Inhibitory effect of epigallocatechin-gallate on brain tumor cell lines in vitro

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We investigated the effect of epigallocatechin-gallate (EGCG), the main constituent of green tea polyphenols, on human glioblastoma cell lines U-373 MG and U-87 MG, rat glioma cell line C6, and rat nonfunctioning pituitary adenoma cell line MtT/E. Cell viability was determined by assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and the extent of apoptosis was studied by flow cytometric analysis. Apoptosis was also characterized by morphology using fluorescent microscopy. The role of insulin-like growth factor-I (IGF-I) was studied by assay with MTT, immunohistochemistry, and immunoradiometric assay. After 72-h exposure, a statistically significant loss of viability ($P = < 0.0001$) was observed at concentrations of 12.5, 25, 50, and 100 μg/ml in U-373 MG cells and U-87 MG cells. EGCG at concentrations of 50 μg/ml and higher significantly reduced the viability of C6 cells. EGCG inhibited viability of MtT/E cells only at a concentration of 100 μg/ml. Quantitative study by flow cytometry demonstrated that lower doses of EGCG (12.5, 25, 50 μg/ml) induced apoptosis in U-373 MG, U-87 MG, and C6 cells; however, only the highest dose (100 μg/ml) induced apoptosis in MtT/E cells. Compared with other cell lines, MtT/E cells showed stronger IGF-I immunoreactivity. Neutralization of IGF-I with an antihuman IGF-I antibody reduced viability of the cell lines. It can be concluded that EGCG has an inhibitory effect on malignant brain tumors, and IGF-I may be involved in the effects of EGCG. *Neuro-Oncology 3, 22–28, 2001 (Posted to Neuro-Oncology [serial online], Doc. 00-026, October 17, 2000. URL <neuro-oncology.mc.duke.edu>)*

Most tea consumed in the world can be classified as green or black. Drinking green tea is an integral part of the Japanese lifestyle. It has been consumed for centuries in Japan, and it has no adverse effects. The inhibitory effects of green tea on carcinogenesis have been investigated in different kinds of cancers. Epidemiologic studies have revealed that cancer incidence is lower in subjects who drink green tea than in those who consume none (Fujiki et al., 1998; Imai et al., 1997; Nakachi et al., 1998). A negative association was reported between green tea consumption and cancer incidence among women drinking more than 10 cups a day (Imai et al., 1997). Consumption of green tea was significantly associated with improved prognosis of breast cancer (Nakachi et al., 1998). These results indicate that green tea can be a potentially valuable agent in tumor prevention (Ahmad et al., 1997).

Green tea contains 4 major polyphenolic compounds: EGCG,[2] epicatechin-gallate, epigallocatechin, and epicatechin (Katiyar and Mukhtar, 1996). The anticarcinogenic effect of EGCG, the major polyphenolic agent, was first found on mouse skin in a 2-stage carcinogenesis experiment (Yoshizawa et al., 1987). The effects of green tea on brain tumors have been investigated, but they have not been clearly elucidated to date (Ahn et al., 1999; Serenelli et al., 1997).

Studies of IGF-I have demonstrated its important role in stimulating tumor cell proliferation and in preventing

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[2]Abbreviations used are as follows: EGCG, epigallocatechin-gallate; IGF-I, insulin-like growth factor-I; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline.

apoptosis (Hirano et al., 1999; Lamm and Christofori, 1998; Singleton et al., 1996; Yokoyama et al., 1998). However, data on the role of IGF-I in EGCG's inhibitory effects on tumors are scant.

In this paper, we document the effect of EGCG, the main constituent of green tea polyphenols, in 4 tumor cell lines including 3 gliomas and a nonfunctioning pituitary adenoma. The mechanism of EGCG-induced cell death was also investigated.

