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Characterization of Angiogenesis Inhibitor Effect of Green Tea Seed Extract

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Abstract: Green tea seed was extracted with absolute ethanol, and the green tea seed extract (GTSE) was subjected to assays for toxicity, antioxidant ability, angiogenesis inhibitory effects and cell adhesion, as well as western blotting, and an *in vivo* experiment against 4 high-ranking adult cancers in Korea. Our series of experimental data demonstrated that GTSE has an antioxidant ability superior to that of EGCG in the green tea leaf, and also exhibits a profound high tumor growth inhibitory activity on a variety of cancer cell lines, as well as nude mice infected with cancer cells. GTSE was identified as a natural anticancer compound showing excellent angiogenesis inhibition and cancer cell suppression abilities. Our preliminary observations also indicate that GTSE may be another potential source of natural dietary antioxidants and also may be applicable as a novel natural anticancer agent.

Key words: green tea seed extract; anti-cancer; anti-angiogenesis; western blotting; *in vivo* animal experiment

INTRODUCTION

Cancer is one of the top 10 leading causes of death worldwide. In 2005, WHO announced that 7.6 million people had died of cancer out of 58 million deaths worldwide. Angiogenesis is a fundamental process by which new blood vessels are formed. Moreover, angiogenesis is known to be a necessary requirement for various diseases, such as proliferation and metastasis in cancer cells, rheumatism arthritis, and diabetic blindness (Folkman and Shing, 1992). Despite the great expenditures thus far put into them, cancer therapies including chemotherapy, radiation therapy, and surgical operation are limited in their ability to cure cancer, as anticancer agent(Bae and Kim, 2008) usually damage not only cancer cells but also normal cells, coupled with severe side effect on immunological function *in vivo*.

Recently, a great many natural resources have been investigated in order to find a new medical substance that can be in an anti-cancer treatment, as their principal benefit is in reducing side effects significantly as compared to synthesized chemicals. Among them, herbs are traditionally used for anticancer treatment, and are anti-angiogenic by virtue of a multiplicity of interdependent processes. Green tea (*Camellia sinensis*) is one of the natural herbs known to exert various beneficial health effects, including anti-inflammatory effects and an amelioration of the risk of cardiovascular disease and cancer (Graham, 1992). As for EGCG in the green tea leaf, a variety of health benefits have been reported, including epidemiological evidence (Kono et al., 1988), animal models against various tumors (Rogers et al., 1998), *in vitro* apoptosis stimulation (Yang et al., 1998). 3142 papers have been published on green tea thus far, but the seed has been the focus of only 60 papers, according to PubMed, NCBI. Among them, only several papers are directly related to extracts of green tea seed. Even though the seed has been utilized as a folk medicine for a long time in Korea, only a few scientists have thus far conducted research on green tea seed (Choi et al., 2006a, Choi et al., 2006b, Hossain et al., 2002, Kim et al., 2008, Lim et al., 2006, Park et al., 2006a, Park et al., 2006b). Green tea seed extract is known to contain many biologically active compounds, including saponins, flavonoids, vitamins, oils, and kaempferol glycosides (Park

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et al., 2006b, Toshikazu et al., 1991).

Here, we describe a new natural anti-cancer candidate from green tea seed, which evidences profound inhibitory effects on cancer cell proliferation, angiogenesis, and cell adhesion.

MATERIALS AND METHODS

Preparation of green tea seed extract (GTSE)

First, the skin was peeled off the seeds of green tea, *Thea sinensis*. The seeds were then pulverized in a mortar and pestle, then homogenized with ethanol at a ratio of 4:6 (w/v). The homogenate was extracted using a heating mantle (Global Lab) for 5 hrs at 60°C. The suspension was filtered with paper filter (Whatman; No2) and membrane filter (Buonovino). The filtered extract was then concentrated in a digital water bath (SB-1000, Eyela), and lyophilized. The green tea leaf extract was prepared as described above.

