Ellagic Acid Enhances the Antitumor Efficacy of Bevacizumab in an In Vitro Glioblastoma Model

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BACKGROUND: **The anticarcinogenic effect of ellagic acid (EA), a natural phenol of fruits and vegetables, has been investigated in several types of tumors. The combined effect of EA with bevacizumab (BEV), a common drug used in treatment of recurrent glioma, on glioblastoma has not been reported. This study observed the combined effect of EA with BEV on the expression profile of the C6 glioma cell line.**

METHODS: **Rat C6 glioma cells were treated with EA at 100 μmol/L concentration in combination with BEV at 100 ng/mL concentration for 24, 48, and 72 hours. Cell proliferation was detected by 5-bromo-2′-deoxyuridine immunohistochemistry, and p53 and caspase-3 protein levels were determined by immunohistochemistry and assessed by the H-Score. Expression profiles for P-glycoprotein (*MDR1*), O^6-methylguanine DNA methyltransferase (*MGMT*), caspase-3, and p53 related proteins were detected by reverse transcriptase polymerase chain reaction after EA treatment with or without BEV.**

RESULTS: **EA combined with BEV conspicuously reduced the cell viability of C6 glioma cells for all incubation times. EA significantly downregulated expression of *MGMT* regardless of combination with BEV even in the early hours after treatment. Combined EA and BEV reduced *MDR1* expression only at 72 hours. EA affected the apoptotic proteins of p53 and caspase-3 at protein level in a time-dependent manner, but not at gene level.**

CONCLUSIONS: **This study suggests successful antiproliferative efficacy of EA combined with BEV, probably through inhibition of *MGMT* expression and time-dependent inhibition of *MDR1*. EA combined with BEV may be an alternative treatment for drug-resistant gliomas.**

INTRODUCTION

Glioblastoma is a common aggressive and malignant tumor of the central nervous system with poor prognosis in children and adults.[1] The disease generally spreads via the cerebrospinal fluid, metastasizes to the normal brain tissue and spinal cord, and establishes a satellite tumor group around the primary tumor owing to its uncontrolled aggressive behavior and invasion.[2] Although it is managed using aggressive procedures, such as surgery, radiation, and chemotherapy, the survival of patients with malignant gliomas has been reported to be between 14 (with no treatment) and 40–50 weeks.[1] This is due to the presence of high-grade gliomas, which generally show recurrence in local regions.[3] Therefore, developing innovative therapeutic strategies and more effective agents is urgently required to prolong patient survival and improve the quality of life.[4] Furthermore, new combinations of agents need to be developed with basic chemotherapeutic effects that are highly efficacious and safe to optimize the efficacy of cancer treatment, resulting in a more suitable choice for chemotherapy.

A combination of chemical compounds, such as phenolic acids and flavonoids, extracted from fruits and vegetables may boost the cytotoxic or antiproliferative effects of cancer medications. One such natural phenolic compound, ellagic acid (EA), is found in strawberries, walnuts, cranberries, raspberries, pecans, pomegranates, and other plant foods.[5] EA has been claimed to have

Key words
- Cancer therapy
- Ellagic acid
- Glioblastoma
- Temozolomide

Abbreviations and Acronyms
BEV: Bevacizumab
BrdU: 5-Bromo-2′-deoxyuridine
EA: Ellagic acid
GBM: Glioblastoma

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anticarcinogenic features that function via cell cycle arrest, tumor formation and growth inhibition, and apoptosis induction[6] or angiogenesis supression.[7] EA is generally used as a dietary supplement to reduce or prevent the risk of cancer. However, its effect when combined with common chemotherapeutics used in the treatment of patients with glioma is still poorly understood.

Bevacizumab (BEV) is generally used in treatment of recurrent gliomas with World Health Organization grades II and III; however, its effects on the expression profiles of glioma cells remain unsupported.[8] BEV has been known to increase intratumoral hypoxia as well as glycolytic activity, resulting in elevated microenvironment acidification and invasiveness. Therefore, therapies that target the metabolic adaptation mechanisms of cells may provide a synergistic effect on BEV therapy. A number of preclinical studies have indicated that BEV combined with inhibitors targeting invasion mechanisms and cellular metabolism reduces tumor progression.[9] To illuminate the mechanism of the possible synergistic activity of EA, we investigated the effect of combining EA with BEV on drug resistance capacity, DNA repair mechanism, and apoptosis levels of C6 glioma cells.