Materials and Methods

Cell Culture and Chemicals

The cells—human glioblastoma cell lines U-373 MG and U-87 MG, rat glioma cell line C6, and rat nonfunctioning pituitary adenoma cell line MtT/E—were used for this study. The MtT/E cells were provided by Drs. Nariaki Fujimoto and Akihiro Ito (Research Institute of Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan). Cell lines U-373 MG, U-87 MG, and C6 were cultured in RPMI-1640 medium (Nikken Bio Medical Lab., Kyoto, Japan) supplemented with 10% fetal bovine serum (GIBCO/BRL, Gaithersburg, Md.) and 2% penicillin-streptomycin (GIBCO/BRL). MtT/E cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (Sigma, St. Louis, Mo.) with 8% horse serum (GIBCO/BRL), 2% fetal bovine serum, and 2% penicillin-streptomycin. Epigallocatechin-gallate (Funakoshi, Tokyo, Japan) was used as a green tea polyphenol in this study. All other chemicals were analytical grade.

Cell Viability

For the study of cell viability, 5×10^3 cells were plated in each well of a round-bottom 96-well culture plate and incubated for 24 h. After 24-, 48-, and 72-h exposure to EGCG (12.5-, 25-, 50-, and 100-μg/ml doses), the cells were treated with 10 μl MTT and were incubated for 3 h. To dissolve the cells, we used 100 μl of 10% sodium dodecyl sulfate and measured the resulting solution's absorbance at 570 nm by microplate reader. The experiments were run 3 times during which 6 samples from each of the doses were examined each time. The viability was determined as the percentage of absorbance of EGCG-treated cultures compared with those of untreated control cultures. To evaluate the difference of cell viability among the various groups, we applied the one-factor ANOVA test and examined all possible pairings by Scheffe's F test.

An antihuman IGF-I polyclonal antibody (R & D Systems, Minneapolis, Minn.) was added to U-87 MG and MtT/E cells at a concentration of 10 μg/ml to neutralize the effect of IGF-I produced by the tumor cells. After 72 h of EGCG treatment (12.5-, 25-, and 50-μg/ml doses), viability was determined by MTT assay. The difference in cell viability between the anti–IGF-I antibody treatment and the control (EGCG alone) was statistically evaluated by factorial ANOVA test.

Quantification of Apoptosis by Flow Cytometry

The tumor cells were grown at a density of 5×10^5 cells in 100-mm culture dishes and were treated with EGCG (0-, 25-, 50-, and 100-μg/ml doses) for 24 h. The cells were then collected and washed by phosphate-buffered saline containing 10 mM EDTA and 0.1% bovine serum albumin, fixed in 70% ethanol, resuspended in phosphate-buffered saline containing 50 μg/ml RNase, and stained with propidium iodide at a concentration of 5 μg/ml. DNA content, as measured by propidium-iodide staining, was evaluated using a EPICS XL Flow Cytometer (Beckman Coulter, Miami, Fla.).

Morphological Study by Fluorescent Microscopy

For fluorescent microscopic observation, cells were cultured in 2-well chamber slides and were treated with EGCG for 24 h. Slides were rinsed 3 times with phosphate-buffered saline, incubated at room temperature for 30 min in the Hoechst 33258 (Molecular Probes, Eugene, Ore.), rinsed 3 times, and mounted with aqueous mounting medium (Immunon, Pittsburgh, Pa.). Apoptosis was characterized by morphologic changes, such as chromatin condensation, nuclear condensation, and formation of apoptotic bodies. Two hundred nuclei were counted and the percentage of apoptotic cells was determined in EGCG-treated cells.

Immunohistochemistry

The tumor cells were cultured on chamber slides, fixed with 10% formalin, and immunostained using the IGF-I antibody (Upstate Biotechnology, Lake Placid, N.Y.). Immunostaining was performed by the avidin-biotin-peroxidase complex method (Hsu et al., 1981). After routine blockage of endogenous peroxidase activity, the slides were incubated for 3 h at room temperature with IGF-I monoclonal antibody. Antigen–antibody complexes were detected with the 3,3′-diaminobenzidine/H_2O_2 reaction and counterstained with Mayer's hematoxylin.

