Suppression of Oxidized LDL-Induced PDGF Receptor β Activation by Ginkgo Biloba Extract Reduces MMP-1 Production in Coronary Smooth Muscle Cells

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Aim: An extract of Ginkgo Biloba L. was shown to have preventive effects on cardiovascular disorders, but the molecular mechanisms of its actions remain to be elucidated. Since matrix metalloproteinases (MMPs) are implicated in the rupture of atherosclerotic plaques and the subsequent occurrence of acute coronary syndrome, we examined the effects of a leaf extract (Ginkgolon-24) on the production of MMP-1 in human coronary smooth muscle cells stimulated with oxidized low-density lipoprotein (oxLDL) and 4-hydroxynonenal, which are factors proposed to play a pivotal role in atherogenesis.

Methods: The production of MMP-1 and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 were estimated by immunoblotting. The tyrosine-phosphorylated form of platelet-derived growth factor receptor β (PDGFR-β) was analyzed by immunoprecipitation of the receptor followed by immunoblotting.

Results: oxLDL and 4-hydroxynonenal accelerated the production of MMP-1 with the preceding phosphorylation of ERK1/2 and PDGFR-β. Pretreatment with Ginkgolon-24 inhibited the production of MMP-1 and phosphorylation of ERK1/2 induced by oxLDL and 4-hydroxynonenal, but did not affect the production and phosphorylation induced by phorbol ester. Furthermore, Ginkgolon-24 prevented tyrosine phosphorylation of the receptor induced by oxLDL and 4-hydroxynonenal.

Conclusion: These results suggest that Ginkgo Biloba extract suppresses the oxLDL- and 4-hydroxynonenal-induced production of MMP-1, probably through the inhibition of PDGFR-β activation in human coronary smooth muscle cells.


Key words: Ginkgo Biloba extracts, Metalloproteinase-1, Oxidized low-density lipoprotein, Smooth muscle cells

Introduction

The progression of atherosclerosis is a complex process, which is mediated by numerous cellular responses in atheroma-associated cells, such as macrophages and vascular smooth muscle cells (SMCs), stimulated with cytokines, atherogenic lipoproteins including oxidized low-density lipoprotein (oxLDL), or bioactive oxidized lipids including oxysterols and 4-hydroxynonenal (4-HNE). Among the cellular responses, the production of matrix metalloproteinases (MMPs) is involved in the degradation of extracellular matrix proteins, such as collagen and fibronectin, leading to the migration of SMCs into the intima and to the rupture of atherosclerotic plaques.

The more than 20 types of MMPs identified to date have been classified into groups including collagenases, gelatinases, stromelysins, membrane-type MMPs, and matrilysins. The expression of collagenases (MMP-1, -8, and -13) is increased in atherosclerotic lesions, especially the shoulder region of vulnerable plaques, in humans and animal models. MMP-1 localizes mainly with SMCs and macrophages, while MMP-8 and -13 are expressed by...
neutrophils and macrophages, respectively. Several cytokines have been shown to stimulate the expression of MMP-1 in SMCs and macrophages. Recently, we reported that oxLDL and 4-HNE accelerated the production of MMP-1 through the activation of platelet-derived growth factor receptor \( \beta \) (PDGFR-\( \beta \)) in human coronary SMCs (hCSMCs). Since oxLDL and bioactive oxidized lipids are well known to mediate the progression and rupture of atherosclerotic lesions, it is conceivable that prevention of atherogenic factor-induced MMP-1 production could be beneficial in atherosclerosis.

An extract of *Ginkgo Biloba L.*, a mixture mainly composed of flavonoid glycosides (glycosides of quercetin and kaempferol) and terpenoids (ginkgolides and bilobalide), is shown to suppress the generation of reactive oxygen species (ROS) as well as the oxidation of LDL, which is a critical event in atherogenesis. The extract and its ingredients, especially ginkgolides, also exhibit an antagonistic effect on platelet-activating factor. These effects of the extract are responsible, in part, for its protective effects on tissue abnormalities including myocardial ischemia-reperfusion injury and ischemic brain damage, being beneficial to patients with cardiovascular, cerebrovascular, and neurological disorders. A previous study showed that Ginkgo Biloba extracts inhibit serum-induced proliferation of rat vascular SMCs, and prevent intimal hyperplasia in balloon-injured abdominal aorta in rabbits fed with high-cholesterol diet; however, it is unclear whether these inhibitory effects are ascribable only to the antioxidative effects or antagonistic effects on platelet-activating factor. Thus, the molecular mechanisms underlying the beneficial effects on cardiovascular disorders, especially atherosclerosis, remain to be fully elucidated. Flavonoids including quercetin and kaempferol, of which glycosides are major constituents of Ginkgo Biloba extracts, are shown to exhibit inhibitory effects on tyrosine kinases, including growth factor receptors. Furthermore, our recent study showed that oxLDL-induced production of MMP-1 is mediated by the activation of PDGFR-\( \beta \), but occurs independently of the generation of ROS in hCSMCs. Taking these findings into account, in the present study, we examined the effects of the leaf extract on the production of MMP-1 in hCSMCs stimulated with oxLDL and 4-HNE.

