Potential of $^{99m}$Tc-LDLs Labeled by Two Different Methods for Scintigraphic Detection of Experimental Atherosclerosis in Rabbits

D.E. Atsma, R.I.J. Feitsma, J. Camps, F.M. van 't Hooft, E.E. van der Wall, W. Nieuwenhuizen, and E.K.J. Pauwels

In this study we evaluated two different $^{99m}$Tc-labeling techniques to produce $^{99m}$Tc-low density lipoprotein (LDL) suitable for the scintigraphic delineation of experimental atherosclerotic lesions. The two methods are (1) a procedure that uses stannous chloride and sodium borohydride (borohydride method) and (2) a procedure that uses sodium dithionite as a reducing agent and that has been successfully applied in previous scintigraphic atherosclerosis detection (dithionite method). $^{99m}$Tc-LDL produced by either method was injected into New Zealand White rabbits with diet-induced atherosclerotic plaques and in control rabbits. Scintigraphic images were taken 10 minutes (t=0) and 1, 4, 8, 16, and 24 hours after injection. Clearance of plasma radioactivity was also studied. Stability of the $^{99m}$Tc-LDL complex in the circulation was examined by size exclusion chromatography of plasma samples. After scintigraphy, the animals were killed, and the biodistribution of radioactivity was determined. The thoracic and abdominal aortas appeared in scintigraphic images to accumulate $^{99m}$Tc over their entire length with either $^{99m}$Tc-LDL preparation. The sparse imaging of focal atherosclerosis was found to be due to the fact that the aortas were covered with confluent atherosclerotic lesions. Scintigraphic image analysis showed that 24 hours after injection, the accumulated radioactivity in the abdominal aorta of the atherosclerotic rabbits was 57% and 54%, respectively, of the accumulated radioactivity in the abdominal aorta at t=0 when the borohydride versus the dithionite method was used. In the control animals this value was 25% for the dithionite method, whereas in the borohydride method the aortas could not be detected in the images at t=24 hours. When the borohydride method was used, radioactivity in the abdominal aorta in the atherosclerotic animals was significantly higher than in control rabbits 4 hours after injection of the $^{99m}$Tc-LDL. For the dithionite method this was 16 hours after injection. Plasma radioactivities in atherosclerotic aortas at t=24 hours were 28% and 41% of the accumulated radioactivity in the abdominal aorta at t=0 for the borohydride and the dithionite method, respectively. Both methods produced relatively stable $^{99m}$Tc-LDL complexes, with 72% and 68% of the $^{99m}$Tc remaining attached to LDL at t=24 hours. In the biodistribution study, uptake of radioactivity in the aortas was comparable for both labeling methods. We conclude that the borohydride method may be useful in the scintigraphic detection of atherosclerosis, since it yields $^{99m}$Tc-LDL that accumulates in atherosclerotic lesions to the same extent as LDL labeled by the dithionite method. In addition, it is cleared from the circulation considerably faster, resulting more rapidly in a higher target-to-nontarget ratio, which facilitates early identification of atherosclerosis. (Arteriosclerosis and Thrombosis 1993;13:78-83)

Key Words • scintigraphy • radiolabeling • LDL • atherosclerosis • technetium-99m

The detection of atherosclerotic plaques at an early stage is considered to be crucial for successful therapeutic intervention. Until now, diagnosis of asymptomatic atherosclerosis has been primarily based on the demonstration of the space-occupying characteristics of well-advanced plaques, as is done in angiography. The need exists for a simple noninvasive test that could be used over time to detect early atherosclerotic lesions and to monitor the effects of long-term therapy. It is known that low density lipoprotein (LDL), the major cholesterol-transporting protein in the circulation, is involved in early atherosclerosis. An increased influx of LDL into the forming plaque leads to deposition of the lipoprotein intracellularly, in macrophages and smooth muscle cells, or extracellularly, when bound to proteoglycans and elastin.1-4

Several authors have reported the use of $^{123}$I-LDL,5,6 $^{111}$In-LDL,7,8 and $^{99m}$Tc-labeled LDL9,10 in the detection of experimental and human atherosclerosis. Since $^{99m}$Tc has distinct advantages over $^{123}$I and $^{111}$In in terms of cost and availability, $^{99m}$Tc is the isotope of choice. We
Animals

Six male New Zealand White (NZW) rabbits weighing 3.1–4.2 kg received an atherogenic diet containing 10% coconut oil and 0.1% cholesterol for 38 weeks (athero group). Six male NZW rabbits weighing 3.1–4.0 kg were fed a control diet containing 0.3 ml/kg body wt i.m. Hypnorm (Janssen Pharmaceuticals, Beerse, Belgium).

LDL Preparation

LDL was isolated from human plasma freshly collected into EDTA and was obtained from healthy volunteers. The LDL was isolated from the plasma by density gradient ultracentrifugation at 284,000g for 18 hours, as described by Terpstra et al. The protein content of the LDL was determined by the method of Lowry et al. LDL was harvested and used without further treatment before radiolabeling.

