No inhibitory effects of (-)-epigallocatechin gallate and lycopene on spontaneous hepatotumorigenesis in C3H/HeN mice

Fukushima J. Med. Sci.,
Vol. 56, No. 1, 2010

[Original Article]

NO INHIBITORY EFFECTS OF (−)-EPIGALLOCATECHIN GALLATE AND LYCOPENE ON SPONTANEOUS HEPATOTUMORIGENESIS IN C3H/HeN MICE

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(Received March 11, 2009, accepted March 3, 2010)

Abstract: Although several studies have indicated that (−)-epigallocatechin gallate (EGCG) and lycopene, representative dietary antioxidants, inhibit chemically induced animal tumorigenesis, only a few studies have examined the inhibitory effects of these compounds on spontaneous liver tumorigenesis in rodents. In this study, we investigated the inhibitory effects of these compounds on the formation of spontaneous liver tumors in C3H/HeN mice. We used xeroderma pigmentosum group A (XPA) gene-deficient mice to simultaneously examine whether the knockout mice could be used as a sensitive animal model. In addition, we examined the levels of 8-hydroxy-2´-deoxyguanosine (8-OHdG) — a marker of reactive oxygen species-induced DNA injury — in liver tissue. Male *XPA* +/+, *XPA* +/−, and *XPA* −/− mice with a C3H/HeN genetic background were divided into 3 groups: control, EGCG, and lycopene. Autopsy at 18 months of age revealed that EGCG and lycopene did not exhibit obvious suppressive effects on the development of liver tumors in any XPA genotype; further, the XPA genotype did not influence any susceptibility to liver tumors. With regard to 8-OHdG levels in non-tumorous liver tissue at 8 months of age, EGCG showed no significant inhibitory effects and lycopene showed significant inhibitory effects only in *XPA* +/− mice. The present study demonstrates that contrary to previous reports of the inhibitory effects of EGCG and lycopene on the development of various carcinogen-induced animal tumors, these compounds exert no chemopreventive effects on spontaneous liver tumorigenesis in C3H/HeN mice. EGCG and lycopene may inhibit carcinogen-induced tumors through properties other than their antioxidant abilities.

Key words: (−)-Epigallocatechin gallate, Knockout mice, Lycopene, Spontaneous liver

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tumor, Xeroderma pigmentosum group A

INTRODUCTION

Liver cancer is a major cause of death in African and Asian countries, including Japan[1]. More than 30,000 Japanese die of liver cancer annually, and 95% of these deaths are due to hepatocellular carcinoma (HCC)[2]. Moreover, the number of HCC patients in Japan is increasing.

Reactive oxygen species (ROS) are considered to be one of the main causes of carcinogenesis in various organs. ROS induce cancer-causing mutations, oxidize lipids and proteins, and alter signal transduction pathways, resulting in increased cancer risk[3,4]. The relationship between ROS and carcinogenesis is supported by the fact that dietary and endogenous antioxidants inhibit carcinogenesis in animal models[5]. Epidemiological studies have revealed that high consumption of antioxidant-rich fruits and vegetables is inversely correlated with the incidence of cancer[6-8].

Lycopene and (−)-epigallocatechin gallate (EGCG) are representative dietary antioxidants. EGCG is the most abundant polyphenolic compound present in green tea (more than 40% of the total polyphenolic mixture)[9], and it is the most powerful antioxidant among green tea catechins[10]. Lycopene is the most abundant carotenoid in tomatoes and the cause of their deep-red color[11]. Lycopene is known to possess high singlet oxygen-quenching capability[12]. Although EGCG and lycopene have been reported to inhibit the formation of carcinogen-induced tumors in various animal models[13-16], only a few studies have examined the inhibitory effects of these compounds on spontaneous liver tumorigenesis in rodents[17,18].

