6． 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表氏名	巻号	ページ	出版年
Suganuma, M., Sueoka, E., Sueoka, N., Okabe, S. and Fujiki, H.	Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-α expression.	BioFactors	13	67-72	2000
Fujiki, H., Suganuma, M., Okabe, S., Sueoka, E., Suga, K., Imai, K. and Nakachi, K.	A new concept of tumor promotion by tumor necrosis factor-α, and cancer preventive agents (-)-epigallocatechin gallate and green tea.	Cancer Detect. Prev.	24	91-99	2000
Inoue, K., Okabe, S., Sueoka, E., Sueoka, N., Tabei, T. and Suganuma, M.	The role of interleukin-6 in inhibition of lung metastasis in subcutaneoustumor-bearing mice.	Oncology Reports	7	69-73	2000
Nakachi, K., Matsuyama, S., Miyake, S., Suganuma, M. and Imai, K.	Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention	Bio Factors	13	49-54	2000
Tori, M., Sono,Y., Nakashiba, Y., Hamada, N., Sono, M., Asakawa, Y., Suganuma,M., Okabe, S., Fujiki, H.	Syntheses of isocitric acid derivatives and biological evalution.	Tetrahedron Letters	41	3095-3098	2000
Suganuma, M., Ohkura, Y., Okabe, S. and Fujiki, H.	Combination cancer chemoprevention with green tea extract and sulindac shown in intestinal tumor formation in Min mice.	J. Cancer Res. Clin. Oncol.	127	69-72	2001
Fujiki, H., Suganuma, M., Okabe, S., Sueoka, E., Sueoka, N., Fujimoto, N., Goto, Y., Matsuyama, S., Imai, K. and Nakachi, K.	Cancer Prevention with green tea and monitoring by a new biomarker, hnRNP B1.	Mutation Res.		in press	2001

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Fujiki, H., Suganuma, M., Okabe, S. et al.	Green tea as a cancer preventive.	I. T. Johnson and G. R. Fenwick eds.	Dietary Anticarcinogen and Antimutagens, Chemical and Biological Aspects.	Royal Society of Chemistry	Cambridge, UK	2000	12-17

BioFactors 13 (2000) 67–72
IOS Press

Original report

Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-α expression

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Abstract. Among various biochemical and biological activities of tea polyphenols, we believe inhibition of the expression and release of tumor necrosis factor-α (TNF-α) is crucial, since our study with TNF-α-deficient mice has revealed that TNF-α is an essential factor in tumor promotion. We found that EGCG dose-dependently inhibited AP-1 and NF-κB activation in BALB/3T3 cells treated with okadaic acid, resulting in inhibition of TNF-α gene expression. Furthermore, treatment with 0.1% green tea extract in drinking water reduced TNF-α gene expression as well as TNF-α protein level in the lung of TNF-α transgenic mice; and IL-1β and IL-10 gene expression in the lung was also inhibited by treatment with green tea extract, indicating that green tea inhibits both TNF-α and the cytokines induced by TNF-α in organs. We recently found synergistic effects of EGCG and cancer preventive agents such as tamoxifen and sulindac, on cancer preventive activity. Taken together, the results show that green tea is efficacious as a non-toxic cancer preventive for humans.

Keywords: EGCG, sulindac, tamoxifen, tumor promotion

1. Introduction

Green tea is rapidly being acknowledged as one of the most practical cancer preventives, based on results of various rodent carcinogenesis experiments on the inhibitory effects of (−)-epigallocatechin gallate (EGCG) and green tea extract, along with results of a prospective cohort study of humans [1–3]. In 1987, we first reported the anti-tumor promoting activity of EGCG, the main constituent of green tea, in a two-stage carcinogenesis experiment on mouse skin [4]. Topical applications of EGCG before treatment with various tumor promoters – teleocidin, one of the 12-*O*-tetradecanoylphorbol-13-acetate – (TPA)-types, or okadaic acid – inhibited tumor promotion on mouse skin initiated with 7,12-dimethylbenz(a)anthracene [1]. Based on these results, we next looked at how EGCG inhibits the process of tumor promotion. Our study of tumor promotion with okadaic acid had provided strong indications that tumor necrosis factor-α (TNF-α) is an endogenous tumor promoter and the essential

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cytokine for tumor promotion [5]. Recently we were able to prove our hypothesis using TNF-α-deficient (TNF-$\alpha^{-/-}$) mice, which were established by Lloyd J. Old's group in 1997 [6,7]: Repeated applications of okadaic acid did not induce any tumors in TNF-$\alpha^{-/-}$ mice by 19 weeks of tumor promotion, whereas 100% of TNF-$\alpha^{+/+}$ mice developed tumors by week 17. These results clearly demonstrated that TNF-α is the key cytokine for tumor promotion in mouse skin. We have also found that EGCG as well as cancer preventive agents inhibited TNF-α release from BALB/3T3 cells treated with okadaic acid [8]. Based on all of our results, we think that, among various biochemical and biological activities of tea polyphenols, inhibition of TNF-α production is crucial. So, we examined inhibitory mechanisms of EGCG on TNF-α production.

Next, inhibitory effects of EGCG on cytokine production in vivo were studied using TNF-α transgenic mice. The TNF-α transgenic mice overexpressed TNF-α only in the lung and developed interstitial pneumonitis resembling idiopathic pulmonary fibrosis in humans [9]. Treatment with green tea extract in drinking water reduced TNF-α mRNA expression and its protein level in the lung of these mice, along with reduction of IL-1β and IL-10 gene expression. This is the first evidence that green tea inhibits TNF-α production as well as other cytokine production in mouse organs.

More recently, we found synergistic effects of EGCG and (−)-epicatechin (EC) on cancer preventive activity [10]. We extended our investigation of synergistic effects by examining EGCG with other cancer preventive agents, such as tamoxifen and sulindac. That is, cotreatment with EGCG and tamoxifen or EGCG and sulindac enhanced cancer preventive activity. Tamoxifen is the first cancer preventive drug for breast cancer, approved by FDA in the United States in 1998 [11]; and sulindac is a cancer preventive agent for colon cancer and a nonsteroidal anti-inflammatory agent [12]. This paper presents our recent findings with tea polyphenols and green tea extract looking mainly at inhibition of TNF-α gene expression in the cells and in TNF-α transgenic mice. The synergistic effects of EGCG and sulindac are also discussed.

2. Materials and methods

2.1. Inhibition of AP-1 and NF-κB activation by EGCG

BALB/3T3 cells were pretreated with EGCG for one hr, and treated with 200 nM okadaic acid for another 8 hr. Binding activity of AP-1 and NF-κB in nuclear extract to each consensus sequence of DNA was examined by electrophoretic mobility gel-shift assay (EMSA) [13].

2.2. Reduction of TNF-α in the lung of TNF-α transgenic mice by consumption of green tea extract

Transgenic mice carry a chimeric gene consisting of the promoter region of the human surfactant protein-C (SP-C) gene and mouse TNF-α gene. Transgenic mice and transgenic negative littermates were given 0.1% green tea extract in drinking water from embryo to 4 month-old. Expression of TNF-α, IL-1β, and IL-10 genes was examined by reverse transcription (RT)-PCR in the presence of ^{32}P-dCTP [9]. TNF-α protein level in the lung was determined by enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α (Genzyme Corporation, USA) [9].

2.3. Induction of apoptosis of PC-9 cells by EGCG and sulindac

PC-9 cells were incubated in sulindac with or without 75 μM EGCG for 2 days. DNA fragmentation was quantitatively measured using ELISA kit (Boehringer Mannheim, Mannheim, Germany) [10].

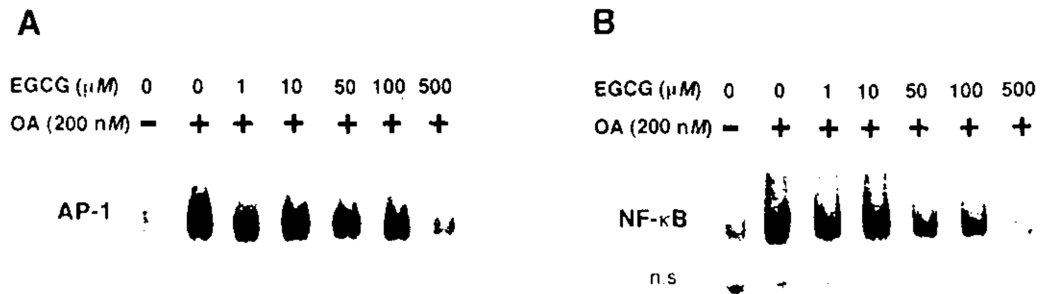

Fig. 1. Inhibition with EGCG of AP-1 and NF-κB activation induced by okadaic acid. Binding activity in nuclear extracts to AP-1 (A) and NF-κB (B) binding sites was examined by EMSA.