Culture of HUVEC, U937, and cancer cell lines

Human umbilical vein endotherial cells (HUVEC) were purchased from Young Science (Seoul, Korea) and cultured in 2% gelatin (Sigma)-coated T75 flask (Cohesion, USA). The medium utilized for HUVEC culture was EBM-2 medium (Clotenics). Additional supplements added to the culture medium were as follows: hydrocortisone, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), ascorbic acid, heparin, and 2% fetal bovine serum (FBS). Cell culture was continuously conducted in a 5% CO_2 incubator at 37°C until it reached to 3-5 passages.

U937 human monocytic cell line (American Type Culture Collection, Rockville, MD) was used for the cell adhesion experiment. The cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) including L-glutamine (2 mM, Life Technologies), penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% FBS.

Four human cancer cell lines from stomach (SNU-719), liver (SNU-423), breast (HCC-1428), and uterus (SNU-1005) were obtained from the Korean Cell Line Bank. The cell lines were cultured in RPMI-1640 medium containing penicillin-streptomycin (100units/mL) and 10% FBS in a 5% CO_2 incubator at 37°C. The medium in the cell lines was exchanged with fresh medium 2-3 times per week. After a week, the cell lines were washed with physiological salt solution and were pooled using 0.05% trypsin-0.02% EDTA. The cells were collected via centrifugation (Combi-514R) and cultured continuously with fresh medium.

Toxic test against GTSE on HUVEC

The GTSE was added to HUVEC at various concentrations ($0.05\sim10.0\times10^{-3}$%) with 0.01% dimethyl sulfoxide (DMSO). After 24 hours, the cells were stained with 0.4% trypan blue, and live cells were counted using a leukocytometer under a microscope.

Antioxidant activity of GTSE

The antioxidant activity of GTSE was determined via the ferric reducing ability assay of plasma (FRAP) method (Szollosi and Varga, 2002). One unit of antioxidant capacity was designated as the equivalent antioxidant capacity of 1 mM Fe (II). EGCG (Wako, Japan) was applied as described above as a positive control.

Inhibitory effect of cancer cell proliferation, angiogenesis, and cell adhesion by GTSE

GTSE was added to four human cancer cell lines from the stomach (SNU-719), liver (SNU-423), breast (HCC-1428), and uterus (SNU-1005). The cells were washed twice with physiological salt solution and were pooled with 0.05% trypsin-0.02% EDTA. Trypsinized cells were collected via centrifugation. The live cells were counted using trypan blue solution and a leukocytometer.

HUVEC (2.5×10^4) was plated onto the Matrigel-coated wells of a 24-well plate (Matrigel, Cohesion). GTSE was added to the well, and cultured for 4 hrs in 5% CO_2 incubator at 37°C. The tube formation of HUVEC was photographed with a digital camera (Nikon, Coolpix 4500), and analyzed the tube length with NIH image software. As a control, EGCG and green tea leaf extract (GTLE) were applied in same manner as GTSE.

HUVEC cells were cultured to confluency in 24-well plates and were treated for 20 hrs with different concentrations of GTSE at 37°C. The cells were then stimulated for 6hrs with recombinant human IL-1β (5 ng/mL). The U397 cells (1×10^6 cell/mL) were seeded over the HUVEC monolayer and incubated for 20 hrs. Cell adhesion was verified by obtaining five random photographs from each well with a digital camera (Nikon, Coolpix 4500), and analyzed by counting the adherent U397 cells.

Immunoprecipitation and Western blot analysis

For the immunoprecipitation of signal molecules (Choi et al., 2006), HUVEC was seeded on 100-mm Petri dishes, and incubated in EBM2 medium with 2% FBS until confluence. GTSE was treated with different doses for 24 hrs and the cells were washed with serum-free EBM2 medium. Control and GTSE-supplemented cells were starved overnight, and then the cells were stimulated for 30 min with 50 ng/mL of recombinant human VEGF (BD Science, USA) at 37°C. In order to protect against protein phosphorylation, the cells were pretreated for the final 7 min prior to lysis with a dilution of 1:1,000 from an equal volume mixture of vanadate (100 μmol/L) and hydrogen peroxide (200 μmol/L) (Kim et al., 2006).

