals. Although there are a number of alternative models, e.g., two-stage carcinogenesis bioassays (Tamano, 2010) and bioassays using genetically modified animals by transgenic or gene targeting technologies (Eastin, 1998), they are also expensive and time-consuming, and have limited target organs. Toxicogenomic approaches for the prediction of carcinogenic potential in each target organ appear promising; however, they are also expensive and require integrative methodologies between different laboratories sharing expression databases (Uehara *et al.*, 2011). Therefore, the development of new and rapid means for evaluation or prediction of the carcinogenic potential of chemicals in the whole body is necessary for efficient detection of carcinogens.

The development of nuclear enlargement is sometimes observed in carcinogenic target cells after repeated administration of carcinogens, irrespective of genotoxic potential, from the early stages of exposure in rodents (Adler *et al.*, 2009; Allen *et al.*, 2004). This nuclear enlargement is typically observed in the liver

and kidney. It has been reported that renal carcinogens are classified into two categories, the renal tubular nephropathy type and the renal tubular karyomegaly-inducing type (Taniai *et al.*, 2012a). Recent studies have shown that ochratoxin A, a renal carcinogen in rodents, can induce karyomegaly, which reflects mitotic disruption and subsequent unstable polyploidy, accompanied by aberrant expression of cell cycle-related molecules, particularly, of G₂ and M phases (Adler *et al.*, 2009). Hepatocarcinogens at the early stage of carcinogenicity bioassays frequently induce hepatocellular cytomegaly/karyomegaly (Allen *et al.*, 2004) suggesting that induction of cell cycle aberration is an early stage signature of carcinogen responses. It has also been reported that escape from

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the G₂/M checkpoint along with the accumulation of DNA damage during cell proliferation may lead to chromosomal mis-segregation and cytokinesis failure, causing tetraploidy/aneuploidy and subsequent chromosomal instability (Ichijima *et al.*, 2010). We therefore hypothesize that an early event, which disrupts cell cycle regulation, triggers the carcinogenic response in the molecular mechanism responsible for the development of cytomegaly/karyomegaly.

We have recently found that carcinogens evoking cell proliferation in carcinogenic target cells after a 28-day administration can also induce aberrant expression of cell cycle-related molecules reflecting activation of G₁/S checkpoint function and apoptosis in many target organs (Taniai *et al.*, 2012a, b; Yafune *et al.*, 2013a, b). Furthermore, by double immunohistochemical analysis, we found that ochratoxin A, targeting renal tubular cells and thioacetamide (TAA), targeting liver cells, induce aberrant expression of ubiquitin D (Ubd), at G₂ phase, suggestive of disruption of the spindle checkpoint at M phase (Taniai *et al.*, 2012b). These results suggest that cell proliferation-inducing carcinogens induce aberrant cell cycle regulation including dysfunction of M phase players, indicating the early cellular events responsible for the carcinogenicity. However, the cellular onset time point of carcinogen-specific cellular proliferation has not been addressed until now.

The present study was performed to identify the cellular onset time point of carcinogen-specific facilitation of aberrant cell cycle during the early time course of repeated carcinogen administration. For this purpose, we repeatedly administered hepatocarcinogens to rats for up to 28 days and examined the temporal changes in cell proliferation activity, expression of G₁/S and spindle checkpoint-related molecules and apoptosis by immunohistochemistry and real-time reverse transcription-polymerase chain reaction (RT-PCR). For comparison, we also examined the liver cell responses under regenerative cell proliferation induced by partial hepatectomy or non-carcinogenic hepatotoxicants.

Materials and Methods

Chemicals

Methyleugenol (MEG; CAS no. 93-15-2, purity ≥ 98.0%), acetaminophen (APAP; CAS no. 103-90-2, purity ≥ 98.0%) and α -naphthyl isothiocyanate (ANIT; CAS no. 551-06-4, purity ≥ 98.0%) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). TAA (CAS no. 62-55-5, purity > 98%), promethazine hydrochloride (PMZ; CAS no. 58-33-3, purity > 98%) and methylcellulose 400 were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Animal Experiments

Five-week-old male F344/NSlc rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), and acclimatized to a powdered basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum for 1 week. Rats were maintained under standard conditions (room temperature, 23 ± 3 °C; relative humidity, 50 ± 20%; 12 h light/dark cycle).

In the present study, two lines of animal experiments were conducted. In both experiments, animals were subjected to two-thirds partial hepatectomy or to administration of hepatocarcinogens (MEG or TAA) or noncarcinogenic hepatotoxicants (APAP, ANIT or PMZ). MEG and TAA were selected as hepatocarcinogens (Becker, 1983; NTP, 2000), and APAP, ANIT and PMZ were selected as non-carcinogenic hepatotoxicants (NTP, 1993a, b; Rees *et al.*, 1962).

Three- and 7-day repeated administration studies were conducted in experiment 1, and a 28-day repeated administration study was conducted in experiment 2. In both experiments, cell proliferation activity, expression of cell cycle-related molecules and apoptosis were examined using immunohistochemistry, and gene expression changes in cell cycle molecules, signaling molecules and repair enzymes were examined by real-time RT-PCR.

Experiment 1

Animals were divided into seven groups based on initial body weights and subjected to a two-thirds partial hepatectomy (PH, $n = 22$), or exposed to MEG (1000 mg kg⁻¹ body weight) daily by gavage in 0.5% methylcellulose ($n = 22$), TAA (400 ppm) in basal diet ($n = 20$), APAP (10 000 ppm) in basal diet ($n = 20$), ANIT (1000 ppm) in basal diet ($n = 20$) or PMZ (200 mg kg⁻¹ body weight) daily by gavage in 0.5% methylcellulose ($n = 22$) for 3 or 7 days. Untreated controls ($n = 20$) were maintained on the basal diet and tap water without any treatment during the experimental period. In the PH group, one animal died at day 6 after PH treatment. In the ANIT group, the animals' body weights decreased at day 3 after the start of treatment, so the dose was reduced to 600 ppm thereafter. In the PMZ group, the animals' body weights decreased at day 3 after the start of treatment, so the dose was reduced to 100 mg kg⁻¹ body weight. One day after the 3- and 7-day treatment period, half of the animals in each group were killed by exsanguination from the abdominal aorta under deep anesthesia with CO₂/O₂ and livers were removed.

