Raman Spectroscopic Evaluation of the Effects of Diet and Lipid-Lowering Therapy on Atherosclerotic Plaque Development in Mice


Abstract—Quantitative characterization of atherosclerotic plaque composition with standard histopathological methods remains limited to sectioned plaques. Raman spectroscopy enables nondestructive quantification of atherosclerotic plaque composition. We used Raman spectroscopy to study the effects of diet and lipid-lowering therapy on plaque development in apolipoprotein (APO) E*3-Leiden transgenic mice. Raman spectra were obtained over the full width and entire length of the ascending aorta and aortic arch. Spectra were modeled to calculate the relative dry weights of cholesterol and calcium salts, and quantitative maps of their distribution were created. In male mice (n = 20) that received a high-fat/high-cholesterol (HFC) diet for 0, 2, 4, or 6 months, Raman spectroscopy showed good correlation between cholesterol accumulation and total serum cholesterol exposure (r = 0.87, P < 0.001). In female mice (n = 10) that were assigned to an HFC diet, with or without 0.01% atorvastatin, a strong reduction in cholesterol accumulation (57%) and calcium salts (97%) (P < 0.01) was demonstrated in the atorvastatin-treated group. In conclusion, Raman spectroscopy can be used to quantitatively study the size and distribution of depositions of cholesterol and calcification in APOE*3-Leiden transgenic mice. This study encourages Raman spectroscopy for the quantitative investigation of atherosclerosis and lipid-lowering therapy in larger animals or humans in vivo. (Arterioscler Thromb Vasc Biol. 2001; 21:1630-1635.)

Key Words: atherosclerosis ▪ Raman spectroscopy ▪ cholesterol ▪ calcification ▪ lipid-lowering treatment

Progression of atherosclerosis is dependent on the amount of lipids that accumulate in the intima of arteries. Several drugs have been developed that successfully lower the plasma cholesterol concentration, thereby reducing the rate of progression of atherosclerosis. However, despite the success of these drugs, a considerable number of treated patients will still encounter an ischemic event. To improve management of patients with atherosclerosis, a more thorough understanding of plaque progression and regression in vivo, at the chemical level, is required.

Raman spectroscopy is a technique that can provide this kind of information. It provides quantitative information about the molecular composition of a sample and enables the nondestructive examination of small volumes of tissue. A Raman method to quantify the relative amounts of protein, cholesterol, adventitial fat, and calcium salts (CS) in the human coronary artery wall has been developed by Brennan et al. Recently, it was demonstrated that high signal-to-noise Raman spectra can be obtained from the aortic arch and arteries of sheep in vivo, in the presence of blood flow, by using specially designed Raman fiber-optic catheters and a transluminal approach.7,8

We studied atherosclerotic plaque formation in transgenic mice that were fed a high cholesterol–containing diet. These mice, carrying the dysfunctional apoE variant from patients with a dominantly inherited form of familial dysbeta-lipoproteinemia (APOE*3-Leiden), develop severe hyperlipidemia that results in rapid atherosclerotic plaque formation with feeding of a diet enriched in saturated fat and cholesterol.9 In vitro quantitative characterization of atherosclerotic plaque composition with standard histopathological methods is laborious and remains limited to sectioned plaques. In contrast, Raman spectroscopy allows evaluation of entire lesions, leaving the morphology of the plaque intact.

In this study, we describe a method of generating chemical maps of the relative distribution of cholesterol and calcification in the aortic arch of APOE*3-Leiden transgenic mice. We used this method to study changes in atherosclerotic plaque composition as a function of cholesterol exposure and treatment with the lipid-lowering drug atorvastatin. We dem-
onstrate in this report that Raman spectroscopy enhances the utility of this atherosclerotic mouse model for studying the progression of atherosclerosis and for the evaluation of antiatherosclerotic treatment.

Methods

APOE*3-Leiden Transgenic Mice

All animal procedures were approved by the institutional review committee on animal experimentation at TNO, the Netherlands. Transgenic mice (n=44) expressing the human APOE*3-Leiden gene were generated as described before. Transgenic mice were the F11 (group I), F13 (group II), or F16 (group III) generation resulting from the breeding of male APOE*3-Leiden transgenic mice with female C57BL/6jico mice (Brockman Institute BV, Someren, the Netherlands). Identification of transgenic mice occurred by using a sandwich ELISA for the presence of human APOE*3-Leiden in serum.

