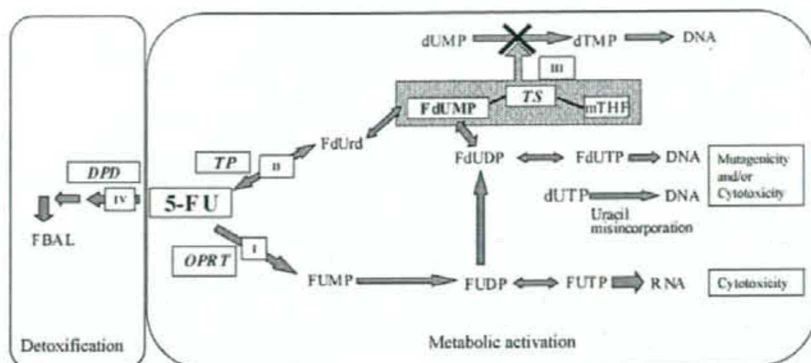

Fig. 5. The intracellular metabolism and catabolism of 5-FU. (I) 5-FU is metabolized by OPRT to FUMP. (II) 5-FU is metabolized to FdUrd by TP, but TP can convert FdUrd to 5-FU. (III) When 5-FU is converted to the active form of 5-FU, FdUMP, it forms complex with TS and 5,10-methylene tetrahydrofolate, and competitively inhibits DNA synthesis. (IV) 5-FU is detoxicated by DPD to FBAL. Thymidine depletion is a trigger for DSBs. DSBs are caused by the misincorporation of dUTP and/or misrepair of the uracil-containing lesion. Furthermore, 5-FU induces DNA strand breaks by the direct incorporation of 5-fluoro-deoxyuridine triphosphate (FdUTP). 5-FU is also misincorporated to RNA, and show the cytotoxicity. Modified from IARC Monographs [26].

genicity in MOLY and WTK-1 cells. Cell cycle analysis showed no clear difference between MOLY cells and the human cell lines, suggesting that p53 gene status did not directly determine the sensitivity of the cells to 5-FU mutagenicity and cytotoxicity.

Fig. 5 shows the intracellular metabolism and detoxification of 5-FU. When 5-FU, a fluorinated pyrimidine base analogue, is converted to 5-fluoro-2-deoxyuridylate monophosphate (FdUMP), it complexes with TS and 5,10-methylene tetrahydrofolate (CH_2FH_4), and competitively inhibits DNA synthesis [23–26]. Thymidine depletion by TS inhibition induces a nucleotide pool imbalance and the misincorporation of dUTP and/or misrepair of the uracil-containing lesion, causing double strand DNA breaks (DSBs) [40–42]. Furthermore, the direct incorporation of 5-fluoro-deoxyuridine triphosphate (FdUTP) inhibits DNA synthesis [24]. Therefore, the intracellular metabolism of 5-FU to FdUMP or FdUTP leads to cytotoxic and mutagenic effects.

5-FU can be metabolized by OPRT to fluoro-uridylate monophosphate and detoxified by DPD to fluoro-β-alanine, or by TP to 5-fluoro-deoxyuridine (FdUrd) and, in turn, back to 5-FU (Fig. 5). In this study, DPD activity was lower and OPRT activity was higher in MOLY cells than in TK6 and WTK-1 cells, suggesting the efficient metabolism of 5-FU in MOLY cells, which could have led to the strong cytotoxic and mutagenic effects that we observed.

Only MOLY cells had no TP activity in this study. TP and OPRT are involved in the intracellular metabolism of 5-FU. Inhibition of OPRT decreases the incorporation of 5-FU into nucleotides and therefore its cytotoxicity, while TP has no effect [43]. Thus, the absence of TP activity in MOLY cells would have little effect on its sensitivity to 5-FU. TS content, on the other hand, was not associated with the cytotoxic and mutagenic effects of 5-FU.

From these findings, we conclude that the difference in DPD and OPRT activity in MOLY, TK6, and WTK-1 cells were responsible for differences in the cells' cytotoxic and mutagenic responses to 5-FU.

The relationship between sensitivity to 5-FU and the intracellular distribution of 5-FU metabolic enzymes indicate that differences in the way that human cells and animal cells metabolize a chemical can cause differences in mutagenicity test results. Thus, potentially mutagenic chemicals should be tested in human cells as well as animal cells.

Compared with WTK-1 cells, TK6 cells had much lower DPD activity, and 5-FU in TK6 cells was efficiently metabolized to FdUMP without being detoxified. But the TS content was higher in TK6 cells than in WTK-1 cells, so 5-FU metabolism would not be very different in TK6 and WTK-1 cells. In this study, however, 5-FU cytotoxicity was higher in TK6 cells than in WTK-1 cells, perhaps because 5-FU is more cytotoxic in p53 normal cells than in p53 mutant or null cells [15–17]. Our results suggest that if 5-FU metabolizing enzyme activity is equivalent, p53 status might influence 5-FU cytotoxicity. Indeed, p53 status might influence responses to cytotoxicity induced by 5-FU as it does to cytotoxicity induced by X-rays [3]. Furthermore 5-FU increased the

MN frequency in MOLY and WTK-1 cells but not TK6 cells, while the ratio of apoptosis cells induced by 5-FU was the highest in TK6 cells. In TK6 cells, the wild type p53 gene might suppress the induction of micronuclei by leading of DNA damaged cells to apoptosis. Thus, 5-FU mutagenicity might be influenced by also the p53 gene status. However, IC_{50} of RS0 was obviously lower and MF was obviously higher in MOLY cells than TK6 and WTK-1 cells. Therefore we suspected that the difference in 5-FU metabolism influenced to 5-FU cytotoxicity and mutagenicity more than the p53 gene status.

In summary, our study showing that MOLY cells were more sensitive than WTK-1 cells to 5-FU cytotoxicity and mutagenicity even though both have a mutated p53 gene suggested that those differences were attributable to differences in 5-FU metabolism rather than the p53 gene status.

Acknowledgments

We thank Drs. Tanaka and Wakuri, Hatano Research Institute, Food and Drug Safety Center, for providing us with MOLY, TK6, and WTK-1 cell lines. We are indebted to Dr. M. Bloom for the precise review of the manuscripts.

References

[1] T.J. Oberly, B.J. Bewsey, G.S. Probst, An evaluation of L5178YTK$^{+/-}$ mouse lymphoma forward mutation assay using 42 chemicals, Mutat. Res. 125 (1984) 291–306.

[2] P. Clay, M.F. Cross, Microwell mutation assays: evaluation of ethyl methanesulfonate, benzo[a]pyrene and benzidine using the tk locus in L5178Y mouse lymphoma cells, Mutagenesis 5 (Suppl.) (1990) 45–54.

[3] M. Honma, M. Hayashi, T. Sofuni, Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells, Mutat. Res. 374 (1997) 89–98.

[4] M. Honma, L.S. Zhang, M. Hayashi, K. Takeshita, Y. Nakagawa, N. Tanaka, T. Sofuni, Illegitimate recombination leading to allelic loss and unbalanced translocation in p53-mutated human lymphoblastoid cells, Mol. Cell. Biol. 17 (1997) 4774–4781.

[5] M. Honma, M. Hayashi, H. Shimada, N. Tanaka, S. Wakuri, T. Awogi, K.I. Yamamoto, N. Kodani, Y. Nishi, M. Nakadate, T. Sofuni, Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test, Mutagenesis 14 (1999) 5–22.

[6] D. Clive, J.F.S. Spector, Laboratory procedure assessing specific locus mutation at the TK locus in cultured L5178Y mouse lymphoma cells, Mutat. Res. 31 (1975) 17–29.

[7] P.M. Leong-Morgenthaler, C.O.H. Velt, E. Jaccaud, R.J. Turesky, Mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in human lymphoblastoid cells, Carcinogenesis 19 (1998) 1749–1754.

[8] Y.Y. Chuang, Q. Chen, J.P. Brown, J.M. Sedivy, H.L. Liber, Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells, Cancer Res. 59 (1999) 3073–3076.

[9] R.F. Branda, J.P. O'Neill, E.M. Brooks, L.M. Trombley, J.A. Nicklas, The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status, Mutat. Res. 473 (2001) 51–71.

[10] L. Bassi, M. Carloni, E. Fonti, N.P. de la Pena, R. Meschini, F. Palitti, Pifithrin-alpha, an inhibitor of p53, enhances the genetic instability induced by etoposide (VP16) in human lymphoblastoid cells treated in vitro, Mutat. Res. 499 (2002) 163–176.

[11] S.A. Amundson, F. Xia, K. Wolfson, H.L. Liber, Different cytotoxic and mutagenic responses induced by X-rays in two human lymphoblastoid cell lines derived from a single donor, Mutat. Res. 286 (1993) 233–241.

[12] F. Xia, X. Wang, Y.H. Wang, N.M. Tsang, D.W. Yandell, K.T. Kelsey, H.L. Liber, Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in two closely related human lymphoblast lines, Cancer Res. 55 (1995) 12–15.

[13] S.M. Morris, A role for p53 in the frequency and mechanism of mutation, Mutat. Res. 511 (2002) 45–62.

[14] J.L. Schwartz, R. Jordan, B.A. Sedita, M.J. Swenningson, J.P. Banath, P.L. Olive, Different sensitivity to cell killing and chromosome mutation induction by gamma rays in two human lymphoblastoid cell lines derived from a single donor: possible role of apoptosis, Mutagenesis 10 (1995) 227–233.

[15] D. Arango, G.A. Corner, S. Wadler, P.J. Catalano, L.H. Augenlicht, c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil in vitro and in vivo, Cancer Res. 61 (2001) 4910–4915.

[16] J.L. Grem, K.D. Danenberg, K. Behan, A. Parr, L. Young, P.V. Danenberg, D. Nguyen, J. Drake, A. Monks, C.J. Allegra, Thymidine kinase, thymidylate synthase, and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's Anticancer Drug Screen, Clin. Cancer Res. 7 (2001) 999–1009.

[17] D.B. Longley, J. Boyer, W.L. Allen, T. Latif, P.R. Ferguson, P.J. Maxwell, U. McDermott, M. Lynch, D.P. Harkin, P.G. Johnston, The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates, Cancer Res. 62 (2002) 2644–2649.

[18] S.K. Greenwood, M.J. Armstrong, R.B. Hill, C.I. Bradt, T.E. Johnson, C.A. Hilliard, S.M. Galloway, Fewer chromosome aberrations and earlier apoptosis induced by DNA synthesis inhibitors, a topoisomerase II inhibitor or alkylating agents in human cells with normal compared with mutant p53, Mutat. Res. 401 (1998) 39–53.

[19] T. Allio, E.M. Donner, R.J. Preston, A comparison of the roles of p53 mutation and AraC inhibition in the enhancement of bleomycin-induced chromatid aberrations in mouse and human cells, Mutat. Res. 447 (2000) 227–237.

[20] T. Allio, R.J. Preston, Increased sensitivity to chromatid aberration induction by bleomycin and neocarzinostatin results from alterations in a DNA damage response pathway, Mutat. Res. 453 (2000) 5–15.

[21] L.S. Zhang, M. Honma, M. Hayashi, T. Suzuki, A. Matsuoka, T. Sofuni, A comparative study of TK6 human lymphoblastoid and L5178Y mouse lymphoma cell lines in the in vitro micronucleus test, Mutat. Res. 347 (1995) 105–115.

[22] R.D. Storer, A.R. Kraynak, T.W. McKelvey, M.C. Elia, T.L. Goodrow, J.G. DeLuca, The mouse lymphoma L5178Y $Tk^{+/-}$ cell line is heterozygous for a codon 170 mutation in the p53 tumor suppressor gene, Mutat. Res. 373 (1997) 157–165.

[23] A.A. Piper, R.M. Fox, Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil, Cancer Res. 42 (1982) 3753–3760.

[24] R.B. Diasio, B.E. Harris, Clinical pharmacology of 5-fluorouracil, Clin. Pharmacokinet. 16 (1989) 215–237.

[25] D.M. Thomas, J.R. Zalcberg, 5-fluorouracil: a pharmacological paradigm in the use of cytotoxics, Clin. Exp. Pharmacol. Physiol. 25 (1998) 887–895.

[26] 5-fluorouracil, IARC Monogr. Eval. Carcinogen. Risk Chem. Hum. 26 (1981) 217–235.

[27] L. Griffiths, I.J. Stratford, Platelet-derived endothelial cell growth factor thymidine phosphorylase in tumor growth and response to therapy, Br. J. Cancer 76 (1997) 689–693.

[28] N. Yajima, K. Kondo, K. Morita, Reverse mutation tests in *Salmonella typhimurium* and chromosomal aberration tests in mammalian cells in culture on fluorinated pyrimidine derivatives, Mutat. Res. 88 (1981) 241–254.

[29] A. Ohuchida, A. Furukawa, J. Yoshida, M. Watanabe, F. Aruga, Y. Miwa, K. Shinkawa, N. Kinae, Micronucleus assays on 5-fluorouracil and 6-mercaptopurine with mouse peripheral blood reticulocytes, Mutat. Res. 278 (1992) 139–143.

[30] T. Sofuni, M. Honma, M. Hayashi, H. Shimada, N. Tanaka, S. Wakuri, T. Awogi, K.I. Yamamoto, Y. Nishi, M. Nakadate, Detection of in vitro clastogens and spindle poisons by the mouse lymphoma assay using the microwell method: interim report of an international collaborative study, Mutagenesis 11 (1996) 349–355.