Experiment 2

Animals were divided into seven groups based on initial body weights and subjected to PH ($n = 12$), or exposed to MEG (1000 mg kg⁻¹ body weight) daily by gavage in 0.5% methylcellulose ($n = 11$), TAA (400 ppm) in basal diet ($n = 10$), APAP (10 000 ppm) in basal diet ($n = 10$), ANIT (600 ppm) in basal diet ($n = 11$) or PMZ (100 mg kg⁻¹ body weight) by gavage in 0.5% methylcellulose ($n = 11$) for 28 days. Untreated controls ($n = 10$) were maintained on the basal diet and tap water without any treatment during the experimental period. In the MEG group, one animal was killed at day 10 after the start of treatment, because of its deteriorating general condition, and then the dose was reduced from 1000 to 800 mg kg⁻¹ body weight. One day after the 28-day treatment period, all animals were killed by exsanguination from the abdominal aorta under deep anesthesia with CO₂/O₂ and livers were removed.

The dose levels of MEG and TAA selected in both experiments even after the dose change were reported to be tumor-inducible (Becker, 1983; NTP, 2000). The initial dose levels of APAP, ANIT and PMZ induced hepatotoxicity after a 13- or 16-week administration in rats (NTP, 1993a, b; Rees *et al.*, 1962).

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology. All efforts were made to minimize animal suffering.

Histology and Immunohistochemistry

Three-micrometer sections of paraffin-embedded tissues from the liver were stained with hematoxylin and eosin for histopathological examination, or subjected to immunohistochemistry. Immunohistochemistry of liver sections was performed using the Vectastain[®] Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3'-diaminobenzidine/H₂O₂ as the chromogen and antibodies against the following proteins: Ki-67, a cell proliferation marker expressed in the nucleus during the G₁ to M phase of

the cell cycle (Scholzen & Gerdes, 2000), cleaved caspase 3, an apoptosis marker expressed during the later stage of apoptosis (Eckle *et al.*, 2004), Topoll α , acting on DNA decatenation at G₂/M phase (Mattila *et al.*, 2007), phosphorylated histone H3 (p-Histone H3), acting on chromatin condensation at the early M phase (Hirota *et al.*, 2005), Mad2, a spindle checkpoint protein (Kops *et al.*, 2004), phosphorylated histone H2AX (γ H2AX), which responds to DNA damage (Burma *et al.*, 2001), p21^{Cip1}, one of the cyclin-dependent kinase inhibitors (Sherr & Roberts, 1995), and Ubd, a molecule that leads to chromosomal instability through reduction in kinetochore localization of checkpoint proteins, such as Mad2, during the G₂/M phase (Herrmann *et al.*, 2007; Lim *et al.*, 2006), phosphorylated Mdm2 (p-Mdm2), a p53 downstream molecule that facilitates degradation of p53 (Malmlof *et al.*, 2007; Mayo & Donner, 2002). Information of antibodies and their antigens used in experiments are shown in Table 1. Sections were counterstained with hematoxylin for microscope examination. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide.

For double immunohistochemistry of Ubd with Topoll α or p-Histone H3, 3,3'-diaminobenzidine was used to visualize Ubd with the Vectastain[®] ABC-AP kit (Vector Laboratories Inc.), and Vector[®] Red Alkaline Phosphate Substrate Kit I (Vector Laboratories Inc.) was used to visualize Topoll α and p-Histone H3.

Analysis of Immunoreactivity

The immunoreactive cells for Ki-67, Topoll α , p-Histone H3, Ubd, Mad2, γ H2AX, p21^{Cip1} or p-Mdm2 were counted in 10 selected areas per animal at 200 \times magnification, and the immunoreactive cells for cleaved caspase 3 were counted in five selected areas per animal at 100 \times magnification, avoiding areas of connective tissues and vasculature. In case of even distribution of immunoreactive cells, area selection was conducted randomly. In case of uneven immunoreactive cell distribution within the liver lobules,

such as accumulation of immunoreactive cells at the periportal area, 5 or 10 adjacent areas were selected for analysis to avoid selection of areas disproportionately. Immunoreactive liver cells were counted visually, and the total number of liver cells in the micrographs was separately counted using the image binarization method in the Win-ROOF image analysis and measurement software (version 6.4.2.; Mitani Corporation, Fukui, Japan). Then, the percentage of total immunoreactive cells was estimated in each animal.

Real-time Reverse Transcription-Polymerase Chain Reaction Analysis

To investigate the expression levels of representative cell cycle-related genes and DNA damage-related genes in the liver, mRNA expression analysis was performed using the StepOnePlus[™] Real-time RT-PCR System (Life Technologies, Carlsbad, CA, USA) with the SYBR[®]Green PCR Master Mix (Life Technologies). The forward and reverse primers listed in Table 2 were designed using the Primer Express 3.0 software (Life Technologies). Using the threshold cycle values of *Hprt1* in the same sample as the endogenous control, the relative differences in gene expression were calculated using the 2^{- $\Delta\Delta C_T$} method (Livak & Schmittgen, 2001).

Statistical Analysis

All data are represented as mean \pm SD. Numerical data were analyzed by the Bartlett's test for the homogeneity of variance. If there was no significant difference in variance, Dunnett's test was performed for comparison between the groups. If a significant difference was found in variance, Steel's test was performed. All numerical data of the treatment groups were compared with those of untreated controls.

