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Effects of (−)-Epigallocatechin-3-gallate on Brain Infarction and the Activity Change of Matrix Metalloproteinase-9 Induced by Middle Cerebral Artery Occlusion in Mice

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Matrix metalloproteinases (MMPs) can degrade a wide range of extracellular matrix components. It has been reported that MMP-9 are activated after focal ischemia in experimental animals. (−)-Epigallocatechin-3-gallate (EGCG), a major constituent of green tea polyphenols, is a potent free radical scavenger and reduces the neuronal damage caused by oxygen free radicals. And it has been known that EGCG could reduce the infarction volume in focal brain ischemia and inhibit MMP-9 activity. To delineate the relationship between the anti-ischemic action and the MMP-9-inhibiting action of EGCG, we investigated the effect of EGCG on brain infarction and the activity of matrix metalloproteinase-9 induced by permanent middle cerebral artery occlusion (pMCAO) in ICR mice. EGCG (40 mg/kg, i.p. 15~30 min prior to MCAO) significantly decreased infarction volume at 24 hr after MCAO. GM 6001 (50 mg/kg, i.p. 15~30 min prior to MCAO), a MMP inhibitor, also significantly reduced infarction volume. In zymogram, MMP-9 activities began to increase at ipsilateral cortex at 2 hr after MCAO, and the increments of MMP-9 activities were attenuated by EGCG treatment. Western blot for MMP-9 also showed patterns similar to that of zymogram. These findings demonstrate that the anti-ischemic action of EGCG in mouse focal cerebral ischemia involves its inhibitory effect on MMP-9.

Key Words: (−)-Epigallocatechin-3-gallate (EGCG), Permanent middle cerebral artery occlusion, Matrix metalloproteinase-9 (MMP-9), Mouse

INTRODUCTION

Stroke is a leading cause of mortality and disability in industrialized countries. Despite intensive efforts to develop new therapeutics for stroke in the past 2 decades, all treatmentshave so far failed to show clinical effect, except thrombolysis with tissue plasminogen activator (Ovbiagele et al, 2003). This outcome made it to shift major targets for stroke drug from neurons to the neurovascular unit composed of endothelial cells, astrocytes and neurons (Lo et al, 2003).

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, can degrade a wide range of extracellular matrix components (Cuzner & Opdenakker, 1999). It has been reported that activation of MMPs is involved in damages of neurovascular unit in ischemic region (Asahi et al, 2001; Gu et al, 2002; Lee et al, 2004b), and that MMP-9 is activated after focal ischemia in not only experimental animals but also humans (Rosenberg et al, 1998; Gasche et al, 1999; Heo et al, 1999; Asahi et al, 2000; Rosell et al 2005; Rosell et al 2006).

(−)-Epigallocatechin gallate (EGCG), a major constituent of green tea polyphenols, is a potent free radical scavenger and reduces the neuronal damage that is caused by oxygen free radicals (Ho et al, 1992; Hanasaki et al, 1994; Salah et al, 1995). And it has been known that EGCG could inhibit MMP-9 activity (Demeule et al, 2000; Sartor et al, 2002).

The present study was performed to delineate the relationship between the anti-ischemic action and the MMP-9-inhibiting action of EGCG. We investigated the effect of EGCG on brain infarction and the activity of MMP-9 induced by permanent middle cerebral artery occlusion (MCAO) in male ICR mice.

METHODS

Animals were cared for in accordance with the guide for Animal Experiments, edited by The Korean Academy of Medical Sciences. Procedures using experimental animals

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ABBREVIATIONS: EGCG, (−)-epigallocatechin-3-gallate; MCAO, middle cerebral artery occlusion; MMP-9, matrix metalloproteinase-9; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery.

were approved by the institutional animal care and use committee.

Permanent focal cerebral ischemia

Male ICR mice (Daehan Biolink Co, Chungbuk, Korea), weighing 25~30 g, were allowed free access to food and water and kept under 12 : 12 light/dark cycle. Anesthesia was induced with 4% enflurane and maintained at 2% in 100% O_2 using rodent mask (Stoelting, USA). The right middle cerebral artery (MCA) was permanantly occluded using intraluminal suture technique. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed through midline cervical incision. MCA occlusion was achieved by introducing a silicon-coated 7-0 nylon monofilament (Ethicon, NJ, USA) into the CCA through ECA and advancing it 9±1 mm via ICA to the origin of MCA. The rectal temperature of the animals was maintained at 37±0.5°C using homeothermic blanket control unit (Harvard, UK) during the operation.

