The Effect of Dietary Carotenoids on Lung Tumorigenesis Induced by Intratracheally Instillated Diesel Exhaust Particles

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Summary The purpose of this study is to examine the carotenoid effects on lung tumorigenesis induced by intratracheal instillation of diesel exhaust particles (DEP) into mice weekly for 20 wk. It was suggested that active oxygen radicals might play an important role in DEP-induced lung tumorigenesis. Mice were divided to 4 groups of diet containing 0.02% of palm oil carotene, 0.02% of β-carotene, or no carotenoid with or without DEP. The BF group (4% fat) and the HF group (16% fat) were prepared for each diet group. The experimental period was 12 mo. By the administration of palm oil carotene, neither adenocarcinoma nor adenoma was found in the BF group. In the HF group with palm oil carotene, no adenocarcinoma was observed, and adenoma was reduced. Adenoma in the HF group was not greatly reduced by β-carotene, but rather increased in the BF group. No adenocarcinoma was found in either the BF or the HF groups with β-carotene. The 8-hydroxydeoxyguanosine/deoxyguanosine ratio in palm carotene groups was lower than in the other groups, while that in β-carotene groups was not. From these results, palm oil carotene was suggested to prevent lung tumorigenesis by its protective effect on DNA from active oxygen. Beta-carotene was supposed to have different effects from palm oil carotene on lung tumorigenesis. Besides the chemopreventive effect, the growth of mice was inhibited by the administration of palm oil carotene. Further studies are necessary to elucidate the mechanisms of carotenoid effects.

Key Words β-carotene, palm oil carotene, lung tumorigenesis, active oxygen, 8-hydroxydeoxyguanosine

Many epidemiological studies have reported the negative relationship between carotenoid intake and cancer incidence (1–3), especially with regard to β-carotene and lung tumorigenesis. To investigate the details of carotenoid effects on cancer, experimental studies are currently underway using ultraviolet (UV) light, chemical carcinogens and so on (see review, 3). Some studies have used diesel exhaust particles (DEP) to induce lung cancer. It is proposed that active oxygen species is generated by the phagocytosis of particles and enzymatic or non-enzymatic reactions in lung (4–6), which are suggested to be involved in some stages of tumorigenesis (7–12). DNA damages caused by active oxygen from DEP are proposed to be a possible mechanism for lung tumorigenesis because a significant correlation between the formation of 8-hydroxydeoxyguanosine (8-OHdG) (an indicator of active oxygen damaged DNA) and lung tumorigenesis was found (13). Various oxygen radical-forming carcinogenic substances were observed to induce the formation of 8-hydroxydeoxyguanosine (8-OHdG) (14, 15) and in vivo after instillation (13) or inhalation of DEP (16, 17). Carotenoid has the effect of trapping active oxygen radicals in biological systems (3). From this knowledge, it was expected that carotenoid containing diet might protect DNA from the attack of active oxygen radicals. In our previous study, β-carotene provided partial prevention against 8-OHdG formation (13, 16).

Here, we fed mice with some kinds of diet containing palm oil carotene or β-carotene to compare the effects of carotenoids on lung tumorigenesis. Both a basal fat
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Materials and Methods