[31] 5-fluorouracil, IARC Monogr. Eval. Carcinogen. Risk Chem. Hum. (Suppl. 6) (1987) 316–318.

[32] T. Matsushima, M. Hayashi, A. Matsuoka, M. Ishidate, K.F. Miura, H. Shimizu, Y. Suzuki, K. Morimoto, H. Ogura, K. Mure, K. Koshi, T. Sofuni, Validation study of the in vitro micronucleus test in a Chinese hamster lung cell line (CHL/IU), Mutagenesis 14 (1999) 569–580.

[33] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.

[34] M. Carbonari, M. Cibati, M. Fiorilli, Measurement of apoptotic cells in peripheral blood, Cytometry 22 (1995) 161–167.

[35] C.P. Spears, A.H. Shahinian, R.G. Moran, C. Heidelberger, T.H. Corbett, In vivo kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas, Cancer Res. 42 (1982) 450–456.

[36] T. Takechi, J. Uchida, F.A.M. Fukushima, Enhancing 5-fluorouracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with uracil in human tumor cells, Int. J. Oncol. 11 (1997) 1041–1044.

[37] F.N. Naguib, M.H. el Kouni, S. Cha, Enzymes of uracil catabolism in normal and neoplastic human tissues, Cancer Res. 45 (1985) 5405–5412.

[38] G.J. Peters, E. Laurensse, A. Leyva, J. Lankelma, H.M. Pinedo, Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes, Cancer Res. 46 (1986) 20–28.

[39] Y. Maehara, H. Nakamura, Y. Nakane, K. Kawai, M. Okamoto, S. Nagayama, T. Shirasaka, S. Fujii, Activities of various enzymes of pyrimidine nucleotide and DNA syntheses in normal and neoplastic human tissues, Gann 73 (1982) 289–298.

[40] H.A. Ingraham, B.Y. Tseng, M. Goulian, Nucleotide levels and incorporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine, Mol. Pharmacol. 21 (1982) 211–216.

[41] N.J. Curtin, A.L. Harris, G.W. Aherne, Mechanism of cell death following thymidylate synthase inhibition: 2′-deoxyuridine-5′-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and dipyridamole, Cancer Res. 51 (1991) 2346–2352.

[42] R.D. Ladner, The role of dUTPase and uracil-DNA repair in cancer chemotherapy, Curr. Protein Pept. Sci. 2 (2001) 361–370.

[43] M. Fukushima, Y. Murakami, N. Suzuki, K. Aiba, The analysis of the innate pathways of 5-fluorouracil phosphorylation in human gastrointestinal cancer cell lines in vitro and in vivo, Oncol. Rep. 4 (1997) 1189–1194.

Genes and Environment

Vol. 28, No. 1, February, 2006

Characterization of Genotoxicity of Kojic Acid by Mutagenicity in Salmonella and Micronucleus Induction in Rodent Liver

Satoko Ishikawa[1], Yu F Sasaki[2], Satomi Kawaguchi[2], Masataka Mochizuki[1] and Minako Nagao[1]

[1]Kyoritsu University of Pharmacy, Tokyo, Japan
[2]Hachinohe National College of Technology, Aomori, Japan

Genes and Environment, Vol. 28, No. 1 pp. 31-37 (2006)

Regular Article

Characterization of Genotoxicity of Kojic Acid by Mutagenicity in Salmonella and Micronucleus Induction in Rodent Liver

Satoko Ishikawa[1], Yu F Sasaki[2], Satomi Kawaguchi[2], Masataka Mochizuki[1] and Minako Nagao[1,3]

[1]Kyoritsu University of Pharmacy, Tokyo, Japan
[2]Hachinohe National College of Technology, Aomori, Japan

(Received December 19, 2005; Accepted January 16, 2006)

Three lots of kojic acid (KA) which were produced for use as a reagent, food additive and in cosmetics were shown to be mutagenic in *S. typhimurium* TA100 with or without S9 mix, with a specific activity of around 100 revertants per mg of KA. Since there are contradictory reports on genotoxicity of KA, we examined, using HPLC, whether the mutagenicity to *S. typhimurium* is due to KA itself, or due to contaminants present in the KA samples. Although two UV absorbing fractions were separated by HPLC, mutagenicity was detected only in the major fraction and the specific mutagenic activity of KA did not change before and after HPLC separation. The material in the major peak fractions on HPLC was confirmed to be KA by NMR. Thus it was demonstrated that KA itself is mutagenic and no mutagenic contaminants were detected in the three lots of samples. Since KA is known to produce liver tumors in mice, we further examined the genotoxicity of KA in the liver of rodents. KA induced micronuclei (MN) in the regenerating liver of adult mice by its gastric intubation at 1 g per kg body weight. However, no MN were induced in young mice (3 weeks old) without partial hepatectomy. Since it was recently found that KA had no tumor-initiating activity in the liver of mice in a two-step carcinogenicity study, there is no evidence that the genotoxicity detected in the mouse liver is involved in liver carcinogenesis.

Key words: mutagenicity, liver micronuclei, genotoxicity, *S. typhimurium*, kojic acid.

Introduction

Kojic acid (KA) [5-hydroxy-2-hydroxymethyl]-4*H*-pyran-4-one; CAS No. 501-30-4; (Fig. 1)] is a natural substance produced by various fungi, *Penicillium* spp., *Aspergillus orizae, flavus and lamarii*, and also by certain bacteria (1). KA which had been used as a food additive for the prevention of enzymatic browning of shell fish, raw crabs and shrimp, owing to its inhibitory activity on tyrosinase, was found to be genotoxic *in vitro*, inducing his^+ reverse mutations in *S. typhimurium* (2-5). It was also found to be genotoxic *in vivo*, inducing micronuclei (MN) in peripheral blood of rats (6).

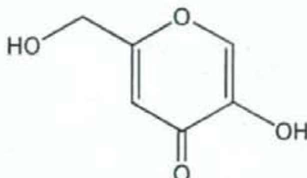

Fig. 1. Structure of kojic acid (KA).

KA had been reported to induce hepatomas and thyroid adenomas in mice (7). Thyroid adenomas or hyperplasia production in the mouse or rat was suggested to be due to promoting activity of KA (7-9). In contrast, the possibility of involvement of genotoxicity of KA in mouse hepatoma development could not be excluded (10). In 2003, the Ministry of Health, Labour and Welfare, Japan noticed that KA was no longer used for prevention of browning of shell fish in market due to the development of modern technology, and KA was withdrawn from the list of existing food additives in the same year. Since KA is a fungal product, soy sauce, miso and sake were suspected to contain KA. Out of 32 samples of fermented foods examined for the presence of KA, three samples were found to be positive, but the levels were not so high, being at the maximum, 1 ppm (personal communication by Dr. Tamio Maitani, National Health Sciences, Tokyo, Japan, Dec. 7, 2005). Further, KA is still used in cosmetics as a skin lightening agent (quasi-drug) at concentrations of up to 1.5% due

[3]Correspondence to: Minako Nagao, Kyoritsu University of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Tel: +81-3-3434-6241, Fax: +81-3-3434-5343, E-mail: mnagao@m8.dion.ne.jp

© The Japanese Environmental Mutagen Society

to its inhibiting action on human melanocyte tyrosinase (11). Thus, the safety of KA needs to be confirmed from various points.

Recently, results of an extensive study on genotoxicity of KA have been reported, in which KA showed only weak mutagenicity without a dose-dependent response in *S. typhimurium* (12). Many contradictory or inconsistent results had been reported for the genotoxicity of KA. KA induced MN in peripheral blood, but not in the liver of young rats by a single application through gastric intubation (6). However, it did not induce MN in bone marrow of mice (12,13) by a single application through intraperitoneal injection. Furthermore, MN were not induced in human keratinocytes SVK14 nor in human hepatocellular carcinoma cells, HepG2, *in vitro* (12) but were induced in human lymphoblastoid cells, TK6 and WTK-1 (personal communication by Dr. Masamitsu Honma, National Institute of Health Sciences, Tokyo Japan, December 12, 2005). KA did not induce $HPRT^-$ mutations in Chinese hamster V79 cells or mouse L5178Y cells (12), but induced TK^- mutation in TK6 ($TK^{+/-}$) and WTK-1 ($TK^{+/-}$, $P53^{-/-}$) cells (unpublished observations, Yu F Sasaki). As for chromosome aberrations and sister chromatid exchanges *in vitro*, positive results were reported in Chinese hamster cells (5).

Since there had been no standards for composition of KA in food hygiene law, while the manufacturers' specifications for cosmetic use was at least 97% pure, there was a possibility that the discordance reported for genotoxicity was due to differences in composition of KA samples used. Thus, it is important to clarify whether the mutagenicities of some KA samples were due to KA itself or contaminant included. In the present study, samples of various lots of KA produced by different companies that are used as a food additive, reagent and cosmetics ingredient were purified by HPLC. The resulting fractions were tested in order to determine whether the reported mutagenicity in *S. typhimurium* was due to KA itself or to another component present in the KA samples. This testing showed that the mutagenicity is due to KA itself and not to contaminants. As KA has been reported to be hepatocarcinogenic in mice (7), we further examined whether KA shows *in vivo* genotoxicity in rodent liver, by MN assay.

Materials and Methods

Chemicals: Kojic acid, reagent grade, lot no. 052K2516 was purchased from Sigma (St. Louis), 5312 used for food additive (content is 100.6%) was supplied from Alps Pharmachemical Industry (Gifu, Japan) and 2Y181 [at least 97% pure, but more typically (by HPLC) was >99% pure] used for cosmetics was supplied from Sansho Seiyaku Co., Ltd (Fukuoka, Japan). For *in vivo* genotoxicity studies, KA for food additive provided by Alps Pharmachemical Industry was used. Cyclophospamide, diethylnitrosamine (DEN), dimethyl sulfoxide (DMSO), 4-nitroquinoline 1-oxide and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 1,2-dimethylhydrazine (1,2-DMH) and benzo[*a*]pyrene were from Tokyo Kasei Kogyo (Tokyo, Japan). Solutions of KA were prepared immediately before use.

HPLC separation: Analytical HPLC was performed under three systems. System 1; an LC-20A series from Shimadzu Co. (Kyoto, Japan) equipped with Shimadzu SPD-M20A photodiode detector. A Mightysil RP-18 GP column (5 μm particle, 3 mm i.d. ×50 mm; Kanto Chemical Co., Tokyo, Japan) was used at 40°C, with an isocratic eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.15 mL/min: System 2; a Shimadzu LC-10A series was used with a Mightysil RP-18 GP column (5 μm particle, 4.6 mm i.d. ×250 mm), an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.7 mL/min: System 3; the same equipment and column with system 2, but the eluent used was 0.1% propionic acid-0.05N perchloric acid and a flow rate of 0.7 mL/min. Preparative HPLC was performed on an LC-8A series from Shimadzu Co., using a large volumetric flow cell in the UV detector. A Mightysil RP-18 GP column (5 μm particle, 10 mm i.d. ×250 mm; Kanto Chemical Co., Tokyo, Japan) and an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) were used with a flow rate of 4.0 mL/min. After separation, each fraction was lyophilized and weighed. All fractions were subjected to mutagenicity testing.

NMR analysis: ^1H and ^{13}C spectra were recorded on an ECP-600 spectrometer (JEOL Ltd., Akishima, Tokyo) using DMSO-d_6 as a solvent.

Mutagenicity assay: The mutation assays were performed using *S. typhimurium* TA100 with and without S9 mix (14). The S9 purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) was prepared from Crj:CD (SD), male rat treated with phenobarbital and 6-naphthoflavone. KA was dissolved in 0.1 mL of distilled water and the assay was performed by the method of preincubation (15). For the mutagenicity test, after separation of samples by HPLC, 1 mg of dried residues was applied to a plate for each of fractions 6, 8 and 9. For fraction 7, a dose-dependent response with 0.5, 1.0 and 1.5 mg/plate was examined and the specific activity was calculated based on the linear regression of the least square method. For other fractions, the residues were dissolved in 500 μL water, and 10 or 100 μL were applied to each plates. Statistic analysis was performed by multiple regression analysis.

