Inhibitory effect of esculetin on migration, invasion and matrix metalloproteinase-9 expression in TNF-α-induced vascular smooth muscle cells

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Abstract. Esculetin, a potent non-competitive inhibitor of lipoxygenase, has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation. However, the effect of esculetin on the matrix metalloproteinase-9 (MMP-9) regulation responsible for cell migration and invasion has not been previously investigated. The results of the present study showed the esculetin (12.5-25 μg/ml) induced the inhibition of migration and invasion in tumor necrosis factor-α (TNF-α)-treated VSMC, as demonstrated by a matrigel invasion assay and wound healing analysis. However, esculetin did not affect cell viability in TNF-α-treated VSMC under 0-25 μg/ml concentration conditions. In addition, both zymographic and immunoblot experiments showed that esculetin suppressed the TNF-α-induced expression of MMP-9 in VSMC in a dose-dependent manner. Furthermore, the treatment of cells with esculetin decreased the activity of the TNF-α-induced MMP-9 promoter, which was driven by a luciferase reporter. Finally, esculetin reduced the binding activities of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are cis-elements present in the promoter of the MMP-9 gene, in TNF-α-treated VSMC. Taken together, these results demonstrated that esculetin decreased the migration and invasion of cells by suppressing MMP-9 expression, which subsequently reduced the binding activities of NF-κB and AP-1 in TNF-α-treated VSMC. These novel findings provide basic information for effective therapeutic treatment with esculetin for atherosclerotic disease.

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Introduction

The growth of arterial lesions, such as vascular remodeling, is a critical step in the pathogenesis of atherosclerosis and restenosis after balloon angioplasty (1,2). The proliferation and migration of vascular smooth muscle cells (VSMC) from the media toward the intima are involved in vascular remodeling in response to injury (1,2). VSMC migration requires the degradation of extracellular matrix (ECM) proteins, which is regulated by matrix metalloproteinases (MMPs) (2,3). Studies on the role of MMPs in the basement degradation of VSMC (3) have focused on the gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa). The expression of MMP-2 and MMP-9 was increased in balloon injury models, which resulted in vascular lesion formation (4). Previous studies have suggested that MMP-9 plays a pluripotent role in the growth of neointimal lesions in atherosclerosis (5-8). For instance, MMP-9 showed low expression in normal arteries, but was up-regulated in rat carotid arteries 6 h after injury, and this condition continued for 6 days (6). Accumulated data have emphasized that the loss of MMP-9 indicates significantly retarded arterial lesion growth through the control of VSMC migration in MMP-9$^{-/-}$ animals (7,8). As a result, a great deal of effort is being directed toward the development of therapies that inhibit VSMC migration to prevent atherosclerosis or restenosis (9).

The formation of vascular lesions occurs during several critical pathological processes, which are associated with the accumulation of inflammatory cells and the secretion of cytokines (10). The presence of cytokine tumor necrosis factor-α (TNF-α) has been found in VSMC after balloon injury (11). In addition, previous studies demonstrated TNF-α expression in intimal VSMC and in atherosclerotic plaques (12,13). Cumulative data have demonstrated that MMP-9 is activated by TNF-α in VSMC (14,15). Additional studies identified the essential proximal activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) binding sites in MMP-9 expression after TNF-α-treatment (15,16).

Esculetin is an active compound derived from *Artemisia scoparia* (17), and is known to be a potent non-competitive inhibitor of lipoxygenase (17). Esculetin also reportedly possesses a number of biological activities, such as

anti-oxidant activity, suppression of xanthine oxidase activity, platelet aggregation, inhibition of human leukemia cell growth and anti-tumor activity (18-22). In addition, esculetin is known to inhibit VSMC prolifertion *in vitro* and neointima formation *in vivo* (23,24). However, there is no evidence thus far of the possible effects of esculetin on migration and MMP-9 regulation in VSMC.

Data from the present study demonstrated the role of MMP-9 expression in the inhibition of VSMC migration and invasion.

Materials and methods

Materials. Esculetin was purchased from Sigma (St. Louis, MO, USA). TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). Polyclonal MMP-9 antibody was obtained from Chemicon International (Temecula, CA, USA).

Cell culture. VSMC were isolated from Sprague-Dawley rats. These explants were grown in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml gentamycin and 50 μl/ml amphotericin-B at 37˚C in a humidified 5% CO_2 atmosphere.

