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Decreased Levels of 2-Amino-3-methylimidazo[4,5-*f*]quinoline-DNA Adducts in Rats Treated with β-Carotene, α-Tocopherol and Freeze-dried Aloe

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To assess mechanisms of chemoprevention of hepatocarcinogenesis by *trans*-β-carotene (β-C), DL-α-tocopherol (α-T), and freeze-dried whole leaves of Kidachi aloe (Aloe), formation of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-DNA adducts was measured by ^{32}P-post-labeling analysis, and CYP1A1 and CYP1A2 protein levels were analyzed by ELISA. Group 1 rats were fed diet containing 0.02% β-C, 1.5% α-T or 30% Aloe over an 8-day period, while group 2 was given basal diet alone. On day 7, all animals were subjected to two-thirds partial hepatectomy (PH). Twelve hours after PH, they received a single dose of the carcinogenic food pyrolysate IQ (100 mg/kg) intragastrically, to initiate hepatocarcinogenesis. Rats were killed 6, 12, 24 and 48 h after IQ administration. The levels of adducts, expressed as relative adduct labeling values in rats treated with β-C, α-T and Aloe, were decreased as compared with the control group at hour 24 (36 h after PH), with a significant difference in the case of the β-C group (46.4% of the control value). Similarly, all showed a tendency for decrease at hour 48. Furthermore, the levels of CYP1A2, known to be responsible for activation of IQ, showed a significant reduction at hour 24. It is concluded that β-C, and possibly also α-T and Aloe, have the potential to reduce IQ-DNA adduct formation, presumably as a result of decreased formation of active metabolites. The results may explain, at least in part, the previously observed inhibitory effects of these compounds on induction of preneoplastic hepatocellular lesions.

Key words: Chemoprevention — Hepatocarcinogenesis — Initiation — β-Carotene — IQ-DNA adduct

There is extensive evidence indicating a chemopreventive role for plant constituents against carcinogenesis, mainly from studies of fruit and vegetable intake.[1–4] Plant foods contain various vitamins and phenolic compounds possessing antioxidant activity.[1,2] Many studies of the chemopreventive effects of such compounds have employed experimental systems featuring administration during the post-initiation stage of carcinogenesis, mostly in the liver and intestines.[5–13] In the rat liver, several naturally occurring antioxidants have also been shown to exert inhibitory effects on the development of early preneoplastic focal lesions when applied before and during the initiation phase.[14] In this latter context, inhibition of mutagenic activity of carcinogens by plant antioxidants may be of direct significance.[15,16] In a previous study, we showed that several naturally occurring antioxidants, including *trans*-β-carotene (β-C), DL-α-tocopherol (α-T) and freeze-dried whole leaves of Kidachi aloe (*Aloe arborescens* Miller var. *natalensis* Berger) (Aloe), exerted inhibitory effects on initiation of hepatocarcinogenesis by the food-pyrolysate carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), and so have potential as chemopreventive agents.[14,17,18] However, there is only limited information available concerning the underlying mechanisms. For the purpose of understanding the causative events, levels of IQ-DNA adducts and cytochrome P-450 species responsible for activation of IQ were studied in rats treated with β-C, α-T and Aloe, which is rich in α-T.[19,20]

MATERIALS AND METHODS

Chemicals and enzymes IQ was purchased from the NARD Institute, Ltd. (Osaka). β-C, apyrase, spermidine and dithiothreitol were from Sigma Chemical Co. (St. Louis, MO). α-T and proteinase K were from E. Merck (Darmstadt, Germany). Ribonuclease A, ribonuclease T_1, micrococcal nuclease and spleen phosphodiesterase were from Worthington Biochemical Co. (Freehold, NJ). [γ-^{32}P]ATP, T_4 polynucleotide kinase and nuclease

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P_1 were obtained from ICN Biomedicals (Irvine, CA), Takara Shuzo Co. (Kyoto) and Yamasa Shoyu Co. (Choshi), respectively. All other chemicals used were of analytical grade. Freeze-dried powder of whole Kidachi aloe leaves was carefully prepared at the Institute of Pharmacognosy, Fujita Health University. β-C, α-T and Aloe were mixed into powdered basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) at three-day intervals and stored at 4°C.

