Inhibitory effect of extracts of *Ginkgo biloba* leaves on VEGF-induced hyperpermeability of bovine coronary endothelial cells *in vitro*¹

Yan QIU, Yao-cheng RUI², Tie-jun LI, Li ZHANG, Peng-yuan YANG

Department of Pharmacology, School of Pharmacy, Second Medical Military University, Shanghai 200433, China

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ABSTRACT

AIM: To study whether extract of *Ginkgo biloba* (EGb) can protect against atherosclerosis. METHODS: Confluent monolayers of bovine coronary endothelial cells (BCECs), bovine coronary smooth muscle cells (BCSMCs), and cocultures of the two were incubated with medium containing VEGF and/or EGb, and flux of ¹²⁵I-labeled oxidized low density lipoprotein (ox-LDL) across the monolayers was measured. RESULTS: Incubation with VEGF significantly increased the permeability of BCEC monolayers to ¹²⁵I-ox-LDL in a time- and concentration-dependent manner, but had no effect on permeability of BCSMCs or endothelial cells-smooth muscle cells cocultures. EGb significantly inhibited the VEGF-induced hyperpermeability of BCECs. CONCLUSION: VEGF was important in the formation and development of atherosclerosis. The inhibition of VEGF-induced permeability by EGb suggests that extracts of *Ginkgo biloba* leaves may have important clinical applications in the treatment of cardiovascular diseases.

INTRODUCTION

Traditional Chinese medicines have attracted great interest as alternative therapies for diseases. In contrast to Western medicines, each of which is a single chemical entity, Chinese medical philosophy is characterized by its emphasis on maintaining and restoring homeostasis through interaction with a wide variety of targets. Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities. Unfortunately, it is often difficult to analyze the biological activities of these extracts because of their complex nature and the possible synergistic effects of their components.

Extracts of *Ginkgo biloba* leaves are currently marketed as therapeutic dietary supplements to counteract a variety of disorders. These leaves are thought to act as antioxidants and/or free radical-scavengers, which may prevent strokes or transient ischemic attacks. Although the putative therapeutic benefits of *Ginkgo biloba* may be due to the synergistic effects of many of its components, isolated constituents have been found to be active in a variety of assays. For example, ginkgo-
linder has been shown to be a potent antagonist of platelet activating factor, and the flavonoid fraction of *Ginkgo biloba*, which contains free radical scavengers, likely contributes to the antioxidant properties of this herb.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), which was initially described as a product of tumor tissues, is also expressed by and released from normal tissues, including the adrenal glands, renal glomeruli, and lung epithelium. In addition to its mitogenic effects, VEGF has shown to increase the permeability of the peripheral circulation and endothelial cell monolayers. Mechanisms that account for the effects of VEGF on vascular and endothelial cell permeability in the circulation are varied but appear to involve an increase in endothelial cell calcium influx, increased synthesis of platelet-activating factor, and/or increased release of products produced by the activation of the cyclooxygenase pathway.

The finding that VEGF may be an important regulator of vascular permeability in the circulation led us to hypothesize that VEGF may be able to induce leakiness in vascular cell monolayers. Along with an increase in permeability and the accumulation of ox-LDL, this may ultimately lead to the formation of coronary atherosclerosis during many pathological conditions. *Ginkgo biloba* extracts may counteract this leakiness, thus acting to reduce coronary atherosclerosis. We therefore assayed the effects of a *Ginkgo biloba* extract on VEGF-induced permeability of cell monolayers to ox-LDL.

**MATERIALS AND METHODS**

**Reagents** Recombinant human VEGF was purchased from Calbiochem (San Diego, CA); LDL, CuSO₄, and ethylenediamine tetraacetic acid disodium salt (EDTA) were purchased from Sigma Chemical Co (St Louis, MO). ox-LDL labeled with ¹²⁵I was performed by the Institute of Atomic Nucleus of the Chinese Academy of Medical Sciences (Shanghai, China). Transwell System cell culture dishes with diameter of 6.5 cm were purchased from Falcon.

**Source of Ginkgo biloba** The standardized *Ginkgo biloba* leaf extract EGB761, which has been used extensively in clinical trials, was the kind gift of Prof Wei-Zhou CHEN (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). This extract contained 24 % flavone glycosides (primarily composed of quercetin, kaempferol, andisorhamnetin) and 6 % terpene lactones (2.8 %-3.4 % ginkgolides A, B, and C, and 2.6 %-3.2 % bilobalide). Other constituents included proanthocyanidins, glucose, rhamnose, organic acids (hydroxykinu-renal, kynurenic, protocatechuic, vanillic, shiki-mic), D-glucaric acid, ginkgolic acid (<5 µmol/L), and related alkylphenols.

**Ox-LDL preparation** LDL (d=1.019-1.063 kg/L) was sterilized by filtration through 0.45 μm Millipore membranes and stored at 4 ºC as described. Edetic acid was removed by dialysis, and the LDL was oxidized by incubating it in CuSO₄, 10 µmol/L for 16 h at 37 ºC and dialyzing it in phosphate buffered saline (PBS) containing EDTA 0.1 mmol/L for 24 h at 4 ºC.

