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Different Responses Other than the Formation of DNA-adducts between the Livers of Carcinogen-resistant Rats (DRH) and Carcinogen-sensitive Rats (Donryu) to 3′-Methyl-4-dimethylaminoazobenzene Administration

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Carcinogen-resistant inbred DRH rats developed from the Donryu strain showed a remarkably low incidence of liver tumors when they were fed diets containing hepatocarcinogens such as 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). In this work, we examined various characteristics of male DRH and Donryu rats during 3′-Me-DAB administration for 8 weeks. ^{32}P-Postlabeling analysis showed that essentially similar levels of DNA-adducts were generated by the metabolites of 3′-Me-DAB in the livers of these two strains of rats at several time points. However, both GADD45 (growth arrest and DNA damage-inducible) and O^6-methylguanine methyltransferase (putatively DNA damage-inducible) mRNA levels were increased significantly in Donryu rat livers, but were increased to a lesser extent in DRH rats. [^{3}H]Thymidine incorporation into hepatic DNA began to increase around 10 to 20 days after the start of 3′-Me-DAB administration in Donryu rats probably due to DNA repair, while no significant change occurred in DRH rats under the same conditions. Furthermore, inductions of heme oxygenase (due to degradation of heme-proteins) and hepatocyte growth factor (HGF; cell death and regeneration of hepatocytes) mRNAs were greater in Donryu rat livers than those of DRH, suggesting that the former were more sensitive to cytotoxic effects of 3′-Me-DAB than the latter. Another remarkable difference observed between these two strains was the significant induction of cytochrome P-450 2E1 mRNA in Donryu rat livers; this may contribute to the generation of reactive oxygen intermediates. Finally, increases of glutathione S-transferase (P-form) and γ-glutamyltranspeptidase mRNAs as marker enzymes of preneoplastic changes of hepatocytes were clearly seen only in Donryu rat livers at 6 to 8 weeks after the start of 3′-Me-DAB administration. These results indicate that the different susceptibility to hepatocarcinogenesis between these two strains of rats may arise from events other than the DNA adduct formation.

Key words: Carcinogen resistance — DNA adduct — 3′-Me-DAB — Glutathione S-transferase — Nongenotoxic effect

The carcinogen-resistant rat strain was isolated from Donryu rats on the basis of examination of selective markers such as reduced induction of γ-glutamyltranspeptidase and lower incidence of liver tumors during 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) administration. After repeated inbreeding for more than 10 years, the carcinogen-resistant inbred DRH rat strain has been established.[1–4] The DRH rats showed a remarkably low incidence of hepatic tumors when they were fed diet containing 0.06% 3′-Me-DAB and they also showed resistance to other aminoazo carcinogens, 3′-hydroxymethyl-4-dimethylaminoazobenzene (3′-CH_2OH-DAB), and DAB, and another type of hepatocarcinogen, 2-acetylaminofluorene (2-AAF).[4] Moreover, DRH rats showed tolerance to 7,12-dimethylbenz(*a*)anthracene (DMBA), which is metabolically activated via a different mechanism from aminoazo carcinogens and 2-AAF and caused tumors in the mammary gland[4] (Fig. 1).

Previous studies demonstrated that DRH rat livers showed somewhat reduced activities of microsomal drug-metabolizing enzymes when compared with Donryu rat livers.[1–4] Although the cumulative effect of the quantitative differences at several steps of metabolic activation of carcinogens may be decisive, we attempted to examine differences in susceptibility to hepatocarcinogenesis between these two strains of rats at a later stage than the steps of metabolic activation of 3′-Me-DAB.