Cells were quickly rinsed with ice-cold PBS including 0.1 mM Na_3VO_4 and then solubilized on ice for 20 min with ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM vanadate, 1 mM EDTA, 1 mM FGTA, 0.2 mM PMSF, 0.5% NP-40) by a gentle side-to-side rocking motion. The cells were scraped and the lysates were centrifuged (14,000 rpm, 10 min, 4°C). The supernatants were collected and immunoprecipitated for 12 hrs with protein G-Sepharose coupled to the polyclonal antibody VE-cadherin (1 μg/mL) at 4°C. The immunoprecipitates were washed four times in lysis buffer containing 1 mM Na_3VO_4, then boiled in Laemmli's sample buffer. The samples were separated on 7.5% SDS-PAGE gel and transferred to 0.2 μm nitrocellulose membrane (Bio-Rad, USA). The membranes were then blocked with 5% skim milk in PBS containing 0.1% Tween-20 and probed for 1 hr at room temperature with either of the following antibodies: VEGFR2, PI3K, β-catenin, and VE-cadherin. Immunoreactive bands were visualized after 1 hr incubation using horseradish-peroxidase-conjugated antigoat antibodies, followed by Western Blotting Luminol reagent (Santa Cruz Biotechnology, USA)

Animal experiment

Human cancer cells (2×10^6 cells in 0.1 mL PBS) from stomach (SNU-719), liver (SNU-423), breast (HCC-1428), and uterus (SNU-1005) were injected subcutaneously into the dorsal part of 6-week-old female nude mice. Cancer tissues were produced for 2 weeks incubation. 5 μg GTSE per body weight (g) was injected into the abdominal cavities of the mice daily for 4 weeks, and injected directly into the cancer tissue once per week.

RESULTS

Toxic test against GTSE on HUVEC

According to the results of our toxic test against GTSE on HUVEC, we observed no significant changes as compared to those of other GTSE concentrations up to 0.01% (Fig. 1). Over 90% of the HUVEC cells survived after the addition of 0.01% GTSE. The toxicity of a compound occurs via the uptake of the compound into the cell or via interaction with the cell membrane and associated molecules. Our result on the cytotoxicity of GTSE tested in this *in vitro* cell culture system shows that at least 0.01% added GTSE is not harmful to cell culture.

Determination of antioxidant ability

Antioxidants have been shown to inhibit the interaction of immune-endothelial cells' (Faruqi et al., 1994), which is mediated via the endotherial expression of several cell surface adhesion molecules and ligands from monocytes

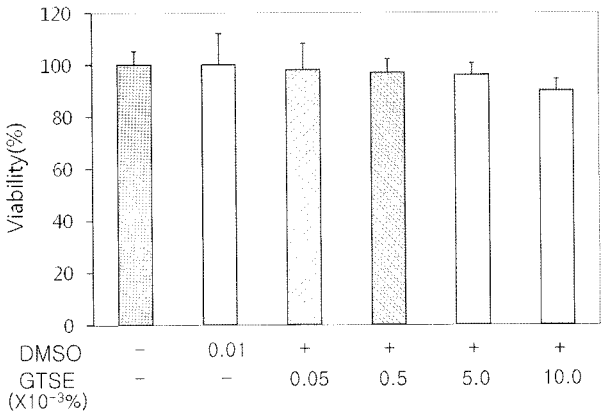

Fig. 1. Toxicity test on HUVEC cells against GTSE with or without DMSO and GTSE. Data is expressed as the mean±standard deviation of three experiments.

