TOXICOLOGICAL SCIENCES **73,** 287–293 (2003)
DOI: 10.1093/toxsci/kfg094

Hepatocellular Tumor Induction in Heterozygous *p53*-Deficient CBA Mice by a 26-Week Dietary Administration of Kojic Acid

Tamotsu Takizawa,*,† Kunitoshi Mitsumori,*,‡ Toru Tamura,* Masahiro Nasu,§ Makoto Ueda,* Toshio Imai,* and Masao Hirose*,[1]

*Division of Pathology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; †Department of Molecular and Environmental Pathology, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan; ‡Laboratory of Veterinary Pathology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan; and §Panapharm Laboratories Co., Ltd., 1285, Kurisaki-machi, Uto, Kumamoto 869-0425, Japan

Received November 5, 2002; accepted January 31, 2003

In order to evaluate the tumorigenic potential of kojic acid (KA), used as a food additive for preventing enzymatic browning of crustaceans and a cosmetic agent for the purpose of skin whitening, heterozygous *p53*-deficient CBA [*p53*(+/–)] mice, which are recognized as useful for detecting genotoxic carcinogens, and wild-type littermates [*p53*(+/+) mice] were fed diet containing 0, 1.5, and 3% KA for 26 weeks. KA induced diffuse hypertrophy and hyperplasia of thyroid follicular epithelial cells with decreased serum thyroxine levels in both *p53*(+/–) and *p53*(+/+) mice, but caused no thyroid tumors. In the liver, the incidence of altered hepatocellular foci was significantly increased at 1.5 and 3% in *p53*(+/–) and 1.5% in *p53*(+/+) mice, and that of hepatocellular adenomas was increased at 1.5 and 3% in *p53*(+/–) and 3% in *p53*(+/+) mice. *p53*(+/–) mice thus appeared to be more susceptible in terms of the tumorigenic dose of KA with a greater prevalence of hepatic proliferative lesions. The results of the present study indicate tumorigenic potential of KA in the liver, but not thyroid follicular epithelial cells in CBA mice and a contribution of genotoxicity on hepatocellular tumor development cannot be ruled out.

Key Words: **kojic acid; hepatocarcinogenesis; heterozygous *p53*-deficient mice.**

Kojic acid (KA; 5-hydroxy-2-(hydroxymethyl)-4-pyrone) is a secondary metabolic product of various species of *Aspergillus* and *Penicillium* (Kwak and Rhee, 1992; Parrish *et al*., 1966). It is known to inhibit polyphenol oxidase (tyrosinase) in mushrooms (Saruno *et al*., 1979), potatoes, apples, and crustaceans (Chen *et al*., 1991). Since polyphenol oxidase catalyzes the conversion of tyrosine to melanin via 3,4-dihydroxyphenylalanine and dopaquinone (Kahn, 1995; Saruno *et al*., 1979), KA has been used as an inhibitor of polyphenol oxidase in foods and as a food additive for preventing enzymatic browning of raw crabs and shrimps. In addition, because of its excellent skin-whitening properties (Ohyama, 1990; Perez-Bernal *et al*., 2000) and inhibitory action on human melanocyte tyrosinase (Maeda and Fukuda, 1991), KA has been used as cosmetic agent for a purpose of skin lightening.

Recently, thyroid follicular cell adenomas were found to develop in B6C3F1 mice fed diet containing 1.5 or 3% KA for 20 months (Fujimoto *et al*., 1998). Proliferative lesions of thyroid follicular cells were also increased in F344 rats fed diet containing 2 or 4 % KA for 12 weeks after initiation with N-*bis*(2-hydroxypropyl)nitrosamine [DHPN] (Mitsumori *et al*., 1999; Tamura *et al*., 1999b), associated with a decline in serum thyroxine (T4) and 3,5,3′-triiodothyronine (T3) and elevation of thyroid stimulating hormone (TSH) levels (Mitsumori *et al*., 1999). Moreover, it has been demonstrated that KA inhibits thyroid iodine uptake and its organification (Fujimoto *et al*., 1999; Tamura *et al*., 1999a). Based on the findings, prolonged TSH secretion from the pituitary in response to decreased circulating thyroid hormones due to the administration of KA is presumably responsible for the thyroid tumor induction, as established for other goitrogenic substances (Hill *et al*., 1998; McClain, 1992). On the other hand, female B6C3F1 mice receiving 3% KA in the diet were also found to develop hepatocellular tumors in the first experiment conducted by Fujimoto *et al.* (1998), but consistent interpretation or underlying mechanisms of liver tumorigenicity remain uncertain. A number of *in vitro* mutagenicity assays have been performed for KA and the Ames test using *Salmonella* strains revealed positive results in the presence and absence of rat S9-mix fraction (Shibuya *et al*., 1982; Wei *et al*., 1991). In addition, positive results were obtained for sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells (Wei *et al*., 1991), but not in the forward gene mutation assay in Chinese hamster lung V79 cells (Shibuya *et al*., 1982) *in vitro*. However, data for genotoxicity of KA *in vivo* are limited, and a negative result was obtained in a dominant lethal test (Shibuya *et al*., 1982). It has been reported that heterozygous *p53*-deficient mice, in which one allele of the *p53* gene is inactivated, derived from C57BL/6 or CBA origins provide a useful model to detect carcinogens, genotoxic car-

[1] To whom correspondence should be addressed. Fax: +81-3-3700-1425. E-mail: m-hirose@nihs.go.jp.

