In search of antioxidants and anti-atherosclerotic agents from herbal medicines

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Abstract. Many recent studies have suggested that low-density lipoprotein (LDL) oxidation, endothelial dysfunction, and inflammation are involved in the pathogenesis of atherosclerosis. Herbal regimens in the treatment of blood stasis, a counterpart of atherosclerosis, commonly use medicinal plants of leguminosae and labiatae. We have developed disease-oriented screening methods to search for bioactive components, particularly isoflavones in leguminosae and polyphenols in labiatae from Chinese herbal medicines. Many bioactive components and active fractions capable of inhibiting a. Cu(II)-induced LDL oxidation, b. oxidized LDL-induced endothelial damage, c. uptake of oxidized LDL by macrophages (J774A.1), and d. expression of cell adhesion molecules (CAMs) have been identified. A polyphenol, namely salvianolic acid B from \textit{Salvia miltiorrhiza} was identified to be a potent antioxidant, endothelial-protecting agent, and an inhibitor to suppress the expression of ICAM and VCAM. This review also briefly describes the strategy for developing herbal medicines as anti-atherosclerotic agents.

Keywords: Atherosclerosis, low-density lipoprotein, antioxidants, leguminosae, labiatae, \textit{Salvia miltiorrhiza}

1. Introduction

Atherosclerosis is the major cause of coronary heart disease (CHD) in humans [6,21]. Many recent studies have indicated that oxidative modification of low-density lipoprotein (LDL), endothelial dysfunction, and inflammation are involved in the pathogenesis of atherosclerosis [10,14]. Mildly oxidized LDL (oxLDL) may appear in the plasma whereas extensively oxidized LDL only occurs in the intimal area of vessel wall. Functionally disturbed endothelial cells express MCP-1, cell adhesion molecules (CAMs), and selectins to recruit the circulating monocytes. Uptake of oxLDL by the macrophage scavenger receptors (MSRs) of monocyte-derived macrophages leads to the formation of foam cells, fatty streaks, and fibrous plaques [4].

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Many potential targets have been identified for therapeutic intervention [16]. Among them, plasma cholesterol-lowering, particularly LDL-cholesterol (LDL-C), remains a major goal of anti-atherosclerotic treatment. As cholesterol-lowering drugs, statins reduce LDL-C by inhibiting HMG-CoA reductase. However, recent clinical studies also have indicated that the beneficial effect of very extensive LDL-cholesterol lowering by combination therapy of lipid-lowering drugs is doubtful [17]. Epidemiological and animal studies have demonstrated that antioxidants that inhibit LDL oxidation may reduce atherosclerosis [3]. However, clinical studies have not been able to demonstrate that antioxidant vitamins, such as vitamin E and C, reduce cardiovascular disease. Lipophilic antioxidants, such as DPPD [22], probucol [12,25], and BO-653 [2], which co-migrate with circulating LDL particles, may protect LDL from oxidative modification in the intimal area (Fig. 1). Hydrophilic antioxidants also reduce atherosclerosis in animal models [5,8,26].

Traditional Chinese medicine (TCM) takes a holistic approach to maintain body homeostasis. In which, diseases are considered as the disturbances in body homeostasis. Atherosclerosis has no precise counterpart in TCM and vise versa. However, blood stasis has been described as dyslipidemia, poor circulation, hypertension, enhanced platelet aggregation and thrombosis, hyperglycemia, and high oxidative stress [15]. Since these disturbances are related to atherosclerosis, it strongly suggests that herbal medicines used in the treatment of blood stasis are good resources to search for anti-atherosclerotic...
Fig. 2. Determination of potential antioxidants using Cu\(^{2+}\)-induced LDL oxidation as an in vitro system. Conjugated diene formation causing by oxidation of polyunsaturated fatty acids (PUFA) in LDL was monitored by the increase of UV absorption at 234 nm. An herbal extract (sample W1) containing lipophilic antioxidants prolonged the lag phase and reduce the rate of oxidation in the time-course of LDL oxidation. Trolox was used as a positive control in this assay. The five curves from left to right indicated the prolongation of lag phases by trolox at 0, 0.5, 1.0, 2.0 and 5.0 µM, respectively. Increased trolox concentrations prolonged the lag phase without affecting the rate of oxidation.

agents. Extensive literature search further suggested that herbal regimens in blood stasis are composed of medicinal plants taxonomically belonging to leguminosae and labiatae. Their potentials as the resources of anti-atherosclerotic agents deserve the greatest attention.

