Vitamin E inhibits cell proliferation and the activation of extracellular signal-regulated kinase during the promotion phase of lung tumorigenesis irrespective of antioxidative effect

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We have already reported that the activation of extracellular signal-regulated kinase (Erk) is critical in the stimulation of cell proliferation during the promotion stage of urethane-induced lung tumorigenesis in mice. Also, we have found that vitamin E suppresses lung tumorigenesis by inhibiting cell proliferation at the promotion stage. However, it is still unclear whether this inhibitory effect at the promotion stage is based on the antioxidative effect of vitamin E or not. In order to address this question, we examined the inhibitory effect of \( \alpha \)-tocopheryloxybutyric acid (TSE), an ether derivative of vitamin E that cannot act as an antioxidant in vivo, on cell proliferation and the activation of Erk during promotion of lung tumorigenesis. On day 30 after urethane injection (750 mg/kg, i.p.) in A/J mice, TSE or vitamin E at 100 \( \mu \)mol/kg, p.o. was administered. Twenty-four hours after the final administration, the mice were killed to analyze cell proliferation and related parameters. The labeling index of proliferating cell nuclear antigen (a marker of cell proliferation) and ornithine decarboxylase activity (a marker of the promotion stage in lungs) were attenuated by treatment with TSE or vitamin E. TSE or vitamin E treatment also inhibited urethane-induced activation of Erk and suppressed the activation of other essential members of the Erk cascade (Ras, Raf and Mek).

These results suggest that vitamin E inhibits cell proliferation and activation of the Erk cascade during promotion of urethane-induced lung tumorigenesis in mice, independent of its antioxidative effect.

Introduction

Many epidemiological and experimental studies have shown that several naturally occurring compounds, including vitamins, can reduce the risk of cancer in various organs (1). The exact role of vitamin E in carcinogenesis has not been yet established, as it has shown opposite effects on carcinogenicity in a variety of model systems (2–5). For example, in rats fed a choline-deficient diet, vitamin E supplementation decreased the number and size of hepatic focal lesions (6), while a recent report showed that vitamin E functioned as a liver tumor promoter in diethylnitroamine-treated mice (7). Thus, vitamin E cannot be used clinically as a chemopreventive agent, because under some pathophysiological conditions it may enhance carcinogenicity. The reason why the enhancement occurs may depend on the prooxidant property of vitamin E but not its antioxidant property (8). Previous in vitro studies have shown that the antioxidative effect of vitamin E is not required for negative growth control of several tumor cell lines (9,10). If this in vitro effect can be extrapolated to an in vivo effect, it could be possible to develop a new chemopreventive agent based on the beneficial effect of vitamin E irrespective of the antioxidative effect.

We have already reported that vitamin E prevents lung cancer in several mouse models, including a urethane-treated mouse model (11–13). In these reports, we indicated that the inhibitory effect of vitamin E on lung tumorigenesis depends on the regulation of cell proliferation supported by the suppression of the induction of ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis in the promotion phase. However, it is still unclear if the inhibitory effect is based on the antioxidative property of vitamin E. In a recent study, we suggested that the constitutive activation of extracellular signal-regulated kinase (Erk), a key kinase in intracellular signal transduction pathways to stimulate cell proliferation, is essential for cell proliferation linked to the induction of ODC in the promotion phase of urethane-induced lung tumorigenesis in mice (13,14). Thus, it is likely that vitamin E inhibits cell proliferation in the promotion phase of lung tumorigenesis in part by inactivating Erk.

The aim of this study was to investigate if the inhibitory effect of vitamin E is a result of its non-antioxidative action. One vitamin E derivative, \( \alpha \)-tocopheryloxybutyric acid (TSE), has no antioxidative effect in vivo, unlike vitamin E, maybe because the ether bond in TSE cannot be hydrolyzed in vivo (14). Results with TSE should show if the inhibitory effect of vitamin E is dependent on its antioxidative or non-antioxidative effects. We therefore compared the inhibitory effect of TSE on cell proliferation and Erk activation in the promotion phase with that of vitamin E. Here, we present the first evidence that non-antioxidative effects of vitamin E contribute to the suppression of cell proliferation and activation of Erk during the promotion phase of urethane-induced lung tumorigenesis in mice.

