Original

Lack of Initiating Activity of Kojic Acid on Hepatocarcinogenesis in F344 Rats

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Abstract: Kojic acid (KA) has been used as a food additive for preventing enzymatic browning of crustaceans and as a cosmetic agent for skin whitening. To date, it has been reported that female B6C3F1 mice receiving 3% KA in the diet were found to develop hepatocellular tumors, but the underlying mechanism of liver tumorigenicity is still not clear. In the present experiments, in order to investigate possible liver initiation activity, partially hepatectomized male F344 rats received a single oral dose of 0, 1000 and 2000 mg/kg body weight of KA followed by dietary administration of 0.015% of *N*-2-acetylaminofluorene (2-AAF) for 2 weeks and a single 0.8 mL/kg body weight dose of CCl₄. Furthermore, male F344 rats were fed a diet containing 0 or 2% KA for 3, 7 and 28 days, and the 8-oxodeoxyguanosine (8-OxodG) levels in nuclear DNA were measured to examine the formation of oxidative DNA adduct and cell proliferating activities of hepatocytes in the liver. In the liver initiation assay, there were no significant differences in the number or area of glutathione S-transferase placental form (GST-P) positive foci, putative preneoplastic lesions, between the KA-treated and control groups. Cell proliferation of hepatocytes in rats given KA for 3 and 7 days was significantly increased as compared with the relevant control values, but no significant elevation in 8-OxodG levels was apparent. The results of the present study suggest that KA has neither liver initiation activity nor capability of 8-OxodG formation, but some evidences suggestive of liver tumor promoting effects in rats.
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Key words: Kojic acid, hepatocarcinogenicity, liver initiation assay, F344 rat

Introduction

Kojic acid (KA; 5-hydroxy-2-(hydroxymethyl)-4-pyrone) is a secondary metabolic product of various species of *Aspergillus* and *Penicillium*^{1,2}. It is known that this acid inhibits polyphenoloxidase (tyrosinase) in mushrooms³, potatoes, apples and crustaceans⁴. Since polyphenol oxidase catalyzes the conversion of tyrosine to melanin via 3,4-dihydroxyphenylalanine and dopaquinone^{3,5}, 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^{6,7} and inhibitory actions on human melanocyte tyrosinase⁸, KA has been distributed as a

cosmetic agent for the purpose of skin lightening.

It has been reported that hepatocellular tumors were induced in B6C3F1 mice fed diet containing 3% KA for 20 months⁹. In our previous study in which diet containing 0, 1.5 or 3% KA was administered to heterozygous *p53*-deficient mice of the CBA strain [*p53* (+/–) mice] and their wild-type littermates [*p53* (+/+) mice] for 26 weeks, the incidences of hepatocellular adenomas as well as altered hepatocellular foci were increased in *p53* (+/–) and *p53* (+/+) mice, with and without initiation, as compared with untreated control mice¹⁰. In addition, the incidences of focal hepatocellular necrosis and inflammatory cell infiltration in the liver appeared to be increased by the 1.5 and 3% KA diets. Since no spontaneous hepatocellular foci were observed in control animals in our earlier 26-week studies using *p53* (+/–) and *p53* (+/+) mice^{11–13}, it was considered that these proliferative lesions in the liver were attributable to KA. In addition, in another study in which F344 rats received diet containing 2 or 0% KA for 20 weeks with or without initiation treatment of *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), the numbers of

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glutathione S-transferase placental form (GST-P) positive foci were significantly increased in the KA groups as compared with the corresponding control group¹⁴. This finding suggested the possibility that KA also has carcinogenic potential in the liver of rats.

Regarding genotoxicity, a number of previous *in vitro* genotoxicity tests of KA showed positive results in the reverse gene mutation assay (Ames test) using microorganisms in the presence or absence of rat S9 mix fraction^{15,16}, and in DNA-damaging and clastogenic ability in cultured rat liver cells^{17,18}, as well as sister chromatid exchange and chromosomal aberration in Chinese hamster ovary cells¹⁶. However, it remains uncertain whether or not the induction of hepatocellular proliferative lesions in rats as well as *p53* (+/-) and *p53* (+/+) mice was the result of genotoxicity of KA.