cinogens in particular, within a short-term treatment period of less than six months (Dunnick *et al*., 1997; Mitsumori *et al*., 2000; Ozaki *et al*., 1998). Spontaneous tumor development during short-term assays is generally rare although a low incidence of lymphomas and lung adenomas or adenocarcinomas is known in both strains of *p53* hemi-knockout mice (Mahler *et al*., 1998, Takizawa *et al*., 2001). A number of tissues or organs are counted as target for carcinogen in *p53*(+/–) mice. These include vascular tissue (Carmichael *et al*., 2000), lymphatic organs (Dunnick *et al*., 1997), urinary bladder (Ozaki *et al*., 1998; Tennant *et al*., 1996), uterus (Mitsumori *et al*., 2000), and liver (French *et al*., 2001). In contrast, *p53* hemi-knockout mice are reported to not respond to nongenotoxic carcinogens that can induce tumors in the liver or kidney in conventional long-term assays (Spalding *et al*., 2000). Heterozygous *p53*-deficient CBA mice were also reported to develop proliferative lesions in response to a single administration of N-dimethylnitrosamine (Onodera *et al*., 2001). It is possible that KA exerts carcinogenic potential via genotoxic effects in the liver or other organs, and the present study was performed using heterozygous *p53*-deficient CBA mice and wild-type littermates in order to further evaluate this question.

MATERIALS AND METHODS

Test substance. Kojic acid (KA) powder obtained from Nagase Biochemical Co. (Tokyo, Japan) was admixed into powdered basal diet (CRF-1®, Oriental Yeast Co., Tokyo, Japan) at 1.5 and 3%. The diet was prepared at least once biweekly and stored until use at 5°C.

Experimental animals. The mice used in the present study, heterozygous *p53*-deficient CBA mice [*p53*(+/–) mice] in which exon 2 of the lateral p53 allele was inactivated, were F1 offspring of heterozygous *p53*-deficient C57BL/6J male mice back-crossed with CBA female mice (Tsukada *et al*., 1993). Twenty-seven male *p53*(+/–) mice and 35 male wild-type littermates [*p53*(+/+) mice], six or seven weeks of age, were purchased from Oriental Yeast Co. and used after an acclimatization period of one week. They were housed at a maximum of five per cage in plastic cages with soft chip bedding in a room that was controlled for light-dark cycle (12–12 h, lights on 0700–1900 h), ventilation (air-exchange rate of 18 times per h), temperature (21–25°C), and relative humidity (50–60%). The cages and the chip bedding were exchanged for new ones twice a week. Each animal had free access to basal diet and tap water. The experiment was carried out in accordance with the Guide for Animal Experimentation of the National Institute of Health Sciences of Japan.

Experimental design. *p53*(+/–) mice were randomly allocated into one group of seven animals and two groups of 10 animals based on their body weights. *p53*(+/+) mice were allocated in a similar manner into three groups consisting of 10, 12, and 13 animals. Groups of *p53*(+/–) and *p53*(+/+) mice were fed the diet containing 1.5 or 3% KA for 26 weeks, and the remaining group, serving as a control, was fed basal diet for the same period. During the study period, the animals were observed once a day for their general condition, and weighed once weekly.

At the end of the treatment period, surviving animals were sacrificed after blood sampling for hormone assays under deep ether anesthesia, and autopsied. Livers and thyroids were dissected out and their weights were recorded. These organs and the pituitary, spleen, lungs, and other tissues or organs with macroscopic lesions were fixed in 10% neutral buffered formalin. Next, all lobes of the liver and other organs and tissues were processed routinely, embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin for histopathological examination. In addition, tissue sections were immunohistochemically stained for proliferating cell nuclear antigen (PCNA) using an anti-PCNA mouse monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1:100 with a blocking reagent (Vector MOM™, Vector Labs, Inc., Burlingame, CA) and an avidin-biotin peroxidase complex kit (DAKO) with 3, 3′-diaminobenzidine as the chromogen followed by counterstaining with hematoxylin. Five thousand hepatocellular nuclei in normal background parenchyma in each animal were counted for determination of PCNA positivity.

Hormone assays. Blood samples were obtained from the abdominal aorta for determination of serum T3, T4, and TSH levels. For this purpose, coat-A-Count Canine T3 and DPC total T4 assay kits (Diagnostic Products Corporation, Los Angeles, CA) and a rat thyroid stimulating hormone [^{125}I] assay system (Amersham Pharmacis Biotech, Buckinghamshire, U.K.) were employed.

Statistical analysis. Body weights, organ weights, serum hormone levels, and multiplicities of proliferative lesions were analyzed by one-way analysis of variance for homogeneity followed by Dunnett's test for comparison with the 0% KA control group. PCNA-positivity was analyzed by a nonparametric Dunnett's type test. Incidences of proliferative lesions observed were analyzed with the Fisher's exact test. The dose-response-relationship was analyzed with the Cochran Armitage test for histopathological findings and by Jonckheer's trend test for other data. Incidences of histopathological findings and PCNA-positivity were compared between *p53*(+/–) and *p53*(+/+) mice using the Fischer's exact test and Wilcoxson rank-sum test, respectively. Significance was inferred at either 5 or 1% levels.