We have developed several disease-oriented, mechanism-based bioassays to search for bioactive components from the medicinal plants in leguminosae and labiatae. The in vitro assays include a. inhibition of LDL oxidation [5], b. inhibition of oxLDL-induced endothelial injury, c. inhibition of foam cell formation and d. inhibition of endothelial expression of ICAM and VCAM [6]. The observations are summarized in this review.

2. Antioxidants to inhibit LDL oxidation

Inhibition of Cu\(^{2+}\)-induced LDL oxidation was used as the assay system to search for potential antioxidants. It is based on the observation that antibodies against Cu\(^{2+}\)-induced oxLDL can react with oxLDL from atherosclerotic plaques in human and experimental animals and vice versa.

Screening for antioxidants was carried out in a 96-well microtiter plate (quartz) and the time course of LDL oxidation was followed [24]. Conjugated diene formation causing by oxidation of polyunsaturated fatty acids (PUFA) in LDL was continuously monitored by the increase of absorption at 234 nm using a microtiter plate reader. The lag phase (T\(_{lag}\)), a commonly used marker of LDL susceptibility to oxidative damage, was defined as intercept of the tangent of the slope of UV absorption curve in the propagation phase with the baseline. The maximum rate of oxidation (tangent) in the propagation phase could also
be determined. The half life ($T_{1/2}$) in LDL oxidation was defined as the time required to cause LDL oxidation to the stage that the increase of conjugated diene formation reached half of its maximum value ($\Delta A_{234}$). The antioxidant potential of a plant extract or a pure natural product can be evaluated by its capability to prolong the lag phase ($\Delta T_{lag}$), as compared with that of a control. Trolox (a water-soluble vitamin E analog) and probucol (a lipophilic antioxidant) were used as the positive controls (Fig. 1).

A representative time course of Cu$^{2+}$-induced LDL oxidation is illustrated in Fig. 2. The dose-response effect of trolox in prolongation of LDL lag phase is also shown. To further demonstrate that PUFA was oxidatively damaged in LDL oxidation, $^1$H-NMR was used to determine the decrease of signal intensity due to the bis-allylic protons ($-\text{CH}=$CH=CH$_2$-CH=CH-) (2.85 ppm in chemical shift) of PUFA in oxLDL [11] (Fig. 3). In the time course of LDL oxidation, arachidonic acid was oxidized ahead of linoleic acid. The reduction in $\alpha$-tocopherol and arachidonic acid can serve as markers for LDL oxidation in the lag phase and propagation phase, respectively.

In the past years, over 2000 plant extracts, enriched fractions, and purified natural products have been screened for potential antioxidants in this laboratory. Since medicines and foods are considered isogenic
Table 1
Prolongation of lag phase by salvianolic acid B (Sal B) and potency of known antioxidants to inhibit Cu²⁺-induced LDL oxidation

<table>
<thead>
<tr>
<th></th>
<th>T_{lag} (min)</th>
<th>∆T_{lag} (min)</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Probucol</td>
<td>302</td>
<td>151</td>
<td>1.00</td>
</tr>
<tr>
<td>Trolox</td>
<td>251</td>
<td>100</td>
<td>0.66</td>
</tr>
<tr>
<td>Quercetin</td>
<td>422</td>
<td>271</td>
<td>1.79</td>
</tr>
<tr>
<td>Genistein</td>
<td>216</td>
<td>65</td>
<td>0.43</td>
</tr>
<tr>
<td>Sal B</td>
<td>1.286</td>
<td>1.135</td>
<td>7.52</td>
</tr>
</tbody>
</table>

1. LDL (50 µg/mL LDL-C) oxidation was induced by 5.0 µM Cu²⁺. The potency of probucol (as a positive control) was set as 1.00. Results are mean values of three determinations.

in TCM, foods and vegetables of leguminosae and labiatae origin were also screened. Results supported the view that medicinal plants and edible vegetables taxonomically belonging to leguminosae (rich in isoflavones) and labiatae (rich in polyphenols) were rich in antioxidants to inhibit LDL oxidation.

