

Effects of *Ginkgo biloba* Extract on the Proliferation of Vascular Smooth Muscle Cells In Vitro and on Intimal Thickening and Interleukin-1 β Expression After Balloon Injury in Cholesterol-Fed Rabbits In Vivo

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Abstract Restenosis may develop in response to cytokine activation and smooth muscle cell proliferation. *Ginkgo biloba* extract (EGb) has been used to treat cardiovascular and cerebrovascular diseases. In the present study, the effects of EGb on the growth of cultured vascular smooth muscle cells (VSMC), as well as on the expression of interleukin-1 β (IL-1 β) and the intimal response in balloon-injured arteries of cholesterol-fed rabbits, were investigated. Using bromodeoxyuridine incorporation as an index of cell proliferation, EGb was found to inhibit serum-induced mitogenesis of cultured rat aorta VSMC in a dose-dependent manner. In vivo, EGb and probucol (positive control) reduced the atheroma area in thoracic aortas of male New Zealand white rabbits fed a 2% cholesterol diet for 6 weeks with balloon denudation of the abdominal aorta being performed at the end of the third week. Intimal hyperplasia, expressed as the intimal/medial area ratio, in the abdominal aortas was significantly inhibited in the both the EGb group (0.61 ± 0.06) and the probucol group (0.55 ± 0.03) compared to the C group (0.87 ± 0.02). In the balloon-injured abdominal aorta, both EGb and probucol significantly reduced IL-1 β mRNA and protein expression and the percentage of proliferating cells. The inhibitory effects of EGb on the intimal response might be attributed to its antioxidant capacity. EGb may have therapeutic potential for the prevention of restenosis after angioplasty. *J. Cell. Biochem.* 85: 572–582, 2002.

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Key words: *Ginkgo biloba* extract (EGb 761); restenosis; interleukin-1 β (IL-1 β); smooth muscle cell; antioxidants

Percutaneous coronary intervention has achieved wide acceptance in the treatment of

coronary artery disease, but is complicated by the development of proliferative restenosis within 3–6 months [Serruys et al., 1988]. Restenosis is characterized by phenotypic conversion of medial smooth muscle cells and their migration into the subendothelium and subsequent proliferation [Hanke et al., 1990; Chen et al., 1997]. The development of restenotic lesions has also been considered as an immunoinflammatory reaction, triggered by a cascade involving the activation of growth factors and cytokines [Clausell et al., 1995; Oemar, 1999]. Interleukin-1 β is a proinflammatory cytokine, which actively stimulates vascular smooth muscle cell proliferation [Libby et al., 1988], leukocyte adhesion to the endothelium [Cavender

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et al., 1986], modulation of low density lipoproteins (LDL) metabolism [Beutler and Cerami, 1985], and secretion of extracellular matrix protein [Montesano et al., 1984], suggesting that it might play an important role in the pathogenesis of restenosis following angioplasty.

Ginkgo biloba extract (EGb761), a defined, but complex, mixture of active compounds extracted from *Ginkgo biloba* leaves, has commonly been used as a therapeutic agent for cardiovascular and neurological disorders [Kleijnen and Knipschild, 1992]. A number of mechanisms have been proposed for the beneficial actions of EGb; these include increased blood flow [Varga et al., 1999], inhibition of platelet aggregation [Akiba et al., 1998], suppression of NO production [Marcocci et al., 1994], and its antioxidant capacity [Yan et al., 1995]. In addition, EGb provides protection in model systems of oxidative stress, including cardiac [Shen and Zhou, 1995; Varga et al., 1999] and retinal [Szabo et al., 1992] ischemia-reperfusion injury. Recent studies have provided considerable support for the occurrence of free radical and lipid peroxidation reactions in peripheral arterial and venous diseases and in central nervous system injury, and it is possible that antioxidants may have antiproliferative and antisecretory effects, thereby attenuating the processes leading to atherosclerosis or restenosis after angioplasty. This concept has been clearly demonstrated using a well-characterized lipid antioxidant, probucol, which reduces neointimal formation after oversized balloon injury in swine and rabbit models of restenosis [Ferns et al., 1992; Schneider et al., 1993] and reduces postangioplasty restenosis in humans [Tardif et al., 1997]. However, the effects of EGb on restenosis is largely unknown. In the present study, its effects on the proliferation of cultured VSMC and on neointimal thickening and IL-1 β expression after balloon injury of aortas in cholesterol-fed rabbits were investigated.

MATERIALS AND METHODS

Inhibition of LDL Oxidation

Probucol was dissolved as a stock solution (2.0 mM) in ethanol, then diluted to 50 μ mol/L in 10% aqueous ethanol before use. EGb stock solution (3.7% in purified water) was purchased from Dr. Willmar Schwabe Arzneimittel Inc., Germany. In vitro LDL oxidation was carried out in a 96-well microtiter plate at 37°C. A 50 μ l

aliquot of LDL (0.9 mg cholesterol/ml), followed by various volume of EGb or probucol were added to the wells, and was adjusted to 100 μ l solution with phosphate-buffered saline (PBS, pH 7.4). The final concentrations of probucol and EGb were equal to 1, 2, 3, 4, 5, 6, 7 μ g/ml and 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7 μ g/ml, respectively. The wells were incubated for 1 h at 37°C, then LDL oxidation was initiated by adding CuSO₄ to a final concentration of 10 μ M. After incubation for 2 h at 37°C, 150 μ l EDTA (2 mM) was added. A 100 μ l portion of the mixture was then transferred to a minivial containing 0.9 ml of 2-propanol and the precipitate was removed by centrifugation for 10 min at 13,600g. The concentration of conjugated dienes in the supernatant was determined by absorption at 234 nm. The potency of EGb or probucol in inhibiting Cu⁺²-induced LDL oxidation was calculated as: $(A - A_{Cu^{2+}}) / A_{Cu^{2+}} \times 100\%$, where A is the absorbance of the sample treated with copper and either EGb or probucol. $A_{Cu^{2+}}$, the absorbance of the sample treated only with copper; and Ac, the absorbance with no treatment.