MATERIALS AND METHODS

Cell Culture

C6 glioma cells obtained from American Type Culture Collection (Manassas, Virginia, USA) were cultured at 37°C in 95% humidified air with 5% carbon dioxide in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were subcultured every third day using trypsin. All concentrations were handled according to dose experiments as a range of 1 µmol/L, 10 µmol/L, and 100 µmol/L for EA and 1 ng/mL, 10 ng/mL, and 100 ng/mL for BEV, which were determined using the doses described in the literature.[10,11] The accepted doses of 100 µmol/L of EA (E2250-10G; Sigma-Aldrich, St. Louis, Missouri, USA) and 100 ng/mL of BEV (Altuzan; Roche, Istanbul, Turkey) were added to the medium, and cells were incubated for 24 hours, 48 hours, and 72 hours. The cells were divided into 4 groups for every incubation time: control, BEV, EA, and combined EA and BEV groups. All experiments were repeated at least 3 times.

5-Bromo-2′-Deoxyuridine Proliferation Assay

5-Bromo-2′-deoxyuridine (BrdU) proliferation analysis was performed according to a previous study based on immunohistochemistry.[12] BrdU (SC-32323; Santa Cruz Biotechnology, Santa Cruz, California, USA) and SensiTek Histostain-Plus Bulk Kit (ScyTek Laboratories, Inc., Logan, Utah, USA) were used for analysis. Mouse monoclonal anti-BrdU antibody (Bu20A, SC-20045; Santa Cruz Biotechnology) was used as the primary antibody (1:200, overnight). Aminoethyl carbazole (ScyTek Laboratories, Inc.) was used as the chromogen. BrdU labeling was assessed by 2 researchers (A.Ç. and B.B.), and the proliferation index was calculated by evaluating at least 3000 cells and scored as the ratio of number of positively stained cells to total number of cells counted.[12]

Immunohistochemistry

C6 glioma cells cultured on coverslips were incubated for 24 hours; subsequently, groups of EA and BEV were established. After 24 hours, 48 hours, and 72 hours of incubation, the experiments were ended at given durations and repeated 3 times. Cells were fixed with 100% cold methanol for 5 minutes, and immunostaining was performed via the indirect streptavidin immunoperoxidase method using SensiTek HRP Anti-Polyvalent Kit (ScyTek Laboratories, Inc.) to detect p53 and caspase-3 proteins. The coverslips were incubated overnight at 4°C with primary antibodies, anti-p53 (orb136435; Biorbyt, San Francisco, California, USA) and anti—caspase-3 (active form, AB3623; Merck KGaA, Darmstadt, Germany), diluted according to the manufacturers' instructions. The antigen-antibody complex was subsequently visualized using the SensiTek AEC Substrate Detection System (ScyTek Laboratories, Inc.). The intensity of immunoreactivity was semiquantitatively evaluated using H-score analysis according to a previous study.[12]

Expression Analysis

Total RNA was extracted using the Total RNA Purification Kit (Jena Bioscience GmbH, Jena, Germany), according to the kit protocol. Complementary DNA was reverse-transcribed using the SCRIPT cDNA Synthesis Kit (Jena Bioscience GmbH), according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction was conducted using qPCR GreenMaster with UNG (Jena Bioscience GmbH) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). Primer pairs were as follows: *MDR1*, F: 5′-CAGTTCATTCGCTCCTGACTAC-3′ and R: 5′-CGTGCTGTAGCTGTCAATCT-3′; *MGMT*, F: 5′-GAAGCCTATTTCCACGAACCT-3′ and R: 5′-CACCTGTCTGGTGAATGAATCT-3′; p53, F: 5′- ACATGACTGAGGTCGTGAGA-3′ and R: 5′-GATTTCCTTCCACCCGGATAAG-3′; caspase-3, F: 5′-CTGACTGGAAAGCCGAAACT-3′ and R: 5′-GTTCCACTGTCTGTCTCAATACC-3′; and glyceraldehyde-3-phosphate dehydrogenase, F: 5′-GCAAGGATACTGAGAGCAAGAG-3′ and R: 5′-GGATGGAATTGTGAGGGAGATG-3′. After normalizing to glyceraldehyde-3-phosphate dehydrogenase levels, the relative amounts of *MDR1*, *MGMT*, p53, and caspase-3 transcripts in treated cells compared with those in controls were calculated as mean ± SEM.