Materials. All-trans β-carotene was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) for the animal experiment (synthetic purity 92–99%), and was kindly donated by Hoffmann-La Roche Japan, Co. (Tokyo, Japan) for the HPLC standard. All-trans α-carotene standard and palm oil carotene were provided by Lion Co. (Tokyo, Japan). Tocopherol standards were purchased from Eisai Co. (Tokyo, Japan), nuclease P1 and dG standard solution from Yamasa Corporation (Chiba, Japan), and alkaline phosphatase, calf thymus DNA, and hematoxylin from Sigma Chemical Co. (St. Louis, USA). The chemicals for high performance liquid chromatography (HPLC) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Diets. Two hundred and eighty male ICR mice (6 wk old) were purchased from Japan Clea Co. (Tokyo, Japan), and divided into 8 groups (35 mice per group). Each group of mice was fed the different type of diet whose basal composition was the same (16). The BF diet (fat content: 4%) was enriched with corn oil to prepare the HF diet (fat content: 16%) (prepared by Funabashi Farm Co., Chiba, Japan). The BF diet and the HF diet were supplemented with 0.02% of β-carotene, 0.02% of palm oil carotene or no carotenoid. They were sealed under nitrogen gas in bags and kept at 4°C. Water was available ad libitum. Less than 12 mice were housed in one plastic cage on soft wood chips. The conditions to keep mice were the same mentioned before (19), and the experimental period was 12 mo. The names of the mice groups in this paper were as follows, according to their diets and DEP intratracheal instillation in glass tubes, filtrated with gauze, and centrifuged vigorously for 5 min in a nitrogen gas atmosphere (21). The hexane layer was once washed with distilled water and evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in the exact amount of methanol. The carotenoids were determined on the same day as the extraction. Until the determination of retinol and tocopherol, the samples were stored at –20°C under nitrogen gas.

Extraction of DNA for the 8-OHdG determination. Five lung right lobes not bearing tumors were chosen in each group and stored at –80°C after macroscopic observation until the determination of 8-OHdG. The method developed by Marmur (22) was modified for the extraction of DNA from the lung. Samples were homogenized with 9 mL of 1.2 M sucrose/3.3 mM CaCl2 solution in glass tubes, filtrated with gauze, and centrifuged at 18,000 rpm for 1 h. The pellets were suspended in 2 mL of 0.34 M sucrose, and centrifuged at 2,500 rpm for 5 min. The pellets (nuclei fraction) were resuspended in 500 µL of 0.34 M sucrose containing 10% ethanol, had 50 µL of 25% SDS added, and were incubated at 37°C for 30 min. For the DNA extraction, 150 µL of 5 M sodium perchlorate and 700 µL of dichloromethane containing 4% isoamylalcohol were added, and the samples were mixed by inverting for one min and centrifuged at 8,000 rpm for 5 min. The DNA in the upper layer was precipitated with chilled ethanol three times, dried, and treated in 80–100 µL of 0.05 mg/mL nuclelease P1 at 37°C for 30 min. The DNA solution was incubated for further digestion with 15 µL of 1 mg/mL nuclease P1 solution for 30 min, and digested into deoxyribonucleotides with 5 µL of alkaline phosphatase in 30 µL of 1 M Tris-HCl (pH 7.5) by 1–2 h incubation. The digested DNA mixture solutions were placed on ice until injection into HPLC. They had 0.1 volume of 10% methanol/10 mM sodium dihydrogen phosphate added in case of keeping for several hours until their determination.

HPLC apparatus and condition. All-trans α-carotene and all-trans β-carotene were determined by HPLC (LC-6A, Shimadzu Corporation, Osaka, Japan) with a Wako pak® HPLC column, 4.0×250 mm (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using 50 mM sodium perchlorate and 2 μM EDTA-4Na in 50% methanol, 48% acetonitrile, and 2% distilled water as a mobile phase. The detector was a BAS LC-4C electro-

(BF) diet and a high fat (HF) diet was prepared for each kind of diet group because more lung tumorigenesis is expected to be induced by the increase of oxygen free radicals by the double effect of DEP and high fat (18). The effect of carotenoid on lung tumorigenesis was examined by histopathological observation, carotenoids, retinol and tocopherol content, and the ratio of 8-OHdG/deoxyguanosine (dG) whose extraction method was improved from our previous study (13, 16).
chemical detector (ECD) (BAS, Tokyo, Japan). The system was maintained at 40°C, the flow rate 1.0 mL/min.

