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Inhibition of Melanoma Growth and Metastasis by Combination With (−)-Epigallocatechin-3-Gallate and Dacarbazine in Mice

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Abstract (−)-Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, was shown to have cancer chemopreventive activity. In this study, we examined the antimetastatic effects of EGCG or the combination of EGCG and dacarbazine on B16-F3m melanoma cells in vitro and in vivo. First, the antimetastatic potentials of five green tea catechins were examined by soft agar colony formation assay, and the results show that EGCG was more effective than the other catechins in inhibiting soft agar colony formation. Second, EGCG dose-dependently inhibited B16-F3m cell migration and invasion by in vitro Transwell assay. Third, EGCG significantly inhibited the spread of B16-F3m cells on fibronectin, laminin, collagen, and Matrigel in a dose-dependent manner. In addition, EGCG significantly inhibited the tyrosine phosphorylation of focal adhesion kinase (FAK) and the activity of matrix metalloproteinase-9 (MMP-9). In animal experiments, EGCG alone reduced lung metastases in mice bearing B16-F3m melanomas. However, a combination of EGCG and dacarbazine was more effective than EGCG alone in reducing the number of pulmonary metastases and primary tumor growths, and increased the survival rate of melanoma-bearing mice. These results demonstrate that combination treatment with EGCG and dacarbazine strongly inhibits melanoma growth and metastasis, and the action mechanisms of EGCG are associated with the inhibition of cell spreading, cell-extracellular matrix and cell-cell interactions, MMP-9 and FAK activities. J. Cell. Biochem. 83: 631–642, 2001.

Key words: EGCG; dacarbazine; melanoma; metastasis; invasion

Green tea contains a large amount of polyphenols, especially flavan-3-ol, also known as catechins. The six main catechins in green tea are (+)-catechin, (+)-gallocatechin (GC), (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and EGCG. Green tea polyphenols and EGCG were reported to have antioxidative [Yen and Chen, 1995; Lin et al., 1996], antimutagenic [Wang et al., 1989], anti-inflammatory [Lin and Lin, 1997], and anticarcinogenic activities [Yang and Wang, 1993; Stoner and Mukhtar, 1995]. Several reports have demonstrated that green tea polyphenols also inhibit the growth of experimental skin tumors or implanted tumors in animal [Wang et al., 1992; Yang and Wang, 1993]. Recently, others and our group have indicated that the anti-tumor promotion mechanisms of EGCG were associated with blocking EGF binding to its receptor [Liang et al., 1997] and inhibiting AP-1 activity induced by TPA or EGF [Dong et al., 1997]. Previous studies showed that the peroral administration of a green tea infusion or EGCG inhibited lung metastasis in mouse melanoma and Lewis lung carcinoma cells [Taniguchi et al., 1992; Sazuka et al., 1995]. In short-term experiments (4 weeks), primary tumor growth was little or not at all inhibited by the administration of a green tea infusion or EGCG; mice survival was not investigated to indicate the cure efficiency, and thus antimetastatic mechanisms of a green tea infusion or EGCG were not well understand [Taniguchi et al., 1992; Sazuka et al., 1995]. (+)-Catechin and (−)-epicatechin may bind to laminin and exhibit anti-invasive activity [Bracke et al., 1987; Bracke et al., 1991]; however, the underlying mechanisms of EGCG or other tea catechins have not been clearly elucidated.

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Metastasis is one of the major causes of mortality in cancer patients. Tumor invasion is the end result of a complex series of steps involving multiple tumor-host interactions [Woodhouse et al., 1997]. One important step is invasion into tissues, which requires proteolytic degradation of extracellular matrix (ECM) components. Matrix metalloproteinases (MMPs) are a family of zinc-binding enzymes that cleave ECM components, and the expression levels of MMPs are correlated with tumor invasiveness [Stetler-Stevenson, 1990]. Both MMP-2 and MMP-9 are postulated to play a critical role in tumor invasion and angiogenesis [Stetler-Stevenson, 1990]. During the metastatic process, tumor cells need to attach to other cells and/or ECM components, such as fibronectin (FN), laminin (LN), and collagen. Several reports have implied that adhesive interactions of tumor cells with ECM components play a critical role in the establishment of metastases [Iwamoto et al., 1987; Yamada et al., 1990]. In addition, several lines of evidence indicated that integrins are a key factor in interactions of cells with ECM components [Giancolti and Mainiero, 1994]. Integrin-mediated cell adhesion induces tyrosine phosphorylation of focal adhesion kinase (FAK) [Miyamoto et al., 1995], which represents key events in signaling for cell movement, cell adhesion, and anchorage-independent growth [Guan and Shalloway, 1992]. Cell movement was inhibited by down-regulation of FAK phosphorylation and signaling [Gilmore and Romer, 1996]. Overexpression of FAK enhanced cell movement [Cary et al., 1996] and the invasive potential of a tumor [Owens et al., 1995].

