Morphological evidence for the antiatherogenic effect of scoparone in hyperlipidaemic diabetic rabbits

Yuh-Lien Chen, Hucii-Chen Huang, Yu-I Weng, Yu-en-Jinn Yu, and Yuan-Teh Lee

Objective: Scoparone (6,7-dimethoxycoumarin), a coumarin isolated from a hypolipidaemic Chinese herb Artemisia scoparia, has vasodilator and antiproliferative activities and possesses free radical scavenging properties in vitro. The aim of the study was to investigate the morphological effects of scoparone in the antiatherogenic process in vivo by using hyperlipidaemic diabetic rabbits as an animal model. Methods: Male New Zealand White rabbits were divided into three groups: control (normal), hyperlipidaemic diabetic, and scoparone treated hyperlipidaemic diabetic. The plasma concentration of total cholesterol and triglycerides were determined. The thickness of the tunica intima was measured on paraffin sections of the aortas stained with Movat's pentachrome. The aortic samples were also processed for scanning and transmission electron microscopy. Results: Neither the lipid profile in the plasma nor the structures of the aortic wall from the control group showed abnormalities. In contrast, the aortas from the hyperlipidaemic diabetic group showed prominent atherosclerotic plaques. Large numbers of monocytes were found adherent to the luminal surface and a markedly thickened intima filled with many lipid laden foam cells was clearly observed. By comparison, the scoparone treated group showed less advanced atherosclerosis with a lower plasma cholesterol. In the scoparone treated rabbits, the proportion of the aortic surface area covered with macroscopic plaques was 30%, and the thickness of the tunica intima 17%, of that of the non-scoparone treated hyperlipidaemic diabetic rabbits. Conclusions: Scoparone has an antiatherogenic action in hyperlipidaemic diabetic rabbits.

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Atherosclerotic lesions are characterised by the accumulation of lipid, the formation of many foam cells, and the proliferation of smooth muscle cells in the vascular wall. Injury to the endothelium has been hypothesised as a key event in atherogenesis. The dysfunction of the endothelium caused by injury, notably at branch points in the arterial tree, includes increased trapping of lipoproteins from plasma in the artery and the appearance of specific adhesive glycoproteins on the surfaces of endothelial cells. Monocytes adhere to the vascular endothelium and migrate into the vessel wall under the influence of growth regulatory factors and chemotactants released by the altered endothelium, by its adherent leucocytes, and possibly by underlying smooth muscle cells. As the process continues, monocytes reach the subendothelial space where they are converted into macrophage derived foam cells, and, together with the actions of accompanying lymphocytes, the fatty streak is formed. These lesions often form at sites where intimal smooth muscle cells migrate from the tunica media. The conversion of macrophages into foam cells requires the modification of low density lipoprotein (LDL) into a form that allows its uptake via the scavenger receptor pathway. Vasoconstrictor and vasodilator responses are progressively reduced with increasing plaque formation. Hence agents which inhibit LDL oxidative modification (free radical scavengers) or abrogate the formation of foam cells might be expected to inhibit the formation and the progression of atherosclerotic lesions. One such compound is scoparone.

Scoparone (6,7-dimethoxycoumarin), a coumarin isolated from the hypolipidaemic Chinese herb Artemisia scoparia, is a cardiovascular drug used in Chinese traditional medicine. The pharmacological activities recently described for the drug include vasodilator and hypotensive actions, immuno-suppressive activities, and free radical scavenging properties. Scoparone has also been shown to exert anti-anginal effects on the heart. Results from these studies raise the possibility that scoparone might inhibit the progression of atherosclerosis. However, there have been no ultrastructural investigations on the morphological changes in atherosclerotic vascular wall following treatment with scoparone. In the present study, we gave scoparone to hyperlipidaemic diabetic rabbits to examine its morphological effects in the atherogenic process in vivo. In this animal model, the cholesterol fed rabbit was treated with a diabetogenic agent, alloxan, which markedly increased plasma lipid and lipoprotein lipid concentrations as compared to animals fed with cholesterol only. Such studies are crucial in establishing that scoparone is a potential therapeutic drug in atherosclerosis treatment.

