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Short communication

The inhibitory effect of vitamin E on K-*ras* mutation at an early stage of lung carcinogenesis in mice

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Abstract

In this study, we investigated the effect of vitamin E on the activation of the K-*ras* oncogene with a 61st codon A → T mutation at an early stage of urethane-induced lung carcinogenesis in mice. Thirty days after urethane injection, the K-*ras* mutation was detected in 64% of lung samples tested by mutant-allele-specific amplification. The consumption of a supplemented diet with about 20-times more vitamin E than the control diet, only during the promotion phase or during both the initiation and promotion phases of lung carcinogenesis, reduced the frequency of the mutation to 36 and 18%, respectively. Also, vitamin E suppressed the level of proliferating cell nuclear antigen as a marker of cell proliferation in the lungs of mice treated with urethane. These results support the notion that vitamin E is a useful chemopreventive agent against lung cancer. © 1997 Elsevier Science B.V.

Keywords: Lung cancer; K-*ras*; Proliferating cell nuclear antigen; Vitamin E; Chemoprevention

1. Introduction

Mutations of K-*ras* genes at codons 12, 13 and 61 are the key to the development of mouse lung tumors as well as of human lung adenocarcinoma (Malkinson, 1992). This report suggests that the mouse model of lung adenocarcinoma is a useful one for analyzing genetic abnormalities involved in the carcinogenic process of humans. Various carcinogens, including urethane, induce particular profiles of K-*ras* gene point mutations in lung carcinogenesis, via carcinogen-specific DNA adducts formed upon direct interaction of the chemical with DNA, indicating that the mutations are concomitant with the initiation of lung carcinogenesis (You et al., 1989). The specific mutation found in the K-*ras* gene of urethane-induced lung tumors of mice is AT → TA transversion at the 61st codon, and we detected the mutation at an early stage of the lung carcinogenesis by means of highly sensitive mutant-allele-specific amplification (Ichikawa et al., 1996). Although epidemiological and experimental studies have indicated that vitamin E has a beneficial effect upon lung adenocarcinoma (Wald et al., 1987; Yano et al., 1993), the modulating effect of this vitamin against genetic abnormalities involved in lung carcinogenesis has not been reported upon.

In this context, we investigated the effect of vitamin E on the frequency of K-*ras* mutation at the early stage of urethane-induced lung carcinogenesis in mice, using mutant-allele-specific amplification, to check the usefulness of the vitamin as a chemopreventive agent against lung adenocarcinoma in humans.

2. Material and methods

2.1. Animals and treatment

Specific pathogen-free, A/J strain male mice (SLC, Shizuoka, Japan) were fed with a control CE-2 diet or a vitamin E-supplemented CE-2 diet (CE-2′) (Clea, Japan) and sterilized water ad libitum. The α-tocopherol contents in the control CE-2 and the CE-2′ diets were 25.5 and 550.9 mg/kg, respectively. All mice were given urethane (Sigma, St. Louis, MO, USA) solution at a dose of 750 mg/kg body weight, or vehicle, as a single intraperitoneal

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injection on the first day of this experiment. The mice were divided into three groups. Group 1 was fed the control diet throughout the experimental period, and the CE-2′ diet was given to groups 2 and 3 from 4 days after and 7 days before the injection, respectively. The animals remained on the diet until the end of the experiment. From our published data on the time-course of changes in pulmonary ornithine decarboxylase activity, we determined that the first week after urethane injection might be associated with the initiation phase, and that the weeks following might be associated with the promotion phase (Yano et al., 1997). Thus, group 2 received the diet only during the promotion phase, and group 3 received it during the initiation and promotion phases. On day 30 after the injection, all mice were killed by pentobarbital anesthesia to isolate genomic DNA from lung tissues.