Vascular Smooth Muscle Cell Cultures

VSMCs were isolated from the thoracic aortas of 200–300 g male Sprague Dawley rats by an enzymatic digestion method [Gunther et al., 1982]. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), and Fungizone (2.5 μ g/ml) in a 5% CO₂ incubator and were used at the 3–5 passages, when they were plated in 96-well culture plates and maintained in medium supplemented with 10% FBS to achieve 80–90% confluency. They were then rendered quiescent by incubation in serum-free medium for 24 h. Subsequently, most wells of these subconfluent cultures were incubated for 24 h in fresh medium supplemented with 2% FBS alone or containing increasing concentrations of EGb (1, 2.5, 5, 15, 25, 50, 100, 200, and 400 μ g/ml), while a few were incubated in fresh medium without FBS or EGb as controls. DNA synthesis was measured using a 5-bromo-2'-deoxyuridine (BrdU)-Labeling and Detection Kit (BrdU-Kit III, Boehringer Mannheim, Ingelheim, Germany) [Kahler et al., 1997]. BrdU (10 μ M) was added to triplicate cultures of cells, then, after 2 h, the cells were fixed and nucleases added to partially digest cellular DNA. Anti-BrdU antibody-peroxidase conjugate was then added for 30 min at 37°C to bind

to BrdU, then the signal was developed with a water-soluble peroxidase substrate enhanced by a substrate enhancer (1 mg/ml) and measured at 405 nm with a reference wavelength at 495 nm on an enzyme-linked immunosorbent assay reader. In preliminary experiments, cell viability after BrdU treatment was measured using the Trypan blue exclusion test and was always > 98%.

Animal Experiments

Twenty-six male New Zealand white rabbits, 3 months of age and weighing about 2.5 kg, were used. After one week on a commercial rabbit chow diet (Scientific Diet Services, Essex, UK), the animals were placed on a 2% cholesterol diet (Purina Mills Inc., St. Louis, MO, USA) and randomly allocated to one of three groups: (1) the negative control group with no drug treatment (group C; n = 8); (2) the positive control group treated by oral ingestion of probucol (100 mg/kg body wt/day) (group P; n = 8); and (3) the experimental group treated by oral ingestion of EGb tablet (16 mg/kg body wt/day) (group EGb; n = 10). The experimental procedures and animal care and handling conformed to the guidelines of the American Physiological Society. The animal study protocols were approved by the Review Board of National Science Council. At the end of the third week of the high cholesterol diet, the animals were anesthetized by intramuscular injection of xylazine (5 mg/kg) and ketamine hydrochloride (35 mg/kg) and surgery and balloon denudation of the lower abdominal aorta performed under sterile conditions as described previously [Feng et al., 1999]. After the surgery, the animals continued with the same diet and treatments and were sacrificed at the end of the sixth experimental week. The abdominal aortas were then harvested, gently dissected free of adhering tissues, rinsed with ice-cold PBS, and cut into six segments. A small part of each arterial segment was taken, immersion-fixed with 4% buffered paraformaldehyde, paraffin-embedded, then cross-sectioned for morphometry and immunohistochemistry, while the remaining larger portion was immediately frozen in liquid nitrogen for RNA and protein isolation. The thoracic aortas were also collected and stained with Sudan IV solution to visualize the atherosclerotic lesion areas. Sudanophilic areas were measured quantitatively by computer-assisted planimetry and the extent of the lesions

expressed as a proportion of the total surface area (surface area of lesions/total surface area of the thoracic aorta).

Biochemical Measurements

Blood samples for biochemical measurements were collected from each animal before, and at 3 and 6 weeks after the start of, the high cholesterol diet. Samples were separated by centrifugation, and the serum stored at -20°C prior to analysis. Serum total cholesterol and triglyceride were measured using Merck assay kits (Parmstadt, Germany). Serum glucose, blood urea nitrogen (BUN), creatinine, glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transferase (GPT), and γ -glutamyl transferase (GGT) were also measured.

Hematoxylin-Eosin Staining and Morphometry

Arterial segments were fixed in 4% buffered formaldehyde for 3 h at 4°C and subsequently dehydrated in sequential alcohol washes, cleared in xylene, and embedded in paraffin. One 5 μm thick cross-section was taken from each segment of the abdominal aorta and stained with hematoxylin and eosin. Morphometric analysis of 6 arterial cross-sections per animal was performed using an LV-2 Image Analyzer (Winhow Instruments, Taipei, Taiwan). The intimal and medial thicknesses of each arterial cross-section specimen were measured at eight separate points, the intima/media thickness ratio calculated at each point, and the overall intimal/medial thickness ratio for each section expressed as the mean of these eight values. The intimal and medial areas for each arterial cross-section specimen were also measured and the intimal/medial area ratio determined.