For the retinol and the tocopherol determination, the analytical column was a 4.6×150 mm Shim-pack CLC-ODS (M) column (Shimadzu Co., Tokyo, Japan) with a Guard-Pak pre-column (Shim-pack G-ODS, 4 mm×1 cm, Shimadzu Co.). They were separated at a flow rate 0.8 mL/min using methanol/acetonitrile/dichloromethane (7 : 7 : 2, v/v/v) as the mobile phase. The detection of retinol was performed by UV detector (SPD-10, Shimadzu Co.) at a wavelength of 325 nm. The detection of tocopherol was performed by spectrofluorometric detector (RF-550, Shimadzu Co.) at an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

For 8-OHdG determination, the mobile phase was composed of 7% methanol, 10 mM sodium dihydrogen phosphate, and 10 mM sodium acetate in distilled water. The flow rate was 1 mL/min. The HPLC column, Intersil ODS-2, 4.6×250 mm (GL Science Inc., Tokyo, Japan) was maintained at 35°C. The content of 8-OHdG in the digested DNA was measured using an ECD (Coulochem II, Esa Inc., Bedford, MA, USA). The total amount of dG was simultaneously detected using a UV detector (UV-8020, Tosoh, Tokyo, Japan) at a wavelength of 290 nm. The content of 8-OHdG in DNA was expressed as the ratio of 8-OHdG to total dG (8-OHdG/dG).

Standard solution was injected every 10 samples to examine the accuracy of the analysis. All chromatographic peaks were identified by comparing retention time against known standards.

Statistics. The significance of difference was calculated using the χ²-test for the examination of lung tumorigenesis, the ANOVA test for the determination of vitamins and 8-OHdG/dG ratio. The regression analysis show significance at p<0.05 (*), p<0.01 (**), and p<0.001 (***)

RESULTS

Body weight and Diet consumption

Growth curves of mice are shown in Fig. 1. The body weight was measured every 1.5 mo. The body weight in the palm oil carotene groups, both in the basal fat group and the high fat group (BFP+DEP and HFP+DEP), was significantly lower than those in no carotenoid fed groups (BF+CONT and HF+CONT). The growth in other groups was normal, and we could not find any significant difference.

As an approach to discover the reason for the growth difference with palm oil carotene administration, the diet consumption was compared in the same diets and DEP instillation groups under the same condition as those used in this study (data not shown). Both the diet consumption and the body weight were measured for 5 mo. The body weight checked 2–4 times per month showed again significantly lower measures for the palm oil carotene groups (BFP+DEP and HFP+DEP). The diet consumption was measured daily for 5d running, and the average of the daily consumption determined. It was measured 4 times, at 2 wk, 5 wk, 10 wk, and 18 wk. No significant difference in diet consumption could be found between the groups.

Histopathological examination of lung tumorigenesis

The mortality rate at the end point of the experiment was shown in Table 1. At the start of the experiment, 35 mice were in each group. Several mice died during the DEP instillation for 20 wk; for example, 9 mice died in the HF+CONT group. Those mice were not analyzed and not included in mortality rate because the cause of the death was supposed not to be the DEP effect. After the last DEP instillation, mice were kept under the same condition for 7 mo to develop tumor (tumor development period). The mice which died during the tumor development period were histopathologically examined for tumorigenesis, and included in “the effective number” in Table 1 if the cause of death was able to be diagnosed. The mortality rate represents the ratio of the number of dead mice during the tumor development period to the number of mice living at the last DEP instilla-
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Table 1. Results of Histopathological examination.

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective numbers</th>
<th>Number surviving at the end of experiment</th>
<th>Mortality rate (%)</th>
<th>Lung tumor bearing mice (%)</th>
<th>Type of Lung tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenoma (%)</td>
</tr>
<tr>
<td>BF+CONT</td>
<td>33</td>
<td>30</td>
<td>9.1</td>
<td>12.2</td>
<td>12.2</td>
</tr>
<tr>
<td>BF+DEP</td>
<td>33</td>
<td>28</td>
<td>15.2</td>
<td>18.8</td>
<td>12.5</td>
</tr>
<tr>
<td>BFB+DEP</td>
<td>30</td>
<td>28</td>
<td>6.7</td>
<td>20.7</td>
<td>20.7</td>
</tr>
<tr>
<td>BFP+DEP</td>
<td>30</td>
<td>19</td>
<td>36.7</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>HF+CONT</td>
<td>26</td>
<td>19</td>
<td>26.9</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>HF+DEP</td>
<td>33</td>
<td>29</td>
<td>12.1</td>
<td>30.3</td>
<td>24.2</td>
</tr>
<tr>
<td>HFB+DEP</td>
<td>30</td>
<td>26</td>
<td>13.3</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>HFP+DEP</td>
<td>30</td>
<td>26</td>
<td>13.3</td>
<td>16.7</td>
<td>13.3</td>
</tr>
</tbody>
</table>