2.2. Mutant-allele-specific amplification assay

Genomic DNA was prepared from whole lung tissues of mice by pronase-sodium dodecyl sulfate (SDS) lysis (Oreffo et al., 1993). At the first polymerase chain reaction (PCR), using 5′-primer, 5′-GACTCCTACAG-GAAACAAGT-3′, and 3′-primer, 5′-CTCCCCAGTTCT-CATGTACT-3′, we amplified the second exon region of the K-*ras* gene including the 61st codon. At the second PCR, the mutant-allele-specific amplification assay was performed to detect activation of the K-*ras* gene with the 61st codon A → T mutation by using 5′-primer, 5′-GACTCCTACAGGAAACAAGT-3′, and 3′-primer, 5′-CATTGCACTGTACTCCTCTA-3′ (Ichikawa et al., 1996). Also, we confirmed the wild-type K-*ras* gene in all samples using 5′-primer,5′-GACTCCTACAGGAAACAAGT-3′, and 3′-primer, 5′-CATTGCACTGTACTCCTCTT-3′. The first PCR was performed as 35 cycles of 0.5 min at 94°C, 1 min at 55°C and 1 min at 72°C, in a 100 μl reaction volume containing 500 ng of DNA, 200 μM of each dNTP, 1 μM of each primer, 2 U of *Taq* polymerase, 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris (pH 8.3). The first PCR product (117 bp) was purified by phenol/chloroform extraction and ethanol precipitation. The second PCR was performed as 30 cycles of 1 min at 94°C and 1 min at 60°C, in a 25 μl reaction volume containing 50 pg of purified DNA, 50 μM of each dNTP, 0.25 μM of each primer, 1.5 U of *Taq* polymerase, 10% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris (pH 8.3). The oligonucleotide probe with which to detect the K-*ras* mutation, 5′-CAGCAGGTCTAGAGGAGTA-3′, was labeled using a DIG Oligonucleotide 3′ End Labeling Kit (Boehringer-Mannheim, Mannheim, Germany). The product (90 bp) was transferred from a 2% agarose gel to a nylon membrane, hybridized with the oligonucleotide probe and detected using a DIG Luminescent Detection Kit for Nucleic Acids (Boehringer-Mannheim). In order to check the PCR products amplified from the wild-type K-*ras* gene, the samples were analyzed by 2% agarose gel electrophoresis, and the gels were stained with ethidium bromide.

2.3. Cell proliferation analysis

Soluble fractions were prepared from 10% lung homogenates in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 20 μM leupeptin and 5 mM sodium vanadate, by centrifugation at 4°C for 60 min at $100\,000 \times g$. The extracts (2 mg of proteins in 1 ml lysis buffer) were incubated overnight under constant rotation at 4°C with 20 μl of $p13^{suc1}$-agarose (UBI, New York, NY, USA). The p13-agarose pellets were washed three times with 1 ml lysis buffer, then resuspended in 40 μl of Laemmli sample buffer and boiled for 3 min. Electrophoresis and immunoblotting against anti-proliferating cell nuclear antigen (Dako, Denmark) proceeded as described (Higaki et al., 1994). The blots were developed using a chemiluminescence detection system (Boehringer-Mannheim).

2.4. Statistical analysis

The K-*ras* mutation frequency in urethane-treated and other groups was compared by χ^2-analysis. A P value of 0.05 or less was considered to be significant.

3. Results

3.1. The frequency of K-ras point mutation

Mutant-allele-specific amplification is based on the rationale that a DNA primer with a 3′ end complementary to a specific gene mutation will only allow chain elongation of mutant DNA sequences during PCR. Furthermore, this method is sensitive enough to detect only a few copies of mutant alleles (Ichikawa et al., 1996). The present study indicated an inhibitory effect of vitamin E on the frequency of K-*ras* mutation with a 61st codon AT → TA transversion in the lungs of mice on day 30 after urethane injection (Fig. 1 A). The frequency of K-*ras* mutation in the urethane-treated group (group 1) was 64% of 11 samples tested. Vitamin E supplementation during the initiation and promotion stages of lung carcinogenesis reduced the frequency of the mutation (group 3). This effect of vitamin E was also evident in group 2, which received the vitamin only during the promotion phase. The frequencies of the K-*ras* mutations in groups 2 and 3 were 36 and 18% of 11 samples tested, respectively, and the difference between groups 1 and 3 was statistically significant ($P < 0.05$). We also confirmed the wild-type K-*ras* gene in all samples with the present mutant-allele-specific amplification, using wild-type primers (Fig. 1B). The PCR

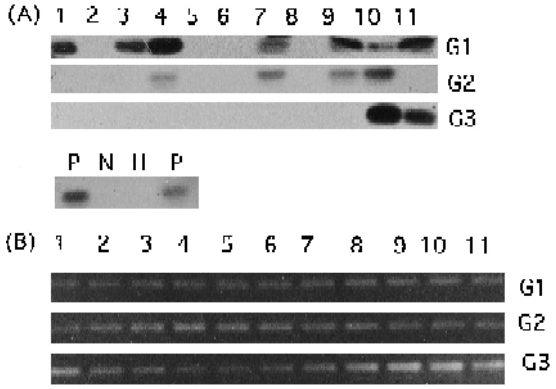

Fig. 1. Southern blot detection of the mutant K-*ras* allele, generated by mutant-allele-specific amplification of lung DNA and agarose gel electrophoresis of the PCR products obtained (A) and ethidium bromide staining of the obtained PCR products by amplification with wild-type primers (B). The PCR primers and oligonucleotide probe for detection of the mutation are described in Section 2. P, positive control (DNA isolated from urethane-induced lung tumors); N, negative control (DNA isolated from normal lung); H, mock reaction (absence of DNA template). G1, group 1 (control); G2, group 2 (vitamin E supplement during promotion phase); G3, group 3 (vitamin E supplement throughout initiation and promotion phases). Lanes 1–11, samples in each group.

product amplified from the mutant allele was not detected in the normal samples or in the absence of DNA template (mock reaction). The mutation was not found in the normal group which was given vehicle and the control diet (data not shown).