Measurement of IGF-I in Culture Medium

Tumor cells were cultured in several dishes (5×10^5 cells/culture dish) for 72 h, and the IGF-I level for each dish was measured by an immunoradiometric kit (somatomedin C II Chiron; Yuka Medias, Ami, Japan). Five samples of each cell line were measured. IGF-I was extracted with extract solution containing HCl/MeOH and mixed with radiolabeled IGF-I antibody. Antibody-coated beads were added to the mixture and incubated for 2 h at room temperature. The radioactivity of the beads was counted with a γ-counter. The cross-reactivity of IGF-II in the immunoradiometric IGF-I mixture was less than 0.1%.

Results

The effect of EGCG at doses of 12.5, 25, 50, and 100 μg/ml on viability of the cell lines by MTT assay is shown

in Fig. 1. Data shown are from a representative experiment repeated 3 times with similar results. The treatment duration was 72 h. A remarkable loss of viability in U-373 MG cells was observed at concentrations of 12.5, 25, 50, and 100 μg/ml (Fig. 1A). All possible comparisons between various dose levels were statistically significant ($P < 0.0001$) except between the 12.5- and 25-μg/ml dose levels. The results of EGCG doses on U-87 MG cells were also compared, and all possible pairings were statistically significant ($P < 0.0001$) (Fig. 1B). With an increase in concentration levels, U-373 MG and U-87 MG cells showed a remarkable loss of viability. In C6 cells (Fig. 1C), the dose levels of 25 μg/ml and lower were statistically significant compared with the dose levels of 50 or 100 μg/ml ($P < 0.0001$). The 50- and 100-μg/ml results were also significant ($P < 0.0001$). EGCG reduced viability on MtT/E cells (Fig. 1D) at a concentration of 100 μg/ml compared with 50 μg/ml and lower ($P < 0.0001$).

As shown in Fig. 2, 50 μg/ml of EGCG significantly reduced cell viability in U-373 MG after 24, 48, and 72 h. Comparisons between all the time points were statistically significant (24 h versus 48 h, $P < 0.05$; others, $P < 0.0001$).

The extent of apoptosis was quantified by flow cytometric analysis of propidium iodide–labeled cells. EGCG doses of 0, 25, 50, and 100 μg/ml for 24 h induced 8.22%, 35.0%, 54.2%, and 59.7%, respectively, of apoptotic cells in the U-373 MG cell line (Fig. 3); 13.1%, 13.4%, 21.3%, and 29.5% in the U-87 MG line; 8.7%, 20.2%, 27.8%, and 36.8% in the C6 line; and 13.6%, 7.2%, 7.6%, and 33.5% in MtT/E. Although lower doses of EGCG induced apoptosis in U-373 MG, U-87 MG, and C6 cells, only the highest dose (100 μg/ml) induced apoptosis in MtT/E cells.

The induction of apoptosis by EGCG was confirmed morphologically by fluorescent microscopy. This method can identify cells in the early phase of apoptosis, which we observed 24 h after EGCG treatment. The control cells showed no changes in nuclear morphology (Fig. 4A). However, with a 50-μg/ml dose of EGCG, condensation and fragmentation of nuclei in brain tumor cells,