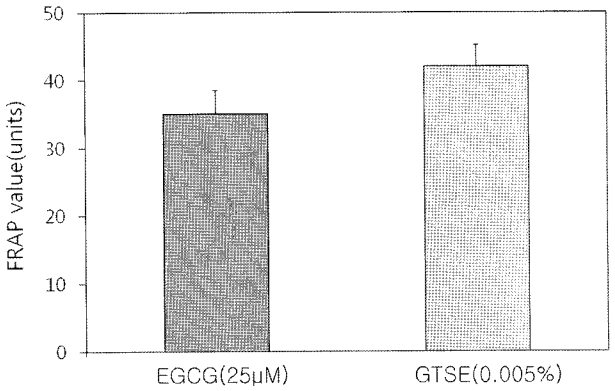

Fig. 2. Antioxidant activity of 0.005% GTSE and 25 μM EGCG. Data is expressed as the mean±standard deviation of three experiments.

during inflammation and atherosclerosis (Meydani, 1998). As shown in Fig. 2, the antioxidant ability of GTSE is 1.2 times higher than that of EGCG, which is the most potent antioxidant and anticancer compound in the green tea leaf (Jankun et al., 1997). EGCG evidences profound antioxdative capacity, high affinity for the lipid bilayer of the cell membrane, and can easily enter the nuclei of cancer cells (Okabe et al., 1997). Moreover, the oxygen-scavenging effects of EGCG are superior to those of ascorbic acid (vitamin C) and tocopherol (vitamin E) with respect to some active oxygen radicals, but are less pronounced with hydroxyl free radicals (Zhao et al., 1989). Thus, GTSE a similar or superior function EGCG which inhibit tumor cell invasion by scavenging oxygen radicals.

Inhibitory effect of cancer cell proliferation by GTSE

Figure 2 shows inhibitory effect of cancer cell lines from stomach (Fig. 3-1), liver (Fig. 3-2), breast (Fig. 3-3), and uterus (Fig. 3-4), indicating gradual decreases with increases in GTSE concentration. A significant inhibitory effect was noted on the cancer cell lines with an addition of 0.01% GTSE, in which over 90% of the HUVEC cells