Animals and treatments A total of 41 male F344 rats, 6 weeks old, were purchased from Charles River Japan Inc. (Atsugi). They were housed in plastic cages on wood chip bedding made of a mixture of Shaglin Spruce, White Spruce and Yezo Spruce (White Flake, Charles River Japan Inc., Yokohama), in an air-conditioned room at 24 $\pm$2°C and 60$\pm$5% relative humidity. The dosing schedule was essentially the same as in a previous study.[14] Group 1 rats were fed on basal diet supplemented with 0.02% β-C (11 rats), 1.5% α-T (9 rats) or 30% Aloe (10 rats) for the initial 8 days, then returned to the basal diet until they were killed. On day 7, rats were subjected to partial hepatectomy (PH). Twelve hours after PH, they were intragastrically administered a single dose of IQ (100 mg/kg, suspended in olive oil) for initiation of hepatocarcinogenesis. Group 2 (11 rats) was treated in the same way as group 1 without feeding of β-C, α-T or Aloe (Fig. 1). Animals were killed at hours 6, 12, 24 and 48 after the carcinogen application (3 or 4 rats each time). The livers were immediately excised, frozen in liquid nitrogen and stored at -80°C until isolation of DNA.

Preparation of DNA samples from rat liver tissues Homogenates were prepared from 200–300 mg weight of rat liver tissue. The methods for the isolation of DNA have been described in detail elsewhere.[21,22] The resultant DNA samples were dissolved in 100 μl aliquots of 0.01$\times$ diluted standard saline citrate (SSC)-0.1 m*M* EDTA (final concentration), and the concentration of solutions was adjusted to 2 mg DNA/ml.

Determination of DNA adduct levels by ^{32}P-post-labeling analysis Three or 4 samples (1 sample from each rat) were analyzed at each time point. Aliquots containing 10 μg of DNA (2 μg/μl) were digested with a nuclease mixture (micrococcal nuclease and spleen phosphodiesterase) in succinate buffer (pH 6.0), then 5 μl was subjected to nuclease P_1 treatment,[23,24] according to Ochiai *et al.*,[24] and the remaining 5 μl was assayed for total 3′-deoxynucleotides.[25] Thereafter, the phosphorylation reaction using a labeling cocktail containing 2.3 μl of [γ-^{32}P]ATP (650 Ci/mmol) and 5U of T_4 polynucleotide kinase in buffer (pH 9.5) was separately carried out for each of these solutions. The ^{32}P-labeled samples described above were next applied to a polyethyleneimine (PEI) cellulose TLC sheet (POLYGRAM CEL 300 PEI; Macherey-Nagel, Duren, Germany) and the chromatographic separation of DNA adducts was carried out by the methods used in studies on IQ-DNA adducts.[24] To determine the amounts of DNA-adduct, PEI sheets were read with a Bio-Image Analyzer (BAS 2000 system; Fuji Photo Film Co., Ltd., Tokyo) after exposure to an imaging plate for 2 h. Levels of adducts were determined by the relative adduct labeling (RAL) method of Gupta.[25] Variance analysis was used for statistical evaluation of the results.

Immunochemical assays of CYP1A1 and CYP1A2 Monoclonal antibodies (MoAbs) APL-1 and APH-3 specifically reacting with CYP1A1 and CYP1A2, respectively[26] were used for enzyme-linked immunosorbent assay (ELISA) analysis. MoAb APL-2 reactive to both CYP1A enzymes was used for Western blotting analysis.[26] To analyze levels of enzymes presumably responsible for metabolizing IQ, CYP1A2 was measured along with CYP1A1, which specifically metabolizes polycyclic aromatic hydrocarbons, by protein A-ELISA and Western blotting methods, as previously described.[27] Briefly, for protein A-ELISA, total microsomes were prepared from the pooled livers of 3 or 4 rats of each group for the 24 h time point, and aliquots (100 μg protein/well) were used for the analysis of CYP1A enzymes using APL-1 and APH-3. Triplicate assays were performed by protein

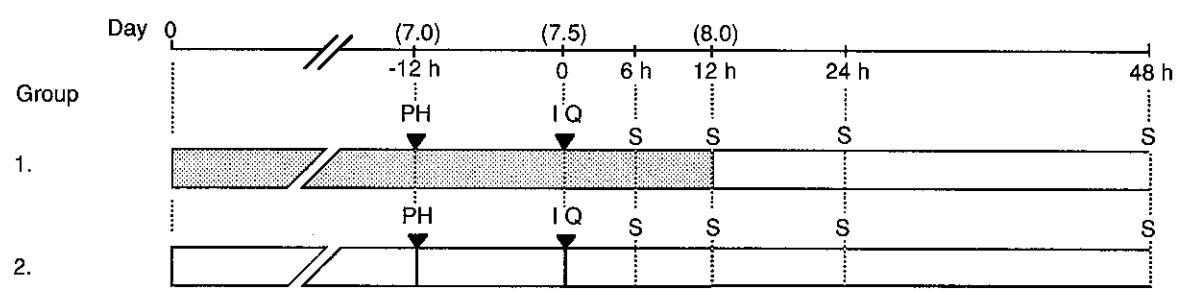

Fig. 1. Experimental protocol (see "Materials and Methods"). ▨ Basal diet supplemented with a, β-C; b, α-T; c, Aloe, □ Basal diet; PH, partial hepatectomy; IQ, IQ administration; S, killed for examination.