**Cell culture** Bovine coronary endothelial cells (BCECs) and smooth muscle cells (BCSMCs) were harvested as described and cultured in minimum essential medium (MEM) containing 10 % fetal bovine serum and gentamicin 50 mg/L. Permeability assays utilized confluent BCEC or BCSMC monolayers in their third to fifth passages.

BCECs and BCSMCs were cultured in a Transwell system, consisting of two compartments, a removable Transwell insert (upper or apical chamber) and a bottom well (lower or basolateral chamber). The two compartments were separated by a microporous polycarbonate membrane (pore size: 0.4 µm) that covered the bottom of the insert and permitted exchange of medium between the two chambers. Cells were seeded at 1×10⁵/L onto the Transwell inserts. The insert chamber contained 100 µL BCEC medium, and the bottom chamber contained 600 µL MEM. The cells were grown in a 37 ºC incubator maintained in an atmosphere of 5 % CO₂ and 95 % humidity. The culture medium was changed on the third day after plating and every other day thereafter.

**Coculture system** BCSMCs (3×10⁶ cells/cm² in MEM containing 10 % FBS) were plated at the bottom of Transwell inserts 6-8 h prior to addition of BCECs (1×10⁶ cells/cm² in MEM containing 10 % FBS) to the upper chambers plates.

**Effect of VEGF on BCEC, BCSMC monolayer, and EC-SMC coculture system** Confluent BCEC monolayers were incubated with culture medium containing recombinant human VEGF at various concentrations and for various periods, and transendothelial ¹²⁵I-ox-LDL flux across the monolayers was measured. To test the effects of VEGF on BCSMC monolayers and cocultured EC-SMC, cells were incubated with
VEGF 100 µg/L for various periods, and transendothelial 
$^{125}$I-ox-LDL flux was measured as above.

We also examined the effect of VEGF on different types of low density lipoprotein of BCEC. Monolayers of endothelial cells were treated with VEGF 100 µg/L for 24 h and pulsed with $^{125}$I-ox-LDL or with $^{125}$I-native-LDL for 1 h, and transendothelial LDL flux was measured as above.

**Transendothelial flux measurement (permeability assay)** To measure the flux of $^{125}$I-ox-LDL across filter-grown cell monolayers, 100 µL MEM containing $^{125}$I-ox-LDL (0.1 mCi/L) was added to the existing medium 100 µL. After incubation for various periods at 37 ºC, the basolateral chamber medium was removed, and radioactivity was measured with an SN-965 automatic liquid scintillation counter. For comparison, $^{125}$I-ox-LDL flux across filters alone was measured. The amount of radiolabeled tracer that penetrated the BCEC monolayer was expressed as $\frac{C_r}{C_d} \times \frac{V_r}{V_d} \times 100$, where $C_r$ is the count in the receptor chamber, $C_d$ is the count in the donor chamber, $V_r$ is the volume (mL) of medium in the receptor chamber, and $V_d$ is the volume (mL) in the donor chamber.

**Inhibitory effect of extracts of Ginkgo biloba leaves** Confluent BCEC monolayers were incubated with culture medium containing VEGF 100 µL and $E_b$ 100 µg/L at various concentrations. $E_b$ 10 µg/L was incubated with VEGF at various concentrations and BCSMC monolayers for 1 d.

**Statistical analysis** All experiments were carried out in triplicate and were repeated at least once. Results were presented as mean±SD. Statistical analysis was performed with ANOVA.

**RESULTS**

**Establishment of BCEC and BCSMC monolayers and the EC-SMC coculture system in the Transwell plates** BCECs cultured on microporous membranes in Transwells were observed to reach confluence after 4 d and formed a tight monolayer approximately on d 4 or d 5 after plating. BCSMCs cultured according to the same protocol reached confluence on d 3 and formed a tight monolayer on d 4-5. In contrast, EC-SMC cocultures did not reach confluence until d 6 and took 1-2 weeks to form tight layers.

**Effect of VEGF on permeability of BCEC monolayers** There was a significant increase in permeability of VEGF-treated BCEC at 45 min compared with cells without VEGF treatment (Fig 1).

VEGF increased monolayer permeability in a concentration-dependent manner, 50 µg/L was required to detect significant increases in monolayer permeability (Fig 2).

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monolayers and EC-SMC cocultures  Incubation of confluent SMCs or cocultured EC-SMC with VEGF 100 µg/L did not cause any further increase in cell permeability to 125I-ox-LDL (Fig 3).

Fig 3. Effect of VEGF on permeability of monolayers of SMCs and EC-SMC cocultures. SMCs or EM-SMC cocultures were incubated in the presence (○, SMC; ▲, EC-SMC) or absence (▲, SMC; ●, EC-SMC) of VEGF 100 µg/L for 2 d, and permeability of 125I-ox-LDL for 1 h was measured. Note that VEGF did not significantly affect cell permeability. n=4 independent determinations. Mean±SD.