Methods

Experimental animals
Male New Zealand White rabbits were used. The investigation conforms with the Guide for the care and use of laboratory animals published by the US National Institute of Health (NIH publication No 85-23, revised 1985). The effects of scoparone were evaluated by using three rabbit groups: control (normal, fed with normal unsupplemented rabbit chow); hyperlipidaemic diabetic (cholesterol fed and alloxan-diabetic); and scoparone treated (cholesterol fed, alloxan-diabetic, and scoparone treated). Each group consisted of six rabbits. Hyperlipidaemia was induced in rabbits weighing 2-2.5 kg by feeding them an atherogenic diet consisting of 1% cholesterol (Sigma), and 20% corn oil. Each rabbit was housed in a single cage and fed daily ad libitum. Diabetes was induced in the second week by intravenous administration of a 10% solution of alloxan monohydrate (Sigma) freshly prepared in saline (60 mg kg-1). In the scoparone treated group, cholesterol fed rabbits were given alloxan and at the same time treated by a daily injection of scoparone (5 mg kg-1 subcutaneously) (Aldrich Chemical Co). Six weeks after the cholesterol feeding, the rabbits were processed for the morphological experiments.

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Blood samples
Blood samples of each animal were collected before and after the treatment. The values of total plasma cholesterol and triglyceride were then measured by Merck assay kits (Parnestad, Germany).

Perfusion fixation for vascular morphology
The animals were anaesthetised with pentobarbitone (50 mg/kg i.v.) intravenously. Both femoral veins were exposed and cannulated for perfusion runoff. A cannula connected to a perfusion apparatus was inserted deeply into the right carotid artery near the heart. Prior to exsanguination each animal was given an intravenous injection of sodium pentobarbitone and the blood was flushed with Ringer solution at a flow rate of 400 ml/min, which was required to maintain the intravascular pressure at 110 mm Hg. When the runoff was clear, 3 litres of 10% formalin were pumped through at the same flow rate. Following the perfusion fixation, the aortic arch and thoracic aorta were removed and processed for morphological studies.

Gross morphological examination
After the aorta was slit open longitudinally, the inner surface was exposed. The vesse was then covered with a clear plastic sheet and the area corresponding to atheromatous plaques on the sheet was delineated. The delineated areas of atherosclerotic plaques and the total surface area were quantitated by using the micro computer imaging device (MCID) system with the BRS-2 feature (Imaging Research Inc, Brock University, Canada). The extent of the lesions was expressed as the proportion of the surface area with atheromatous plaque. The aorta was then divided into six segments. Each segment was further cut into three parts. Two 2 mm sections were taken for light microscopy and transmission electron microscopy; the remaining larger portion was processed for scanning electron microscopy.

Light microscopy
Tissue samples (six segments per animal) were immersion fixed in a 10% formalin fixative overnight. Following dehydration through a graded series of ethanol, the samples were embedded in paraffin, cut into 5 µm sections, and stained with Movat’s pentachrome stain. Histological observations were observed and photographed under the light microscope. The thickness of the intima was determined by computerised planimetry. Some areas in the tunica intima from the aortic cross sections of the hyperlipidaemic diabetic group and the scopolamine treated group showed abnormal thickness. Three data points (the thickest, middle range, and thinnest area from the abnormal region) from each section were collected.

Scanning electron microscopy
The arterial segments were examined for monocyte adherence and the extent of atherosclerosis. The segments were washed in buffer and processed in 1% osmium tetroxide (O₃O₄) in 0.1 M phosphate buffer for 2 h at room temperature. Tissue samples were then dehydrated through a graded ethanol series and critical point dried with liquid carbon dioxide, mounted in stubs, and coated with gold palladium. The specimens were examined with a JEOL JSM-5400 scanning electron microscope at 15 kV.

