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Green tea polyphenol (−)-epigallocatechin gallate reduces matrix metalloproteinase-9 activity following transient focal cerebral ischemia☆

Jong-Wook Park[a,*], Jung-Seok Hong[b,e], Kyoung-Suk Lee[c], Hahn-Young Kim[d], Jung-Jeung Lee[a], Seong-Ryong Lee[a,b,*]

[a]Chronic Disease Research Center and Institute for Medical Science, School of Medicine, Keimyung University, Taegu 700-712, South Korea
[b]Department of Pharmacology, School of Medicine and Brain Research Institute, Keimyung University, Taegu 700-712, South Korea
[c]Department of Psychology, Catholic University of Daegu, 712-702 Taegu, South Korea
[d]Department of Neurology, Konkuk University Hospital, 143-729 Seoul, South Korea
[e]Department of Emergency Medicine, Ulsan University Hospital, 682-714 Ulsan, South Korea

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Abstract

Green tea polyphenol (−)-epigallocatechin gallate (EGCG) has been reported to reduce neuronal damage after cerebral ischemic insult. EGCG is known to reduce matrix metalloproteinase (MMP) activity. MMP can play an important role in the pathophysiology of neurological disorders including cerebral ischemia. The purpose of the current study was to investigate whether EGCG shows an inhibitory effect on MMP activity and neural tissue damage following transient focal cerebral ischemia. In the present study, C57BL/6 mice were subjected to 80 min of focal ischemia induced by middle cerebral artery occlusion (MCAO). Animals were killed 24 h after ischemia. EGCG (50 mg/kg) was administered intraperitoneally immediately after ischemia. Gelatin gel zymography showed an increase in the active form of MMP-9 after ischemia. EGCG reduced ischemia-induced up-regulation of the active form of MMP-9. In *in situ* zymography, EGCG reduced up-regulation of gelatinase activity induced by cerebral ischemia. Co-incubation with EGCG reduced gelatinase activity directly in postischemic brain section. In 2,3,5-triphenyltetrazolium chloride (TTC) assay, brain infarction was remarkable in the middle cerebral artery territory after focal cerebral ischemia. In EGCG-treated mice, infarct volume was significantly reduced compared with vehicle-treated mice. These results demonstrate that EGCG, a green tea polyphenol, may reduce up-regulation of MMP-9 activity and neuronal damage following transient focal cerebral ischemia. In addition to its antioxidant effect, MMP-9 inhibition might be a possible mechanism potentially involved in the neuroprotective effect of a green tea polyphenol, EGCG.

Keywords: Focal cerebral ischemia; Green tea polyphenol; Matrix metalloproteinase; Neuroprotection

1. Introduction

Highly complex pathophysiologic processes are involved in neuronal damage pathway in cerebral ischemia. Previously, intracellular processes have been suggested to explain the causative pathology of ischemic insult. In addition to intracellular pathologic events, the dysregulation of pericellular signaling such as cell-to-matrix or cell-to-cell interaction has been emphasized as a possible mechanism leading to neural tissue damage in cerebral ischemia [1,2]. In particular, structural alteration in the neurovascular unit may attribute to the main pathophysiology such as edema and hemorrhage in focal cerebral ischemia [3,4].

Abnormal regulations in the expression and activity of matrix metalloproteinases (MMPs) are closely related to various types of brain diseases. MMPs are up-regulated and play an important role in the pathologic processes of focal cerebral ischemia [5,6]. In particular, the gelatinases MMP-2 and MMP-9 have been implicated specifically in cerebral ischemia [7]. Two gelatinases, MMP-9 and MMP-2, degrade the matrix components of basement membrane that maintain cerebral vasculature integrity, resulting in neuroinflammation, brain edema, and hemorrhagic transformation in focal brain ischemia [6,8,9]. Genetic and pharmacological inhibitions of MMP-9 have been shown to reduce neuronal damage in focal and global brain ischemia [5,10,11].