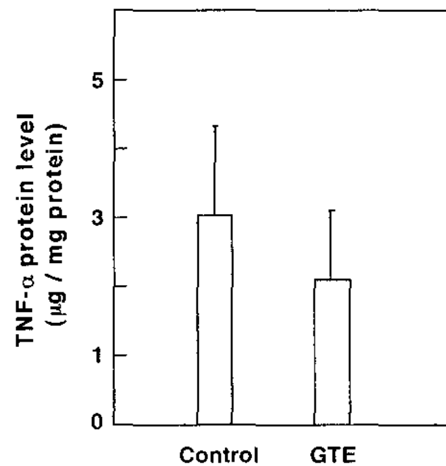

Fig. 2. Reduction of TNF-α protein level in the lung of TNF-α transgenic mice. Transgenic mice were treated with 0.1% green tea extract in drinking water from embryo to 4 month-old. GTE: green tea extract.

3. Results

3.1. Inhibition of TNF-α gene expression by EGCG

Tea polyphenols, such as EGCG, (−)-epicatechin gallate (ECG) and (−)-epigallocatechin (EGC), inhibit TNF-α release from BALB/3T3 cells and KATO III cells treated with okadaic acid [8,13]. TNF-α gene expression was enhanced by treatment with okadaic acid in both BALB/3T3 cells and KATO III cells, and EGCG dose-dependently inhibited this TNF-α gene expression, just as it inhibited TNF-α release. Since TNF-α gene expression is regulated by several transcription factors, such as AP-1 and NF-κB, we examined inhibition of AP-1 and NF-κB activation with EGCG using EMSA: 200 nM okadaic acid significantly enhanced AP-1 and NF-κB binding to each consensus sequence of DNA in BALB/3T3 cells, and pretreatment with 500 μM EGCG clearly inhibited these binding activities, as shown in Fig. 1(A) and Fig. 1(B) [13]. These results demonstrated that EGCG inhibits TNF-α production by inhibiting AP-1 and NF-κB activation.

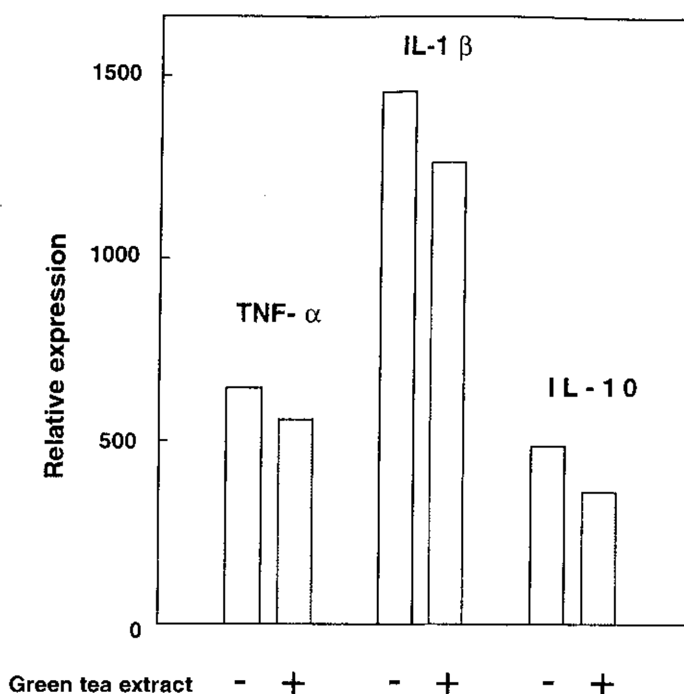

Fig. 3. Effects of EGCG on expression of TNF-α, IL-1β, and IL-10 genes in the lung of TNF-α transgenic mice. Transgenic mice were treated with 0.1% green tea extract in drinking water from embryo to 4 month old.

3.2. Reduction of TNF-α in the lung of TNF-α transgenic mice by consumption of green tea extract

TNF-α transgenic mice had enhanced TNF-α gene expression just after birth and continuously overexpressed it in the lung. At 4 month-old, TNF-α protein level in the lung of TNF-α transgenic mice was significantly reduced, from 3.0 to 2.1 μg/mg protein, by treatment with 0.1% green tea extract in drinking water (Fig. 2). This reduction level was well correlated with reduction of TNF-α gene expression (Fig. 3). Treatment with green tea extract also reduced expression of IL-1β and IL-10 genes about 15–20% in the lung (Fig. 3). These results indicated that green tea inhibited TNF-α gene expression as well as expression of other cytokine genes induced by TNF-α.

3.3. Enhanced effects of EGCG and tamoxifen or EGCG and sulindac on cancer preventive activity

Based on our discovery that synergistic induction of apoptosis by EC and other tea polyphenols, EGCG, ECG and EGC, we examined synergistic effects of tea polyphenols with other cancer preventive agents, such as tamoxifen and sulindac. We found that cotreatment with EGCG and tamoxifen inhibited growth of MCF-7 cells and induction of apoptosis of PC-9 cells even more strongly than either did alone (data not shown) [10]. These results suggest that green tea enhances tamoxifen's preventive activity against breast cancer in humans.

We next looked at the synergistic effects of EGCG and sulindac or its metabolites, sulindac sulfide and sulindac sulfone, on induction of apoptosis of PC-9 cells. Sulindac at a concentration of 10 μM induced almost no apoptosis of PC-9 cells, while 10 μM sulindac with 75 μM EGCG induced apoptosis more than 20 times as strongly as sulindac alone (Fig. 4). Sulindac sulfide, an inhibitor of cyclooxyganase (COX)-1

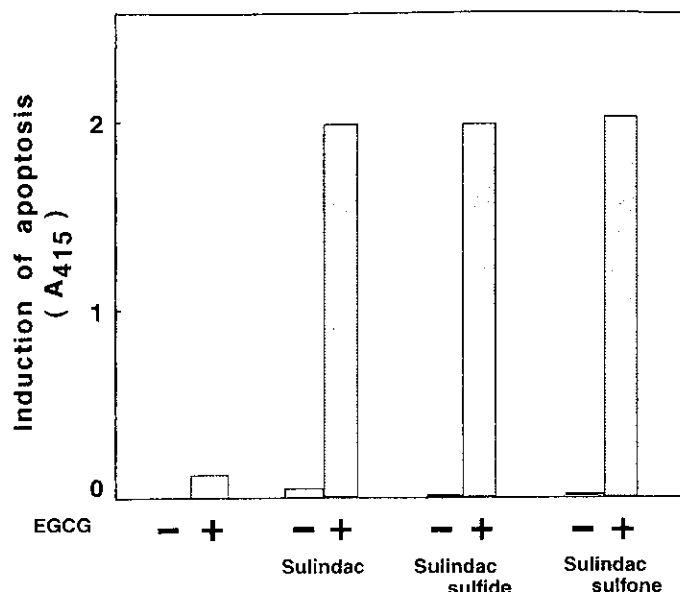

Fig. 4. Synergistic induction of apoptosis of PC-9 cells by cotreatment with EGCG and sulindac or its metabolites.

and COX-2, and sulindac sulfone, an inactive metabolite, both synergistically induced apoptosis of the cells in the presence of 75 μM EGCG, whereas neither was effective alone (Fig. 4). We therefore believe that the synergistic effects on apoptosis by cotreatment with EGCG and sulindac are not directly related to inhibition of COX. Clearly, cotreatment with EGCG and sulindac will result in increased cancer preventive activity in humans.