The genetics of tumor susceptibility has been extensively studied in the mouse. Among the strains studied for

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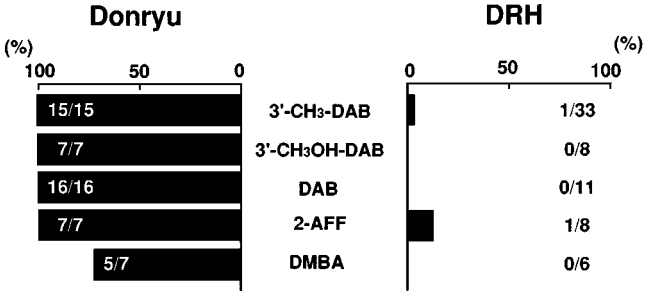

Fig. 1. Carcinogenic activities of 3′-CH_3-DAB, 3′-CH_2OH-DAB, DAB and 2-AAF in the livers of male, and of DMBA in the mammary glands of female carcinogen-sensitive Donryu and resistant DRH rats. Male rats were given diets containing 0.06% 3′-CH_3-DAB, 0.064% 3′-CH_2OH-DAB, 0.057% DAB or 0.06% 2-AAF from 4 weeks of age and killed after 20, 15, 60 and 16 weeks, respectively. DMBA (66.7 mg) was given intragastrically to female rats at 7 and 8 weeks of age. Rats were given basal diet and killed after 15 weeks. Tumors were observed macroscopically. The numbers in this figure show the number of tumor-bearing rats/total number of rats killed. Fig. 1 is taken from ref. 4, with permission.

induction of liver tumors, C3H/MeJ and CBA/J were the most susceptible to several hepatocarcinogens, whereas A/J, C57BL/6J and SWR/J were highly resistant to them.[5–7] Our knowledge of hepatocarcinogenesis in inbred strains of rats is much less than in the case of mice. However, the process of hepatocarcinogenesis between the administration of a carcinogen and the actual appearance of pathologically manifested neoplasm has been extensively studied in rats for many years.[8–10]

In the present work, we compared the expression of a variety of genes which might contribute to the different susceptibility to 3′-Me-DAB carcinogenic action between DRH and Donryu rats during long-term administration of 3′-Me-DAB. Unexpectedly, we found essentially similar patterns of DNA-adducts in DRH and Donryu rat livers. In spite of this similarity, Donryu rat livers were more sensitive to noxious effects of 3′-Me-DAB other than the formation of DNA adducts.

MATERIALS AND METHODS

Animals and treatments Male Donryu and inbred DRH rats (8-week-old) were purchased from Seac Yoshitomi, Ltd. (Fukuoka). They were fed on commercial rat chow (Clea, Tokyo) and tap water *ad libitum*, and were used one week after acclimation. These rats were then maintained on a diet containing 0.06% 3′-Me-DAB. Animals were killed at 1, 2, 3, 7, and 10 days and 2, 4, 6 and 8 weeks (3 rats at each time from both DRH and Donryu groups). All animal handling was performed in accordance with protocols approved by the Animal Use and Care Committee of University of Occupational and Environmental Health. Livers were perfused with ice-cold saline and aliquots were used in the following individual analysis. This series of experiments was repeated twice in the present study.

^{32}P-Postlabeling analysis DNA samples were prepared from the livers according to the method described by Wang *et al*.[11] DNA contents were calculated from the absorbance at 260 nm, based on 1 mg/ml DNA giving an A_{260} of 20.

The butanol extraction method was used for the analysis of DNA adducts.[12] For a single analysis, 5 μg of DNA was digested with micrococcal nuclease and spleen phosphodiesterase. Adducted nucleotides were selectively extracted from the hydrolysate with butanol. The extracted material was dried and taken up in a total volume of 2 μl of T4 polynucleotide kinase labeling mixture containing [γ-^{32}P]ATP. The labeled samples were spotted and developed on polyethyleneimine-cellulose thin layer chromatography plates (Macherey-Nagel, Postiach, Germany) using three solvent systems: D1, 1 *M* sodium phosphate, pH 6.0; D3, 3.6 *M* lithium formate, 8.5 *M* urea, pH 3.4; and D4, 0.8 *M* lithium chloride, 0.5 *M* Tris, 8.5 *M* urea, pH 8.0. DNA adducts were detected using a Bio-Image Analyzer (BAS 2000; Fuji Photo Film Co., Tokyo) after exposing the thin layer plates to the Fuji imaging plate.