Experimental Setup

Because of the difference in response to high-cholesterol feeding, male and female mice were assigned to different groups. Group I mice (female, n=14) were used in a feasibility study to illustrate that the distribution of cholesterol and calcification as measured by Raman scanning is in agreement with the distribution of these compounds as determined by von Kossa (calcification) staining and oil red O (lipids) staining. During the first 8 to 10 weeks after birth, these mice were kept on standard mouse chow (SRM-A Hope Farms) and were then randomly assigned to a group (n=7) receiving normal chow or to a group (n=7) receiving a high-fat/high-cholesterol (HFC) diet. An extra 0.5% cholate was added to the HFC diet to facilitate intestinal uptake of fat and cholesterol, thereby increasing the total plasma cholesterol (TC) levels. Serum cholesterol concentrations of these mice were obtained post mortem.

Group II, 3-month-old male mice (n=20) were used to evaluate plaque progression in time. These mice were randomly assigned to 4 subgroups, which were fed the same HFC diet with 0.5% cholate for 0, 2, 4, or 6 months. One subgroup (n=5) was kept on normal chow for 6 additional months. One subgroup (n=5) was assigned to 4 months of normal chow and 2 months of the HFC/0.5% cholate diet. Five mice received 2 months of normal chow and subsequently 4 months of the HFC/0.5% cholate diet. The last subgroup (n=5) received the HFC/0.5% cholate diet for the entire 6 months. All mice were humanely killed at 6 months from the start of the randomization.

Group III, female mice (n=10) were used to quantify the effect of atorvastatin on the contents of cholesterol and calcification in the aortic wall. Before the start of this study, these mice were kept on standard chow for ~3 months. During a 3-week run-in period, these animals received the HFC diet with 0.05% cholate. Because female mice develop more severe hyperlipidemia, a lower concentration of cholate was chosen to prevent liver damage. After the run-in period, TC concentrations were measured. On the basis of age and cholesterol level, the animals were matched and assigned to 2 equal groups, with approximately the same age and cholesterol level. The control group (n=5) received the HFC/0.05% cholate diet. The atorvastatin group received the same diet but with the addition of 0.01% (wt/wt) atorvastatin (Pfizer Inc), equaling a daily dose of ~15 mg/kg body weight. All mice in group III were humanely killed at 28 weeks.

Tissue Handling

Mice were humanely killed with an overdose of pentobarbital (Nembutal®) anesthesia. The thorax was opened and the heart and aorta were dissected, flushed with PBS (pH 7.4), snap-frozen in liquid N2, and stored at ~80°C until use. At the time of spectroscopic investigation, the heart and aorta of each mouse were thawed to reach room temperature. The aorta was dissected distally from the aortic valve. The first 5 to 8 mm of each aorta was used for spectroscopic investigation because this area is the most prone to atherosclerotic lesion development. The aorta was cleaned of its adventitial fat to facilitate opening the aorta through its outer curvature, longitudinally through the brachiocephalic, carotid, and subclavian arteries. The samples were then moistened with PBS and placed between 2 (0.8-mm-thick) CaF2 windows (Crystalan). CaF2 was used because it does not exhibit Raman bands in the 800- to 1800-cm−1 wavelength region, which was chosen for our study.

Raman Instrumentation and Data Acquisition

To collect Raman spectra, near-infrared laser light (~847 nm) was coupled to a microscope that was rebuilt for Raman spectroscopic measurements. The microscope was equipped with a motorized, computer-controlled sample stage, which enables automatic scanning of the sample. The aorta and CaF2 windows were positioned underneath the microscope with the internal side of the artery facing up. Raman spectra were obtained in 0.25-mm steps over the full width and entire length of the excised aorta. For mouse aortas in the feasibility group (group I), a coarser grid of data sampling points was used (0.50 mm). All samples were irradiated with 80 to 100 mW of near-infrared laser light (~847 nm). The collection time per measurement was 10 seconds.