RESULTS

In-Life Examination and Organ Weights

One *p53*(+/+) male receiving 3% KA was found dead at week 13. The 3% KA groups of both *p53*(+/–) and *p53*(+/+) mice showed reduction in body weight gain as compared with the 0% KA control groups (Fig. 1), terminal body weights being 8 and 15% lower, respectively with statistical significance at $p < 0.05$ and $p < 0.01$. In contrast, absolute thyroid weights were significantly ($p < 0.01$) increased in a dose related fashion by 209 and 444% in the 1.5 and 3% KA groups, respectively, in *p53*(+/–) mice, and by 140 and 374% in *p53*(+/+) mice (Table 1). Absolute and relative liver weights in the KA-treated groups showed somewhat higher values in both *p53*(+/–) and *p53*(+/+) mice than in the respective control groups, but the difference was not significant except for the relative weight in the 3% *p53*(+/+) mice.

Serum Hormone Levels

Serum T3 levels were not altered by KA treatment, but serum T4 levels declined dose dependently by 35 and 58% in the 1.5 and 3% KA groups of *p53*(+/–) mice, respectively, and by 50 and 65% in *p53*(+/+) mice with statistical significance ($p < 0.01$) in all KA-treated groups (Fig. 2). Serum TSH level was significantly ($p < 0.05$) elevated in the 1.5% group of *p53*(+/–) mice, but no significant alterations were observed in other KA-treated *p53*(+/–) or *p53*(+/+) mice.

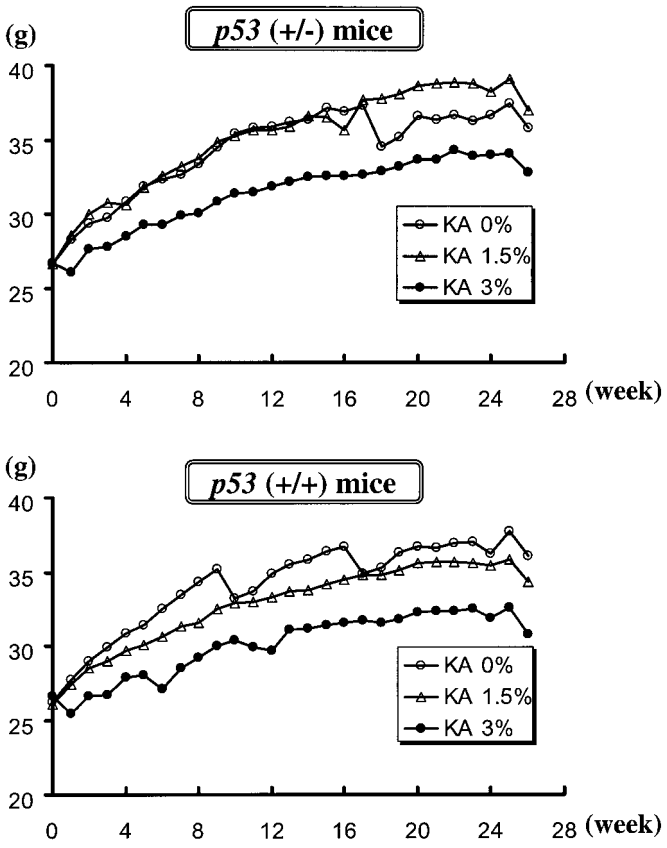

FIG. 1. Body weight changes of *p53*(+/–) and *p53*(+/+) mice fed diet containing KA for 26 weeks

Histopathology

Histopathological examination revealed changes attributable to the KA treatment in the thyroid and liver. In the thyroid, diffuse hypertrophy and hyperplasia of the follicular epithelial cells accompanied by increase in cytoplasmic colloid-like droplets were observed in all KA-treated *p53*(+/–) and *p53*(+/+) mice (Figs. 3, 4). There were no benign or malignant neoplasms of the thyroid in any groups. In the liver, hepatocellular adenomas as well as altered hepatocellular foci of eosinophilic cell-, clear cell-, and/or mixed cell-types were observed in the 1.5 and 3% KA groups of both *p53*(+/–) and *p53*(+/+) mice (Fig. 5). The incidences of hepatic tumors were significantly increased in both 1.5% ($p < 0.01$) and 3% ($p < 0.05$) groups of *p53*(+/–) mice, while those of *p53*(+/+) mice were significantly ($p < 0.05$) increased only in the 3% group (Table 2). When compared for percent incidences of hepatic proliferative changes, the *p53*(+/–) mice showed greater prevalence than wild-type mice, and the difference was significant for adenomas in the 1.5% group and altered foci in the 3% group. As nonproliferative lesions in the liver, focal hepatocellular necrosis and inflammatory cell infiltration appeared to be enhanced in the 1.5 and 3% groups of *p53*(+/–) and *p53*(+/+) mice (Table 2). The animals with necrotic changes in the liver showed elevated PCNA expression in hepatocytes of background parenchyma, and the average of PCNA-positivity in the 3% KA group of *p*53(+/–) mice was significantly higher than that in the 0% group (Table 3). In *p53*(+/+) mice, effects of the compound were masked by the strong increase in PCNA-positive nuclei in animals showing hepatic necrosis. There were no remarkable findings that could be attributed to the KA-treatment in any of the other tissues and organs examined.

DISCUSSION

Tumorigenic activity of KA in the thyroids of B6C3F1 mice was earlier demonstrated after dietary treatment for 20 months (Fujimoto *et al*., 1998), while no thyroid tumors were found in *p53*(+/–) or *p53*(+/+) CBA mice in the present experiment, despite the high susceptibility of heterozygous *p53*-inactivated mice to genotoxic carcinogens (Mitsumori *et al*., 2000; Tennant *et al*., 1995, 1996). This might be due to variation in the strain of mice used and duration of the administration period.