Materials and methods

Animals and treatment

Female A/J mice (Japan SLC, Shizuoka, Japan) were used in this study. The mice were fed a CE-2 diet (Japan Clea, Tokyo, Japan) and sterilized water ad libitum. Tumor initiation was achieved by a single i.p. injection of urethane dissolved in saline (750 mg/kg body weight) at the age of 6 weeks. TSE was prepared according to an established method (15). TSE was >99% pure as judged by high performance liquid chromatography analysis. We confirmed that TSE was not hydrolyzed in vivo by showing that TSE treatment did not affect the concentration of \( \alpha \)-tocopherol in lungs or serum. On day 30 after...
the final injection, TSE and α-tocopherol suspended in vitamin E-stripped corn oil (100 μmol/kg body weight, p.o.) were given to the mice. Control mice were treated with vehicle only. Day 30 was chosen as the start point for treatment because this is when the activation of Erk and induction of ODC are maximal (14). Preliminary experiments showed that these treatment conditions were sufficient to inhibit Erk activation and ODC induction without affecting their basal concentrations. Twenty-four hours after the final treatment, all mice were killed and several parameters were measured.

**Cell proliferation assay**

The lungs were fixed with 10% buffered formalin. The tissues were embedded in paraffin wax and sectioned. Deparaffinized sections of tissue were stained by an avidin–biotin–peroxidase method with an anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Newk, CA, USA). Sections were counterstained with methyl green. The PCNA labeling index (LI) was determined by counting >500 nuclei in randomly selected microscopic fields of the alveolar region. This area was selected for estimation of the LI because we previously found that the activation of Erk and the induction of ODC during the promotion phase of urethane-induced lung tumorigenesis occurred mainly in alveolar epithelial cells, progenitor cells of lung tumors (14).

**Assay of ODC activity**

ODC activity was assayed as previously described (16). A 20% (w/v) lung homogenate in 0.05 M Tris–HCl, pH 7.5 was centrifuged at 100,000 × g for 30 min, and the supernatant was used to assay ODC activity by measuring the amount of radioactive CO2 liberated from L-[1-14C]ornithine. The assay mixture contained 0.4 mM L-[1-14C]ornithine, 0.18 mM pyridoxal phosphate, 0.25 mM EDTA, 2 mM dithiothreitol and 25 mM sucrose in 0.05 M Tris–HCl (pH 7.5).

**Analysis of the Ras signaling pathway**

The Erk signal cascade was analyzed by immunoprecipitation and immunoblotting as previously described (14). In brief, a 20% lung homogenate in lysis buffer (20 mM HEPES pH 7.0, 1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 25 mM β-glycerophosphate, 20 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) containing 0.25 M sucrose was centrifuged at 2000 × g for 10 min. The supernatant was used as a postnuclear fraction. The pellet was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. Immunoblotting with an antibody against phosphorylated Erk (Promega, Madison, WI, USA) was used to estimate the activation of Erk and with anti-Erk antibody (New England Biolabs, Beverly, MA, USA) to check the level of Erk. The postnuclear fraction was centrifuged at 100,000 × g for 60 min, yielding the cytoplasmic fraction and the membrane pellet. The cytoplasmic fraction was subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an antibody against phosphorylated Mek (New England Biolabs) to estimate the activation of Mek and with anti-Mek antibody (New England Biolabs) to check the level of Mek. The pellet was solubilized in lysis buffer containing 0.25 M sucrose and 1% Triton X-100. The solubilized fraction was incubated with an anti-c-Raf-1 antibody (Santa Cruz, CA, USA) or anti-Ras antibody (UBI, Lake Placid, NY, USA) and precipitated with protein G–Sepharose. The immuno-complex was separated on 5% or 7.5% SDS–PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with an anti-Ras monoclonal antibody (Oncogene Science, Cambridge, NY, USA) or an anti-c-Raf monoclonal antibody (Transduction Laboratories, Lexington, NY, USA) to check the level of Ras–Raf complex as an index of the activation of Ras and Raf (17). All immunoreactive bands were detected using the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK). Molecular sizing was carried out using the Rainbow molecular weight marker (Amersham).

**Statistical analysis**

Statistical analysis was performed by one-way analysis of variance followed by Duncan’s multiple-range test. A P value of ≤0.05 was considered significant.

**Results**

The effects of TSE or vitamin E on PCNA LI and ODC activity in lung are shown in Figure 1 panels A and B, respectively. PCNA LI and ODC activity in urethane-treated group increased by 60–80% and were significantly higher than in the control group. The increase induced by urethane treatment was significantly inhibited by TSE or vitamin E administration. There was no statistically significant difference between control and urethane+vitamin E-treated groups or between control and urethane+TSE-treated groups.

![Fig. 1. The effects of TSE and vitamin E on pulmonary PCNA LI (A) and ODC activity (B) at the promotion stage of urethane-induced lung tumorigenesis. Values are expressed as the mean from five mice; vertical lines indicate SEM. *Significantly different from the control group. **Significantly different from the urethane-treated group.](image-url)
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Fig. 2. The effects of TSE and vitamin E at the level of Ras–Raf complex at the promotion stage of urethane-induced lung tumorigenesis. (A) Immunoblot analysis of Ras protein immunoprecipitated with anti-Raf antibody. (B) Immunoblot analysis of Raf protein immunoprecipitated with anti-Ras antibody. This result is representative of three independent experiments.