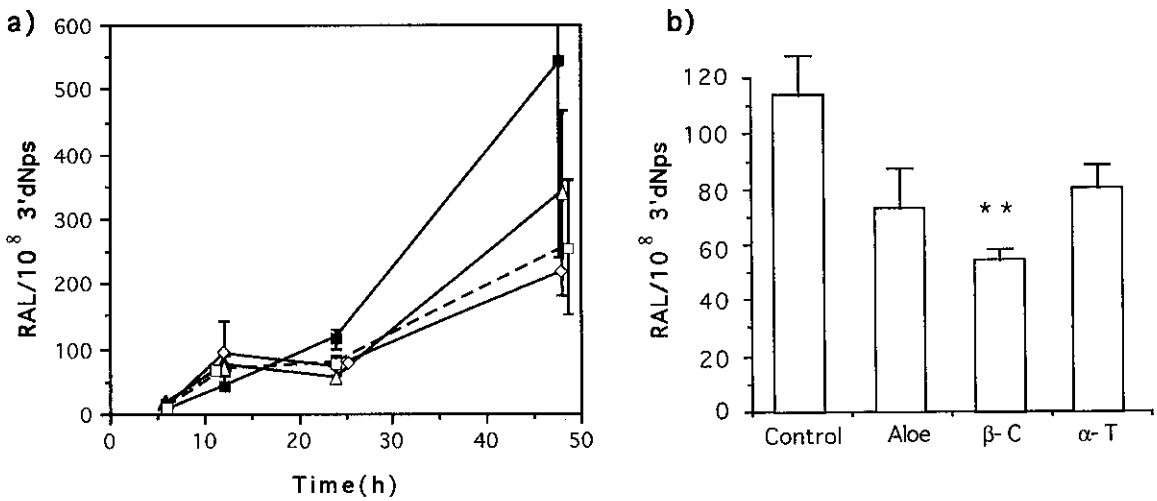

Fig. 2. (a) Time course analysis of RAL values after initiation by IQ in the livers of F344 rat groups treated with β-C ($\triangle$), α-T ($\square$) or Aloe ($\diamond$), as compared with the control group ($\blacksquare$). Liver samples of three rats per group were assayed at one time point for IQ-DNA adduct levels and results represent the mean$\pm$SE of duplicate determinations. (b) Comparison of DNA adducts levels at hour 24 in 3'dNps, 3'-deoxynucleotides. **, $P<0.02$ as compared to control group.

A-ELISA to generate mean$\pm$SE values for optical densities at 414 nm. Student's t test was used for statistical evaluation of the results. For western blotting, microsomal preparations (30 μg protein/lane) were solubilized with sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose sheets and then immunostained using APL-2.

RESULTS

The RAL values increased time-dependently in groups 1 and 2 (Fig. 2) but were reduced in group 1 at hours 24 and 48, as compared to group 2 (Fig. 2a). The differences in levels relative to the carcinogen control at hour 24 were β-C, 46.4%; α-T, 71.5%; Aloe, 62.9% and at hour 48 they were β-C, 62.1%; α-T, 46.6%; and Aloe, 39.7%. The β-C value at hour 24 was significantly lowered ($P<0.02$) (Fig. 2b). A representative chromatographic profile of ^{32}P-postlabeled hepatic DNA adducts in the β-C and control groups is shown in Fig. 3. The profile shows loss of one major adduct spot (arrow), which rapidly moved in direction 4 on the PEI-sheet in the case of liver from rats treated with β-C (Fig. 3a and b). Fig. 4 shows a representative Western blot profile of hepatic microsomal CYP1A proteins. The microsomal CYP1A protein was decreased in groups treated with β-C, α-T and Aloe. The results of Western blot analysis were confirmed by quantitative analyses with protein A-ELISA using the antibodies APL-1 and APH-3, which are specifically reactive with CYP1A1 and CYP1A2,