Green tea polyphenols are flavanols, generally known as catechins. (−)-Epigallocatechin gallate (EGCG) is the major green tea polyphenols. EGCG has shown to have marked pharmacological effects,

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* Corresponding authors. Seong-Ryong Lee is to be contacted at 194 Dongsan dong, Junggu, Department of Pharmacology, School of Medicine, Keimyung University, Taegu 700-712, South Korea. Tel.: +82 53 250 7478. Jong-Wook Park, 194 Dongsan dong, Junggu, Chronic Disease Research Center and Institute for Medical Science, School of Medicine, Keimyung University, Taegu 700-712, South Korea, Tel.: +82 53 250 7796.
E-mail addresses: j303nih@dsmc.or.kr (J.-W. Park), srlee@kmu.ac.kr (S.-R. Lee).

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such as anti-oxidant [1], anti-inflammatory [12] and antimutagenic [13] activities. In addition, EGCG is a neuroprotective agent against focal and global brain ischemia [11,14,15].

The capabilities of green tea catechins including EGCG to inhibit MMP activities are well documented. EGCG has been shown to inhibit MMPs in a variety of experimental models [16-19]. To our knowledge, however, there are no previous studies on the effect of EGCG against MMP activity following transient focal cerebral ischemia. In the present study, we examined the effect of EGCG on the up-regulation of MMP-9 activity following transient focal cerebral ischemia.

2. Materials and methods

2.1. Animals and production of transient focal brain ischemia

All procedures were approved by the Keimyung University School of Medicine Animal Care and Use Committee. Male C57BL/6 mice (Koatec-Harlan, Korea) weighing 25–30 g were used in this study. Mice were kept in cages under a light–dark cycle with the light on from 6:00 a.m. to 6:00 p.m. Mice were anesthetized with a mixture of N_2O and O_2 (70:30) and 3% isoflurane, and anesthesia was maintained with the inhalation of 1.7% isoflurane. Under the operating microscope, the external carotid artery was ligated and the internal carotid artery isolated. A microvascular clip was placed across the right common carotid artery. Through an external carotid stump, a 7-0 surgical nylon monofilament with silicon coating was advanced into the right internal carotid artery up to the origin of the middle cerebral artery. A laser Doppler flowmeter (Perimed 5000 system, Järfälla, Sweden) was used to measure cerebral cortical microperfusion (4 mm lateral to bregma). The core temperature was monitored and maintained at 37±0.5°C with a feedback-controlled heating pad. The filament was left in place for 80 min and then withdrawn. Animals were then placed to a warm box at 30°C temperature condition for 3 h to avoid the biased results by hypothermia. At the end of the reperfusion period [24 h after reperfusion following 80 min of middle cerebral artery occlusion (MCAO)], the mice were killed for the subsequent experiments.

2.2. Drug treatment

EGCG (50 mg/kg, Sigma, St. Louis, MO, USA) was administered intraperitoneally immediately after ischemia. Saline was injected to mice as vehicle according to same volume and time schedule of EGCG. The dosage of EGCG used in this study was determined based on a previous study [15].

2.3. Gelatin gel zymography

Mice were anesthetized deeply with choral hydrate and then perfused transcardially with ice-cold PBS (pH 7.4). The brains were removed quickly and divided into ipsilateral and contralateral hemispheres under ice-cold condition and stored at −75°C. Brain sample extracts were prepared as described previously [10]. Briefly, brain tissues were homogenized in lysis buffer including protease inhibitors. After centrifugation, the supernatant was collected and total protein concentration was determined using the Bradford assay. Samples were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After separation by electrophoresis, the gel was renaturated and then incubated in a developing buffer at 37°C for 24 h. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min and then destained appropriately. Proteolytic bands in zymography were quantified by scanning densitometry (Quantity One, Bio-Rad).

2.4. In situ zymography

In situ zymography was performed as described previously [2]. The gelatin with a fluorescent tag remains caged (no fluorescence) until the gelatin is cleaved by gelatinase activity. Fresh ischemic brain slices (20 μm) were incubated with a reaction solution including FITC-labeled gelatin (Molecular Probes, Eugene, OR, USA) and vehicle or EGCG (200 μM) to test the direct inhibitory effect of EGCG on gelatinase activity after ischemia. A broad-spectrum MMP inhibitor doxycycline (200 μM) was used as a standard metalloproteinase inhibitor. For a quantitative analysis of the inhibitory effect of EGCG on *in situ* gelatinase activity, different concentrations of EGCG (0, 50, 100, 200 or 500 μM) were used in *in situ* zymography. *In situ* gelatinolysis was revealed by the appearance of fluorescent brain constituents. Reaction products were visualized by a fluorescence microscope. For quantitative data, images of high-power field were acquired by a Leica DM 3000 microscope and a CCD camera (Leica DFC 480). Quantification of fluorescent intensity was performed by a blind examiner (J.-H.P.) using an image analyzing software (Carl Zeiss LSM Image Examiner version 4.2.0.121).