4. Discussion

This paper reports that green tea extract in drinking water inhibits TNF-α gene expression and reduces TNF-α protein level in the lung of TNF-α transgenic mice. Since treatment with green tea extract did not inhibit expression of SP-C gene, we think that green tea inhibits expression of TNF-α gene and other cytokine genes subsequently induced by TNF-α through cytokine network. This important evidence strongly suggests that administration of green tea extract somehow inhibits TNF-α gene expression, resulting in reduction of an endogenous tumor promoter in the lung. We previously reported that ^{3}H-EGCG orally administered was distributed into the lung as well [14], so it is reasonable to suppose that tea polyphenols reach the lung and inhibit TNF-α gene expression mediated through inhibition of AP-1 and NF-κB activation in the lung. It is now known that various cancer preventive agents, such as green tea polyphenols, sodium salicylate and curcumin, inhibit AP-1 and NF-κB activation [15–17]: Our results here indicate that inhibition of AP-1 and NF-κB activation is a further step toward inhibition of TNF-α gene expression.

The synergistic effects of EGCG and sulindac were significant for cancer preventive activity in cells, and we recently demonstrated their synergistic effects on inhibition of tumor formation in C57BL/6J Min mice (manuscript in preparation). All our results show that green tea is a promising candidate for use in combination with cancer preventive agents such as sulindac and tamoxifen, for the purpose of reducing their adverse effects [10].

For cancer prevention in Japan, 10 Japanese-size cups of green tea per day, roughly 2.0 to 2.5 g green tea extract, is recommended based on the results of a prospective cohort study [3]. For those who cannot drink 10 cups per day, we have now prepared green tea tablets in collaboration with the Tea Experiment Station in Saitama Prefecture [18]. To determine whether the average person can consume tea polyphenols equal to ten cups of green tea per day without any changes in life-style, we conducted a trial using green tea tablets with 102 healthy volunteers in Saitama Prefecture. The number of green tea tablets was decided by each volunteer based on daily consumption of green tea, and most of the volunteers (91%) continued taking the tablets for 3 months, indicating that they can indeed drink the amounts of green tea for 10 cups per day.

In summary, our concept for cancer prevention with green tea is as follows: For the purpose of cancer prevention, green tea consumption should be increased from the level for a daily beverage to the amount of a cancer preventive beverage: 10 Japanese-size cups of green tea daily is acceptable for most people, and will provide protection against cancer.

Acknowledgments

This work was supported by the following Grants-in-Aid: for Cancer Research from the Ministry of Health and Welfare, Scientific Research on Priority Areas for Cancer Research from the Ministry of Education, Science, Sports and Culture, Japan: for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare, Japan: for Selectively Applied and Developed Research from Saitama Prefecture, Japan; and by the Smoking Research Fund.

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Cancer Detection and Prevention, 24(1): 91–99 (2000)

A New Concept of Tumor Promotion by Tumor Necrosis Factor-α, and Cancer Preventive Agents (-)-Epigallocatechin Gallate and Green Tea—A Review

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Accepted for publication September 1, 1999.

ABSTRACT: The study of tumor promotion in rodent carcinogenesis using chemical tumor promoters has revealed various tumor promotion pathways, such as the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) pathway mediated through activation of protein kinase C, and the okadaic acid pathway mediated through inhibition of protein phosphatases 1 and 2A (PP-1 and PP-2A). We previously demonstrated that application of TPA and okadaic acid induced tumor necrosis factor-α (TNF-α) gene expression in mouse skin, but that tautomycin, which is an inhibitor of PP-1 and PP-2A and not a tumor promoter on mouse skin, did not. Moreover, we found that TNF-α stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene 1,000 times stronger than did TPA (Cancer Res. *53*, 1982-1985, 1993). This evidence demonstrates a link between the okadaic acid pathway and the endogenous tumor promotion pathway of TNF-α. Recently we presented the first evidence that tumor promotion in TNF-$\alpha^{-/-}$ mice was significantly depressed compared with TNF-$\alpha^{+/+}$ mice. Thus, in human carcinogenesis, we think that TNF-α and other inflammatory cytokines in preneoplastic lesion stimulate tumor promotion and progression of initiated cells as well as premalignant cells. The first part of this paper reports on this TNF-α tumor promotion pathway.

In the second part, we report a promising screening method for cancer preventive agents, based on evidence that pretreatment with agents such as tamoxifen, sulindac, 1α, 25-$(OH)_2$ vitamin D_3, quercetin, caffeic acid phenethyl ester, and (-)-epigallocatechin gallate (EGCG) commonly inhibited TNF-α release from BALB/3T3 cells induced by okadaic acid. EGCG, the main constituent of Japanese green tea, and green tea itself are acknowledged cancer preventives in Japan, and this paper presents evidence of their effectiveness in both a high-risk group and the general population.

KEY WORDS: okadaic acid, tautomycin, protein phosphatases 1 and 2A, cancer prevention.

INTRODUCTION

About five years ago, we reported tumor promotion by the okadaic acid class of compounds, under the title of "A New Tumor Promotion Pathway and Its Inhibitors," in this Journal.[1] In the article we showed that the tumor promotion pathway mediated through the inhibition of protein phosphatases 1 and 2A (PP-1 and PP-2A) is different from that of activation of protein kinase C (PKC) by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) type tumor promoters, and, furthermore, that inhibitors of protein phosphatases, such as okadaic acid, dinophysistoxin-1, calyculin A, microcystin-LR and nodularin, demonstrated tumor promotion in three different organs: mouse skin, rat glandular stomach, and rat liver. We called it a new tumor promotion pathway, or the okadaic acid pathway, in contrast to the classical TPA pathway.[2] This okadaic acid pathway is significant in that it is a general biochemical and molecular process applicable to various organs, and it gave us the answer to the historical question, which molecule is essential for tumor

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promotion in rodents? The answer, as we reported, is as follows: (1) a tumor promoter induces expression of tumor necrosis factor-α (TNF-α) gene in the target organ, and (2) TNF-α itself is both the essential molecule for tumor promotion and an endogenous tumor promoter.[3] Thus, in this paper we present a new concept of tumor promotion in which TNF-α is significantly involved.

In aiming at cancer prevention, it is obviously important to identify any common mechanisms of action of cancer preventive agents. In our recent research, we found that various cancer preventive agents commonly inhibit TNF-α release from the cells.[4] In addition, this paper reviews the important features of (-)-epigallocatechin gallate (EGCG) and green tea as cancer preventives. Green tea is now an acknowledged cancer preventive in Japan, based on an epidemiological study conducted in Saitama Prefecture.[5]

TUMOR PROMOTION BY THE OKADAIC ACID CLASS OF COMPOUNDS

In 1988, we first reported that okadaic acid, isolated from a black sponge, *Halichondria okadai,* is a tumor promoter as potent as TPA on mouse skin initiated with 7,12-dimethylbenz (a)anthracene (DMBA).[6] The evidence provided new insight into the understanding of the mechanisms of tumor promotion in relation to a unique signal transduction pathway mediated through inhibition of PP-1 and PP-2A.[7] The okadaic acid class compounds were classified into four structurally different types: okadaic acid itself, calyculin A, microcystin-LR and tautomycin (Figure 1).[2] To them, cantharidin was recently added as a fifth type of the okadaic acid class. These compounds are all potent inhibitors of PP-1 and PP-2A, and all inhibit the receptor binding with ^{3}H-okadaic acid.