Northern analysis Total RNA was extracted by the guanidine thiocyanate procedure.[13] Twenty micrograms of the total RNA, obtained by mixing equal amounts from 2–3 rats at the same time point to minimize individual variations, was electrophoresed in a 1.0% agarose gel and transferred onto nylon membrane (Hybond N, Amersham Pharmasia Biotech, Backinghamshire, UK). The membrane had been prehybridized in a solution containing 4× saline sodium citrate (SSC), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 20 μg/ml salmon sperm DNA.[14] The membrane was then hybridized with one of the following ^{32}P-labeled nick-translated probes: GADD45,[15] O^6-methylguanine methyltransferase (MGMT),[16] cytochrome P-450 2E1,[17] heme oxygenase,[18] hepatocyte growth factor (HGF),[19] glutathione S-transferase placental form (GST-P)[20] or γ-glutamyltranspeptidase (GGT).[21] The hybridization reaction was carried out at 42°C for 16–24 h. The membrane was washed in l× SSC containing 0.1% SDS at 42°C for 30 min, and this was repeated 2 or 3 times.[14] The radioactivity in the hybridized membrane was quantitated using the bio-imaging analyzer (BAS2000).

RESULTS

^{32}P-Postlabeling analysis of carcinogen-DNA adducts ^{32}P-Postlabeling analysis was carried out to see whether

the carcinogen-resistant DRH rat liver had a smaller amount of DNA adducts than Donryu rat liver during 3′-Me-DAB administration. Unexpectedly, essentially the same profiles of DNA adducts were observed in the liver DNAs from both strains of rats at 3 days, 2 weeks and 8 weeks after the start of 3′-Me-DAB administration (Fig. 2). The time courses of changes of individual spots were also roughly similar between the two strains under these conditions, although we did not identify the components of individual spots. For comparison, we tentatively regarded them as five major spots, as shown in Fig. 2B, and quantitated the radioactivities of the individual areas at 2 and 8 weeks (Table I). Although the intensities of some spots vary somewhat between DRH and Donryu, there are no significant differences of DNA-adducts which would be sufficient to explain the remarkable difference in tumor susceptibility between DRH and Donryu rats (Fig. 1).

