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The leaf extract of *Ginkgo Biloba L.* suppresses oxidized LDL-stimulated fibronectin production through an antioxidant action in rat mesangial cells

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1 The leaf extract of *Ginkgo Biloba L.* exhibits a variety of pharmacological effects through an antioxidant action. We examined the effects of the leaf extract (Ginkgolon-24) on the production of fibronectin induced by oxidized low-density lipoprotein (oxLDL) in rat mesangial cells.

2 Stimulation with oxLDL accelerated the production of fibronectin with the preceding generation of reactive oxygen species (ROS). Pretreatment with Ginkgolon-24 inhibited the oxLDL-induced fibronectin production as well as ROS generation.

3 oxLDL also elicited the activation of SP-1, nuclear factor-κB, and cAMP response element-binding protein, which are transcription factors involved in the fibronectin production. Among these activated transcription factors, Ginkgolon-24 inhibited the activation of SP-1 only.

4 Furthermore, 7-ketocholesterol, an oxidized lipid in oxLDL particles, induced the production of fibronectin and the activation of SP-1, which were also suppressed by Ginkgolon-24.

5 These results suggest that the leaf extract of *Ginkgo Biloba L.* inhibits the oxLDL-induced production of fibronectin probably through inhibitory effects on ROS generation and SP-1 activation in rat mesangial cells.
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Abbreviations: CREB, cAMP response element-binding protein; DCFH, 2′,7′-dichlorodihydrofluorescein; LDL, low-density lipoprotein; NF-κB, nuclear factor-κB; oxLDL, oxidized LDL; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance

Introduction

Glomerular lipid deposition resulting from hyperlipidemia, a common feature in patients with nephrotic syndrome and diabetic mellitus, is associated with the development and progression of chronic renal diseases. Accumulation of atherogenic lipoproteins such as low-density lipoprotein (LDL) within the mesangium is implicated in the proliferation of mesangial cells and the overproduction by mesangial cells of mesangial extracellular matrix proteins, which are major pathobiological processes in progressive glomerular damage (Keane *et al*., 1988; Diamond, 1991; Wanner *et al*., 1997; Kamanna, 2002). While LDL stimulates the production of extracellular matrix proteins, such as fibronectin and collagen, in mesangial cells (Rovin & Tan, 1993; Lee *et al*., 1999), it undergoes oxidative modification mediated by mesangial cells (Wheeler *et al*., 1994), resulting in the formation of oxidized LDL (oxLDL). In addition to LDL, oxLDL has been shown to accelerate the production of fibronectin in human (Chana & Wheeler, 1999), mouse (Roh *et al*., 1998), and rat (Chen *et al*., 2002) mesangial cells. Thus, oxLDL in the glomeruli is also involved in the pathogenesis of glomerular damage, leading to glomerulosclerosis (Diamond, 1991; Wanner *et al*., 1997; Kamanna, 2002).

An extract of the leaves of *Ginkgo Biloba L.*, a mixture mainly composed of flavonoid glycosides and terpenoides (ginkgolides and bilobalide), has been shown to exhibit a variety of pharmacological actions. The leaf extract acts as a scavenger of reactive oxygen species (ROS), that is, superoxide radical (Pincemail *et al*., 1989), peroxyl radical (Maitra *et al*., 1995), and nitric oxide (Marcocci *et al*., 1994), hence suppressing oxidation of LDL (Maitra *et al*., 1995; Yan *et al*., 1995) and cellular lipids (Rong *et al*., 1996). Previously, we reported that the leaf extract suppresses platelet aggregation induced by *tert*-butyl hydroperoxide and hydrogen peroxide through its antioxidant action (Akiba *et al*., 1998). Furthermore, the extract and its ingredients exhibit an antagonistic effect on platelet-activating factor (Lamant *et al*., 1987), and inhibitory effects on the expression of inducible nitric oxide synthase as well as nitric oxide production (Kobuchi *et al*., 1997; Cheung *et al*., 1999; 2001). They also exhibit protective effects on tissue abnormalities that include myocardial ischemia–reperfusion injury (Shen *et al*., 1998), ischemic brain damage (Zhang *et al*., 2000), and neuronal apoptosis (Ahlemeyer *et al*., 1999). These effects are supposed to be

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beneficial in cardiovascular, cerebrovascular, and neurological disorders (DeFeudis, 1991; Yoshikawa *et al*., 1999).