Tumor promotion in mouse skin: Like okadaic acid, repeated topical applications of dinophysistoxin-1 and calyculin A induced tumor promotion on the skin of female CD-1 mouse,[8,9] whereas applications of tautomycin did not,[10] probably due to lack of TNF-α gene expression in basal cell layers and TNF-α release from them. Tumor promotion of cantharidin was not tested at this time, since it was well established.[11]

Tumor promotion in rat glandular stomach: Okadaic acid and dinophysistoxin-1 were once given special attention as causative agents of diarrhetic shellfish poisoning in humans. In support of this, intubation of okadaic acid or dinophysistoxin-l into the stomach of rats caused diarrhea with similar symptoms of diarrhetic shellfish poisoning.[6,8] Furthermore, okadaic acid in drinking water induced promotion of neoplastic changes in the glandular stomach initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in male Wistar rats.[12] Taken together, these results with the skin and stomach demonstrated that inhibition of PP-1 and PP-2A by okadaic acid is involved in tumor promotion of two different organs initiated with two different initiators.[2] However, tautomycin in the diet did not induce tumor promotion in rat glandular stomach, but, rather, inhibited tumor development with MNNG, suggesting that tautomycin has additional function in addition to inhibition of PP-l and PP-2A.[10]

Tumor promotion in rat liver: Microcystins and nodularin are potent hepatotoxic compounds, because their i.p. injections mainly cause damage to the liver. Microcystins are cyclic heptapeptides and nodularin is a cyclic pentapeptide. Repeated i.p. injections of microcystin-LR and nodularin, twice a week, stimulated induction of positive foci of the glutathione-*S*-transferase placental form in the liver of F344 male rats initiated with diethylnitrosamine.[13,14]

We concluded that the okadaic acid class of compounds induced tumor promotion in three different organs initiated with three different initiators, and that inhibitors of protein phosphatases provided the first

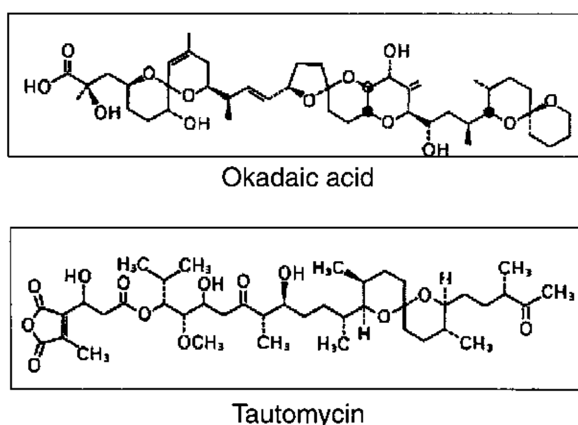

FIGURE 1. Structures of okadaic acid and tautomycin.

evidence of a general tumor promotion pathway applicable to various organs.[1]

A BIOCHEMICAL LINK BETWEEN TUMOR PROMOTION BY OKADAIC ACID AND TNF-α

The first indication of a link was obtained by biochemical studies of protein phosphorylation. Guy *et al.* reported in 1992 that a total of ~ 116 proteins exhibited significant and concordant changes in phosphorylation or dephosphorylation within 15 min in human fibroblasts activated by either okadaic acid, TNF, or IL-1.[15] Knowing that hyperphosphorylation of vimentin and heat shock protein 27 (HSP 27) was one of the most significant biochemical effects of okadaic acid on primary human fibroblasts, we confirmed that TNF-α induced phosphorylation of the same proteins, i.e., vimentin and HSP 27, as did okadaic acid, but that the time-courses of their phosphorylation were different.[16,17] Although okadaic acid and TNF-α induce similar biochemical effects in the cells, okadaic acid and TNF-α were found in completely unrelated experiments. Okadaic acid is a marine natural product associated with potent inhibitory activity of PP-1 and PP-2A,[18] and TNF-α with 17 kDa was orginally identified as a serum factor inducing hemorrhagic necrosis of transplanted solid tumors in mice,[19] but is now known to be a primary mediator and cytokine associated with inflammatory response.[20] However, there was still no clear evidence that okadaic acid was directly involved in tumor promotion of human cancer development. Based on these results and others, we thought that, if okadaic acid induces TNF-α in the target tissue of tumor promotion and subsequently TNF-α activates cytokine network through the autocrine and paracrine systems, then, if TNF-α has tumor promoting activity similar to that of okadaic acid, TNF-α is an endogeneous tumor promoter. Moreover, if TPA also induced TNF-α in mouse skin, the tumor promotion pathway by TNF-α would be considered a general pathway for tumor promotions induced by various classes of tumor promoters (Figure 2). Classical theory of tumor promotion had always looked at the relationship to inflammation, wound-healing and clonal expansion,[21] but now it would be possible to reconsider classical tumor promotion by TNF-α, which would provide the answers to all these historical puzzles of tumor promotion.

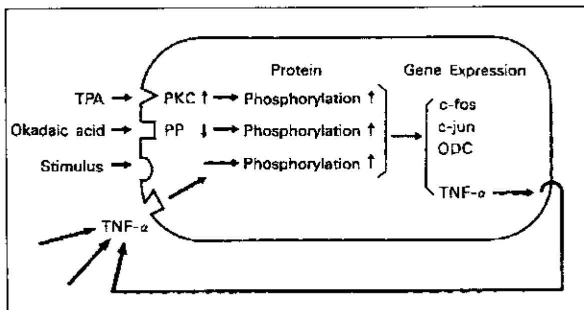

FIGURE 2. Mechanisms of tumor promotion.

GENE EXPRESSION AND TUMOR PROMOTION BY TNF-α

Mouse skin: Various doses of tumor promoters in mouse skin, with TPA and okadaic acid as positive controls and tautomycin as a negative control, were applied to the backs of mice. Total RNA was isolated from the skin 4 h after the applications. TNF-α mRNA amplified by reverse transcription-polymerase chain reaction (RT-PCR) was expressed as a relative change in expression (- fold) in comparison with that of the non-treated control.[10] Figure 3 shows that topical application of TPA and okadaic acid stimulated TNF-α mRNA level dose-dependently, whereas that of tautomycin up to 300 nmol (224 μg) per application did not. Thus, TNF-α gene expression has a close correlation with tumor promoting activity in mouse skin.

Stomach cancer cell line: KATO III cells of human stomach cancer cell line were incubated with various concentrations of okadaic acid as a positive control and tautomycin as a negative control for 24 h.[10] The concentration of TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) kit. Okadaic acid at a concentration of 50 nM induced TNF-α release at maximal level, whereas tautomycin even at concentrations of up to 2.0 μM did not (Figure 4). We assumed that tumor promotion of okadaic acid was mediated by TNF-α in glandular stomach, and that the absence of tumor promoting activity of tautomycin was related to its inability to induce TNF-α and TNF-α gene expression.

Rat liver and rat hepatocytes: A single i.p. administration of nodularin, a potent liver tumor promoter, 50 μg/kg body weight, induced TNF-α gene

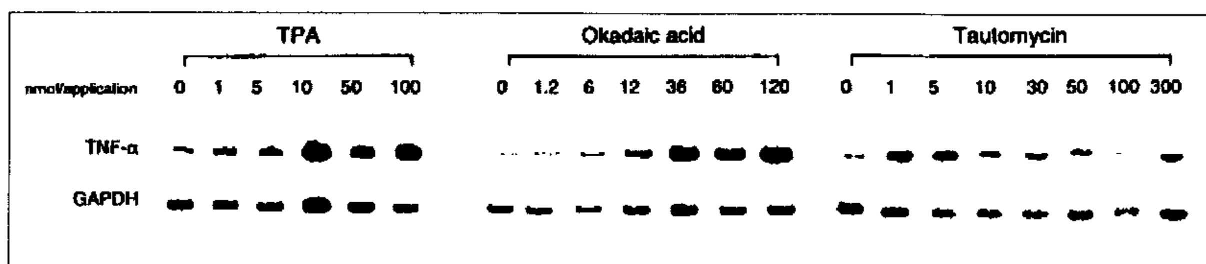

FIGURE 3. Difference in induction of TNF-α gene expression in mouse skin by TPA, okadaic acid and tautomycin. Various doses of TPA, okadaic acid and tautomycin in 0.2 ml acetone were applied to the skin of the backs of mice. Total RNA was isolated from the skins 4 h later by the acid guanidinium thiocyanate/phenol/chloroform extraction method, as reported previously.[10] TNF-α mRNA was determined by reverse transcription/polymerase chain reaction, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as a control.[10]

expression in rat liver (Figure 5).[22] We concluded that this tumor promoter expresses TNF-α gene in its target organs. To determine the time-course of TNF-α gene expression and TNF-α release, primary cultured rat hepatocytes were treated with various concentrations of nodularin for up to 24 h (Table 1).[23] Expression of TNF-α gene reached a maximum at 10 h, which correlated well with that in rat liver. Various concentrations of nodularin induced TNF-α release from rat hepatocytes into the medium (Table 1), but TPA, which is a tumor promoter in mouse skin, but not in the liver, did not induce any significant TNF-α gene expression in primary cultured rat hepatocytes. All the results in the experiments with rat hepatocytes closely reflected those with rat liver *in vivo*.