Table 1. Antibodies used for immunohistochemistry

Antigen	Abbreviated name	Host species	Clone name	Dilution	Antigen retrieval ^a	Manufacturer (city, state, country)
Ki-67 antigen	Ki-67	Mouse	Monoclonal (MIB-5)	1: 200	Autoclaving in citrate buffer	Dako (Glostrup, Denmark)
Cleaved caspase 3 (Asp175)	-	Rabbit	Polyclonal	1: 500	Autoclaving in target retrieval solution	Cell Signaling Technology, Inc. (Danvers, MA, USA)
p21 ^{Cip1}	-	Mouse	Monoclonal (CP74)	1: 1000	Microwaving in citrate buffer	Abcam (Cambridge, UK)
Ubiquitin D	Ubd	Rabbit	Polyclonal	1: 400	Autoclaving in citrate buffer	Proteintech Group, Inc. (Chicago, IL, USA)
Topoisomerase II alpha	Topoll α	Rabbit	Monoclonal (EP1102Y)	1: 400	Autoclaving in citrate buffer	Epitomics, Inc. (Burlingame, CA, USA)
Phosphorylated histone H3 (Ser10)	Phospho-Histone H3	Rabbit	Polyclonal	1: 400	Autoclaving in citrate buffer	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
Histone H2AX (phospho-Ser139)	γ H2AX	Rabbit	Monoclonal (EP854(2)Y)	1: 1000	Autoclaving in citrate buffer	Abcam
Mitotic arrest deficient-2	MAD2	Mouse	Monoclonal (48/MAD2)	1: 400	Microwaving in citrate buffer	BD Transduction Laboratories (Lexington, KY, USA)
Phosphorylated Mdm2 (Ser166)	p-Mdm2	Rabbit	Polyclonal	1: 400	Autoclaving in target retrieval solution	Cell Signaling Technology, Inc.

^aAntigen retrieval was applied for immunohistochemistry. Retrieval conditions were either autoclaving at 121 °C for 10 min in 10 mm citrate buffer (pH 6.0) or in target retrieval solution (3-in-1; pH 9.0, Dako), or microwaving at 90 °C for 10 min in 10 mm citrate buffer (pH 6.0).

Table 2. Sequence of primers used for real-time RT-PCR

Gene	Accession no.	Forward primer (5' → 3')	Reverse primer (5' → 3')
G₁/S checkpoint-related genes			
<i>Cdkn1a</i>	NM_080782	ACCAGCCACA GGCACCAT	CGGCATACTT TGCTCCTGTG T
<i>Cdkn2a</i>	NM_031550	CAAACGCCCC GAACACTT	CACITTTGACG TTGCCCATCA
<i>Rb1</i>	NM_017045	CACCAGGCCT CACTACCTGT C	AGGAATCCGC AAGGGTGAAC
<i>Rbl2</i>	NM_031094	AAGTGAATCG CCTGCAAAAA G	CTCGGTTCATT AGCTACATCT TGGA
<i>Mdm2</i>	NM_001108099	GAAGGAGGAC ACACAAGACA AAGA	ATGGCTCGAT GGCCTTCA
<i>Tp53</i>	NM_030989	CATGAGCGTT GCTCTGATGG T	GATTTCTTC CACCCGGATA A
Spindle checkpoint and M phase-related genes			
<i>Aurka</i>	NM_153296	AAGAGAGTCA TCCACAGAGA CATCAA	CGATCTTCAA CTCCCCATTT G
<i>Aurkb</i>	NM_053749	CGGATGCATA ATGAGATGGT AGAT	TCCCCACCAT CAGTTCATAG C
<i>Bub1</i>	NM_001106507	CCTAGCTCC CAGTCCTAAA AGT	TTGTGGAATG GTGTAGATGA AAGC
<i>Mad111</i>	NM_001109387	TCCAGGAGTT CCGCAAGGT	GAGGCCGTAT TGGCTCTCAG T
<i>Mad211</i>	NM_001106594	ACAGCCACTG TGACATTTCT ACCA	CCCGATTCTT CCCACTTTTC A
<i>Plk1</i>	NM_017100	TCCCACCAAG GTTTTCAATA GC	TGTGAGAGGC TTCCTGTTGC T
DNA damage-related genes			
<i>Atm</i>	NM_001106821	AGGCTGTCGG CAGGTGTTT	TGGTGTACGG CGTATCTTTG C
<i>Brca1</i>	AF036760	TGATGTGGGA CTGGGTGTTG	CTGTACCAGG TAGGCATCCA GAT
<i>Brca2</i>	NM_031542	AGGCTTTCGG TTGGCAGAT	AGAGACCCAG ACGCTAGAAA TCA
<i>Brc3</i>	NM_001127300	CCACATCCAC TCGGTCATCA T	AAATCTCCAC GCGTCTCTT
<i>Chek1</i>	NM_080400	TGGCAGCTGG CAAAGGA	AATCCCAGTC TTCCACAAAA GG
<i>Chek2</i>	NM_053677	TTGCTTCGAT GGACCACTGT T	GATGCGAAAG TGCTTCTTGC T
<i>Esco1</i>	NM_001126299	CCAAATCCCA CTGCCGTTA	GCTGCCTCTT TTGCTCTTTC C
<i>Gadd45a</i>	NM_024127	CACCATAACT GTCGGCGTGT A	GGCACAGGAC CACGTTGTC
<i>Rad17</i>	NM_001024778	GACTGGGTAG ATCCGGCATT T	AAACGGTGAT GGTGGTGACA
<i>Rad50</i>	NM_022246	TGGCCCTGG CAGTGA	AACTTCGCAC GCCCAGAGT
Housekeeping gene			
<i>Hprt1</i>	NM_012583	GCCGACCGGT TCTGTCAT	TCATAACCTG GTTCATCATC ACTAATC

