Note

Effect of Acerola Cherry Extract on Cell Proliferation and Activation of Ras Signal Pathway at the Promotion Stage of Lung Tumorigenesis in Mice

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Summary The present study was undertaken to estimate the effect of acerola cherry extract (ACE) pretreatment on cell proliferation and the activation of Ras signal pathway at a promotion stage of lung tumorigenesis in mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Pretreatment with ACE (dose, 70 mg/kg body weight and 700 mg/kg body weight) inhibited increases in the levels of proliferating nuclear cell antigen and ornithine decarboxylase at the promotion stage. This treatment of ACE also suppressed the activation of Ras signal pathway at the same stage. These results suggest that ACE regulates abnormal cell growth at the promotion stage of lung tumorigenesis in mice treated with NNK as a result of suppression of the initiation stage.

Key Words acerola, cell proliferation, Ras signal pathway, lung tumorigenesis, mice

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent carcinogen formed from nicotine during tobacco processing and cigarette smoking (1). The activation of NNK mainly occurs via α -hydroxylation, leading to the formation of promutagenic adduct, O⁶-methylguanine (O⁶MG), and the formation of O⁶MG is critical in the NNK-induced lung tumorigenesis of mice (2, 3). Furthermore, we have demonstrated that the mutational activation of K-ras oncogene caused by the formation of O⁶MG occurs at an early promotion stage of the NNK-induced lung tumorigenesis and that the constitutive activation of Ras signal pathway based on the ras gene mutation is required for abnormal cell proliferation at the promotion stage and the development of lung tumors (4, 5). These reports mean that the attenuation of NNK-induced events during the initiation stage of the lung tumorigenesis finally leads to the suppression of the development of tumors. In fact, we succeeded in reducing the development of lung tumors in mice treated with NNK, through inhibition in the formation of O⁶MG by some food factors (6, 7).

To reduce the formation of O⁶MG, it is very useful to activate the NNK detoxification pathways (8). Besides some food factors (6, 7), it has been recently reported that vitamin C enhances the detoxification pathways of NNK through reduction in vitro (9). In this reduction, vitamin C stimulates the formation of a reduced NNK metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and this enhancement may contribute to the elimination of a final NNK metabolite, NNAL-glucuronide, in urine (9).

Overall, it is assumed that vitamin C suppresses cell proliferation and the activation of the Ras signal pathway through the inhibition of O⁶MG-induced mutational activation of the K-ras gene during the lung tumorigenic process of mice treated with NNK.

Acerola cherry is considered to be one of the richest sources of vitamin C (10) and has shown in human trials to be a better source of bioavailable vitamin C (11). Besides vitamin C, ACE contains several food factors such as carotenoids useful for lung cancer prevention (unpublished data). Taken together, it seems that ACE has anticarcinogenic activity against lung cancer based on the additive and/or synergistic effects of vitamin C and other food factors in ACE. In this context, the present study was carried out to determine if ACE really inhibited cell proliferation and the activation of the Ras signal pathway at the promotion stage of the lung tumorigenesis as a result of the suppression of the initiation stage.

Materials and Methods

Animals, diets, and feeding. Six-week-old female, specific pathogen-free A/J mice (CLEA Japan, Tokyo, Japan) were used. The mice were fed a CE-2 diet and sterilized water ad libitum. Dry powder was prepared from acerola cherry produced in Okinawa, the powder was suspended in sterilized saline, and the suspension was used as ACE. Each group (one group, $n=8$) was administered ACE (70 mg/kg body weight and 700 mg/kg body weight) or saline by gavage for 5 d. One sample was prepared from the lungs of two mice, and a total of 4 samples in one group were used for cell proliferation

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and tumor promotion marker, the activation of Ras, the activation of Mek and extracellular signal-regulated kinase (Erk), and the level of O⁶MG. This dose was determined because of the recommended dietary allowance for vitamin C in Japan and the content of vitamin C in ACE (2%). All the mice were given NNK solution (100 mg/kg body weight) or saline by a single i.p. injection 4 h after the final gavage. Since our previous report showed that day 30 after NNK injection was the best point to estimate the markers of tumor promotion in lung (12), we selected the day to determine the parameters. There was no difference in body weight change during this experiment among all groups (data not shown). All animal experiments were performed in strict accordance with the institution's criteria for the care and use of laboratory animals as approved by an institutional governing committee.

The assay of cell proliferation and tumor promotion marker. Samples were prepared from lungs according to our previous report (6). The extracts were incubated overnight at 4°C with P13suc1-agarose (UBI, NY, USA). The pellets were washed and resuspended in Laemmli's sample buffer. Electrophoresis and immunoblotting against antiproliferating nuclear cell antigen (PCNA) antibody (Dako Japan, Tokyo, Japan) were performed as previously described (13, 14). The blots were developed by the use of the ECL system (Amersham, NJ, USA). Since PCNA-P13 complex was useful as a sensitive marker of cell proliferation during tumorigenesis (13), we used the complex as the marker. We also used the ornithine decarboxylase (ODC) protein level as a marker of tumor promotion. The protein level was analyzed by electrophoresis and immunoblotting with an anti-ODC antibody (Sigma, MS, USA) (15). The detection of each band was accomplished by the use of an ECL system (Amersham) and a cooled camera-linked Cool Saver system (Atto, Tokyo, Japan). A two-dimensional densitometric evaluation of each band was performed with an ATTo Image Analysis Soft (Atto). Protein content was determined with a Bradford reagent (Bio-Rad, CA, USA), and molecular sizing was done with a Rainbow molecular weight marker (Amersham).