Male C3H mice exhibit high susceptibility to spontaneous and chemically induced hepatotumorigenesis ; these mice spontaneously develop liver tumors late in their life, with an incidence as high as 70%[19]. Genetic linkage analysis using C3H mice and hepatotumorigenesis-resistant strains have revealed that 6 different regions on chromosomes 2, 5, 7, 8, 12, and 19 showed significant linkage with hepatocellular tumor development ; these regions were named "hepatocarcinogen sensitivity (Hcs) loci"[20]. Thus, C3H mice are considered to be a good model of polygenic inheritance for predisposition to liver cancer.

Xeroderma pigmentosum (XP) is a hereditary disease characterized by impairment of the nucleotide excision repair (NER) pathway, which is one of the main DNA repair pathways[21,22]. XP is classified into 8 types. Previously, we established XP group A gene-deficient mice (*XPA*-deficient mice) on a C3H/HeN genetic background and confirmed that *XPA* −/− mice were more susceptible to spontaneous and carcinogen-induced liver tumorigenesis than *XPA* +/+ and *XPA* +/− mice[23]. This observation suggests that *XPA*-deficient C3H mice may be a more sensitive animal model than wild-type C3H mice in identifying liver carcinogens or liver tumor-preventing compounds.

In this study, we administered EGCG or lycopene to *XPA*-deficient mice with a C3H/HeN genetic background for a long period and examined the inhibitory effects of these compounds on the development of spontaneous liver tumors. We also examined the levels of 8-hydroxy-2´-deoxyguanosine (8-OHdG) — a marker of ROS-induced DNA injury — in

liver tissue.

MATERIALS AND METHODS

Mice

XPA-deficient mice were originally produced by Tanaka *et al.*; these mice had a hybrid genetic background[24]. We established *XPA*-deficient congenic mice with a C3H/HeN genetic background by repeated back-crossing with inbred C3H/HeN mice[23]. In the present study, we used an F12 line because we maintained the mice of this line. The method of genotype analysis has previously been described[23]. The mice were maintained in the Laboratory Animal Center of Teikyo University School of Medicine at 21°C and 53% humidity in accordance with the rules of the animal center.

Effects of EGCG and lycopene on spontaneous liver tumor

The *XPA*-deficient mice with a C3H/HeN genetic background were divided into 3 groups : control, EGCG, and lycopene. Each group contained 15-20 male mice for each XPA genotype. Only male mice were used because male C3H mice are more susceptible to spontaneous and chemically induced hepatotumorigenesis than female C3H mice[25-27]. The mice in the control group were fed a CRF-1 diet (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum until the end of the experiment. The mice in the EGCG group were fed a CRF-1 diet and tap water containing 0.05% EGCG ad libitum from 6 weeks of age until the end of the experiment. Pure EGCG was extracted from green tea as previously reported[10], and stored in a refrigerator at 4°C. Tap water containing EGCG was administered from light-shielded, polyvinyl bottles, and the bottles were refilled with fresh solution twice a week. The mice in the lycopene group were fed a CRF-1 diet containing 0.005% lycopene and tap water ad libitum from 6 weeks of age until the end of the experiment. Lycopene was donated by LycoRed Natural Products Industries (Beer-Sheva, Israel) as a natural tomato extract containing 6% lycopene (Lyc-O-Mato). The Lyc-O-Mato and CRF-1 diet containing lycopene were stored in a refrigerator at 4°C. Feed containers for mice were refilled with fresh lycopene diet twice a week. The doses of EGCG and lycopene in the present study are comparable to those in previous studies in which these compounds inhibited chemically induced and spontaneous mouse tumorigenesis[16,17].

All the mice were killed by anesthesia at 18 months of age, and a complete autopsy was performed. At autopsy, the total body weight and weight of the major organs were measured. The surface of the livers was grossly examined, and tumor nodules were counted. After fixation in 10% formaldehyde solution, each liver lobe was completely sectioned into 2-mm-thick slices and internal tumor nodules were counted. The total number of tumor nodules was calculated as the sum of the surface and internal tumor nodules. All sections were mounted on slides for light microscopy. Hepatocellular adenomas (HCAs) and carcinomas were diagnosed microscopically on the basis of established diagnostic criteria[28]. Major organs, except the liver, were also grossly examined, and representative sections were

mounted on slides.

