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Immunohistochemical cellular distribution of proteins related to M phase regulation in early proliferative lesions induced by tumor promotion in rat two-stage carcinogenesis models

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ABSTRACT

We have previously reported that 28-day treatment with hepatocarcinogens increases liver cells expressing p21^{Cip1}, a G_1/S checkpoint protein, and M phase proteins, i.e., nuclear Cdc2, Aurora B, phosphorylated-Histone H3 (p-Histone H3) and heterochromatin protein 1α (HP1α), in rats. To examine the roles of these markers in the early stages of carcinogenesis, we investigated their cellular distribution in several carcinogenic target organs using rat two-stage carcinogenesis models. Promoting agents targeting the liver (piperonyl butoxide and methapyrilene hydrochloride), thyroid (sulfadimethoxine), urinary bladder (phenylethyl isothiocyanate), and forestomach and glandular stomach (catechol) were administered to rats after initiation treatment for the liver with *N*-diethylnitrosamine, thyroid with *N*-bis(2-hydroxypropyl)nitrosamine, urinary bladder with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, and forestomach and glandular stomach with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Numbers of cells positive for nuclear Cdc2, Aurora B, p-Histone H3 and HP1α increased within preneoplastic lesions as determined by glutathione *S*-transferase placental form in the liver or phosphorylated p44/42 mitogen-activated protein kinase in the thyroid, and hyperplastic lesions having no known preneoplastic markers in the urinary bladder, forestomach and glandular stomach. Immunoreactive cells for p21^{Cip1} were decreased within thyroid preneoplastic lesions; however, they were increased within liver preneoplastic lesions and hyperplastic lesions in other organs. These results suggest that M phase disruption commonly occur during the formation of preneoplastic lesions and hyperplastic lesions. Differences in the expression patterns of p21^{Cip1} between thyroid preneoplastic and proliferative lesions in other organs may reflect differences in cell cycle regulation involving G_1/S checkpoint function between proliferative lesions in each organ.

1. Introduction

Carcinogenicity testing using rodent animals is one of the most important endpoints for evaluating the carcinogenic potential of chemicals. However, regular carcinogenic bioassays requiring 1.5 or 2 years for conducting animal experiments are time-consuming and expensive, and require the use of many experimental animals. A number of alternative methods have been developed to predict carcinogenic potential in short-term assays. There are genetically modified animals using transgenic or gene targeting technologies (Eastin, 1998) or medium-term carcinogenesis bioassays (Tamano, 2010). However, these are expensive and time-consuming or have limited target organs. In contrast, recently developed toxicogenomic approaches for the prediction of carcinogenic potential in target organs appear promising (Jonker et al., 2009; Uehara et al., 2011). Unfortunately, toxicogenomic approaches are also expensive and require integrative methodologies between different laboratories sharing an expression database. Thus, there are no commonly used rapid assays for evaluating the carcinogenic potential of chemicals.

It has been reported that nuclear enlargement is occasionally found from the early stages in target cells after repeated administration of carcinogens, irrespective of their genotoxic potential, in toxicity studies using rodent animals (Allen et al., 2004; Adler et al., 2009). This nuclear enlargement is typically observed in the liver and kidney, and often called as cytomegaly in case of liver cells characterized by the presence of hepatocytes that are enlarged due to increased cytoplasmic volume and as karyomegaly when it

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occurs in renal tubular cells. Recent studies have shown that ochratoxin A, a renal carcinogen that typically induces karyomegaly, induces aberrant expression of cell cycle-related molecules in proximal tubular areas exhibiting karyomegaly (Adler et al., 2009). These observations suggest that this aberrant expression might eventually cause carcinogenicity in association with the development of chromosomal instability. Therefore, we hypothesize that an early event that disrupts cell cycle regulation initiates the carcinogenic response in the molecular mechanism responsible for cytomegaly/karyomegaly development.

We have previously reported that carcinogens that induce cell proliferation after 28-day treatment in rats concurrently increase the number of carcinogenic target cells, irrespective of target organs, suggestive of disrupting spindle checkpoint at M phase that may eventually lead to chromosomal instability linked to carcinogenesis (Taniai et al., 2012a). Further, we performed immunohistochemical analysis of cell cycle-related proteins after 28 days of repeated administration of hepatocarcinogens that do or do not induce cytomegaly in rat liver cells (Yafune et al., 2013a). In that study, we found that hepatocarcinogens, irrespective of their cytomegaly-inducing potential, increased the number of immunoreactive liver cells for $p21^{Cip1}$, a G_1/S checkpoint protein, and Aurora B, a M phase protein, suggestive of increased cell populations undergoing G_1 arrest or showing chromosomal instability, respectively. We also found that hepatocarcinogens that induce liver cell proliferation might cause M phase arrest of hepatocytes, judging from increased cell population expressing nuclear Cdc2, phosphorylated Histone H3 (p-Histone H3) and heterochromatin protein 1α (HP1α), accompanied by apoptosis. Therefore, we hypothesize that disruption of cell cycle regulation may be the common feature of carcinogenic target cells at the early time point before forming preneoplastic or hyperplastic lesions after treatment with carcinogens that induce cell proliferation. In another study that we have recently performed, we found that M phase proteins may be early prediction markers of carcinogens evoking cell proliferation in many target organs in a scheme of 28-day treatment (Yafune et al., 2013b).