Animal and KA administration: Male ddY mice of 3 and 8 weeks old, and male Fischer 344 rats of 8 weeks

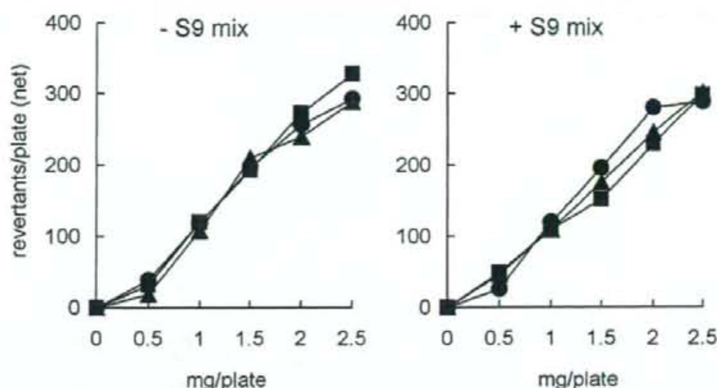

Fig. 2. Mutagenicities of kojic acid (KA) in *S. typhimurium* TA 100 in the absence (left) or presence (right) of S9 mix. The values were averages of two plates, subtracted with numbers of spontaneous revertants of 128 (−S9 mix) or 167 (+S9 mix). Positive control, 0.03 μg of 4-nitroquinoline 1-oxide induced 400 revertants (−S9 mix) and 5 μg of benzo[*a*]pyrene induced 1545 revertants (+S9 mix) over spontaneous one. No statistic differences were observed among these three samples. ●; lot.052K2516, ■; lot.5312, ▲; lot.2Y181.

old were purchased from SLC Japan (Shizuoka, Japan). Eight week old animals were acclimatized for one week at 24±2°C with a relative humidity of 55±5%, with basal diet of MF pellets (Oriental Yeast Industries, Tokyo, Japan) and tap water ad libitum, and were used at age 9 weeks. Three week old mice were used immediately. For gastric intubation, KA was suspended at 0, 50 and 100 mg/mL in 0.5% sodium carboxymethyl-cellulose and immediately administered to animals at a dose of 10 mL/kg, corresponding to KA doses of 0, 500 and 1000 mg/kg. The doses administered to the animals were based on the approximate maximum tolerated dose for each species determined by simple acute toxicity experiments: oral gavage with 2000 mg/kg KA resulted in death of 4/4 mice and 4/4 rats within 3h.

Micronucleus assay: Four mice and rats were used for each KA-dose group and three for positive control groups, following the reported method (16,17). Twenty-four hours after administration of KA, partial hepatectomy (two-thirds) was performed on 9 week old animals by removing three major lobes of the liver, the left lateral, left medial and the right lateral lobes under ether-anesthetized conditions. After four days, the animals were anesthetized with ether, and their livers were perfused *in situ* for 5 min with Liver Perfusion Medium [Hanks' balanced salt solution (Ca^{2+}/Mg^{2+} free) containing 0.5 mM EGTA and 10 mM HEPES (pH 7.2-7.3)] (GIBCO-Invitrogen, Carlsbad, CA), followed by 5 min with Liver Digest Medium (Hanks' balanced solution (Ca^{2+}/Mg^{2+} free) containing 0.05% collagenase type IV, 50 μg/mL trypsin inhibitor, 10 mg/mL bovine serum albumin, 10 mM HEPES, and 560 μg/mL $CaCl_2$) (GIBCO-Invitrogen). Both solutions were maintained at 37°C and delivered at a flow rate of 14 mL/min. The perfused livers were minced in a Petri dish containing Liver Digest Medium; the minced tissue was then passed through gauze, centrifuged at 200 g for 1 min, and the cell pellet obtained was fixed with 1 mL of 10% neutral formaldehyde. Ten μL of the cell suspension were mixed with 10 μL of 500 μg/mL acridine orange, the mixture placed on a glass slide and covered with a coverslip. The number of micronucleated hepatocytes (MNHEPs) among 1000 hepatocytes was recorded for each animal under a fluorescence microscope (×400 or greater), with a blue excitation filter and a yellow barrier filter. MNHEP was defined as a hepatocyte with two nuclei, one being less than 1/4 in diameter of the other nucleus. For 3 week old mice, MN assays were performed by the same procedure without partial hepatectomy, and livers were removed at 72, 96 and 120 h. The differences between the frequencies of MN in KA treated and KA 0 control animals were analyzed by one-way ANOVA followed by pairwise comparisons using the Dunnett test. A P-value of less than 0.05 was considered statistically significant.

All animal experiments were carried out following the guidelines set out by Hachinohe National College of Technology in the Guide for the Care and Use of Laboratory Animals.

Results

Mutagenicity in *S. typhimurium* of KA for reagent, food additive and cosmetics: Three lots of KA, 052K2516 (reagent), 5312 (food additive) and 2Y181 (cosmetics) showed similar mutagenic activities in TA 100, under conditions without or with S9 mix (Fig. 2). All samples showed linear dose-dependent response between 0.5 and 1.5 mg/plate of KA. The specific mutagenic activities calculated by linear regression by

Table 1. Weight and mutagenicity in *S. typhimurium* TA100 of each fraction obtained by HPLC of KA samples

Fr. No.	Lot. 052K2516			Lot. 5312			Lot. 2Y181		
	Weight (mg/Fr.)	Revertants/Fr.		Weight (mg/Fr.)	Revertants/Fr.		Weight (mg/Fr.)	Revertants/Fr.	
		−S9 mix	+S9 mix		−S9 mix	+S9 mix		−S9 mix	+S9 mix
1	0.2	0	0	0.6	0	20	0.1	0	0
2	0.2	0	0	0.4	0	0	0.1	0	0
3	0.2	0	0	0.3	0	0	0.0	0	0
4	0.2	0	0	0.4	0	0	0.1	0	0
5	0.0	0	0	0.0	0	30	0.1	0	0
6	11.3	1402	1232	14.2	2321	1734	10.2	1129	946
7	22.8	2686	3051	27.0	4260	3504	33.4	3176	3377
8	15.1	1595	1700	17.4	2153	2205	26.6	2258	2471
9	7.4	749	853	9.0	945	1359	11.6	1170	961
10	2.9	292	213	2.3	259	94	4.1	410	250
11	2.0	0	0	0.3	25	0	1.2	0	0
12	0.8	0	0	0.3	85	0	0.2	0	0
13	0.4	0	0	0.4	85	0	0.2	0	0
14	0.4	0	0	0.1	40	0	0.2	0	0
Total weight	63.9			72.5			88.1		

the least square method for 052K2516, 5312 and 2Y181 were 116, 115 and 106 revertants/mg, respectively, without S9 mix and 113, 106 and 111, respectively, with S9 mix. No statistic differences were detected among these different sources of samples at significance of ≤ 0.05.

Separation of mutagenic substance in KA samples by HPLC: To clarify whether the mutagenicity was derived from KA itself or some contaminants in the samples, HPLC separations were performed. A KA sample solution in distilled water of 0.5 μg/0.5 μL was eluted by analytical HPLC under the conditions described in Materials and Methods (systems 1-3). The chromatograms under system 1 of three lots of KA monitored by absorption at 270 nm were similar, and revealed a single peak at 3.8 min (Fig. 3a). Only when a very large amount of KA was applied, a small shoulder peak was detected at the foot of the major peak (data not shown). Using two other different HPLC systems 2 and 3, similar chromatograms were obtained with three samples of KA (data not shown), with their small and very large amounts. These results indicated only a minor UV absorbing material(s) are contaminated in all three samples at similar levels.

Preparative HPLC was then applied to each lot of KA to facilitate the determination of the mutagenicity of the various constituents of the KA samples. A 500 μL aliquot of 25 mg/mL KA solution in distilled water was applied to a column, under the conditions described in Materials and Methods. Typical 270 nm chromatograms of the three samples are shown in Fig 3b. From the chromatograms, it can be seen that all of the samples include an impurity detected as a small peak shoulder, which was eluted after the major peak (arrows in Fig 3b). Circa 100 mg of each KA sample was separated by repeating the HPLC eight times, then all corresponding fractions of the eight runs were pooled and lyophilized to dryness. The contaminant distributed in fractions 10 and 11 in all three samples. The residual weight of each fraction (Table 1) correlated well with its absorption at 270 nm (Fig 3b).

When each fraction was examined for mutagenicity in *S. typhimurium* TA100, fractions 6–10 of all three samples were mutagenic with and without S9 mix, while no significant mutagenicity was detected in the other fractions, under either condition. The total mutagenicity of each fraction of the three samples (Table 1) correlated well with its absorption at 270 nm (Figs 3). All three lots of KA supplied were slightly colored, but after preparative HPLC, the purified KA (fractions 6–9) was almost colorless, and fractions 10 and 11 were strongly colored according to the original color shade.

Table 2 shows the specific activities (revertants/mg) of fractions 6–10. Since fraction 10 contained significant amounts of contaminant, the average specific activities of fractions 6–9 of each sample are also indicated, together with those of the original KA samples, calculated from the data presented in Fig 2, by linear regression by least square method. The specific activities of the three samples were almost the same before and after separation by HPLC, with and without S9 mix.

Structure confirmation by NMR: The dried residues of fractions 6–9 of 052K165, 5312 and 2Y181 were subjected to ^{1}H and ^{13}C-NMR analysis. All of the protons in the KA structure (Fig. 1) were detected in the ^{1}H-NMR spectra, with the following chemical shifts; 4.29 (doublet, CH_2), 5.65 (triplet, CH_2-OH, exchanges with D_2O), 6.33 (singlet, 5-H), 8.02 (singlet, 2-H), and

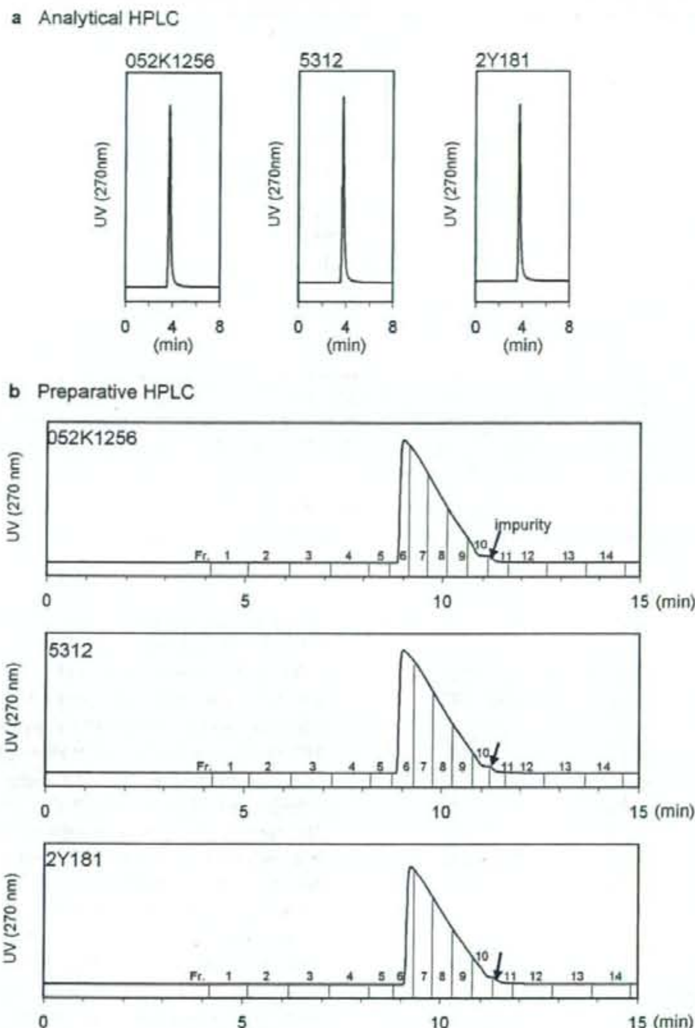

Fig. 3. Analytical (a) and preparative HPLC chromatograms (b) of three samples of KA (lot. 052K1256, 5312, 2Y181) (a): A 0.5 μL aliquot of KA solution (1 mg/mL) in distilled water was loaded on an analytical column (Mightysil RP-18 GP, 3 × 50 mm), and separated by an eluent of methanol − 0.05 v/v% trifluoroacetic acid (3:97) with a flow rate of 0.15 mL/min and absorption at 270 nm was recorded. (b): A 500 μL aliquot of KA solution (25 mg/mL) was applied to a preparative column, Mightysil RP-18 GP (5 μm, 10 × 250 mm), and separated by the same eluent as that used for analytical HPLC with a flow rate of 4 mL/min. In addition to the major peak of KA, a small shoulder peak of impurity was detected as indicated by arrow.

9.05 (singlet, C=C-OH, exchanges with D_2O) ppm. Further, the KA carbon skeleton was detected by ^{13}C-NMR at 60.01 (exocyclic carbon (CH_2)), 110.37 (C3), 139.79 (C6), 146.25 (C5), 168.61 (C2), and 176.09 (C4(C=O)) ppm. No impurity peaks were detected in any of the samples analyzed. Thus, the major 270 nm absorbing substances in these three samples were confirmed to be KA (data not shown).

***In vivo* genotoxicity of KA:** *In vivo* genotoxicity of KA was examined by MN assay in regenerating livers of mice and rats. Four days after partial hepatectomy (120 h after KA administration), mean values of MNHPCs in mice increased dose dependently and with 1000 mg/kg of KA, the value was significantly increased as compared with 0 dose of KA (Table 3). In rats, however, no increase was observed. Furthermore, KA was found to have no MN inducing ability in infant mice without partial hepatectomy (3 weeks old) (Table 4).

Table 2. Specific mutagenicity in *S. typhimurium* TA100 of KA samples after HPLC separation

Fraction No.	Revertants/mg					
	Lot. 052K1652		Lot. 5312		Lot. 2Y181	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
6	124	109	164	120	111	93
7	118	134	158	130	95	101
8	106	113	124	127	85	93
9	101	115	105	151	101	83
10	95	71	113	41	128	76
Average ± SEM*	112 ± 5.3	118 ± 5.6	138 ± 14	132 ± 6.7	98 ± 5.4	93 ± 3.7
Before HPLC**	116	113	115	106	106	111

Fractions 6, 8 and 9 were analyzed with 1 mg/plate of dried substance. Fraction 7 was tested with 0.5, 1.0 and 1.5 mg/plate of the dried substance, specific mutagenicity was calculated from the linear regression by the least square method. Other fractions were tested with 2 and 20% of dried substances, and results of tests with 20% of total residue of each fraction are indicated. With 2% weight of each fraction, no more than 20% of spontaneous mutagenicity was detected. For fraction 10, the mg amount applied to a plate is; lot.052K1652, 0.58 mg; lot. 5312, 0.46 mg; lot. 2Y181, 0.64 mg. Means of duplicate assays are indicated.