* Compared BF+DEP with BFP+DEP, p<0.05, by χ² test.

tion. Lung tumorigenesis was determined by both pathological diagnosis and light microscopical observation of histopathological samples. The results of the incidence of the lung tumors were shown in Table 1. Lung tumors were diagnosed as adenoma or adenocarcinoma ("type of lung tumor" in Table 1) by the examination. Adenoma was a solid lump with moderate compression of surrounding parenchyma. Adenocarcinoma showed a tubular papillary growth pattern and distortion of the alveolar spaces by neoplastic cells with cellular atypia. A large portion of tumor had invaded a bronchus. "Lung tumor bearing mice" in Table 1 represents the total percentage of adenoma and adenocarcinoma in "effective numbers".

In the BF groups, the percentages of the total lung tumor were 12.2% for the BF+CONT group, 18.8% for the BF+DEP group, 20.7% for the BFB+DEP group and 0% for the BFP+DEP group. Adenocarcinoma was found only in the BF+DEP group (6.3%). The incidence of lung tumor in the BF+DEP group was significantly lower than that in the BF+DEP group. In the HF groups, the percentages of total lung tumor were 12.5% for the HF+CONT group, 30.3% for the HF+DEP group, 20.0% for the HFB+DEP group and 16.7% for the HFP+DEP. In the HF+DEP and the HFP+DEP groups, 6.1% and 3.3% showed adenocarcinoma, respectively. In the HFB+DEP group, no adenocarcinoma was found.

We found few liver tumors in this study (data not shown). The tumors found in the lungs were not metastases of tumors that had developed in other organs.

Fat-soluble vitamin levels in plasma

The precision of the vitamin extraction was 89–115% by the standard recovery experiment. The correlation between the amount of standard samples and the area of peaks was 0.97–0.99 in these vitamin standard curves. Alpha-carotene, β-carotene, retinol and tocopherols levels were determined in plasma collected from mice examined for lung tumorigenesis. Both α-carotene and β-carotene were below the sensitivity of assay method used in this study in the non carotenoids fed groups. α-carotene was detected only in the palm oil carotene fed groups (BFP+DEP and HFP+DEP). The α-carotene amount in the HFP+DEP group was about 3 times greater than that in the BFP+DEP group, although individual differences were very large. Beta-carotene was observed in both β-carotene and palm oil carotene-fed groups. The β-carotene content in the HFB+DEP group was about 4 times greater than in the BFB+DEP group. The β-carotene amount in palm oil carotene groups (BFP+DEP and HFP+DEP) was lower than that in β-carotene groups (BFB+DEP and HFB+DEP). The ratio of β-carotene content in β-carotene groups to palm oil carotene groups (BFB+DEP: BFP+DEP and HFB+DEP: HFP+DEP) was not same between basal fat and high fat. The β-carotene amount in the HFP+DEP group was about 2.2 times greater than that in the BFP+DEP group.

No difference of retinol content between groups was found in the BF groups. In the HF groups, the retinol content in the HFB+DEP was greater than that in other groups. Significant difference was found between HF+DEP and HFB+DEP groups, and HFB+DEP and HFP+DEP groups.