In Caucasian populations, the worldwide incidence of invasive primary cutaneous melanoma has increased during the past decade, and the main reason is believed to be increased recreational exposure to ultraviolet radiation from sunlight [Singletary and Balch, 1991]. There was no effective standard treatment to cure patients with metastatic melanoma, and median survival time of these patients was only 4–6 months. [Dummer et al., 1994]. Presently, dacarbazine is the only chemotherapeutic agent and the most effective cytostatic drug, but patients usually survive only a short time. Combinations of dacarbazine with other cytostatic drugs has not improved response rates or survival [Mulder et al., 1994]. Some results have revealed a significant improvement compared to that reported with dacarbazine alone, such as combination therapy with dacarbazine and interferon α-2b [Falkson et al., 1991].

The purpose of the present study was (i) to investigate the antimetastatic mechanisms of EGCG with special reference to the inhibition of the adhesion and spread of tumor cells, suppression of MMP-9 secretion, and serum-induced tyrosine phosphorylation of FAK; and (ii) to examine the efficacy of combination treatment with EGCG and dacarbazine on primary tumor growth, lung metastases, and the survival rate of tumor-bearing mice.

METHODS

Materials

EGCG was purified from Chinese tea (Longjing tea, *Camellia sinensis*) by the method of Nonaka et al. [1983] with some modifications as described in our previous report [Lin et al., 1996]. Dacarbazine (5-[3,3-dimethyl-1-triazenyl]imidazole-4-carboxamide; DTIC) was purchased from Sigma Chemical Co. (St. Louis, MO). Gelatin was purchased from ICN Biochemicals (Cleveland, OH).

Cells and Animals

Mouse B16 melanoma cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS, Life Technologies, Gaithersburg, MD) in a humidified atmosphere of 95% air-5% CO_2 at 37°C. The high-metastatic B16 cell line was isolated from the lung of Balb/c mouse, which had been inoculated intraperitoneally with B16 cells. Cells were cloned by the limiting dilution methods and selected clones were inoculated into mice. This method were repeated thrice, and the final clone with high metastatic potential was obtained as B16-F3m. Male Balb/c mice (6–7 weeks old) were obtained from the Animal Center of National Taiwan University Hospital (Taipei, Taiwan), and maintained on standard chow and water ad libitum.

Soft Agar Colony Formation Assay

The effects of EGCG and other catechins on soft agar colony formation of B16-F3m cells were investigated. Briefly, single-cell suspensions of B16-F3m cells were treated with or without different concentrations of inhibitors (EGCG and other catechins), and then mixed with agarose in a final concentration of 0.33%.

Aliquots of 1.5 ml containing 10^3 cells and 10% FBS were plated in triplicate in 35-mm culture dishes over a base layer of 0.6% agarose and allowed to gel. Colonies of > 8 cells were counted after 21 days of incubation [Glinsky et al., 1996].

In Vitro Migration/Invasion Assay

B16-F3m was seeded with or without different concentrations of EGCG into the upper compartment of a Transwell cell culture chamber. After 12 h of incubation, the migrating or invading cells on the lower surface were visually counted. Cell migration was assayed in a 48-well modified Boyden chamber (Neuroprobe, Bethesda, MD) with an 8-μm pore size polycarbonate membrane (Transwell, Costar, Cambridge, MA) as described previously [Lin et al., 1998] with some modifications. Briefly, 1×10^4 cells were resuspended in culture medium and seeded into the upper wells. Subsequently, 3T3 fibroblast-conditioned medium was placed in the lower compartment of the chemotaxis chambers to serve as a source of chemoattractants. Cells remaining on the upper surface of the membrane were removed with a cotton swab after a 12-h migration at 37°C. Cells that migrated to the underside of the membrane were fixed, stained with hematoxylin and eosin, and then counted with a light microscope at high power (×200) using a gridiron objective. Tumor cell invasion was assayed by the same procedure, except that the polycarbonate membrane was precoated with 10 μg of Matrigel, a reconstituted basement membrane gel (Collaborative Biomedical Products, Bedford, MA).

Cell Aggregation and Adhesion/Spreading Assay

The cell aggregation assay was performed essentially as described previously [Glinsky et al., 1996]. Briefly, a single-tumor cell suspension was obtained from subconfluent cultures using standard trypsinization procedures. Subsequently, a total of 2×10^5 cells in 1 ml of DMEM (serum-free) with different concentrations of EGCG was placed in polystyrene microtubes and shaken gently every 5 min for 1 h at 37°C. At the end, glutaraldehyde (at a final concentration of 2%) was added to the tubes to stop the aggregation process, and aggregation was quantified as described in Glinsky et al. [Glinsky et al., 1996].

The cell adhesion assay was performed in 6-well plates that had been pre-coated with different ECM proteins: fibronectin, laminin, collagen type IV, and Matrigel (Biocoat, Becton Dickinson, Bedford, MA). B16-F3m cells were suspended in serum-free DMEM to form a single-cell suspension. The tumor cell suspension (2×10^5) was treated with or without different concentrations of EGCG in a volume of 2 ml/well, then seeded into the pre-coated wells. After a 45-min incubation at 37°C, the wells were washed three times with PBS to remove non-adherent cells. Following cell fixation and staining with 0.5% crystal violet in 20% methanol, quantitation of adhesion was achieved by washing the cells, eluting the dye with 1 ml/well of 33% acetic acid and measuring the absorbance at 650 nm. For spreading assay, cells were added to the pre-coated wells as described above. After 45 min of incubation at 37°C, the cells were fixed with cold methanol for 5 min, photographed, and the percentage of spread cells was determined as described previously [Yamada et al., 1990; Pasqualini et al., 1996].