Statistical Analysis

Semiquantitative and quantitative data from all groups were statistically evaluated using GraphPad InStat 3.06 (GraphPad Software, San Diego, California, USA). All data were presented as mean ± SEM. The means of continuous variables were compared using one-way analysis of variance, and variations between the groups were compared using Tukey-Kramer multiple comparison test. $P < 0.05$ and $P < 0.001$ were accepted as statistically significant.

RESULTS

Treatment with Combined EA and BEV Enhances Cell Proliferation Inhibition

To determine whether EA could potentiate the inhibitory effects of BEV on the proliferation of C6 glioma cells, we semiquantitatively

analyzed the effects of EA with or without BEV using the BrdU proliferation assay. As shown in Figure 1, compared with the control group, treatment with EA alone significantly suppressed cell proliferation in a time-dependent manner ($P < 0.001$). Moreover, combined treatment with EA and BEV significantly enhanced BEV-mediated inhibition of cell proliferation compared with BEV treatment alone ($P < 0.001$).

EA Affects Apoptotic Proteins of p53 and Caspase-3 at Protein Level in a Time-dependent Manner but Not at a Gene Level

To determine the apoptotic effects of EA on glioma cells, p53 and caspase-3 gene expression and protein levels were investigated using quantitative polymerase chain reaction (Figure 2) and immunohistochemistry (Figures 3 and 4), respectively. After 24 hours and 48 hours of incubation, BEV significantly upregulated the expressions of p53 and caspase-3 ($P < 0.001$) as well as increasing the immunoreactivities of these proteins. However, after 24 hours of incubation, EA did not significantly alter the gene and protein levels of p53 and caspase-3 even when combined with BEV. Although there were slight increases in the expressions of p53 and caspase-3 in the treatment group compared with the control group after 48 and 72 hours of incubation, the differences were not significant; however, EA with or without BEV increased the reactivity of p53 protein, probably by mediating post-translational modifications ($P < 0.05$). A similar time-dependent modification of caspase-3 protein was also observed at 72 hours after treatment with EA and BEV ($P < 0.05$).

Combined EA and BEV Downregulates Expression of *MDR1 (ABCB1)* in a Time-dependent Manner

As a modulatory gene in drug resistance, *MDR1* (*ABCB1*) is often involved in the nonresponsive features of glioma cells.[13] The present study investigated whether EA combined with BEV affected the expression of *MDR1*. The results indicated that EA alone or in combination with BEV failed to reduce the expression of *MDR1* after 24 hours and 48 hours of incubation (Figure 5). However, its expression was significantly downregulated by EA and BEV application at 72 hours of treatment, suggesting that only long-term use of EA in combination with BEV could provide an inhibitory effect on the multiple drug resistance capacity of the cells.

EA Downregulates Expression of *MGMT* Regardless of Presence of BEV

To determine whether the expression of *MGMT*, which is highly encountered in solid types of cancer and proved to remove drug-induced cytotoxic O^6-alkylguanine DNA adducts, is regulated by EA, we investigated the expression levels of *MGMT* in EA-applied C6 glioma cells. At 24 hours of incubation, EA suppressed BEV-induced overexpression of *MGMT* ($P < 0.001$ compared with BEV group) (Figure 5). At 48 hours and 72 hours of incubation, EA significantly downregulated the expression of *MGMT* whether or not it was combined with BEV ($P < 0.001$ vs. control and BEV groups), suggesting that the antiproliferative features of EA could be related to the modulatory effect of EA on *MGMT*.

DISCUSSION

Up to 95% of patients with glioblastoma have died within 5 years after initial diagnosis. Therefore, investigating novel strategies to treat glioblastoma is crucial, including targeting metabolic adaptation mechanisms and using combination therapies of chemotherapeutic drugs with inhibitors targeting evasion mechanisms or those with various anti-inflammatory, antineoplastic, apoptotic, or toxic agents.[14] Another chemotherapeutic approach to target glioblastoma is to use antiangiogenic agents such as anti–vascular endothelial growth factor monoclonal antibodies,