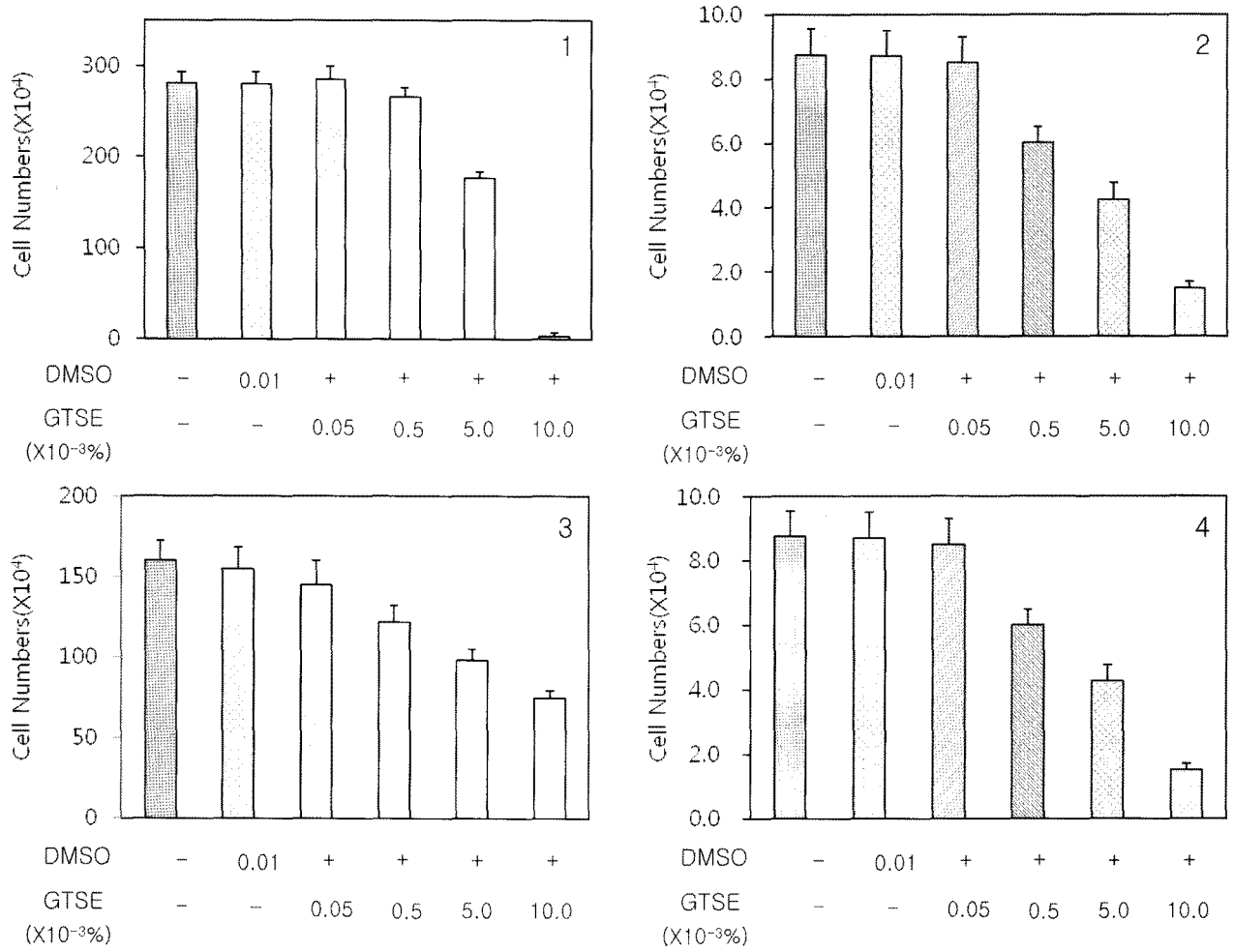

Fig. 3. Inhibitory effect of GTSE on cancer cell lines from stomach (1), liver (2), breast (3), and uterus (4). Data is expressed as the mean±standard deviation of three experiments.

survived. In particular, stomach cancer cells were largely suppressed with an addition of 0.01% GTSE. The data indicated that GTSE should be the most effective on stomach cancer cells (Fig. 3-1). Moreover, all cancer cell lines became stressed, were morphologically lengthened, and were finally removed by increasing the GTSE concentration (data not shown).

Inhibitory effect of angiogenesis

The quantitative analysis of tube formation by endothelial cells on Matrigel was conducted by determining the tube lengths of five photographs obtained from the random field of cell cultures in each well. We assessed the maximum safe and nontoxic doses (0.5 LD50) of GTSE for its anti-angiogenic effect. Fig. 4 shows the differential suppression of GTSE on tubular structure formations in VEGF-stimulated HUVEC on Matrigel. As it was determined earlier that EGCG inhibits angiogenesis (Tang and Meydani, 2001), we used 25 μM EGCG as a positive control, which inhibited angiogenesis by 86.9%. Our results demonstrated that at a maximum safe dose of 0.005%, the green tea leaf inhibited angiogenesis by 20.0%, and was as effective as 25 μM of EGCG for the inhibition of *in vitro* angiogenesis, whereas the maximum safe dose of 0.005% GTSE significantly suppressed the HUVEC angiogenesis by 92.0%.

It was assumed that the low molecular weight of GTSE would inhibit the interaction of immune and endothelial cells, which are mediated via the endothelial expression of cell surface adhesion molecules and ligands, thereby as receptor molecules (Faruqi, 1994).

Inhibitory effect of U397 cell adhesion to IL-1β-stimulated HUVEC

As shown in Fig. 5, the monocyte-like U397 cells did not adhere to unstimulated HUVEC cells to a significant degree. However, U397 adhesion rate increased by up to 4-fold when HUVEC was stimulated with IL-1β. The stimulated adhesion was clearly inhibited by GTSE addition, and this effect occurred in a dose-dependent manner. GTSE at 5, 12.5, 25, and 50×10^{-5}% suppressed