*Average of specific activities of fractions 6 ~ 9 ± standard error of means (SEM).
**Values were calculated from Fig. 2, based on the linear regression by the least square method.

Table 3. MN induced in regenerating liver of mice and rats treated with single gavages of KA

Species	Chemical	Dose (mg/kg)	MNHPCs/1000 HPCs (mean ± SEM)
Mouse	KA	0	2.33 ± 0.33
		500	5.00 ± 1.00
		1000	10.3 ± 1.45*
	DEN	160	15.7 ± 1.20*
Rat	KA	0	1.67 ± 0.33
		500	2.00 ± 0.58
		1000	1.33 ± 0.33
	DEN	160	17.3 ± 2.01*

KA, kojic acid; DEN, diethylnitrosamine
MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from control: $p < 0.05$.

Table 4. Micronuclei induced by KA in the liver of young mice

Chemical	Dose (mg/kg)	MNHPCs/1000HPCs (Mean ± SEM)		
		72 h	96 h	120 h
KA	0	0.75 ± 0.48	1.75 ± 0.75	1.5 ± 0.65
	500	0.75 ± 0.25	2.00 ± 0.82	1.75 ± 0.75
	1000	2.00 ± 0.41	2.00 ± 0.41	2.75 ± 0.75
Cyclophosphamide	20		8.75 ± 1.38*	

MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from KA 0 control: $p < 0.05$ by student *t* test.

Discussion

In the present study, KA samples used as a reagent, food additive and cosmetics ingredient showed mutagenicity in *S. typhimurium* TA100. The mutagenic activities of the samples were almost the same with or without S9 mix. The specific activity was weak, being around 100 revertants per mg (Table 2). The major, 270 nm absorbing component of each sample was isolated by preparative HPLC at a retention time of 8.5–10.5 min (Fig. 3b) and identified as KA by NMR analysis. Each KA sample supplied contained some UV absorbing materials which eluted later than KA (indicated by arrow in Fig. 3b). However, the impurity fraction, fraction 11, did not show any mutagenicity. When the mutagenic activities per unit weight of the three lots of KA supplied are compared with those of the major peak fractions 6–9 obtained by HPLC separation, there are no appreciable differences (Table 2). Although preparation methods of these three KA samples supplied are not available, features of these three samples are different and it is suspected that their preparation methods are different. Although a possibility of contamination of mutagenic non-UV absorbing material(s) which behave in the same way as KA on HPLC could not be completely negated, this possibility can be expected as negligible. Thus, it is indicated that all three lots did not contain mutagenic contaminants, and the mutagenic activity of the KA samples is solely derived from KA.

KA, at a dose of 1 g/kg, induced MN in the regenerating liver of mice. Thus, it seems that KA is genotoxic to liver. However this genotoxicity of KA was not detected in the liver of infant mice, on which partial hepatectomies were not performed. The reason for not detecting genotoxicity of KA in infant mice might have been because, while the mitotic index is expected to rise to a very high level after partial hepatectomy, it would be much lower in infant mice (3 weeks old). Further, differences in the metabolisms of infant mice and adult mice having received partial hepatectomies may play

some roles in this difference in genotoxicity (18). It is also noteworthy that KA did not induce MN in bone marrow of mice (12,13).

In contrast, KA was not genotoxic in regenerating rat's liver (Table 3). KA was also recently reported by Suzuki *et al.* (6). not to induce MN in young rats (4 weeks old). However, KA induced MN in peripheral blood of young rats (6). At present, neither the molecular mechanism of the genotoxicity of KA, nor the difference in metabolisms between rats and mice is known.

During preparation of this manuscript it has been reported in a two step carcinogenesis study, in which mice were fed a diet containing 3% KA at initiation step and phenobarbital was used as a tumor promoter, that there is no liver tumor initiating activity (19). At present, it is not clear whether the partial hepatectomy which was performed two weeks after the beginning of phenobarbital administration was at an appropriate time to detect initiating activity of KA or not. It is possible that the difference in the timing of the partial hepatectomy after cessation of KA administration might be one of the reasons for the discrepancy between KA being positive in MN induction and negative in initiating activity.

Although in the present study, genotoxicity of KA was detected in the mouse liver, it was not proved that this genotoxicity is involved in hepatic tumor development in mice.

Acknowledgement: Dr. Makoto Hayashi of National Institute of Health Sciences is thanked for his critical reading of this manuscript. This study was supported by Grants-in-Aid for the Health and Labour Sciences from the Ministry of Health, Labour and Welfare, Japan.

References

1 Manabe M, Goto T, Tanaka K, Matsuura S. The capabilities of the *Aspergillus flavus* group to produce aflatoxins and kojic acid. Rept Natl Food Res Inst. 1981; 38: 115–20 (Japanese).

2 Wehner FC, Thiel PG, van Rensburg SJ, Demasius IP. Mutagenicity to *Salmonella typhimurium* of some *Aspergillus* and *Penicillium* mycotoxins. Mutat Res. 1978; 58: 193–203.

3 Shibuya T, Murota T, Sakamoto K, Iwahara S, Ikeno M. Mutagenicity and dominant lethal test of kojic acid–Ames test, forward mutation test in cultured Chinese hamster cells and dominant lethal test in mice. J Toxicol Sci. 1982; 7: 255–62.

4 Bjeldanes LF, Chew H. Mutagenicity of 1,2-dicarbonyl compounds: maltol, kojic acid, diacetyl and related substances. Mutat Res 1979; 67: 367–71.

5 Wei CI, Huang TS, Fernando SY, Chung KT. Mutagenicity studies of kojic acid. Toxicol Lett. 1991; 59: 213–20.

6 Suzuki H, Ikeda N, Kobayashi K, Terashima Y, Shimada Y, Suzuki T, Hagiwara T, Hatakeyama S, Nagaoka K, Yoshida J, Saito Y, Tanaka J, Hayashi M. Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/ Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). Mutat Res. 2005; 583: 133–45.

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

8 Fujimoto N, Onodera H, Mitsumori K, Tamura T, Maruyama S, Ito A. Changes in thyroid function during development of thyroid hyperplasia induced by kojic acid in F344 rats. Carcinogenesis. 1999; 20: 1567–71.

9 Mitsumori K, Onodera H, Takahashi M, Funakoshi T, Tamura T, Yasuhara K, Takegawa K, Takahashi M. 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. 1999; 20: 173–6.

10 Takizawa T, Mitsumori K, Tamura T, Nasu M, Ueda M, Imai T, Hirose M. Hepatocellular tumor induction in heterozygous p53-deficient CBA mice by a 26-week dietary administration of kojic acid. Toxicol Sci. 2003; 7: 3287–93.

11 Maeda K, Fukuda M. *In vitro* effectiveness of several whitening cosmetic components in human melanocytes. J Soc Cosm Chem. 1991; 42: 361–8.

12 Nohynek GJ, Kirkland D, Marzin D, Toutain H, Leclerc-Ribaud C, Jinnai H. An assessment of the genotoxicity and human health risk of topical use of kojic acid [5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one]. Food Chem Toxicol. 2004; 42: 93–105.

13 Nonaka M, Omura H, Sofuni T, Hayashi M. Kojic acid did not induce micronuclei in mouse bonemarrow hematopoietic cells. MMS Commun. 1996; 4: 109–12.

14 Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983; 113: 173–215.

15 Yahagi T, Nagao M, Seino Y, Matsushima T, Sugimura T, Okada M. Mutagenicities of N-nitrosamines on Salmonella, Mutat Res. 1977; 48: 121–30.

16 Suzuki H, Shirotori T, Hayashi M. A liver micronucleus assay using young rats exposed to diethylnitrosamine: methodological establishment and evaluation. Cytogenet Genome Res. 2004; 104: 299–303.

17 Parton JW. Garriott ML, An evaluation of micronucleus induction in bone marrow and in hepatocytes isolated from collagenase perfused liver or from formalin-fixed liver using four week-old rats treated with known clastogens. Environ Mol Mutagen. 1997; 29: 379–85.

18 Rossi AM, Romano M, Zaccaro L, Pulci R, Salmona M. DNA synthesis, mitotic index, drug-metabolising systems and cytogenetic analysis in regenerating rat liver. Comparison with bone marrow test after '*in vivo*' treatment with cyclophosphamide. Mutat Res. 1987; 2: 75–82.

19 Moto M, Mori T, Okamura M, Kashida Y, Mitsumori K. Absence of liver tumor-initiating activity of kojic aid in mice. Arch Toxicol. 2005; Oct 18 Epub ahead of print.

Increased DNA Damage in ALDH2-Deficient Alcoholics

Tomonari Matsuda,*,† Hisatoshi Yabushita,† Robert A. Kanaly,† Shinya Shibutani,‡ and Akira Yokoyama§

Department of Technology and Ecology, Graduate School of Global Environmental Studies, Kyoto University, Kyoto, Japan, Department of Pharmacological Sciences, State University of New York, Stony Brook, New York, and National Hospital Organization Kurihama Alcoholism Center, Yokosuka, Japan

Received May 26, 2006

Drinking alcohol is a risk factor for cancers of the oral cavity, pharynx, larynx, and esophagus. Although many studies suggest that acetaldehyde, a major metabolite of orally ingested alcohol, plays a crucial role in cancer initiation, the link between the aldehyde dehydrogenase-2 (ALDH2) genotype and acetaldehyde-derived DNA damage has not yet been explored. We have developed a sensitive and quantitative method for detecting the acetaldehyde-derived DNA adducts, N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG), α-*S*- and α-*R*-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG), and N^2-(2,6-dimethyl-1,3-dioxan-4-yl)-deoxyguanosine (N^2-Dio-dG), by using liquid chromatography electrospray tandem mass spectrometry (LC/ESI-MS/MS) and stable-isotope internal standards. We determined the DNA adducts in 44 blood DNA samples from Japanese alcoholic patients. The levels of three acetaldehyde-derived DNA adducts, N^2-Et-dG, α-*S*-Me-γ-OH-PdG, and α-*R*-Me-γ-OH-PdG, were significantly higher in alcoholics with the *ALDH2*1/2*2* genotype compared to those with the *ALDH2*1/2*1* genotype. N^2-Dio-dG was not detected in any of the DNA samples analyzed. These results provide molecular evidence that the ALDH*2* genotype affects the genotoxic damage caused by acetaldehyde.

Introduction

Alcoholic beverages are a risk factor for several cancers including oral cavity, pharynx, larynx, and esophagus cancers (*1*). Acetaldehyde, an oxidized metabolite of ethanol, is suspected to be the ultimate carcinogen in alcohol-related cancers. Acetaldehyde reacts with DNA *in vitro*, resulting in the formation of DNA adducts, such as N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG)[1] (*2*), α-*S*- and α-*R*-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG), and N^2-(2,6-Dimethyl-1,3-dioxan-4-yl)-deoxyguanosine (N^2-Dio-dG) (*3*). The exposure of acetaldehyde to mammalian cells increases the frequency of sister chromatid exchanges and chromosomal aberrations (*4, 5*) and induces DNA interstrand-cross-links (*6*) and DNA-protein-cross-links (*7*). Acetaldehyde has shown in shuttle-vector pMY189 propagated in human cells to induce tandem base substitutions that are most likely produced via DNA-intrastrand cross-links (*6*). Inhalation of acetaldehyde significantly increased the incidence of nasal tumors (*8*). These experimental evidences suggested that acetaldehyde acts as a tumor initiator.

Most of the acetaldehyde generated during alcohol ingestion is eliminated by aldehyde dehydrogenase-2 (ALDH2) (*9*). The mutant *ALDH2*2* allele (Glu487Lys) encodes a catalytically inactive subunit (*10*). Because ALDH2 is a homotetrameric enzyme, individuals with the *ALDH2*1/2*2* genotype should have only 6.25% of the normal protein; other molecules containing one or more *ALDH2*2* subunits are considered to be inactive (*11*). Distribution of the *ALDH2*2* allele varies by race (*12*), and it is most prevalent among East Asians. Approximately 40% of Japanese have the *ALDH2*2* allele, but it is absent in Caucasians and Africans. When ALDH2 is inactive, the body fails to rapidly metabolize acetaldehyde, leading excessive accumulation. In fact, alcohol challenge tests showed that after drinking a small amount of ethanol (0.1 g/kg body weight), the average peak of blood acetaldehyde concentrations in *ALDH2*1/2*2* individuals was five times greater than that of active *ALDH2*1/2*1* homozygotes who consumed a moderate amount of ethanol (0.8 g/kg body weight) (*13*).

A link between the ALDH2 genotype and cancer rates has been suggested (*14, 15*), but no direct evidence exists. In the present study, we explored a relationship between the ALDH2 genotype and the formation of acetaldehyde-derived DNA adducts in the blood of 44 alcoholics. In order to quantify low levels of the DNA adducts, we employed a sensitive method using stable isotope dilution coupled with liquid chromatography electrospray tandem mass spectrometry (LC/ESI-MS/MS).