Invasion assay. VSMC were resuspended with TNF-α (100 ng/ml) alone or together with esculetin in 100 μl of medium and placed in the upper chamber of the transwell plate. The cells were then incubated for 24 h. Cells had to pass through a polycarbonate memebrane with a thin layer of ECM-like material and 8-μM pores. The ability of VSMC to invade the ECM-like material was determined with a commercial cell invasion assay kit (Chemicon), as described previously (25).

Wound migration assay. VSMCs were plated on 6-well dishes and grown to 90% confluence in 2 ml of growth medium. The cells were damaged using a 2-mm-wide tip, then treated with TNF-α (100 ng/ml) alone or together with esculetin. The cells were allowed to migrate and photographs were captured through an inverted microscope (magnification, x40).

Immunoblotting assays. Growth-arrested VSMC were treated with TNF-α in the presence or absence of esculetin for specified time periods at 37˚C. Cell lysates were prepared and immunoblotting was performed as described previously (15).

Transient transfection. Each plasmid was transfected into VSMC using a Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions (15). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activity was standardized to β-galactosidase activity.

Zymography. The conditioned medium was electrophoresed in a polyacrylamide gel containing 1 mg/ml gelatin. The gel was then washed at room temperature for 2 h with 2.5% Triton X-100, and then at 37˚C overnight in a buffer containing 10 mM $CaCl_2$, 150 mM NaCl and 50 mM Tris-HCl (pH 7.5). The gel was stained with 0.2% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Creation of MMP-9 promoter reporter construct. A 0.7-kb segment in the 5'-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (Accession no. D10051): 5'-ACATTTGCCCGAGCTCCTGAAG (forward/*Sac*I) and 5'-AGGGGCTGCCAGAAGCTTATGGT (reverse/*Hind*III). The pGL2-Basic vector containing a polyadenylation signal upstream of the luciferase gene was used to construct the expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the *Sac*I/*Hind*III site of the pGL2-Basic vector (15). The size of the PCR products was confirmed by electrophoresis and DNA sequencing.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared essentially as described elsewhere (15). Cultured cells were collected by centrifugation, washed and suspended in a buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF for 15 min at 4˚C.

The nuclear extract (10-20 μg) was pre-incubated at 4˚C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the -79 MMP-9 cis-element of interest. The sequences were as follows: AP-1, CTGACCCCTGAGTCAGCACTT; and NF-κB, CAGTGGAATTCCCCAGCC. The reaction mixture was then incubated at 4˚C for 20 min in a buffer containing 25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol, with 2 μg of poly dI/dC and 5 fmol ($2x10^4$ cpm) of a Klenow end-labeled (^{32}P-ATP) 30-mer oligonucleotide, which spans the DNA binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4˚C on a 6% polyacrylamide gel using a TBE (89 mM Tris, 89 mM boric acid and 1 mM EDTA) running buffer. The gel was rinsed with water, dried and exposed to X-ray film overnight.

Statistical analysis. When appropriate, data were expressed as the mean ± SE. Data were analyzed by factorial ANOVA and Fisher's least significant difference test where appropriate. Statistical significance was set at $P<0.05$.

Results

Esculetin suppressed TNF-α-induced migration and invasion in VSMC. The effect of esculetin on TNF-α-induced VSMC invasion and migration was first examined. As shown in Fig. 1A, treatment with TNF-α increased VSMC invasion through a Matrigel-coated membrane after 48 h of culture, compared to the untreated control. This TNF-α-induced VSMC invasion was strongly inhibited by the addition of esculetin (12.5-25 μg/ml) in a dose-dependent manner (Fig. 1A). In addition, TNF-α treatment significantly induced the migration of VSMC, as determined using a wound migration assay (Fig. 1B). The migration induced by TNF-α was also attenuated by esculetin in a dose-dependent manner (Fig. 1B). However, under 25 μg/ml concentration conditions, esculetin did not affect cell viability (data not shown).