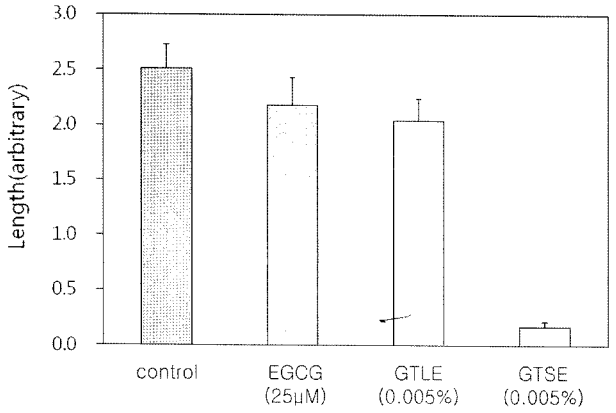

Fig. 4. Inhibitory effect on angiogenesis of 25 μM EGCG, 0.005% GTLE, and 0.005% GTSE. Data is expressed as the mean± standard deviation of three experiments.

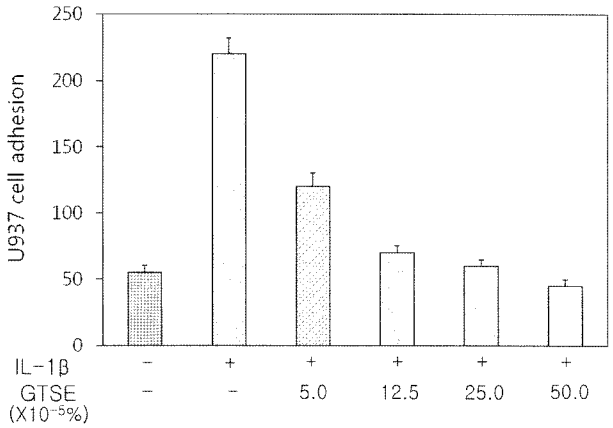

Fig. 5. Inhibitory effects on cell adhesion of GTSE. Data is expressed as the mean±standard deviation of three experiments.

U937 cell adhesion by 50.0, 94.5, 100.0, and 105.5%, respectively. This result showed that GTSE exerts an inhibitory effect on cancer cell metastasis, and also evidences an angiogenesis inhibition effect.

Western blot of signal molecules for GTSE

In order to determine the GTSE inhibition of tubular formation in HUVEC via the suppression of the signal pathways of VEGFR2, PI3K, β-catenin, and VE-cadherin, the expression of signal molecules was evaluated using anti-signal molecule antibodies including anti-VEGFR-2, anti-PI3K, anti-β-catenin, and anti-VE-cadherin, respectively. As shown in Fig. 6, VEGF effected a significant increase in the number of signal molecules, and with pre-supplementation of HUVEC cells with dose-dependent dosages of 25 and 50 $\times 10^{-5}$%, GTSE inhibited the expression of all four types of signal molecules. As GTSE suppressed the pathways from four kinds of signal molecules to NF-κB, angiogenesis was decreased. Specifically, VE-cadherin is a crucial molecule in cell-cell recognition and also strengthen cell contact during vascular morphogenesis (Tang et al., 2003).

Recently, Carmelit et al (1999) reported that VE-cadherin molecules are also involved in VEGF signaling for vascular endothelial cell survival and proliferation in angiogenesis. VE-cadherin and its associated molecule, β-catenin, cross-link chemically with VEGFR2-PI3 kinase and support the survival of endothelial cells and the development of new capillaries. VEGF, which has been demonstrated to be a