It is now important to know whether $p21^{Cip1}$ and M phase proteins play roles in the formation of proliferative lesions during carcinogenic processes. The present study aimed to clarify the involvement of these proteins in the early carcinogenic processes in the formation of preneoplastic or hyperplastic lesions in different target organs. For this purpose, we employed two-stage carcinogenesis models targeting different organs utilizing organ-specific tumor initiator and following promoter to allow selective proliferation of initiated cells forming proliferative lesions in each organ for short-term. We analyzed the immunohistochemical cellular distribution of these proteins at early stages of tumor promotion in the liver, thyroid, urinary bladder, forestomach, and glandular stomach.

2. Materials and methods

2.1. Chemicals

Methapyrilene hydrochloride (MP; CAS No. 135-23-9), *N*-diethylnitrosamine (DEN; CAS No. 55-18-5, >99.0%), and sulfadimethoxine sodium salt (SDM; CAS No. 122-11-2) were purchased from Sigma–Aldrich Japan K. K. (Tokyo, Japan). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; CAS No. 70-25-7, >95.0%), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; CAS No. 3817-11-6, >90.0%), and phenylethyl isothiocyanate (PEITC; CAS No. 2257-09-2, ≥97.0%) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Catechol (CC; CAS No. 120-80-9, >99.0%) was purchased from Wako Pure Chemicals Industries (Osaka, Japan). *N*-Bis(2-hydroxypropyl)nitrosamine (DHPN; CAS No. 53609-64-6) was purchased from Nacalai Tesque (Kyoto, Japan). Piperonyl butoxide (PBO; CAS No. 51-03-6, 90%) was obtained from Nagase & Co. (Osaka, Japan).

2.2. Animal experiments

Animals and experimental design were identical to those previously reported (Taniai et al., 2012b). Animal studies were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology. Briefly, five-week-old male F344/NSIc rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and acclimatized to a powdered basal diet (CRF-1 diet; Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. They were housed in stainless steel cages in a barrier-maintained animal room on a 12-h light–dark cycle at 23 ± 3 °C with a relative humidity of 50 ± 20%.

After a 1-week acclimatization period, animals were subjected to two-stage carcinogenesis bioassays as described in Fig. 1. To study the liver as the target organ, a medium-term liver bioassay was employed (Shirai, 1997). All animals were initiated with a single intraperitoneal injection of DEN (200 mg/kg body weight). Two weeks later, animals were divided into three groups and fed the basal diet (DEN-alone) or a diet containing either PBO at 20,000 ppm (DEN + PBO) or MP at 1000 ppm (DEN + MP) for 6 weeks. The animals were subjected to a two-thirds partial hepatectomy at week 3. The doses of PBO and MP have been shown to promote induction of preneoplastic lesions as determined by glutathione *S*-transferase placental form (GST-P) in the liver after a 6-week administration in a two-stage model (Horn et al., 1996; Ichimura et al., 2010). To study the thyroid as the target organ, all animals were initiated with a single subcutaneous injection of DHPN (2800 mg/kg body weight). One week later, animals were given drinking water with (DHPN + SDM) or without (DHPN-alone) SDM at 1500 ppm for 4 weeks. This dose of SDM has been shown to promote induction of follicular cell carcinomas after a 13-week administration in a two-stage model (Kemmochi et al., 2012). To study the urinary bladder as the target organ, all animals were initiated with BBN at 500 ppm in drinking water for 4 weeks. Animals were then given either the basal diet (BBN-alone) or a diet containing PEITC at 1000 ppm (BBN + PEITC) for 8 weeks. This dose of PEITC has been shown to promote induction of transitional cell carcinomas for 32-week administration in a two-stage model (Hirose et al., 1998). To study the forestomach and glandular stomach, all animals were initiated with a single gavage of MNNG (150 mg/kg body weight). One week later, animals were fed either the basal diet (MNNG-alone) or a diet containing CC at 8000 ppm (MNNG + CC) for 12 weeks. This dose of CC has been shown to promote induction of both forestomach and glandular stomach carcinomas after a 51-week administration in a two-stage model (Wada et al., 1998). After cessation of tumor promotion, all animals were sacrificed by exsanguination from the abdominal aorta under deep anesthesia and target organs were removed.

Tissue fixation and following preparation for histopathological assessment were described previously (Taniai et al., 2012b).

2.3. Histopathology and immunohistochemistry

From paraffin-embedded tissues of the liver, thyroid, urinary bladder, and stomach (forestomach and glandular stomach), 3-μm sections were stained with hematoxylin and eosin for histopathological examination and subjected to immunohistochemistry.

Immunohistochemistry was performed using the Vectastain® Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) with