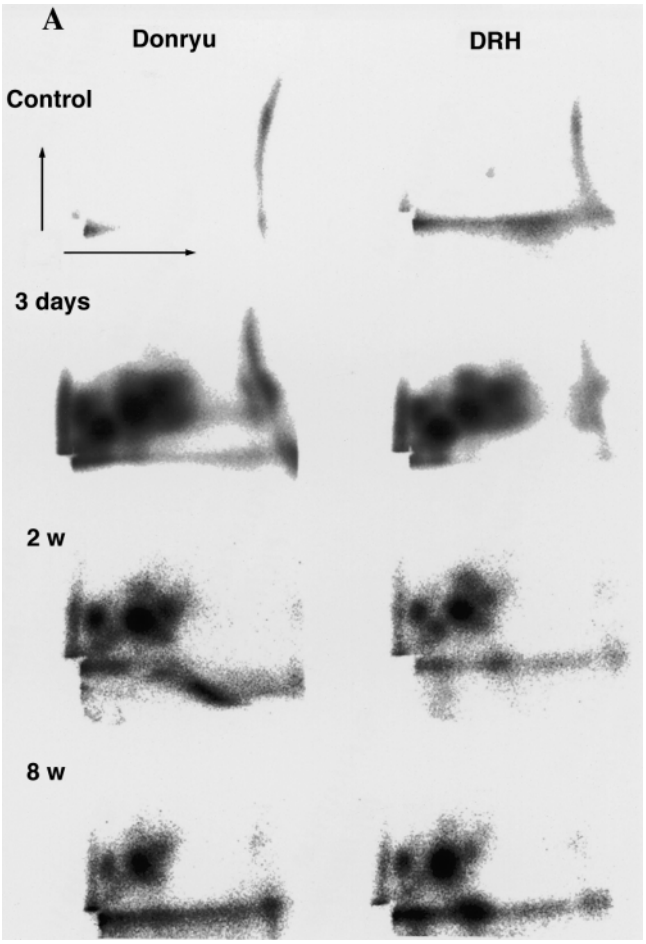

Fig. 2. ^{32}P-Postlabeling analysis of carcinogen-DNA adducts in hepatic DNA from Donryu and DRH rats during 3′-Me-DAB administration. (A) Autoradiograms of the spots developed by 2-dimensional thin-layer chromatography were obtained as described in "Materials and Methods." (B) The spots were tentatively separated into five areas and total radioactivities in the closed area were employed for calculation (see Table I).

Induction of DNA-damage-inducible genes The formation of DNA adducts is a necessary step for cellular transformation, but it is not sufficient for hepatocarcinogenesis. Some adducts are removed rapidly and others persist.[22, 23] To investigate subsequent changes in the hepatocytes after the formation of DNA adducts, we examined the behavior of DNA-damage-inducible genes by northern blot analysis. The levels of mRNA of GADD45, growth arrest and DNA-damage-inducible gene,[15, 24] increased significantly in Donryu rat liver, that is, to more than 20 times the basal levels, at around 6–8 weeks of 3′-Me-DAB administration, while the GADD45 mRNA was hardly detectable in DRH rat liver under the same conditions (Fig. 3).

It was reported that MGMT activity was induced when rat cells were exposed to various DNA-damaging agents such as N-methyl-N′-nitro-N-nitrosoguanidine, ultraviolet light and γ-rays,[25, 26] although an increase in MGMT also occurred in rat liver after partial hepatectomy.[27] In the liver of Donryu rats, MGMT mRNA began to increase as early as the 3rd day of 3′-Me-DAB administration, reaching a level of 7-fold above the control level of untreated rat liver and it gradually increased further thereafter to approximately 15-fold above the control (Fig. 3). On the other hand, only slight increases of MGMT mRNA were observed at 7 to 14 days (lanes 5–7) in DRH rat liver.

Table I. Quantitation of Individual Spots of DNA Adducts

Spot	2 weeks		8 weeks	
	Donryu	DRH	Donryu	DRH
A	10.5[a)]	5.9	4.6	4.6
B	3.8	4.5	1.7	2.2
C	24.8	15.6	12.0	20.1
D	4.1	4.2	2.2	2.1
E	9.4	4.7	3.7	3.6

a) The intensity of each spot is represented in an arbitrary unit. The analyses at 2 and 8 weeks were carried out with the same specific radioactivity of ^{32}P-ATP.