ROS are involved in the production of extracellular matrix proteins, thus, being associated with the pathogenesis of renal diseases (Shah, 1995; Mason & Wahab, 2003). A previous report showed that oxLDL induces generation of ROS accompanied by the production of fibronectin in rat mesangial cells (Chen *et al*., 2002). Recently, we demonstrated that oxLDL-induced fibronectin production is dependent on ROS generation and subsequent activation of SP-1, a transcription factor involved in fibronectin production in rat mesangial cells (Akiba *et al*., 2003a). Therefore, it is possible that the leaf extract of *Ginkgo Biloba L.* exhibits inhibitory effects on oxLDL-induced production of fibronectin through its antioxidant action. In order to clarify this possibility, the present study was undertaken to examine the effects of the leaf extract on oxLDL-accelerated production of fibronectin in rat mesangial cells.

Methods

Cell culture

Rat mesangial cells were prepared as described previously (Hayama *et al*., 2002). Briefly, mesangial cells were obtained from a culture of glomeruli isolated from Sprague–Dawley rats (100–150 g) by sieving, and grown in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 100 units ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 5 $\mu g\,ml^{-1}$ insulin, 5 $\mu g\,ml^{-1}$ transferrin, and 5 $ng\,ml^{-1}$ selenious acid in collagen (type I)-coated culture dishes (Asahi Techno glass, Japan). The cells, derived from one preparation, were used between the third and sixth passages. For the following experiments, mesangial cells were plated in 35-mm dishes at 1×10^5 cells or in 100-mm dishes at 5×10^5 cells in RPMI 1640 medium, and made quiescent by incubation for 48 h. The quiescent cells were placed in fresh Dulbecco's modified Eagle's medium for the following experiments. Each experiment was performed using three separate cell preparations.

Preparation of oxLDL

The oxidation of LDL was performed as described previously (Akiba *et al*., 2003b). Human native LDL, prepared from plasma from multiple donors, was dialyzed against PBS at 4°C. LDL (2.5 mg protein ml^{-1}) was oxidized with 10 μM $CuSO_4$ at 37°C for 3 h. The degree of LDL oxidation was evaluated by a thiobarbituric acid-reactive substance (TBARS) assay according to the method of Yagi (1976). The oxLDL contained 10–15 nmol TBARS mg $protein^{-1}$, while native LDL (before oxidation) contained 0.6–1.2 nmol TBARS mg $protein^{-1}$. The oxLDL was further dialyzed against PBS containing 200 μM EDTA at 4°C, and was used within 10 days.

Immunoblot analysis for fibronectin

The production of fibronectin by mesangial cells was determined as described previously (Hayama *et al*., 2000). The quiescent mesangial cells (35-mm dishes) were treated with Ginkgolon-24 for 1 h, and stimulated with oxLDL or 7-ketocholesterol for 12 h as described in the figure legends. The extracellular medium was removed and centrifuged. The supernatant obtained was solubilized with a buffer (2% sodium dodecyl sulfate, 1 mM EDTA, 2 mM EGTA, 2% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, and 100 mM Tris–HCl, pH 6.8), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 5% gel. The separated proteins were transferred onto a nitrocellulose membrane. Anti-rat fibronectin antibodies (1/2000 dilution) were applied. The bound antibodies were visualized using a peroxidase-conjugated secondary antibody (1/20,000 dilution) and enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech). To estimate the amount of fibronectin, the density of the fibronectin band on a film was analyzed using a film scanner and NIH image analysis software.

Measurement of the generation of ROS

The quiescent mesangial cells (100-mm dishes) were collected and resuspended at 1×10^5 cells ml^{-1} in Hanks' solution. The cells were treated with Ginkgolon-24 at 37°C for 1 h, and further incubated with DCFH diacetate (100 μM), as a probe, for 5 min. The cells were stimulated with oxLDL for 30 min. The fluorescence of 2′,7′-dichlorofluorescein resulting from oxidation of DCFH was measured with a flow cytometer (FACSCalibur with CellQuest software, Becton-Dickson Biosciences). The generation of ROS was expressed as the mean fluorescence intensity of 10,000 cells determined in each sample.