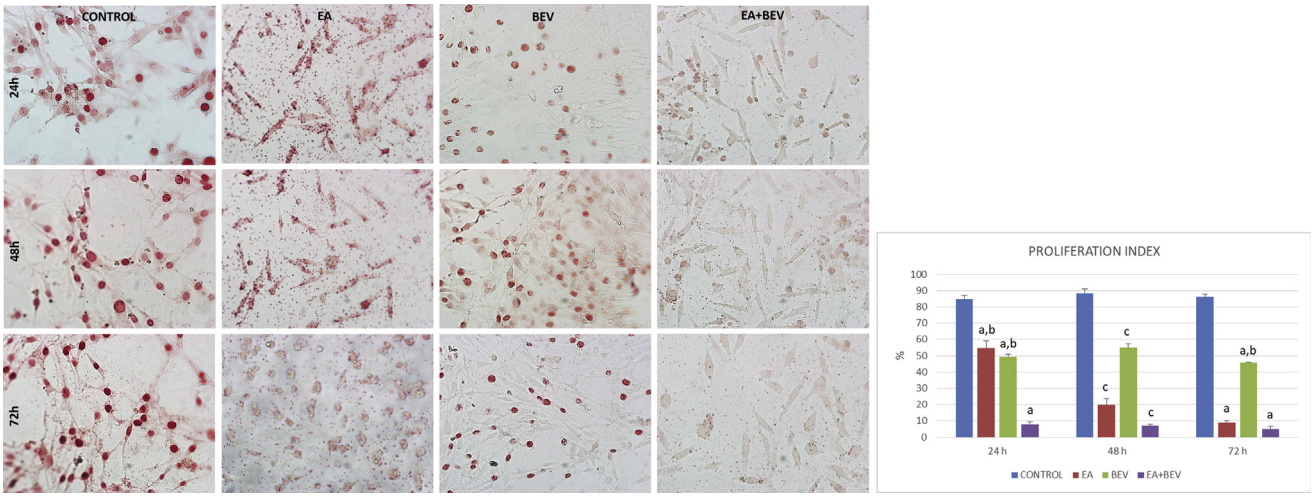

Figure 1. Microphotographs and graphic presentation of 5-bromo-2′-deoxyuridine proliferation assay for ellagic acid and bevacizumab treatment of C6 glioma cells. Magnification: 400×. [a]$P < 0.001$ versus control group. [b]$P < 0.001$ versus ellagic acid and bevacizumab group. [c]$P < 0.001$ versus all groups. EA, ellagic acid; BEV, bevacizumab.

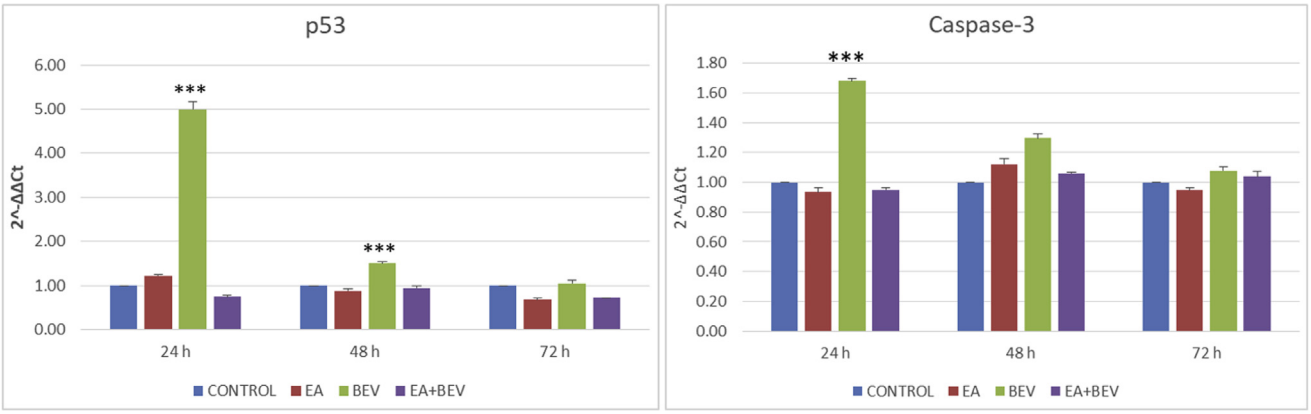

Figure 2. Graphic presentation of p53 and caspase-3 expression results of ellagic acid and bevacizumab treatment of C6 glioma cells. $^{**}P < 0.01$ versus ellagic acid group. $^{***}P < 0.001$ versus all groups. EA, ellagic acid; BEV, bevacizumab.

of which BEV is an example.[15] In recent years, BEV has been extensively used for the treatment of recurrent glioblastomas.[9] However, recent findings of 2 double-blind, placebo-controlled phase III trials have shown that BEV therapy does not affect overall survival, but rather results in a minor increase in progression-free survival.[16] EA, a naturally occurring dietary polyphenolic compound, has been known to have antioxidant, antifibrotic, and anticarcinogenic properties.[17] Therefore, we examined the effect of combining EA with BEV on the proliferation and expression profiles of C6 glioma cells.