Fig. 3. The effects of TSE and vitamin E on the phosphorylation state of Erk and Mek at the promotion stage of urethane-induced lung tumorigenesis. The phosphorylation level of each protein was analyzed by immunoblotting, using antibodies against phosphorylated Erk or Mek. This result is representative of three independent experiments.

Fig. 4. The effects of TSE and vitamin E on the level of Erk and Mek at the promotion stage of urethane-induced lung tumorigenesis. Immunoblot analysis was performed using the same sample as in Figure 3. This result is representative of three independent experiments.

Discussion

Lung cancer causes a great many deaths worldwide, and is increasing in incidence in Japan (19), for example, warranting research into drugs that could prevent it. Our previous animal studies suggest that vitamin E could be useful for preventing lung cancer (11–13,20,21). However, as mentioned above, vitamin E is not suitable for clinical use as a chemopreventive agent, because it acts as a tumor promoter in liver and skin, maybe because of its function as a pro-oxidant (7,8). To investigate whether this disadvantage can be overcome, we investigated whether vitamin E can suppress the promotion of lung tumorigenesis in mice based on its non-antioxidative effect. The present study demonstrated that vitamin E inhibits cell proliferation and its related signal transduction during promotion of lung tumorigenesis irrespective of its anti-oxidative effect, so it is possible that a new chemopreventive agent for lung cancer could be developed based on the beneficial effect of vitamin E.

Ras proteins, which are located upstream of the Erk signaling cascade, normally serve as molecular switches in mitogenic signal transduction and regulate cell growth and differentiation under the control of growth factor receptor-dependent signaling. Activating mutations in ras genes result in proteins being trapped in the active state and constitutively transmit growth signals whether or not the growth factor receptor is activated (22,23). In a previous study, we showed that urethane-induced ras mutations cause constitutive activation of the Erk.
signal pathway in the promotion stage of lung tumorigenesis without activating epidermal growth factor receptor (EGFR) (14). In addition to the ras mutation-dependent activation, we have also demonstrated that activation of EGFR stimulates activation of the Ras–Erk cascade (14). Our present study indicates that activation of the Erk cascade was almost completely inhibited by vitamin E and that inhibition by TSE was similar. Thus, vitamin E can inhibit both EGFR- and ras mutation-dependent Erk activation caused by urethane treatment without its antioxidative effect. Although the inhibitory mechanism is still unclear, we propose that vitamin E may suppress the EGFR-dependent Erk activation by altering membrane function via a non-antioxidative effect. Irrespective of antioxidative effect, vitamin E and its derivatives stabilize membrane physicochemically, and inhibit membrane-dependent signal pathways, e.g. growth factor receptor-dependent mitogenic signaling (24–26). Another finding that supports this possibility is that a tea polyphenol, a lipid-soluble compound, shuts down membrane-dependent cell growth signal by a so-called ‘sealing effect’; it inhibits the interaction of tumor promoters, hormones and growth factors with their receptors (27). However, this proposed mechanism cannot explain how vitamin E inhibits ras mutation-induced Erk activation, because this type of Erk activation occurs without any activation of EGFR. Previous reports indicate that Ras precursors are synthesized as biologically inactive forms in cytoplasm and undergo multiple post-translational modifications before they become mature Ras proteins capable of mediating intracellular signal transduction (28,29). Farnesylation is the first step in this Ras maturation process; several steps later, the Ras protein is inserted into the cell membrane and finally becomes biologically active (30). Since the non-antioxidative effect of vitamin E is mainly dependent on membrane stabilization (25), it seems that vitamin E inhibits ras mutation-induced Erk activation by blocking the insertion of Ras protein into the cell membrane in a similar manner to the sealing effect mentioned above.

The ODC gene is now recognized as a proto-oncogene required for cell-cycle progression and cell transformation (31,32), but little is known about the signal-transduction pathways involved in the induction of ODC. More recently, it was reported that Erk is involved in regulating the expression of ODC in leukaemia cells by affecting its transcription (33). The mouse ODC gene is a principal transcription target for AP-1, c-Myc, SP1 and CREB (34). Erk mediates the increase in AP-1 level through c-fos gene expression based on the phosphorylation of Elk-1 (35), so this pathway may be an Erk-dependent signal for ODC induction. If activation of Erk during the promotion of lung tumorigenesis induces ODC through this pathway, the inhibitory effect of vitamin E on the activation of Erk leads to ODC induction to be suppressed due to the modulation of transcriptional level. However, further study is needed to clarify the mechanism. Understanding of this mechanism may help in the development of new drugs to prevent lung cancer based on the non-antioxidative effect of vitamin E.

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References

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