Transmission electron microscopy
Examination of transmission electron micrographs from the three groups confirmed and extended the observations made by light microscopy. After fixation, each arterial segment was washed thoroughly in 0.1 M phosphate buffer and postfixed in 1% buffered OsO₄ for 3 h at room temperature. The samples were dehydrated completely through a graded ethanol series followed by propylene oxide. Each sample was then embedded in Epon 812. The thin sections (70 nm) were cut with an ultramicrotome (Reichert Jung Ultracut E) and placed on formvar grids. The sections were counterstained with uranyl acetate and lead citrate and were examined with a JEOL JEM-2000EXII transmission electron microscope.

Statistics
All values are expressed as the mean±SEM. Differences between means from three groups were tested for statistical significance by use of analysis of variance followed by a Duncan’s multiple range test. p values of less than 0.01 are considered significant.

Results
For the convenience of comparison, the light microscopical results for three rabbit groups are all presented in fig 1, the scanning electron micrographs are in fig 2, and the transmission electron micrographs are in fig 3.

Control animals
In the control group, the plasma cholesterol and triglyceride concentrations were 72±SEM 9 and 80±7 mg/dl, respectively (table I). The concentrations did not change significantly during the six week period. Gross examination of the arterial segments from all six normal animals showed no visible abnormalities (table II). No lesions were observed in the vascular wall by light microscopy (fig 1A). The thickness of the tunica intima was too thin to measure by light microscopy, and was therefore measured by transmission electron microscopy (table II). The thickness of the intima was only about 0.003 mm. Scanning electron microscopy revealed no atherosclerotic plaques on the luminal surface. The surfaces of the aortas were flat and smooth and were covered by a structurally intact endothelium (fig 2A). Endothelial cells were aligned with their long axes in the direction of blood flow. The endothelial cells appeared intact and formed a single layer by transmission electron microscopy (fig 3A). No foam cells were observed in the tunica intima. The endothelial cells and underlying smooth muscle cells contained no lipid droplets.

Hyperlipidaemic diabetic group
The plasma became milky after the six week period. The total plasma cholesterol and triglyceride levels increased 32-fold and 2.5-fold, respectively, as compared with the control group (table I). Grossly visible lesions covered a

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of scopolamine on plasma lipid concentrations in hyperlipidaemic diabetic rabbits. Values are means(SEM) at the end of six weeks.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total cholesterol (mg/dl)</td>
</tr>
<tr>
<td>Control group</td>
<td>72(9)</td>
</tr>
<tr>
<td>Hyperlipidaemic diabetic group</td>
<td>238(320)*</td>
</tr>
<tr>
<td>Scopolamine treated group</td>
<td>1620(182)*</td>
</tr>
</tbody>
</table>

The hyperlipidaemic diabetic group was fed with a specific diet containing 1% cholesterol and 20% corn oil and diabetes was then induced by a bolus intravenous injection of 10% alloxan solution (60 mg/kg) beginning in the second week. The scopolamine treated group was fed with the same diet as detailed above, injected with alloxan, and treated by daily injections of scopolamine (5 mg/kg i.d) subcutaneously starting at the time of alloxan administration.

The values in square brackets express the percentage of the equivalent values in the hyperlipidaemic diabetic group.

Significantly different from control values: p<0.01 (ANOVA, Duncan’s test).

<table>
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<tr>
<th>Table II</th>
<th>Percentage of plaque surface area and the thickness of the tunica intima of the aorta of the three groups. Values are means(SEM).</th>
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<tr>
<td></td>
<td>Surface area of plaque (%)</td>
</tr>
<tr>
<td>Control group</td>
<td>0</td>
</tr>
<tr>
<td>Hyperlipidaemic diabetic group</td>
<td>15.2(2.8)*</td>
</tr>
<tr>
<td>Scopolamine treated group</td>
<td>4.3(1.7)*</td>
</tr>
</tbody>
</table>

The thickness of the tunica intima was measured by light microscopy. The plaque surface area is expressed as the sum of areas of the athero-arterotic plaques × 100 / total surface area.

The values in square brackets express the percentage of the equivalent values in the hyperlipidaemic diabetic group.

*The normal group did not have an abnormal region in the aortic cross section so the thickness of the tunica intima is very low and was thus measured only through transmission electron microscopy.

Significantly different from control values: p<0.01 (ANOVA, Duncan’s test).