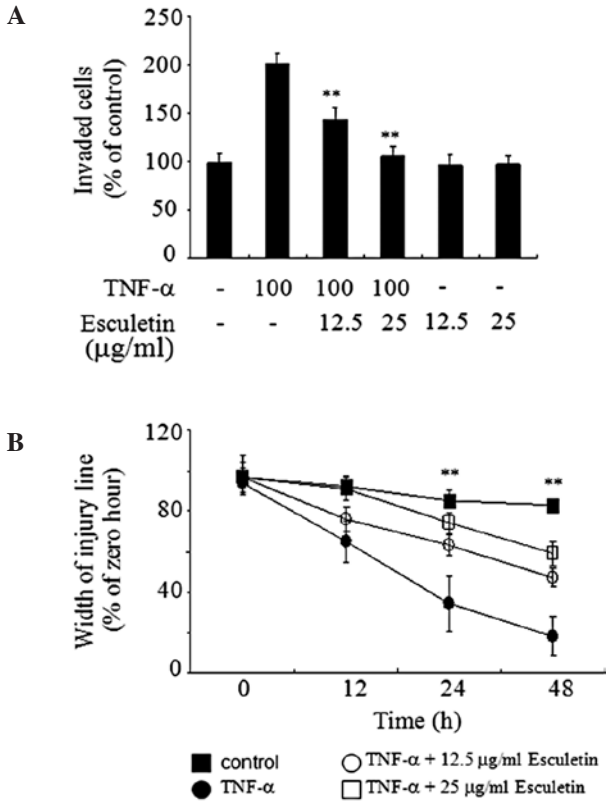

Figure 1. Effect of esculetin on inhibition of TNF-α-induced invasion and migration in VSMC. (A) Cells seeded in serum-free medium were incubated on Matrigel-coated membranes of transwell plates and then exposed to 12.5 and 25 μg/ml esculetin for 40 min, followed by the addition of TNF-α (100 ng/ml) for 48 h. Results are expressed as the number of invaded cells relative to the untreated control, as determined from three independent experiments. **P<0.05 compared to TNF-α treatment. (B) Confluent cells in serum-free medium were incubated with 12.5 and 25 μg/ml esculetin for 40 min, followed by the addition of TNF-α (100 ng/ml). The widths of injury lines made in cells were measured at 0, 12, 24 and 48 h. Results are expressed as the widths of injury lines relative to the untreated control at 0 h, as determined from three independent experiments. **P<0.05 compared to TNF-α treatment.

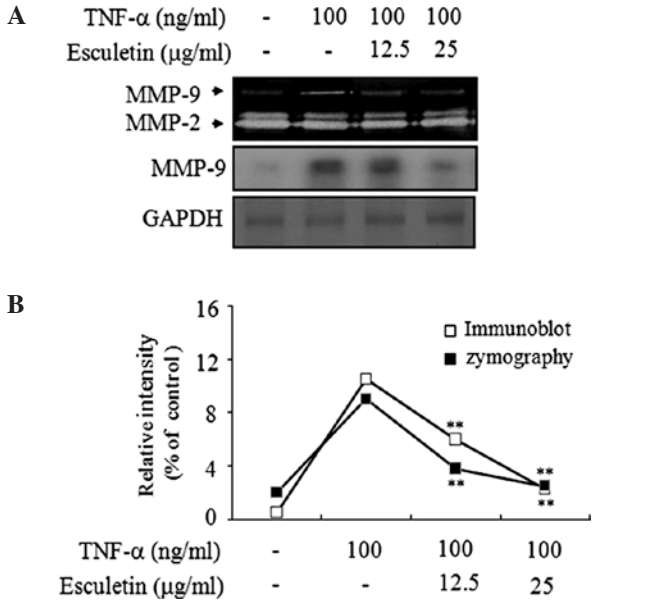

Figure 2. Effect of esculetin on TNF-α-induced MMP-9 expression in VSMC. (A) Cells seeded in serum-free medium were incubated with TNF-α (100 ng/ml) for 24 h after 40 min of pre-treatment at the indicated concentrations (μg/ml) of esculetin, and then the conditioned media were analyzed zymographically for MMP activity. Similarly, an immunoblot analysis was conducted with antibodies specific for MMP-9. **P<0.05 compared to TNF-α treatment. (B) The expression of GAPDH was normalized by densitometric analysis for the relative intensity of MMP-9 expression. **P<0.05 compared to TNF-α treatment.