2.5. Measurement of brain infarct volume

Mice were killed for determination of infarct volume 24 h after focal ischemia. The brains were removed and then sliced into six 1-mm coronal sections. The sections were incubated in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 30 min at 37°C. When the stain had developed, the sections were fixed with 10% formalin solution [20]. TTC-stained coronal sections were then photographed by a digital camera (Nikon, Coolpix 5200, Tokyo, Japan). In areas where infarction occurs, TTC does not stain and the tissue remains white. The infarct volume was quantified with ImageJ version 1.30 (National Institute of Health) by an investigator blind to the animal treatments (H.Y.K.) (n=7, vehicle-treated animals; n=8, EGCG-treated animals). To avoid artifact in volume measurement from brain edema within the infarcted tissue, the corrected infarct volume was calculated by measuring and subtracting the volume of the noninfarcted part of the ipsilateral hemisphere from the volume of the contralateral hemisphere.

2.6. Statistics

Data were expressed as mean±S.E. Nonparametric statistical analyses were performed by the Mann–Whitney U test (gelatin gel zymography and infarct volume). For quantitative evaluation of the inhibitory effect of EGCG on *in situ* gelatinase activity, two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was used. Significance refers to results for which P<.05 was obtained.

3. Results

3.1. Gelatin gel zymography for evaluation of the effect of EGCG on gelatinase activity

Gelatin gel zymography was performed to evaluate the protein levels of MMP-9 and MMP-2 in both ipsilateral and contralateral hemispheres. Within the limits of our sensitivity, sham-operated animals showed very low levels of the active form of MMP-9 (97 kDa) and of the latent form of MMP-2 (72 kDa) (Fig. 1A). After transient focal cerebral ischemia, the active form of MMP-9 in the ipsilateral hemisphere of vehicle-treated animals markedly increased and EGCG administration significantly inhibited the induction of the active form of MMP-9 (Fig. 1A and B; P<.01). Although both MMP-2 and MMP-9 may contribute to gelatinase activity, the amount of the latent form of MMP-2 (72 kDa) was not affected by ischemia and there was no active form of MMP-2 in this study (Fig. 1).

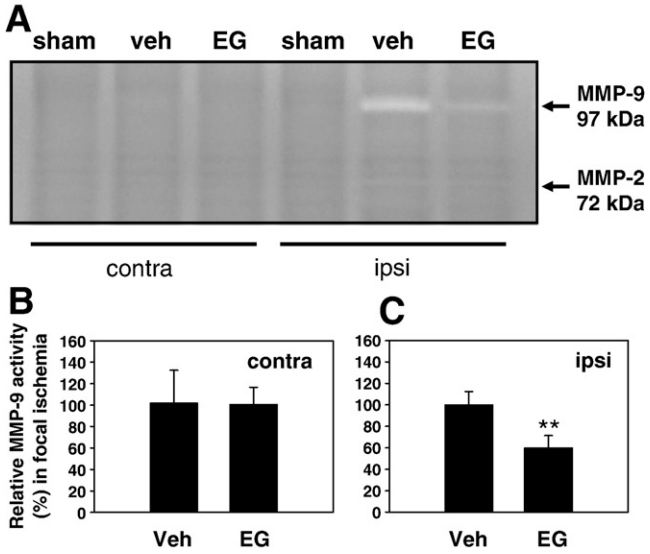

Fig. 1. Representative gelatin gel zymogram of the ipsilateral and contralateral hemispheres following transient focal cerebral ischemia. Zymogram gel showing an increase in the active form of MMP-9 in brain homogenates following transient focal cerebral ischemia. EGCG reduces the increase of MMP-9 activity in the ipsilateral hemisphere (A). Bar graph of relative optical density of MMP-9 bands. Increased optical density of MMP-9 bands (97 kDa) was attenuated by EGCG (mean±S.E.; n=7) (B). Sham: Sham-operated animals; veh: animals used in vehicle treatment and ischemia; EG: animals used in EGCG treatment and ischemia; contra: contralateral hemisphere; ipsi: ipsilateral hemisphere. **P<.01 vs. vehicle-treated animal.