RNA Isolation and Northern Blot Analysis

The aortas were homogenized and total RNA isolated from the aortic lysate by repeated phenol-chloroform extraction followed by ethanol precipitation [Chomczynski and Sacchi, 1987]. IL-1 β mRNA expression was examined using Northern blot analysis as described previously [Feng et al., 1999]. Following electrophoresis, the RNA was transferred onto a nylon membrane and fixed by ultraviolet irradiation, the membranes were hybridized with ^{32}P -labeled IL-1 β cDNA probes, then the blots were washed, covered with Saran wrap, and exposed to X-ray film at -70°C for autoradiography. The intensity of each hybridized band on XAR

film was measured using optical densitometry (Molecular Dynamics, Sunnyvale, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels served as the internal standard to normalize the IL-1 β signals [Alwine et al., 1997]. The DNA fragments used as probes were a 0.75 kb EcoRI and Pst I fragment containing full-length IL-1 β cDNA and a 1.3 kb PstI fragment containing GAPDH cDNA (American Type Culture Collection, Rockville, MD).

Immunohistochemical Analysis

To identify the cell type undergoing proliferation and IL-1 β expression, three serial sections were examined by immunohistochemistry for, respectively, IL-1 β , smooth muscle cells, and proliferation. The arterial cross-sections (5 μ m) were placed on poly-L-lysine-coated slides, deparaffinised, rehydrated, and washed with PBS. Non-specific binding was blocked by preincubation for 1 h at room temperature with PBS containing 5 mg/ml of serum albumin. One section was incubated for 1 h at 37°C with goat anti-rabbit IL-1 β antibody (1:50 dilutions, R&D systems, Minneapolis, MN, USA), for 1 h at room temperature with biotinylated horse anti-goat IgG (1:400 dilutions, Vector), and for 1.5 h at room temperature with avidin-biotin-horseradish peroxidase complex, then with 0.5 mg/ml 3,3'-diaminobenzidine/0.01% hydrogen peroxide in 0.1 M Tris-HCl buffer, pH 7.2 as chromogen (Vector Lab, Burlingame, CA, USA). The second section was incubated for 1 h at 37°C with mouse anti- α -smooth muscle actin (1:100 dilution, Neomarkers, Fremont, CA, USA), then for 1.5 h at room temperature with FITC-conjugated goat anti-mouse antibody (1:400 dilution, ICN, Aurora, OH, USA) and observed by fluorescent microscopy. For Ki-67 immunostaining which detects proliferating cells [Wohrley et al., 1995], the third slide was boiled for 20 min in 0.01 M citrate buffer, pH 5.0, to unmask the Ki-67 antigen, cooled at room temperature for 15 min, and incubated for 24 h at 4°C with mouse anti-human Ki-67 antibody (Zymed, South San Francisco, CA, USA), then with peroxidase-conjugated goat anti-mouse secondary antibody for 2 h at room temperature (1:50 dilution, Sigma, St. Louis, MO, USA), followed by 0.5 mg/ml 3,3'-diaminobenzidine/0.01% hydrogen peroxide in 0.1 M Tris-HCl buffer, pH 7.2 (Vector Lab, Burlingame, CA, USA); the percentage of proliferating cells was measured quantitatively using the Image-Pro Plus Data Analysis Pro-

gram (Media Cybernetics, Silver Spring, MD). Eight separate areas (length: 0.3 mm; width: variable) from each cross section were quantitatively analyzed. Proliferating cells scanned in each area were estimated by Ki-67 immunopositive brown cells before methyl green staining. In the same area, the total cells scanned were counted after methyl green staining. The percentage of proliferating cells was calculated for each area, and the overall percentage of proliferating cells expressed as a mean of these eight values. Negative controls were performed by omitting the primary antibodies.

Western Blots

The abdominal aortas from each experimental group of rabbits were frozen in liquid nitrogen, homogenized and sonicated in a tissue homogenizer in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.05% SDS, 0.5% Triton X-100, and 1 mM PMSF, pH 7.4), and the lysate centrifuged at 13,500g for 30 min at 4°C. The protein content of the supernatant was measured using the Bradford assay (Bio-Rad), then samples were applied to 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (NEN), which were then blocked for 1 h at room temperature with PBS-Tween 20 (0.05%)/2% skimmed milk, then incubated for 1 h at room temperature with goat anti-human-IL-1 β antibody (diluted 1:1,000; R&D), washed, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG monoclonal antibody (diluted 1:3,000; R&D). Bound antibody was detected using Chemiluminescence Reagent Plus (NEN) and exposure to Biomax MR film (Kodak), the intensity of the band being quantified on a densitometer.

Statistical Analysis

Values were expressed as the mean \pm SEM. The data for the three groups of animals were compared using one-way ANOVA. When ANOVA indicated a significant difference between groups, the data for individual groups were compared using the Dunnett test; a probability of $P < 0.05$ was considered significant.

RESULTS

Effects of EGb and Probucol on Cu⁺²-Induced LDL Oxidation

Both EGb and probucol inhibited copper-induced LDL oxidation. The IC₅₀ values, ob-

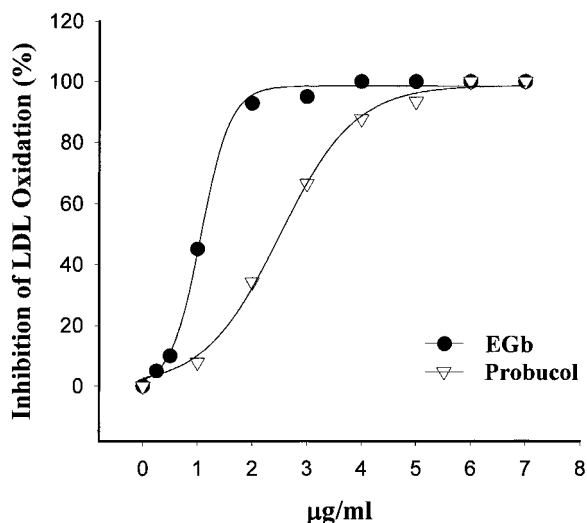


Fig. 1. Inhibition by EGb and probucol of Cu^{2+} -induced plasma LDL oxidation. Based on the IC_{50} values, the antioxidant potency of EGb is 223% that of probucol.

tained from the concentration-response curves (Fig. 1), were 1.15 $\mu\text{g}/\text{ml}$ for EGb and 2.58 $\mu\text{g}/\text{ml}$ for probucol. Based on the IC_{50} values, the antioxidative potency of EGb was about 223% of that of probucol in the test assay.