For further assessment, a liver initiation assay, sensitive for the detection of genotoxic carcinogens in F344 rats^{19,20}, and a 28-day feeding study were performed to evaluate the liver initiating potential of the compound and to examine the formation of oxidative DNA adduct and cell proliferating activity in the livers of F344 rats exposed to KA.

Materials and Methods

2.1. Test substance

Kojic acid (KA; more than 99.5% pure), kindly provided by Alps Pharmaceutical Industry Co., Ltd. (Gifu, Japan), was admixed with powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) to give an average concentration of 2%. Diets were prepared at least bi-weekly and stored until use at 5°C.

Experimental animals

Sixty male F344 rats, thirty of five weeks of age (28-day study) and thirty of seven weeks of age (5-week study), were purchased from Japan SLC, Inc., Shizuoka, Japan, and used after an acclimatization period of one week. Rats were housed at a maximum of 5 per plastic cage with wood chips for bedding and at a maximum of 3 per steel cage, respectively, under standard conditions (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; 12 h-light: 12 h-dark cycle), and given a pulverized basal diet (Oriental CRF-1, Oriental Yeast Co., Ltd.) and tap water *ad libitum*. The bedding was changed twice a week. The experiment was carried out in accordance with the Guide for Animal Experimentation of Tokyo University of Agriculture and Technology.

Twenty eight-day dietary administration study in F344 rats

Thirty male rats were split into two groups and fed a diet containing 0 or 2% KA for 3, 7 and 28 days. Five rats were sacrificed in each group under ether anesthesia at each end point. All animals were injected with bromodeoxyuridine (BrdU: 100 mg/kg, Sigma Chemical Co., St Louis, MO) i.p. once a day for the final 2 days of the

exposure and once on the day of termination, 2 hours before sacrifice. Body weight was measured on the day of necropsy in order to calculate the liver / body weight ratio. At autopsy, the livers were immediately removed and weighed, and slices taken from each lobe were fixed in 10% buffered formalin for BrdU immunostaining. The remaining pieces of the livers were frozen in liquid nitrogen and stored at -80°C until measurement of 8-oxodeoxyguanosine (8-OxodG) in nuclear DNA. BrdU immunostaining was performed using the avidin-biotin complex method, and for each animal at least 3000 hepatocytes were counted. Labeling indices (LIs) were calculated as the percentages of cells positive for BrdU incorporation divided by the total number of cells counted. Nuclear DNA was extracted with a DNA Extractor WB kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and digested to deoxynucleotides with nuclease P1 (Yamasa Shoyu Co., Ltd., Chiba, Japan) and alkaline phosphatase (Sigma Chemical Co., St Louis, MO). 8-OxodG levels (8-OxodG/10⁵ deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (ECD) (HPLC pump 420, Kontron AG, Zurich Inst., Switzerland; ECD Coulochem Model-5100A, ESA, Bedford, MA).

Five-week, 2-stage liver initiation assay in male F344 rats

The *in vivo* short-term liver initiation assay was performed according to the method of Tsuda *et al.*¹⁹. All animals were subjected to two-thirds partial hepatectomy on day 0, divided into 3 groups (Group 1, 8; Group 2, 12; Group 3, 10) and 12 hr after the completion of surgery, were treated once orally with vehicle (0.5% carboxymethyl cellulose, CMC), 1000 (half of the LD₅₀ value) and 2000 mg/kg (LD₅₀ value of KA), respectively. The vehicle and suspensions of KA were given orally at a volume of 10 mL/kg body weight. Subsequently, the rats were fed basal diet for 2 weeks, and then diet containing 0.015% of *N*-2-acetylaminofluorene (2-AAF, Japan Clea Co., Ltd., Osaka, Japan) for the following 2 weeks. Three weeks after KA administration, all animals received a single 0.8 mL/kg body weight dose of CCl₄. At the end of week 5, the survivors were sacrificed under ether anesthesia, and slices of all liver lobes were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained immunohistochemically for glutathione S-transferase placental form (GST-P), a marker of preneoplastic foci in the rat liver, using the avidin-biotin complex method. Additionally, hematoxylin-eosin staining of serial sections was performed for microscopic examination. With a computer-assisted image analyzer (NIH image), we measured the area and number of GST-P positive foci (> 0.1 mm in diameter) and the total area of each liver section examined in each animal, and then calculated the mean area and number of GST-P positive foci per unit area of all the liver sections examined.