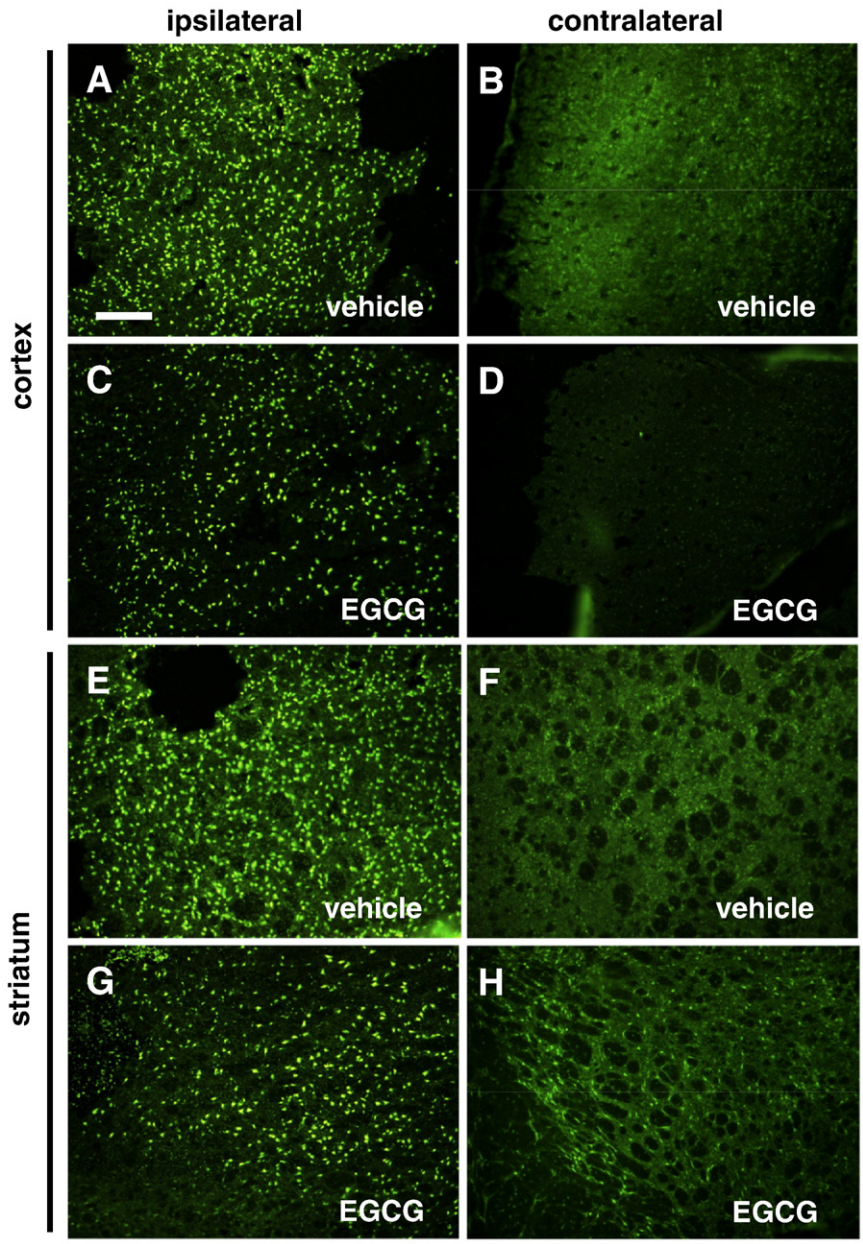

Fig. 2. Representative *in situ* gelatin zymograms in the cortex and striatum following transient focal cerebral ischemia. Gelatinase activity is very weak in the contralateral cortex and striatum in vehicle-treated animals (B, F). *In situ* gelatinase activity markedly increased in the ipsilateral cortex and striatum following transient focal cerebral ischemia (A, E). EGCG administration markedly reduced *in situ* gelatinase activity in the ipsilateral side cortex and striatum (C, G) and the contralateral side cortex and striatum (D, H). Scale bar=200 μm.