Quantification of 8-OHdG

The *XPA*-deficient mice with a C3H/HeN genetic background were divided into 3 groups as in the tumorigenesis experiment, and each group contained 6 male mice for each XPA genotype. The treatments were the same as those in the tumorigenesis experiment. All the mice were killed at 8 months of age, and approximately 1 g of non-tumorous liver tissue was excised and stored at −80°C. The mice were killed at that age because, in our experience, we have found that spontaneous liver tumors begin to occur around that age. DNA samples isolated from the frozen liver by phenol and chloroform were digested into deoxynucleosides by a combined treatment with nuclease P1 and alkaline phosphatase. The 8-OHdG levels were determined using high-performance liquid chromatography as described previously[29] and expressed as the number of 8-OHdG residues for every 10^5 deoxyguanosines.

The protocol for this research project was approved by the Ethics Committee of Teikyo University School of Medicine, and it conformed to the provisions of the 1995 Declaration of Helsinki (as revised in Edinburgh in 2000). The experiment was ethically acceptable and conformed to national guidelines for animal usage in research.

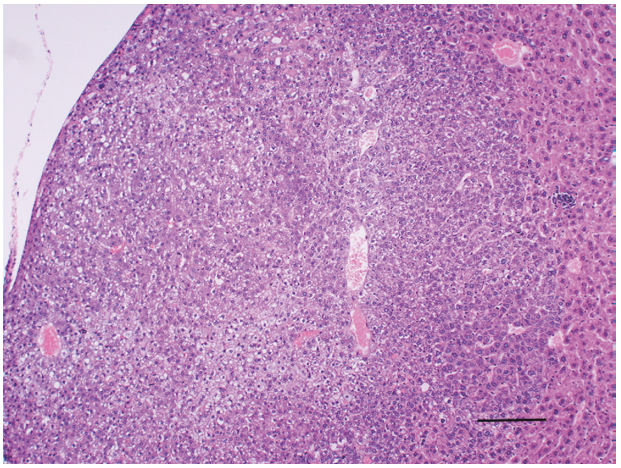
Statistical analysis

Group differences were assessed for statistical significance by using the χ^2 test for the incidence of tumors and the *t*-test for the number of tumors per mouse, diameter of tumors, and 8-OHdG levels. A *p*-value of <0.05 was considered to be significant.

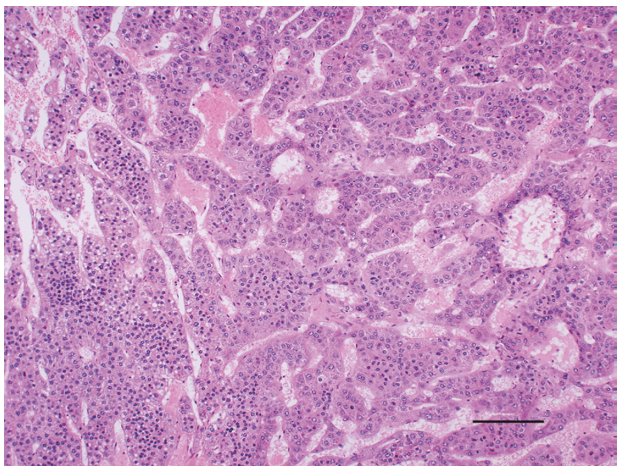
RESULTS

General observations

No statistically significant differences were observed in the average body weight and average food or water consumption among the 3 experimental groups or the 3 XPA genotypes (data not shown). In addition, there were no statistically significant differences in the weight of the major organs among the 3 experimental groups or the 3 XPA genotypes (data not shown). Only a few mice died during the experiments, and in most cases, liver tumor was not the cause of death ; these mice were excluded from the study. On the basis of autopsy findings of these mice, we speculated that liver tumors began to occur at 8 or 9 months of age. Most liver tumors that occurred around this age were HCAs. In addition to liver tumors, lung adenomas were observed, although the incidence was low (14%, in total). With regard to the incidence of lung adenoma, there were no statistically significant differences among the 3 experimental groups or the 3 XPA genotypes. Previously, the incidence of spontaneous lung adenoma in C3H/HeN mice was reported to be 12% until 28 months of age[30]. Neither the liver nor the lung tumors had metastasized. Pathological examinations confirmed that no tumors occurred in other major organs.