Experimental Procedures

Subjects and ALDH2 Genotyping. The participants in this study were 44 cancer-free, male Japanese alcoholic patients. This study was approved by the Ethics Committee of the National Hospital Organization Kurihama Alcoholism Center of Japan, and informed consent was obtained from participating patients. Information on the subjects' drinking profiles and smoking habits was obtained from the patients as described previously (*16*). Subjects' blood samples were obtained on the day of admission for alcoholism treatment, and ALDH2 genotyping was performed on blood DNA by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) analysis as described previously (*16*).

* Corresponding author. Phone: +81-75-753-5171. Fax: +81-75-753-3335. E-mail: matsuda@eden.env.kyoto-u.ac.jp.
† Kyoto University.
‡ State University of New York.
§ National Hospital Organization Kurihama Alcoholism Center.

[1] Abbreviations: HPLC, high performance liquid chromatography; LC/ESI-MS/MS, liquid chromatography electrospray tandem mass spectrometry; ALDH2, aldehyde dehydrogenase-2; N^2-Et-dG, N^2-ethyl-2′-deoxyguanosine; α-Me-γ-OH-PdG, α-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine; N^2-Dio-dG, N^2-(2,6-dimethyl-1,3-dioxan-4-yl)-2′-deoxyguanosine.

10.1021/tx060113h CCC: $33.50 © 2006 American Chemical Society
Published on Web 09/23/2006

Synthesis of DNA Adduct and Stable Isotope Standards. Analytical standards of N^2-Et-dG were prepared as described by Vaca et al. (*7*). Two diastereoisomers of α-Me-γ-OH-PdG and two diastereoisomers of N^2-Dio-dG were prepared as described by Wang et al. (*8*). The [U-$^{15}N_5$]-labeled adducts were also synthesized following previous methods (*7, 8*) using [U-$^{15}N_5$]-dG instead of dG and employed as the internal standards for LC/MS/MS.

Preparation of a Standard DNA Sample Containing N^2-Et-dG and α-Me-γ-OH-PdG. To make N^2-Et-dG-containing DNA, calf thymus DNA (73 μg in 100 μL) was mixed with 10 μL of acetaldehyde and incubated at 37 °C for 1 h. This was followed by treatment with approximately 3 mg of $NaBH_3CN$, and the mixture was incubated for another hour at 37 °C, yielding DNA containing N^2-Et-dG lesions. A similar approach was used to make calf thymus DNA containing α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG except that the DNA was reacted with 10 μL of crotonaldehyde and incubated for 2 h, and the reduction by $NaBH_3CN$ was unnecessary. The DNA samples were ethanol precipitated and gel-filtrated by using MicroSpin G-25 Columns (Amersham Biosciences, U.K.) mixed with untreated calf thymus DNA, and the final standard concentrations were determined as indicated in the Results section.

Isolation of DNA from Blood and Digestion with Internal Standards. DNA isolation was performed by the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany). Blood DNA samples (20 μg) were digested to their corresponding 2′-deoxyribonucleoside-3′-monophosphates by the addition of 15 μL of 17 mM citrate plus 8 mM $CaCl_2$ buffer that contained micrococcal nuclease (22.5 units) and spleen phosphodiesterase (0.075 units) plus internal standards. Solutions were mixed and incubated for 3 h at 37 °C, after which alkaline phosphatase (3 units), 10 μL of 0.5 M Tris HCl (pH 8.5), 5 μL of 20 mM $ZnSO_4$, and 67 μL distilled water were added and incubated further for 3 h at 37 °C. The digested sample was extracted twice with methanol. The methanol fractions were evaporated to dryness, resuspended in 100 μL of distilled water, and subjected to LC/ESI-MS/MS.

Instrumentation. LC/ESI-MS/MS analyses were performed using a Shimadzu LC system (Shimadzu) interfaced with a Quattro Ultima triple stage quadrupole MS (Waters-Micromass, Manchester, U.K.). The LC column was eluted over a gradient that began at a ratio of 2% methanol to 98% water and was changed to 40% methanol over a period of 40 min, changed to 80% methanol from 40 to 45 min, and finally returned to the original starting conditions, 2:98, for the remaining 15 min. The total run time was 60 min. Sample injection volumes of 50 μL each were separated on a Shim-pack FC-ODS column (4.6 × 150 mm; Shimadzu, Japan) and eluted at a flow rate of 0.4 mL/min. Mass spectral analyses were carried out in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C, and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 L/h), and cone gas (35 L/h) and argon were used as the collision gas at a collision cell pressure of 1.5×10^{-3} mBar. Positive ions were acquired in MRM mode. The MRM transitions monitored were as follows: N^2-Dio-dG, *m/z* 382 → 266; [$^{15}N_5$]- α-Me-γ-OH-PdG, *m/z* 343 → 227; α-Me-γ-OH-PdG, *m/z* 338 → 222; [$^{15}N_5$]-N^2-Et-dG, *m/z* 301 → 185; N^2-Et-dG, *m/z* 296 → 180. The amount of each DNA adduct was quantified by the ratio of the peak area of the target adducts and of its stable isotope (stable isotope-dilution method). QuanLynx (ver. 4.0) software (Waters-Micromass, Manchester, U.K.) was used to create standard curves and calculate the adduct concentrations. The amount of deoxyguanosine (dG) was monitored by a Shimadzu SPD-10A UV-Visible detector that was in place before the tandem mass spectrometer. The adduct level is shown as femtomoles of adducts per micromoles of dG. The number of DNA adducts per 10^9 bases was calculated by the following equation: Number of DNA adducts per 10^9 bases = adduct level (fmol/ μmol dG) × 0.218 (μmol dG/μmol dN), where 0.218 is the average ratio of guanine bases relative to total base number in human blood DNA calculated from this experiment (data not shown).

Table 1. Genotype, Age and Drinking and Smoking Habits of 44 Japanese Alcoholics[a]

ALDH2 genotype	n	age (years)	ethanol (g/day)	duration of drinking (years)	cigarettes (no/day)
*2*1/2*1*	19	52 ± 11	130 ± 54	26 ± 13	22 ± 13
*2*1/2*2*	25	51 ± 11	105 ± 59	24 ± 12	24 ± 15

[a] Information was obtained from patients and, when available, their partners upon admission to the program. The information included the daily alcohol consumption during the year preceding admission, the duration of habitual drinking, and the daily number of cigarettes currently smoked. Daily alcohol consumption was expressed in grams per day of ethanol using standard conversion for alcoholic beverages: beer was considered to be 5% ethanol (v/v); wine, 12%; sake, 16%; shochu, 25%; and whiskey, 40%.

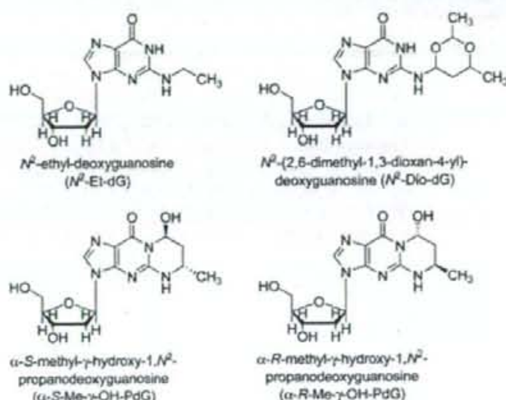

Figure 1. Structure of DNA adducts induced by acetaldehyde.

Results

We enrolled 19 alcoholic patients with the *ALDH2*1/2*1* genotype and 25 alcoholic patients with the *ALDH2*1/2*2* genotype. The averages of age, daily ethanol consumption, duration of drinking, and daily cigarette consumption were not significantly different between the ALDH2 genotypes (Table 1).

Figure 2 shows the MS/MS chromatogram (raw data, without smoothing) of five acetaldehyde-derived DNA adduct standards: two diastereoisomers of N^2-Dio-dG, two diastereoisomers of α-Me-γ-OH-PdG, and N^2-Et-dG. The amount of injection on column, for each, was 250 fg, corresponding to 2 to 3 adducts per 10^8 bases in 10 μg of DNA. Sufficient signal-to-noise ratios was observed in each peak, indicating the high sensitivity of this analysis.

To make a standard DNA solution containing N^2-Et-dG and α-Me-γ-OH-PdG, acetaldehyde-treated and crotonaldehyde-treated calf thymus DNA and untreated calf thymus DNA were mixed in proper proportions. Then, 10 fmol each of the three stable isotope standards, [U-$^{15}N_5$]-N^2-Et-dG, [U-$^{15}N_5$]-α-*S*-Me-γ-OH-PdG, and [U-$^{15}N_5$]-α-*R*-Me-γ-OH-PdG, were spiked into 20 μg of the standard DNA and enzymatically digested to deoxynucleosides; half of the digests were subjected to LC/ESI-MS/MS analysis. Repeated analysis (eight times) of the same standard DNA gave 613 ± 52 fmol/μmol dG (CV = 8.5%), 592 ± 40 fmol/μmol dG (CV = 6.7%), and 845 ± 81 fmol/μmol dG (CV = 9.6%) for N^2-Et-dG, α-*S*-Me-γ-OH-PdG, and α-*R*-Me-γ-OH-PdG, respectively.

Next, the DNA samples extracted from the blood of the 44 alcoholics were analyzed by the same procedures. Representative chromatograms of acetaldehyde-derived DNA adducts in blood

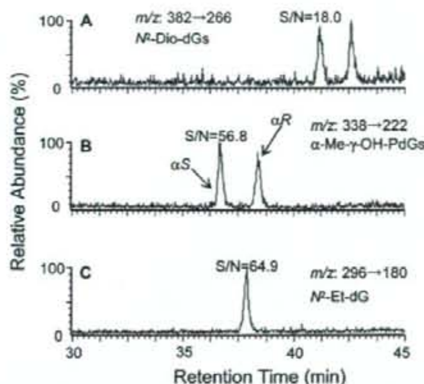

Figure 2. MS/MS chromatograms of five acetaldehyde-derived DNA adduct standards. (A) Two diastereomers of N^2-Dio-dG (*m/z*: 382 → 266); (B) two diasteromers of α-Me-γ-OH-PdG (*m/z*: 338 → 222); (C) N^2-Et-dG (*m/z*: 296 → 180). A Quattro Ultima Pt triple stage quadrupole MS spectrometer was used. The analytical settings are as described in Experimental Procedures. Fifty microliters of each 5 pg/mL solution of a 5-adduct mixture was injected (250 fg on column). The signal-to-noise ratio (S/N) was calculated by using the MassLynx (ver 4.0) software.

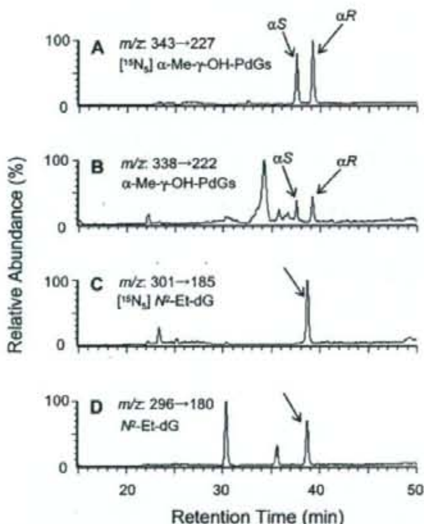

Figure 3. LC/ESI-MS/MS detection of acetaldehyde-derived DNA adducts from human blood. Stable isotope internal standards [$^{15}N_5$]-α*S*- and α*R*-Me-γ-OH-PdG (A) and [$^{15}N_5$]-N^2-Et-dG (C) were spiked into DNA samples and monitored at transitions *m/z* 343 → 227 and 301 → 185 as indicated and were used for the unambiguous confirmation of α*S*- and α*R*-Me-γ-OH-PdG (B) and N^2-Et-dG (D) at transitions *m/z* 338 → 222 and 296 → 180. In the sample shown in the Figure, N^2-Et-dG, α*S*-, and α*R*-Me-γ-OH-PdG were detected at levels of 51, 8, and 13 adducts per 10^9 bases, respectively, illustrating the sensitivity of the method.

DNA are shown in Figure 3. The peaks of N^2-Et-dG, α-*S*-Me-γ-OH-PdG, and α-*R*-Me-γ-OH-PdG were clearly observed at the same retention time as that of their stable isotope internal standards. Peaks corresponding to N^2-Dio-dG were not detected in any of the samples analyzed.