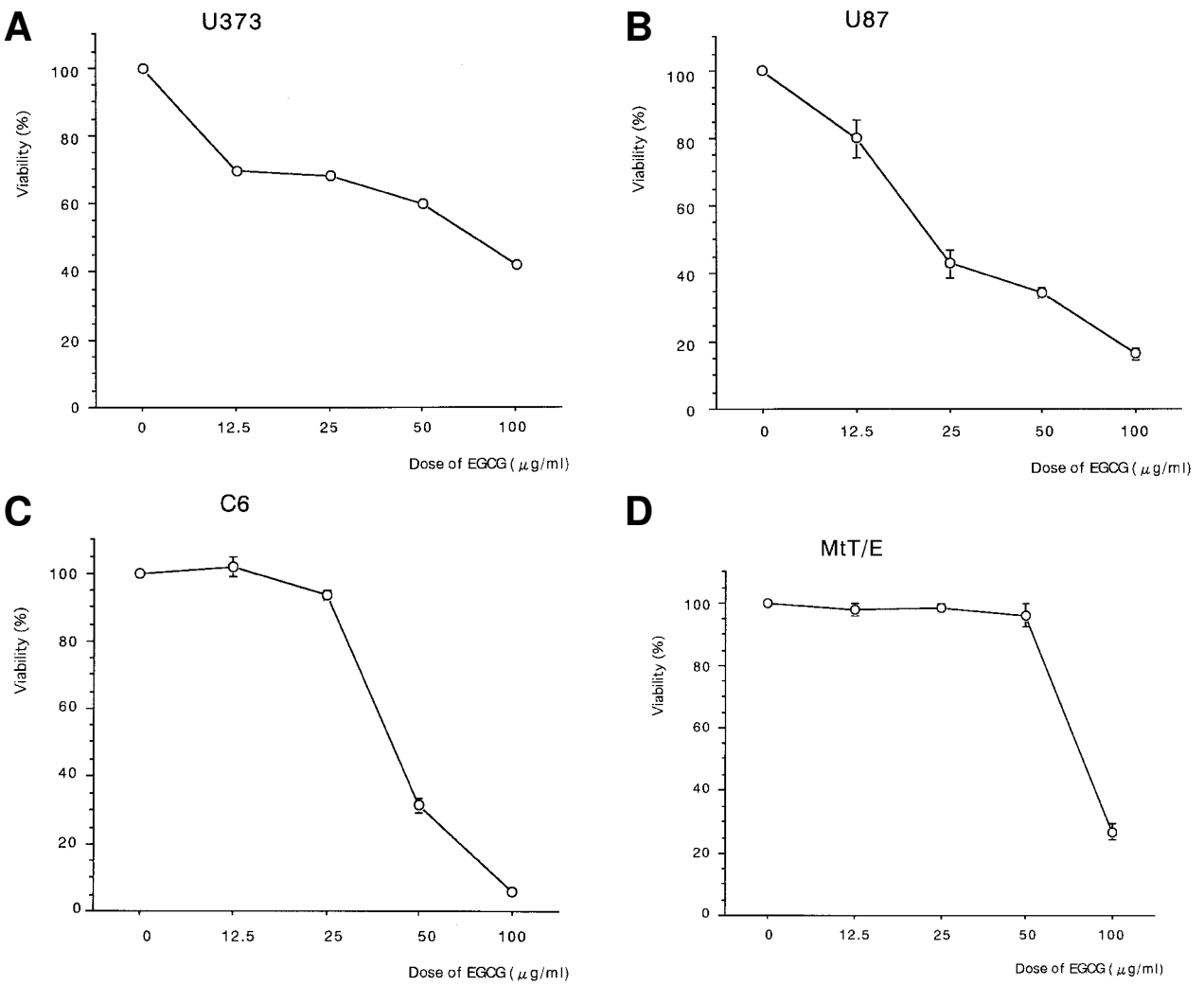

Fig. 1. Viability of brain tumor cell lines as determined by MTT assay after 72-h treatment. A. Remarkable loss of viability at EGCG concentrations of 12.5, 25, 50, and 100 μg/ml in U-373 MG cells ($P < 0.0001$). B. At an EGCG concentration of 12.5 μg/ml and higher, significantly reduced viability in U-87 MG cells ($P < 0.0001$). C. A significant loss of viability ($P < 0.0001$) at 50 μg/ml and 100 μg/ml of EGCG in C6 cells. D. Significantly inhibited viability only at EGCG concentration of 100 μg/ml ($P < 0.0001$) in MtT/E cells. No suppressing effect was observed at lower concentrations. Each value is the mean of 6 samples; error bars are ±SE.