1a



1b

Fig. 1. (a) Representative histological appearance of HCA. Small and monotonous tumor cells proliferate, forming a thin trabecular pattern. Scale bar represents 200 μm. (b) Representative histological appearance of HCC. Tumor cells show a thick trabecular growth pattern with dilated sinusoids. Scale bar represents 200 μm.

Liver tumors

Representative histological samples of HCA and HCC are shown in Figures 1a and 1b, respectively. Table 1 shows the occurrence of tumorigenesis in the 18-month-old mice. The incidence of liver tumor was 65-89%, and the average number of liver tumors per mouse was 1.2-2.9. In many mice, HCAs and HCCs coexisted. Most HCAs were less than 10 mm in diameter and the majority of HCCs were more than 5 mm in diameter. HCC nodules occasionally contained a component of HCA, and a differential diagnosis between HCA and HCC was very difficult in some cases. These observations imply that a considerable number of HCCs developed from HCAs.

EGCG and lycopene did not exhibit obvious tumor-suppressive effects for any XPA gen-

Table 1. Liver Tumors of the Mice at the Age of 18 Months

Control group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	16	18	18
tumor-bearing mice (%)	14 (88%)	16 (89%)	12 (67%)
HCA-bearing mice (%)	12 (75%)	11 (61%)	12 (67%)
HCC-bearing mice (%)	8 (50%)	11 (61%)	4 (22%)
tumors per mouse (average ± S.D.)	1.8 ± 1.2	2.2 ± 1.9	1.3 ± 1.3
HCAs per mouse (average ± S.D.)	1.1 ± 0.8	1.4 ± 1.9	1.1 ± 1.1
HCCs per mouse (average ± S.D.)	0.7 ± 0.9	0.8 ± 0.7	0.3 ± 0.6[b]
diameter of tumors (mm, average ± S.D.)	8.1 ± 8.0	8.2 ± 6.8	5.7 ± 5.0[c]
HCAs (mm, average ± S.D.)	3.3 ± 2.4	3.8 ± 2.0	3.5 ± 2.2
HCCs (mm, average ± S.D.)	15.6 ± 8.0	16.2 ± 4.6	13.8 ± 4.1

EGCG group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	15	17	17
tumor-bearing mice (%)	13 (87%)	11 (65%)	15 (88%)
HCA-bearing mice (%)	11 (73%)	10 (59%)	13 (76%)
HCC-bearing mice (%)	10 (67%)	4 (24%)	7 (41%)
tumors per mouse (average ± S.D.)	2.9 ± 2.0	1.2 ± 1.3	2.0 ± 1.5
HCAs per mouse (average ± S.D.)	2.0 ± 1.8	0.9 ± 0.9	1.4 ± 1.3
HCCs per mouse (average ± S.D.)	0.9 ± 0.9	0.4 ± 0.7	0.6 ± 0.9
diameter of tumors (mm, average ± S.D.)	7.6 ± 7.0	6.7 ± 6.7	7.4 ± 6.7
HCAs (mm, average ± S.D.)	3.9 ± 2.5	3.3 ± 2.4	3.7 ± 2.1
HCCs (mm, average ± S.D.)	15.6 ± 6.7	15.3 ± 6.2	16.3 ± 5.3