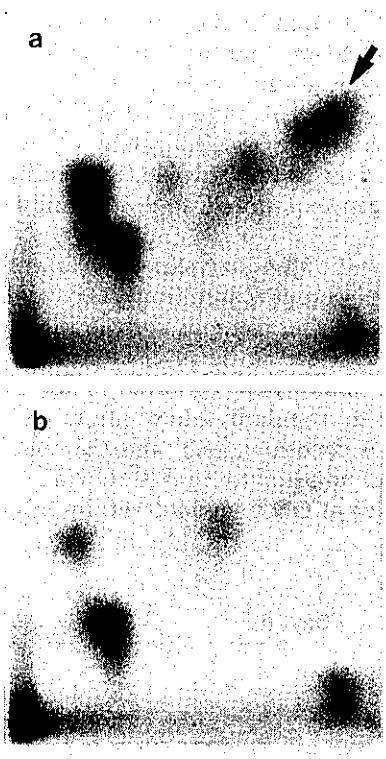

Fig. 3. Representative chromatographic profiles on PEI cellulose TLC maps of ^{32}P-postlabeled hepatic DNA adducts at hour 24 after IQ administration. (a) DNA from the liver of a control rat. (b) DNA from the liver of a rat fed 0.02% β-C; one of the major spots in control DNA (arrow) is not observed in the β-C-treated liver DNA (see "Materials and Methods").

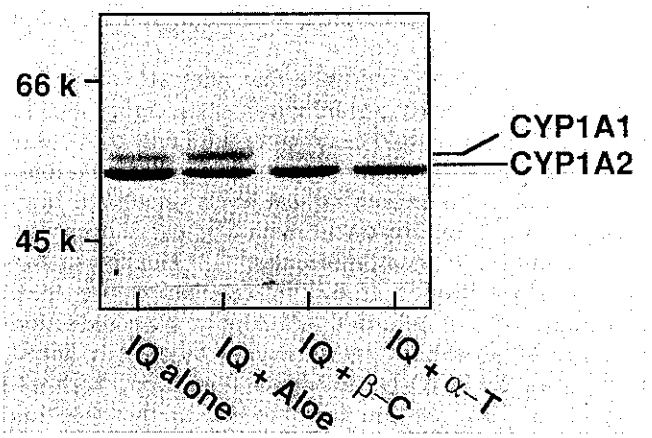

Fig. 4. Results of Western blot analysis of CYP1A2 and CYP1A1 using MoAb APL-2 in the groups fed diet with or without test compounds. The levels of microsomal CYP1A protein showed a decrease in groups treated with Aloe, β-C and α-T.

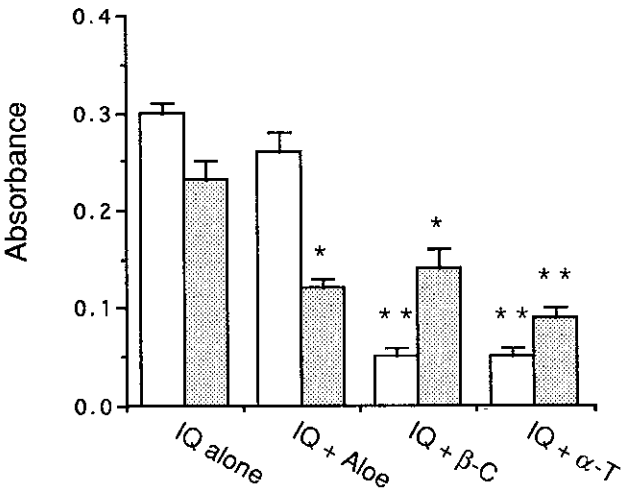

Fig. 5. Protein A-ELISA reactivity of microsomes with anti CYP1A MoAbs, CYP1A2 MoAb (APH-3) (▨) and CYP1A1 MoAb (APL-1) (□) at hour 24. Protein levels of CYP1A2 and CYP1A1 in all treated groups fed β-C, α-T and Aloe are significantly decreased as compared to the corresponding controls by Student's t test. * $P<0.01$; ** $P<0.001$. Results represent the mean±SE from three rats.

respectively. CYP1A2 levels at hour 24 were significantly decreased in group 1 rats treated with β-C, α-T or Aloe, as compared with group 2 control rats ($P<0.01$, 0.001 and 0.01, respectively) (Fig. 5). Similarly, the levels of CYP1A1 were significantly decreased in groups treated with β-C and α-T (both $P<0.001$) (Fig. 5).