Leguminosae in herbal medicines, foods and vegetables rich in antioxidants are shown as it follows. A. Herbal medicines: Glycyrrhiza uralensis Fisch.; Astragalus membranaceus Bunge; Dalbergia odorifera T. Chen.; Cassia obtusifolia L.; Psoralea corylifolia L.; Pueraria lobata (Willd.) Ohwi; Sophora flavescens Ait.; Sophora japonica L.; Dolichos lablab L. B. Foods and vegetables: Glycin max (L.) Merr.; Glycine tomentella; Arachis hypogaea L.; Medicago hispida Gaertn.; Vigna radiata (L.) R. Wilczak; Ormosia hosiei Hems. et Wils.; Pisum sativum L.; Phaseolus vulgaris L.

Labiatae in herbal medicines, foods and vegetables rich in antioxidants are listed as it follows. A. Herbal medicines: Salvia miltiorrhiza Bunge.; Agastache rugosa (Fisch. et Mey.) O. Kuntze.; Leonurus japonicus Houtt.; Scutellaria baicalensis Georgi.; Prunella vulgaris L.; Schizonepeta tenuifolia (Benth.) Briq. B. Foods, vegetables, and others: Rosmarinus officinalis L.; Perilla frutescens (L.) Britt.; Ocimum basilicum L.; Rabdosia longitubata (Miq.) Hara; Scutellaria barbata D. Don (S. rivularis Wall.). Among them, S. miltiorrhiza was found particularly rich in antioxidants.

Bioassay-guided purification leads to the identification of bioactive antioxidants. Salvianolic acid B (Sal B), a water-soluble phenolic natural product, was identified from S. miltiorrhiza as a very potent antioxidant to inhibit Cu²⁺-induced LDL (Table 1). S. miltiorrhiza is a commonly used medicinal plant in the herbal regimens for the treatment of blood stasis [15,19]. It is particularly rich in water-soluble polyphenols (Fig. 4). Sal B prolonged the lag phase (T_{lag}) in the time course of LDL oxidation without affecting the rate of LDL oxidation. This trend of Sal B to inhibit LDL oxidation was similar to that of trolox, a water-soluble analogue of α-tocopherol. Sal B was 11.4 times more potent than trolox. Lipophilic antioxidants, such as probucol, reduced the rate of oxidation and prolonged the lag phase. Judged by prolongation of lag phase per se, Sal B was also more potent than probucol (Table 1).

3. Agents protecting oxLDL-induced endothelial damage

Endothelial dysfunction and injuries are the key events in the pathogenesis of atherosclerosis. Agents capable of reducing endothelial injuries, causing by oxLDL particularly, may have the potential to reduce atherosclerosis. In this study, cultured human aortic endothelial cells (HAECs) were used as the cellular model. Treatment with extensively oxLDL (at the range of 20–80 µg oxLDL-cholesterol/mL) induced apoptosis of HAECs in a dose-dependent manner. At the same concentration range and higher, native LDL was not harmful to HAECs.
Results showed that many antioxidants capable of inhibiting LDL oxidation also inhibited oxLDL-induced endothelial injuries. Most of them are water-soluble flavones, isoflavones, and polyphenols. A typical microscopic observation is shown to illustrate that Sal B (5–40 \( \mu \) M) rescued HAECs damaged by extensively oxidized LDL (Fig. 5). Sal B alone, at 100 \( \mu \) M, was not cytotoxic to HAECs.

Agents that stimulate endothelial cell proliferation could also be identified in this assay system. The aqueous ethanolic extract of *Salvia miltiorrhiza* (SME) was found to stimulate the proliferation of HAECs. However, Sal B was not the major active component in SME to stimulate HAECs proliferation. Identification of the major endothelial proliferating agents in SME is currently underway.