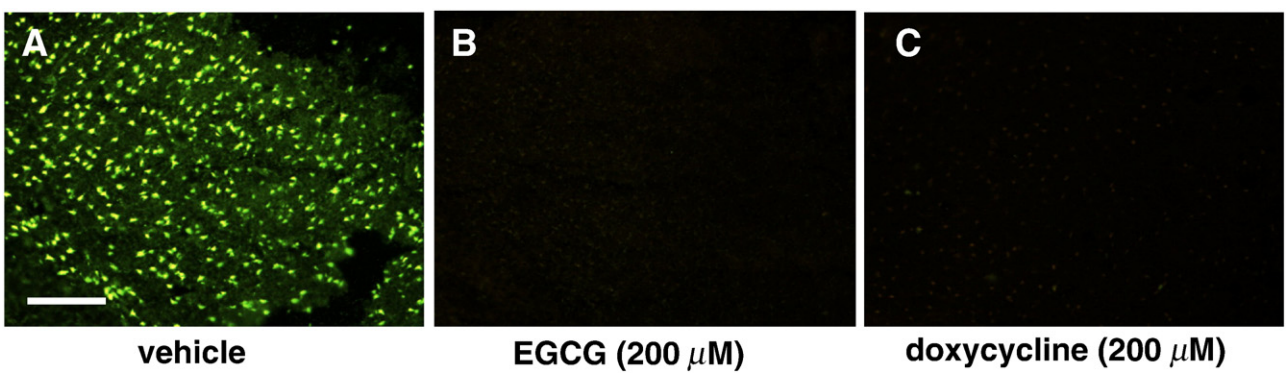

Fig. 3. Direct inhibitory effect of EGCG on *in situ* gelatinase activity in the postischemic brain section. Transient focal cerebral ischemia induces the gelatinase activity in the ipsilateral cortex (A). Suppression of gelatinolytic activity in the postischemic cortex after co-incubation with 200 μM of EGCG (B) or 200 μM of doxycycline (C). Scale bar=200 μm.

3.2. In situ gelatin zymography evaluation of the effect of EGCG on gelatinase activity

To study the histological distribution of gelatinolytic activity, *in situ* gelatin zymography was performed with fresh postischemic brain slices. FITC signal representing gelatinase activity was clearly observed in the damaged cortical and striatal areas of the ipsilateral hemisphere. Gelatinase activity was reduced in the ipsilateral hemisphere of EGCG-treated animal (Fig. 2).

To examine whether EGCG has direct inhibitory effect on gelatinase activity in our model, we also tested its effect on *in situ* gelatinase activity in postischemic brain slices. EGCG (200 μM) co-incubation clearly reduced the intensity and density of gelatinase activity (Fig. 3). Doxycycline (200 μM), a broad-spectrum MMP inhibitor, was used as a standard metalloproteinase inhibitor (Fig. 3). For quantitative analysis of the inhibitory effect of EGCG on *in situ* gelatinase activity, we tried co-incubating several concentrations of EGCG (0, 50, 100, 200 or 500 μM) for use *in situ* zymography. At