We found that the average levels of three acetaldehyde-derived adducts were significantly higher in *ALDH2*1/2*2*

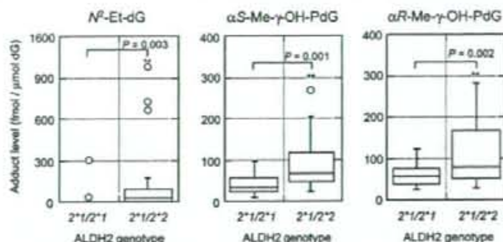

Figure 4. Box plots illustrating that the average levels of three acetaldehyde-derived adducts were significantly higher in the blood DNA of alcoholics who possessed the *ALDH2*1/2*2* genotype compared to alcoholics who possessed the *ALDH2*1/2*1* genotype. The *P* values are given for the Wilcoxon–Mann–Whitney test (N^2-Et-dG) and *t*-test (α-Me-γ-OH-PdGs).

alcoholics. N^2-Et-dG was detected in 14 out of 25 blood DNA samples from the *ALDH2*1/2*2* alcoholics but in only 2 out of 19 from the *ALDH2*1/2*1* alcoholics. The average level of N^2-Et-dG in the blood samples from the *ALDH2*1/2*2* alcoholic patients (average ± SEM: 130 ± 52 fmol/μmol dG, $n = 25$) was significantly higher than that from the *ALDH2*1/2*1* alcoholics (17.8 ± 15.9 fmol/μmol dG, $n = 19$) (Figure 4). α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG adducts were also detected in all analyzed blood samples. As was shown for N^2-Et-dG, the levels of α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG in DNA samples from the *ALDH2*1/2*2* alcoholics (average ± SEM: 92.4 ± 12.9 and 114 ± 15 fmol/μmol dG, $n = 25$) were also significantly higher than those of the *ALDH2*1/2*1* alcoholics (42.9 ± 6.0 and 61.3 ± 6.4, $n = 19$) (Figure 4).

Discussion

Using stable isotope internal standards, we determined acetaldehyde-derived DNA adducts, N^2-Et-dG, α-Me-γ-OH-PdG, and N^2-Dio-dG, in the blood DNA of alcoholic patients by LC/ESI-MS/MS. N^2-Et-dG and α-Me-γ-OH-PdG were detected; however, N^2-Dio-dG was not detected in any of the blood DNA samples. Because N^2-Dio-dG is produced from three acetaldehyde molecules, a high concentration of acetaldehyde may be required in the human body to form this adduct.

The reaction of acetaldehyde with dG results in the formation of an unstable Schiff base at the N^2 position of dG (N^2-ethylidene-dG). The formation of the stable N^2-Et-dG adduct requires a subsequent reduction step that must be accomplished by some reducing agent such as vitamin C, glutathione, and so forth. Using a ^{32}P-postlabeling procedure in combination with HPLC-radioisotope detection, Fang and Vaca (*7*) detected 2 to 3 adducts per 10^7 bases of N^2-Et-dG in white blood cell DNA obtained from Swedish alcoholic patients. In the present study, we found, using a more reliable LC/ESI-MS/MS and internal standards, that the average level of blood N^2-Et-dG adducts in *ALDH2*1/2*2* and *ALDH2*1/2*1* alcoholics were 28.3 and 3.9 adducts per 10^9 bases, respectively; the level was at least 1 or 2 orders of magnitude lower in Japanese alcoholic patients than that observed with the Swedish alcoholic patients by ^{32}P-postlabeling. This year, Wang et al. (*17*) showed that N^2-ethylidene-dG in human liver DNA is relatively stable and that the presence of this adduct could be confirmed by the detection of N^2-Et-dG after the reduction of DNA during isolation and enzymatic hydrolysis. They showed that when the reduction step was included during these steps approximately a few 100 times more N^2-Et-dG was detected in some cases. Although the biological significance of N^2-ethylidene-dG has not yet been

determined, this new adduct may be a good biomarker to evaluate exposure to acetaldehyde, and we will consider evaluating this adduct in future studies.

The mutagenicity of N^2-Et-dG has been reported previously (*15*). The primer extension assay using site-specifically modified oligodeoxynucleotides containing a single N^2-Et-dG revealed that dGMP is incorporated opposite the N^2-Et-dG adduct during DNA synthesis and is catalyzed by the *Escherichia coli* DNA polymerase I Klenow fragment (40% of fully extended primer contained dGMP opposite to N^2-Et-dG in template), indicating that this adduct induces G to C mutations (*18*). N^2-Ethyl-2′-deoxyguanosine triphosphate (N^2-Et-dGTP) was effectively utilized during DNA synthesis catalyzed by mammalian DNA polymerases α and δ (*19*). N^2-Et-dG strongly blocks polymerization of DNA polymerase α, but DNA polymerase η bypassed the lesion in an error-free manner (*20*). However, miscoding and the mutagenic potential of the N^2-Et-dG adduct have not so far been extensively explored in mammalian cells.

The exposure of DNA to acetaldehyde *in vitro* resulted in the formation of α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG (*8*). The formation of α-Me-γ-OH-PdGs via acetaldehyde is facilitated by basic amino acids, such as arginine and lysine, histones, or polyamines, such as spermine and spermidine, indicating the possibility of the formation of these adducts by acetaldehyde *in vivo* (*21*, *22*). When a single-strand shuttle vector containing a single diastereoisomer of α-Me-γ-OH-PdG was propagated in a mammalian cell line (*23*), the mutational frequency was 5 to 6%; G to T transversions were dominantly detected. In addition, α-Me-γ-OH-PdGs are thought to be the precursor lesions to DNA−DNA or DNA−protein cross-links. In the nucleoside form or in single-stranded DNA, α-Me-γ-OH-PdGs exist in cyclic form. However, in double-stranded DNA, the ring-opened form of the lesion containing a free aldehyde group is dominant. Kozekov et al. (*24*) demonstrated that double-stranded oligonucleotides that contain a single α-*S*-Me-γ-OH-PdG or α-*R*-Me-γ-OH-PdG spontaneously formed DNA interstrand cross-links. The reaction of forming DNA interstrand cross-links was very slow, taking a few days to a few weeks, although the reaction with α-*R*-Me-γ-OH-PdG was significantly faster than that with α-*S* diastereomer. When double stranded oligonucleotides containing a single α-*S*-Me-γ-OH-PdG or α-*R*-Me-γ-OH-PdG were mixed with the peptide (Lys-Trp-Lys-Lys), the DNA−protein cross-links were also formed (*25*) within an hour. DNA interstrand cross-links and DNA−protein cross-links have been documented in acetaldehyde-exposed mammalian cells and may lead to chromosomal aberrations and sister chromatid exchanges. α-Me-γ-OH-PdG adducts may contribute to the development of alcohol-induced cancers.

Studies of various Japanese drinking populations have shown that deficient ALDH2 encoded by the *ALDH2*1/2*2* genotype is a high risk factor for esophageal cancer. For example, heavy drinkers with the *ALDH2*1/2*2* genotype have a high odd ratio (16.4) of getting esophageal cancer (*14*). We demonstrated that the level of acetaldehyde-derived DNA adducts in the alcoholics with the *ALDH2*1/2*2* genotype is much higher than that in alcoholics with the *ALDH2*1/2*1* genotype. Our results strongly indicate that the ALDH2 genotype plays a crucial role in the formation of acetaldehyde-derived DNA adducts.

Ishikawa et al. demonstrated that micronuclei frequency in peripheral blood lymphocytes of ALDH2-deficient habitual drinkers were slightly, but significantly higher than that of ALDH2-proficient habitual drinkers (*26*). Morimoto and Takeshita reported that lymphocytes from habitual drinkers with the deficient ALDH2 enzyme had significantly higher frequencies of sister chromatid exchanges than those from ALDH2-proficient individuals (*27*). These observations suggest that the ALDH2 genotype may affect the risk of cancer initiation by acetaldehyde in humans. In this study, we showed more direct evidence that the formation of acetaldehyde-derived DNA adducts, N^2-Et-dG and α-Me-γ-OH-PdG, was closely related to the ALDH*2* genotype in populations that are continuously exposed to high levels of acetaldehyde by alcohol consumption. Taken together, the observations from biochemical, epidemiological, and molecular epidemiological studies discussed above, in conjunction with this study, well fit the scenario that acetaldehyde is a primary causative factor for alcohol-induced cancers.

Acknowledgment. This research was supported in part by Grants-in-aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan and Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

(1) IARC (1988) Alcohol Drinking. *IARC Monograph on the Evaluation of the Carcinogenic Risk to Humans*, Vol. 44, pp 153−246, IARC, Lyon, France.
(2) Fang, J. L., and Vaca, C. (1997) Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers. *Carcinogenesis 18*, 627−632.
(3) Wang, M., McIntee, E. J., Cheng, G., Shi, Y., Villalta, P. W., and Hecht, S. S. (2000) Identification of DNA adducts of acetaldehyde. *Chem. Res. Toxicol. 13*, 1149−1157.
(4) Obe, G., Natarajan, A. T., Meyers, M., and Hertog, A. D. (1979) Induction of chromosomal aberrations in peripheral lymphocytes of human blood *in vitro*, and SCEs in bone-marrow cells of mice in vivo by ethanol and tits metabolite acetaldehyde. *Mutat. Res. 68*, 291−294.
(5) Obe, G., and Ristow, H. (1977) Acetaldehyde, but not ethanol, induces sister chromatid exchanges in Chinese hamster cells *in vitro*. *Mutat. Res. 56*, 211−213.
(6) Matsuda, T., Kawanishi, M., Yage, T., Matsui, S., and Takebe, H. (1998) Specific tandem GG to TT base substitutions induced by acetaldehyde are due to intra-strand crosslinks between adjacent guanine bases. *Nucleic Acids Res. 26*, 1769−1774.
(7) Kuykendall, J. R., and Bogdanffy, M. S. (1992) Efficiency of DNA-histone crosslinking induced by saturated and unsaturated aldehydes *in vitro*. *Mutat. Res. 283*, 131−136.
(8) IARC (1985) Allyl Compounds, Aldehydes, Epoxides and Peroxides. *IARC Monograph on the Evaluation of the Carcinogenic Risk to Humans*, Vol. 36, pp 101−132, IARC, Lyon, France.
(9) Bosron, W. F., and Li, T. K. (1986) Genetic polymorphism of human liver alcohol and aldehyde dehudrogenases, and their relationship to alcohol metabolism and alcoholism. *Hepatology 6*, 502−510.
(10) Yoshida, A., Hsu, L., and Yasunami, M. (1991) Genetics of human alcohol-metabolizing enzymes. *Prog. Nucleic Acids Res. Mol. Biol. 40*, 255−287.
(11) Crabb, D. W., Edenberg, H. J., Bosron, W. F., and Li, T. K. (1989) Genotype for aldehyde dehydrogenase deficiency and alcohol sensitivity: the inactive ALDH22 allele is dominant. *J. Clin. Invest. 83*, 314−316.
(12) Goedde, H. W., Agarwal, D. P., Fritze, G., et al. (1992) Distribution of ADH2 and ALDH2 genotyps in different populations. *Hum. Genet. 88*, 344−346.
(13) Enomoto, N., Takase, S., Yasuhara, M., and Takada, A. (1991) Acetaldehyde metabolism in different aldehyde dehydrogenase-2 genotypes. *Alcohol Clin. Exp. Res. 15*, 141−144.
(14) Yokoyama, A., and Omori, T. (2003) Genetic polymorphisms of alcohol and aldehyde dehydrogenases and risk for esophageal and head and neck cancers. *Jpn. J. Clin. Oncol. 33*, 111−121.
(15) Brooks, P., and Theruvathu, J. A. (2005) DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. *Alcohol 35*, 187−193.
(16) Yokoyama, A., Muramatsu, T., Ohmori, T., Yokoyama, T., Okuyama, K., Takahashi, H., Hasegawa, Y., Higuchi, S., Maruyama, K., Shirakura, K., and Ishii, H. (1998) Alcohol-related cancers and aldehyde dehydrogenase-2 in Japanese alcoholics. *Carcinogenesis 19*, 1383−1387.

(17) Wang, M., Nanxiong, Y., Li, C., Villalta, P. W., Hochalter, J. B., and Hecht, S. S. (2006) Identification of an acetaldehyde adduct in human liver DNA and quantitation as N^2-ethyldeoxyguanosine. *Chem. Res. Toxicol. 19*, 319−324.

(18) Terashima, I., Matsuda, T., Fang, T., Suzuki, N., Kobayashi, J., Kohda, K., and Shibutani, S. (2001) Miscoding potential of the N^2-ethyl-2′-deoxyguanosine DNA adduct by the exonuclease free Klenow fragment of Escherichia coli DNA polymerase I. *Biochemistry 40*, 4106−4114.

(19) Matsuda, T., Terashima, I., Matsumoto, Y., Yabushita, H., Matsui, S., and Shibutani, S. (1999) Effective utilization of N^2-ethyl-2′-deoxyguanosine triphosphate during DNA synthesis catalyzed by mammalian replicative DNA polymerases. *Biochemistry 38*, 929−935.

(20) Perrino, F. W., Blans, P., Harvey, S., Gelhaus, S. L., McGrath, C., Akman, S. A., Jenkins, G. S., LaCourse, W. R., and Fishbein, J. C. (2003) The N^2-ethylguanine and the O^6-ethyl-and O^6-methylguanine lesions in DNA: Contrasting responses form the "bypass" DNA polymerase η and the replicative DNA polymerase α. *Chem. Res. Toxicol. 16*, 1616−1623.

(21) Sako, M., Inagaki, S., Esaka, Y., and Deyashiki, Y. (2003) Histones accelerate the cyclic 1, N^2-propanoguanine adduct-formation of DNA by the primary metabolite of alcohol and carcinogenic crotonaldehyde. *Bioorg. Med. Chem. Lett. 13*, 3497−3498.

(22) Theruvathu, J. A., Jaruga, P., Nath, R. G., Dizdaroglu, M., and Brooks, P. J. (2005) Polyamines stimulate the formation of mutagenic 1, N^2-propanodeocyguanosine adducts from acetaldehyde. *Nucleic. Acids. Res. 33*, 3513−3520.