EGb Inhibits Proliferation of Rat VSMC In Vitro

The results of a trypan blue exclusion test performed on VSMCs indicated that the EGb

concentrations (1, 2.5, 5, 15, 25, 50, 100, 200, and 400 $\mu\text{g}/\text{ml}$) used in this study were not cytotoxic and did not affect cell viability (data not shown). As shown in Figure 2, treatment of control cells for 24 h with 2% serum resulted in a 2-fold increase in BrdU incorporation above baseline and this effect was inhibited in a dose-dependent manner by EGb. Statistically significant inhibition of BrdU incorporation was seen at EGb concentrations equal to or greater than 5 $\mu\text{g}/\text{ml}$.

Biochemical Measurements in Experimental Rabbits

During the experimental period, weight gain and final weight did not differ significantly between the groups of animals. Serum glucose, GOT, GPT, GGT, BUN, and creatinine levels also showed no significant difference between groups.

As shown in Table I, in the negative control group, serum cholesterol levels were increased after 3 weeks of feeding a 2% cholesterol diet ($1,746 \pm 192$ mg/dl compared to 54 mg/dl before the high cholesterol diet) and increased further after 6 weeks of feeding ($2,421 \pm 152$ mg/dl), while the corresponding concentrations in the probucol group were $1,726 \pm 186$ and $1,326 \pm 176$ mg/dl and those in the EGb group

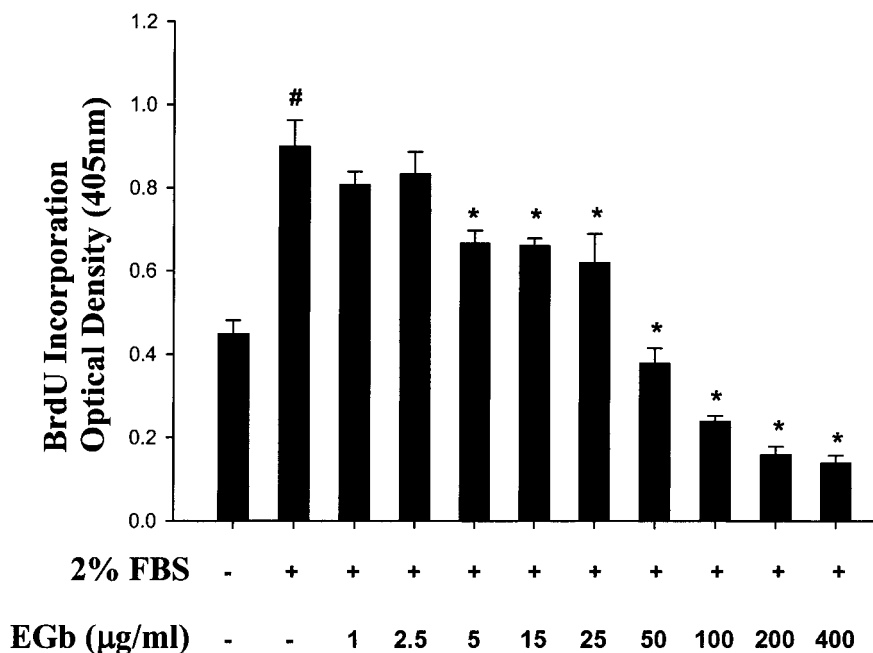


Fig. 2. BrdU incorporation into cultured VSMCs. EGb inhibits serum-stimulated DNA synthesis in a dose-dependent manner. # $P < 0.05$ compared to untreated VSMCs; * $P < 0.05$ compared to 2% FBS-treated VSMCs.

TABLE I. Plasma Lipid Levels at Baseline, and 3, 6 Weeks After High Cholesterol Diet Feeding in Different Groups of Rabbits

	Control		Probucol		<i>Ginkgo bibola</i> extract	
	TG	CHOL	TG	CHOL	TG	CHOL
Baseline	58 \pm 9	54 \pm 5	53 \pm 8	65 \pm 9	62 \pm 6	55 \pm 5
3 weeks	59 \pm 13	1,746 \pm 192	43 \pm 9	1,726 \pm 186	47 \pm 10	1,576 \pm 163
6 weeks	62 \pm 12	2,421 \pm 152	44 \pm 6	1,326 \pm 176*	42 \pm 7	1,772 \pm 236*

* $P < 0.05$ compared with control group at 6 weeks.

TG indicates triglycerides (mg/dl); CHOL, cholesterol (mg/dl). Values are mean \pm SEM.

1,576 \pm 163 and 1,772 \pm 236 mg/dl. Compared to the control group, both the probucol and EGb groups showed significantly lower cholesterol levels at week 6 ($P < 0.05$). In contrast, serum triglyceride levels did not increase significantly during the experimental period in any group.