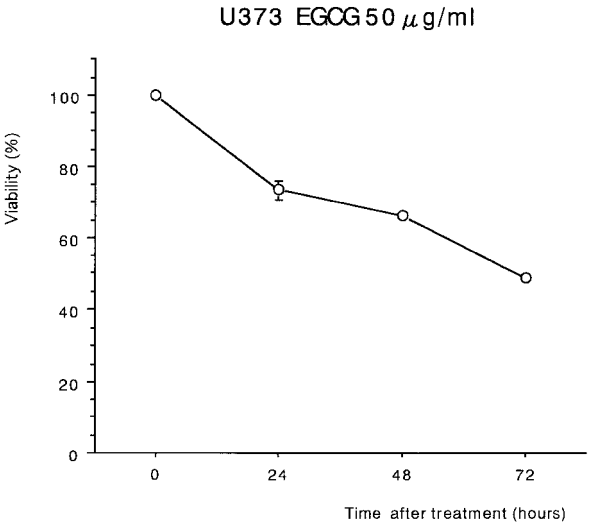

Fig. 2. At a concentration of 50 μg/ml, EGCG significantly reduced U-373 MG cell viability after 24, 48, and 72 h. All pairings of the various time points were statistically significant (24 h vs. 48 h, $P < 0.05$; others, $P < 0.0001$). Each value is the mean of 6 samples; error bars are ±SE.

indicating apoptosis, were evident (Fig. 4B). The number of apoptotic cells was counted, and the percentage was 51.6% with the 50-μg/ml dose of EGCG.

IGF-I levels in the culture media of U-373 MG, U-87 MG, C6, and MtT/E cells were 14.8 ± 2.2 ng/ml, 13.3 ± 0.4 ng/ml, 18.5 ± 0.6, and 19.5 ± 1.4 ng/ml (mean ± SE), respectively. MtT/E cells, which were resistant to EGCG, released the highest amount of IGF-I, followed by C6, U-373 MG, and U-87 MG. This result was consistent with that of cell viability in MTT assay. Immunohistochemical analysis showed strong positive IGF-I immunoreactivity in MtT/E cells. Although the other 3 cell lines also expressed IGF-I, immunostaining was not as strong as in MtT/E cells (Fig. 5). The addition of antihuman IGF-I polyclonal antibody to U-87 MG cells ($P < 0.005$) and MtT/E cells ($P < 0.0001$) significantly reduced the viability of the cells with EGCG treatment compared with EGCG alone as assessed by the factorial ANOVA test (Fig. 6A and B). The effect was much more noticeable in MtT/E cells. The cell viability after treatment with antihuman IGF-I antibody alone in U-87 MG and MtT/E cell lines was 102.7 ± 1.4% and 100.3 ± 1.5% (mean ± SE), respectively.

Discussion

Our results indicate that the green tea polyphenol EGCG can affect growth of gliomas. The antitumor effects of EGCG were heterogeneous in the 3 glioma cell lines. By MTT assay, U-373 MG and U-87 MG cells showed a higher sensitivity to EGCG than did C6 cells. The pituitary adenoma cell line MtT/E was more resistant to EGCG treatment than was the glioma cell lines. The MtT/E cell line was established from an estrogen-induced mammotropic tumor and secreted no pituitary hormones (Inoue et al., 1990). Ahmad et al. (1997) reported that EGCG caused apoptosis only in malignant tumor cell lines and did not affect normal cells. These results suggest that EGCG inhibits cell proliferation in malignant, highly proliferative brain tumors, but has no effect on normal cells or benign tumor cells. EGCG, the main constituent of green tea polyphenols, may be useful in the management of patients with malignant brain tumors.

Green tea is a widely consumed beverage worldwide. In both laboratory experiments and human epidemiologic studies, it promotes cancer prevention effects (Ahmad et al., 1997; Fujiki et al., 1998; Imai et al., 1997). However, it is not well documented whether the protective effect of green tea can be observed in vivo. For an in vivo study, the relationship between tea polyphenol (EGCG) ingestion and its level in plasma should be clarified. Lee et al. (1995) report that after 4 human volunteers ingested 1.2 g of decaffeinated green tea in warm water, plasma samples collected 1 h later contained 46 to 268 ng/ml of EGCG. The effective dose of green tea for cancer prevention is more than 10 cups per day as demonstrated by epidemiological studies (Imai et al., 1997), corresponding to 2.25 g of green tea extract or 337.5 mg EGCG (Suga et al., 1998). Our in