TABLE 1
Dose response of tumor necrosis factor-α (TNF-α) gene expression and TNF-α release into the medium in primary cultured rat hepatocytes following treatment with nodularin

Treatment	Dose (nM)	TNF-α gene expression (-fold)	TNF-α release (pg/ml)
Control	0	1	0
Nodularin	1	1	0
	10	3	12.3 ± 2.5
	100	10	26.7 ± 5.8
	500	40	27.3 ± 6.8
	1,000	40	25.0 ± 8.7

Hepatocytes were prepared from the liver of male Fischer 344 rats as reported previously.[23] Primary cultured rat hepatocytes (2 x 10^6) were treated with nodularin as reported in the text. The expression of rat TNF-α gene was determined by the qualitative reverse transcription/polymerase chain reaction (RT-PCR) method.[23] Expression of the TNF-α gene is recorded as -fold induction compared with non-treated hepatocytes (control). The results were obtained by three independent experiments. TNF-α release into the medium was determined using a rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camerillo, CA, USA) and expressed as mean ± SD. Two independent experiments were performed in duplicate assays.[17,30]

Cell transformation by TNF-α: A tumor promoter in mouse skin usually dissolves in organic solution and then penetrates into the basal cell layers.[24] Since TNF-α is a protein, we thought it would not be effective on mouse skin. If so, two-stage cell transfor-

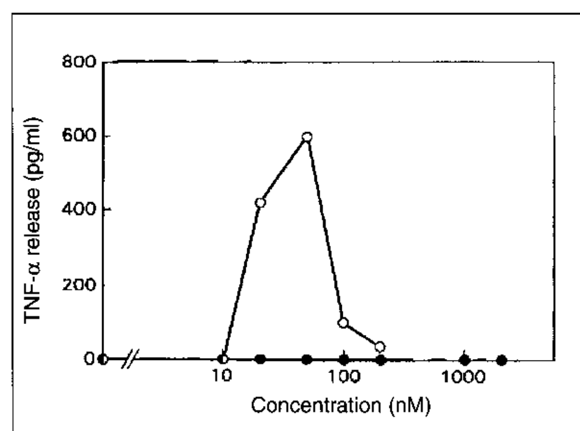

FIGURE 4. Difference in TNF-α release from KATO III cells treated with okadaic acid (o) and tautomycin (●). KATO III cells (5 x 10^5) were incubated with 0.5 ml medium containing various concentrations of okadaic acid or tautomycin for 24 h. The concentration of TNF-α was measured by an enzyme-linked immunosorbent assay kit, as described previously.[10]

mation would be the best test of the tumor promoting activity of TNF-α. However, before going on to possible cell transformation by TNF-α, we first confirmed that okadaic acid at a concentration of 20 ng/ml (24.9 μM), and dinophysistoxin-1 at a concentration of 3.0 ng/ml (3.7 μM), induced 1.58 foci/dish and 1.21 foci/dish, respectively, in a cell transformation experiment with BALB/3T3 cells initiated with 3-methylcholanthrene (MCA) at a concentration of 0.5 μg/ml.[25] And 0.2 μM okadaic acid induced TNF-α release from BALB/3T3 cells into the medium 12 h after treatment.[17] Next, the tumor promoting activity of TNF-α was tested by *in vitro* transformation of BALB/3T3 cells. Using a similar experimental procedure to that described above, initiation was achieved by MCA at a concentration of 0.1 μg/ml this time. Human TNF-α at a concentration of 0.1 μg/ml (0.6 nM) significantly stimulated transformation of BALB/3T3 cells with 1.83 foci/dish, and TPA at a concentration of 300 ng/ml (0.5 μM) as a positive control induced 1.92 foci/dish. Thus, TNF-α was about 1,000 times stronger than TPA at their molar concentrations.[17]

Lung: Mycobacterial cord factor, trehalose 6-monomycolate (TMM) enhances PKC activation and induces granuloma formation in the lung and interstitial pneumonitis, suggesting that TMM is a new tumor promoter in the lung.[26] As a control we examined PKC activation by cord factors of *Rhodococcus ruber*, and found that the cord factors with $C_{44\text{-}46}$ mycolic acids of *R. ruber* did not significantly activate PKC. Thus, we assume that PKC activation was correlated with induction of various biological activities.[26] To test our hypothesis, we wanted to determine whether TMM induces TNF-α in the lung, a possible target organ. We found that a single i.p. administration of TMM at a concentration of 10 μg into mice induced TNF-α release in serum, reaching a maximum level around four and five days after treatment; in the lung, TNF-α levels continuously increased up to seven days.[26] The evidence indicated that TMM, a possible lung tumor promoter, induces TNF-α, an endogenous tumor promoter, in the target organ.

Screening method: Besides the okadaic acid class of compounds, we had previously studied tumor promotion with teleocidin derivatives and the core structure (-)-indolactam-V, which are activators of PKC and belong to the TPA-type tumor promoters.[27] Based on our previous findings, that tumor promoters induce TNF-α release from target cells mediated through TPA pathway and okadaic acid pathway, four different teleocidin derivatives, teleocidin B-4, (-)-benzolactam-V8-310, (-)-indolactam-V and (+)-benzolactam-V8-310, as a negative control, were administered to HL-60 cells.[28] The potency of these compounds in inducing TNF-α release was closely associated with the potency of their tumor promoting activity on mouse skin (Figure 6).[28] This strongly indicates, we think, that TNF-α release is a useful biomarker for screening chemical tumor promoters. All the results showed that TNF-α is an endogenous tumor promoter and a central mediator of cancer development in rodents and humans. Recently we showed that TNF-$\alpha^{-/-}$ mice treated with DMBA plus okadaic acid developed no tumor up to 19 weeks compared with 100% for TNF-$\alpha^{+/+}$ mice, and TNF-α $^{-/-}$ mice treated with DMBA plus TPA developed tumors four weeks later than those with TNF-$\alpha^{+/+}$ CD-1 mice. Thus, we concluded that TNF-α is the key cytokine for tumor promotion on mouse skin.[29]

INHIBITION OF TNF-α RELEASE

TNF-α is synthesized in the cells as a precursor of 26 kDa protein, and released from the cells as 17 kDa protein after proteolytic cleavage. In the culture

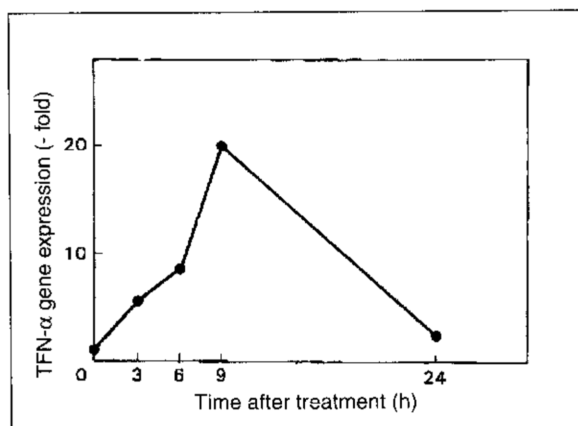

FIGURE 5. Induction of TNF-α gene expression in rat liver treated with a single i.p. administration of nodularin (●). Total RNA was prepared from rat liver treated with nodularin 50 μg/kg body weight. Samples of 1 μg total RNA were subjected to reverse transcription and then cDNAs obtained were amplified by PCR. The amount of TNF-α mRNA expression was analyzed by BAS 2000 image analyzer and normalized by GAPDH mRNA as control.[22]

medium, TNF-α can be easily detected after it is released from the cells, such as BALB/3T3, KATO III, and HL-60 cells induced by okadaic acid.[10,17,28,30] To compare the inhibitory potency of various compounds, we used TNF-α release from BALB/3T3 cells treated with 0.2 μM okadaic acid as a standard experimental criterion.[17,30]

Sixty-four compounds so far have been subjected to the BALB/3T3 cell system and their results compared with those of inhibition of tumor promotion in mouse skin two-stage carcinogenesis experiments. As Table 2 shows, 45 compounds inhibited TNF-α release, 15 compounds did not, and four compounds enhanced TNF-α release rather than inhibiting it.