3.2. The analysis of cell proliferation

To determine the effect of vitamin E on pulmonary cell proliferation after urethane injection, we analyzed the level of proliferating cell nuclear antigen precipitated by cell division control (cdc)2 protein kinase using $p13^{suc1}$, a subunit of the protein kinase, as a marker of cell proliferation (Said and Medina, 1995) (Fig. 2). Like the frequency of the K-*ras* mutation, the level was decreased by the vitamin E-supplemented diet. Furthermore, the level in group 3 was lower than that in group 2. These results indicated that vitamin E reduced cell proliferation during the early stage of urethane-induced lung carcinogenesis in mice.

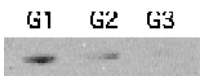

Fig. 2. Immunoblots of the level of proliferating cell nuclear antigen precipitated with $p13^{suc1}$-agarose. G1, group 1 (control); G2, group 2 (vitamin E supplement during promotion phase); G3, group 3 (vitamin E supplement throughout initiation and promotion phases). Four samples in each group were combined, and proliferating cell nuclear antigen was precipitated with $p13^{suc1}$-agarose, using a combined sample. The mice used for this analysis were different from the mice used for Southern blot analysis.

4. Discussion

The mutational activation of the *ras* oncogenes plays important roles in the early stage of animal carcinogenesis (Nelson et al., 1992). It is also proposed that initiated cells having mutated oncogenes remain latent among normal cells and begin to proliferate in response to growth stimulation, leading to the development of tumors (Kumar et al., 1990). We clarified this notion in a mouse model of urethane-induced lung carcinogenesis, using K-*ras* point mutation as a marker of initiated cells (Ichikawa et al., 1996). From these reports, it is assumed that the reduction in the number of the initiated cells in the early stage of lung carcinogenesis finally leads to inhibition of the development of lung tumors. We also reported that vitamin E supplementation throughout the initiation and promotion phases reduced the incidence of lung tumors by 20% and lung tumor multiplicity by 41% compared with those in urethane-treated group (Yano et al., 1997). In order to find if the vitamin E-induced reduction in the number of initiated cells could contribute to the inhibitory effect against lung carcinogenesis, we investigated the effect of this vitamin supplementation during the initiation and promotion phases on the frequency of urethane-induced K-*ras* mutation in the lungs. We found that the frequency of the K-*ras* mutation in group 2 given the vitamin during the promotion phase only, and in group 3 given the vitamin E-supplemented diet throughout the initiation and promotion phases, tended to be lower than that in group 1 given the control diet. Taking together our previous and present results, it seems that a vitamin E-dependent reduction in the frequency of the K-*ras* mutation, that is, the decrease of the initiated cell numbers at the early stage of the lung carcinogenesis contributes in part to the suppression of the development of lung tumors.

In the present study, the frequency of the K-*ras* mutation in group 2 also showed a tendency to be higher than that in group 3. It is likely, based on these data, that vitamin E has at least two different inhibitory effects at the initiation and promotion phases of the lung carcinogenesis. As it has been reported that the mutation of the oncogene based on the formation of DNA-adduct is a major event in the initiation phase (Nelson et al., 1992), the inhibitory effect of vitamin E at this phase may be based on the reduction in the frequency of the K-*ras* mutation. It is also known that abnormal cell proliferation during the promotion phase is a determining factor for the development of tumors (Kumar et al., 1990). Thus, the inhibitory effect of vitamin E at the promotion phase may be due to the reduction in the proliferation rate of the initiated cells. In order to clarify this possibility, we investigated whether vitamin E could inhibit cell proliferation at the promotion phase. Proliferating cell nuclear antigen is a useful marker of cell proliferation during carcinogenesis (Matsui-Yuasa et al., 1992). A recent report indicated that the level of proliferating cell nuclear antigen-cdc2 complex was more

useful than that of proliferating cell nuclear antigen as a marker of cell proliferation during carcinogenesis (Said and Medina, 1995). We therefore examined whether or not vitamin E suppresses cell proliferation in the promotion phase of lung carcinogenesis by using the level of the complex as a marker of cell proliferation. In the present study, the level of the complex paralleled the frequency of the K-*ras* mutation, indicating that vitamin E-dependent inhibition of cell proliferation during lung carcinogenesis was closely related to the decrease in the number of initiated cells. In addition, another group reported that the arrest of tumor cells at the G1 phase of the cell cycle by vitamin E treatment leads to cytostasis (cell death) and reduced cell numbers (Kline and Sanders, 1991). This report can also support the notion mentioned above. Overall, it is likely that vitamin E inhibits the development of lung tumors, through a reduction in the number of initiated cells having the K-*ras* mutation throughout the initiation and promotion phases in mice.

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