Electrophoretic mobility shift assay

The quiescent mesangial cells (100-mm dishes) were treated with Ginkgolon-24 for 1 h, and stimulated with oxLDL or 7-ketocholesterol for 3 h as described in the figure legends. After the cells had been washed, nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, U.S.A.), and subjected to an electrophoretic mobility shift assay using a commercial assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. Briefly, oligonucleotides containing a consensus binding site for SP-1, nuclear factor-κB (NF-κB), or cAMP response element-binding protein (CREB) were prelabeled with [γ-^{32}P]ATP. The assay was carried out by incubating the nuclear extracts (3 μg protein) with each labeled oligonucleotide for 20 min in a final volume of 10 μl. The reaction mixture was subjected to native polyacrylamide gel electrophoresis on a 4% gel. After the gel had dried, radioactive signals were detected with an image analyzer (BAS-5000, Fuji Photo Film Co., Ltd, Japan).

Materials

The leaf extract of *Ginkgo Biloba L.* (Ginkgolon-24), a mixture containing flavonoid glycosides (14.2% quercetin-3-*O*-rutinoside, 11.3% kaempferol-3-*O*-rutinoside, and 2.0% isorhamnetin-3-*O*-rutinoside) and terpene lactones (2.3% bilobalide, and 3.8% ginkgolide A, B, and C), was donated by Tokiwa Phytochemical Co., Ltd (Chiba, Japan). *N*-acetylcysteine, 7-ketocholesterol, and fetal bovine serum were purchased from Sigma (St Louis, MO, U.S.A.). Human native LDL

(BT-903) was from Biomedical Technologies Inc. (Stoughton, MI, U.S.A.), the anti-rat fibronectin antibody was from Biogenesis Ltd (Poole), 2′,7′-dichlorodihydrofluorescein (DCFH) diacetate was from Molecular Probes, Inc. (Eugene, OR, U.S.A.), and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was from Amersham Pharmacia Biotech (Buckinghamshire). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Statistical analysis

Values are expressed as the mean ± s.e.m. Data were analyzed by one-way analysis of variance and Dunnett's test. $P<0.05$ was considered statistically significant.

Results

Effects of Ginkgolon-24 on oxLDL-induced fibronectin production and ROS generation

As shown in Figure 1a, stimulation of rat mesangial cells with oxLDL (20 μg ml^{-1}) for 12 h induced a significant increase in the production of fibronectin. In this study, we examined the effects of the leaf extract of *Ginkgo Biloba L.* (Ginkgolon-24), known to exhibit an antioxidant action, on the fibronectin production. The results in Figure 1 showed that, when mesangial cells were pretreated with Ginkgolon-24 (10–30 μg ml^{-1}), the oxLDL (20 μg ml^{-1})-induced fibronectin production was suppressed dose-dependently with an apparent inhibition at 30 μg ml^{-1}. We confirmed that *N*-acetylcysteine (1–5 mM), an antioxidant, also inhibited the oxLDL (20 μg ml^{-1})-induced fibronectin production (data not shown), as shown recently (Akiba *et al*., 2003a). Figure 2 indicates that oxLDL (20 μg ml^{-1}) induced the generation of ROS, which was estimated by flow cytometric analysis with DCFH as a probe. As shown

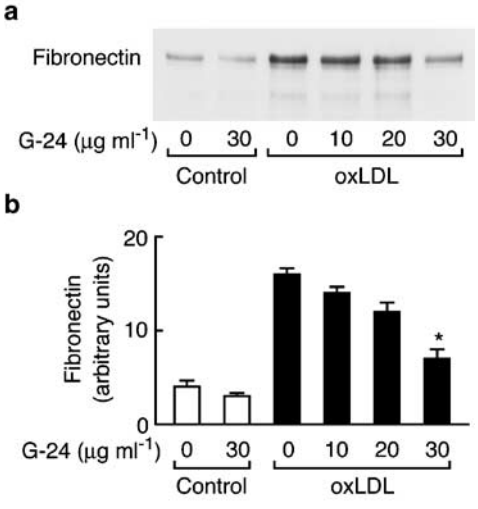

Figure 1 Inhibitory effects of Ginkgolon-24 on oxLDL-induced fibronectin production. (a) Rat mesangial cells were treated with various concentrations of Ginkgolon-24 (G-24) for 1 h, and then stimulated with oxLDL (20 μg ml^{-1}) or vehicle (Control) for 12 h. (b) The density of the fibronectin band shown in (a) was measured. Each value shown in (b) represents the mean ± s.e.m. of three separate experiments. *$P<0.01$, relative to the response of oxLDL alone.