**Materials and methods**

**Materials**

Antibodies against extracellular signal-regulated kinase (ERK) 1/2, phosphorylated ERK1/2, PDGFR-\( \beta \) (sc-432), and the phosphorylated tyrosine residue of PDGFR-\( \beta \) (sc-17173), and protein A-agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MCDB131 medium, basic fibroblast growth factor, epidermal growth factor, insulin, and the anti-MMP-1 antibody were from Sigma (St. Louis, MO, USA). PD98059, 4-HNE, and AG1295 were from Calbiochem (La Jolla, CA, USA). Human native LDL (BT-903) was from Biomedical Technologies Inc. (Stoughton, MI, USA). The leaf extract of *Ginkgo Biloba L.* (Ginkgolgon-24), a mixture containing flavonoid glycosides (14.2% quercetin-3-\( \beta \)-rutinoside, 11.3% kaempferol-3-\( \beta \)-rutinoside, and 2.0% isorhamnetin-3-\( \beta \)-rutinoside) and terpene lactones (2.3% bilobalide, and 3.8% ginkgolide A, B, and C), was donated by Tokiwa Phytochemical Co., Ltd. (Chiba). All other reagents were obtained from Wako Pure Chemical Industries (Osaka).

**Cell Culture**

hCSMCs (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were cultured in MCDB131 medium supplemented with 5% heat-inactivated fetal bovine serum, 1 ng/mL basic fibroblast growth factor, 0.5 ng/mL epidermal growth factor, 5 \( \mu \)g/mL insulin, 100 units/mL penicillin, and 100 \( \mu \)g/mL streptomycin in collagen (type I)-coated culture dishes (Asahi Techno Glass, Corp. Tokyo). The cells, between the 6th to 8th passage, were plated in 35-mm dishes at \( 2 \times 10^5 \) cells, and made quiescent by incubating with MCDB131 medium for 24h.

**Preparation of oxLDL**

Human native LDL was dialyzed against phosphate-buffered saline at 4\( ^\circ \)C, and oxidized as described previously. Briefly, LDL (2.5 mg protein/mL) was oxidized with 10 \( \mu \)M CuSO\(_4\) at 37\( ^\circ \)C for 3h. The oxLDL contained 10-15 nmol thiobarbituric acid-reactive substances/mg protein. The oxLDL was further dialyzed against phosphate-buffered saline containing 200 \( \mu \)M EDTA at 4\( ^\circ \)C, and used within 10 days.

**Immunoblot Analysis**

hCSMCs were treated and stimulated with various reagents, including oxLDL and 4-HNE, as described in the figure legends. The culture medium and cells, as samples, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. The separated proteins were transferred onto a nitrocellulose membrane. Antibodies against MMP-1, ERK1/2, and phosphorylated ERK1/2 were applied. The bound antibodies were visualized using a peroxidase-conjugated secondary antibody and enhanced...
Effects of Ginkgolon-24 on the Production of MMP-1 and the Activation of ERK1/2

Stimulation of hCSMCs with oxLDL (50 μg/mL) for 24h induced an increase in MMP-1 protein in the culture medium, as estimated by immunoblotting. The amount of MMP-1 was estimated by measuring the density of the MMP-1 band. Each value represents the mean ± SEM of three separate experiments. *p<0.01, relative to the response of oxLDL or 4-HNE alone.

Immunoprecipitation of PDGFR-β

hCSMCs were incubated with Ginkgolon-24 for 30 min or AG1295 for 1h in the presence of 5 mM Na₃VO₄, and stimulated with oxLDL or 4-HNE as described in the figure legends. After being washed, the cells were lysed and subjected to immunoprecipitation of PDGFR-β as described previously. The lysate was incubated with anti-PDGFR-β antibodies overnight at 4°C and further with protein A-agarose for 3h. After centrifugation, the pellet was subjected to immunoblotting (a 5% gel) with antibodies against PDGFR-β or the phosphorylated tyrosine residue of PDGFR-β. Representative results of three separate experiments are shown. To estimate the phosphorylation of PDGFR-β, the density of the bands for PDGFR-β and its phosphorylated form on a film was analyzed as above. Results are adjusted for PDGFR-β levels and expressed in arbitrary units (mean ± SEM, n = 3).