Various studies have revealed that EA has a significant role in the inhibition of cell proliferation, metastasis, and invasion into tumor cells. In a study by Wang et al.,[18] EA was shown to inhibit the cell viability, proliferation, and invasion of human glioblastoma cells, suggesting that cell cycle arrest and DNA damage induced by EA could promote the inhibitory effects of EA. EA has been demonstrated to reduce the occurrence of a variety of carcinogen-induced tumors,[19] including distinctive anticarcinogenic effects in several types of cancers.[6,11,18-20] Wang et al.[11,18] also indicated that EA drastically reduced the cell viability of glioblastoma cells at doses of 25–200 μmol/L, suggesting its antiproliferative efficacy. Most importantly, EA downregulated the expression of the antiapoptotic proteins survivin and Bcl-2, but elevated the expression of caspase-3 and the proapoptotic protein Bax in a dose-dependent manner.[18] Another study showed that EA induced G_1 arrest and apoptosis via p53-mediated activation in cells[21] and inhibited angiogenesis by inactivating metalloproteinases.[22] In the present study, BEV significantly

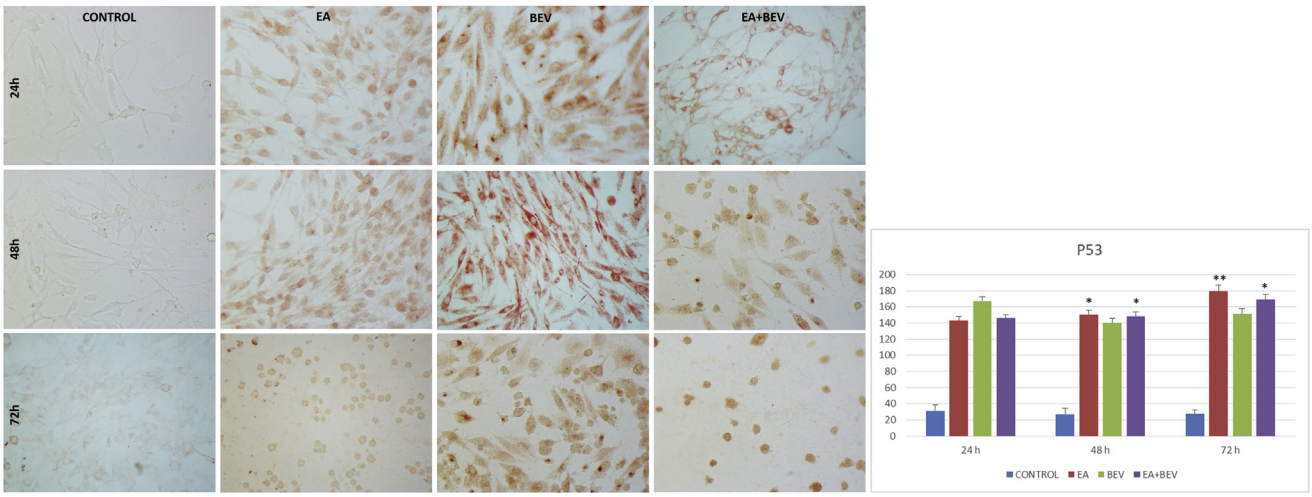

Figure 3. Microphotographs and graphic presentation of p53 immunohistochemical results of ellagic acid and bevacizumab treatment of C6 glioma cells. Magnification: 400×. $^{*}P < 0.05$ versus control group. $^{**}P < 0.01$ versus control group. EA, ellagic acid; BEV, bevacizumab.