Raman Data Processing

At each location of the grid, the Raman spectrum was modeled as a linear combination of the Raman spectra of delipidated artery, cholesterol, cholesterol esters, β-carotene, triglycerides and phospholipids, calcification and β-carotene, according to the method developed by Brennan et al (Figure 1). In this way, the molecular composition of the tissue could be determined and expressed in relative weight percentages. In this study, we explicitly focused on the distribution of cholesterol and CS. Therefore, we adapted the analysis protocol in the following way. After calculation of the relative weight percentages (normal mouse aorta, cholesterol, cholesterol esters, β-carotene, triglycerides and phospholipids), calcification and β-carotene, according to the method developed by Brennan et al (Figure 1). In this way, the molecular composition of the tissue could be determined and expressed in relative weight percentages. In this study, we explicitly focused on the distribution of cholesterol and CS. Therefore, we adapted the analysis protocol in the following way. After calculation of the relative weight percentages (normal mouse aorta, cholesterol, cholesterol esters, β-carotene, adventitial fat, calcification, and optical instrument background), the sum of the contributions of protein (normal mouse aorta), TC (the sum of cholesterol and cholesteryl esters), and CS was scaled to 100%. Subsequently, the relative quantities of protein, CS, and TC were recalculated. In this way, the otherwise interfering and varying signal contributions of the remaining adventitial fat, β-carotene, and instrument background signal were eliminated. Maps reflecting the relative distribution of these arterial components in the aortic samples could then be reconstructed.
Lipid Analysis

At 3-week time intervals and at post mortem, the serum lipid levels of mice in groups II and III were measured. Blood samples were obtained at baseline and in 3-week time intervals from the tail vein, always after 4 hours of fasting. Blood samples were obtained at baseline and in 3-week time intervals from the tail vein, always after 4 hours of fasting. Post mortem blood samples were obtained from the ophthalmic artery after enucleation. Levels of TC and triglycerides were measured enzymatically by using commercially available kits (Boehringer). TC exposure was calculated as the sum of the serum values and is expressed in mmol/L \times \text{week}.

In mice in group III, VLDL, IDL/LDL, and HDL fractions were determined at 25 weeks from the start of randomization. For size fractionation, 40 μL of pooled plasma per group was injected onto a Superose 6 column (3.2 × 30-mm Smart-system, Pharmacia) and eluted at a constant flow rate of 50 μL/min with PBS (pH 7.4, containing 1 mmol EDTA). 10

Histology

After spectral examination, aortas of group I mice were positioned on a silicone surface in a 60-mm-diameter Petri dish and held in place with 0.15-mm-diameter steel pins. The aortas were subsequently stained with oil red O 13,14 and von Kossa stains. 15 Digital images were made en face with a HitachiHV-C20M color CCD camera equipped with a 55-mm Nikon lens.

Statistical Analysis

For statistical analysis, spss 9.0 software (SPSS) was used. Because the data were not normally distributed, nonparametric tests were used for comparisons between groups. Overall comparison between groups was performed with the Kruskal-Wallis test. When only 2 groups were compared, Mann-Whitney rank-sum tests were used. Differences were regarded as statistically significant when \( P < 0.05 \).

Results

Histological Validation

Figure 1 shows Raman spectra obtained from the inner curvature of a mouse aortic arch (group I). Also shown are the results of the fit with model spectra (see Methods) and a table of the fit contributions. Raman maps of TC and CS from a mouse in feasibility group I and corresponding stains with oil red O (lipids) and von Kossa stain (calcification) are shown in Figure 2. The Raman maps show the distribution of choles-
terol and CS, and the stains confirm the presence and location of lipids and calcified deposits in the artery wall. In all samples in group I, there was a good correlation between the location of TC as detected in the Raman maps and the lipids stained by oil red O.

In all 7 mice (group I) that were fed the HFC diet, atherosclerotic plaques were observed in the center of the inner curvature of the aortic arch and at the origin of the aortic side branches. In all atherosclerotic samples, calcification accumulated roughly in the center of the cholesterol-rich areas. In mice that were fed the normal chow, atherosclerotic lesions were practically absent. In some areas with high quantities of cholesterol, a calcium-phosphate stretch bond (at 960 cm$^{-1}$) was clearly present in the Raman spectra, whereas von Kossa staining did not visualize calcification in the aortic sample. Previous histological studies have clearly demonstrated the power of Raman spectroscopy to detect microcalcifications.$^{15,16}$ The discrepancies between the Raman maps and the von Kossa stains can therefore be explained by the presence of overlying tissue, which hinders appropriate staining of the calcific deposits.