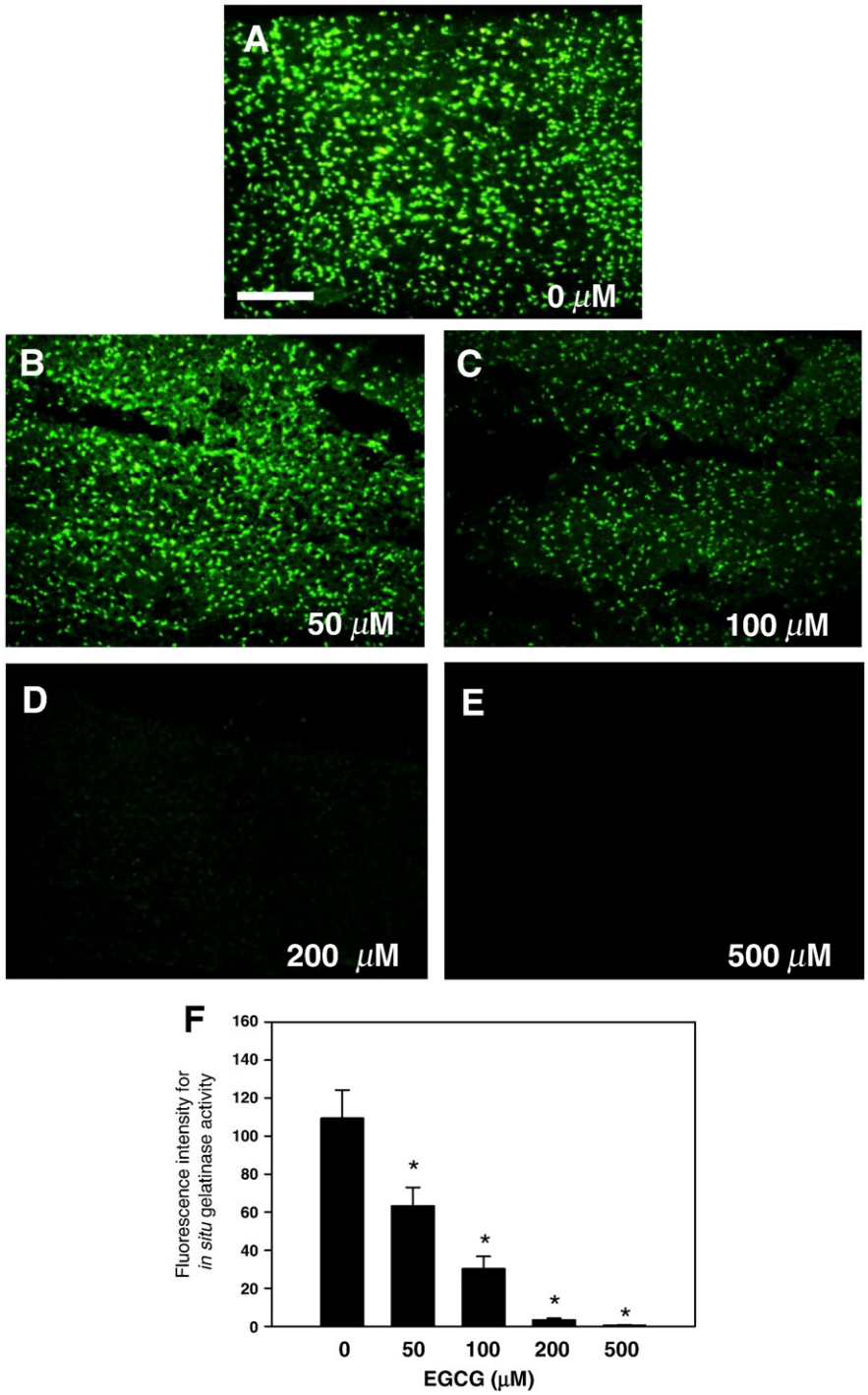

Fig. 4. Inhibitory effect of different concentrations of EGCG on *in situ* gelatinase activity in the postischemic brain section. Transient focal cerebral ischemia induces the gelatinase activity in the ipsilateral cortex (A). Gelatinolytic activity in the postischemic cortex was suppressed by co-incubation with 50, 100, 200 or 500 μM of EGCG (B–E). Quantitative analysis of the inhibitory effect of EGCG on *in situ* gelatinase activity (F). Scale bar=200 μm. The data were expressed as mean±S.E., $n=6$. *$P<.05$ vs. EGCG (0 μM).