TABLE 1
Terminal Body, Thyroid, and Liver Weights for *p53* (+/–) and *p53* (+/+) Mice Fed Diet Containing Kojic Acid (KA) for 26 Weeks

Group	No. of animals (g)	Body weight	Thyroid weight		Liver weight	
			mg	mg/100g bw	g	g/100g bw
p53 (+/–) mice						
0% KA	7	35.8 ± 5.3	4.3 ± 1.4++	12.0 ± 4.8++	1.55 ± 0.18	4.39 ± 0.81++
1.5% KA	10	37.0 ± 2.8	13.3 ± 2.3**	36.2 ± 7.1**	2.19 ± 1.00	5.95 ± 2.83
3% KA	10	32.8 ± 2.5*	23.4 ± 2.8**	71.6 ± 8.5**	1.75 ± 0.19	5.33 ± 0.21
p53 (+/+) mice						
0% KA	10	36.1 ± 3.5++	4.7 ± 1.1++	13.1 ± 2.8++	1.47 ± 0.15++	4.10 ± 0.63++
1.5% KA	12	34.3 ± 4.2	11.3 ± 3.6**	33.2 ± 9.8**	1.72 ± 0.54	4.98 ± 1.36
3% KA	12	30.8 ± 1.9**	22.3 ± 4.5**	73.0 ± 17.0**	1.70 ± 0.13	5.53 ± 0.30**

++Statistically significant at $p < 0.01$ in dose proportionality analysis.
*,**Significantly different from the 0% KA group at $p < 0.05$, $p < 0.01$, respectively.

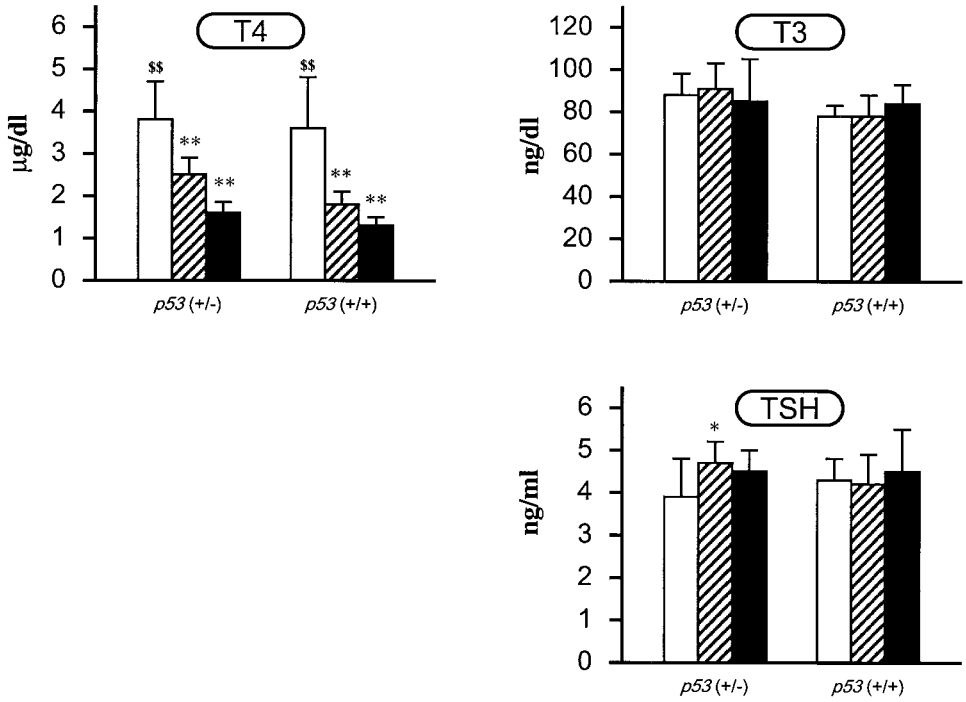

FIG. 2. Serum T4, T3 and TSH levels in p53(+/–) and *p53*(+/+) mice fed diet containing KA for 26 weeks. $$$p < 0.01$ in analysis of dose proportionality. $^{**}p < 0.01$ as compared with the 0% KA group. Open bar: 0% KA; hatched bar: 1.5% KA; black bar: 3% KA.

However, the KA-treated CBA mice showed diffuse hypertrophy and hyperplasia of thyroid follicular cells, which were typical histopathological features associated with goitrogenic substances in rodents (Capen, 1996; Gopinath *et al*., 1987), as reported in B6C3F1 mice. KA may thus exert tumorigenic potential in the thyroids of *p53*(+/–) and *p53*(+/+) CBA mice with more prolonged exposure.