Gel Zymography

Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 9% polyacrylamide gels containing 0.1% gelatin. Cell culture medium was obtained from cultured B16-F3m cells in serum-free DMEM with or without different concentrations of EGCG for 24 h. Cultured media were concentrated 2-fold and mixed with Laemmeli's sample buffer in the absence of β-mercaptoethanol, loaded onto gels and electrophoresed. After PAGE, the gel was washed with 2.5% Triton X-100 and incubated for 24 h at 37°C in 50 mM Tris/10 mM $CaCl_2$/10 μM $ZnCl_2$ buffer, pH 7.4. Clear bands of gelatinolytic activity were visualized after staining the gel with Coomassie blue. The intensities of the bands were evaluated by using an IS-1000 Digital Imaging System (Kaiser Alpha Innotech, San Lendro, CA).

Immunoprecipitation and Immunoblotting Procedures

Cells were first serum-starved for 48 h and pretreated with or without different concentrations of EGCG for 30 min, and then with 2% FBS for 1.5 h. Total cell lysates (300 μg) were immunoprecipitated with 2.5 μg of anti-FAK mAb (Upstate Biotechnology, Lake Placid, NY) and protein A/G plus-agarose (Santa-Cruz Biotechnology, Santa Cruz, CA) as described

[Liang et al., 1999]. Total cell lysate (40 μg) or immunoprecipitated protein (FAK) was resolved by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Amersham, Arlington, IL), followed by immunoblotting with anti-FAK (1:1000 dilution) or anti-phosphotyrosine first antibody (PY-20; 1:1000 dilution, Transduction Laboratory, Lexington, KY) and then secondary antibody-conjugated horseradish peroxidase (Transduction Lab.). The immunocomplexes were visualized using the enhanced chemiluminescence kits (ECL) (Amersham, Oakville, Canada).

HPLC Analysis of EGCG in Blood Extracts

EGCG (2.0 mg) was dissolved in 0.1 ml of normal saline and given to Balb/c mice by intraperitoneal injection. Immediately prior to sacrifice, mice were anesthetized with ether at indicated times after dosing, and bled from the heart. Serum was obtained from the coagulated blood by centrifugation, and 400 μl of serum was incubated with 20 μl of 5 N HCl for 30 s, then extracted three times with 500 μl of ethyl acetate. The extract was evaporated to dryness and dissolved in 50% methanol, then analyzed by reverse-phase HPLC, as described in our previous paper [Lin et al., 1996].

Assay of Tumor Metastasis in an Animal Model

For injection into mice, cultured B16-F3m melanoma cells were harvested with 2 mM EDTA in phosphate-buffered saline (PBS), then washed three times with PBS. Inoculation of this single-cell suspension was then carried out intraperitoneally (i.p.) into Balb/c mice at 2×10^6 cells per animal. After 3 days, the drug treatment was started according to the following treatment protocol. Animals were divided into four groups of six animals each. The same experiment was repeated two times. Drugs were dissolved in normal saline, and injected intraperitoneally into mice. Group 1 was injected with normal saline (0.1 ml) five times a week for 11 weeks. Group 2 was injected with EGCG (2 mg/0.1 ml) three times a week for 11 weeks. Group 3 was injected with dacarbazine (200 μg/0.1 ml) three times a week for 11 weeks. Group 4 was injected with EGCG (2 mg/0.1 ml) three times and dacarbazine (200 μg/0.1 ml) twice a week for 11 weeks. Drug solutions were prepared daily by suspension of preweighed aliquots in sterile normal saline to a final concentration of 2 mg/0.1 ml of EGCG and 200 μg/0.1 ml of dacarbazine. The solutions were filtered through a 0.2-μm syringe filter before use. Mice were sacrificed at the end of the treatment schedule or when moribund; the total number of visible nodules on the lung surface per mice was scored, and the primary tumors were weighed.

Statistical Analysis

Results were expressed as mean ± SE for each study. Data were analyzed by Dunnett's test or Student's test and a P value of 0.05 or less was considered statistically significant.

RESULTS

In Vitro Evaluation of Antimetastatic Activity of Tea Catechins

Effect of tea catechins and EGCG on colony formation in soft agar. It has been reported that the ability of tumor cells to grow in semisolid agarose is correlated with their metastatic potential [Price, 1986]. Therefore, we screened the antimetastatic activity of five green tea catechins using a clonogenic growth assay. Following exposure to a panel of tea catechins (10 μM), the inhibition of colony formation was determined and is shown in Figure 1A. Both ECG and EGCG significantly inhibited colony formation of B16-F3m. EGCG is the major component of green tea polyphenols and was shown to be more effective than ECG. It was selected for further antimetastatic studies in vitro and in vivo. The results of dose-response tested groups indicated that 10 μM of EGCG was sufficient to inhibit colony formation by about 63% (Fig. 1B). The inhibition of colony formation was not due to toxicity, as determined by the trypan blue exclusion assay (Data not shown).