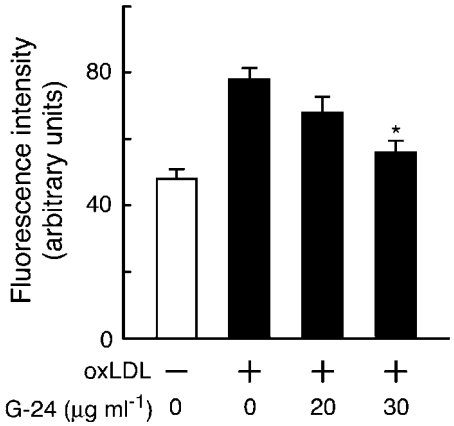

Figure 2 Inhibitory effects of Ginkgolon-24 on oxLDL-induced ROS generation. Rat mesangial cells were treated with or without Ginkgolon-24 (G-24, 20 or 30 μg ml^{-1}) for 1 h, and further incubated with DCFH diacetate (100 μM) for 5 min. The cells were stimulated oxLDL (20 μg ml^{-1}) or vehicle for 30 min. The fluorescence of 2′,7′-dichlorofluorescein resulting from DCFH oxidation was measured. Each value represents the mean ± s.e.m. of three separate experiments. *$P<0.05$, relative to the response of oxLDL alone.

in Figure 2, Ginkgolon-24 (20 or 30 μg ml^{-1}) apparently prevented the oxLDL-induced ROS generation. These results suggest that Ginkgolon-24 suppresses oxLDL-stimulated production of fibronectin probably through impairment of ROS generation.

Effects of Ginkgolon-24 on oxLDL-induced SP-1 activation

Since the production of fibronectin is mediated through the activation of several transcription factors including SP-1 (Dean *et al*., 1987; Suzuki *et al*., 1998), NF-κB (Lee *et al*., 2002; Chen *et al*., 2003), and CREB (Dean *et al*., 1990; Reddy *et al*., 2002), we examined the effects of Ginkgolon-24 on the activation of the transcription factors in response to oxLDL. The results in Figure 3 demonstrated that stimulation with oxLDL (20 μg ml^{-1}) induced the activation of SP-1, NF-κB, and CREB, as estimated by electrophoretic mobility shift assay. Pretreatment with Ginkgolon-24 (30 μg ml^{-1}) inhibited markedly the oxLDL-induced SP-1 activation, but had no effect on the activation of NF-κB or CREB (Figure 3). These results indicate that inhibition by Ginkgolon-24 of the oxLDL-stimulated production of fibronectin occurred in parallel with suppression of SP-1 activation.

Effects of Ginkgolon-24 on 7-ketocholesterol-induced fibronectin production and SP-1 activation

Oxidized lipids in oxLDL particles act as a biological activator responsible for oxLDL-induced events. Recently, we reported that, when rat mesangial cells were stimulated with 7-ketocholesterol, 25-hydroxycholesterol, and 4-hydroxynonenal, as oxidized lipids observed in oxLDL, the production of fibronectin was accelerated only by 7-ketocholesterol (Akiba *et al*., 2003a). Therefore, the present study examined the effects of Ginkgolon-24 on fibronectin production in response to 7-ketocholesterol. As shown in Figure 4a and b, pretreatment with Ginkgolon-24 (10–30 μg ml^{-1}) significantly suppressed 7-ketocholesterol (5 μM)-induced fibronectin production. We

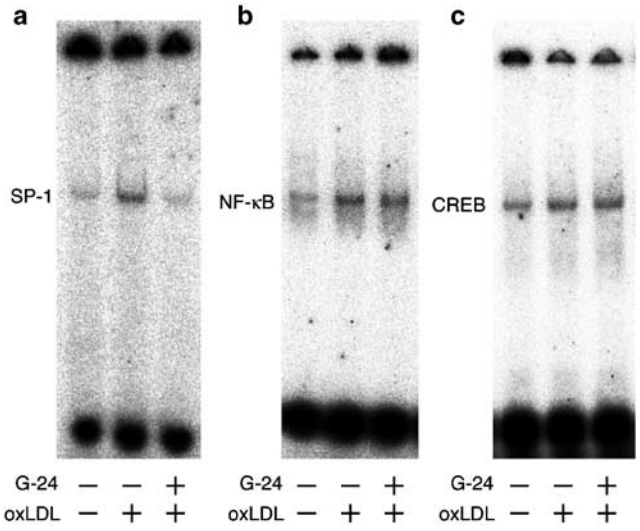

Figure 3 Effects of Ginkgolon-24 on oxLDL-induced activation of SP-1, NF-κB, and CREB. Rat mesangial cells were treated with or without Ginkgolon-24 (G-24, 30 μg ml^{-1}) for 1 h, and then stimulated oxLDL (20 μg ml^{-1}) or vehicle for 3 h. Nuclear extracts from the cells were subjected to electrophoretic mobility shift assay using ^{32}P-labeled oligonucleotides containing a consensus-binding site for SP-1 (a), NF-κB (b), or CREB (c). Radioactive signals were detected with an image analyzer. The results are representative of three separate experiments.