*Significantly different from the hyperlipidaemic diabetic group: p<0.01 (ANOVA, Duncan’s test).
mean of 15% of the total aortic surface (table I). Atherosclerotic lesions were distributed prominently near branch sites, bifurcations, and dorsal portions. A markedly thickened intima filled with many lipid laden foam cells and some pericellular lipids in the intimal-medial layers were clearly visible under the light microscope (fig 1B). Many significant fatty streaks were observed by scanning electron microscopy (fig 2B). Endothelial cells over the atherosclerotic lesions were irregularly arranged, and numerous monocytes were adjacent to the luminal surface in most areas (fig 2C). The surfaces of the atherosclerotic lesions often contained substantial irregularities associated with marked disruption of the endothelial cells, resulting in the exposure of the underlying foam cells and connective tissues in the subendothelial space (fig 2D). Platelet microthrombi were frequently seen to be attracted to the surface of the exposed foam cells. Transmission electron microscopy revealed that the endothelial cells were generally not intact and were denuded at the luminal surface. The smooth muscle cells and macrophages of the lesion had taken up large quantities of lipids (fig 3B). Multiple layers of subendothelial foam cells were observed and contained large numbers of lipid droplets (fig 3C).

The antiatherogenic effect of scoparone

The total plasma cholesterol in the scoparone treated animal was 69% of that of the hyperlipidemic diabetic group, but the total plasma triglyceride concentration was similar between these two groups (table I). The observed lesion area covered a mean of 4% of the total aortic area (table II). The lesions consisted of fewer layers of intimal foam cells overlying an otherwise normal looking arterial wall by light microscopy (fig 1C). The mean intima thickness in the atherosclerotic lesions of the scoparone treated rabbits was 83% less than that of the scoparone untreated hyperlipidemic diabetic rabbits (table II). The atherosclerotic lesions were fewer and smaller and were usually observed only near branch sites and bifurcations by scanning electron microscopy (fig 2E). Endothelial cells appeared intact and few monocytes adhered to the luminal surface (fig 2F). Few foam cells in the intima were observed by transmission electron microscopy. Endothelial cells and smooth muscle cells had less lipid accumulation in the cytoplasm (fig 3D).

Discussion

To the best of our knowledge, this is the first study to provide morphological evidence of a significant retardation of atherosclerotic lesions in cholesterol fed alloxan-diabetic rabbits following the scoparone treatment. These results indicate that scoparone is effective in inhibiting athemomatous lesion formation in the aortae of hyperlipidemic diabetic rabbits.

In this study, plasma cholesterol levels in the hyperlipidemic diabetic rabbits were over 2000 mg/dL and severe progressive atherosclerotic lesions occurred after six weeks. Although the scoparone treated rabbits did not have total cholesterol levels as low as those in the normal rabbits, the group showed both a lower plasma cholesterol and a retarded progression of atherosclerosis after five weeks of treatment. Thus the preventive effects of scoparone on the formation of atherosclerotic plaques may be partially attributed to lower cholesterol levels.

Free radicals and lipid peroxidation play an important role in the pathogenesis of atherosclerosis. Proscanol, an antioxidant, was shown to retard the progression of atherosclerosis by limiting oxidative LDL modification and
Figure 2  Scanning electron micrographs of the luminal surface of aortas from three rabbit groups. (A) The control group shows no visible atherosclerotic plaques. Endothelial cells (E) are intact and aligned parallelly. (B) The hyperlipidemic diabetic group shows atherosclerotic plaques (arrows) that are strikingly raised above the luminal surface. (C) A higher magnification of atherosclerotic lesions from the hyperlipidaemic diabetic group. Many monocytes (M) are adherent to the luminal surface and seem to be in transit between the endothelial cells. (D) The hyperlipidemic diabetic group shows an expanding fatty streak and endothelial denudation that exposes the underlying foam cells (F), connective tissue, and subendothelial space. Platelet microthrombi (arrowhead) are adherent to these areas. (E) The scopolamine treated group shows an atherosclerotic lesion (arrows) at the right side near the branch point of an intercostal artery (asterisk). The left side of the branch of the intercostal artery shows a normal luminal surface. (F) The scopolamine treated group shows intact endothelial cells and several monocytes adherent to them in the atherosclerotic lesion. A: ×480; B: ×280; C: ×1300; D: ×3200; E: ×150; F: ×1700.