Atm, ATM serine/threonine kinase; Aurka, aurora kinase A; Aurkb, aurora kinase B; Brca1, breast cancer 1, early onset; Brca2, breast cancer 2, early onset; Brc3, BRCA1/BRCA2-containing complex, subunit 3; Bub1, BUB1 mitotic checkpoint serine/threonine kinase; Cdkn1a, cyclin-dependent kinase inhibitor 1A; Cdkn2a, cyclin-dependent kinase inhibitor 2A; Chek1, checkpoint kinase 1; Chek2, checkpoint kinase 2; Esco1, establishment of sister chromatid cohesion N-acetyltransferase 1; Gadd45a, growth arrest and DNA-damage-inducible, alpha; Hprt1, hypoxanthine phosphoribosyltransferase 1; Mad111, MAD1 mitotic arrest deficient-like 1 (yeast); Mad211, MAD2 mitotic arrest deficient-like 1 (yeast); Mdm2, MDM2 proto-oncogene, E3 ubiquitin protein ligase; Plk1, polo-like kinase 1; PMZ, Rad17, RAD17 homolog (*S. pombe*); Rad50, RAD50 homolog (*S. cerevisiae*); Rb1, retinoblastoma 1; Rbl2, retinoblastoma-like 2; RT-PCR, reverse transcription-polymerase chain reaction; Tp53, tumor protein p53.

Results

Body and Liver Weight

At day 3, the final body weights significantly decreased in the PH, MEG, TAA, ANIT and PMZ groups, compared with untreated controls (Table 3). The absolute liver weight after PH was significantly lower than untreated controls. The absolute liver weights of rats in the MEG and APAP groups were significantly higher than untreated controls. The relative liver weights of rats in the MEG, TAA, APAP, ANIT and PMZ groups were significantly higher than untreated controls.

At day 7, the body weights significantly decreased in all treatment groups, compared with untreated controls (Table 3). The absolute liver weights of rats in the PH and ANIT groups were significantly lower than untreated controls. The relative liver weights of rats in the MEG, TAA, APAP, ANIT and PMZ groups were significantly higher than untreated controls.

At day 28, the body weights significantly decreased in the MEG, TAA, APAP, ANIT and PMZ groups compared with untreated

controls (Table 3). The absolute liver weights of rats in the PH, TAA and ANIT groups were significantly lower than untreated controls. The absolute liver weight of rats in the PMZ group was significantly higher than untreated controls. The relative liver weights of rats in the MEG, TAA, APAP, ANIT and PMZ groups were significantly higher than untreated controls.

Histopathological Changes

MEG treatment revealed no apparent histopathological changes at day 3, centrilobular liver cell hypertrophy associated with cytoplasmic ground glass appearance at day 7 and diffused distribution of liver cells showing cytomegaly and anisokaryosis at day 28, as previously reported (NTP, 2000). TAA treatment caused centrilobular liver cell hypertrophy associated with nucleolar enlargement at days 3 and 7, and periportal vacuolar degeneration of liver cells at day 7. At day 28, TAA treatment resulted in diffused liver cell cytomegaly often associated with anisokaryosis, aberrant mitosis, and apoptosis and periportal oval cell proliferation, as previously

Table 3. Initial and final body weights and liver weight of rats after partial hepatectomy, or after treatment with hepatocarcinogens or hepatotoxicants

Group	Number of animals	Initial body weight (g)	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (g 100 g ⁻¹ BW)
Day 3 (experiment 1)					
CONT	10	123.8 ± 3.8 ^a	147.7 ± 5.1	6.40 ± 0.31	4.33 ± 0.13
PH	11	121.3 ± 4.4	124.5 ± 4.9**	3.91 ± 0.38**	3.14 ± 0.22
MEG	11	121.8 ± 4.7	129.0 ± 5.4**	6.92 ± 0.36*	5.37 ± 0.24**
TAA	10	123.0 ± 6.7	134.3 ± 4.9**	6.39 ± 0.36	4.76 ± 0.18**
APAP	10	122.2 ± 2.7	142.1 ± 4.5	6.94 ± 0.54*	4.88 ± 0.25**
ANIT	10	122.4 ± 3.0	125.8 ± 4.0**	6.28 ± 0.43	4.99 ± 0.27**
PMZ	11	121.2 ± 4.4	118.3 ± 9.0**	6.03 ± 0.44	5.11 ± 0.38**
Day 7 (experiment 1)					
CONT	10	124.2 ± 9.9	167.4 ± 13.9	6.95 ± 0.71	4.15 ± 0.14
PH	10	121.1 ± 10.4	141.8 ± 9.0**	5.19 ± 0.65**	3.66 ± 0.34
MEG	11	122.7 ± 10.0	139.4 ± 5.8**	7.21 ± 0.42	5.17 ± 0.30**
TAA	10	122.7 ± 7.4	141.5 ± 8.0**	7.21 ± 0.38	5.10 ± 0.19**
APAP	10	123.3 ± 8.4	150.4 ± 12.0**	6.82 ± 0.88	4.52 ± 0.29*
ANIT	10	123.1 ± 8.2	121.7 ± 5.4*	5.71 ± 0.36**	4.70 ± 0.23**
PMZ	11	121.0 ± 9.4	136.2 ± 7.1**	7.22 ± 0.42	5.30 ± 0.29**
Day 28 (experiment 2)					
CONT	10	128.0 ± 7.8	250.8 ± 10.5	10.20 ± 0.48	4.07 ± 0.14
PH	12	128.1 ± 5.3	240.5 ± 10.2	9.40 ± 0.52*	3.91 ± 0.07
MEG	10	128.3 ± 5.8	186.0 ± 16.3**	10.54 ± 1.20	5.65 ± 0.22**
TAA	10	126.9 ± 7.3	152.4 ± 10.4**	7.71 ± 0.79**	5.05 ± 0.30**
APAP	10	127.4 ± 5.9	218.6 ± 8.6**	9.48 ± 0.62	4.33 ± 0.13**
ANIT	11	126.3 ± 7.2	162.8 ± 11.3**	8.79 ± 0.64**	5.40 ± 0.16**
PMZ	11	126.7 ± 6.8	212.4 ± 13.2**	11.39 ± 0.90**	5.36 ± 0.20**

ANIT, α -naphthyl isothiocyanate; APAP, acetaminophen; CONT, untreated controls; MEG, methyleugenol; PH, partial hepatectomy; PMZ, promethazine hydrochloride; TAA, thioacetamide.