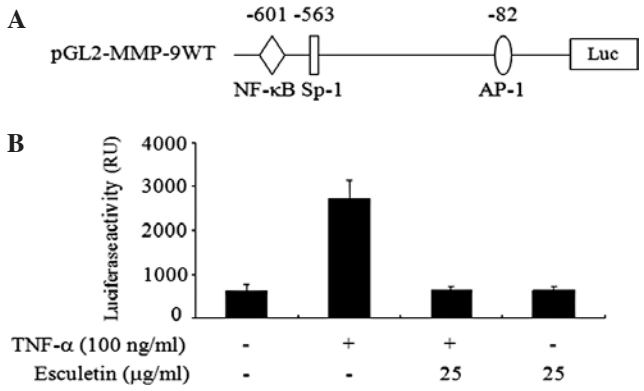

Figure 3. Inhibitory effect of esculetin on TNF-α-induced MMP-9 promoter activity in VSMC. (A) Schematic presentation of the MMP-9 promoter, showing the cis-regulatory elements. (B) pGL2-MMP-9WT, which contains 710 bp of the 5'-promoter region of the MMP-9 gene, was transfected into VSMC together with β-gal for the normalization of transfection efficiency. After 40 min of pre-treatment with or without the indicated concentrations of esculetin, the cells were incubated with TNF-α (100 ng/ml) for an additional 24 h. Luciferase activity was determined from cell lysates, as described in Materials and methods. **P<0.05 compared to TNF-α treatment.

Esculetin inhibited TNF-α-induced MMP-9 expression. To determine the effect of esculetin on TNF-α-induced MMP-9 expression in VSMC, a gelatin zymographic assay was carried out. As shown in Fig. 2, treatment of cells with TNF-α induced MMP-9 expression in VSMC in the conditioned media. By contrast, the expression of MMP-2 was not affected by TNF-α. Increased MMP-9 expression in response to TNF-α was attenuated by the addition of esculetin in a dose-dependent manner. Similar results were observed using immunoblotting. In addition, esculetin did not significantly affect the level of MMP-2 (Fig. 2A).

Esculetin inhibited MMP-9 promoter activity. To determine the exact mechanism of TNF-α-induced MMP-9 expression at the transcriptional level, MMP-9 promoter activity was measured by transient transfection. TNF-α-mediated MMP-9 promoter activation was performed in the absence or presence of esculetin using a pGL2-MMP-9WT plasmid in which the 710 bp human MMP-9 promoter segment was linked to the luciferase reporter gene (Fig. 3A). Treatment of cells transiently transfected with the pGL2-MMP-9WT plasmid with TNF-α markedly increased the reporter activity of this promoter construct (Fig. 3B). In addition, esculetin treatment caused a marked reduction in MMP-9 promoter activity induced by TNF-α (Fig. 3B).

Esculetin attenuated TNF-α-induced MMP-9 promoter activity via suppression of NF-κB and AP-1 binding activity.

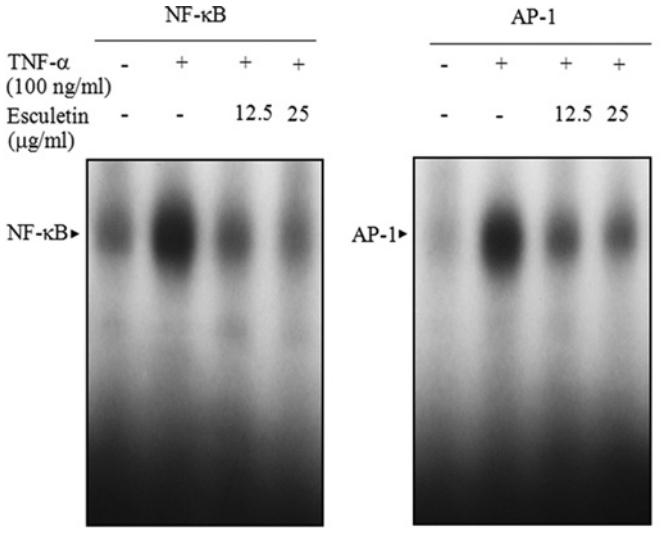

Figure 4. Dose-dependent inhibitory effect of esculetin on the DNA binding activity of NF-κB and AP-1 in TNF-α-induced VSMC. Cells incubated in serum-free medium were pre-treated with the indicated concentrations of esculetin for 40 min, followed by TNF-α (100 ng/ml) stimulation for 24 h. After incubation, nuclear extracts from the cells were subjected to EMSA for activated NF-κB and AP-1 using radiolabeled oligonucleotide probes.