EGb and Probucol Inhibit Formation of Atherosclerotic Lesions in the Thoracic Aorta

Sudanophilic atherosclerotic lesions in the thoracic aortas of the control group were prominent under *en face* observation, the fraction of the total area stained being 0.49 \pm 0.11. In contrast, the corresponding values were 0.16 \pm 0.04 and 0.23 \pm 0.09 in the probucol and EGb groups, respectively (Fig. 3), i.e., probucol or EGb treatment resulted, respectively, in a 67% or 54% decrease in atherosclerotic area, both decreases being statistically significant compared to the control group ($P < 0.05$).

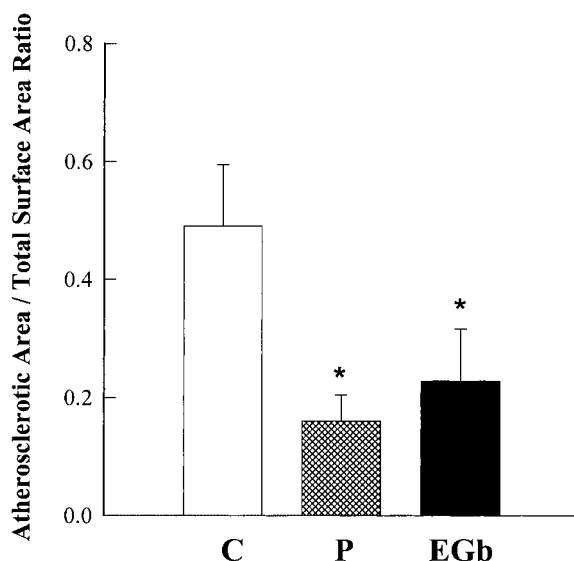


Fig. 3. Extent of atherosclerotic lesions in thoracic aortas from control, probucol-treated, and EGb-treated rabbits. C: control; P: probucol. The atherosclerotic lesions are significantly reduced in the probucol and EGb groups. * $P < 0.05$.

EGb and Probucol Reduce Neointima Formation in the Abdominal Aorta After Balloon Injury

Histopathological sections of abdominal aortas 3 weeks after balloon injury are shown in Figure 4. The control group (C) showed a markedly thickened neointima with accumulation of foam cells, and treatment with either probucol (P) or EGb (EGb) significantly reduced the neointimal hyperplasia. As shown in Figure 5, both the neointimal/medial thickness and area ratios in the probucol and EGb groups were significantly lower than in the control group (thickness: control group 0.99 \pm 0.05, probucol 0.58 \pm 0.03, EGb 0.70 \pm 0.09; area: control group 0.87 \pm 0.02, probucol 0.55 \pm 0.03, EGb 0.61 \pm 0.06).

EGb and Probucol Attenuate IL-1 β mRNA and Protein Expression in the Rabbit Aorta

The control group showed strong expression of IL-1 β mRNA on Northern blots, whereas IL-1 β mRNA expression was significantly reduced in the probucol-treated and EGb-treated animals (0.83-fold and 0.82-fold expression compared to the control group) (Fig. 6). As shown in Figure 7, the markedly thickened intima of the control group showed strong IL-1 β protein expression (CA), which was associated with smooth muscle cells (CB). Many proliferating cells were also found in the thickened intima (CC). The percentage of proliferating cells was 56.0% \pm 8.9%. Staining for IL-1 β protein was weaker in the probucol (PA) and EGb (EGbA) groups, but was also associated with smooth muscle cells (PB and EGbB); proliferating cells were rarely observed (22.8 \pm 9.9% and 5.5 \pm 3.8%, respectively) and were usually detected in the superficial region (PC and EGbC). Consistent with the *in situ* findings, Western blots showed higher levels of IL-1 β