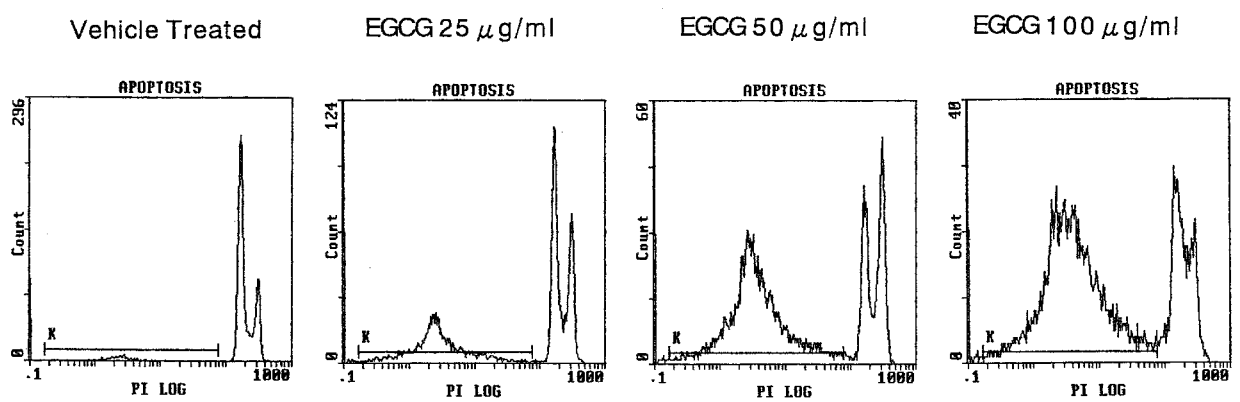

Fig. 3. U-373 MG cells treated with EGCG, stained with propidium iodide, and analyzed by flow cytometry. Vehicle treatment and EGCG treatments (0, 25, 50, and 100 μg/ml for 24 h) resulted in 8.22%, 35.0%, 54.2%, and 59.7% apoptotsis in cells, respectively. The left peaks showing the apoptotic fraction indicate the dose dependence of the apoptosis-inducing effect of EGCG on U-373 MG cells.

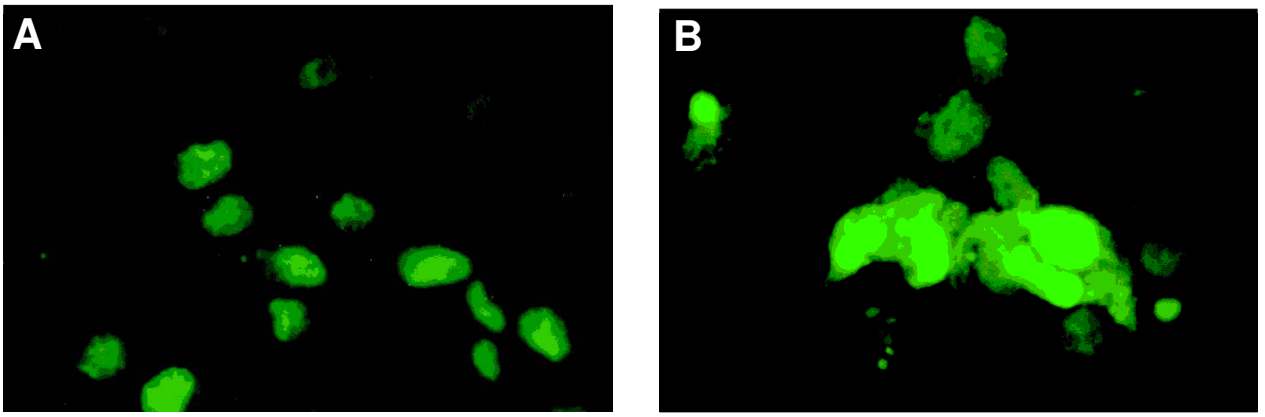

Fig. 4. Morphologic changes in U-373 MG cells shown by fluorescent microscopy. A. The control cells showing no changes in nuclear morphology. Original magnification X400. B. Condensation and fragmentation of nuclei evident after 50 μg/ml of EGCG treatment. Original magnification X400.

vitro data suggest that EGCG should be present in the tumor at concentrations of approximately 12.5 μg/ml or higher to cause apoptosis in brain tumor cells, and the plasma level needed to achieve this level in tumors would be higher. Similar doses are required to obtain inhibition on other cancer cells (Ahmad et al., 1997). It appears that for treating brain tumors, green tea extract or EGCG should be given in much higher doses than the amount obtained from usual dietary ingestion. EGCG may be useful in combination therapy with other chemotherapeutic agents. More studies are required to clarify whether single ingestion of EGCG is effective or whether long-term dietary consumption is necessary to prevent brain tumors in vivo.