^aValues are expressed as mean ± SD.

* $P < 0.05$, ** $P < 0.01$ vs. untreated controls (Dunnett's or Steel's test).

reported (Clawson *et al.*, 1992). Nucleolar enlargement was apparent in these cytomegalic cells at this time point. Treatment with APAP revealed no apparent histopathological changes at day 3. In contrast, APAP treatment for 7 or 28 days revealed slight centrilobular liver cell hypertrophy, as previously reported (NTP, 1993a). ANIT treatment resulted in periportal bile duct proliferation from day 3. From day 7, scattered focal liver cell necrosis was observed, as previously reported (Rees *et al.*, 1962). PMZ treatment resulted in centrilobular liver cell hypertrophy and ground glass appearance at day 7 and day 28, as previously reported (NTP, 1993b).

Cell Proliferation and Apoptosis

Ki-67 was immunolocalized in the nucleus of liver cells (Fig. 1A,C,E). Ki-67⁺ cells distributed evenly within the liver lobules, except for preferential distribution at the periportal area in the TAA group at days 3 and 7 and in the PMZ group at day 28. Cleaved caspase 3 was immunolocalized in the nucleus and cytoplasm of liver cells (Fig. 1B,D,F). Cleaved caspase 3⁺ cells preferentially distributed at the periportal area in any group at any time point.

At day 3, the number of Ki-67⁺ cells significantly increased in the PH, TAA, APAP and ANIT groups and significantly decreased in the PMZ group, compared with untreated controls (Fig. 1A). The number of cleaved caspase 3⁺ cells significantly increased in the TAA

group and significantly decreased in the PMZ group, compared with untreated controls (Fig. 1B).

At day 7, the number of Ki-67⁺ cells significantly decreased in the PH, APAP and ANIT groups, compared with untreated controls (Fig. 1C). The number of cleaved caspase 3⁺ cells significantly increased in the MEG, TAA, APAP and ANIT groups, compared with untreated controls (Fig. 1D).

At day 28, the number of Ki-67⁺ cells significantly increased in the MEG, TAA and PMZ groups, compared with untreated controls (Fig. 1E). The number of cleaved caspase 3⁺ cells significantly increased in the MEG and TAA groups, compared with untreated controls (Fig. 1F).

Immunoreactive Cellular Distribution

TopoII α , p-Histone H3, Mad2, γ H2AX, p21^{Cip1} and p-Mdm2 were immunolocalized in the nucleus of liver cells, and Ubd was immunolocalized in the cytoplasm or mitotic spindle of liver cells (Fig. 2A–G). Immunoreactive cells for TopoII α , p-Histone H3, Mad2 and γ H2AX distributed evenly within the liver lobules, except for preferential distribution at the periportal area in the TAA group at days 3 and 7 and in the PMZ group at day 28. Immunoreactive cells for p21^{Cip1} and p-Mdm2 preferentially distributed at the periportal area in any group at any time point.