Nineteen of the 45 which inhibited TNF-α release were tested for inhibition of tumor promotion on mouse skin and 15 of these compounds demonstrated inhibition: EGCG, sarcophytol A, α-CBT, compound 1 (belonging to cembranoid), canventol, C44, C46 and C47 (belonging to canventol analogs), cryptoporic acid E, discodermin A, 6,6'-di-*O*-octanoyl-α,α–trehalose (SS555), *n*-dodecyl-β-D-maltoside and tobacco extract. Three compounds, compound 4 (belonging to cembranoid), S11 (belonging to canventol analog) and oxa-1α,25-$(OH)_2$ vitamin D_3, did not inhibit tumor promotion on mouse skin, probably because of difficulty in penetrating into basal cell layers. One compound, sunscreen, stimulated tumor promotion but significantly inhibited TNF-α release.

TABLE 2
Results of screening test on TNF-α release from BALB/3T3 cells

	No. of compounds	
Tested compounds in total	64	
Inhibited	45	
Tumor promotion		
inhibited		15
not inhibited		3
enhanced		1
not tested		26
Not inhibited	15	
Enhanced	4	
Tumor promotion		
enhanced		1
not tested		3

BALB/3T3 cells (2 x 10^5/well) were first incubated with preventive agent at various concentrations. After 1 h, 0.2 μM okadaic acid was added and incubated for another 24 h. The concentration of TNF-α in medium was measured using the ELISA of mouse TNF-α (Genzyme, Cambridge, MA).[18] The method of tumor promotion was previously reported.[6]

It is important to note that four compounds enhanced TNF-α release, and one of the four, cryptoporic acid D, significantly stimulated tumor promotion on mouse skin from 73.0% to 93.3% of tumor-bearing mice; three other compounds have not yet been investigated. Thus, inhibition of TNF-α release is clearly a useful method of screening for cancer preventive agents. Table 3 shows IC_{50} values of the cancer preventive agents reported previously.

EGCG AND GREEN TEA

Research in cancer prevention began in Japan in 1983. First, we screened original Japanese cancer preventive agents, looking for a new inhibitor of tumor promotion derived from medicinal plants.[31] Among 30 tannins, we found that repeated topical applications of (-)-epigallocatechin gallate (EGCG), the main constituent of green tea, inhibited tumor promotion in a two-stage carcinogenesis experiment on mouse skin.[32] This was our initial work on green tea which was published in 1987.

TABLE 3
Inhibition of TNF-α release from BALB/3T3 cells treated with okadaic acid

	IC_{50} (μM)
Sarcophytol A	2.5
1 α, 25-$(OH)_2$ Vitamin D_3	4.7
Tamoxifen	5.8
Quercetin	12.5
9-*cis*-Retinoic acid	16.0
Vitamin D_3	17.5
EGCG	20.0
Canventol	21.0
Sulindac	28.0
All-*trans*-Retinoic acid	> 10.0
Aspirin	> 2000.0

Determination of TNF-α release into the medium is reported in Table 2.[17,30]

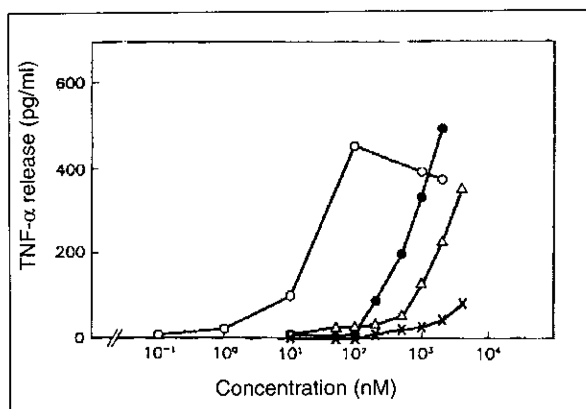

FIGURE 6. Induction of TNF-α release from HL-60 cells treated with three tumor promoters, teleocidin B-4 (○), (-)-BL-V-310 (●), and (-)-IL-V (Δ), along with (+)-BL-V8-310 (x) as a negative control.HL-60 cells (2 x 10^5/ml) grown in RPMI-1640 medium containing 10% fetal calf serum were incubated with various concentrations of tumor promoters for 24 h. TNF-α in the medium was determined by an ELISA kit, as described previously.[28]

Many scientists have since reported the inhibitory effects of EGCG and green tea extract (dried tea) on carcinogenesis in various organs of rodents, such as skin, esophagus, stomach, duodenum, colon, liver, lung, pancreas and bladder.[4] Of particular significance is the fact that EGCG and green tea extract were not toxic for rodents. EGCG and other tea polyphenols also inhibited growth of human cancer cell lines, such as PC-9 cells of human lung cancer cell line with G_2/M arrest.[33] Next, the bioavailability of EGCG was studied using ^{3}H-EGCG (48.1 GBq/mmol). Direct administration of ^{3}H-EGCG into mouse stomach revealed the distribution of radioactivity in organs where inhibition of carcinogenesis by EGCG and green tea extract had previously been shown, as mentioned above.[34] This confirmed that EGCG and green tea have a wide range of target organs.

Mechanisms of action of green tea polyphenols should be discussed briefly. When we discovered that applications of EGCG to mouse skin inhibited tumor promotion of two different tumor promoters, teleocidin, one of the TPA-type tumor promoters and okadaic acid, associated with different mechanisms of action, we found that a membrane fraction of mouse skin treated with a single topical application of EGCG showed immediate reduction of specific binding of both TPA and okadaic acid to their receptors.[35] We named this phenomenon, inhibition of the interaction of tumor promoters with their receptors, as well as those of hormones, growth factors and cytokines, the sealing effect of EGCG.[35] Recently, this sealing effect was confirmed by physicochemical studies using liposomes showing that EGCG is distributed into the surface region of phospholipid bilayer membrance.[36] The sealing effect of tea polyphenols is the key to its inhibitory action.

In addition, as we demonstrated previously, inhibition of TNF-α release by a compound is a key element in inhibition of tumor development, and probably cancer prevention itself.[30] We found strong evidence that EGCG inhibits TNF-α gene expression in the cells and TNF-α release from the cells.[37] Thus, we feel strongly that inhibition of TNF-α expression is one of the most important activities of EGCG and green tea polyphenols for cancer prevention.

EPIDEMIOLOGICAL STUDIES WITH GREEN TEA

Does high daily consumption of green tea prevent cancer in humans? The answer is found in the results of a prospective cohort study conducted by two of our authors (K. N. and K. I.) with 8,552 individuals in Yoshimi town in Saitama Prefecture beginning in 1986.[38] The 10-year follow-up study identified 175 female and 244 male cancer patients, who presented the first evidence for patients who had consumed over 10 cups of green tea per day of an association between delayed cancer onset and high consumption of green tea; cancer onset was 7.3 years later among females, and 3.2 years later among males, compared with patients who had consumed under three cups per day.[5] (The difference between females and males is partly due to higher tobacco consumption by males.) Since a total of 419 cancer cases were diagnosed during the 10 years, the relative risk of major cancers by consumption of green tea was determined: Individuals who consumed over 10 cups of green tea per day showed remarkably low relative risk for lung cancer, colon cancer and liver cancer.[5] The relative risk for stomach cancer was also lower, although not statistically significant. The results of this prospective cohort study showed that many cancers can be prevented, or at least the onsets delayed, by daily consumption of a natural beverage familiar to all Japanese.

Moreover, the same authors (K. N. and K. I.) recently presented results from 472 breast cancer patients showing an association between decreased recurrence of breast cancer and increased consumption of green tea.[39] Specifically, the disease-free period of the group consuming over five cups of green tea per day was 3.6 years, 10 months longer than that of the group consuming less four cups per day (2.8 years), an improved prognosis for breast cancer patients with high consumption of green tea.[39]

To extend the results of these epidemiological studies on green tea to an intervention study, a Phase I trial with green tea tablets was conducted with 108 Japanese healthy volunteers for six months.[40] Each volunteer took 15 green tea tablets per day (2.25 g green tea extract, 337.5 mg EGCG and 135 mg caffeine), and underwent various examinations. The blood examination revealed that none of the volunteers had any strong adverse effects.[40] These results encouraged us, and Phase I clinical trials with capsules of green tea, which are manufactured by ITO EN Ltd, in Japan, are now being conducted at the M. D. Anderson Cancer Center in Texas and the Memorial Sloan-Kettering Cancer Center in New York.