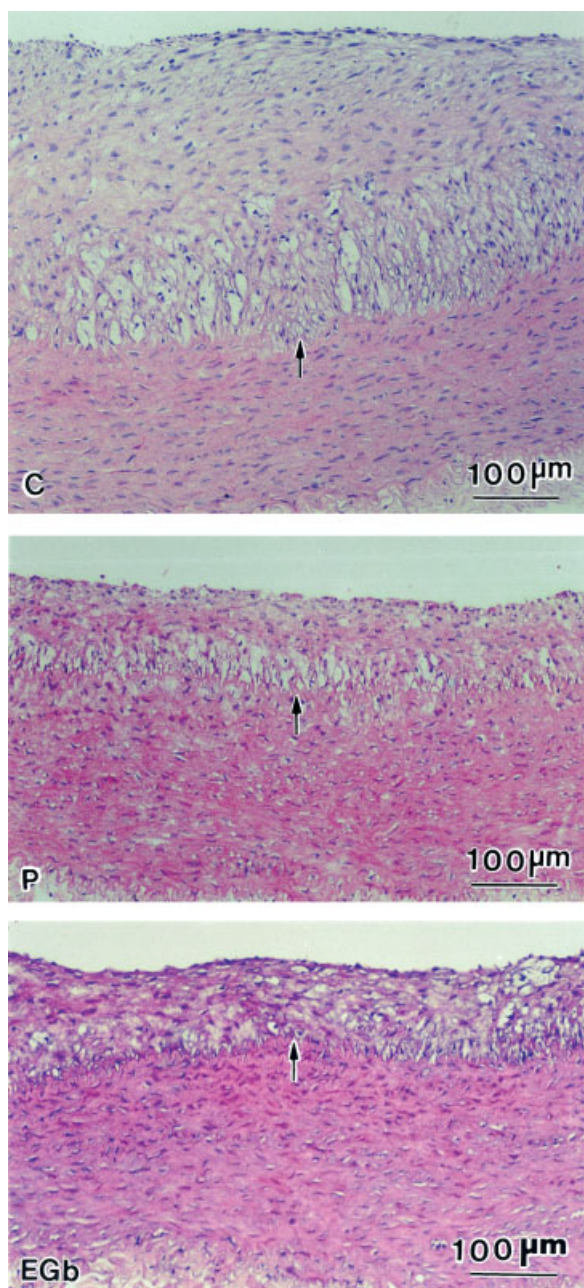


Fig. 4. Histopathological features of cross-sections of abdominal aortas from control, probucol-treated, and EGb-treated rabbits. C: control; P: probucol. The lumen is uppermost in all sections. The internal elastic lamina is indicated by arrows. A markedly thickened intima with accumulation of foam cells is seen in the C group and significantly less intimal hyperplasia is noted in both the P and EGb groups.

expression in the control group, and that probucol or EGb treatment significantly reduced IL-1 β expression (0.69-fold and 0.48-fold expression compared to the control group) (Fig. 8).

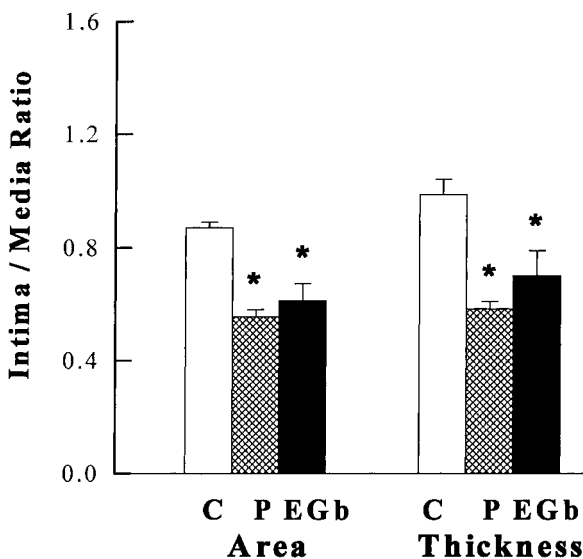


Fig. 5. Extent of intimal hyperplasia, expressed as the intimal/medial area ratio and intimal/medial thickness ratio in abdominal aortas from different groups of rabbits. Both ratios were significantly reduced in the probucol (P) and EGb groups compared to the control (C) group. * $P < 0.05$.

DISCUSSION

The major findings of this study were that EGb inhibited the proliferation of cultured VSMCs and decreased the intimal response to balloon injury of abdominal aortas by inhibiting intimal hyperplasia and reducing IL-1 β expres-

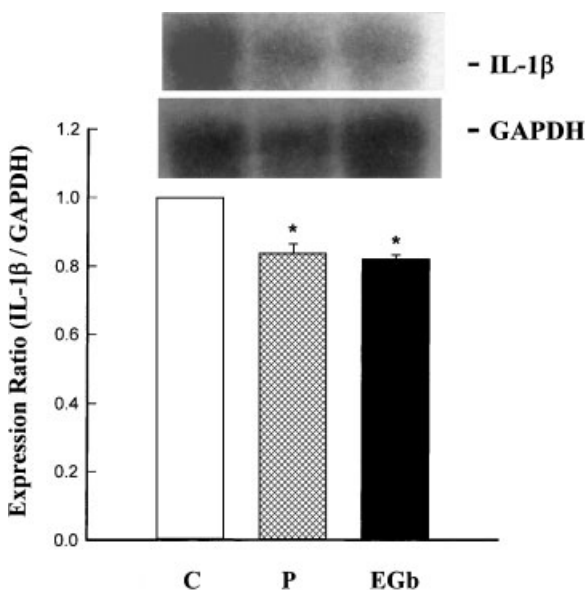


Fig. 6. Northern blot analysis of IL-1 β mRNA expression. The expression ratio (IL-1 β /GAPDH) was significantly decreased in the probucol (P) and EGb groups compared to the control (C) group. * $P < 0.05$.