Effect of EGCG on the migration/invasion of B16-F3m cells in an in vitro assay. To further evaluate the antimetastatic activity of EGCG, we assessed the inhibition of EGCG on melanoma migration and invasion by the Transwell assay. The migration-stimulating activity of the fibroblast-conditional medium was inhibited in a dose-dependent manner by EGCG, and the IC_{50} dose was about 8 μM (Fig. 1C). We also tested the ability of B16-F3m cells to invade through a reconstituted basement membrane barrier (Matrigel) with or without EGCG. Similar to the migration assay,

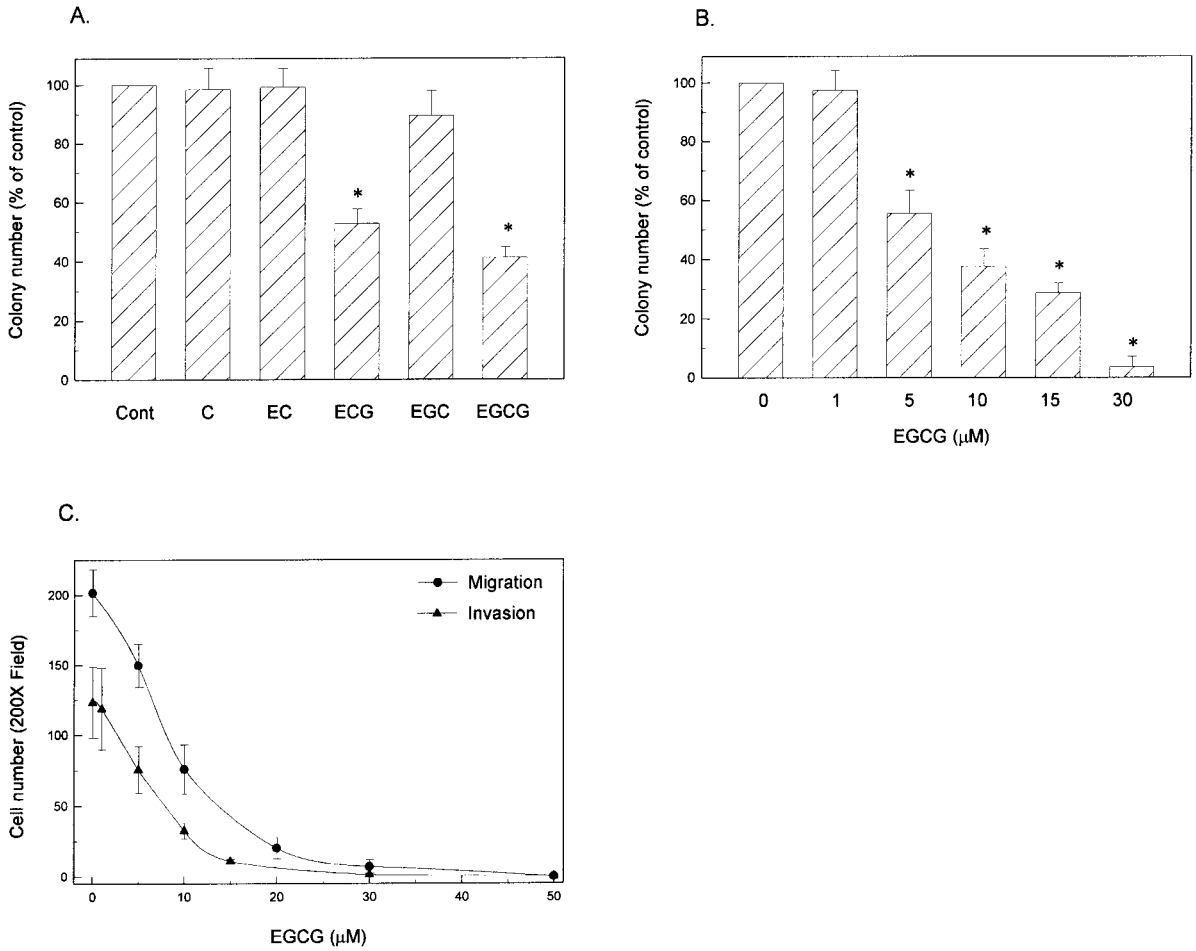

Fig. 1. Effects of tea catechins on colony formation in soft agar, growth, and migration/invasion of B16-F3m cells. B16-F3m cells were treated with or without different kinds of tea catechins (10 μM) (**A**), or different concentrations of EGCG (**B**) in 0.33% agarose containing 10% FBS over 0.6% agarose containing 10% FBS. Cell colonies were counted after a 21-day incubation at 37°C in 5% CO_2. B16-F3m was seeded with or without different concentrations of EGCG into the upper compartment of a Transwell cell culture chamber (**C**). After 12 h of incubation, the migrating or invading cells on the lower surface were visually counted. All data were presented as the mean ± SE of three independent experiments in duplicate. $^*P < 0.01$, compared with the control group (Student's *t*-test). Cont, control; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate.

EGCG also inhibited the invasion of B16-F3m cells in a dose-dependent manner at doses of 1–20 μM, and by 74% at 10 μM EGCG (Fig. 1C).