**Plaque Development in Time**

For mice in groups II and III, maps at higher spatial resolution were created. This is demonstrated in Figure 3. In group II mice, the time dependence (0 to 6 months) of atherosclerotic plaque formation is demonstrated by a progressive increase of plaque areas in the first 5 mm of the aortic arch with $>10\%$ TC (10% to 100%), $>20\%$ TC (20% to 100%), $>30\%$ TC (30% to 100%), and $>40\%$ TC (40% to 100%) (the Table), as detected with Raman spectroscopy. Figure 4 shows the time-dependent growth of plaques with $>10\%$ TC. Because of the large variation within each subgroup of mice, the standard error is relatively high (see error bars in Figure 4). As demonstrated in the Table, the average cholesterol exposure for each subgroup is related to the size of the plaque in the aortic wall. The individual relation between cholesterol exposure and the size of the atherosclerotic plaque with $>10\%$ TC is demonstrated in the inset of Figure 4. From this semilogarithmic plot, it appears that the rate of plaque development in the period studied (0 to 6 months of an HFC/0.5% cholate diet) increased with accumulative cholesterol exposure ($R=0.87$, $P<0.001$). Only 2 mice in the group that was exposed to 6 months of the HFC/0.5% cholate diet exhibited calcified lesions. Therefore, no time-dependent correlation for the development of calcification can be shown here.

Although there was a tendency for plasma triglyceride levels to decrease in mice that were fed the HFC/0.5% cholate diet, these differences were not statistically different (see the Table). In addition, no correlation could be observed between the average level of plasma triglycerides and plaque size as determined by Raman spectroscopy ($R=0.001$). This result is in agreement with previous studies in APOE*3-Leiden transgenic mice.$^{10}$

**Effect of Treatment**

Female mice (group III) developed more severe hyperlipidemia and atherosclerotic lesions than did male mice (group II), including calcification. Cholesterol in the control group was mainly present in VLDL (11.95 mmol/L) and IDL/LDL.

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**Table 1**: TC Exposure and Plaque Surface Calculated by Raman Spectroscopy for Male Mice (Group II) on an HFC Diet and for Female Mice (Group III) Treated With or Without Atorvastatin

<table>
<thead>
<tr>
<th>Group* (n=5)</th>
<th>TC Exposure, mmol/L × Week</th>
<th>Average TG/Week, mg/dL</th>
<th>Plaque Surface, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 months</td>
<td>57±0.6</td>
<td>0.84±0.07</td>
<td>$&gt;10%$ TC</td>
</tr>
<tr>
<td>2 months</td>
<td>117±23</td>
<td>0.87±0.22</td>
<td>$&gt;20%$ TC</td>
</tr>
<tr>
<td>4 months</td>
<td>281±19</td>
<td>0.68±0.09</td>
<td>$&gt;30%$ TC</td>
</tr>
<tr>
<td>6 months</td>
<td>490±60</td>
<td>0.74±0.10</td>
<td>$&gt;40%$ TC</td>
</tr>
<tr>
<td><strong>III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>417±37</td>
<td>1.76±0.43</td>
<td>$&gt;10%$ TC</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>180±13*</td>
<td>1.07±0.15</td>
<td>$&gt;20%$ TC</td>
</tr>
<tr>
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TG indicates triglyceride.

$^*$P<0.01 vs control.
Discussion

This report demonstrates that Raman spectroscopy can be applied to the study of atherosclerotic plaque development in terms of plaque area and composition in a quantitative manner, both as a function of time and as a function of drug therapy. By calculating the relative contributions of cholesterol, calcification, and protein derived from the arterial Raman spectra, we were able to generate maps reflecting the distribution of these components in the aortic arch of mice. To our knowledge, this study is the first to evaluate the time-dependent and treatment-dependent changes in individual plaque components over an entire, intact (ie, unsectioned) atherosclerotic lesion. Recent studies have investigated the lesion surface or lesion type in the ascending aorta, including the aortic arch. Our results demonstrate that the 2 most important chemical components in atherosclerotic plaque, ie, cholesterol and CS, appear to accumulate just distally from the aortic valve and at the base of the major branches of the aortic arch. This concept is in agreement with previous studies. Calciﬁed deposits are located within the area of cholesterol accumulation, similar to the situation in the human coronary artery, and in aortic or peripheral arterial atherosclerotic lesions. This study therefore demonstrates a novel, reliable, and nondestructive method to investigate atherosclerotic plaque formation in a well-deﬁned and relatively inexpensive animal model. This study also suggests an exponential relationship between size of the plaque in the inner curvature of the mouse aortic arch and cholesterol exposure. This suggestion is concordant with previous studies on plaque development in the aortic root of APOE*3-Leiden transgenic mice and other apoE-deﬁcient mice.