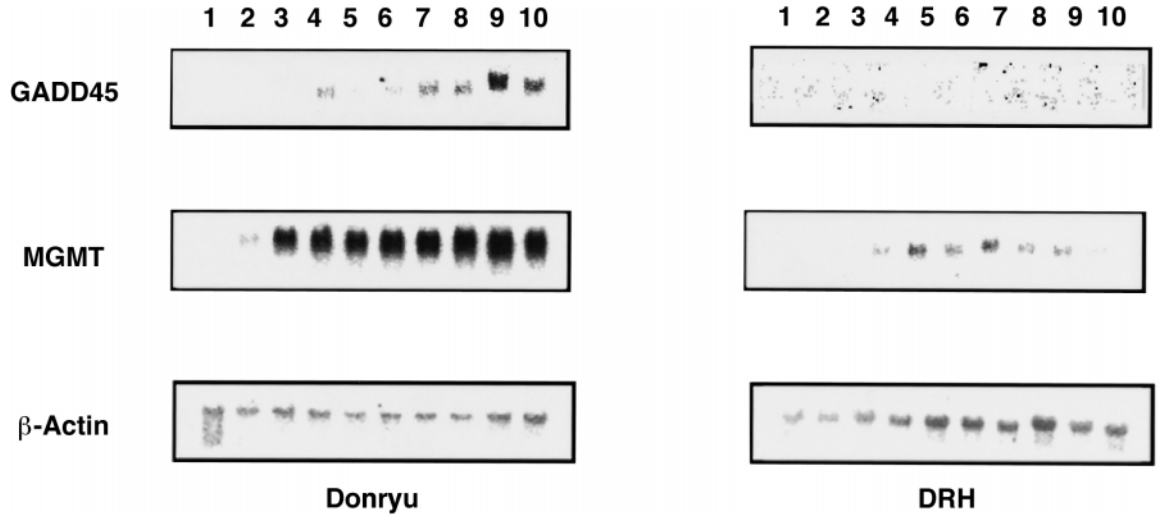

Fig. 3. Induction of GADD45 and O^6-methylguanine-DNA methyltransferase genes in rat liver during 3′-Me-DAB administration. Total RNA (mixtures of RNA from 2 or 3 rats at the same time point; lanes 1 to 10 correspond to 0, 1, 2, 3, 7, 10 days, 2, 4, 6 and 8 weeks, respectively) were analyzed as described in "Materials and Methods."

Incorporation of [^{3}H]thymidine into hepatic DNA in Donryu rats was clearly increased during 10 to 20 days after the start of 3′-Me-DAB administration, probably due to DNA repair. However, no significant change was observed in DRH rat liver under the same conditions (Fig. 4).

Cytotoxic effects of 3′-Me-DAB administration To obtain clues to the significance of the different responses between these two strains of rats, we examined the cytotoxic effects of 3′-Me-DAB other than the direct effects of DNA damage, although some of them might occur indirectly due to the DNA damage in the nucleus.

Previously, we have shown that total content of microsomal cytochrome P-450s in Donryu rat liver gradually declined with time during the long-term administration of 3′-Me-DAB, while the change was smaller in DRH.[28] In the present study, the induction of heme oxygenase mRNA, probably due to the degradation of intracellular heme proteins[18] and also oxidative stress in the cells,[29] was apparently greater in Donryu rat liver (7 times the control) than in DRH rat liver (Fig. 5).

Furthermore, marked induction of HGF mRNA (10 times the control) at around 6 weeks (lane 9) in Donryu rat liver indicates severe damage to hepatocytes[30] and compensatory regeneration of them,[19] but the induction of HGF mRNA was hardly detectable in DRH rat liver under the same conditions (Fig. 5, right lower panel).

Another interesting finding was the significant induction of cytochrome P-450 2E1 (CYP 2E1) mRNA in the carcinogen-sensitive Donryu rat liver as compared with DRH rat liver (Fig. 5, left upper panel). This CYP 2E1 is thought to contribute to the production of reactive oxygen intermediates and may cause oxidative injury to intracellular macromolecules.[31]

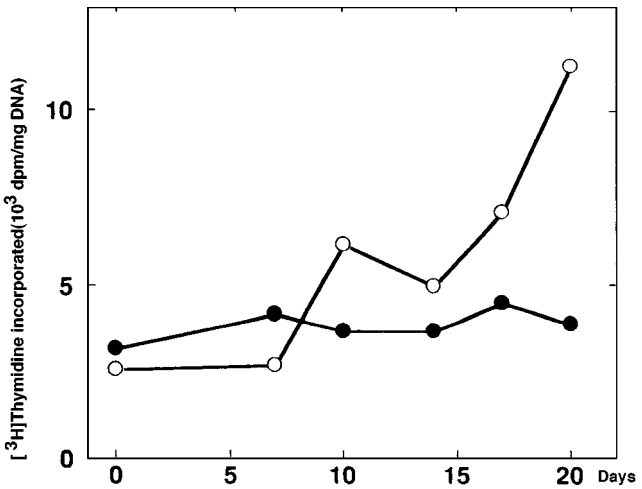

Fig. 4. Time-course of [^{3}H]thymidine incorporation into hepatic DNA of Donryu and DRH rats during 3′-Me-DAB administration. Two male rats (8 weeks of age) were i. p. injected with 50 μCi of [^{3}H]thymidine and killed after 3 h for each time point during 3′-Me-DAB administration. Means of specific radioactivities from two rats are shown for each time point. Open circles show Donryu and closed circles show DRH rats.