Action Mechanisms of Antimetastasis of EGCG

Effects of EGCG on adhesion/spread of B16-F3m cells. First, we examined the effects of EGCG on B16-F3m cell adhesion to ECM proteins. Tumor cell adhesion to the components, such as FN and LN of extracellular matrices and the basement membrane has been shown to be an important step in tumor metastasis. As shown in Figure 2A, EGCG exhibited inhibitory effects on the adhesion of tumor cells to LN or collagen IV with IC_{50} values > 30 μM with 45 min of treatment, and inhibitory effect, on the adhesion of tumor cells to FN with IC_{50} values of about 30 μM. However, EGCG showed a specific, concentration-related inhibition of Matrigel-mediated attachment of B16-F3m cells with IC_{50} values of 12 μM.

Next, the cell-spreading ability of B16-F3m cells treated with EGCG on various substrates was examined. As shown in Figure 2B, EGCG significantly inhibited cell spreading on various substrates in a dose-dependent manner. Moreover, EGCG-treated cells were easily washed out at the end of the 45-min incubation. The most remarkable inhibition in this study resulted from EGCG-inhibited cells spreading

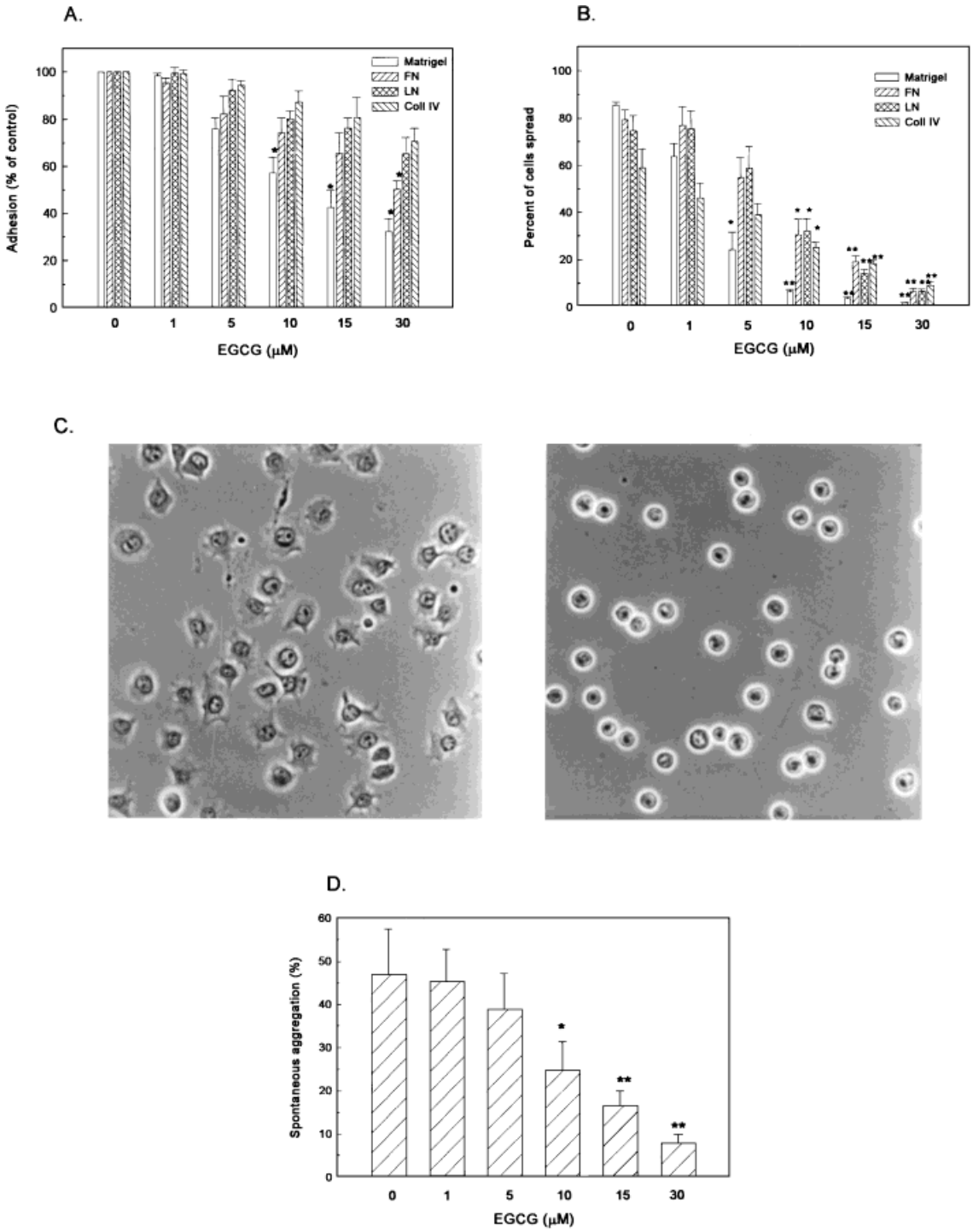

Fig. 2. Effects of EGCG on cell adhesion or spreading on various substrates and spontaneous B16-F3m cell aggregation. B16-F3m cell suspensions were treated with or without different concentrations of EGCG in serum-free DMEM and placed onto the wells coated with Matrigel, fibronectin (FN), laminin (LN), or collagen type IV (Coll IV). After a 45-min incubation at 37° C, the percentage of adhering (**A**) or spreading (**B**) cells were determined as described in ''Materials and Methods''. **C**: Cells were fixed and photographed from (B). Untreated B16-F3m cells (left) and B16-F3m cells treated with 10 μM EGCG (right) were placed in Matrigel-coated wells for 45 min, then fixed and photographed. **D**: B16-F3m cell suspensions were treated with different concentrations of EGCG in polystyrene microtubes containing serum-free DMEM for 1 h, then cells were fixed, and the percentage of aggregated cells was counted as described in ''Materials and Methods''. All data were presented as the mean ± SE of three independent experiments in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with the control (Student's *t*-test).

on Matrigel, with an IC_{50} value below 5 μM. Figure 2C showed that untreated cells spread well on Matrigel substrates (Fig. 2C, left), whereas most of the cells treated with 10 μM EGCG for 45 min remained round and loose (Fig. 2C, right). During the incubation period (45 min), 1–30 μM of EGCG showed no cytotoxic effect against B16-F3m cells by trypan blue exclusion assay [Lin et al., 1996; Okabe et al., 1997].

Effect of EGCG on Spontaneous Homotypic Cell Aggregation

Previous studies have demonstrated that heterophilic and homotypic cell-cell interactions play important roles in tumor growth and metastasis [Albelda, 1993; Glinsky et al., 1996]. Blocking of cell surface galectin-mediated cell-cell interactions resulted in induction of apoptosis in highly metastatic B16-F10 murine melanoma cell variants [Glinsky and Glinsky, 1996]. As shown in Figure 2D, EGCG significantly inhibited homotypic cell aggregation in a dose-dependent manner. Moreover, the main form of cell aggregation was as polymers and dimers in cells treated with vehicle and EGCG, respectively (data not shown).