A lesser amount of α-tocopherol was found in the palm oil carotene fed group in both the BF and the HF groups than in the other groups. In the BF groups, significant difference was found between BFB+DEP and BFP+DEP. Alpha tocopherol amount in the HFP+DEP groups was significantly lower than that in other HF groups. In the HF groups, the content of α-tocopherol in the HFB+DEP group was significantly greater than that in other groups. The other tocopherols, (β+γ)-tocopherol and δ-tocopherol, were not detectable under the present assay condition.

8-OHdG/dG ratio in lungs

No generation of active oxygen during the DNA extraction was confirmed by nuclear magnetic resonance (data not shown). To check the precision of the DNA extraction, rat liver spiked with various amounts of the modified DNA (the high 8-OHdG content DNA, the 8-OHdG/dG ratio in modified DNA was 27×10⁻⁵) was used for the recovery experiment. Between the experimental data and the theoretically calculated value, the correlation efficiency was 0.98, and the slope was 1.08. The correlation between the amount of standard sam-
Fig. 2. Vitamin content in plasma from mice examined for lung tumorigenesis (n=the same as "effective number" in Table 1). (a) α-carotene, (b) β-carotene, (c) retinol, and (d) α-tocopherol. ***, p<0.005.

The 8-OHdG/dG ratio in lung examined for tumorigenesis were shown in Fig. 3. The 8-OHdG/dG ratio in the control groups (BF+CONT and HF+CONT) were greater than that in the other groups. Especially, in the HF groups, the difference was significant. The ratios in the palm oil carotene fed groups (BFP+DEP and HFP+DEP), tended to be lower than the ratios in other groups. In the BF groups, 8-OHdG/dG ratio in BFP+DEP was significantly lower than that in BF+CONT and BF+DEP. The 8-OHdG/dG ratio in the HF groups tended to be greater than that in each matched BF group. A significant difference was found only between the BF+CONT group and the HF+CONT group.

**DISCUSSION**

Lung tumorigenesis was completely inhibited in the BFP+DEP group. The effect of palm oil carotene was so strong that even spontaneous lung tumorigenesis was inhibited in the BF+CONT group (background). The in-
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Fig. 3. 8-OHdG/dG ratio in right lobes of lungs bearing no tumor. (a) basal fat groups, and (b) high fat groups (n=5). *, p<0.05, **, p<0.01, ***, p<0.005.

cidence of lung tumorigenesis in the HFP+DEP group was also lower than the other HF groups although 3.3% of adenocarcinoma was induced. These results suggested that palm oil carotene was effective for the protection of lung tumorigenesis induced by the DEP instillation. The formation of 8-OHdG was also reduced in the palm oil carotene fed groups. Taken together, it was suggested that palm oil carotene might prevent the DNA damage that would develop lung tumorigenesis. Beta-carotene was not so effective on lung adenoma, but adenocarcinoma was not found in these groups. The ratio of 8-OHdG/dG was not reduced by β-carotene administration. Beta-carotene may regulate the development of tumor by actions other than the prevention of the formation of 8-OHdG. It was supposed that palm oil carotene and β-carotene have different chemopreventive effects on lung tumorigenesis. Murakoshi et al. reported that palm oil carotene was more effective on liver carcinogenesis in CH3/He mice than β-carotene or α-carotene (23). Palm oil carotene is the mixture of 30% α-carotene, 60% β-carotene (30% all-trans β-carotene, 25% 9-cis β-carotene, 13% 13-cis β-carotene: the data from the intact palm oil carotene), 3% γ-carotene and 4% lycopene. In palm oil carotene fed groups, although both α-carotene and β-carotene were found in plasma, we have not determined other kinds of carotenoids or β-carotene isomers. Singlet oxygen quenching ability of the various carotenoids was reported as follows: lycopene>γ-carotene>α-carotene>β-carotene (24). Thus, the singlet oxygen quenching ability of palm oil carotene is expected stronger than that of β-carotene. That is supposed to be one of the reasons why palm oil carotene was more effective than β-carotene on the DNA protection from active oxygen attack generated from DEP. Lesser amount of carotenoids, i.e., lycopene, γ-carotene, or α-carotene, or isomers of β-carotene may have important role in the protective effects of palm oil carotene (23). The combination and/or interaction of different kinds of carotenoid may be important for the palm oil carotene effects on lung tumorigenesis.