### 4. Inhibition of oxLDL uptake by macrophage scavenger receptors

Since foam cell formation and fatty streaks are the key events in early atherosclerosis, inhibition of the uptake of oxLDL by MSRs may reduce the formation of foam cells and fatty streaks in the subendothelial space [4].

Murine macrophages (J774A.1), after 24-h culture in a medium supplemented with lipoprotein-deprived serum (LPDS), were used as the cellular model to search for MSR inhibitors [7]. J774A.1 macrophages cultured in a LPDS-containing medium expressed LDL-receptor as well as scavenger receptors. Without fetal calf serum in the medium, macrophages could only uptake extensively oxLDL.
Fig. 5. Microscopic observations of Sal B to protect extensively oxidized LDL-induced cell death in cultured human aortic endothelial cells (HAECs). Cell viability was determined by MTT assay. 1. Control; 2. HAECs treated with extensively oxidized LDL (40 µg/mL); 3. HAECs treated with oxLDL (40 µg/mL) and Sal B (20 µM); 4. HAECs treated with oxLDL (40 µg/mL) and Sal B (50 µM). Extensively oxidized LDL was used in this study.

via MSRs. The accumulation of oxLDL in macrophage-derived foam cells was readily determined by Oil Red O staining. Helvolic acid (HVA) inhibited oxLDL uptake was used as a positive control [20] (Fig. 6). In this assay system, inhibitors to MSRs and inhibitors to ACATs can not be distinguished. Since this assay was performed in a kinetic manner and the findings that addition of excess LDL did not compete against oxLDL uptake, inhibition of ACAT was less likely.

As illustrated in Fig. 7, an active fraction reduced the contents of lipid droplets of macrophage-derived foam cells significantly. We have identified several organic anionic compounds, which contained a rigid ring skeleton and at least one anionic carboxyl group, as mild to potent MSR inhibitors (SRIs). Ganodermic acid S (GAS), a major oxygenated triterpene identified from the medicinal fungus *Ganoderma lucidum* [18], fulfilled the structural requirement as a MSR inhibitor [20]. GAS was more potent than HVA. The common structural character (a rigid ring skeleton and a negatively-charged carboxyl functional group) is evident in HVA and GAS (Fig. 7).

Macrophages play a key role in the immunological system in the defense against infection and surveillance of neoplasm. The ligands capable of binding to MSRs (namely, SRA, SRB and CD36) include oxLDL. However, oxLDL-specific MSR inhibition, without interference to the binding of bacterial endotoxins, is desirable. Further study is required to establish the potential of MSR inhibitors in reducing atherosclerosis.

5. Inhibition of the expression of ICAM and VCAM

Adhesion and infiltration of monocytes into the vessel wall can be observed in early atherosclerosis. In which, expression of cell adhesion molecules (CAMs) by the arterial endothelium plays a key role. Agents inhibiting the expression of CAMs may slow down the progression of early atherosclerosis.
Fig. 6. Screening for macrophage scavenger receptor inhibitors. Uptake of extensively oxLDL by J774A.1 macrophages was determined by Oil Red staining. A. Control (cells without treatment); B. Cells treated with extensively oxLDL (40 µg/mL); C. Cells treated with oxLDL (40 µg/mL) and helvolic acid (HVA) (50 µM); D. Cells treated with oxLDL (40 µg/mL) and an active herbal extract (VGH-801-W1). VGH-801-W1 inhibited the uptake of oxLDL by macrophages more effectively than HVA without affecting cell viability.

Fig. 7. Helvolic acid (HVA) and ganodermic acid S (GAS) as scavenger receptor inhibitors.