In summary, green tea is now an acknowledged cancer preventive in Japan and will possibly soon be recognized as such in other countries. Moreover, since green tea inhibits TNF-α gene expression and TNF-α release, which are involved in various diseases,[41] green tea might be beneficial in cancer prevention.

ACKNOWLEDGEMENT

This work was supported in part by Grants-in-Aid for Cancer Research, Special Cancer Research, from the Ministry of Education, Science and Culture; a grant from the Ministry of Health and Welfare for a 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, Japan; and grants from the Foundation of Promotion of Cancer Research, the Smoking Research Fund, the Uehara Memorial Life Science Foundation, Suzuken Memorial Foundation, the Plant Science Research Foundation of the Faculty of Agriculture, Kyoto University, and both Cutting Edge Fundamental Research and Selectively Applied and Developed Research from Saitama Prefecture.

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The role of interleukin-6 in inhibition of lung metastasis in subcutaneous tumor-bearing mice

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Received July 6, 1999; Accepted September 20, 1999

Abstract. Metastasis is the most important factor for prognosis in cancer patients, and its occurrence is largely associated with host immune response. We found that the presence of a growing tumor of colon 26, a mouse colon cancer cell line, completely inhibited lung colony formation in a mouse injected with colon 26 intravenously, whereas depletion of effector cells, such as natural killer and T cell subsets, did not affect antimetastasis of colon 26. Since colon 26 releases large amounts of interleukin-6 (IL-6) spontaneously, we studied the association of IL-6 with lung metastasis. Serum IL-6 level increased gradually and reached 12.6 pg/ml five days after inoculation of colon 26 in the back of mice, while at the same time, lung colony formation was inhibited. Moreover, expression of IL-6 mRNA in lung was observed to be associated with elevated serum IL-6 level. We show the first evidence that inhibition of lung metastases in tumor-bearing mice by colon 26 is closely associated with an increase in serum IL-6, but not in cellular immunity.

Introduction

The incidence of colon and rectal cancers is remarkably increasing in Japan, and the prognosis in such cases is closely associated with metastasis. It is well known that the immunological situation in a tumor-bearing host is different from that in a normal, healthy situation, and that host immunity can affect the occurrence of metastasis. An experimental model has revealed that tumor-bearing animals have antimetastatic potential: artificial metastasis in lungs by intravenous injection with tumor cells is inhibited in tumor-bearing mice, and the growth of a second inoculum of tumor is often inhibited if an earlier inoculum of tumor is growing in the animal (1-5). Bashford *et al* (6), named this phenomenon 'concomitant immunity', and this has been accepted as the prevalent theory. Concomitant immunity is achieved by the action of effector cells such as lymphocytes (2,3), natural killer cells (7-9), macrophages (10), and granulocytes (11). Involvement of humoral factors (12,13), such as cytokines or angiostatin, has also been proposed (14,15), on evidence that when the primary tumor is present, metastatic growth is suppressed by a circulating angiogenesis inhibitor. Although humoral factors have been much discussed (1,16,17), the precise mechanisms of this phenomenon remain largely unknown. In this study, we first confirmed concomitant tumor resistance by demonstrating that the presence of a growing colon 26 subcutaneous tumor inhibited the development of lung colony formation in mice injected with the same colon 26 cells intravenously. Next, we found that IL-6 in the serum was elevated in tumor-bearing mice, and that the amount was proportional to both the size of primary tumor and the intensity of inhibition of lung colony formation. Based on this evidence, we also discuss the expression of IL-6 gene in the subcutaneous tumor and the lung.

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Key words: interleukin-6, lung metastasis

Materials and methods

Mice. Inbred BALB/cA Jcl male mice used in the experiments were obtained from CLEA Japan, Co., Shizuoka, Japan. Five to 8-week-old male mice were used.

Cell line. Colon 26, a colon adenocarcinoma cell line (18), was a gift from Nippon Roche Research Center. It was cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum.

Animal experimental protocol. Colon 26 cells ($5x10^5$ per mouse) were first inoculated subcutaneously into the back of BALB/cA Jcl mice, and various days after, $2x10^5$ cells suspended in 0.2 ml of phosphate buffer pH 7.4 were next injected into the tail veins. The diameter of subcutaneously developed tumors was measured by caliper. Mice were subjected to a count of the number of tumor colonies in the lungs 8 or 14 days after injection. Approximately 2 ml of India ink was injected into the trachea. The lung was then placed in Fekete's solution, which had been previously mixed with 100 ml 70% ethanol, 10 ml formaldehyde, and 5 ml

Table I. Absence of lung colony formation after intravenous injection of colon 26 cells in subcutaneous tumor-bearing mice and IL-6 level in sera.

Group	n	Subcutaneous tumor	Average diameter of subcutaneous tumors at	Intravenous injection of colon 26 cells day 10 (mm)	Number of lung colonies 14 days after injection	IL-6 in sera (pg/ml)
1	5	-	0	+	52±30	3.2±1.2
2	5	+	8.4±0.6	-	0	952.1±729.4
3	5	+	7.1±1.4	+	0	607.0±538.4

Colon 26 cells ($2x10^5$ per mouse) were injected intravenously (groups 1 and 3). Colon 26 cells ($5x10^5$ per mouse) were inoculated subcutaneously into the back of mice, and allowed to grow for ten days (groups 2 and 3). The mice were sacrificed 14 days (groups 1 and 3) and 24 days later (group 2).

glacial acetic acid. Tumor colonies more than 0.1 mm was counted under a magnifying glass (19,20).

In vivo depletion of $CD4^-$, $CD8^-$ T cells, and NK cells. Anti-CD4 monoclonal antibody was obtained from rat GK1.5 hybridoma, and anti-CD8 antibody was obtained from 53-6.72 rat hybridoma, both partially purified by ammonium sulfate precipitation (21). Purified monoclonal antibodies (1 mg) were injected into mice intraperitoneally for 3 consecutive days. Depletion of $CD4^-$ and $CD8^-$ T cells in the spleen was examined by flow cytometry (21). Anti-asialo GM1 (Wako Junyaku Co., Ltd., Osaka) (20 µl) was injected intravenously into mouse to eliminate NK cells (22).

Expression of mouse IL-6 gene in mouse skin and lung, and concentration of IL-6 sera. Total RNA was isolated from the skin and lung by acid guanidinium thiocyanate/phenol/ chloroform extraction method (23). Poly(A)-rich RNA was isolated from 1 mg total RNA using Oligotex-dT30 (Super) (Nippon Roche, Japan). Samples of 10 ng poly(A)-rich RNA were subjected to reverse transcription with the reverse transcriptase of murine leukemia virus at 37°C for 1 h using an RNA PCR kit (Roche Molecular System Inc., USA). One µl of cDNA was amplified in 10 µl Gene Amp PCR reaction mixture containing 200 nM 5' and 3' primers of IL-6, 200 mM deoxynucleotide triphosphate, 55.5 kBq α^{32}P-dCTP, and 0.25 units Taq polymerase. PCR was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norfolk, CT) for 30 cycles: 30 sec denaturation at 94°C, 45 sec annealing at 60°C, and 1 min extension at 72°C. The reaction product was subjected to 5% polyacrylamide gel electrophoresis in 0.5X Tris/boric acid/EDTA buffer. The radioactivity of amplified products was counted by BAS 2000 image analyzer (Fuji Photo Film Co., Japan). At the same time, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control. The relative amount of IL 6 mRNA was quantified using GAPDH mRNA. After being normalized by the amount of GAPDH mRNA, IL-6 mRNA was expressed as a relative expression (-fold) in comparison with that of the non-treated control (24). IL-6 levels in the sera of mice were determined by ELISA kit (Intertest-6x Genzyme. Co., Ltd.).

Results

Absence of lung colony formation in colon 26 tumor-bearing mice. Colon 26 cells were injected intravenously into normal mice (group 1), and the mice developed 52±30 lung colonies 14 days after injection (Table I). In group 2, colon 26 cells were inoculated subcutaneously into the back of mice, resulting in development of subcutaneous tumors, the average diameter after 10 days was 8.4±0.6 mm. Next, group 3 was given subcutaneous injection of colon 26 cells followed by intravenous injection of same cells, and in the same amount as group 1. Tumor growth was not affected by intravenous injection (Table I), the average diameter of subcutaneous tumors in group 2 increased from 8.4±0.6 to 17.8±1.2, and in group 3 from 7.1±1.4 to 15.4±2.2 mm, at 24 days. The average level of IL-6 in sera 24 days after subcutaneous inoculation of the cells was 952.1±729.4 pg/ml for group 2, and 607.0±538.4 pg/ml for group 3, whereas that in group 1 was 3.2±1.2 pg/ml. Thus, serum IL-6 in subcutaneous tumor bearing mice was remarkably elevated.