In spite of the positive effects of palm oil carotene, the normal growth of mice was impaired in these groups. The diet consumption of mice was not different by groups. Components or metabolites of palm oil carotene might inhibit the normal metabolism of nutrients, or keep metabolic turnover slow. Mice were not supposed to get enough energy or elements to synthesize the body components for their normal growth. Further studies are needed for the administration of palm oil carotene since the growth defect is a serious problem. The intake of n-6 polyunsaturated fatty acids, such as linoleic acid present in corn oil (we used corn oil to prepare the high fat diet) has been reported to enhance tumor development (25). By oxygen free radicals produced during lipid peroxide formation and its degradation process in vivo (26), incidence of lung tumorigenesis and 8-OHdG/dG ratio are supposed to be increased in the HF groups. The content of carotenoids in plasma of HF groups was also increased because more fat soluble vitamins should be absorbed with fat (27). It was supposed that increased carotenoids content was not enough to protect DNA from active oxygen attack.

The individual difference of carotenoid content was great in plasma. Beta-carotene was suggested to be absorbed and transferred from the intestine into chylomicrons (28). It was also reported to degrade in the intestine, and the content of the β-carotene intact form was different by the individual (29). It may be necessary to obtain the exact carotenoid absorption data (amount, metabolism, and so on) for their administration as chemopreventive chemicals. Only in the HFB+DEP group were retinol and α-tocopherol content greater. More β-carotene should be absorbed and available in this group with the absorption of more fat, and the excess amount of β-carotene might convert to retinol. The effect of this phenomenon on lung tumorigenesis or DNA was not found in this study. The content of α-tocopherol was less in palm oil carotene fed groups, and was higher in β-carotene fed groups. There are some reports that carotenoid treatment affected tocopherol metabolism in plasma and organs (30, 31). Carotenoids and tocopherol may compete for absorption in intestine (32, 33) or for sharing their role in lipoproteins (28). If
α-tocopherol storage in lung was increased by the supplementation of carotenoids in this system, it might be protected more effectively from active oxygen attack because a combination of β-carotene and α-tocopherol was reported to inhibit lipid peroxidation to a significantly greater degree in isolated membranes (34).

In the present system, some “body defense system” seemed to be activated and/or induced. The incidence of lung tumorigenesis was not so different between the BF+CONT and the BF+DEP group. Ichinose et al., who instilled 0.01 mg of DEP once a week for 10 wk, could induce lung tumorigenesis 13.3% for the BF+CONT, and 31.0% for BF+DEP (16). With our system of instilling 0.005 mg DEP for 20 wk, the incidence of tumorigenesis was 12.2% for the BF+CONT and 18.8% for BF+DEP. When diluted DEP was instilled for a longer period, its effect on lung may have been more moderate than that of concentrated DEP for a short period. This phenomenon is proposed to be a “body defense system” (17), which is the system to protect normal physiological condition from active oxygen toxicity, for example, induction and/or activations of scavengers for active oxygen species, antioxidation enzymes, antioxidation vitamins, and enzymes of DNA repair system. The body defense system is supposed to be activated by the chronic and moderate, but not acute, exposure of diesel exhaust (17). In the case of the study by Ichinose et al., 0.01 mg DEP per week may have been too strong for the body system to defend against active oxygen toxicity. The 8-OhDg/dG ratio in non DEP instilled groups was greater than that in the DEP instilled groups. The time course of the formation of 8-OHdG was reported after the 3 mg single instillation of DEP (13). The 8-OHdG content was increased after 3 h to 2 d, and then decreased to a lower level than the normal condition in their system.

Besides the chemopreventive effect of palm oil carotene, the growth was inhibited by its administration. Thus, we should pay attention to the possibility that the reduction of body weight may play some role in suppression of lung tumorigenesis. Further studies are necessary to elucidate the mechanisms of carotenoid effects.

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