The mechanism of cell death by chemotherapeutic agents currently used in cancer therapy is controversial (Ahmad et al., 1997; Dunn et al., 1997). Induction of apoptosis may be preferable in cancer management compared with necrosis. Our results on brain tumor cells and previous reports on other cancers have shown that EGCG inhibits cancer growth by inducing apoptosis (Ahmad et al., 1997; Watanabe et al., 1998). Conversely, several tumor promoters, including IGFs, have been shown to inhibit apoptosis (Dunn et al., 1997). IGFs appear to have a role as stimulators of tumor cell proliferation: they can protect tumor cells from apoptosis (Hirano et al., 1999; Lamm and Christofori, 1998; Singleton et al., 1996; Yokoyama et al., 1998). Dunn et al. (1997) showed that lower serum IGF-I concentration improves the efficacy of chemotherapeutic drugs for the treatment of breast cancer. However, the effect of these growth factors on EGCG has not yet been reported. The present study shows that MtT/E cells, which are resistant to EGCG, release the highest levels of IGF-I in the culture medium and express the strongest IGF-I immunostaining. IGF-I and IGF-I mRNA were detected in almost all types of pituitary adenomas (Yokoyama et al., 1997). The addition of antihuman IGF-I antibody accelerates the inhibitory effect of EGCG in the tumor cell lines, especially in MtT/E. IGF-I may be involved in the effects of EGCG on brain tumor growth by suppressing apoptosis, and it also may be responsible for the lack of a dose response curve with EGCG inhibition of MtT/E cells. Additionally, there may be a dose response curve between 50 μg/ml and 100 μg/ml of EGCG.

Another possible mechanism of the cancer inhibitory effect of EGCG is its effect on lipid metabolism. An aqueous methanol extract taken from green tea and containing EGCG shows potent acetyl-CoA carboxylase inhibitory activity (Watanabe et al., 1998). Acetyl-CoA carboxylase is considered to be the rate-limiting step in lipogenesis and appears to play an important role in the

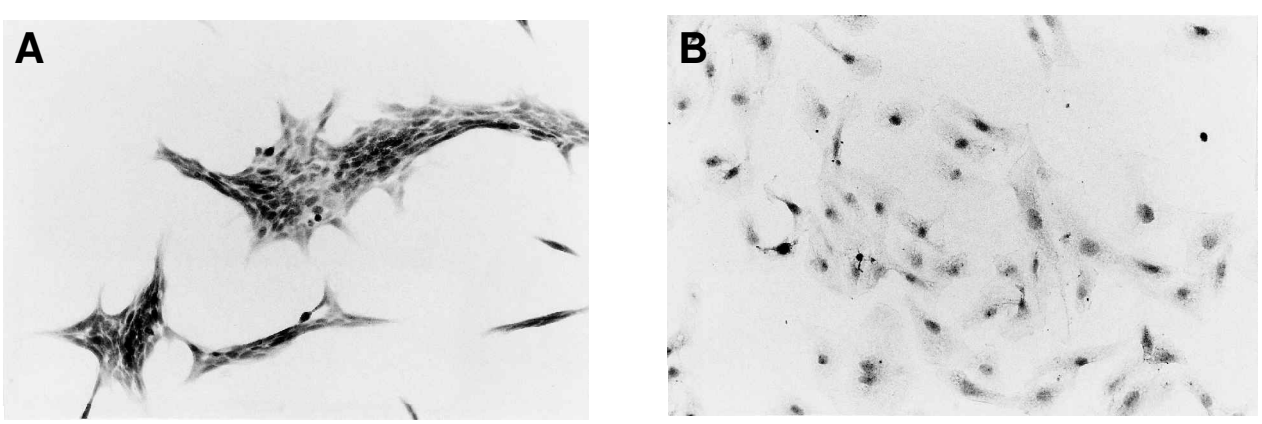

Fig. 5. IGF-I reactivity in MtT/E (A) and U373 MG (B). Strong IGF-I immunoreactivity is shown in MtT/E, whereas U-373 MG cells express weak immunoreactivity. Original magnification X200.