Statistical analysis

The data obtained from both experiments were

expressed as mean $\pm$ S.D. and analyzed by one-way analysis of variance for homogeneity followed by Dunnett's test²¹ for comparison with the 0% KA control group. For the initiation assay, the significance of differences between the control and KA groups was determined by Student's *t*-test²². A *p*-value less than 0.05 was considered statistically significant in both cases.

Results

Twenty eight-day dietary administration study in F344 rats

Body weight gain of the 2% KA group was significantly decreased on Day 28 as compared to the control group (Fig. 1). Relative liver weights of the 2% KA groups were significantly increased as compared to those of the control group at each necropsy point (Fig. 2). The LIs of hepatocytes of rats exposed to KA in the diet for 3 ($3.44 \pm 1.13\%$) and 7 ($1.32 \pm 0.68\%$) days were significantly increased as compared with the relevant control values (1.88 ± 0.64 , $0.18 \pm 0.10\%$), but there were no changes in the LIs between the groups (KA: 0.31 ± 0.31 , Control: $0.28 \pm 0.19\%$) on Day 28 (Fig. 3). 8-OxodG levels in the liver DNA of rats given KA were slightly higher than the relevant control values throughout the experimental period (Day 3: KA 0.58 ± 0.15 , Control 0.52 ± 0.18 ; Day 7: KA 0.73 ± 0.26 , Control 0.58 ± 0.18 ; Day 28: KA 0.65 ± 0.12 , Control 0.53 ± 0.06 8-OxodG/ 10^5 dG), but without statistical significance (Fig. 4).

Liver initiation assay in F344 rats

One rat in the control group died during the experimental period. On immunohistochemical examination, no significant differences were observed in the mean area (Control 1.53 ± 0.74 ; 1000 mg/kg KA 1.69 ± 0.97 ; 2000 mg/kg KA 1.91 ± 1.72) or number (Control $4.38 \pm$

Fig. 2. Absolute liver weights and liver / body weight ratio of F344 rats treated with 2% KA for 3, 7 and 28 days. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, Significantly different from the corresponding control group.

Fig. 3. BrdU labeling indices of hepatocytes of F344 rats treated with vehicle (open column) or 2% Kojic acid (closed column) for 3, 7 and 28 days. *: $p < 0.05$, **: $p < 0.01$, Significantly different from the corresponding control group.

Fig. 1. Mean body weight changes in F344 rats treated with 2% Kojic acid. Five rats each were measured on Day 3, 7 and 28. *: $p < 0.05$, ***: $p < 0.001$, Significantly different from the corresponding control group.

Fig. 4. 8-OxodG levels in liver DNA of F344 rats treated with vehicle (open column) or 2% Kojic acid (closed column) for 3, 7 and 28 days.

Fig. 5. Area and number of GST-P positive liver cell foci (>0.1 mm in diameter) in partially hepatectomized rats treated with vehicle (open column) or kojic acid (striped or closed columns). Data represent the mean $\pm$ S.D. of 7, 12 and 10 animals.

1.51; 1000 mg/kg KA 5.36 ± 3.04 ; 2000 mg/kg KA 6.80 ± 3.32) of GST-P positive foci between the KA and control groups (Fig. 5).