Effect of EGCG on MMP Activity

We further tested the effect of EGCG on the activity of MMPs in B16-F3m cells. Gelatin zymography of concentrated serum-free conditioned medium revealed one major band of lysis: the 92-kDa gelatinase (MMP-9) (Fig. 3). MMP-9 activity was inhibited (58%) when cells were cultured with 10 μM of EGCG. Casein zymography of the same conditioned medium revealed no band of lysis (data not shown).

Effect of EGCG on Tyrosine Phosphorylation of Focal Adhesion Kinase

Several focal adhesion proteins become tyrosine phosphorylation, including one of the major tyrosine phosphorylated, focal adhesion kinase (FAK), as cells adherence to ECM protein [Burridge et al., 1992]. On the other hand, tyrosine kinase inhibitors prevent the formation of focal adhesion [Romer et al., 1994]. In the present study, B16-F3m cells were incubated with various concentrations of EGCG for 24 h. As shown in Figure 4 (upper), FAK was tyrosine phosphorylated upon 2% serum stimulation for 1.5 h (lane 2), and 10 μM of EGCG was sufficient to inhibit tyrosine phosphorylation by 70.2% (lane 5). There was no effect of EGCG treatment on the expression of FAK protein in either test (Fig. 4, lower panel).

Treatment of Tumor-Bearing Animals With EGCG in Combination With Dacarbazine

Concentrations of EGCG in mouse blood. Following a single i.p. administration of 2 mg EGCG in 0.1 ml normal saline, plasma concentrations of EGCG were measured, and were shown in Figure 5. During a period of 48 h, mean concentrations of free-form EGCG were initially low, rose to a peak, and then declined (Fig. 5). At 8 h after injection, the concentration of free-form EGCG in mice blood was 7.68 ± 0.82 μM, which was reached to the maximal level. Even 48 h after injection, there was 0.65 ± 0.11 μM of free-form EGCG in the blood. Although the plasma concentration of free-form EGCG was determined to be lower than 9 μM by ethyl acetate extraction, we believed that the available EGCG which could possibly act on tumor cells exceeded 9 μM. Because some EGCG might be adsorbed on plasma protein, blood cells, tumor cells, and others to form conjugated forms of EGCG, it could not be extracted quantitatively by the method used in this study [Okushio et al., 1996].

Effect of Combination Treatment on Spontaneous Pulmonary Metastasis and Host Survival

In consideration of the low plasma EGCG levels in previous experimental rats and mice [Unno and Takeo, 1995; Wang et al., 1995; Yang

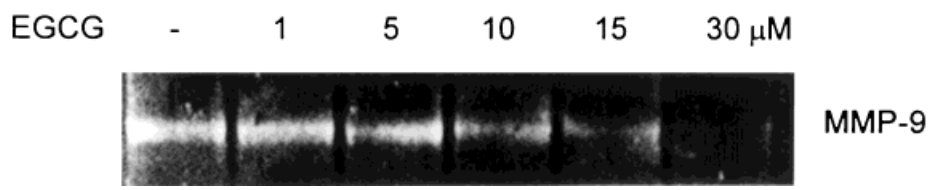

Fig. 3. Effects of EGCG on MMP-9 production in B16-F3m cells. Cells were treated with or without different concentrations of EGCG for 24 h in serum-free DMEM, and concentrated aliquots of media were electrophoresed on gelatin gels and incubated in reaction buffer as described in ''Methods''.