Measurement of infarct volume

The animals were euthanized at 24 hr after ischemia and decapitated. Brains were rapidly removed and then sliced into 2-mm coronal sections using a mouse brain matrix (Zivic-Miller Lab. Inc., U.S.A.). The brain slices were incubated in phosphate-buffered saline (PBS; pH 7.4) containing 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma, MO. U.S.A.) at 37°C for 15 min and then washed with saline and fixed with 10% formalin. The caudal face of each section was scanned using a flatbed color scanner and the images were stored. Ischemic and non-ischemic hemisphere infarct areas were measured using Image-Pro Plus (Image & Graphics, U.S.A.).

Preparation of tissue extracts

At 2, 8, 24 hrs after the onset of ischemic insult, mice were deeply anesthetized with enflurane and then transcardially perfused with ice-cold PBS (pH 7.4). Sham-operated control mice were similarly perfused at 24 hrs. Brains were removed quickly, and divided into ipsilateral ischemic hemispheres and contralateral nonischemic hemispheres, frozen immediately in liquid nitrogen and stored at −80°C. Brain samples were homogenized in Nonidet p-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM benzamidine, 1% Nonidet P-40, 5 mM EDTA, pH 8.0, 1 μg/ml trypsin inhibitor, 1 mM PMSF) on ice using a Teflon glass homogenizer. After centrifugation (14,000 g) for 15 min at 4°C, the supernatant was collected and aliquoted into cryovial. Total protein concentration of each sample was determined using the Bradford assay (Bio-Rad Laboratories, CA, U.S.A.).

Western blot analysis

To investigate the effects of EGCG on the MMP-9 protein expression, Western blot analysis for MMP-9 was performed. Protein samples were separated in 10% polyacrylamide gel and transfered to a membrane The membrane was blocked with 10% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). MMP-9 was detected with rabbit anti-mouse polyclonal antibody (Chemicon International, U.S.A.) at a dilution rate of 1 : 1,000. After washing with PBS-T, the membrane was incubated with peroxidase-conjugated secondary antibody at room temperature for 1 hr. Finally, MMP-9 protein was detected by using chemical luminescence (ECL; Amersham Pharmacia Biotech, NJ, U.S.A.).

Zymogram

To investigate the effects of ECGC or GM6001 on the activity of MMP-9, zymogram was performed. Similarly prepared protein samples (as in Western blot analysis) were loaded and separated in a polyacrylamide gel containing 1 mg/ml gelatin. After separation by electrophoresis, the gel was incubated in renaturing buffer (2.5% Triton X-100 in distilled water) at room temperature for 3 hrs and then overnight in developing buffer (100 mM $CaCl_2$, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) at 37°C with gentle agitation. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min, destained appropriately and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Drug used

EGCG and GM6001 were purchased from Sigma (MO, U.S.A.) and Biomol International LP (PA, USA), respectively. EGCG was dissolved in physiologic saline and GM6001 in 4% [w/v] carboxymethylcellose in water. These drugs were administered intraperitoneally at 15~30 min before MCA occlusion.

Statistical analysis

The data are expressed as mean±standard error (SEM). To compare multiple means of infarct volume, one-way ANOVA followed by post-hoc Tukey test was used. All analyses were performed using Instat (GraphPad Software, CA, USA), and differences were considered to be statistically significant when p value was less than 0.05.

RESULTS

Effect of EGCG and GM6001 on the infarct volume and neurologic deficit

The occlusion of MCA for 24 hrs using intra-arterial filament yielded reproducible infarction in the MCA territory of the frontoparietal cortex and caudatoputamen, which was clearly delineated by TTC staining (Fig. 1A).

In the saline-treated control group, the cerebral infarction was found on all of 5 coronal sections and the percent of infarction was 23.4±0.76 (n=17). Pretreatment of animals with EGCG (40 mg/kg, ip) significantly reduced the cerebral infarction (18.5±1.21% infarction n=10) ($p<0.01$). Pretreatment of animals with a non-selective MMP inhibitor, GM 6001 (50 mg/kg, ip), also showed significant reduction of the cerebral infarction size (18.2±1.38% infarction n=7) ($p<0.01$) (Fig. 2).

