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Aberrant cell cycle regulation in rat liver cells induced by post-initiation treatment with hepatocarcinogens/hepatocarcinogenic tumor promoters

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ABSTRACT

The present study aimed to determine the onset time of hepatocarcinogen/hepatocarcinogenic tumor promoter-specific cell proliferation, apoptosis and aberrant cell cycle regulation after post-initiation treatment. Six-week-old rats were treated with the genotoxic hepatocarcinogen, carbadox (CRB), the marginally hepatocarcinogenic leucomalachite green (LMG), the tumor promoter, β-naphthoflavone (BNF) or the non-carcinogenic hepatotoxicant, acetaminophen, for 2, 4 or 6 weeks during the post-initiation phase using a medium-term liver bioassay. Cell proliferation activity, expression of G_2 to M phase- and spindle checkpoint-related molecules, and apoptosis were immunohistochemically analyzed at week 2 and 4, and tumor promotion activity was assessed at week 6. At week 2, hepatocarcinogen/tumor promoter-specific aberrant cell cycle regulation was not observed. At week 4, BNF and LMG increased cell proliferation together with hepatotoxicity, while CRB did not. Additionally, BNF and CRB reduced the number of cells expressing phosphorylated-histone H3 in both ubiquitin D (UBD)$^+$ cells and Ki-67$^+$ proliferating cells, suggesting development of spindle checkpoint dysfunction, regardless of cell proliferation activity. At week 6, examined hepatocarcinogens/tumor promoters increased preneoplastic hepatic foci expressing glutathione *S*-transferase placental form. These results suggest that some hepatocarcinogens/tumor promoters increase their toxicity after post-initiation treatment, causing regenerative cell proliferation. In contrast, some genotoxic hepatocarcinogens may disrupt the spindle checkpoint without facilitating cell proliferation at the early stage of tumor promotion. This suggests that facilitation of cell proliferation and disruption of spindle checkpoint function are induced by different mechanisms during hepatocarcinogenesis. Four weeks of post-initiation treatment may be sufficient to induce hepatocarcinogen/tumor promoter-specific cellular responses.

1. Introduction

Evaluation of chemical carcinogenicity is crucial for assessment of chemical safety. However, regular carcinogenicity bioassays in which hundreds of rodent animals are administered test compounds for years are time-consuming and costly. Although alternative *in vivo* bioassay models, such as the two-stage carcinogenesis models (Tamano, 2010) and genetically modified animal models produced by transgenic or gene-targeting technologies (Eastin, 1998), have been developed, these models are also laborious and costly, or have limited target organs. Toxicogenomic approaches for the prediction of carcinogenic potential in each target organ may be promising (Jonker et al., 2009; Matsumoto et al., 2014; Uehara et al., 2011). However, these assays also require integrative methodologies between different laboratories sharing expression databases. Therefore, there is a need to develop new rapid assays for predicting chemical carcinogenicity based on the molecular responses induced by carcinogens irrespective of target organs.

We have previously reported that a 28-day administration of carcinogens facilitating cell proliferation in carcinogen target cells

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induced expression changes in cell cycle-related molecules reflecting the activation of G_1/S and G_2/M checkpoint functions, resulting in cell cycle arrest and apoptosis in many target organs in rats (Taniai et al., 2012a,b; Yafune et al., 2013a,b). We have also found that the renal carcinogen, ochratoxin A, and the hepatocarcinogen, thioacetamide, induced aberrant expression of ubiquitin D (UBD), a molecule interfering with spindle checkpoint function (Herrmann et al., 2007; Lim et al., 2006), at G_2 phase in each target cell, suggesting disruption of spindle checkpoint function (Taniai et al., 2012b). Furthermore, we have recently found that hepato- and renal carcinogens specifically induce disruption of spindle checkpoint function as suggested by a reduced ratio of UBD^+ cells and proliferative cells at M phase at day 28 and/or at day 90 after starting administration, which may contribute to the early stage of carcinogenesis (Kimura et al., 2015, 2016a,b). However, carcinogens exerting marginal carcinogenicity and tumor promoters did not induce both facilitation of cell proliferation and disruption of spindle checkpoint function in this administration model. Conversely, we have previously reported that the hepatocarcinogenic piperonyl butoxide, which did not facilitate cell proliferation after a 28-day administration, increased cell proliferation and the number of cells expressing G_1/S checkpoint- and M phase-related molecules within preneoplastic lesions, similar to other hepatocarcinogens that facilitated cell proliferation after a 28-day treatment in the analysis of post-initiation treatment in a rat two-stage hepatocarcinogenesis model (Taniai et al., 2012c; Yafune et al., 2014). These results suggest that examination of cell cycle regulation in two-stage models may be useful for detection of carcinogens/tumor promoters lacking facilitation of cell proliferation activity after a 28-day administration. For establishing a new rapid bioassay for the prediction of carcinogenicity, it is now important to estimate the critical onset time point of carcinogen/tumor promoter-specific cellular responses by administration during the post-initiation phase.