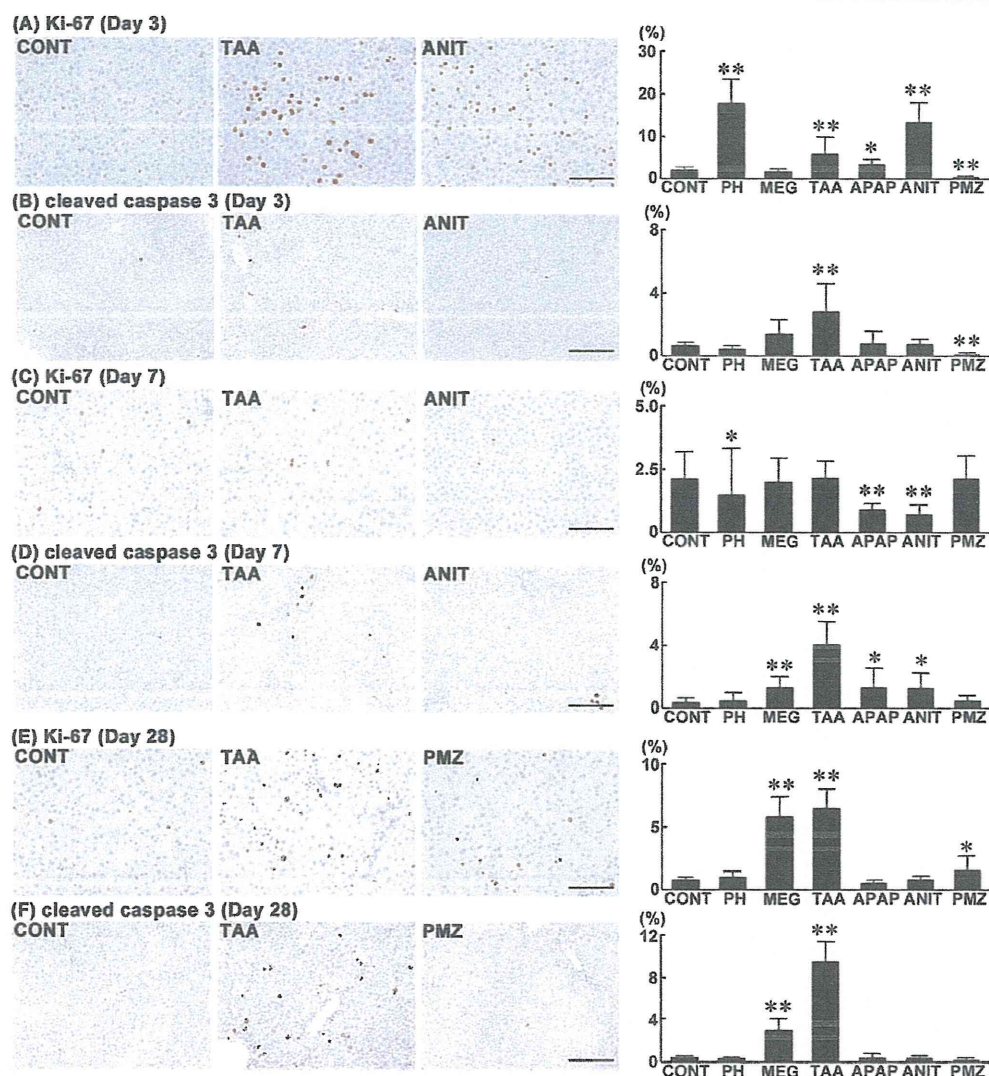


Figure 1. Distribution of Ki-67⁺ and cleaved caspase 3⁺ cells in the liver of rats at days 3, 7 and 28 after treatment with PH, noncarcinogenic hepatotoxics or hepatocarcinogens. Photomicrographs show the distribution of Ki-67⁺ and cleaved caspase 3⁺ cells in the liver of representative cases from untreated controls (A–F), animals treated with TAA or ANIT (A–D), and animals treated with TAA or PMZ (E,F). The graphs show the positive cell ratios of hepatocytes per total cells counted in 10 animals in each group. Values represent mean + SD. (A) Ki-67 (day 3), (B) cleaved caspase 3 (day 3), (C) Ki-67 (day 7), (D) cleaved caspase 3 (day 7), (E) Ki-67 (day 28) and (F) cleaved caspase 3 (day 28). Bar = 100 μ m (A,C,E) or 200 μ m (B,D,F). * P < 0.05, ** P < 0.01 vs. untreated controls (Dunnett's or Steel's test). ANIT, α -naphthyl isothiocyanate; APAP, acetaminophen; CONT, untreated controls; MEG, methyleugenol; PH, partial hepatectomy; PMZ, promethazine hydrochloride; TAA, thioacetamide.

At day 3, the number of TopoII α ⁺ cells, p-Histone H3⁺ cells and Mad2⁺ cells significantly increased in the PH, TAA and ANIT groups, and significantly decreased in the MEG and PMZ groups, compared with untreated controls (Fig. 2A–C). The number of Ubd⁺ cells and γ H2AX⁺ cells significantly increased in the PH, TAA and ANIT groups, and significantly decreased in the PMZ group, compared with untreated controls (Fig. 2D,E). The number of p21^{Cip1}⁺ cells significantly increased in the MEG, TAA and APAP groups, compared with untreated controls (Fig. 2F). The number of p-Mdm2⁺ cells significantly increased in the MEG and TAA groups, compared with untreated controls (Fig. 2G).

At day 7, the number of TopoII α ⁺ cells, p-Histone H3⁺ cells and Mad2⁺ cells significantly decreased in the PH, MEG, APAP and ANIT groups, compared with untreated controls (Fig. 3A–C). The number of Ubd⁺ cells significantly decreased in the PH, MEG, TAA, APAP and ANIT groups, compared with untreated controls (Fig. 3D). The number of γ H2AX⁺ cells significantly decreased in the PH, APAP and

ANIT groups, compared with untreated controls (Fig. 3E). The number of p21^{Cip1}⁺ cells significantly increased in the MEG, TAA, APAP and ANIT groups, compared with untreated controls (Fig. 3F). The number of p-Mdm2⁺ cells significantly increased in the MEG, TAA, APAP and PMZ groups, compared with untreated controls (Fig. 3G).

At day 28, the number of TopoII α ⁺ cells significantly increased in the PH, MEG, TAA and PMZ groups, and significantly decreased in APAP group, compared with untreated controls (Fig. 4A). The number of p-Histone H3⁺ cells, Mad2⁺ cells and γ H2AX⁺ cells significantly increased in the MEG, TAA and PMZ groups, compared with untreated controls (Fig. 4B,C,E). The number of Ubd⁺ cells significantly increased in the PH, MEG, TAA and PMZ groups, compared with untreated controls (Fig. 4D). The number of p21^{Cip1}⁺ cells significantly increased in the MEG, TAA and APAP groups, compared with untreated controls (Fig. 4F). The number of p-Mdm2⁺ cells significantly increased in the MEG and TAA groups, compared with untreated controls (Fig. 4G).