Statistical Analysis

Values are expressed as the mean ± SEM. Data were analyzed with one-way analysis of variance and Dunnett’s test or with Student’s t-test. P<0.05 was considered statistically significant.
Effects of Ginkgolon-24 on the phosphorylation of ERK1/2 induced by oxLDL (A), 4-HNE (B), and PMA (C).

hCSMCs were treated with various concentrations of Ginkgolon-24 (G-24) for 30 min, and then stimulated with (+) or without (−) oxLDL (50 μg/mL), 4-HNE (20 μM), or PMA (200 nM) for 10 min. ERK1/2 and its phosphorylated form (p-ERK1/2) in the cells were analyzed by immunoblotting. The results are representative of three separate experiments.

G-24 (μg/mL) 0 2.5 5 10 oxLDL − + + + − + + + PMA − + + + PD98059 (μM) 0 5 10

Effects of Ginkgolon-24 on the phosphorylation of ERK1/2 induced by oxLDL (A) and 4-HNE (B). hCSMCs were treated with various concentrations of Ginkgolon-24 (G-24) or PD98059 (5 μM) for 30 min, and then stimulated with (+) or without (−) PMA (200 nM) for 24h. MMP-1 in the culture medium was analyzed by immunoblotting. The results are representative of three separate experiments.

Fig. 2. Effects of Ginkgolon-24 on the phosphorylation of ERK1/2 induced by oxLDL (A), 4-HNE (B), and PMA (C).

MMP-1

As shown in Fig. 4A, stimulation with oxLDL (50 μg/mL) for 10 min induced the tyrosine phosphorylation of PDGFR-β, as estimated based on the immunoprecipitation of PDGFR-β and immunoblotting with antibodies against PDGFR-β bearing a phospho-tyrosine residue. Ginkgolon-24 (10 μg/mL) apparently suppressed the tyrosine phosphorylation of PDGFR-β induced by oxLDL (Fig. 4A) to a similar extent to AG1295 (100 μM), an inhibitor of tyrosine kinase of PDGFR (Fig. 4C). Furthermore, 4-HNE (40 μM) stimulated the tyrosine phosphorylation of PDGFR-β, which was also prevented by Ginkgolon-24 (5 or 10 μg/mL) (Fig. 4B).

Discussion

The formation of atherosclerotic plaques is associated with the development of a lipid core and the formation of fibrous caps to cover the lipid core, events which are mediated by oxLDL-induced biological responses, including the transformation of macrophages into lipid-laden foam cells and the migration of SMCs into the intima. In the advanced stages of atherosclerosis, the stability of atherosclerotic plaques decreases with the thinning of the fibrous caps, the resulting vulnerable plaques being susceptible to rupture. Disruption of the plaques triggers the formation of a thrombus, which is the cause of acute coronary syndromes. Among these events in the progression of atherosclerosis, the migration of SMCs and the rupture of plaques occur with the degradation of extracellular matrix proteins, such as collagen, catalyzed by MMPs including MMP-1. The expression of MMP-1 is increased in human atherosclerotic plaques, where SMCs and macrophages are colocalized. Our recent study with hCSMCs showed that the production of MMP-1 was accelerated by oxLDL and 4-HNE, which are atherogenic factors. Since collagenases, including MMP-1, are the only MMPs that can degrade native collagen, an increase in MMP-1 is a criti-

Effects of Ginkgolon-24 on the Activation of PDGFR-β

The activation of ERK1/2 is triggered by the stimulation of receptors for growth factors such as PDGFR-β in a variety of cell types including SMCs, oxLDL and 4-HNE have been shown to induce the activation of PDGFR-β in hCSMCs and rabbit arterial SMCs. We further reported that inhibition of the oxLDL-induced activation of PDGFR-β resulted in the suppression of ERK1/2 phosphorylation and MMP-1 production, suggesting that PDGFR-β mediates the ERK1/2-dependent production of MMP-1 induced by oxLDL; therefore, the effects of Ginkgol-
cal cellular response in the progression of atherosclerosis. It is possible, therefore, that preventing the production of MMP-1 induced by atherogenic factors could be beneficial in cases of atherosclerosis. The current study showed that Ginkgolon-24, an extract of Ginkgo Biloba L., suppressed the production of MMP-1 induced by oxLDL and 4-HNE in hSCMs, suggesting that this inhibitory activity is also involved in the therapeutic effects of the extract on cardiovascular disorders and intimal hyperplasia in balloon-injured abdominal aorta in an animal model.