The effect of lipid-lowering therapy on plaque composition could also be evaluated by Raman spectroscopy. Female mice fed a high-cholesterol diet in combination with atorvastatin showed a 57% reduction in TC exposure and a 97% reduction in atherosclerotic plaque size (expressed as the area of the aortic arch, in which TC accounts for >10% of the dry weight of the intima and media). No signiﬁcant calcification was observed in the atorvastatin-treated group, whereas calcification was abundant in the control group. These atorvastatin-induced changes are expected, as it is well known that owing to the lipid-lowering effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors such as atorvastatin, atherosclerotic plaque formation is inhibited. However, the present study quantiﬁed the effects of atorvastatin on chemical changes in an entire, unsectioned, intact plaque.

As can be observed from the Table, the female mice in group III that received atorvastatin were exposed to 180 mmol/L x week of plasma cholesterol but seemed to produce less plaque than did those mice in group II at 2 months with 117 mmol/L x week. Because atorvastatin did not inﬂuence the HDL cholesterol level in our study, this effect is surprising and might be due to an unanticipated effect of atorvastatin. In this study, however, it is difﬁcult to draw conclusions from this observation, because the mice in group II were male and were fed an HFC diet with 0.5% cholate, whereas the mice in group III were female and were fed the HFC diet with a much lower concentration (0.05%) of cholate. Further animal studies in combination with Raman spectroscopy would allow quantiﬁcation of this pleiotropic effect of statins on the arterial wall.

Ideally, the chemical composition of a plaque is monitored in vivo, allowing repeated measurements of the same location at staining of structures in deeper layers. Moreover, decalcification before sectioning and staining leads to inaccurate visualization and quantification of CS that are present in the plaque. Likewise, quantification with other techniques, such as staining intact plaques with oil red O or visualizing calcification with von Kossa stain, is of limited value because lipids and calcium are mostly stained superficially. In contrast, the penetration depth of Raman spectroscopic investigation of arterial tissue allows evaluation of deeper deposits.

Most studies on atherosclerosis in the aortas of APOE*3-Leiden transgenic mice have focused on the aortic root, where atherosclerotic changes become apparent soon after starting a high-cholesterol diet. We studied plaque formation in the ascending aorta, including the aortic arch. Our results demonstrate that the 2 most important chemical components in atherosclerotic plaque, ie, cholesterol and CS, appear to accumulate just distally from the aortic valve and at the base of the major branches of the aortic arch. This concept is in agreement with previous studies. Calciﬁed deposits are located within the area of cholesterol accumulation, similar to the situation in the human coronary artery, and in aortic or peripheral arterial atherosclerotic lesions. This study therefore demonstrates a novel, reliable, and nondestructive method to investigate atherosclerotic plaque formation in a well-deﬁned and relatively inexpensive animal model. This study also suggests an exponential relationship between size of the plaque in the inner curvature of the mouse aortic arch and cholesterol exposure. This suggestion is concordant with previous studies on plaque development in the aortic root of APOE*3-Leiden transgenic mice and other apoE-deﬁcient mice.

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Figure 5. Mean plaque area ±SEM (mm2) with >10% cholesterol or CS in the aortic arch of female mice that were fed an HFC/0.05% cholate diet for 6 months, with or without 0.01% (wt/wt) atorvastatin. ■ Control, □ atorvastatin. *P<0.01 vs control.

(6.22 mmol/L) fractions and not in the HDL (1.80 mmol/L) fractions. In the atorvastatin-treated group, these values were 4.25, 2.7, and 1.80 mmol/L, respectively. There were no statistical differences between the triglyceride levels of the 2 groups (see the Table).

When female mice were treated with 0.01% (wt/wt) atorvastatin during 6 months of the HFC diet, cholesterol-rich lesions (TC>10%) were dramatically smaller (97%, P<0.01; Figure 5). The effect of atorvastatin on the development of atherosclerosis can largely be ascribed to a 57% (P<0.01) drop in TC exposure (see the Table), with the reduction of cholesterol mainly in the VLDL (66%) and IDL/LDL (55%) fractions and not in the HDL fraction, as described above. For plaques containing higher quantities of cholesterol or CS (>20%, >30%, or >40%), analogous results were observed (the Table).
various times during the atherosclerotic process. Recently, we have demonstrated that with specially developed optical catheters, remote Raman spectra can be acquired intravascularly from the arteries of sheep in vivo, in the presence of blood flow, and from human atherosclerotic plaques at the time of vascular surgery. This, in combination with the present study, demonstrates the unique potential of Raman spectroscopy to study and monitor the effects of diet and lipid-lowering drugs or other antiatherosclerotic interventions on plaque formation.