Dithionite method. We used a modification of the method described by Vallabhajosula et al. To a mixture of 18 mg LDL protein and 50 mCi $^{99m}$TcO$_4^-$ in 0.15 M NaCl, 0.1 ml of a freshly prepared sodium dithionite solution (100 mg/ml glycine buffer, pH 10) was added (total volume was 6.4 ml). This is five times less $^{99m}$TcO$_4^-$ than that used in the original method described by Vallabhajosula et al. After incubation at room temperature for 30 minutes, complete separation of $^{99m}$Tc-LDL from unbound $^{99m}$TcO$_4^-$ was achieved by means of size exclusion chromatography on 10×1-cm Sephadex G25 columns (Pharmacia, Uppsala, Sweden). These columns were prewashed with 4 mg albumin in 2 ml of 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-buffered saline (HBS), pH 7.4. The eluant used was 20 mM HBS, pH 7.4. No evidence for aggregation of the LDL as a result of the labeling procedures was found on size exclusion chromatography by fast protein liquid chromatography on Superose 6 prep-grade columns.

Borohydride method. This newly developed method, for which a patent application has been filed, will be described in further detail elsewhere. In short, 18 mg LDL was added to a mixture of 55 mCi $^{99m}$TcO$_4^-$, stannous chloride (4 μl/mg protein of the DTPA Teshnes kit, Mallinckrodt, Petten, The Netherlands, containing 25 mg calcium trisodiumpentetrate, 0.21 mg SnCl$_2$, and 0.25 mg gentisic acid in 4.2 ml distilled water) and sodium borohydride (4 μl/mg protein of 5 mg KBH$_4$ per milliliter of a 0.1 M NaOH solution). Volume of the reaction mixture was 6.3 ml. After incubation at 37°C for 2 hours, 1 ml sodium citrate, 3.8% (wt/vol), pH 7.5, was added, and the reaction mixture was incubated at 37°C for 1 hour. The $^{99m}$Tc-LDL was separated from free $^{99m}$TcO$_4^-$ on 10×1-cm Sephadex G25 columns as described above.

Labeling Yield and Specific Activity

Labeling yield was determined by measuring the radioactivity recovered in the LDL fraction after Sephadex G25 chromatography and expressed as percentage of the total amount of radioactivity applied to the column. Specific radioactivity was expressed as radioactivity per milligram of protein after measuring the protein content of this fraction.

Scintigraphy In Vivo

Scintigraphy was performed on a Toshiba 40A gamma camera fitted with a high-energy, super-high-resolution collimator with a spatial resolution of 1.2 mm that was connected to an MDS-A$^<$2$>$ computer. After anesthesia, the rabbits were positioned on their right flank to ensure a projection of the abdominal aorta that was unobstructed by the spine and small bowel. After injection of 2.2–2.5 mg $^{99m}$Tc-LDL (3.3–3.8 mCi) in the marginal ear vein, the radiolabeled LDL was allowed to circulate for 10 minutes. Whole-body images for which 1 million counts were acquired were taken at that moment (t=0) and at 1, 4, 8, 16, and 24 hours after injection. Concomitant imaging of an external $^{99m}$Tc standard allowed correction for physical decay of the $^{99m}$Tc.

Image Analysis

The resulting scintigrams were reviewed by independent observers. A hand-drawn region of interest was defined for an area over the abdominal aorta. A second region of interest was placed over the external $^{99m}$Tc standard, and dedicated software performed statistical analysis of the regions of interest. To study the kinetics of radioactivity decrease in the aorta, the values of the radioactivity measured in the aorta in the images taken 1, 4, 8, 16, and 24 hours after injection were expressed as a percentage of the value at t=0.
**Plasma Decay**

To study the clearance of radioactivity from the circulation, 0.9-ml blood samples were collected from the central ear artery before each image was taken, with 0.1 ml of 3.8% (wt/vol) sodium citrate as the anticoagulant. In this manner, the relation between blood pool radioactivity and the observed radioactivity in the abdominal aorta in the scintigraphic images could be studied. Blood pool radioactivities measured at different intervals after injection of the $^{99m}$Tc-LDL were expressed as a percentage of the blood pool radioactivity at $t=0$. Blood sample radioactivities were measured in a Scalar Ratemeter SR4 well-type gamma counter.