With regard to the mechanisms responsible for the production of MMP-1, the induction of MMP-1 gene expression was mediated by ERK1/2 in canine fibroblast-like synoviocytes. The activation of ERK1/2 was mediated by the activation of receptors for growth factors such as PDGFR-β in SMCs. We and other investigators showed that oxLDL stimulated the activation of PDGFR-β in hSCMs and rabbit arterial SMCs. Our recent study further demonstrated that the production of MMP-1 induced by oxLDL and 4-HNE was suppressed under conditions where the activation of PDGFR-β and ERK1/2 was inhibited by an inhibitor of tyrosine kinase (AG1295 and genistein) and by an inhibitor of mitogen-activated protein kinase/ERK kinase (PD98059), respectively. Moreover, suppression of the oxLDL-induced activation of PDGFR-β resulted in the inhibition of ERK1/2 phosphorylation. Considering these findings, it is conceivable that oxLDL accelerates the production of MMP-1 through the phosphorylation of PDGFR-β followed by the activation of ERK1/2. The present study showed that Ginkgolon-24 inhibited the activation of PDGFR-β and ERK1/2 induced by oxLDL and 4-HNE. However, the extract had no effect on the activation of ERK1/2 or the production of MMP-1 induced by PMA, which activates ERK1/2 independently of the activation of receptors, indicating that the Ginkgolon-24–caused inhibition of MMP-1 production is not ascribable to a direct suppressive effect on the activation of ERK1/2. Consequently, the present results suggest that Ginkgolon-24 inhibits the oxLDL- and 4-HNE-induced production of MMP-1 by suppressing the activation of PDGFR-β.

The leaf extract of Ginkgo Biloba L. has beneficial effects on the prevention and treatment of cardiovascular, cerebrovascular, and neurological disorders. These therapeutic effects are thought to result, in part, from antioxidative actions. In fact, the extract has been shown to act as a scavenger of ROS. Our previous study with rat mesangial cells demonstrated that Ginkgolon-24 suppresses the oxLDL-induced production of fibronectin by preventing the generation of ROS. In contrast, oxLDL-stimulated hSCMs produce MMP-1 independently of the generation of ROS, because N-acetylcysteine, an antioxidant, had no effect on the oxLDL-induced production of MMP-1 or activation of ERK1/2 despite inhibiting the generation of ROS. It is possible, therefore, that the antioxidative effects of Ginkgolon-24 are not involved in its inhibitory effect on the oxLDL-induced production of MMP-1 in SMCs. Major constituents of Ginkgo Biloba extracts are quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside, which are flavonoid glycosides. Some flavonoids (quercetin, kaempferol) and

Fig. 4. Inhibitory effects of Ginkgolon-24 and AG1295 on the phosphorylation of PDGFR-β.

hSCMs were treated with various concentrations of Ginkgolon-24 (G-24) for 30 min (A, B) or AG1295 (100 μM) for 1 h (C), and then stimulated with (+) or without (−) oxLDL (50 μg/mL) (A, C) or 4-HNE (40 μM) (B) for 10 min. PDGFR-β (PDGFR) and its tyrosine-phosphorylated form (p-PDGFR) in the cells were detected by the immunoprecipitation of PDGFR-β followed by immunoblotting. The degree of PDGFR phosphorylation was estimated by measuring the density of PDGFR and p-PDGFR bands (mean ± SEM). Each value represents the mean ± SEM of three separate experiments. *p < 0.02, **p < 0.01, relative to the response to oxLDL or 4-HNE alone.
isoflavonoids (genistein) are used as inhibitors of tyrosine kinase as well as antioxidants. In our recent report, genistein inhibited the tyrosine phosphorylation of PDGFR-β induced by oxLDL and 4-HNE in hCSMCs\(^5\). Quercetin and kaempferol were shown to prevent the tyrosine phosphorylation of epidermal growth factor receptor in A431 tumor cells\(^6\) and of PDGFR-β in rat aortic vascular SMCs\(^3\), respectively. Considering these findings and their structures, we speculate that constituents of Ginkgo Biloba extracts, probably quercetin and/or kaempferol derivatives, act as inhibitors of tyrosine kinase of PDGFR-β rather than antioxidants, thus exhibiting inhibitory effects on the oxLDL- and 4-HNE-induced tyrosine phosphorylation of PDGFR-β and subsequent production of MMP-1.

In summary, the present study showed that Ginkgolon-24, an extract of *Ginkgo Biloba L.*, suppressed the production of MMP-1 and activation of PDGFR-β and ERK1/2 in response to oxLDL and 4-HNE in hCSMCs. The inhibition of MMP-1 production probably results from an impairment of PDGFR-β activation. In addition to the antioxidative actions of Ginkgo Biloba extracts, the suppressive effects of Ginkgolon-24 shown here may be involved in their beneficial effects.

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