Discussion

Fujimoto *et al.*⁹ first reported that hepatocellular tumors were induced in female B6C3F1 mice receiving 3% KA in the diet for 20 months, but they did not pay special attention to the positive result of the liver tumorigenicity of KA. In our previous study in which *p53* (+/–) mice, that have high susceptibility to genotoxic carcinogens^{11,23–25}, and *p53* (+/+) mice were fed diet containing 0, 1.5 or 3% KA for 26 weeks, the incidence of hepatocellular adenomas and altered hepatocellular foci were increased in the 1.5 and 3% KA groups of *p53* (+/–) mice (7/10 and 5/10) and *p53* (+/+) CBA mice (2/10 and 5/10) as compared to the control group (0/10)¹⁰. In addition, in the rat two-stage carcinogenesis model using DHPN, or the medium-term liver bioassay initiated with DEN, KA demonstrated clear promoting activity of hepatocarcinogenesis¹⁴. Furthermore, although the numbers and areas were small, KA increased GST-P-positive foci after 20-week dietary administration of 2% KA without any prior initiation treatment¹⁴. These results indicate that KA is a promoter and possibly also a complete hepatocarcinogen in rats as well as in mice. With respect to the genotoxicity, KA has been shown not to be mutagenic to *Salmonella typhimurium* TA1537 or TA1538 strains, but it was mutagenic to the TA100, TA1535 and TA98 strains in the presence or absence of rat S9 mix^{15,16}. While positive and negative responses were noted for KA in the rec-assay and SOS spot test, respectively, chromosomal aberrations and sister chromatid exchanges were induced in Chinese hamster ovary CHO cells with and without S9 mix¹⁶. Chromosomal aberrations in Chinese hamster lung CHL/IU cells were also found at 1000 μ g/mL KA in the absence of S9 mix²⁶, but

reproducibility of clastogenicity could not be confirmed at concentrations up to 5000 μ g/mL KA. In addition, KA gave positive results for DNA-damaging and clastogenic ability in cultured rat liver cells^{17,18}. Therefore, KA's contribution to genotoxicity in the induction of hepatocellular tumors in mice and rats cannot be ruled out.

In the present liver initiation assay using F344 rats, no initiation activity was observed at 2000 mg/kg (LD₅₀ value) of KA. Since it has been shown that genotoxic carcinogens, which do not normally target the liver, can cause induction of GST-P positive foci in an *in vivo* liver initiation assay²⁷, it is generally recognized that genotoxic carcinogenesis can be detected by this initiation assay, regardless of the target organs in long-term carcinogenicity studies in rats and mice. This initiation assay seems to be a useful method for clarifying whether or not chromosome aberrations and/or gene mutations are fixed as a result of DNA damage, therefore, we examined the initiation effects of kojic acid in a short-term liver initiation assay in partially hepatectomized rats. Based on the many findings obtained by this approach, it can be concluded that KA does not have any liver initiation activity in rats, a conclusion which is supported by our present data showing that 8-OxodG formation in the liver DNA of rats exposed to KA for 28 days failed to increase. However, since it is not known whether the liver initiation assay using rats can reliably detect almost all the genotoxic carcinogens in mice, it is premature to draw a conclusion on the negativity of liver initiation activity of KA. Considering species difference between mice and rats, additional studies of the liver initiation assay using mice are now in progress to define whether KA is a genotoxic carcinogen.

In the present study, transient increases of cell proliferation in hepatocytes were observed in rats given KA for 3 and 7 days, but not for 28 days. In a previous study in which F344 rats were given diet containing 0 or 2% KA for 20 weeks without any initiation treatment, liver weights

increased without affecting the body weights in non-initiated rats receiving 2% KA, together with significant increases in GST-P-positive foci¹⁴. These findings may suggest that KA has a liver tumor promoting effect. A number of chemicals inducing hepatic cytochrome P-450s, particularly of the phenobarbital type, are known to be hepatic tumor promoters^{28–30}. Although it is not known whether KA exerts phenobarbital-type induction of cytochrome P-450s, it has been demonstrated that hepatic uridine diphosphate glucuronosyl transferase was not increased in the livers of rats given KA^{31,32}. Based on the data of that report, the tumor promoting effect of KA might not be exerted via the induction of hepatic cytochrome P-450s. Further studies are necessary to clarify the mechanism of the liver tumor promoting effect of KA.

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