Effect of EGCG and GM 6001 on ischemia-induced MMP-9 activity

In zymogram, MMP-9 activities began to increase at ipsilateral cortex 2 hr after MCAO and continuously increa-

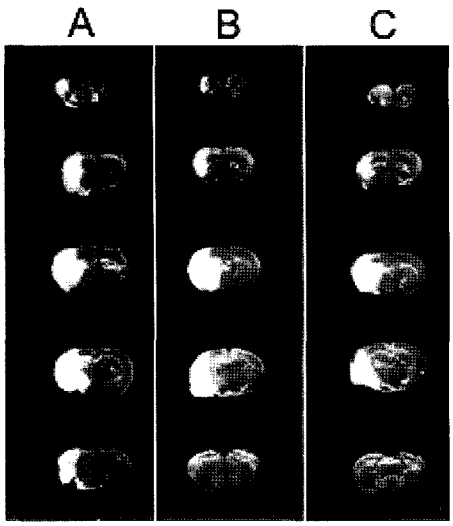

Fig. 1. Representative brain slices showing the inhibitory effects of EGCG (40 mg/kg, i.p., 15~30 min before MCAO) or GM6001 (50 mg/kg, i.p., 15~30 min before MCAO) on the infarct at 24 hr after MCAO in mice. The slices were stained with 2% triphenyltetrazolium chloride. A, Control; B, EGCG; C, GM 6001.

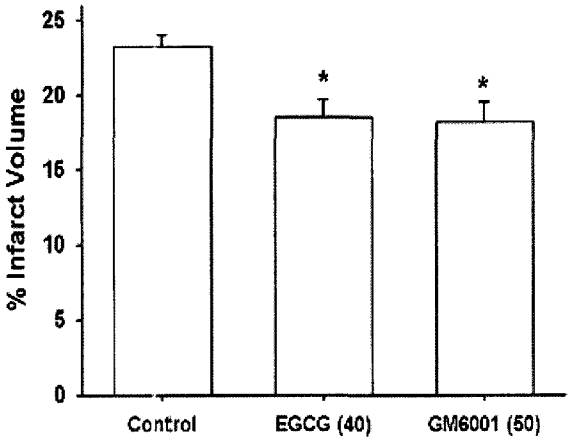

Fig. 2. Effects of treatment with EGCG (40 mg/kg, i.p., 15~30 min before MCAO) or GM6001 (50 mg/kg, i.p., 15~30 min before MCAO) on the infarct volume at 24 hr after MCAO in mice. Each column and vertical bars represent mean±SEM from 7~17 animals. Asterisk indicates significant difference from control group ($p < 0.01$).

sed up to 24 hrs. Treatment with EGCG (40 mg/kg, ip) attenuated the increments of MMP-9 activities (Fig. 3). The inhibitory effects of 40 mg/kg EGCG on the MMP-9 activity was comparable to that of 50 mg/kg GM 6001 (Fig. 4).

Effect of EGCG on ischemia-induced MMP-9 protein expression

In Western blot analysis, MMP-9 protein expression was detected as early as 2 hrs after MCAO, and continuously increased up to 24 hrs. Treatment with EGCG also inhibited the increased expression of MMP-9 protein (Fig. 5).

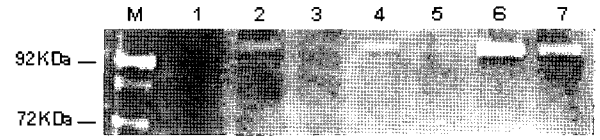

Fig. 3. Gelatin-zymogram showing the effect of EGCG on MMP-9 activity. EGCG inhibited the MMP-9 activity induced by MCAO. Samples were obtained from ipsilateral cortex at 2, 8, and 24 hrs after MCAO. Lane M, positive control for MMP-2 (72 kDa) and MMP-9 (92 kDa); lane 1, sham-operated control; lane 2, 2 hrs after MCAO; lane 3, 2 hrs after MCAO, EGCG-pretreated; lane 4, 8 hrs after MCAO; lane 5, 8 hrs after MCAO, EGCG-pretreated; lane 6, 24 hrs after MCAO; lane 7, 24 hrs after MCAO, EGCG-pretreated.