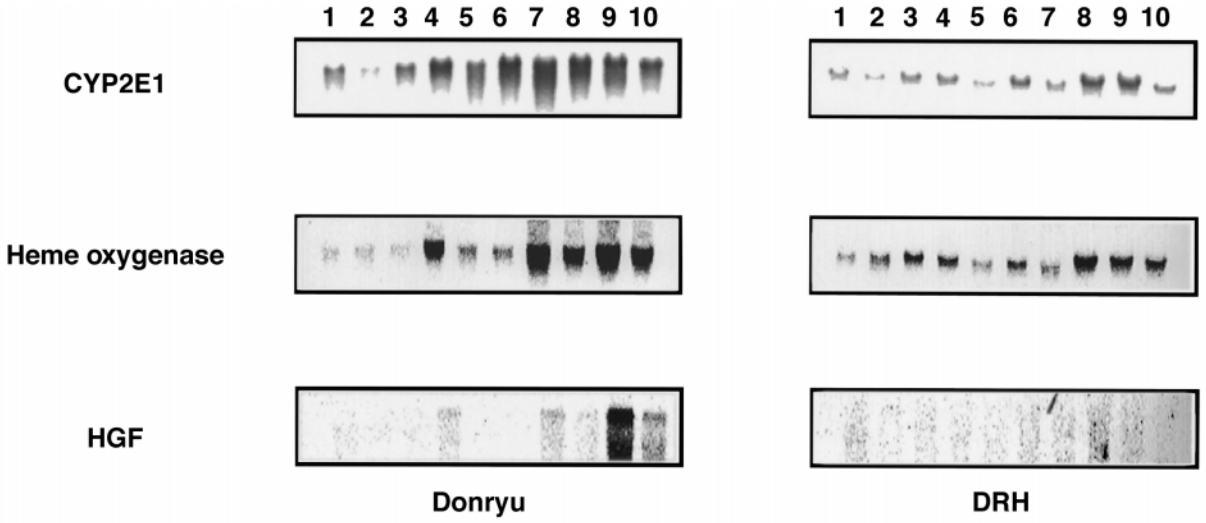

Fig. 5. Alterations of cellular responses during 3′-Me-DAB administration. Total RNAs were obtained from either Donryu or DRH rats at the time points indicated in Fig. 3 during 3′-Me-DAB administration and analyzed by northern blot analysis using probes for cytochrome P-450 2E1 (CYP 2E1), heme oxygenase and HGF (hepatocyte growth factor) as described in "Materials and Methods."