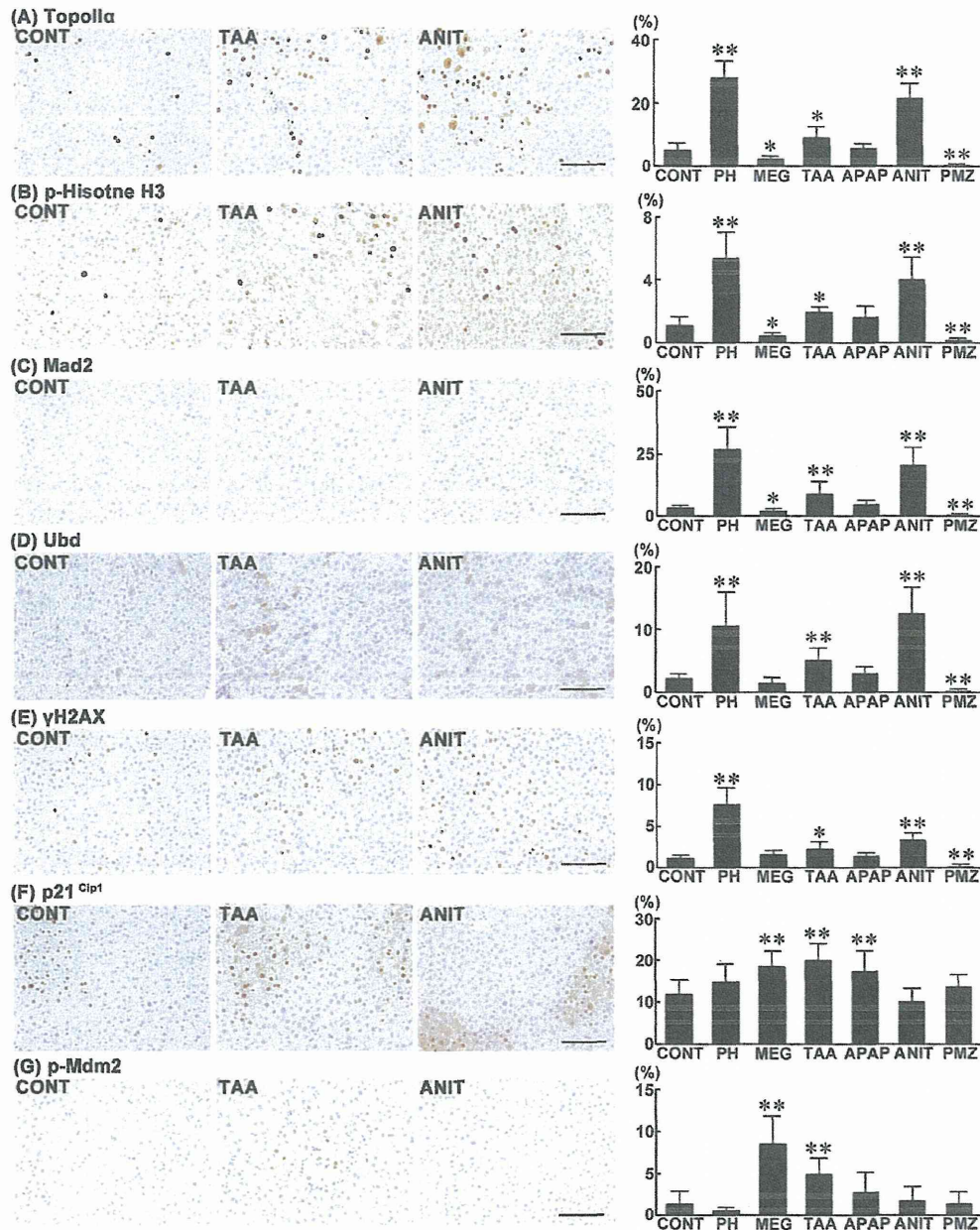


Figure 2. Distribution of TopoII α ⁺, p-Histone H3⁺, Mad2⁺, Ubd⁺, γ H2AX⁺, p21^{Cip1}⁺ and p-Mdm2⁺ cells in the liver of rats at day 3 after treatment with PH, noncarcinogenic hepatotoxicants or hepatocarcinogens. Photomicrographs show the distribution of TopoII α ⁺, p-Histone H3⁺, Mad2⁺, Ubd⁺, γ H2AX⁺, p21^{Cip1}⁺ and p-Mdm2⁺ cells in the liver of representative cases from untreated controls and animals treated with TAA or ANIT. The graphs show positive cell ratios of hepatocytes per total cells counted in 10 animals in each group. Values represent mean + SD. (A) TopoII α , (B) p-Histone H3, (C) Mad2, (D) Ubd, (E) γ H2AX, (F) p21^{Cip1} and (G) p-Mdm2. Bar = 100 μ m. * P < 0.05, ** P < 0.01 vs. untreated controls (Dunnett's or Steel's test). ANIT, α -naphthyl isothiocyanate; APAP, acetaminophen; CONT, untreated controls; MEG, methyleugenol; PH, partial hepatectomy; PMZ, promethazine hydrochloride; TAA, thioacetamide.

The ratio of the number of p-Histone H3⁺ cells to that of Ki-67⁺ cells significantly decreased in the MEG and TAA groups, compared with untreated controls (Fig. 5).

Double Immunohistochemistry of Ubd with TopoII α or p-Histone H3

At day 3, the number of Ubd⁺ cells within the population of the TopoII α ⁺ cells significantly increased in the TAA and ANIT groups, compared with untreated controls (Fig. 6A). In contrast, the ratio of the TopoII α ⁺ cells to the total number of Ubd⁺ cells did not change in any of the treatment groups. The number of

Ubd⁺ cells within the population of p-Histone H3⁺ cells significantly decreased in the PH group, compared with untreated controls (Fig. 6B). On the other hand, the number of p-Histone H3⁺ cells within the population of Ubd⁺ cells significantly decreased in the PH and ANIT groups, compared with untreated controls.

At day 7, the number of Ubd⁺ cells within the population of TopoII α ⁺ cells significantly increased in all treatment groups, compared with untreated controls (Fig. 6C). In contrast, the ratio of the TopoII α ⁺ cells to the total number of Ubd⁺ cells did not change in any of the treatment groups. The number of Ubd⁺ cells within the population of p-Histone H3⁺ cells and the number of p-Histone