In the present experiment, serum T4 levels in mice receiving KA were reduced in a dose-related manner, but dose-proportional effects on T3 or TSH were not observed. Similar inconsistent alteration of thyroid hormones or TSH was also reported in F344 rats receiving KA at dietary concentrations up to 2% for 20 weeks (Tamura *et al*., 2001) and B6C3F1 mice given 1.5 or 3% KA for 20 months (Fujimoto *et al*., 1998). Moreover, in our previous study in rats, administration of sulfadimethoxine

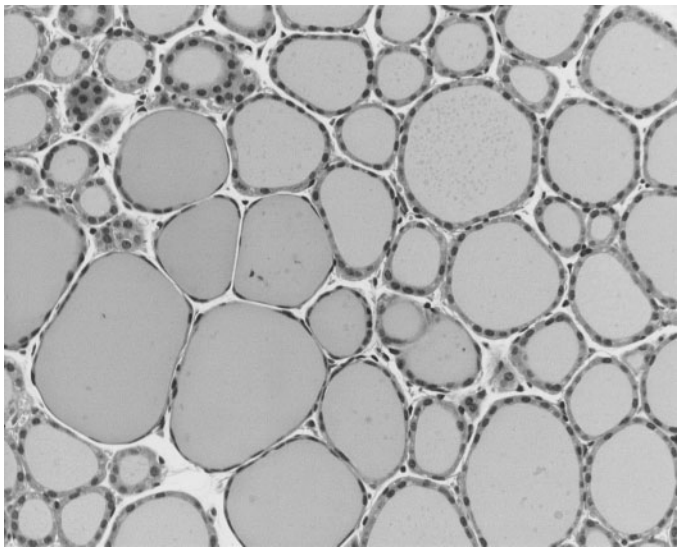

FIG. 3. Thyroid of a *p53*(+/–) mouse fed diet containing 0% KA for 26 weeks; various sizes of round follicles with flattened to cuboidal epithelial cells are visible, H.E. staining, ×13.

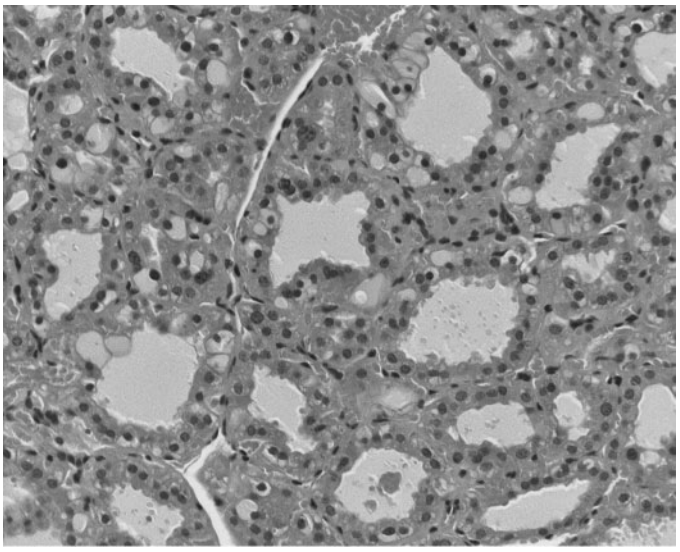

FIG. 4. Thyroid of a *p53*(+/–) mouse fed diet containing 3% KA for 26 weeks; irregular-shape follicles with columnar epithelial cells and reduced colloid are visible. Several follicular cells contain large amounts of colloid-like substance. H.E. staining, ×13

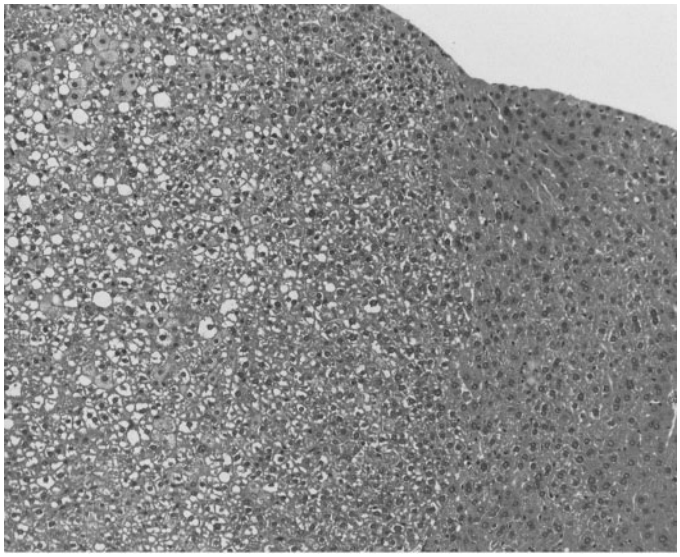

FIG. 5. Liver of a *p53*(+/–) mouse fed diet containing 3% KA for 26 weeks; a hepatocellular adenoma protruded above the surface is visible. H.E. staining, ×6.6.

TABLE 3
PCNA Positivity for Hepatocytes in Background Parenchyma of the Liver of *p53* (+/–) and *p53* (+/+) Mice

Group	No. of animals	No of PCNA-positive cells in 5000 hepatocytes
p53 (+/–) mice		
0% KA	7	1.71 ± 1.11+
1.5% KA	10	3.70 ± 3.50
3% KA	10	5.00 ± 3.59*
p53 (+/+) mice		
0% KA	10	6.40 ± 14.06
1.5% KA	12	4.42 ± 4.93
3% KA	12	9.00 ± 10.58

Note. Mice were fed diet containing kojic acid for 26 weeks. Data are mean ± SD values. KA, kojic acid.
+Statistically significant at $p < 0.05$ in dose proportionality analysis.
*Significantly different from the 0% KA group at $p < 0.05$.