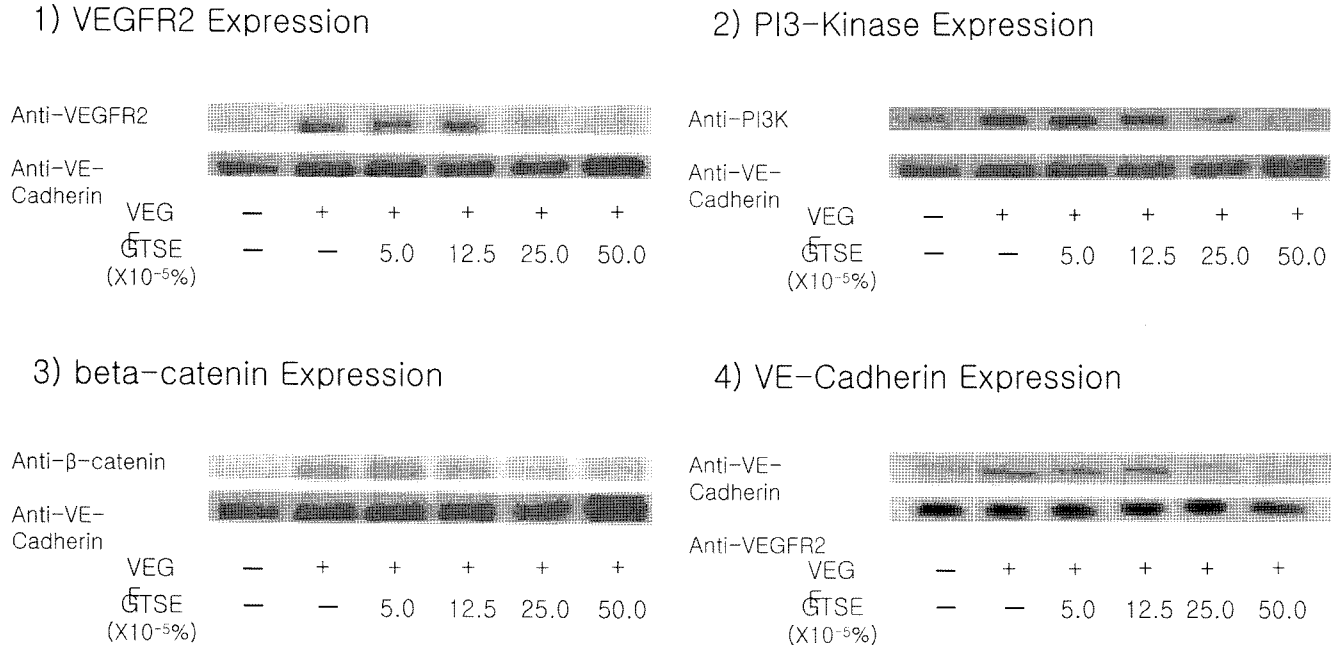

Fig. 6. Effects of GTSE on interaction of VE-cadherin with VEGFR-2, PI3-kinase, and β-catenin upon cell activation with VEGF. HUVEC cell extracts were immunoprecipitated with VE-cadherin antibodies (1-3) and immunoblotted with antibodies to VEGFR2, PI3-kinase, and β-catenin, IP with VEGFR2 (4) and IB, VE-cadherin.