The assay of Ras signal pathway. The activation of Ras was determined by the use of an established method (16). To detect the active form of Ras (Ras-GTP), we used GST-B-Raf RBD protein precoupled to glutathione-sepharose beads. After the incubation of lung extract with the beads at 4°C for 2 h and a subsequent washing step, the beads were analyzed by electrophoresis and immunoblotting by the use of an anti-Ras antibody (Transduction Labs, KY, USA) (17). The residual lung extract was used to detect total Ras level by immunoblot analysis. The activation of Mek and Erk was estimated by electrophoresis and subsequent immunoblot analysis with antiphosphorylated Mek and Erk antibodies (New England Biolabs, MA, USA) (17). We determined the level of total Mek and Erk by the same method as that to determine activation, using anti-Mek and Erk antibodies (New England Biolabs) (17). Each protein band was detected and analyzed as mentioned above.

Results and Discussion

As shown in Fig. 1, the levels of ODC as well as PCNA at the promotion stage of NNK-induced lung tumorigenesis increased, and by ACE pretreatment they returned to a level similar to control before NNK injection. This result indicates that the inhibition of the initiation stage by ACE contributes to a reduction of abnormal cell proliferation at the promotion stage. To further confirm this inhibitory effect of ACE, we investigated the effect of ACE pretreatment on the activation of the Ras signal pathway related to an enhancement of cell proliferation at the promotion stage. As shown in Fig. 2, members of the Ras signal pathway (Ras, Mek, and Erk) were activated by NNK injection as previously reported (5), and the ACE treatment reduced the NNK-elevated level to that of control. These results support the above notion from the result in Fig. 1.

During the initiation stage of carcinogenesis, the fixation of DNA damage induced by carcinogen exposure occurs (the appearance of the initiated cells); then the initiated cells begin to proliferate in response to various

Fig. 1. Level of PCNA precipitated with P13suc1-agarose and ODC. (A) Immunoblot analyses. C, control; N, NNK-treated group; A1, NNK+ACE (70 mg/kg)-treated group; A2, NNK+ACE (700 mg/kg)-treated group. This result is representative one of three independent experiments. (B) Densitometric analyses. Densitometric analysis was performed as mentioned in Materials and Methods, and each intensity was shown as the mean of three independent experiments. Each value was expressed as a relative ratio against group N (NNK-treated group), and the value in group N was shown as 100.

Fig. 2. Effect of ACE pretreatment on the activation of Ras (A), Mek (B), Erk (C), and their densitometric analyses (D). The activation of Ras was examined by determining the level of the active form of Ras precipitated with GST-B-Raf RBD protein to the level of total Ras. We carried out the activation of Mek and Erk by estimating the level of the phosphorylated form of each protein to that of each total protein. C, control; N, NNK-treated group; A1, NNK+ACE (70 mg/kg)-treated group; A2, NNK+ACE (700 mg/kg)-treated group. This result is representative one of three independent experiments. Densitometric analysis was performed as mentioned in Fig. 1.

growth stimuli such as a promoter, finally leading to the development of tumors (18). In general, it is considered that the suppression on the fixation of DNA injury at the initiation stage reduced the number of initiated cells, leading to a decrease of cells showing abnormal growth at the promotion stage and a reduction of the development of tumors (19). We also observed a reduction of the O^6 MG level by ACE treatment: The levels ($n=3$) were NNK-treated group (100%), NNK+ACE (70 mg/kg)-treated group (46%), and NNK+ACE (700 mg/kg)-treated group (40%), respectively. This result indicates that ACE reduces the number of NNK-initiated cells at the initiation stage. As a mechanism of the ACE inhibitory effect observed in this study, we can propose the following scheme based on our present data and previous reports (4–7): ACE reduces the frequency of K-ras mutation caused by the formation of O^6 MG, directly leading to a suppression of the mutation-induced Ras signal activation and related cell proliferation at the promotion stage.

An epidemiological study shows that the risks of tobacco-induced lung cancer are lower among persons in the highest dietary intake of vitamin C (20); vitamin C may therefore contribute to a part of ACE-dependent inhibition of the initiation stage. However, the inhibitory effect by ACE reached a plateau at its low dose (70 mg/kg), suggesting that food factors other than vitamin C in ACE also played important roles in suppressing the initiation stage. Of food factors in ACE, carotenoids have been known to inhibit the initiation stage of tumorigenic process (21). Thus carotenoids may also act as inhibitory factors against NNK-induced lung tumorigenesis through the suppression of the initiation stage. Overall, it seems that acerola cherry is a promising food for lung cancer prevention.

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