Lycopene group	XPA genotype		
	+/+	+/−	−/−
number of mice in experiment	14	15	16
tumor-bearing mice (%)	11 (79%)	11 (73%)	14 (88%)
HCA-bearing mice (%)	10 (71%)	11 (73%)	11 (69%)
HCC-bearing mice (%)	6 (43%)	6 (40%)	9 (56%)
tumors per mouse (average ± S.D.)	1.6 ± 1.3	2.3 ± 2.0	2.0 ± 1.3
HCAs per mouse (average ± S.D.)	1.1 ± 0.9	1.7 ± 1.6	1.1 ± 0.9
HCCs per mouse (average ± S.D.)	0.5 ± 0.7	0.6 ± 0.9	0.9 ± 1.0[b]
diameter of tumors (mm, average ± S.D.)	6.9 ± 7.1	5.7 ± 5.2[a]	9.7 ± 8.1[a,c]
HCAs (mm, average ± S.D.)	3.0 ± 2.4	3.1 ± 1.7	3.2 ± 2.0
HCCs (mm, average ± S.D.)	15.3 ± 6.4	13.3 ± 4.6	17.1 ± 5.8

a, b, c Significantly different ($p<0.05$). HCA, hepatocellular adenoma ; HCC, hepatocellular carcinoma.

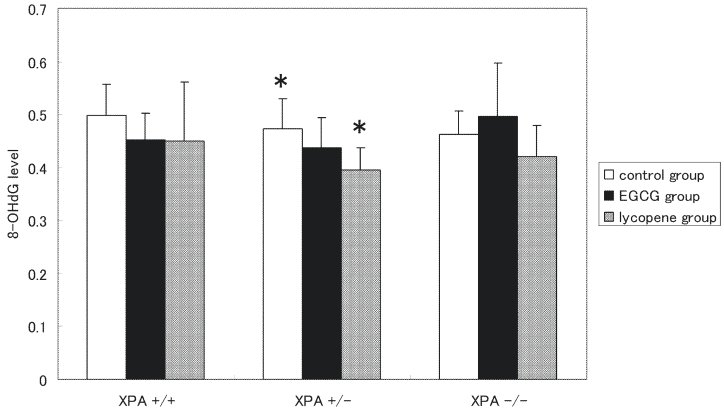


Fig. 2. Levels of 8-OHdG in non-tumorous liver tissues of mice at 8 months of age. The *XPA* +/− mice in the lycopene group showed a significantly lower 8-OHdG level than those in the control group (*) ($p<0.05$), but no other significant differences were observed.

otype. Conversely, among the *XPA* −/− mice, the average number of HCCs per mouse was significantly higher in the lycopene group than in the control group ($p<0.05$). Contrary to our previous report, the *XPA* −/− mice did not show a higher incidence or multiplicity of liver tumors than the *XPA* +/+ and *XPA* +/− mice in the control group. With regard to tumor size, the average diameter of tumors in the *XPA* −/− mice of the lycopene group was significantly larger than that of the tumors of the *XPA* +/− and *XPA* −/− mice of the lycopene and control groups, respectively ($p<0.05$). However, no other significant differences were observed.

Formation of 8-OHdG

Figure 2 shows the levels of 8-OHdG, a biomarker of oxidative DNA damage, in non-tumorous liver tissues at 8 months of age. The *XPA* +/− mice of the lycopene group demonstrated significantly reduced 8-OHdG formation than those in the control group ($p<0.05$) ; however, no other significant differences were observed.

DISCUSSION

In addition to its powerful antioxidative activity, EGCG has been proposed to possess various other properties, including the ability to induce antiangiogenesis, apoptosis, cell cycle regulation, and antimicrobial activity[31]. EGCG has been reported to inhibit carcinogen-induced mouse duodenal[13] and skin[14] tumors and preneoplastic foci of rat liver[32]. Contrary to our results, Nishida *et al*. reported that a dose of 0.05% and 0.1% EGCG in drinking water inhibited the formation of spontaneous hepatoma in C3H/HeNCrj mice[17]. The EGCG used in their experiments was not pure and contained 10% (−)-epigallocatechin and 5% (−)-epicatechin gallate, whereas the EGCG used in our experiments was pure. Catechins other than EGCG might be the primary cause of tumor inhibition, or EGCG and other catechins might synergistically inhibit spontaneous mouse hepatotumorigenesis. In fact, Yan *et al*. reported

that polyphenon E, a mixture of green tea polyphenols, inhibited lung tumorigenesis in A/J mice although pure EGCG did not[33].