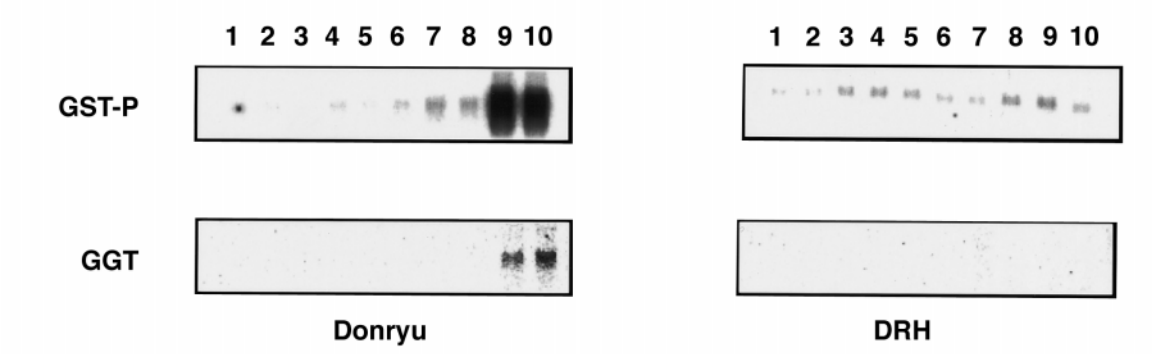

Fig. 6. Induction of mRNAs of marker enzymes for putatively preneoplastic lesions during 3′-Me-DAB administration. RNA samples were prepared and electrophoresed in individual lanes as described in Fig. 3. Lanes 9 and 10 are 6 and 8 weeks after the start of 3′-Me-DAB administration, respectively.

Appearance of marker enzyme mRNAs for preneoplastic nodules The surface of Donryu rat liver became macroscopically rough after 6 to 8 weeks of 3′-Me-DAB administration, while that of DRH rat liver remained smooth throughout the 8 weeks. GST-P form and GGT are marker enzymes for foci showing altered enzyme expression, which represent preneoplastic changes, during hepatocarcinogenesis.[32, 33] The livers from Donryu rats after 6 to 8 weeks of 3′-Me-DAB administration showed more than 50 fold induction of GST-P mRNA above the control level, while only 2–3 fold induction was observed in DRH rat liver at around 6 weeks (Fig. 6). A similar difference of inducibility was observed in the case of GGT (Fig. 6).

DISCUSSION

Previous studies on metabolic activation of mainly 3′-Me-DAB in the livers of DRH and Donryu rats showed lower activities of cytochrome P-450s and hydroxysteroid sulfotransferase in DRH rat liver compared with Donryu rat liver.[1–4] On the other hand, the contents of detoxifica-

tion enzymes such as uridine diphosphoglucuronosyltransferase and GST activities were greater in DRH rat liver than in Donryu rat liver.[4] These changes would lower the susceptibility of carcinogen-resistant DRH rats to the carcinogenic action of 3′-Me-DAB, and probably to that of other carcinogens as well. In spite of these findings, we observed essentially similar profiles of 3′-Me-DAB DNA adducts in the livers of both DRH and Donryu rats as early as 3 days after the start of a diet containing 0.06% 3′-Me-DAB (Fig. 2). Therefore, we focused our attention in the present study on events after the formation of carcinogen-DNA adducts.

The major carcinogen-DNA adducts formed in the liver by the hepatocarcinogenic aminoazo dye N-methyl-4-aminoazobenzene were identified by Tullis *et al.*[23] Among them, 3-(deoxyguanosin-N^2-yl)-MAB appeared to be a relatively persistent adduct, while N-(deoxyguanosin-8-yl)-MAB disappeared from hepatic DNA rather rapidly.[23] In the present study, both DRH and Donryu rats were exposed to 3′-Me-DAB continuously throughout 8 weeks. Although we did not identify the components of individual spots in ^{32}P-postlabeling analysis, the total profiles of the modified nucleotides were somewhat changed at different time points, but showed essentially the same time course for the hepatic DNA from both DRH and Donryu rats (Fig. 2). These results indicate that there was no significant difference in the repair systems for these carcinogen-DNA adducts and no significant dilution by cell proliferation in the livers of both rat strains. It is not known, however, which DNA adduct plays a critical role in the initiation of hepatocarcinogenesis.