or thiourea, a goitrogenic anti-thyroidal compound, was associated with a reduction of thyroid hormones and elevation of TSH after a one-week treatment but no apparent alteration of T3, T4, or TSH was evident at 19 weeks (Onodera *et al*., 1994). Hood *et al.* (1999) also reported only transient alterations of serum T3, T4, and/or TSH levels on prolonged administration to rats of phenobarbital, propylthiouracil or pregnenolone-16α-carbonitrile. These findings suggest that hormonal desensitization may be induced by prolonged anti-thyroidal treatments (Shimo *et al*., 1994; Wynford-Thomas *et al*., 1982). KA is reported to interfere with thyroid iodine uptake and its organification (Fujimoto *et al*., 1999; Tamura *et al*., 1999a) but not elicit any changes in the activity of hepatic uridine diphosphate glucuronosyl transferase or histopathological hypertrophy or swelling of hepatocytes in F344 rats (Mitsumori *et al*., 1999). There was also no hepatocellular hypertrophy in the present study, although the KA-treated animals showed somewhat

TABLE 2
Incidences (Percent Incidence) of Histopathological Findings and Multiplicities of Proliferative Lesions in the Liver for *p53* (+/–) and *p53* (+/+) Mice Fed Diet Containing Kojic Acid (KA) for 26 Weeks

	Incidence (percent incidence) of histopathological findings					
	p53 (+/–) mice			*p53* (+/+) mice		
Group/(no. of animals)	0% KA (7)	1.5% KA (10)	3% KA (10)	0% KA (10)	1.5% KA (12)	3% KA (12)
Thyroid						
Diffuse follicular cell hypertrophy	0++ (0)	10** (100)	10**(100)	0++ (0)	12** (100)	12** (100)
Follicular cell hyperplasia	0++ (0)	10** (100)	10** (100)	0++ (0)	12** (100)	12** (100)
Liver						
Hepatocellular adenoma	0 (0)	7**# (70)	5* (50)	0+ (0)	2 (17)	5* (42)
Altered hepatocellular foci	0++ (0)	5* (50)	8**# (80)	0 (0)	5* (42)	2 (17)
Focal necrosis of hepatocytes	0 (0)	3 (30)	2 (20)	1 (10)	1 (8)	3 (25)
Inflammatory cell infiltration	0 (0)	2 (20)	1 (10)	2 (20)	2 (17)	3 (25)
	Multiplicity of hepatic proliferative lesions[a]					
Liver						
Hepatocellular adenoma		1.2 ± 1.1	0.6 ± 0.7		0.2 ± 0.4	0.7 ± 0.9
Altered hepatocellular foci		0.9 ± 1.1	0.9 ± 0.6		0.5 ± 0.7	0.2 ± 0.4

[a]Values indicate the mean ± SD.
+,++Statistically significant at $p < 0.05$ and $p < 0.01$, respectively in dose proportionality analysis.
*,**Significantly different from the 0% KA group at $p < 0.05$, $p < 0.01$, respectively.
#Significantly different from *p53*(+/+) mice at $p < 0.05$.

elevated liver weights. Considering the results, KA might exert goitrogenic action via hormonal mechanisms in *p53*(+/–) and *p53*(+/+) mice of CBA-background, as observed in B6C3F1 mice and F344 rats. The fact that KA failed to form DNA adducts in the thyroid glands of rats by dietary feeding at 2% (unpublished data) supports our inference.

Hepatocellular adenomas as well as altered hepatocellular foci were observed in KA-treated groups not only in *p53*(+/–) mice but also in their wild-type littermates. Since no spontaneous hepatocellular proliferative lesions were observed in control animals in line with the previous 26-week studies (Mitsumori *et al*., 2000; Onodera *et al*., 2001; Takizawa *et al*., 2001), these proliferative lesions in the liver could be attributed to the treatment with KA. The fact that focal hepatocellular necrosis and inflammatory cell infiltration were enhanced in the 1.5 and 3% KA groups suggests a hepatotoxic potential of KA. The elevated proliferation indices for hepatocytes were roughly parallel the occurrence of necrotic lesions both in *p*53(+/–) and *p*53(+/+) mice, and might make the large variability in the *p53*(+/+) mice. Although the effects of KA were masked by the relatively variable control level of the 0% KA group in *p53*(+/+) mice, the elevated proliferation index in the 3% group of *p53*(+/–) mice might also be indicative of the hepatic regeneration. In the 20-month carcinogenicity study conducted by Fujimoto *et al.* (1998), a slight (10%) but significant increase in the incidence of hepatocellular carcinomas was observed in female B6C3F1 (C57BL × C3H/He) mice receiving 3% KA in the diet, but no hepatic disorders were observed on histopathological examination as well as serum biochemistry. The findings suggest a high susceptibility of CBA-background mice particularly *p53*(+/–) mice to hepatotoxicity of KA, and thus associated secondary cell proliferation might influence the induction of hepatocellular tumors. In addition, significant tumorigenic dose was lowered and the prevalence of hepatic proliferative lesions was higher in the *p53*(+/–) mice as compared to their wild-type counterparts. In particular, incidences of hepatic tumors at a dose of 1.5% and altered foci at a dose of 3% KA in *p53*(+/–) mice were significantly higher than in *p53*(+/+) mice. Since *p53*(+/–) mice are sensitive to genotoxic carcinogens (Mitsumori *et al*., 2000; Tennant *et al*., 1995, 1996), the possibility that KA exerts carcinogenic action through genotoxicity cannot be ruled out. With respect to the genotoxicity of KA *in vitro*, there have been positive results reported in the reverse gene mutation assay (Ames test) using microorganisms in the presence or absence of rat S-9 mix fraction (Shibuya *et al*., 1982; Wei *et al*., 1991). KA was also reported to induce DNA breaks and a clastogenic effect in cultured rat liver cells (Kinoshita *et al*., 1968; Stark, 1980) as well as sister chromatid exchange and chromosomal aberration in Chinese hamster ovary cells (Wei *et al*., 1991). However, KA failed to induce positive response in dominant lethal test *in vivo*. From these facts, mutagenic potential of KA *in vitro* is indicated, but *in vivo* genotoxicity and mechanisms of action of liver tumorigenicity remain to be clarified. Further investigations focusing on induction or promotion activity of KA in the liver as well as *in vivo* genotoxicity toward hepatocytes need to be performed.