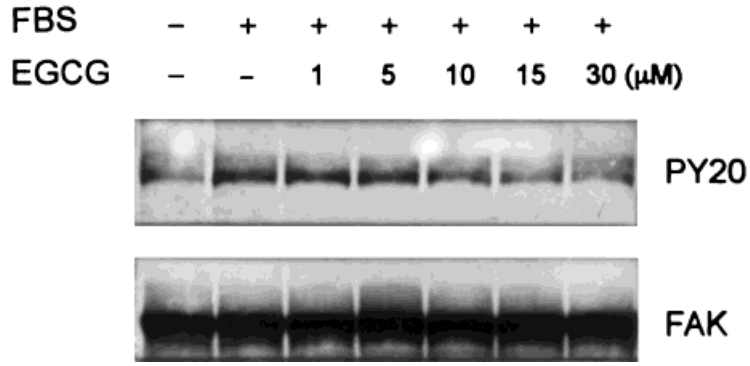

Fig. 4. Effects of EGCG on tyrosine phosphorylation of FAK. Cells were first serum-starved for 48 h and pretreated with or without different concentrations of EGCG for 30 min, and then with 2% FBS for 1.5 h. FAK was immunoprecipitated with anti-FAK mAb and blotted with anti-phosphotyrosine antibody (PY-20, upper). Total cellular proteins (40 μg) were separated on SDS–PAGE and blotted with anti-FAK antibody (lower). Immunocomplexes were detected by horseradish peroxidase second antibody and then by ECL kits.

et al., 1995], and our results (Fig. 5), we combined EGCG and dacarbazine in an effort to inhibit solid melanoma cells in further animal experiments, and mice were given EGCG and dacarbazine by i.p. administration. As expected, EGCG (Group 2) reduced pulmonary metastases to 41.1% ($P < 0.01$) of control values (Group 1) (Table I). As the current most-effective single agent against melanoma, dacarbazine did not significantly reduce pulmonary metastases ($P = 0.1207$). However, the combination of EGCG and dacarbazine further decreased metastases to 27.9% ($P < 0.01$) of control values (Group 1) (Table I). EGCG alone was more effective than dacarbazine in suppressing lung metastasis. The combination of EGCG and dacarbazine treatment provide to be more effective than EGCG alone in suppressing lung metastasis ($P < 0.05$) and in inhibiting primary tumor growth (Table I, 7.68 ± 1.05 g (Group 2); 5.68 ± 1.23 g (Group 4)). In addition, the survival rate of mice was elevated from 1/12 (Group 1, control), 5/12 (Group 2, EGCG), to 7/12 (Group 4, EGCG and dacarbazine) at the end (Table I).

DISCUSSION

Chemoprevention has traditionally been defined as the use of one or several natural products or synthetic compounds to prevent the occurrence of cancer [Kelloff et al., 1994]. Many studies have demonstrated the inhibitory actions of tea catechins and EGCG against carcinogenesis in rodent models [Yang and Wang, 1993; Katiyar and Mukhtar, 1996], and tea catechins are believed to be useful chemopreventive agents. Recently, anti-invasion

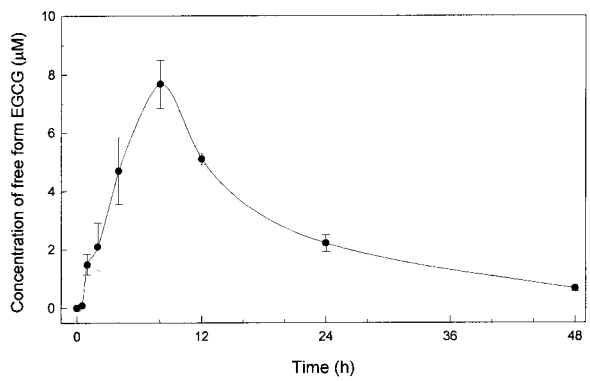

Fig. 5. Concentrations of EGCG in blood of mice following a single intraperitoneal administration of EGCG. EGCG (2 mg/0.1 ml) was injected intraperitoneally, and aliquots obtained from heart bleeds were analyzed by reverse-phase HPLC at the indicated time intervals to monitor the concentration. An extract was prepared and analyzed by HPLC as described in ''Methods''.

TABLE I. Inhibitory Effects of EGCG and/or Dacarbazine on Mice-Bearing Melanoma

Group	Treatment	Number of lung nodules	Primary tumor weight (g)[b]	Survival (alive/total)[c]
1	Control	14.00 ± 2.06[a]	7.82 ± 0.42	1/12
2	EGCG	5.75 ± 1.20	7.68 ± 1.05	5/12
3	Dacarbazine	9.75 ± 1.67	5.69 ± 1.56	7/12
4	EGCG + Dacarbazine	3.91 ± 0.94	5.68 ± 1.23	7/12

[a]Mean ± SE for each group of mice.
[b]Primary tumor weight was measured at the end of treatment schedule, or when moribund during last 3 weeks, and is expressed as the mean ± SE.
[c]The numbers indicated the surviving mice at the end of the experiment.

agents have been defined as a new class of cancer chemopreventive agent [Kohn and Liotta, 1995]. During the metastatic process, interaction between cells and ECM is the important step, which requires three distinct events: (i) attachment and spreading of cells to matrix components, (ii) local degradation of the matrix by proteinases, and (iii) migration of cells through the matrix. The multiple repetitions of these three events permits cells to invade surrounding tissue [Liotta, 1986]. In the present study, we focused on this step and demonstrated that EGCG has anti-invasion activity in vitro and in vivo, and thus, it might represent a new strategy for cancer chemoprevention.