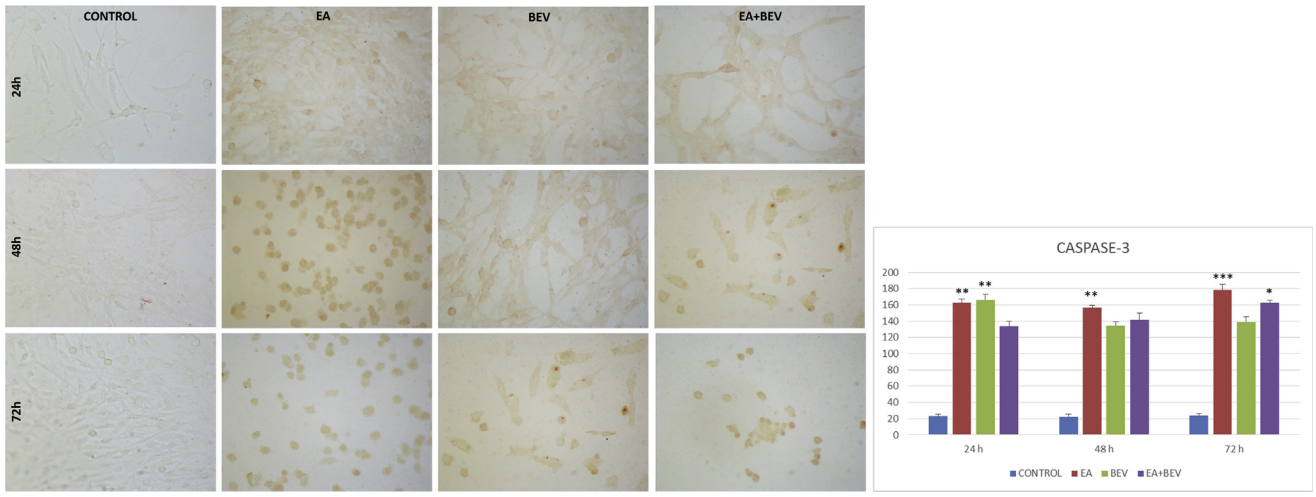

Figure 4. Microphotographs and graphic presentation of caspase-3 immunohistochemical results of ellagic acid and bevacizumab treatment of C6 glioma cells, Magnification 400X. $*P < 0.05$ versus control group. $**P < 0.01$ versus control group. $***P < 0.001$ versus control group. EA, ellagic acid; BEV, bevacizumab.

upregulated the expressions of p53 and caspase-3 ($P < 0.001$) and increased the immunoreactivities of these proteins in cells after 24 hours and 48 hours of incubation. However, EA did not significantly affect the gene and protein levels of p53 and caspase-3 after 24 hours of incubation, even when combined with BEV. A slight increase in the expressions of p53 and caspase-3 after 48 hours and 72 hours of incubation in the EA and BEV treatment group was not significant; however, EA alone increased the reactivity of p53 protein, suggesting a modulatory role of EA in posttranslational modifications. A similar time-dependent modification of caspase-3 protein was also observed after 72-hour treatment with EA and BEV in C6 glioma cells.

Drug resistance, including multiple mechanisms, is the cause of >90% of treatment failures in aggressive metastatic cancers such as glioblastoma.[23,24] Multiple drug resistance pathways may be developed by elevated release of the drug outside the cells; therefore, drug absorption is decreased in cancer cells.[25] Multiple drug resistance characterized by increased expression of P-glycoprotein may be a remarkable obstacle for cancer therapies,[26] which is related to drug nonresponse in malignant tumors. In vitro studies have shown that untreated glioblastomas principally express MDR1 even if they are at least partially chemosensitive.[27] One such study tested the chemosensitivity of glioblastomas to some antineoplastic drugs on cultured tissues and demonstrated the