ACKNOWLEDGMENT

This work was supported by a Grant-Aid from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

Capen, C. C. (1996). Hormonal imbalances and mechanisms of chemical injury of thyroid gland. In *Monographs on Pathology of Laboratory Animals; Endocrine System,* 2nd ed. (T. C. Jones, C. C. Capen, and U. Mohr, Eds.), pp. 217–238. Springer-Verlag, Berlin.

Carmichael, N. G., Debruyne, E. L. M., and Bigot-Lasserre, D. (2000). The p53 heterozygous knockout mouse as a model for chemical carcinogenesis in vascular tissue. *Environ. Health Perspect.* **108,** 61–65.

Chen, J. S., Wei, C. I., Rolle, R. S., Otwell. W. S., Balaban, M. O., and Marshall, M. R. (1991). Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidase. *J. Agr. Food Chem.* **39,** 1396–1401.

Dunnick, J. K., Hardisty, J. F., Herbert, R. A., Seely, J. C., Furedi-Machacek, E. M., Foley, J. F., Lacks, G. D., Stasiewicz, S., and French, J. E. (1997). Phenolphthalein induces thymic lymphomas accompanied by loss of the p53 wild type allele in heterozygous *p53*-deficient (+/–) mice. *Toxicol. Pathol.* **25,** 533–540.

French, J. E., Lacks, G. D., Trempus, C., Dunnick, J. K., Foley, J., Mahler, J., Tice, R. R., and Tennant R. W. (2001). Loss of heterozygosity frequency at the Trp53 locus in p53-deficient (+/–) mouse tumors is carcinogen- and tissue-dependent. *Carcinogenesis* **21,** 99–106.

Fujimoto, N., Onodera, H., Mitsumori, K., Tamura, T., Maruyama, S., and Ito, A. (1999). Changes in thyroid function during development of thyroid hyperplasia induced by kojic acid in F344 rats. *Carcinogenesis* **20,** 1567–1571.

Fujimoto, N., Watanabe, H., Nakatani, T., Roy, G., and Ito, A. (1998). Induction of thyroid tumors in (C57BL/6N × C3H/N)F1 mice by oral administration of kojic acid. *Food Chem. Toxicol.* **36,** 697–703.

Gopinath, C., Prentice, D. E., and Lewis, D. J. (1987). The endocrine glands. In *Atlas of Experimental Toxicological Pathology* (C. Gopinath, D. E. Prentice, and D. J. Lewis, Eds.), pp. 104–121. MTP Press Limited, Lancaster.

Hill, R. N., Crisp, T. M., Hurley P. M., Rosenthal S. L., and Singh D. V. (1998). Risk assessment of thyroid follicular cell tumors. *Environ. Health Perspect.* **106,** 447–457.

Hood, A., Liu, J., and Klaassen, C. (1999). Effects of phenobarbital, pregnenolone-16α-carbinitrile, and propylthiouracil on thyroid follicular cell proliferation. *Toxicol. Sci.* **50,** 45–53.

Kahn, V. (1995). Effect of kojic acid on the oxidation of DL-DOPA, norepinephrine and dopamine by mushroom tyrosinase. *Pigment Cell Res.* **8,** 234–240.

Kinoshita, R., Ishino, T., Sugiyama, S., Seto, T., Igarashi, S., and Goetz, I. E. (1968). Mycotoxins in fermented food. *Cancer Res.* **28,** 2296–2311.

Kwak, M. Y., and Rhee, J. S. (1992). Cultivation characteristics of immobilized *Aspergillus oryzae* for kojic acid production. *Biotechnol. Bioeng.* **39,** 903–906.

Maeda, K., and Fukuda, M. (1991). *In vitro* effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosm. Chem.* **42,** 361–368.

Mahler, J. F., Flagler, N. D., Malarjet, D. E., Mann, P. C., Haseman, J. K., and Eastin, W. (1998). Spontaneous and chemically induced proliferative lesions

in Tg.Ac transgenic and p53-heterozygous mice. *Toxicol. Pathol.* **26,** 501–511.

McClain, R. M. (1992). Thyroid gland neoplasia: Non-genotoxic mechanisms. *Toxicol. Lett.* **64/65,** 397–408.

Mitsumori, K., Onodera, H., Shimo, T., Yasuhara, K., Takagi, H., Koujitani, T., Hirose, M., Maruyama, C., and Wakana, S. (2000). Rapid induction of uterine tumors with *p53* point mutations in heterozygous *p53*-deficient CBA mice given a single intraperitoneal administration of N-ethyl-N-nitrosourea. *Carcinogenesis* **21,** 1039–1042.