Treatment of patients with metastatic malignant melanoma remains unsatisfactory. Dacarbazine is generally considered the most active agent for treating malignant melanoma [Philip et al., 1994]. The anti-tumor activities of dacarbazine resulted from the methylation of nucleic acids or direct DNA damage, and resulted in arrest of cell growth or cell death [Friedman et al., 1996]. Our previous studies demonstrated that inhibition of A431 cell proliferation by EGCG mainly mediated suppression of extracellular signal transduction, such as blocking of EGF binding to its receptor [Liang et al., 1997]. It is possible that EGCG bound to multiple sites of the extracellular membrane surface, and thus blocks the binding of EGF. In addition, EGCG was able to block cell cycle progression in the G1 phase of MCF-7 breast cancer cells [Liang et al., 1999]. In this study, EGCG (< 20 μM) was not efficient in inhibiting the growth of B16-F3m melanoma cells in vitro or in vivo. However, EGCG might bind to the extracellular membrane of B16-F3m cells, and block some signal transduction, which may be correlated with metastasis and adhesion. Dacarbazine exist its anti-tumor effect through the formation of methylated DNA adducts. But, it is mutagenic to eukaryotic cells and may cause liver and lung cytotoxicities at high dosing. A combination of EGCG and dacarbazine not only decreased the dose and the possible cytotoxicity of dacarbazine (2 injection/week) but also increased the efficacy of antimetastasis. Previous studies indicated that oral administration of green tea, i.p. administration of the green tea polyphenols fraction, or i.p. administration of EGCG inhibited the growth and/or caused the regression of established experimentally induced skin papillomas [Wang et al., 1992]. However, the present study and previous other studies showed that i.p. administration of EGCG only or peroral administration of green tea infusion and EGCG slightly or negligibly inhibited the growth of implanted melanoma and lung carcinomas [Taniguchi et al., 1992; Sazuka et al., 1995]. These results suggested that the combination of EGCG and other drugs might provide a more effective way for combating melanoma growth and metastases.

In recent years, some animal studies have suggested that green tea infusion and EGCG exhibited anti-invasive activities. However, the mechanisms of action by which these chemicals inhibit invasion are not clear. In the present study, single EGCG (2 mg) i.p. administration produced blood concentrations of free-form EGCG in the range of 0.6–8 μM (Fig. 5), with no toxicity. Okushio et al. [1996] demonstrated that the recovery rates of EGCG from blood were low. We believed that the dose of EGCG acting on tumor cells must be higher than 0.6–8 μM, because some of the EGCG might be absorbed and retained on the tumor cells. Our previous study have indicated that EGCG might bind to EGF receptors and then blocked EGF binding to its receptor [Liang et al., 1997]. In the present study, EGCG prevented tumor cells from adhering on and spreading onto ECM

substrates, possibly because of blocking interactions of adhesion molecules between cells and ECM substrates. Since recent studies have indicated that tyrosine phosphorylation of focal contact proteins, such as paxillin and $pp125^{FAK}$, plays a critical role in the regulation of cell adhesion to ECM, cell spreading and migration [Burridge et al., 1992; Sankar et al., 1995], it is possible that the inhibitory effects of EGCG on cell adhesion and spreading were correlated with the inhibition of tyrosine phosphorylation of FAK.

It has been reported that gelatinase expression correlates positively with invasive potential [Dyke et al., 1993], and that elevated expression of activated species of MMP-9 and MMP-2 is associated with tumor spread [Brown et al., 1993]. Secretion of MMP-2 by B16-F3m cells was undetectable in our detection system. There are binding sites for both activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcription factors in the conserved regions of the rabbit MMP-9 gene promoter [Fini et al., 1994]. In addition, several growth factor and cytokine regulatory pathways converge at the AP-1 and NF-κB binding site, including EGF, TPA, and LPS [Hung et al., 1996]. Our previous studies [Lin and Lin, 1997; Liang et al., 1997] and an other study [Dong et al., 1997] have indicated that EGCG inhibited AP-1 activity induced by EGF and TPA, and NF-κB activity induced by LPS. Thus, the action mechanism of EGCG might be through those pathways.

In conclusion, this study demonstrated that combination treatment with EGCG and dacarbazine strongly inhibited lung metastasis of B16-F3m melanoma and increased the survival rate of tumor-bearing mice. The antimetastatic effects of EGCG were associated with the inhibition of melanoma adhesion and spreading as well as suppression of MMP-9 secretion and tyrosine phosphorylation of FAK.

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