Change of lung colony formation in tumor-bearing mice by depletion of NK cells, and $CD4^-$ and $CD8^-$ T cells. We next studied the effects of cellular immunity on metastasis in tumor-bearing mice by treatment with anti-asialo GM1, and $CD4^-$ and $CD8^-$ antibodies. As Fig. 1 shows, lung colony formation by intravenous injection of colon 26 cells was increased in normal mice and reduced in subcutaneously tumor-bearing mice. The three antibodies were given to normal mice, and it was apparent that treatment with anti-asialo GM1 antibody increased lung colony 2 fold in normal mice compared with non-treated mice. However, treatment with anti-CD4, or anti-CD8 antibodies, or their combination, did not induce enhancement of lung colony formation in normal mice. As for tumor-bearing mice, treatment with all three antibodies, as well as a combination of anti-CD4 and anti-CD8 antibodies, significantly reduced the number of lung colonies. Our pathological examinations revealed that accumulation of mononuclear cells was not observed in the lungs of either tumor bearing-mice or normal mice. These results suggested that inhibition of lung colony formation is

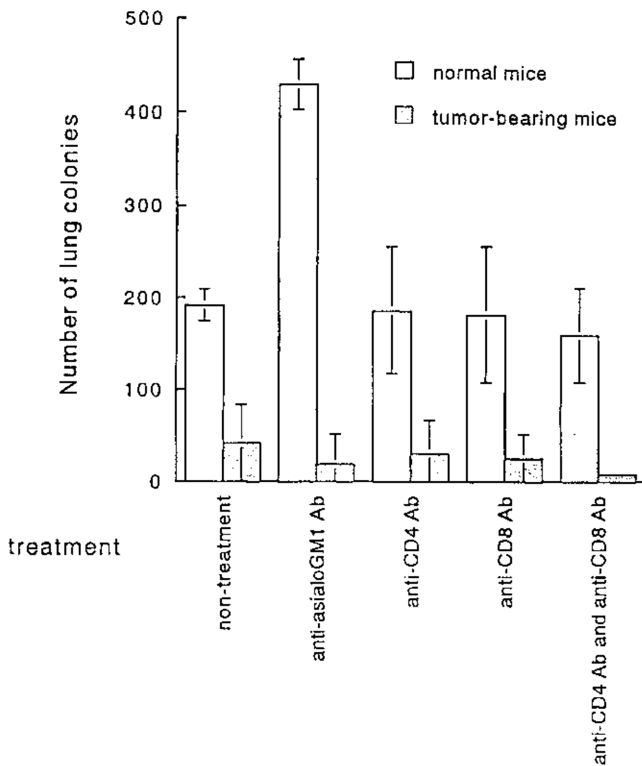

Figure 1. Colon 26 tumor cells ($5x10^5$ per mouse) were inoculated subcutaneously into the back of mice, and allowed to grow for ten days. Anti-asialo GM1 antibody was given intravenously on day 7. Anti-CD4 or/and anti-CD8 antibodies (1 mg) were given intraperitoneally on day 5 to 7. Colon 26 cells ($2x10^5$ per mouse) were challenged 10 days after subcutaneous inoculation of the cells, and lung colony formation was examined 14 days later.

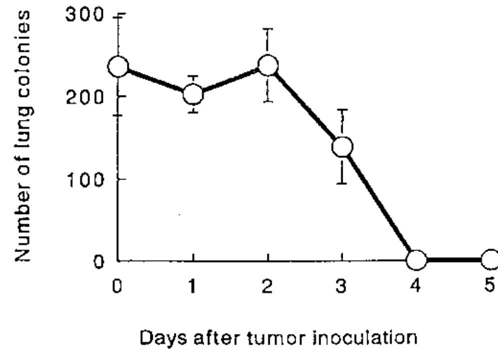

Figure 2. Changes in lung colony formation in tumor-bearing mice. Lung colony formation was examined in tumor-bearing mice after injection of colon 26 cells intravenously. Colon 26 cells ($2x10^5$ cells per mouse) were injected intravenously to normal mice or tumor-bearing mice at various days after subcutaneous inoculation of colon 26 cells. Lung colony was counted at 8 days after intravenous challenge of the colon 26 cells.

not regulated by cellular immunity mediated by NK, CD4 and CD8 cells (Fig. 1).

Changes in lung colony formation, tumor growth, and serum IL-6 level in tumor-bearing mice. Intravenous injection of colon 26 cells into normal mice, 0 day after subcutaneous inoculation of colon 26 cells induced lung colony formation, with the average number of lung colonies being 236±59 at 8 days after the intravenous challenge (Fig. 2). Similarly, intravenous injection of colon 26 cells was conducted various days after subcutaneous inoculation, and the number of lung colonies was examined at 8 days after each intravenous injection. As Fig. 2 shows, the average number of lung colonies was 203±22 in mice 1 day after subcutaneous inoculation, and 139±45 in mice 3 days after subcutaneous inoculation. That is, a 60% reduction was observed in the mice. Furthermore, complete inhibition of lung colony formation was observed in mice 4 and 5 days after subcutaneous inoculation. Therefore, intravenous injection of colon 26 cells was not effective in inducing lung colony formation in mice which had previously received subcutaneous inoculation of colon 26 cells.

Since colon 26 cells release a high concentration of IL-6 in culture medium (10 fg/cells for 8 h-culture, data not shown), we next examined the role of IL-6 in lung colony formation. Fig. 3A shows the average diameter of subcutaneous

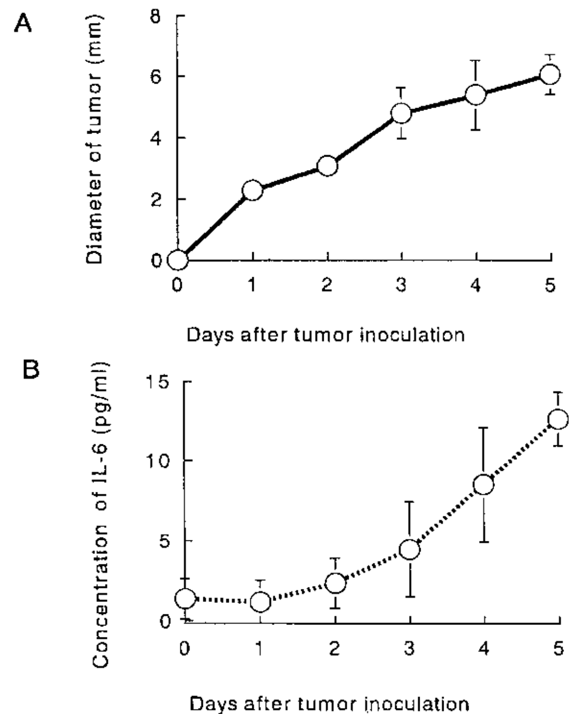

Figure 3. Changes in tumor growth and serum IL-6 level in tumor-bearing mice. Diameter of tumor (A) and concentration of serum IL-6 (B) in mice after subcutaneous inoculation of colon 26 cells ($5x10^5$ cells per mouse).

tumors from 1 to 5 days after tumor inoculation. As expected, the tumors grew gradually: colon 26 cells ($2x10^5$ cells per mouse) produced tumors 2.3±0.5 mm in diameter 1 day, and 6.1±0.7 mm 5 days later. In addition, concentration of serum IL-6 increased steadily, i.e. 1.4±1.3 pg/ml, 1 day, and 12.6±1.7 pg/ml 5 days later. Thus, we found that serum IL-6 increased simultaneously with growth of tumor in mice. These results indicated that inhibition of lung colony formation is reciprocally associated with tumor growth and elevation of serum IL-6 in tumor-bearing mice. However, treatment with IL-6 did not inhibit the growth of colon 26 cells *in vitro*.