DISCUSSION

In our previous studies, dietary administration of Aloe and naturally occurring plant antioxidants, such as β-C and α-T, was found to exert an inhibitory effect on the initiation stage of hepatocarcinogenesis by the food pyrolysate IQ, as assayed in terms of development of preneoplastic placental-form glutathione *S*-transferase-positive foci.[14, 18] The present investigation indicates that this inhibitory effect is probably due to alteration in the metabolism of IQ, causing a reduction in the amount of active form. Thus, although the IQ-DNA adduct levels in all groups showed a time-dependent increase from time 0 to hour 48, the levels in the groups treated with β-C, α-T and Aloe were reduced as compared to the control group, with a significant difference in the β-C group. It should be noted that the degree of reduction at hour 24 parallels the values for preneoplastic placental-form glutathione *S*-transferase-positive foci presented in the previous work, corresponding to the degree of inhibition of carcinogenesis.[14, 18] The decrease in IQ-DNA adducts formation can be explained, at least in part, by the decrease of CYP1A enzyme species, mainly CYP1A2, which is considered responsible for the metabolic activation of IQ.[19, 20, 28, 29] The levels of IQ-DNA adducts at hour 24 paralleled the levels of CYP1A2, with the β-C group being the lowest (Figs. 2, 4 and 5). The current study revealed a reduction in the protein levels of CYP1A1 and CYP1A2, in line with the earlier indication that treatment with antioxidants causes decreased cytochrome P-450 activity.[30] Since Aloe is rich in α-T and also contains β-C and other carotenoids, the observed effects in this case may be partly attributed to these constituent antioxidant compounds (total carotenoid, 6.68 mg/100 g; α-T, 68.1 mg/100 g; unpublished data by Fujita *et al.*). However, since Aloe is a crude material, further studies of various other constituents will be necessary to clarify its effects on carcinogenesis (see ref. 18).

Furthermore, since antioxidants are known to induce phase II species including glutathione *S*-transferase and UDP-glucuronyl transferase activities in liver cells *in vitro* and *in vivo*, and this action correlates with their chemopreventive characteristics, this aspect of the action of β-C and α-T also requires elucidation.[31–36] It is generally accepted that cellular proliferation is critical for initiation of hepatocarcinogenesis and PH has often been utilized as an effective tool for this purpose in cases where hepatocarcinogens are not potent or not obviously necrogenic.[11, 37–40] Enhancement under such circumstances is presumably due to the fact that cells in S-phase are more susceptible to carcinogen exposure in terms of fixation of DNA lesions. Relative low levels of IQ-DNA adducts as compared to the control were observed from hours 24 to

48 (36 and 60 h after PH) in the treatment groups in the present experiment. Although the levels of IQ-DNA adducts at hour 12 (24 h after PH), corresponding to the S-phase of cellular proliferation, appear slightly increased, the differences were small and not statistically significant. If only the S-phase peak were important for initiation, the observed reduction of IQ-DNA adducts at hour 24 (36 h after PH) would obviously merit attention. However, there is evidence of liver tumor development in rats treated with carcinogens administered later than the conventionally postulated S-phase. For example, benzo[*a*]pyrene was found to induce hepatocellular carcinomas when administered 24 h after PH,[40] and since it takes 12 h for benzo[*a*]pyrene to be metabolized to active forms by drug-metabolizing enzymes, including P-450 species,[39] the time of maximal binding of activated benzo[*a*]pyrene to DNA is approximately 36 h after PH. Furthermore, another report indicated dimethylnitrosamine injected 31 h after PH is still effective in inducing hepatocellular carcinomas.[41] Since carcinogens are known to exert a delaying effect on the cell cycle, it is possible that IQ caused suppression of DNA synthesis, so that cell entry into the S-phase was later than in the normal case.[42–44] The present results indicate that the levels of carcinogen-DNA adducts assayed at 36 h after PH may be closely correlated with initiation of lesion development.

There is increasing evidence that naturally occurring and synthetic antioxidants inhibit liver carcinogenesis in the rat.[8, 34, 45–47] It should be noted in this context that one of the reasons proposed for such inhibition has been their lowering effect on oxygen radical levels since such radicals, produced by treatment with the carcinogen IQ, cause damage to macromolecules.[48–52] This possibility also requires further study. In conclusion, we have demonstrated that β-C, α-T and Aloe cause a reduction of CYP1A2 protein levels, which may be directly linked to a reduction in IQ-DNA adduct formation. Both parameters appear relevant to inhibition of the initiation stage of neoplastic lesion development by IQ.

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