(23) Fernandes, P., Kanuri, M., Nechev, L. V., Harris, T. M., and Lloyd, R. S. (2005) Mammalian cell mutagenesis of the DNA adducts of vinyl chloride and crotonaldehyde. *Environ. Mol. Mutagen. 45*, 455−459.

(24) Kozekov, I. D., Nechev, L. V., Moseley, M. S., Harris, C. M., Rizzo, C. J., Stone, M. P., and Harris, T. M. (2002) DNA interchain cross-links formed by acrolein and crotonaldehyde. *J. Am. Chem. Soc. 125*, 50−61.

(25) Kurtz, A. J., and Lloyd, R. S. (2003) 1, N^2-Deoxyguanosine adducts of acrolein, crotonaldehyde, and trans-4-hydroxynonenal cross-link to peptides via schiff base linkage. *J. Biol. Chem. 278*, 5970−5976.

(26) Ishikawa, H., Yamamoto, H., Tian, Y., Kawano, M., Yamauchi, T., and Yokoyama, K. (2003) Effects of ALDH2 gene polymorphisms and alcohol-drinking behavior on micronuclei frequency in non-smokers. *Mutat. Res. 541*, 71−80.

(27) Morimoto, K., and Takeshita, T. (1996) Low Km aldehyde dehydrogenase (ALDH2) polymorphism, alcohol-drinking behavior, and chromosome alterations in peripheral lymphocytes. *Environ. Health Perspect. 104*, 563−567.

TX060113H

Antiestrogens and the Formation of DNA Damage in Rats: A Comparison

Sung Yeon Kim,[†,‡] Naomi Suzuki,[†] Y. R. Santosh Laxmi,[†] Atsushi Umemoto,[‡] Tomonari Matsuda,[∥] and Shinya Shibutani[*,†]

Laboratory of Chemical Biology, Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651, Department of Surgery, School of Medicine, University of Tokushima, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan, and Department of Technology and Ecology, Graduate School of Global Environmental Studies, Kyoto University, Kyoto, 606-8501, Japan

Received March 7, 2006

Tamoxifen (TAM) has been used as an agent for the treatment and prevention of breast cancer. However, long-term treatment of TAM in women increases the risk of developing endometrial cancer. The secondary cancer may be due to the genotoxicity of TAM. To find safer alternatives, four selective estrogen receptor modulators (SERMs), 4-hydroxytamoxifen (4-OHTAM), toremifene (TOR), raloxifene (RAL), and ICI 182,780, were administered to rats with an equimolar dose of TAM [54 μmol/kg (20 mg/kg)/day, p.o. for 7 days]. To evaluate the genotoxicity of each SERM, the presence of bulky DNA adducts was determined by ^{32}P-postlabeling/polyacrylamide gel electrophoresis and ^{32}P-postlabeling/high-performance liquid chromatography. The formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG) was analyzed as a marker of typical oxidative damage, using liquid chromatography electrospray tandem mass spectrometry. Among the SERMs, bulky DNA adducts were detected in the livers of rats treated with TAM; the total amount of TAM−DNA adducts was 26.1 adducts/10^7 nucleotides. However, with a detection limit of ~2 adducts/10^9 nucleotides, no bulky DNA adducts were observed with 4-OHTAM, TOR, RAL, or ICI 182,780. In addition, no significant increase of hepatic 8-oxodG lesions was detected in rats treated with any of the antiestrogens. Therefore, TOR, RAL, and ICI 182,780 are likely to be less genotoxic than TAM.

Introduction

Selective estrogen receptor modulators (SERMs)[1] have been used as agents for the treatment and prevention of breast cancer (*1*). Tamoxifen (TAM, the structure in Figure 1), the first clinically relevant SERM, has been widely used as a first-line endocrine therapy for breast cancer patients with positive estrogen receptors (ERs) and is also a prophylactic agent for women at high risk of this disease. Besides this significant benefit, long-term treatment of TAM in women increases the risk of developing endometrial cancer (*2*−*5*). The carcinogenic effects may be due to the estrogenic activity of TAM through the ER (*6*, *7*) and/or genotoxicity of TAM (reviewed by *8*).

In rats treated with TAM, a high level of TAM−DNA adducts are produced in the liver (*9*−*12*) and initiate the development of hepatocellular carcinomas (*13*, *14*). TAM−DNA adducts are formed through *O*-sulfonation of α-hydroxylated TAM metabolites (*8*); α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-TAM) and α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen (dG-N^2-*N*-desTAM) are detected as major hepatic DNA adducts using mass spectroscopic and ^{32}P-postlabeling/HPLC analyses (*12*, *15*). There is a controversy regarding the detection of TAM−DNA adducts in human tissues (*16*, *17*). TAM−DNA adducts were detected in endometrial tissues of breast cancer patients treated with TAM, using ^{32}P-postlabeling analysis (*18*) and accelerator mass spectrometry (*19*). Other groups did not detect TAM−DNA adducts in the endometrial tissues (*20*−*22*). A high frequency of K-*ras* mutations was recently observed in the endometrium of women treated with TAM (*23*); the mutational specificity was consistent with that which occurred at the TAM−DNA adduct (*24*).

In addition, 4-hydroxytamoxifen (4-OHTAM), a principle TAM metabolite, may be activated metabolically to the quinone methide and quinone that can react with DNA and protein (Figure 2A) (*25*−*27*). An in vitro activation of 4-OHTAM regenerated reactive oxygen species resulted in the increased formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG), an abundant mutagenic DNA lesion (*28*, *29*). Such oxidative DNA damage may also be involved in TAM-induced cancers.

Toremifene (TOR, Figure 1), a chlorinated TAM derivative, has been used in breast cancer therapy. The clinical efficacy of TOR in treating advanced breast cancer was similar to that of TAM (*30*). Although TOR is metabolized similar to TAM (*8*), this drug is not a hepatocarcinogen in rats (*14*, *31*). There has not been any significant clinical report that TOR increases the incidence of endometrial cancer.

Raloxifene (RAL, Figure 1) is currently used for osteoporosis but has not yet been approved by the Food and Drug Administration (FDA) for breast cancer therapy. A recent

* To whom correspondence should be addressed. Tel: 631-444-7849. Fax: 631-444-3218. E-mail: shinya@pharm.stonybrook.edu.
† State University of New York at Stony Brook.
‡ Present address: Department of Pharmacy, Wonkwang University, Iksan Chonbuk 570-709, South Korea.
§ University of Tokushima.
∥ Kyoto University.

[1] Abbreviations: SERM, selective estrogen receptor modulator; ER, estrogen receptor; TAM, tamoxifen; α-OHTAM, α-hydroxytamoxifen; 4-OHTAM, 4-hydroxytamoxifen; TOR, toremifene; RAL, raloxifene; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)tamoxifen; dG-N^2-*N*-desTAM, α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen; 8-oxodG, 7,8-dihydro-8-oxodeoxyguansine; PAGE, polyacrylamide gel electrophoresis; LC/MS/MS, liquid chromatography electrospray tandem mass spectrometry.

10.1021/tx060052n CCC: $33.50 © 2006 American Chemical Society
Published on Web 05/16/2006

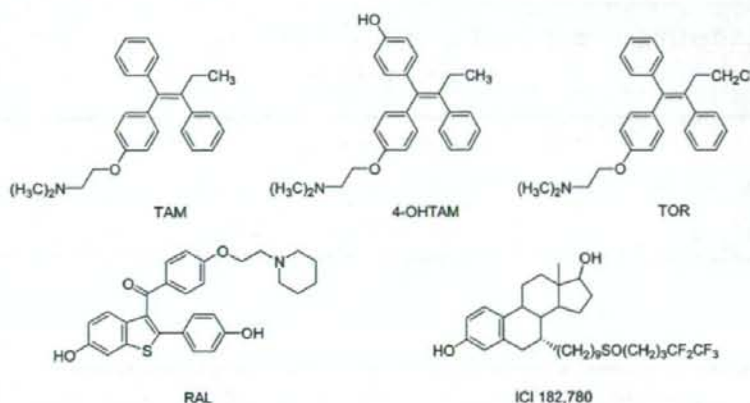

Figure 1. Structure of antiestrogens.

chemoprevention trial showed that RAL significantly reduced the incidence of breast cancer in women at high risk of developing this disease (*32*). RAL has been shown to be metabolically activated to electrophilic and redox active quinoids that can react with protein including GST (Figure 2B) (*33*). 8-OxodG may be produced through redox cycling between RAL catechols and *o*-quinones. These results suggest that electrophilic species of RAL may have the potential to damage macromolecules to cause the toxicity.

ICI 182,780 (Figure 1) is a pure antiestrogen, which blocks the tropic action of estradiol in the rat uterus and is free from estrogen agonist activity (*34*). ICI 182,780 is approved for treatment of postmenopausal breast cancer patients who fail to respond to TAM therapy (*35*). Because the structure of ICI 182,780 resembles 17β-estradiol, this drug may be similarly metabolized and could react with DNA. However, the contribution of reactive oxygen species generated from ICI 182,780 to genotoxicity has not been explored.

To determine the genotoxic potential of SERM, female rats were treated for a week with 4-OHTAM, TOR, RAL, or ICI 182,780 and compared with TAM. The level of hepatic DNA adducts induced by each drug was examined using ^{32}P-postlabeling/polyacrylamide gel electrophoresis (PAGE) and ^{32}P-postlabeling/HPLC analyses. The formation of 8-oxodG was analyzed by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS) analysis.

Materials and Methods

Materials. TAM, TOR, and RAL were purchased from LKT Laboratories, Inc. (St. Paul, MN). ICI 182,780 was obtained from TOCRIS (Ellisville, MO). 4-OHTAM, calf thymus DNA, and potato apyrase were purchased from Sigma-Aldrich (St. Louis, MO). Micrococcal nuclease and spleen phosphodiesterase were obtained from Worthington Biochemical Corp. (Lakewood, NJ). Nuclease P1 was obtained from United States Biological (Swampscott, MA). 3′-Phosphatase-free T4 PNK was obtained from Roche Applied Science (Indianapolis, IN). Trans forms (fr-1 and fr-2) and cis forms (fr-3 and fr-4) of dG 3′-monophosphate-N^2-tamoxifen (dG$_3$′p-N^2-TAM) were prepared as described previously (*36*). [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from GE Healthcare (Piscataway, NJ).

Animal Study. Fisher 344 rats (female, 8 weeks old) were purchased from Taconic (Germantown, NY). The use of animals was in compliance with the guidelines established by the NIH Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled rooms with a 12 h light−dark cycle for at least 1 week prior to use. Regular laboratory chow and tap water were allowed ad lib. Rats were treated orally with TAM [20 mg (54 μmol)/kg/day] for 7 days. 4-OHTAM (21 mg/kg), TOR (22 mg/kg), RAL (26 mg/kg), or ICI 182,780 (33 mg/kg) was given to rats in an equimolar dose of TAM (54 μmol/kg). Control rats were treated with an identical volume of corn oil. TAM-treated rats were euthanized by CO_2 asphyxiation at 5 h after the final treatment and open thoracotomy. All tissues were removed quickly, frozen, and stored at −80 °C until DNA extraction.

Digestion of DNA Samples. The tissue DNA was extracted using a Qiagen DNA isolation kit (Valencia, CA) following the manufacturer's protocol. The concentration of DNA was determined by UV spectroscopy as 50 μg/mL = OD$_{260nm}$1.0. A DNA sample (2.5 μg) was enzymatically digested overnight at 37 °C in 100 μL of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM $CaCl_2$, using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (*37*). The reaction mixture was incubated for another 1 h with nuclease P1 (1 unit). After the incubation, 150 μL of water was added. The reaction samples were then extracted twice with 200 μL of butanol. The butanol fractions were combined, back-extracted with 50 μL of distilled water, and evaporated to dryness.

^{32}P-Postlabeling/PAGE Analysis. The DNA digests were incubated at 37 °C for 40 min with 10 μCi of [γ-^{32}P]ATP and 3′-phosphatase-free T4 polynucleotide kinase (10 units) and then incubated for another 30 min with apyrase (50 milliunits), as described previously (*37*). Known amounts (0.076, 0.0076, 0.00076, or 0.000076 pmol) of dG-N^2-TAM-modified oligodeoxynucleotide were mixed with 2.5 μg of calf thymus DNA (7600 pmol) and served as a standard (1 adduct/10^5 nucleotides, 1 adduct/10^6 nucleotides, 1 adduct/10^7 nucleotides, or 1 adduct/10^8 nucleotides). A part of the ^{32}P-labeled sample was electrophoresed for 4−5 h on a nondenaturing 30% polyacrylamide gel (35 cm × 42 cm × 0.04 cm) with 1400−1600 V/20−40 mA. The position of ^{32}P-labeled adducts was established by β-phosphorimager analysis (Molecular Dynamics Inc.). To quantify the level of ^{32}P-labeled products, integrated values were measured using a β-phosphorimager and compared with the standards. The detection limit for 5 μg of DNA was approximately 7 adducts/10^9 nucleotides.