Effect of Ginkgo biloba extract on VEGF-induced permeability  Incubation of EGb at concentrations of 10-100 µg/L strongly inhibited the hyperpermeability induced by VEGF 100 µg/L (P<0.01). VEGF 100-200 µg/L reduced the inhibitory effects of EGb 10 µg/L (Fig 4).

DISCUSSION

Patients with diabetes mellitus, hypertension, and other illnesses associated with aging are prone to develop vascular lesions. Evidence has shown that chronic intake of a high-fat diet induces a dramatic extravasation of proteins.[10] Ox-LDL and other substances associated with atherogenesis may participate in transformation of the monocytes into macrophage. Uptake of ox-LDL by the macrophages through scavenger receptors will lead to foam cells formation. Identification of the factors that regulate ox-LDL uptake by the vascular wall under physiological conditions and its accumulation during atherosclerosis is crucial to an understanding of the process by which the vascular wall acts as a barrier against plasma proteins and is the most active ox-LDL-catabolizing tissue in the body. Several other studies have suggested that increased arterial wall ox-LDL was related to changes in endothelial cell barrier function. Studies using perfused blood vessels have shown that a number of factors, including nicotine, hydrostatic pressure, endotoxin and phorbol esters[14-16], increase the flux of molecules from the lumen into the arterial wall.

Endothelial cell is not merely a simple physiological barrier, but also is active in the metabolic pool. For example, it is generally agreed that the vascular endothelium plays a key role in the initiation of atherosclerotic processes. Furthermore, functional and structural changes in the endothelium are associated with the onset and development of cardiovascular diseases, and endothelial injury may be responsible for the initiation of atherosclerotic disease processes. Thus, we sought to determine whether VEGF could induce ox-LDL hyperpermeability in cultured BCECs, and if there was a relationship to atherosclerosis.

Among the growth factors, only VEGF can induce protein extravasation[10], and it is likely that its an-
giogenic effects are mediated in large part by inducing leakage of plasma proteins[17]. In this study, we have shown that the addition of purified VEGF to the BCEC monolayer markedly increased the permeability of the latter, thus confirming the potency of VEGF as a mediator of protein vascular permeability.

In previous work[9] about the effects of VEGF on the permeability of monolayers of bovine aortic endothelial cells (BAEC) and cerebral microvascular endothelial cells (CMEC), we found that, while VEGF increased the permeability of these monolayers, its potency was dependent on the identity of the target cell. Utilizing identical experimental conditions, we have shown that coronary endothelial cells are more easily affected than the other two endothelial cell types, thus suggesting that it is easier to initiate atherosclerosis in coronary arteries than in the aortae or microvasculature (Tab 2).

Tab 2. 125I-ox-LDL flux in three types of endothelial cell monolayers in the presence or absence of VEGF. n=4 determinations. Mean±SD. *P<0.01 vs control.

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<tr>
<th>Monolayer</th>
<th>Control (no VEGF)%</th>
<th>VEGF 100 µg·L⁻¹ group%</th>
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<tbody>
<tr>
<td>BCEC</td>
<td>7.29±0.26</td>
<td>11.25±0.15 c</td>
</tr>
<tr>
<td>BAEC</td>
<td>21.7±1.2</td>
<td>28.1±0.4 c</td>
</tr>
<tr>
<td>BCMEC</td>
<td>4.55±0.25</td>
<td>6.0±0.6 c</td>
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</tbody>
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We were successful in establishing and maintaining long-term cocultures of bovine cerebral microvascular endothelial cells and smooth muscle cells. Previously developed cocultures of EC and SMC have been shown to more closely mimic the architecture of the vessel wall and therefore represent a significant advance over homogeneous cultures[5]. Our result that VEGF could not induce further permeability on BCSMC monolayers or cocultured BCEC and BCSMC, is a further indication that VEGF may be responsible for the initiation of atherosclerotic disease processes.

_Ginkgo biloba_ is a Chinese traditional herb widely used to treat atherosclerosis-related disorders and acute cerebral ischemia[18,19]. An extract of this herb, _EGb_, is a potent scavenger of several reactive oxygen species, including singlet oxygen, superoxide anions, and hydroxyl radicals[20,21]. In normal endothelial cells, low concentrations of _EGb_ can stimulate epoprostenol production and release endothelium-derived relaxing factor (EDRF)[22]. While in other in vitro study, _EGb_ could protect rat aortic endothelial cells (RAEC) against the lysophosphatidylcholine-induced damage and down-regulate VEGF protein and VEGF mRNA expression in cultured RAEC[23]. A key and novel finding of the present study was that extracts of _Ginkgo biloba_ leaves, at concentrations of 1×10⁻³ to 1×10⁻⁴ g/L, could dose-dependently inhibit the permeability-increasing activity of VEGF on BCEC monolayers. The ability of _EGb_ to enhance the resistance of lipids to oxidation is probably related to its effective antioxidant protection and anti-PAF action. Our results suggest that _EGb_ may have potential clinical applications in the treatment of cardiovascular diseases.

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