The aqueous ethanolic extract of S. miltiorrhiza (SME) and Sal B also inhibited the expression of CAMs in tumor necrosis factor-α (TNF-α)-treated HAECs. SME or Sal B treatment inhibited TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1). Dose-dependent lowering of expression of intercellular cell adhesion molecule-1 (ICAM-1) was also observed with SME or Sal B [1]. However, the expression of endothelial cell selectin (E-selectin) was not affected. Sal B also significantly reduced the binding of the human monocytic cell line, U937, to TNF-α-stimulated HAECs. Both SME and Sal B inhibited TNF-α-induced activation of nuclear factor kappa B (NF-kB) in HAECs [1]. These findings suggest that SME and Sal B have anti-inflammatory activities.
6. Discussion

In this study, extensive search for antioxidants, endothelium-protecting agents, macrophage scavenger receptor inhibitors, and CAMs expression inhibitors have been carried out. The screening methods are based on the following bioassays in vitro. a. Cu(II)-induced LDL oxidation, b. oxLDL-induced endothelial damage, c. uptake of oxLDL by macrophages (J774A.1), and d. expression of CAMs in HAECs. The above-mentioned events are considered as the key steps in the pathogenesis of atherosclerosis. Semi-automation has been designed for these assays, especially in the screening for antioxidants. For antioxidant assay, over 2000 herbal extracts, enriched fractions, and pure natural products have been screened. Many herbal regimens in the treatment of blood stasis use medicinal plants of leguminosae and labiatae. Our results support the speculation and further provide scientific basis that herbal regimens in the treatment of blood stasis contain antioxidants and anti-atherosclerotic agents. Although different species may be used, *Glycyrrhiza* (licorice) contains potential antioxidant and anti-atherosclerotic agents. It has been reported that licorice extract and its major polyphenol glabridin protect LDL against oxidation in vitro and ex vivo [5].

Atherosclerosis is the key underlying mechanism in CHD. Beyond hypercholesterolemia, multiple risk factors are involved in this degenerative disease [6]. Besides, the disease pathogenesis may take twenty years or longer until clinical symptoms become obvious. It can be anticipated that a single chemical entity alone is less likely to be effective to interrupt the progression of atherosclerosis. It may also partly explain the observations that antioxidant vitamins are not effective for CHD patients in clinical studies. Combination therapy is an emerging trend and should be a better strategy for the management of atherosclerosis. A current study has predicted that a polypill containing a statin, three antihypertensives, folic acid, and aspirin may reduce cardiovascular disease by more than 80% [23]. Since the outcome of intensive reduction of a single risk factor, such as cholesterol-lowering by a combination therapy, remains controversial and risky [17], it may not be the most appropriate strategy for combination therapy.

It is worthwhile to mention that TCM uses combination therapy. A regimen usually contains four or more herbal materials and their soluble components in hot water or water-ethanol are orally consumed. Our results also showed that most antioxidant activities, MSR inhibitors, and endothelial-protecting agents in these herbal regimens could be extracted readily by an aqueous ethanolic solution. It is anticipated that herbal medicines, if effective, may exhibit therapeutic potential in the early, reversible stage of atherosclerosis. Development of combined, enriched active herbal preparations to inhibit the multiple key events in early atherosclerosis is the best strategy. Further pharmacological evaluation and clinical studies are yet required to prove the concept and demonstrate the potential for treatment of atherosclerosis.

In this study, Sal B was identified as a potent antioxidant and an effective endothelium-protecting agent from *Salvia miltiorrhiza*, one of the most popular medicinal herbs in TCM [19]. Therapeutic potentials of Sal B alone or a Sal B-enriched fraction of *S. miltiorrhiza* deserves great attention. New Zealand white (NZW) rabbits fed with a high-cholesterol diet and apolipoprotein E-deficient mice are the two animal models commonly used for the evaluation of pharmacological function of ant-atherosclerotic agents [9, 13,27]. The anti-atherosclerotic potential of Sal B-enrich fraction using cholesterol-fed NZW rabbits as the model has been studied [26]. Treatment of Sal B-enriched fraction reduced atherosclerotic lesion area, inhibited LDL oxidation ex vivo, and preserved vitamin E in LDL. The Sal B-enriched fraction also induced neointimal cell apoptosis in a rabbit angioplasty model [8].

In conclusion, this study demonstrated the fruitfulness to search for potential antioxidants and anti-atherosclerotic agents from herbal medicines traditionally used in the treatment of blood stasis. Based on recent advances in atherosclerotic research, this study also provided platform technology for the evaluation of herbal medicines which are originated from ancient wisdom.
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