^{32}P-Postlabeling/HPLC Analysis. When the ^{32}P-labeled products were developed on the gel as described above, the bands of ^{32}P-labeled products were cut from the gel and put into 2 mL of distilled water overnight at room temperature. The ^{32}P-labeled products extracted from the gel were evaporated to dryness. Recovery of ^{32}P-labeled products was ~95%. The ^{32}P-labeled products were solved in 20 μL of distilled water and subjected to a Hypersil BDS C_{18} analytical column (0.46 cm × 25 cm, 5 μm, Shandon) and eluted at a flow rate of 1.0 mL/min with a linear gradient of 0.2 M

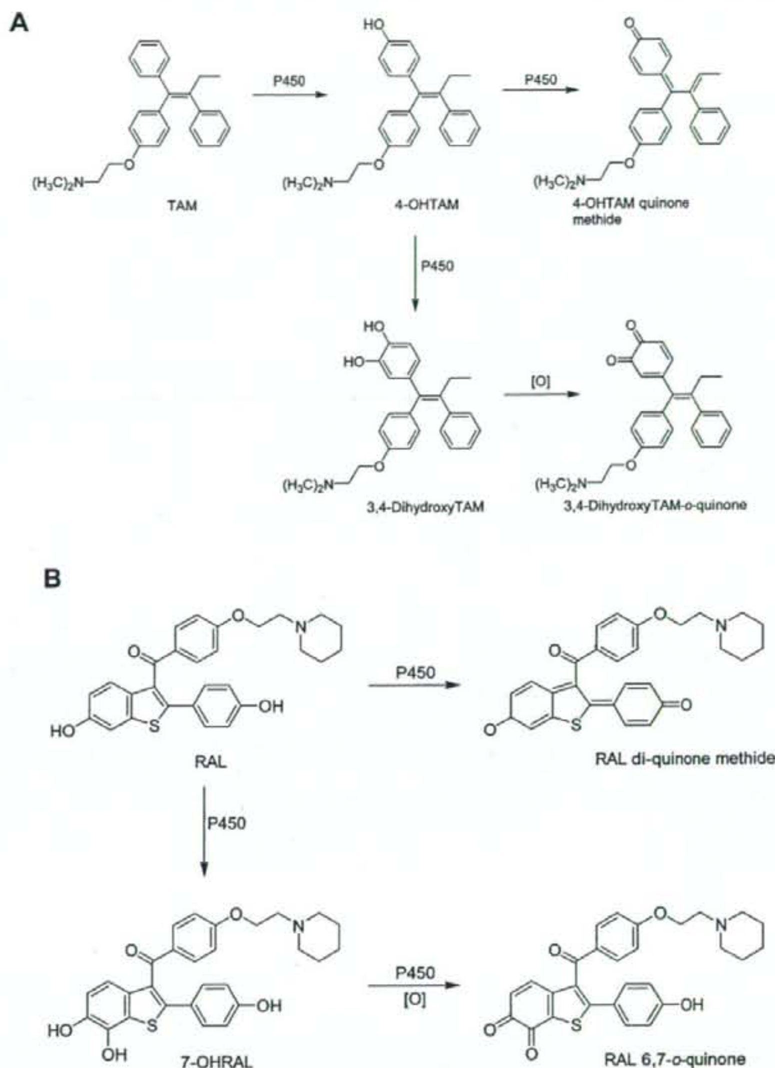

Figure 2. Proposed mechanism of forming TAM−DNA adducts derived from 4-OHTAM.

ammonium formate and 20 mM H_3PO_4, pH 4.0, containing 20−30% acetonitrile for 40 min, 30−50% acetonitrile for 5 min, followed by an isocratic condition of 50% acetonitrile for 15 min (*37*). The radioactivity was monitored using a radioisotope detector (Berthold LB506 C-1, ICON Scientific Inc.) linked to a Waters 990 HPLC instrument. Known amounts (0.076−0.000076 pmol) of dG-N^2-TAM-modified oligodeoxynucleotide were mixed with 2.5 μg of calf thymus DNA (7600 pmol) and served as a standard. As described previously (*37*), the amount of TAM−DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. The detection limit of this assay was ~2 adducts/10^9 nucleotides for 5 μg of DNA.

Determination of 8-OxodG by LC/MS/MS Analysis. The hepatic DNA was extracted by a modified method of Wang et al. (*38*). Liver tissue (100 mg) was homogenated at 4 °C in 0.2 mL of lysis solution A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM desferrioxamine, pH 7.5, and 1% Triton X-100) by disposable pellet pestle (Kimble/Kontes, Vineland, NJ). After tissue grinding, the homogenate was subjected to centrifugation at 10000*g* for 20 s at 4 °C. One milliliter of lysis solution A was added to the pellet and agitated by vortexing. This process was repeated twice. After centrifugation, 200 μL of solution B (10 mM Tris, 5 mM EDTA-Na_2, and 0.15 mM desferrioxamine, pH 8.0) and 20 μL of 10% SDS were added to the pellet. The mixture was vortexed for 10 s and incubated at 37 °C for 10 min for suspension of the pellet and to allow for complete lysis of the nuclear membrane. After the addition of 2.7 μL of RNase T_1 (1.0 unit/μL) and 10 μL of RNase A (1 μg/μL) in a buffer (10 mM Tris, 1 mM EDTA, and 2.5 mM desferrioxamine, pH 7.4), the samples were incubated at 50 °C for 15 min. Following incubation, 10 μL of Qiagen proteinase K solvent was added and the samples were incubated for 1 h at 37 °C. After the addition of 0.3 mL of NaI solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA-Na_2, and 0.3 mM desferrioxamine, pH 8.0), DNA was precipitated by the addition of 500 μL of 2-propanol. The DNA lump was fished out and washed twice with 40% 2-propanol. Finally, the recovered DNA was dried and dissolved in 500 μL of

distilled water. The concentration of DNA was determined by UV spectroscopy.

The level of 8-oxodG was determined using LC/MS/MS (HPLC, a Shimadzu LC-10ADvp pump and SIL-10AD auto injector; MS/MS, Waters-Micromass Quattro Ultima Pt Triple Quadropole mass spectrometer). To quantify 8-oxodG accurately, $^{15}N_5$-8-oxodG was prepared from $^{15}N_5$-dG; all five Ns in guanine base were replaced by ^{15}N (Cambridge Isotope Lab), following a method developed previously (*39*), and used as an internal standard. The DNA (50 μg) was mixed with $^{15}N_5$-8-oxodG (500 pg), digested at 37 °C for 3 h with nucleaseP1 (4 unit) in 114 μL of buffer mixture [100 μL of 30 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, pH 5.3; 5 μL of 20 mM $ZnSO_4$; 5 μL of $^{15}N_5$-8-oxodG solution (5 ng/mL); 4 μL of nuclease P1 solution (1 unit/μL); and 3 units of alkaline phosphatase]. After incubation, 20 μL of 0.5 M Tris-HCl, pH 8.5, was added and incubated for 3 h. The enzymes were precipitated using methanol, and the supernatant containing the nucleoside was evaporated and reconstituted with 100 μL of water.

The LC column was eluted over a gradient that began at a ratio of 2% methanol to 98% water and was changed to 40% methanol over a period of 40 min, changed to 80% methanol from 40 to 45 min, and finally returned to the original starting conditions, 2:98, for the remaining 15 min. The total run time was 60 min. Sample injection volumes of 50 μL each were separated on a Shim-pack FC-ODS column (150 mm × 4.6 mm) and eluted at a flow rate of 0.4 mL/min. Mass spectral analyses were carried out in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C, and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 L/h) and cone gas (35 L/h), and argon was used as the collision gas at a collision cell pressure of 1.5×10^{-3} mBar. Positive ions were acquired in MRM mode. The MRM transitions monitored were as follows: $^{15}N_5$-8-oxodG (*m/z* 288.8 → 172.8) and 8-oxodG (*m/z* 283.8 → 167.8), respectively. The amount of 8-oxodG in DNA sample was determined by comparing with $^{15}N_5$-8-oxodG. The detection limit was approximately 0.1 adducts in 10^6 bases using 50 μg of DNA. The amount of dG in the DNA digest was monitored by a Shimadzu SPD-10A UV−visible detector prior to MS/MS analysis. The level of 8-oxodG lesions was estimated by the following equation: the level of 8-oxodG lesion = (amount of 8-oxodG)/[(amount of dG) × 4].

Statistical Analysis. Results were expressed as means ± SD. Two-sided Student's *t*-test was used to evaluate the difference. Values of $p \leq 0.05$ were considered statistically significant from the control group.

Results

To investigate the genotoxicity of each SERM, rats were treated orally for 7 days with TAM, 4-OHTAM, TOR, RAL, or ICI 182,780 at an equimolar dose of TAM (54 μmol/kg/day). The level of hepatic DNA adduct was determined using ^{32}P-postlabeling/PAGE. A high amount of TAM−DNA adducts (total 26.1 ± 9.5 adducts/10^7 nucleotides) was detected in all of the rats treated with TAM (Figure 3). To determine the TAM−DNA adducts, the adduct was recovered from the gel and subjected to HPLC in-line with a radioisotope detector. As compared with standards of TAM−DNA adducts (Figure 4A), trans forms (fr-2) of dG$_3$'p-N^2-TAM and dG$_3$'p-N^2-*N*-desTAM were major adducts at the levels of 12.1 ± 4.6 adducts/10^7 nucleotides and 11.0 ± 4.4 adducts/10^7 nucleotides, respectively (Figure 4B). Using both ^{32}P-postlabeling/PAGE and ^{32}P-postlabeling/HPLC, no DNA adducts were detected in liver of rats treated with 4-OHTAM, TOR, RAL, or ICI 182,780 (Figures 3 and 4).

The level of hepatic 8-oxodG lesions induced by SERM was determined using LC/MS/MS analysis and an internal standard,

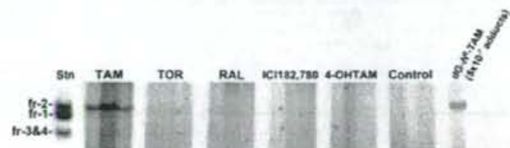

Figure 3. ^{32}P-Postlabeling/PAGE analysis for determination of DNA adducts in rat liver. Three rats were treated orally with either TAM, TOR, RAL, ICI 182,780, 4-OHTAM, or vehicle at a molecular equivalent dose of TAM [54 μmol (20 mg)/kg/day] for 7 days. The hepatic DNA was used for analysis of DNA adducts using ^{32}P-postlabeling/PAGE, as described in the Materials and Methods. Standard (Stn.) is 2.5 μg of calf thymus DNA (0.76×10^7 fmol dNs) containing a known amount (3.8 fmol dNs) of a trans-form (fr-2) of dG-N^2-TAM-modified oligomer, representing 5 adduct/10^7 dNs.

$^{15}N_5$-8-oxodG. Analysis of $^{15}N_5$-8-oxodG and 8-oxodG was achieved by monitoring the MS/MS transitions corresponding to the loss of deoxyribose from $^{15}N_5$-8-oxodG (*m/z* 288.8 → 172.8) and 8-oxodG (*m/z* 283.8 → 167.8), respectively (Figure 5). The amount of 8-oxodG in DNA samples was determined by comparing with $^{15}N_5$-8-oxodG. The detection limit for 50 μg of DNA sample was approximately 0.1 adduct/10^6 nucleotides. The levels of 8-oxodG in the liver of rats treated with TAM and 4-OHTAM were 2.17 adducts/10^6 nucleotides and 1.97 adducts/10^6 nucleotides, respectively; those were slightly higher than that (1.78 adducts/10^6 nucleotides) of rats treated with vehicle; however, no significant difference was observed (Table 1). The levels of 8-oxodG induced by TOR, RAL, and ICI 182,780 were similar to that of the control.

Discussion

To determine the genotoxic potential, we have selected 4-OHTAM, TOR, RAL, and ICI 182,780, which are currently approved by the FDA for breast cancer therapy or under clinical trial, and compared their genotoxic potentials with that of TAM. Rats were treated with each antiestrogen at an equimolar dose of TAM (54 μmol/kg/day). The level of bulky and oxidative DNA damage in the liver was determined using ^{32}P-postlabeling analyses and LC/MS/MS, respectively.

TAM induced a high level of TAM−DNA adducts in rat liver; major adducts were confirmed as a trans isomer (fr-2) of dG-N^2-TAM and dG-N^2-*N*-desTAM (Figure 4), as observed previously by several research groups including us (*8*). Interestingly, the formation of hepatic TAM−DNA adducts was not observed with a trans isomer of 4-OHTAM, a principle TAM metabolite having antiestrogenic activity. Our results are consistent with that observed with rats treated orally with a mixture of trans and cis isomers (~1:1) of 4-OHTAM (21 mg/kg/day for 7 days) (*40*), indicating that 4-OHTAM is not involved in the formation of TAM−DNA adducts. On the basis of the results obtained in in vitro experiments (*28, 29*), 4-OHTAM is suspected to oxidize cellular DNA through the redox cycling between the hydroxyl and the quinone forms (Figure 2A). However, a significant increase of 8-oxodG adducts was not observed in the liver of rats treated with either TAM or 4-OHTAM. A similar result was reported using a different strain in rats treated with TAM; however, no detailed information, including the dose, duration, and route of TAM treatment were described in this study (*41*). These results support that 4-OHTAM-derived quinone methide and/or *o*-quinone (*25−27*) are not significantly involved in the formation of oxidative and bulky TAM−DNA adducts in vivo.

Because TOR is a chlorinated TAM, TOR may be metabolized similarly to TAM (*8*). However, treatment of TOR to rats