Transcriptional binding motifs, such as NF-κB, Sp-1 and AP-1, have been identified as transactivators of MMP-9 (Fig. 3A). Previous studies have demonstrated that TNF-α induces the activation of the MMP-9 promoter via NF-κB and AP-1 binding sites in VSMC (15,16). To determine whether these two types of transcription factors are involved in the inhibition of MMP-9 expression induced by esculetin, the effect of esculetin on the DNA binding activity of NF-κB and AP-1 was examined using EMSA. As shown in Fig. 4, TNF-α significantly enhanced the binding activities of the NF-κB and AP-1 motifs. In addition, treatment of cells with esculetin inhibited TNF-α-induced NF-κB and AP-1 binding activity in VSMC (Fig. 4). These results demonstrate that the transcription factors NF-κB and AP-1 are likely to be involved in the repressive effect of esculetin on TNF-α-induced MMP-9 expression.

Discussion

Esculetin reportedly has beneficial effects on cardiovascular disease *in vitro* and *in vivo* (23,24). However, the exact molecular mechanisms of MMP-9 regulation in response to esculetin remain unknown. The purpose of the present study was to investigate the mechanism of MMP-9 regulation by esculetin in TNF-α-treated VSMC.

First, the effect of esculetin on cell viability was examined using an MTT assay. The data showed that esculetin treatment (<25 μg/ml concentration) had no effect on cell death in TNF-α-treated VSMC (data not shown). Next, the effects of esculetin on VSMC invasion and migration were investigated, because VSMC migration is a critical event in the development of neointima after vascular remodeling (1,2). The inhibition of the invasion and migration of cells was observed following esculetin treatment in TNF-α-treated VSMC in dose-dependent manner. MMPs play important roles in the degradation of the ECM to enable pathophysiological processes, such as vascular remodeling (2,3). Among the various MMPs previously examined, it has generally been concluded that MMP-9 is one of the most important factors in VSMC physiology and vascular remodeling, and its expression can be stimulated by cytokine TNF-α (5-8,14,15). Elevated levels of MMP-9 were found to be integrally linked to the invasion and migration of VSMC (6-8). To understand how esculetin mediates the inhibition of VSMC migration and invasion in the presence of TNF-α, we focused on MMP-9 expression. Treatment with esculetin inhibited TNF-α-stimulated MMP-9 expression in a dose-dependent manner, as shown by zymographic and immunoblot analyses. The inhibition of MMP-9 expression induced by esculetin provides a convincing explanation for its suppression of VSMC migration and invasion. However, esculetin treatment did not affect MMP-2 expression. These results suggest that esculetin-mediated suppression of MMP-9 expression may cooperate with reduced VSMC migration and invasion via the degradation of the ECM.

The control of MMP-9 expression is largely regulated at the transcriptional stage (26). The present study found that esculetin significantly suppressed TNF-α-induced transcriptional activity using MMP-9 promoter analysis. There are several cis-elements, NF-κB, AP-1 and Sp-1 motifs, located at -710 bp in the promoter region of the MMP-9 gene (15,16,27). Previous studies of the MMP-9 promoter found that pivotal NF-κB and AP-1 binding sites in TNF-α-treated VSMC are essential (15,16). Increasing evidence suggests a critical regulatory role of natural resources in the inhibition of MMP-9 expression in several cell lines (28-30). In the same vein, to further investigate the transcriptional regulation of esculetin in the suppression of MMP-9 expression in VSMC, the role of cis-elements in the esculetin-mediated inhibition of the MMP-9 gene in response to TNF-α was examined. The results showed that esculetin markedly inhibited NF-κB and AP-1 binding activity induced by TNF-α. This suggests that esculetin inhibits MMP-9 expression through the suppression of NF-κB and AP-1 binding activity in TNF-α-treated VSMC. A previous study showed that esculetin suppressed MMP-1, MMP-3 and MMP-13 expression in interleukin-1α-treated chondrocyte cells (31). Another report demonstrated the effect of esculetin on the inhibition of MMP-1 and MMP-3 expression in rabbit joint cartilage explants (32). However, the present study is the first to show the regulatory effect of esculetin in the inhibition of MMP-9 expression via the decreased binding activity of the NF-κB and AP-1 motifs.

In conclusion, the present data demonstrate that esculetin (<25 μg/ml) potently inhibits the migration and invasion of TNF-α-treated VSMC without toxicity, by suppressing MMP-9 expression through the down-regulation of NF-κB and AP-1 binding activity. These findings suggest a potential therapeutic use of esculetin in the treatment of cardiovascular diseases and atherosclerosis.

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