In spite of the similarity of ^{32}P-postlabeled DNA adducts between Donryu and DRH rat livers, comparable doses of the carcinogen are more toxic and produce a greater response of DNA-damage-inducible genes such as GADD45 and MGMT in Donryu rats than in DRH rats (Fig. 3). Potter *et al*. reported an increase in the mRNA for alkyltransferase in rat liver in response to 2-AAF.[34] We can not rule out completely the possibility that apparent induction of MGMT in the present study is a secondary response to the cellular damage caused by 3′-Me-DAB. Subsequent to the formation of carcinogen-DNA adducts, some cell proliferation is needed for *fixation* of DNA alterations as *mutations*. Covalent carcinogen-DNA adducts can cause errors during replication, with base pair substitutions (point mutations) as a result. Repair excision of modified bases may similarly produce base pair substitution by faulty base insertion in the opposite DNA strand. The idea that mitogenesis increases mutagenesis helps to explain promotion and other aspects of carcinogenesis.[35–39] Our data showed that for the initial 20 days of 3′-Me-DAB administration, enhancement of [^{3}H]thymidine incorporation into the hepatocellular DNA was detectable only in Donryu rat liver, while little change was observed in DRH rat liver. Sneider *et al*.[40] reported increased DNA synthesis in rat liver during azo dye-induced hepatocarcinogenesis. The magnitude of [^{3}H]thymidine incorporation into hepatic DNA in the present study (Fig. 4) was significantly smaller than that of hepatocytes after partial hepatectomy, suggesting that it might be due to unscheduled DNA synthesis. However, we can not exclude the possibility that only a small population of liver cells is in S phase during the early period of azo dye-administration.

We can not reconcile the different responses between these two strains of rats with the apparent similarity in the levels of DNA adducts at present. However, there are several plausible explanations, as follows. i) DNA synthesis of hepatocytes in DRH is highly suppressed via a different mechanism from that of Donryu rats. ii) Hepatocytes in DRH are insensitive to the presence of covalent carcinogen-DNA adducts and are unable to induce some DNA-damage-inducible genes[41] even in the presence of DNA damage. iii) DNA repair might be coordinated with processes such as transcription.[42, 43] The DNA-adducts detected in the present study might be those remaining after removal of DNA adducts localized at physiologically important loci of DNA. Hepatocytes in DRH rats may not repair such “unimportant” adducts. Further studies are needed to clarify these possibilities.

It is likely that 3′-Me-DAB is a complete carcinogen, that is, it acts as both initiator and promoter. It is therefore possible that the differences between DRH and Donryu rat strains result from post-initiation events. Different sensitivity to the cytotoxic effects of 3′-Me-DAB, such as cell injury, inflammation and generation of reactive oxygen species[43, 44] may contribute to the different responses of hepatocytes exposed to 3′-Me-DAB, as reflected by the different inductions of heme oxygenase and HGF in the two strains of rat (Fig. 4), which may reflect different sensitivity to the non-genotoxic effects[44, 45] of 3′-Me-DAB. We should also consider the possible participation of CYP 2E1[31] as an inducer of oxidative stress at the promotion stage of hepatocarcinogenesis.[46]

Taken together, these results suggest that the different susceptibility to hepatocarcinogenesis between DRH and Donryu rats is mediated through events other than the formation of carcinogen-DNA adducts.

Hepatocarcinogenesis in inbred strains of mice has been extensively studied as a genetic model of tumor development. Both male DBA/2J and C3H/HeJ mice are highly susceptible to hepatocarcinogens such as N,N-diethylnitrosamine relative to male C57BL/6J mice.[5–7] The sensitivity loci for mouse hepatocarcinogenesis are the *Hcs* (hepatocarcinogen sensitivity) loci and the two major resistance loci are *Hcr-1* and *-2* (hepatocarcinogen resistance).[7] It is likely that the susceptibility to hepatocarcinogens is determined by the combined effects of these

multiple loci.[7] These genes are reported to influence greatly the promotion stage of hepatocarcinogenesis.

However, the genetic basis for the differences in susceptibility to hepatocarcinogens in rats is poorly understood at present. Two strains of rats resistant to liver preneoplasia were reported recently, that is, Copenhagen (Cop) rats[47] and Brown Norwegian (BN) rats,[48] although genetic linkage mapping of hepatocarcinogenesis resistance loci has not yet been carried out with these rats. It is interesting that most of the mechanisms for hepatocarcinogenesis resistance so far identified in both mice and rats, including DRH, are related to events downstream from the establishment of initiated cells by genotoxic agents. DRH rats should be useful as a rat genetic model to investigate the suppression of promotion in hepatocarcinogenesis.

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