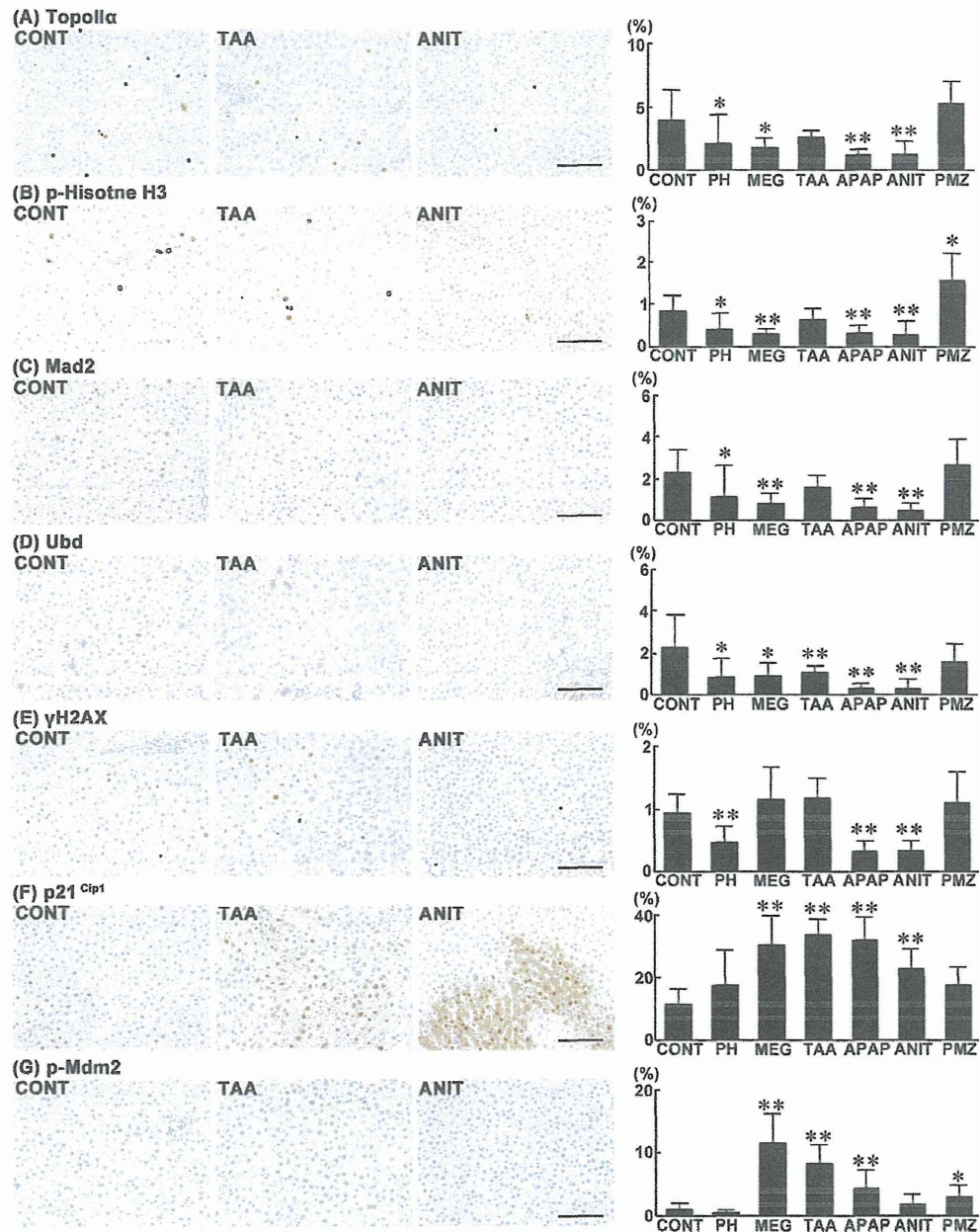


Figure 3. Distribution of TopoII α ⁺, p-Histone H3⁺, Mad2⁺, Ubd⁺, γ H2AX⁺, p21^{Cip1}⁺ and p-Mdm2⁺ cells in the liver of rats at day 7 after treatment with PH, noncarcinogenic hepatotoxicants or hepatocarcinogens. Photomicrographs show the distribution of TopoII α ⁺, p-Histone H3⁺, Mad2⁺, Ubd⁺, γ H2AX⁺, p21^{Cip1}⁺ and p-Mdm2⁺ cells in the liver of representative cases from untreated controls and animals treated with TAA or ANIT. The graphs show positive cell ratios of hepatocytes per total cells counted in 10 animals in each group. Values represent mean + SD. (A) TopoII α , (B) p-Histone H3, (C) Mad2, (D) Ubd, (E) γ H2AX, (F) p21^{Cip1} and (G) p-Mdm2. Bar = 100 μ m. * P < 0.05, ** P < 0.01 vs. untreated controls (Dunnett's or Steel's test). ANIT, α -naphthyl isothiocyanate; APAP, acetaminophen; CONT, untreated controls; MEG, methyleugenol; PH, partial hepatectomy; PMZ, promethazine hydrochloride; TAA, thioacetamide.

H3⁺ cells within the population of Ubd⁺ did not change in any of the treatment groups (Fig. 6D).

At day 28, the number of Ubd⁺ cells within the population of TopoII α ⁺ cells significantly increased in the MEG and TAA groups, compared with untreated controls (Fig. 6E). In contrast, the ratio of the TopoII α ⁺ cells to the total number of Ubd⁺ cells did not change in any of the treatment groups. The number of Ubd⁺ cells within the population of p-Histone H3⁺ cells significantly increased in the PMZ group, compared with untreated controls (Fig. 6F). Furthermore, the ratio of the p-Histone H3⁺ cells to the total number of Ubd⁺ cells significantly decreased in the MEG and TAA groups, compared with untreated controls.

Real-time Reverse Transcription–Polymerase Chain Reaction Analysis

Transcript levels of the genes listed in Table 2 at days 3, 7 and 28 were determined by real-time RT-PCR in the MEG, TAA and PMZ groups, which showed significant increase of cell proliferation at day 28, and compared with the levels in untreated controls (Table 4).

At day 3, *Cdkn1a* showed a significant increase of expression in the MEG and TAA groups compared with those in untreated controls, among the G₁/S checkpoint-related genes. In contrast, the transcript level of *Cdkn1a* was significantly lower in the PMZ group