Mitsumori, K., Onodera, H., Takahashi, M., Funakoshi, T., Tamura, T., Yasuhara, T., Takegawa, K., and Takahashi, M. (1999). Promoting effects of kojic acid due to serum TSH elevation resulting from reduced serum thyroid hormone levels on development of thyroid proliferative lesions in rats initiated with N-*bis*(2-hydroxypropyl)nitrosamine. *Carcinogenesis* **20,** 173–176.

Ohyama, Y. (1990). Melanogenesis-inhibitory effect of kojic acid and its action mechanism. *Fragrance J.* **6,** 53–58.

Onodera, H., Mitsumori, K., Takagi, H., Yasuhara, K., Koujitani, T., Tamura, T., and Hirose, M. (2001). Susceptibility of liver proliferative lesions in heterozygous *p53* deficient CBA mice treated with Phenobarbital after initiation of dimethylnitrosamine. *J. Toxicol. Pathol.* **14,** 273–278.

Onodera, H., Mitsumori, K., Takahashi, K., Shimo, T., Yasuhara, K., Kitaura, K., Takahashi, M., and Hayashi, Y. (1994). Thyroid proliferative lesions induced by anti-thyroid drugs in rats are not always accompanied by sustained increase in serum TSH. *Toxicol. Sci.* **19,** 227–234.

Ozaki, K., Sukata, T., Yamamoto, S., Uwagawa, S., Seki, T., Kawasaki, H., Yoshitake, A., Wanibuchi, H., Koide, A., Mori, Y., and Fukushima, S. (1998). High susceptibility of *p53* (+/–) knockout mice in N-butyl-N-(4-hydroxybuytl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in residual allele. *Cancer Res.* **58**(Suppl. 1), 3806–3811.

Parrish, F. W., Wiley, B. J., Simmons, E. G., and Long, Jr., L. (1966). Production of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium*. *Appl. Microbiol.* **14,** 136–139.

Perez-Bernal, A., Munoz-Perez, M. A., and Camacho, F. (2000) Management of facial hyperpigmentation. *Am. J. Clin. Dermatol.* **1,** 261–268.

Saruno, R., Kato, F., and Ikeno, T. (1979). Kojic acid, a tyrosinase inhibitor from *Aspergillus albus*. *Agric. Biol. Chem.* **43,** 1337–1338.

Shibuya, T., Murata, T., Sakamoto, K., Iwahara, S., and Ikeno, M. (1982). Mutagenicity and dominant lethal test of kojic acid. *Toxicol. Sci.* 7, 255–262.

Shimo, T., Mitsumori, K., Onodera, H., Takahashi, K., Funakoshi, T., Yasuhara, K., Takegawa, K., Takagi, H., and Hirose, M. (1994). Time course observation of thyroid proliferative lesions and serum TSH levels in rats treated with thiourea after DHPN-initiation. *Cancer Lett*. **85,** 141–149.

Spalding, J. W., French, J. E., Stasiewicz, S., Furedi-Machacek, M., Conner, F., Tice, R. R., and Tennant R. W. (2000). Responses of transgenic mouse lines p53+/– and Tg.Ac to agents tested in conventional carcinogenicity bioassays. *Toxicol Sci*. **53,** 213–223.

Stark, A. A. (1980). Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. *Annu. Rev. Microbiol.* **34,** 235–262.

Takizawa, T., Mitsumori, K., Takagi, H., Onodera, H., Yasuhara, K., Tamura, T., and Hirose, M. (2001). Modifying effects of flumequine on dimethylnitrosamine-induced hepatocarcinogenesis in heterozygous *p53* deficient CBA mice. *J. Toxicol. Pathol.* **14,** 135–143.

Tamura, T., Mitsumori, K., Onodera, H., Fujimoto, N., Yasuhara, K., Takegawa, K., Takagi, H., and Hirose, M. (2001). Dose-threshold for thyroid tumor-promoting effects of orally administered kojic acid in rats after initiation with N-*bis*(20hydroxypropyl)nitrosamine. *J. Toxicol. Sci.* **26,** 85–94.

Tamura, T., Mitsumori, K., Onodera, H., Fujimoto, N., Yasuhara, K., Takegawa, K., and Takahashi, M. (1999a). Inhibition of thyroid iodine uptake and organification in rats treated with kojic acid. *Toxicol. Sci.* **47,** 170–175.

Tamura, T., Mitsumori, K., Onodera, H., Takahashi, K., Funakoshi, T., Yasuhara, K., Takegawa, K., Takagi, H., and Hirose, M. (1999b). Time course observation of thyroid proliferative lesions and serum levels of related hormones in rats treated with kojic acid after DHPN initiation. *Toxicol. Sci.* **24,** 145–155.

Tennant, R. W., French, J. E., and Spalding, J. W. (1995). Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. *Environ. Health Perspect.* **103,** 942–950.

Tennant, R. W., Spalding, J., and French, J. E. (1996). Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutat. Res.* **365,** 119–127.

Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe S., *et al.* (1993). Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene* **8,** 3313–3322.

Wei, C. I., Huang, T. S., Fernando, S. Y., and Chung, K. T. (1991). Mutagenicity studies of kojic acid. *Toxicol. Lett.* **59,** 213–220.

Wynford-Thomas, D., Stringer, B. M. J., and Williams E. D. (1982) Dossociation of growth and function in the rat thyroid during prolonged goitrogen administration. *Acta Endocrinol*. **101,** 562–569.