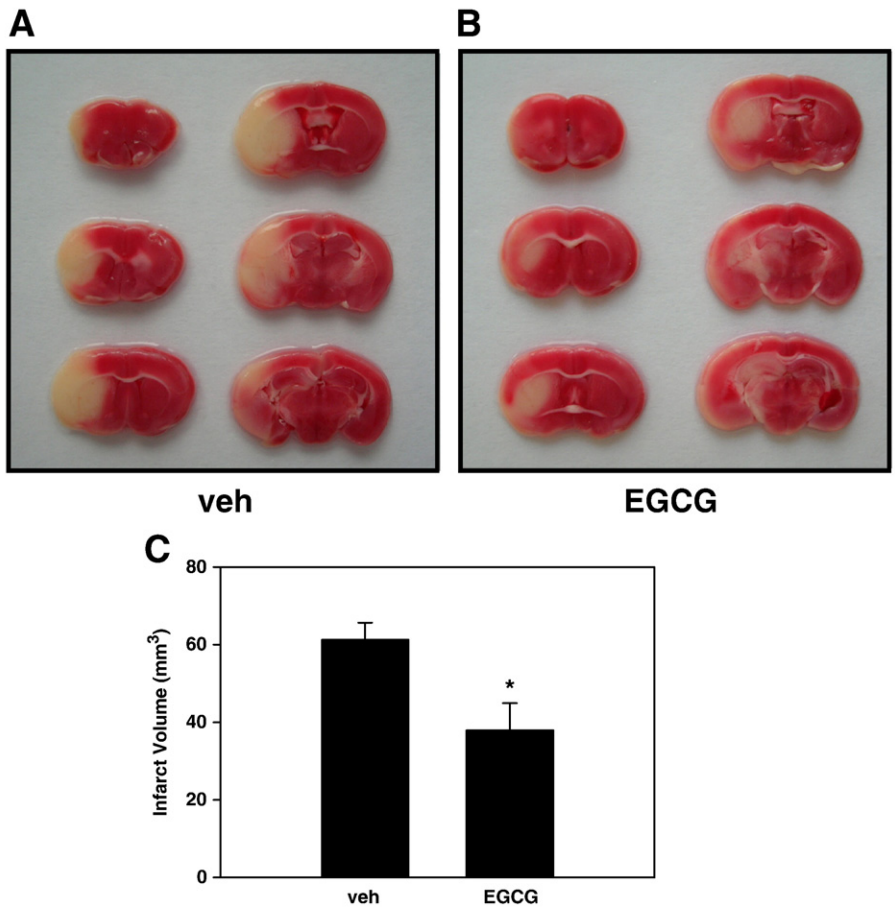

Fig. 5. EGCG reduces infarct volume after 80 min of focal ischemia and 24 h of reperfusion. Representative sections of the brains stained with TTC showing infarction areas in the middle cerebral artery territory of vehicle-treated animals (A) and EGCG-treated animals (B). Quantitative data of infarct volume in the vehicle- and EGCG-treated animals (C). Veh: Vehicle-treated animals; EGCG: EGCG-treated animals. The data were expressed as mean±S.E.; n=7 mice per vehicle-injected animals; n=8 mice per EGCG-injected animals mice per group; *P<.05.

concentrations of 50, 100, 200 and 500 μM, EGCG significantly reduced *in situ* gelatinase activity in a concentration-dependent manner (P<.05, respectively) (Fig. 4).

3.3. Effect of EGCG on infarct volume following focal brain ischemia

We tested the effect of EGCG on brain infarct volume with TTC staining. EGCG administration (50 mg/kg) immediately after MCAO significantly reduced infarct volume compared with vehicle-treated animals (P<.05) (Fig. 5).