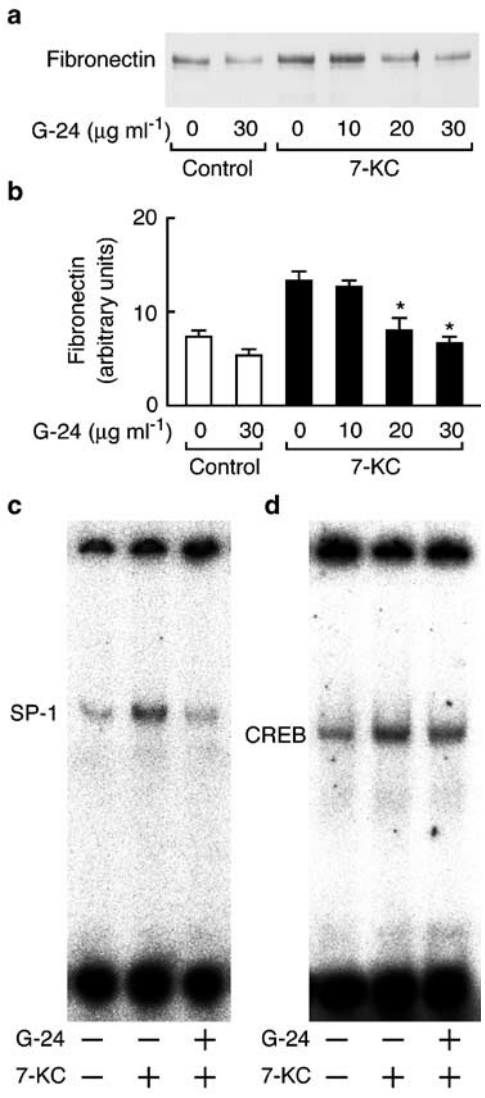

Figure 4 Inhibitory effects of Ginkgolon-24 on 7-ketocholesterol-induced fibronectin production and SP-1 activation. (a) Rat mesangial cells were treated with various concentrations of Ginkgolon-24 (G-24) for 1 h, and then stimulated with 7-ketocholesterol (7-KC, 5 μM) or vehicle (Control) for 12 h. Fibronectin in the extracellular medium was analyzed by immunoblotting. (b) The density of the fibronectin band shown in (a) was measured. Each value shown in (b) represents the mean $\pm$ s.e.m. of three separate experiments. *$P<0.01$, relative to the response of 7-KC alone. (c,d) Cells were treated with or without Ginkgolon-24 (G-24, 30 μg ml^{-1}) for 1 h, and then stimulated with 7-KC (5 μM) or vehicle for 3 h. Nuclear extracts from the cells were subjected to electrophoretic mobility shift assay using ^{32}P-labeled oligonucleotides containing a consensus-binding site for SP-1 (c) or CREB (d). Radioactive signals were detected with an image analyzer. The results are representative of three separate experiments.

further observed that Ginkgolon-24 (30 μg ml^{-1}) almost completely inhibited 7-ketocholesterol (5 μM)-induced SP-1 activation, but did not affect CREB activation (Figure 4c and d). Under our experimental conditions, NF-κB was not activated upon stimulation with 7-ketocholesterol (results not shown).

Discussion

A number of studies have shown that 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 supposed to result from antioxidant actions (Yoshikawa *et al.*, 1999). Since ROS are involved in the deposition of mesangial extracellular matrix proteins associated with the pathogenesis of progressive decline in renal function (Shah, 1995; Mason & Wahab, 2003), it is possible that antioxidant effects of the leaf extract may be beneficial in chronic renal diseases. Therefore, the present study examined the effects of the leaf extract (Ginkgolon-24) on the production of fibronectin, a matrix component, using rat mesangial cells stimulated with oxLDL, which is implicated in the progression of glomerular damage (Diamond, 1991; Wanner *et al.*, 1997; Kamanna, 2002).