In addition to its ROS scavenging activity, lycopene has been proposed to possess various other properties, including interference of cell proliferation, inhibition of cell cycle progression, induction of gap-junctional communication, modulation of signal transduction pathways, and upregulation of carcinogen detoxification[34]. Astorg *et al*. reported that dietary lycopene inhibited diethylnitrosamine (DEN)-induced liver preneoplastic foci in rats, but it did not inhibit 2-nitropropane (2-NP)-induced liver preneoplastic foci[15] ; 2-NP is a hepatocarcinogen that has been shown to induce DNA oxidative damage[35,36]. Astorg *et al*. speculated that lycopene did not act through its antioxidant properties but through its modulating effect on the liver enzyme that activates DEN. Watanabe *et al*. reported that long-term lycopene administration did not inhibit hepatocarcinogenesis in Long-Evans Cinnamon (LEC) rats[18]. They speculated that the antioxidative activities of lycopene might be insufficient to prevent hepatocarcinogenesis in LEC rats. Also, in the present study, lycopene administration did not inhibit spontaneous liver tumorigenesis in the C3H/HeN mice. Conversely, among the *XPA* −/− mice, the average number of HCCs per mouse was significantly higher and the average diameter of tumors was significantly larger in the lycopene group than in the control group. This result might suggest the possibility that lycopene caused earlier occurrence of tumors or lycopene promoted tumor proliferation in *XPA* −/− mice.

An important question that requires attention is why EGCG and lycopene did not inhibit spontaneous liver tumorigenesis in C3H/HeN mice in the present study, although these compounds have been previously reported to inhibit the formation of various carcinogen-induced tumors[13-16]. The concentration of EGCG and lycopene in the present study was comparable to that used in previous studies in which these compounds inhibited chemically induced tumorigenesis. In addition, EGCG and lycopene were administered for a long period in the present study. With regard to the 8-OHdG levels in liver tissues, the administration of EGCG and lycopene had limited effects in the present study. This result implies that EGCG and lycopene showed only limited antioxidant activity in liver tissues. Thus, EGCG and lycopene may inhibit tumorigenesis not through their antioxidant property but through their other properties. The mechanism of spontaneous liver tumorigenesis of C3H mice might be different from that of various chemically induced animal tumor models.

In our previous study, *XPA* −/− mice showed a higher susceptibility to spontaneous liver tumorigenesis than *XPA* +/+ and *XPA* +/− mice[23]. Thus, the results of the present study were contradictory to those of our previous study. The two studies were performed in different laboratories (the previous study was performed at Animal Center of Department of Medicine, University of Tokyo ; the present study was performed at Animal Center of Teikyo University School of Medicine). We speculate that the different experimental conditions caused the discrepancy. In particular, the amount of contaminating genotoxic agents might be important. It was suggested that *XPA*-deficient mice could not be used as a sensitive animal model for the conditions used in the present study.

In conclusion, EGCG and lycopene exerted no obvious inhibitory effects on spontaneous hepatotumorigenesis in C3H/HeN mice, although these compounds have been reported

to inhibit the formation of various carcinogen-induced tumors. The mechanisms underlying the inhibitory effects of these compounds on carcinogen-induced tumorigenesis should be investigated in the future.

ACKNOWLEDGEMENTS

We thank Dr. Zohar Nir (LycoRed Natural Products Industries, Beer-Sheva, Israel) for the generous gift of natural tomato lycopene extract. We are grateful to Mr. Masato Watanabe, Ms. Arisa Kumagai, and Ms. Yurie Soejima for providing expert technical assistance. This work was supported by a grant from Mitsui Norin Co., Ltd.

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