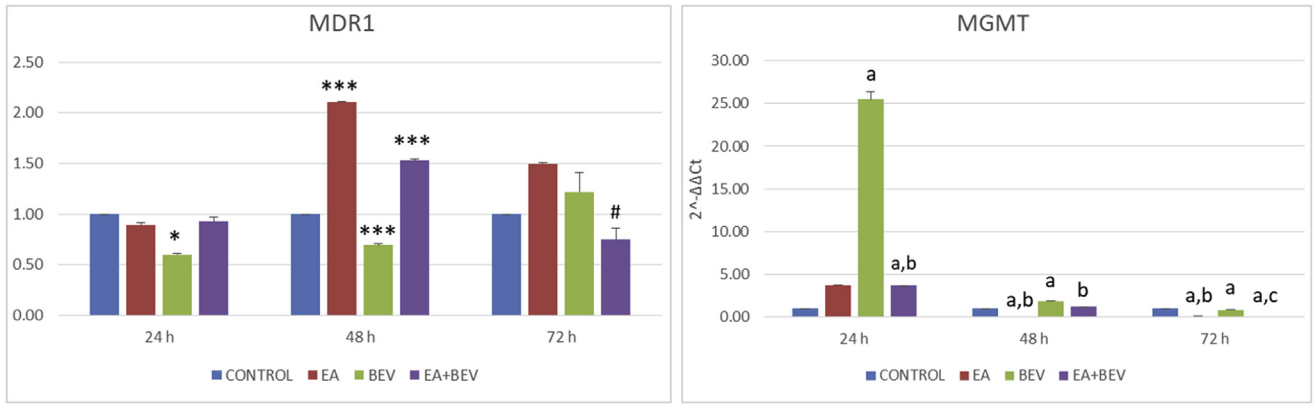

Figure 5. Graphic presentation of *MDR1* and *MGMT* expression results of ellagic acid and bevacizumab treatment of C6 glioma cells. $*P < 0.05$ versus control group. $***P < 0.001$ versus control group. $\#P < 0.05$ versus EA group. $^{a}P < 0.001$ versus control group. $^{b}P < 0.001$ versus BEV group. $^{c}P < 0.05$ versus EA group. EA, ellagic acid; BEV, bevacizumab.

expression of *MDR1* in 16 sensitive and 5 highly resistant glioblastomas.[27] All tumors identically expressed P-glycoprotein, suggesting that untreated glioblastomas mostly express P-glycoprotein, resulting in the existence of cell populations with early drug resistance in these tumors. This phenomenon may explain the disappointing overall long-term efficacy of chemotherapy. In the present study, we investigated whether EA in combination with BEV modulated the expression profile of *MDR1* in C6 glioma cells. We found that 72-hour treatment with EA and BEV significantly downregulated the expression of *MDR1*, suggesting that only long-term application of EA combined with BEV may have an inhibitory role on the multiple drug resistance capacity of the glioma cells. The high expression levels of *MDR1* after 48 hours of incubation are probably due to the posttranslational modulation adaptation of the protein to EA, leading to drug resistance.

Glioblastoma is resistant to some chemotherapeutic drugs because of the presence of the *MGMT* gene in glioma cells,[28] which is highly expressed in solid types of cancer, resulting in the removal of drug-induced cytotoxic O^6-alkylguanine DNA adducts. This unique DNA repair protein, MGMT, has the ability to prevent the formation of DNA interstrand cross-links in most human tumors, which are considered to be critical lethal lesions induced by these drugs. The methylation status of *MGMT* promoter is a crucial indicator of the prognosis of glioblastoma.[29] There is an immediate necessity to develop well-tolerated drugs with the capacity to reverse drug resistance. Various agents targeting drug resistance with the capacity to mediate *MGMT* expression levels are being actively studied. Therefore, we focused on the combination treatment of BEV with EA to inhibit unforeseen DNA repair by mediating *MGMT* expression. We found that EA suppressed BEV-induced overexpression of *MGMT* in a time-dependent manner, suggesting a modulatory effect of EA combined with BEV on the expression of *MGMT*.

In our previous study, the effect of combining EA with BEV was observed by altering the cadherin switch and angiogenesis of C6 glioma cells.[30] As a result, we concluded the successful therapeutic efficacy of EA combined with BEV was probably due to the inhibition of the cadherin switch and vascular endothelial growth factor expression. However, we exhibited the antiproliferative and apoptotic activities of EA in the same cell line by activating p53 and caspase-3 proteins in the present study. The limitation of these 2 studies is the lack of a human glioma cell line to show the reproducible effect of EA combined with BEV in human cells. Animal experiments as well as in vivo human studies are warranted to demonstrate the exact antiproliferative, apoptotic, and antiangiogenic effects of EA combined with BEV. However, together with the former study, we suggest a modulatory effect of EA combined with BEV in cancer therapy.

CONCLUSIONS

The present study suggests that EA exhibits antiproliferative and apoptotic activities in C6 glioma cells by activating p53 and caspase-3 proteins. Long-term application of EA combined with BEV may have an inhibitory role in the multiple drug resistance capacity of the glioma cells and a suppressive role in BEV-induced DNA repair in a time-dependent manner, suggesting a modulatory effect of EA combined with BEV in cancer therapy. EA may be added to drug development and treatment protocols to overcome low drug delivery to develop a novel chemotherapy strategy in sensitizing cancer cells to BEV.

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