The present study aimed to determine the critical onset time point of hepatocarcinogen/hepatocarcinogenic tumor promoter-specific aberration of cell cycle regulation by chemical administration during the post-initiation phase. For this purpose, rats were repeatedly administered hepatocarcinogens, hepatocarcinogenic promoters or non-carcinogenic hepatotoxicants at the post-initiation phase for 2 or 4 weeks, and then subjected to immunohistochemical analysis of the time course response in cell proliferation activity, expression of G_2 to M phase- and spindle checkpoint-related molecules, and apoptosis in the liver. Tumor promotion activity was also examined by exposing groups of animals for 6 weeks.

2. Materials and methods

2.1. Chemicals

Acetaminophen (APAP; CAS No. 103-90-2, purity ≥98.0%) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). β-Naphthoflavone (BNF; CAS No. 6051-87-2, purity ≥98%), carbadox (CRB; CAS No. 6804-07-5, purity 98%) and leucomalachite green (LMG; CAS No. 129-73-7, purity 98%) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). *N*-Diethylnitrosamine (DEN; CAS No. 55-18-5, purity >99.0%) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

2.2. 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 one week. Rats were housed in plastic cages with paper chip in a barrier-maintained animal room under standard conditions (room temperature, 23 ± 3 °C; relative humidity, 50 ± 20%; 12-h light/dark cycle). After a 1-week acclimatization period, animals were subjected to animal experiments.

All animals were initiated with a single intraperitoneal injection of DEN (200 mg/kg body weight). Two weeks later, animals were divided into five groups and fed the basal diet (DEN-alone) or a diet containing either BNF at 10,000 ppm (DEN + BNF), CRB at 300 ppm (DEN + CRB), LMG at 1160 ppm (DEN + LMG) or APAP at 10,000 ppm (DEN + APAP) for 2, 4 or 6 weeks. The animals were subjected to a two-thirds partial hepatectomy (PH) after one week from starting administration. In the experiment of 2 or 4 weeks of post-initiation treatment, 12, 13, 13, 12 and 12 rats were used as the DEN-alone, DEN + BNF, DEN + CRB, DEN + LMG and DEN + APAP groups, respectively. In the experiment of 6 weeks of post-initiation treatment, 15, 16, 16, 16 and 15 rats were used as the DEN-alone, DEN + BNF, DEN + CRB, DEN + LMG and DEN + APAP groups, respectively. After the 2, 4 or 6 weeks of post-initiation treatment, rats were euthanized by exsanguination from the abdominal aorta under deep anesthesia with CO_2/O_2 and livers were removed. Two slices from the quadrate liver lobe were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4;

Table 1
Antibodies used for immunohistochemistry.

Antigen	Abbreviated name	Host species	Clone name	Dilution	Antigen retrieval	Manufacturer (City, State, Country)
Cleaved caspase 3 (Asp175)	–	Rabbit	Polyclonal	1:500	Autoclaving in target retrieval solution	Cell Signaling Technology, Inc. (Danvers, MA, USA)
Glutathione *S*-transferase placental form	GST-P	Rabbit	Polyclonal	1:1000	None	Medical & Biological Laboratries (Nagoya, Japan)
Ki-67 antigen	Ki-67	Mouse	Monoclonal (MIB-5)	1:200	Autoclaving in citrate buffer	Dako (Glostrup, Denmark)
Phosphorylated histone H3 (Ser10)	p-Histone H3	Rabbit	Polyclonal	1:400	Autoclaving in citrate buffer	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
Topoisomerase II alpha	TOP2A	Rabbit	Monoclonal (EP1102Y)	1:400	Autoclaving in citrate buffer	Epitomics, Inc. (Burlingame, CA, USA)
Ubiquitin D	UBD	Rabbit	Polyclonal	1:400	Autoclaving in citrate buffer	Proteintech Group, Inc. (Chicago, IL, USA)

Antigen 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).