In this study, we chose to collect Raman spectra over a relatively large area of the mouse aorta, with a small step size (0.25 mm). To limit the total scanning time of the aortas to ~60 minutes per sample, we reduced the collection time per Raman spectrum to 10 seconds per pixel. The signal-to-noise ratio of each individual Raman spectrum that was collected in this way did not allow for precise discrimination between cholesterol and cholesteryl esters, whereas successful separation has been reported in previous Raman spectroscopic research from other laboratories. This distinction is important, because it is hypothesized that the accumulation of noncrystalline cholesteryl esters may soften the lipid core, making plaques with a thin, fibrous cap more prone to rupture. Other laboratory techniques such as magic spinning nuclear magnetic resonance have also successfully quantified cholesteryl esters in research related to plaque vulnerability. With ongoing improvements in the signal-to-noise ratio of Raman spectra, we will be able to discriminate between the various phases of cholesterol in this mouse model ex vivo and eventually in larger animals in vivo.

In conclusion, this study has demonstrated that Raman spectroscopy allows quantitative mapping of chemical constituents in entire atherosclerotic plaques in the aortas of APOE3-Leiden transgenic mice. Atherosclerotic plaques that are located in the aortic arch of these mice contain calcific deposits, surrounded by high quantities of cholesterol, similar to human plaques. The size of plaque in the aortic arch, as measured by Raman spectroscopy, is well correlated with serum cholesterol exposure and the duration of high-cholesterol feeding. The inhibitory effect of atorvastatin on plaque development in the aortic arch of APOE*3-Leiden transgenic mice has also been demonstrated with Raman spectroscopy. This technique, in combination with an atherosclerotic animal model, may enhance our knowledge of the pathophysiology of atherosclerosis and the effects of lipid-lowering agents. As the feasibility of remote catheter-based Raman spectroscopy in vivo has been demonstrated, this study encourages the investigation of atherosclerotic plaque development and the evaluation of antiatherosclerotic drugs in larger animals and humans in vivo.

References
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Materials and Methods

Raman Instrumentation
To collect Raman spectra, near infrared laser light (~847 nm) provided by an Argon-ion pumped Titanium: Sapphire laser system (Spectra Physics, Mountain View, CA) was coupled into a scanning microscope (DM-RXE, Leica, Cambridge, UK) via a single mode optical fiber and a high pass filter (HPF) (figure I). A x80 NIR optimized objective (Olympus, Japan) with a working distance of approximately 1.6 mm was used to focus the laser light onto the tissue sample, and to collect light that was scattered by the sample. A chevron type dielectric filter set (HPF) was used for optical coupling of the laser, the microscope and the spectrometer, and for suppression of the laser light that was scattered back by the tissue sample.\(^1\) The inelastically scattered Raman light was focused onto the 100 µm ∅ core of an optical fiber, which coupled the light into a spectrometer (System 100, Renishaw, Wotton under Edge, UK), equipped with a thermoelectrically cooled deep-depletion CCD camera. The CCD camera was connected to a personal computer where data were stored and analyzed.

Figure I  Schematic drawing of the Raman spectroscopic setup used in the experiments. 847 nm laser light from an Argon-ion pumped Titanium:Sapphire laser system is coupled via an optical fiber into a microscope, equipped with a motorized sample stage via a dielectric bandpass filter. A microscope objective focuses the light on the arterial sample and collects light which is scattered by the sample. Elastically scattered laser light is filtered out by a chevron type high pass filter. The remaining scattered Raman light is coupled into a second optical fiber, which is connected to a Raman spectrometer. The spectrometer and the microscopic sample stage are controlled by a personal computer. For details, see text. (L1 L2: Lenses; OF1 OF2: optical fibers; S: Spectrometer; HPF: High pass filter; M: Microscope; PC: Personal computer).
The microscope was equipped with a motorized, computer controlled sample stage, which enables automatic scanning of the sample. The area to be scanned and the scanning step size could be programmed. Acquisition of Raman spectra and microscopic stage movement was controlled by Grams/32 Spectral Notebase Software (Galactic Industries Corp., Salem, NH). Spectral resolution of the system was approximately 8 cm\(^{-1}\).