It has been shown that stimulation of human mesangial cells with hydrogen peroxide induces the production of fibronectin and collagen (Iglesias-de La Cruz *et al.*, 2001), suggesting that ROS is involved in the production of extracellular matrix proteins in mesangial cells. A previous report showed that oxLDL induces the generation of ROS as well as the production of fibronectin in rat mesangial cells (Chen *et al.*, 2002). We showed recently that oxLDL-induced production of fibronectin is inhibited by *N*-acetylcysteine, known to act as an antioxidant, in rat mesangial cells (Akiba *et al.*, 2003a). These findings suggest that fibronectin production in response to oxLDL is dependent on the generation of ROS. The present study demonstrated that pretreatment of rat mesangial cells with Ginkgolon-24 suppressed oxLDL-induced fibronectin production as well as ROS generation. The leaf extract has been shown to act as a scavenger of ROS (Pincemail *et al.*, 1989; Maitra *et al.*, 1995). In fact, Ginkgolon-24 used here contains flavonoids as antioxidants. Considering these findings, we suggest that Ginkgolon-24 inhibits the

oxLDL-induced production of fibronectin through suppression of ROS generation by radical scavenging in mesangial cells.

The transcription factors implicated in the production of fibronectin include SP-1 (Dean *et al*., 1987; Suzuki *et al*., 1998), NF-κB (Lee *et al*., 2002; Chen *et al*., 2003), and CREB (Dean *et al*., 1990; Reddy *et al*., 2002). In this study, we demonstrated that Ginkgolon-24 did not affect the oxLDL-induced activation of NF-κB or CREB despite the suppression of fibronectin production. Recently, we reported that *N*-acetylcysteine has no effect on the activation of NF-κB or CREB induced by oxLDL (Akiba *et al*., 2003a). These findings suggest that activation of the two transcription factors is probably necessary but insufficient to produce fibronectin in response to oxLDL. A similar inability of the leaf extract of *Ginkgo Biloba L*. has been shown in RAW264.7 macrophages, in which the extract has no effect on cytokine-induced NF-κB activation (Kobuchi *et al*., 1997). In contrast, our recent report showed that *N*-acetylcysteine inhibits the activation of SP-1 induced by oxLDL in rat mesangial cells (Akiba *et al*., 2003a). Similarly, interleukin 4-induced activation of SP-1 is inhibited by antioxidants in human umbilical vein endothelial cells (Lee *et al*., 2001). These indicate that the activation of SP-1 is dependent on the generation of ROS. Furthermore, we reported that mithramycin A, an inhibitor of SP-1, inhibits oxLDL-induced fibronectin production (Akiba *et al*., 2003a), suggesting that oxLDL stimulates fibronectin production through ROS-dependent SP-1 activation. The present study showed that Ginkgolon-24 suppressed the oxLDL-induced activation of SP-1 under conditions where ROS generation was prevented. Based on these findings, we concluded that the suppression by Ginkgolon-24 of oxLDL-induced fibronectin production results from impairment of ROS-dependent SP-1 activation.

Biological activities of oxLDL are thought to be mediated, in part, through the action of oxidized lipids, such as 7-ketocholesterol (Carpenter *et al*., 1994), in oxLDL particles. Recently, we reported that 7-ketocholesterol stimulates fibronectin production and SP-1 activation, which are inhibited by *N*-acetylcysteine, in rat mesangial cells, and further that mithramycin A suppressed 7-ketocholesterol-induced fibronectin production (Akiba *et al*., 2003a). In the present study, we found that Ginkgolon-24 also prevented the 7-ketocholesterol-induced fibronectin production as well as SP-1 activation. Based on these findings, we speculate that 7-ketocholesterol in oxLDL particles is involved in the Ginkgolon-24-sensitive production of fibronectin in response to oxLDL. On the other hand, it has been shown that mesangial cells induce the oxidation of LDL (Wheeler *et al*., 1994), and further that the leaf extract of *Ginkgo Biloba L*. suppresses LDL oxidation (Maitra *et al*., 1995; Yan *et al*., 1995). Thus, the leaf extract exhibits inhibitory effects on the generation of oxidized lipids and on their ability to induce fibronectin production in mesangial cells. It is possible, therefore, that the leaf extract efficiently interferes with the overproduction of extracellular matrix proteins by mesangial cells associated with glomerular deposition of oxLDL.

In summary, the present study suggests that Ginkgolon-24, a leaf extract of *Ginkgo Biloba L*., suppresses SP-1-dependent fibronectin production in response to oxLDL, through impairment of ROS generation in rat mesangial cells. The effects of Ginkgolon-24 may be beneficial in hyperlipidemia- and ROS-dependent progression of glomerular damage.

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