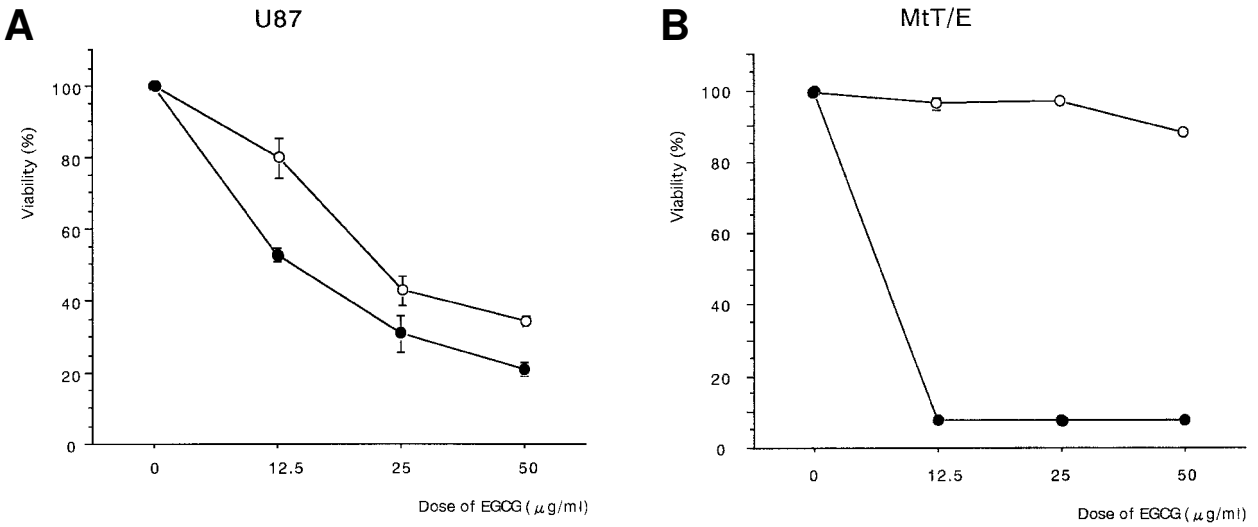

Fig. 6. A. Accelerated inhibitory effect of EGCG by antihuman IGF-I antibody in the U-87 MG cell line. Addition of an antihuman IGF-I antibody significantly reduced the viability of cells with EGCG treatment ($P < 0.005$ by factorial ANOVA) compared with EGCG alone. The error bar is ±SE. B. More noticeable inhibitory effect in MtT/E cell line ($P < 0.0001$ by factorial ANOVA). (○) EGCG alone; (●) EGCG and antihuman IGF-I antibody. Each value is the mean of 6 samples; error bars are ±SE.

maintenance of cell activity (Volpe and Vagelos, 1976). Enhanced synthesis of long-chain fatty acids, which are necessary for tumor proliferation, occurs in many types of cancer and predicts a poor prognosis (Moncur et al., 1998). It has been demonstrated that IGF-I stimulates the activity of acetyl-CoA carboxylase (Flint and Vernon, 1998), which may mediate the effects of both EGCG and IGFs. Further research is necessary to elucidate the role of IGFs in the effects of EGCG.

The present study suggests that green tea polyphenol (EGCG) can be a potentially useful compound for brain tumor prevention. EGCG may inhibit malignant brain tumor cells through apoptosis. IGF-I might play a role in the effects of EGCG on brain tumor cells as well. Further research, especially in vivo studies, are necessary to elucidate the value of EGCG in brain tumor management.

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