Raman data acquisition
The aorta and CaF\(_2\) windows were positioned underneath the microscope with the intimal side of the artery facing up. For each longitudinally opened aorta, two-dimensional maps of the sample were reconstructed, based on the relative Raman signal contributions of the various constituents of the aorta samples. Raman spectra were obtained in 0.25 mm steps over the full width of the aorta, starting 1-2 mm distally from the aortic valve until 3-4 mm distally from the subclavian artery. For mouse aorta in the feasibility group (group I) a coarser grid of data sampling points was employed (0.50x0.50 mm). All samples were irradiated with 80-100 mW of NIR laser light (~847 nm). The depth resolution of the system was 100 µm. The laser light was focused below the surface of the tissue at such a depth that signal intensity was maximized. In order to obtain an average of the molecular composition of the aorta for the entire pixel in the Raman maps, the pixel area was scanned through the laser focus in both lateral directions (an area of 250x250 µm\(^2\)) during each measurement. The entire volume that was sampled per spectrum was therefore 0.25x0.25x0.1 = 0.00625 mm\(^3\). Scanning of the data point did not affect the axial resolution of the system. Signal collection times were 10 s per pixel for all measurements.

Raman data processing
Wavenumber calibration of the spectra was performed using four known Raman calibration standards (4-acetamidophenol, p-bis(o-methylstyrlyl)benzene, gamma-caprolactone (Sigma-Aldrich, St Louis, MO), naphthalene (ICN Biochemicals, Aurora, OH)) and the emission lines of a neon and a neon-argon lamp. After subtraction of the background signal, originating from the optical elements in the laser light delivery pathway, cosmic ray removal, and intensity correction using a calibrated tungsten lamp of a known temperature, the spectra were processed as described previously.\(^2\) Accordingly, to subtract interfering fluorescence background, a fourth order polynomial was fitted through the spectra, and subsequently subtracted.\(^2,3,4\)

For each location of the grid (i.e. for each pixel), the Raman spectrum was modeled as a linear combination of the Raman spectra of basic arterial components according to the method introduced by Brennan et al\(^2\). From the fit results the molecular composition of the tissue was determined and expressed in relative weight percentages of these components (manuscript, figure 1). For the present study, the least squares spectral model included spectra of delipidized artery, free cholesterol, cholesteryl esters, calcium salts, beta-carotene, adventitial fat, and background signal originating from the collection pathway (optics sand CaF\(_2\)). Instead of using human delipidized coronary artery (as in the model used by
Brennan), we used a spectrum from normal delipidized mouse aorta. This spectrum was obtained from an APOE*3 Leiden transgenic mouse that was fed a normal chow for 28 weeks. The mouse aorta was delipidized in a 1:2 methanol: chloroform solution for 48 hours. After rinsing in saline, Raman spectra were obtained with the described experimental setup. These spectra were averaged to obtain a high quality Raman spectrum. Subsequently, this spectrum was scaled according to the model described by Brennan et al7.

In this study, we have explicitly focused on the distribution of total cholesterol (TC) and calcium salts (CS). Therefore we have adapted the analysis protocol2 in the following way: after calculation of the original least square fit-contributions (normal mouse aorta, cholesterol, cholesteryl esters, beta-carotene, adventitial fat, calcification and optical instrument background), the sum of the contributions of protein (P, normal mouse aorta), TC (the sum of cholesterol and cholesteryl esters) and CS were scaled at 100%. Subsequently the relative quantities of P, CS and TC were recalculated. In this way the otherwise interfering and varying signal contributions of the remaining adventitial fat, beta-carotene and instrument background signal were eliminated. Maps, reflecting the relative distribution of these arterial components in the aortic samples could then be reconstructed (manuscript, figures 2 and 3). From these Raman maps, the first 5 mm of the aortic arch were selected for further evaluation.

The thickness of the arterial wall in the mouse aorta is only 100-200 µm. According to a previous study, the maximum signal attenuation would be approximately 50% for a cholesterol deposit covered with 300 µm of normal artery.5 Signal attenuation effects have therefore been neglected in this study.

Reference List