4. Discussion

Studies have shown an important role of MMPs in cerebral ischemia [6,7,21]. It has been suggested that transient focal ischemia-induced neuronal injury coincided with dysregulated extracellular proteolysis involving MMP enzymes [10,22,23]. MMPs, especially gelatinases MMP-2 and -9, have been shown to be clearly increased in animal models of focal cerebral ischemia [8,9,23] and in human focal ischemic stroke [24]. Gelatinase-induced breakdown of neurovascular matrix and extracellular matrix leads to edema, bleeding, increased inflammatory influx and neuronal death in cerebral ischemia [9-11]. MMP enzyme-related blood–brain barrier permeability alteration or vasogenic brain edema is an important pathophysiology of neuronal damage following focal cerebral ischemia [23]. Another possible pathological process by MMP in ischemic insult is the anoikis-type neuronal damage, which is induced by degradation of the cell-to-matrix interaction in focal cerebral ischemia [22]. Loss of neuronal cell-to-matrix interaction can produce the inhibition of integrin signal transduction and result in neurotoxicity [25]. In transient global cerebral ischemia, MMP-induced degradation of perineuronal matrix protein also plays a role in the production of anoikis-type neuronal cell death [2,11]. At least in mouse systems, a dominant role has been attributed to MMP-9 because MMP-9 knockout mice showed neuroprotection against focal ischemia [27], whereas MMP-2 knockout mice did not show neuroprotection on brain damage after focal ischemia [10]. In a previous study [26], it was shown that transient focal cerebral ischemia can induce MMP-9 expression in the damaged area and that pharmacological inhibition or MMP-9 depletion reduces focal ischemia-induced infarction. We performed *in situ* zymography here to examine the distribution of gelatinolytic activity. Although both MMP-2 and MMP-9 are known to contribute to gelatinolytic activity, MMP-9 might be a dominant gelatinase in mice brain ischemia [11]. In gel zymography, the active form of MMP-9 markedly increased after focal ischemia and EGCG reduced MMP-9 activity. There were no active forms of MMP-2 bands in our gel zymography data.

EGCG is the most abundant catechin in green tea [27] and has been known as a potent antioxidant [1,28]. The triphenolic group of EGCG structure contributes to its prominent antioxidant effect [29]. There

are a number of works regarding the neuroprotective effect of EGCG. EGCG reduced the dysfunction of retina following retinal ischemia [30], the beta-amyloid protein-induced neuronal damage [31] and the neuronal damage following focal and global cerebral ischemia [14,15,32]. As previously mentioned, EGCG has been known to inhibit MMP enzyme activity and expression in a number of experimental models. EGCG inhibits MMP-9 activity [33] and MMP-9 expression [34,35] and MMP-1 and MMP-13 expression [16]. Based on these MMP inhibitory effects of EGCG, in addition to its antioxidant effect, it might be possible for EGCG to reduce the tissue damage related to MMP activation.

To our knowledge, there were no reports regarding the effect of EGCG on gelatinase activity after transient focal cerebral ischemia. Actually, a number of studies documenting the inhibitory effect of EGCG against MMP activities have been focused mainly on cancer studies including carcinogenesis, cancer metastasis and cellular invasion. Gelatinases A and B (MMP-2 and MMP-9) seem to play an important role in cancer infiltration and metastasis [36]. The activities of MMP-2 and MMP-9 were strongly inhibited by green tea polyphenol EGCG and ECG [18]. EGCG affected many cellular mechanisms and has been shown to decrease MMP-2 and MMP-9 activities [37,38].

Previous reports have shown that MMP activation can be modulated by various mechanisms. After secretion from the cytoplasm as an inactive proenzyme state, MMPs require an activation process by other types of proteolytic enzymes and free radicals. Free radical reaction has been known as an important process of MMP activation [39,40]. Endogenous reactive oxygen species are necessary in fibronectin fragment-induced MMP activation [40]. In fact, it is no wonder to consider that the anti-oxidant effect of EGCG can affect MMP activity and tissue damage in cerebral ischemia. MMP activity may be inhibited by EGCG administration. According to our *in situ* zymography data and previously reported studies regarding various types of disease, it is apparent that EGCG has a direct inhibitory effect on MMP activities. In the present study, we performed *in situ* zymography with co-incubation of EGCG on the postischemic brain section in which gelatinase was already activated and then EGCG directly inhibited gelatinase activity. In quantification analysis of the inhibitory effect of EGCG, we tried different concentrations of EGCG in *in situ* zymography. It was obvious that EGCG co-incubation markedly reduced the fluorescence intensity regarding gelatinase activity in the postischemic brain section.

Based on our results, we can add that EGCG has a potential to inhibit MMP activities induced by transient focal cerebral ischemia. Therefore, in addition to its well-known antioxidant effect, MMP-9 inhibition might be a possible mechanism potentially involved in the neuroprotective effect of a green tea polyphenol, EGCG. To further evaluate the therapeutic potential of EGCG, future investigation thus needs to focus on permanent focal stroke models and poststroke treatments. In addition, to define the protective mechanism or causality of EGCG, further studies need to examine samples at earlier time points and evaluate other biomarkers as well.

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