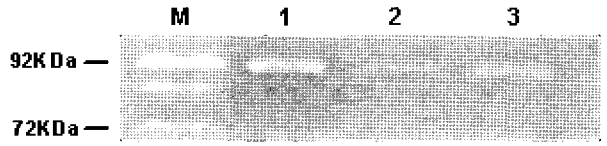

Fig. 4. Gelatin-zymogram showing the effect of EGCG and GM6001 on MMP-9 activity. EGCG or GM6001 inhibited the MMP-9 activity induced by MCAO. Samples were obtained from ipsilateral cortex at 24 hrs after MCAO. Lane M, positive control for MMP-2 (72 kDa) and MMP-9 (92 kDa); lane 1, 24 hrs after MCAO; lane 2, 24 hrs after MCAO, EGCG-pretreated; lane 3, 24 hrs after MCAO, GM 6001-pretreated.

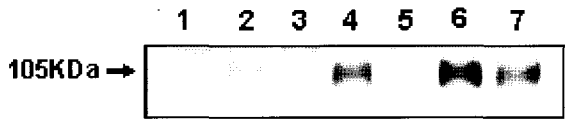

Fig. 5. Western blot analysis showing the effect of EGCG on MMP-9 expression. EGCG-pretreatment inhibited the MMP-9 expression by MCAO. Samples were obtained from ipsilateral cortex at 2, 8, and 24 hrs after MCAO. Lane 1, sham-operated control; lane 2, 2 hrs after MCAO; lane 3, 2 hrs after MCAO, EGCG-pretreated; lane 4, 8 hrs after MCAO; lane 5, 8 hrs after MCAO, EGCG- pretreated; lane 6, 24 hrs after MCAO; lane 7, 24 hrs after MCAO, EGCG-pretreated.

DISCUSSION

In this study, we induced focal brain ischemia using mouse permanent MCAO model. There are several reports to show that EGCG could protect experimental animals against ischemic insults. EGCG at dosages of 25, 50 mg/kg significantly and dose-dependently reduced hippocampal CA1 pyramidal neuronal death in gerbil transient global ischemia model (Lee et al, 2000; Hong et al, 2001). Even delayed administration of EGCG also attenuated the hippicampal neuronal death in gerbils (Lee et al, 2003). Choi et al (2004) reported that 50 mg/kg EGCG significantly reduced infarct in rat transient MCAO model which consisted of 2-hour ischemia and 22-hour reperfusion. Lee et al (2004a) also showed that EGCG attenuated infarct

in gerbil brain ischemia/reperfusion model. EGCG has been shown to display potent antioxidant properties (Lee et al, 2003).

The neuroprotective effects of EGCG on the ischemic insults of the above mentioned reports may mainly be due to its antioxidative action. The major difference between transient and permanent ischemic insults is the existence of reperfusion period. One of major pathophysiologic mechanism of ischemia/reperfusion is the production of reactive oxygen species (ROS) during reperfusion period (Chan, 1996). Therefore, we tried to use the ischemia model in which ROS production is minimally involved in pathogenesis of the insults, in order to rule out the antioxidative action of EGCG, and we used permanent MCAO model. Even though the magnitude of inhibitory effects was smaller than those in ischemia/reperfusion model, EGCG significantly attenuated brain infarct in our permanent ischemia model. To the best of our knowledge, we hardly found any reports to demonstrate the protective effects of EGCG in permanent focal brain ischemia.

GM 6001, a well-known MMP inhibitor, also significantly reduced the brain infarct, and the magnitude of anti-infarction was almost the same as that of EGCG. In gelatin-zymogram, EGCG and GM 6001 attenuated the increased MMP-9 activities which was induced by permanent MCAO. These results demonstrated that EGCG inhibited not only MMP-9 activity, but also MMP-9 protein expression. Although EGCG attenuated brain infarct and MMP-9 activity both of which were induced by MCAO in the present study, it is difficult to interpret an anti-MMP-9 activity of EGCG as the anti-ischemic action.

Taken together, the above findings demonstrate that the anti-ischemic action of EGCG involves its inhibitory effect on MMP-9 in mouse permanent focal cerebral ischemia.

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