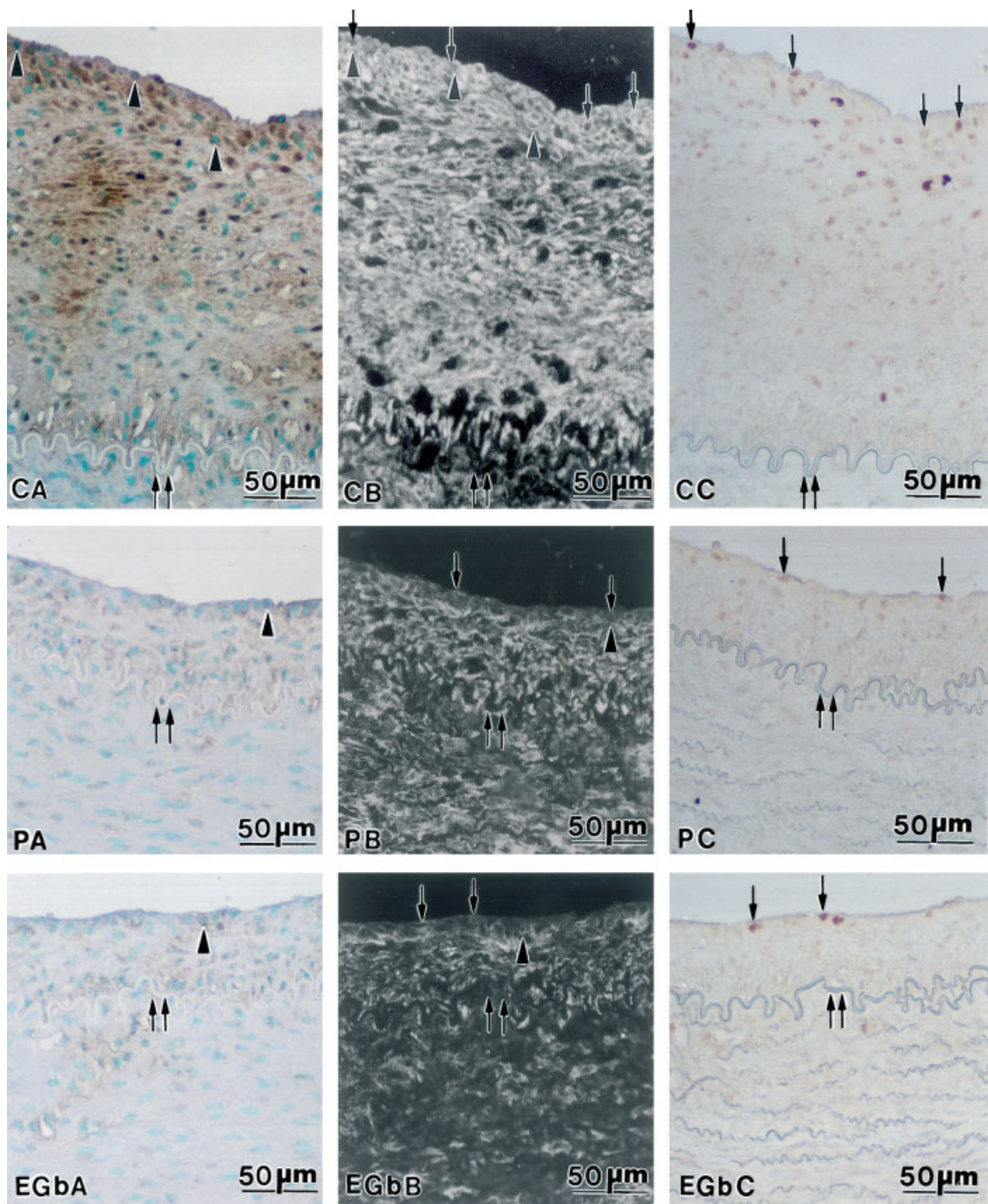


Fig. 7. Immunohistochemical detection of IL-1 β protein expression, smooth muscle cells, and Ki-67 in abdominal aortas from the control, probucol, and EGb groups. **C:** control; **P:** probucol. The lumen (L) is uppermost in all sections. The internal elastic lamina is indicated by double arrows. The arrowheads and single arrows indicate, respectively, colocalization of smooth muscle cells with IL-1 β expression and Ki-67-immunopositive cells. **A:** Staining for IL-1 β expression (brown reaction products),

counterstaining with methyl green. **B:** Staining for smooth muscle-specific α -actin. **C:** Ki-67 staining for proliferating cells. The C group shows a greatly thickened neointima, strong IL-1 β expression, and a large number of Ki-67 immunopositive cells, while the EGb and probucol groups show a less thickened neointima, weak IL-1 β expression in some regions of the neointima, and a decreased number of Ki-67 immunopositive cells.

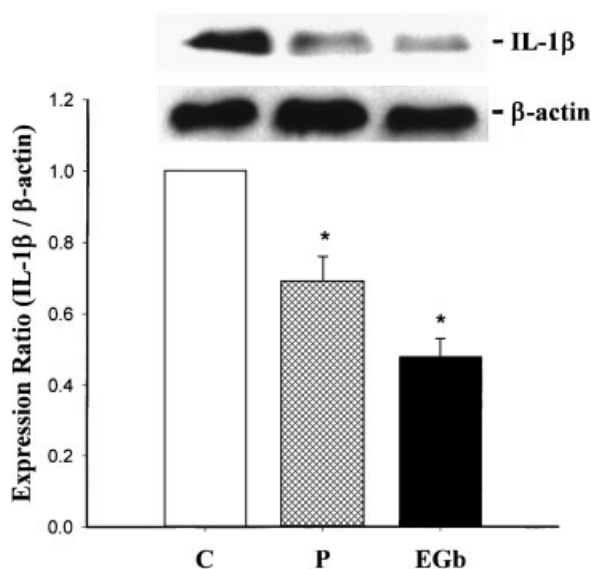


Fig. 8. Western blot analysis of IL-1 β protein levels. The expression ratio (IL-1 β / β -actin) was significantly decreased in the probuocol (P) and EGb groups compared to the control (C) group. * $P < 0.05$.

sion in cholesterol-fed rabbits. EGb treatment also resulted in a marked reduction in atherosclerotic lesion areas in the thoracic aortas, which had not undergone endothelial denudation. In addition, serum cholesterol levels were significantly lowered after 6 weeks of daily EGb treatment when compared to control rabbits. This cholesterol-lowering effect may partly explain the reduced atherosclerotic lesion formation in the thoracic aorta and the decreased intimal response in the balloon-injured abdominal aorta of EGb-treated rabbits.