subsequently foam cell transformation of macrophages in hyperlipidaemic rabbits.19 The antiatherogenic effect of probucol was due to its ability to act as a superoxide radical scavenger. Probufol has also been shown to lower plasma LDL levels, to enhance the fractional catabolic rate of LDL, to augment reverse cholesterol transport through modifications to HDL
and apo E synthesis, and to prevent the oxidative modification of LDL. Since scoparone has been found to have free radical scavenging activity in rat aortic rings, it may protect against elevation of the lipoprotein levels and hence retard the progression of atherosclerotic lesions in hyperlipidaemic diabetic rabbits in a similar manner to probucol.
The atherosclerotic lesions in the hyperlipidaemic diabetic rabbits in this study are mainly composed of fatty streaks, which occur during earlier stages of atherosclerosis. The surface of atherosclerotic lesions contains large numbers of adherent leucocytes, many of which can be observed apparently entering the arterial wall. The fatty streaks are thicker, and frequent disruption of endothelial cells is seen, thus resulting in the exposure of the subendothelial foam cells and adherent platelet microthrombi. In the scaporane treated groups the above phenomena were not prominent. It is of interest to note that the occurrence of the atherosclerotic lesions was low in the scaporane treated group, and the majority of these lesions were preferentially located at branch sites and bifurcations. This suggests that the regional distributions of atherosclerotic lesions were partly determined by haemodynamic factors, for example, shear stress and disturbed flow at the vessel wall.21 The undulating endothelium and elastic lamellae in the specimens observed by light microscopy and scanning electron microscopy may result from vessel recoil during fixation, which was found to occur when glutaraldehyde was not added as a fixative.22 However, this apparent artefact is not likely to interfere with the differences observed among different groups since the same route of fixation and consistently monitored intra-arterial pressure applied to all the animals during the perfusion fixation.

Monocyte adhesion/migration and the deposition of foam cells in the vascular wall in vivo depends in part on the activation state of circulating monocytes and on the expression of adhesion molecules by both mononuclear cells and endothelial cells.5, 23 It is also dependent on the secretion of chemotactic agents and inhibitors of adhesion (such as nitric oxide) by the endothelium or smooth muscle cells.24 In this study we found that scaporane has an inhibitory effect on monocyte adhesion/migration and the deposition of foam cells in vivo. The possibility that scaporane inhibits the activation of monocytes from hyperlipidaemic diabetic animals is supported by the studies of Huang et al.22 in which they showed that scaporane inhibits interleukin-1 (IL-1) and interleukin-2 (IL-2) secretion from phytohaemagglutinin stimulated monocytes. IL-1 and IL-2 are potentially important cytokines in atherogenesis, being indirect mitogens for smooth muscle cells23 and inducers of cell adhesion molecule expression on endothelial cells.22 Oxidised LDL itself causes cell adhesion molecules to be induced on monocytes.25 Products of lipoprotein oxidation are also reported to be chemotactic for monocytes.26 It is conceivable that scaporane may inhibit monocyte chemotaxis, adhesion, and deposition by inhibiting LDL/LDL oxidation. It is also possible that scaporane may inhibit the uptake of modified LDL and VLDL by macrophages. In this context, scaporane has been reported to suppress the proliferative response of human mononuclear cells to the stimulation by phytohaemagglutinin and in mixed lymphocyte reactions.13 Whether the suppression of monocyte proliferation is involved in the protective effect of scaporane against atherogenesis remains to be determined.

In conclusion, this study shows that scaporane significantly retards the progression of visible aortic atherosclerosis, the tunica intima thickness of the aorta, and the stimulated monocyte-endothelium adhesion in hyperlipidaemic diabetic rabbits. Our results suggest that the antiatherogenic action of scaporane might be associated with its hypolipidaemic effects and suppression of foam cell deposition.

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Key terms: scaporane; hyperlipidaemic diabetic rabbit; antiatherogenesis; ultrastructure.

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