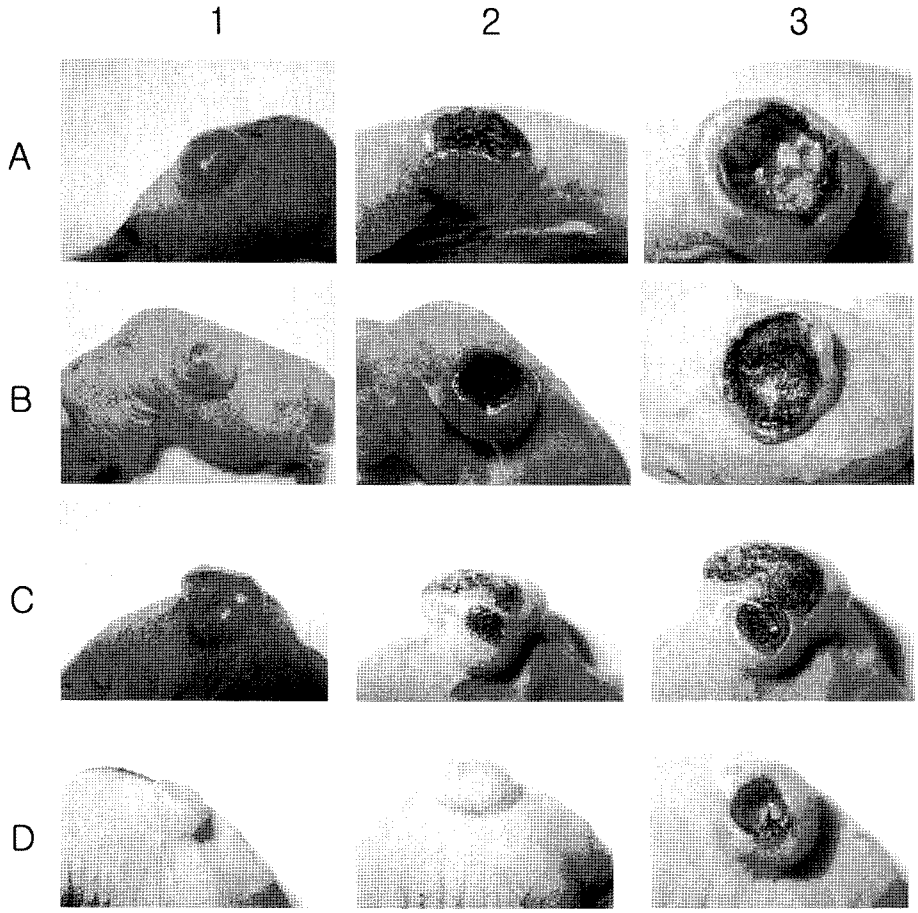

Fig. 7. Animal experiment involving injection of cancer cells from stomach (A), liver (B), breast (C), and uterus (D). The tissues were produced for 2-weeks time intervals for the first 2 weeks (1), the second 2 weeks (2), and the third 2 weeks (3) after injection of each cancer cell.

major factor in tumor growth and other growth factors produced from tumor cells and blood vessel cells, performs a crucial function in the expansion of the microvascular network required to supply oxygen and nutrients for the rapid growth of tumor masses (Folkman and Shing, 1992).

In vivo animal experiment

Cancer tissues of stomach (Fig. 7-A), liver (Fig. 7-B), breast (Fig. 7-C), and uterus (Fig. 7-D) in nude mice were produced for 2 weeks incubation (Fig. 7-1). The tumors began to become necrotic a week after injecting the GTSE into the nude mice (Fig. 7-2). These results show that the provision of nutrients to the tumor was blocked by GTSE. Moreover, the tumors became completely necrotic 4 weeks later (Fig. 7-3). The GTSE attacked cancer cells selectively, but did not attack any other normal tissues.

DISCUSSION

In the present study, we have assessed the effects of the GTSE on the biological functions *in vitro*: anti-angiogenesis, immune-endothelial cell adhesion, and *in vivo* anti-cancer functions in an animal model. Since we first determined the dose-response relationship of the GTSE on the viability of HUVEC cells, we attempted to determine the maximum safe and nontoxic dose in the context of HUVEC angiogenesis. We determined that the maximum safe and nontoxic dose (0.5 LD50) of GTSE at 0.005% in a cell culture system suppressed VEGF-induced angiogenesis, which was comparable to the effect of 25 μM of EGCG. It is important to note that during angiogenesis, in addition to cell migration and tube formation, the proliferation of endothelial cells is a necessary step. We determined that the proliferation of endothelial cells as observed in our *in vitro* cell culture system is affected principally by GTSE, which may have contributed to its efficacy on the inhibition of angiogenesis significantly more than was observed with EGCG and green tea leaf. It is important to note that the "maximum safe dose" of the GTSE utilized in this study was based on the viability of cells in culture, and should not have directly occurred *in vivo*. However, the results of our animal experiment have shown that GTSE can inhibit tumor growth *in vivo*, possibly by inhibiting the formation of new blood vessels. These findings may partially explain the antineoplastic effects associated with the injection of GTSE. A complete knowledge of the molecular mechanism

or mechanisms involved with the anti-tumor efficacy of GTSE may be useful in the development of better strategies for cancer therapy.

The intestinal degradation, absorption and metabolism of the GTSE occur before reaching the targeted tissues. Nevertheless, the information from this study provides a basis for the exploration of the molecular mechanism of action and the potential applications of this compound via different administration routes to target specific tissues in which angiogenic and atherosclerotic inhibition are required. Further research will be to identify more effective compounds in GTSE bearing the anti-angiogenesis effect than that of EGCG in the green tea leaf.

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