Smooth muscle cell migration and proliferation, which lead to intimal hyperplasia, is a major mechanism involved in the development of atherosclerosis and postangioplasty restenosis [Schwartz, 1997]. The results from our *in vitro* experiments showed that BrdU incorporation in VSMCs was reduced by EGb treatment. In addition, the number of Ki-67-immunostained cells, which are representative of proliferating cells, was also reduced by EGb treatment in these *in vivo* studies. The mechanisms underlying the anti-proliferative action of EGb are yet not clear. However, EGb has been shown to block serotonin-induced mitogenesis of bovine pulmonary artery smooth muscle cells by its superoxide scavenging effect [Lee et al., 1998].

A number of antioxidants, including probuocol [Ferns et al., 1992; Schneider et al.,

1993], butylated hydroxytoluene [Freyschuss et al., 1993], and vitamin E [Konneh et al., 1995], have been shown to inhibit neointimal hyperplasia after balloon injury in cholesterol-fed animals. In the present study, probuocol treatment, used as a positive control for comparative purposes, not only significantly reduced atherosclerotic lesions in the thoracic aortas, but also inhibited intimal hyperplasia and IL-1 β expression in the abdominal aorta after balloon injury. This is the first report of an effect of probuocol on IL-1 β expression in this animal model. EGb was used in this study, because it has been used in the treatment of cardiovascular and cerebrovascular diseases, particularly those related to oxidative stress. EGb contains two major active constituents, namely flavanoids (24%) and terpenoids (6%), which are potent antioxidants and are probably responsible for the therapeutic benefits. EGb protects human LDL against copper-induced oxidative modification [Varga et al., 1999] and, in the myocardial ischemia-reperfusion injury model, has also been shown to have cardioprotective effects due to its antioxidant properties [Haramaki et al., 1994; Shen and Zhou, 1995; Varga et al., 1999]. EGb inhibits oxidative stress-induced platelet aggregation [Akiba et al., 1998], acts as a scavenger of free radicals and NO *in vitro* [Maccoci et al., 1994; Maitra et al., 1995], and attenuates the expression of iNOS in cultured endothelial cells [Cheung et al., 1999]. In this study, we demonstrated that EGb administration effectively decreased copper-induced plasma LDL oxidation *in vitro*, in agreement with the report of Yan et al [1995], the antioxidant activity of EGb being 223% that of probuocol. Thus, it is possible that the EGb effects seen in our *in vivo* experiments are, at least in part, related to the antioxidant properties.

Atherosclerosis has been considered as an inflammatory disease [Ross, 1999]. Because of the presence of inflammatory cells, expression of inflammatory cytokines, and accumulation of fibronectin [Clausell et al., 1995], restenotic lesions are also considered to be immuno-inflammatory reactions. IL-1 β , a pro-inflammatory cytokine with pleiotropic effects, appears to be involved in the progression of atherosclerosis and restenosis [Oemar, 1999]. Several lines of evidence suggest that it participates actively in regulating vascular cell functions, acting as a mitogen for human vascular SMCs [Libby et al.,

1988], increasing the binding of lymphocytes to the endothelium [Cavender et al., 1986], suppressing lipoprotein lipase activity [Beutler and Cerami, 1985], inducing extracellular matrix production [Montesano et al., 1984], and increasing vascular permeability [Martin et al., 1988]. IL-1 β expression has previously been detected in iliac arteries of diet-induced atherosclerotic plaques in monkeys [Moyer et al., 1991] and in balloon-injured porcine coronary arteries [Chamberlain et al., 1999]. Increased expression of IL-1 β has also demonstrated in coronary arteries from patients with ischaemic heart disease [Galea et al., 1996] and in restenotic lesions of human coronary artery retrieved by atherectomy [Clausell et al., 1995]. These findings indicate that the level of IL-1 β expression is likely to be a major factor during the formation of atherosclerotic and restenotic lesions. In the present study, marked IL-1 β mRNA and protein expression was seen in balloon-injured abdominal aorta of cholesterol-fed rabbits, whereas the expression of both was significantly lower in EGb-treated or probucol-treated animals. In the EGb group, protein expression was decreased to a greater extent than mRNA expression (52% compared to 18%, Figs. 6 and 8), suggesting that the effect of EGb is mainly on translation. These results suggest that inhibition of IL-1 β expression by EGb may reduce the intimal response to balloon injury of the vessel wall.

Our previous study showed that macrophages and smooth muscle cells were present in the neointima after balloon injury of the abdominal aorta in cholesterol-fed rabbits [Feng et al., 1999]. This feature depended on the activation of circulating monocytes and smooth muscle cells and their subsequent migration into the subendothelial space. It is possible that EGb inhibits the activation of monocytes and smooth muscle cells by its ability to reduce IL-1 β expression. Since IL-1 β production by endothelial cells and smooth muscle cells is stimulated by oxidized LDL [Grasing et al., 1997], IL-1 β expression in the neointima after balloon injury might be controlled locally by oxidized LDL levels. The significant IL-1 β expression seen in the neointima of the balloon-injured aorta, also noted in the present study, might be an important factor in the further recruitment of monocytes and smooth muscle cells and in the progression of restenosis. EGb, via its antioxidant capacity, might decrease oxidized

LDL levels and attenuate IL-1 β expression in the arterial wall, resulting in a reduced intimal response and, therefore, prevention of restenosis.

In conclusion, this study provides the first evidence that EGb treatment attenuates intimal thickening in the balloon-injured abdominal aorta of cholesterol-fed rabbits. The reduced intimal hyperplasia may be explained by the observed decrease in cholesterol levels, suppression of IL-1 β expression, and inhibition of VSMC proliferation, which are partly due to the antioxidant potency of EGb. These data suggest that EGb may be of potential benefit in the prevention of restenosis after percutaneous coronary intervention, particularly in patients with hypercholesterolemia.

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