**In Vivo Stability**

To study whether the $^{99m}$Tc remained attached to the LDL particle while in the circulating blood, gel permeation chromatography of plasma from an atherosclerotic rabbit from both the dithionite group and the borohydride group was performed. After collection of the blood samples as described above, plasma was prepared by centrifugation at 3,000 rpm for 15 minutes. Then 500 μl plasma was chromatographed on a 80×1.6-cm Superose 6 prep-grade column (Pharmacia), with 20 mM HBS containing 0.38% (wt/vol) sodium citrate as the eluant. Fractions eluting at positions that corresponded to those of very low density lipoprotein, LDL, high density lipoprotein plus albumin, and low-molecular-weight material were identified by measurement of total cholesterol and triglycerides (Monotest and Peridochrom, respectively, Boehringer Mannheim, Mannheim, FRG) and by absorbance measurements at 280 nm. The radioactivities in these fractions were expressed as a percentage of the total applied radioactivity. This allows quantification of a possible shift of the radiolabel to molecules other than LDL.

**Biodistribution**

After completion of scintigraphic data acquisition, the rabbits were killed by intracardiac administration of an overdose of Nembutal (Sanofi BV, The Netherlands). The aorta and various other organs were removed and washed in saline. The aorta was divided into a thoracic and an abdominal part at the hiatus aorticus of the diaphragm. Each organ was weighed and counted in a Scalar Ratemeter SR4 well-type gamma counter.

**Aortic Staining**

The aortas were opened longitudinally and pinned down. After fixation in 4% formaldehyde, the arteries were stained with Sudan IV. To estimate the areas of the aortas affected by the atherosclerotic process, a grid of 2-mm squares was positioned over the luminal surface of the aortas. The number of squares overlying atherosclerotic lesions was expressed as a percentage of the number of squares covering the entire luminal surface of the aorta.

**Statistics**

Data are represented as mean±SD. The results from the biodistribution study and the scintigraphic study in control and athero animals for both methods were compared with an analysis of variance. The Bonferroni $t$ test was used to identify the groups that were statistically significantly different ($p<0.05$).

**Results**

**Aortic Staining**

After staining of the aortas with Sudan IV, the area covered with fatty streaks was estimated to be 90–100% in the thoracic aorta and 75–95% in the abdominal aorta of all the rabbits in the atherosclerotic group. In the control group no fatty streaks were observed in the aorta.

**Labeling Yield and Specific Activity**

The observed labeling yield and specific activity were 53% and 1.57 mCi/mg LDL protein for the dithionite method, whereas for the borohydride method these values were 47% and 1.51 mCi/mg, respectively.

**Scintigraphy and Plasma Decay**

With LDL labeled by either method, the entire thoracic and abdominal aortas were visualized in both the athero group and control group. When the borohydride method was used, focal accumulation of radioactivity within some areas of the atherosclerotic abdominal aorta was occasionally observed on the scintigraphic images (Figure 1A). The kinetics of radioactivity decrease in the abdominal aorta at different intervals after injection of the $^{99m}$Tc-LDL are shown in Figure 2 for the LDL labeled by the dithionite method and in Figure 3 for the borohydride method. Also shown in these figures are the curves of blood radioactivity. In both the athero and control
groups and with LDL labeled with either method, the decrease in aortic radioactivity was slower than the decrease in blood radioactivity (Figures 2 and 3).

When the borohydride method was used, radioactivity in the abdominal aorta in the athero group was significantly higher than in the control group 4 hours after injection of the radiolabeled LDL. For the dithionite method, this difference became significant 16 hours after injection (Figures 2 and 3). At 16 and 24 hours after injection of the \(^{99m}\text{Tc}\)-LDL, it was no longer possible to discriminate the abdominal aortas in the rabbits injected with LDL that was labeled by the borohydride method in the scintigraphic images (Figure 2).

In the control group, the decrease in blood radioactivity relative to the corresponding aortic radioactivity did not differ much between the two labeling methods during the first 8 hours after injection. However, 4 hours after injection both blood radioactivity and aortic radioactivity decreased significantly faster in the control group when the borohydride method was used compared with the dithionite method. In the athero group, the mean radioactivity in the aortas at 24 hours was comparable for both labeling methods (57% and 54% of aortic radioactivity at \(t=0\), respectively). In the control group, the value for the dithionite method at this interval was 25%. The aortas in the borohydride method group could no longer be visually discerned in the images taken at 16 and 24 hours.

Blood radioactivity in the athero group at 24 hours was lower when the borohydride method was used in comparison with the dithionite method (28% and 41% of blood radioactivity at \(t=0\), respectively, \(p=0.08\)). In the control group, these values were 6% and 18%, respectively (\(p<0.05\)).

**In Vivo Stability**

There was a loss of \(^{99m}\text{Tc}\) from LDL labeled by either method during the first hour after injection into the circulation. These losses were 14% for the dithionite method and 11% for the borohydride method (Table 1). During the experiment, only a small additional loss was observed, i.e., 68% and 72% of the circulating radioactivity remained attached to LDL labeled by the dithionite method and the borohydride method, respectively, 24 hours after injection into circulation.

**Biodistribution**

Although accumulation of radioactivity in the thoracic and abdominal aortas in the athero group was somewhat higher when we used \(^{99m}\text{Tc}\)-LDL labeled by the borohydride method compared with the dithionite method, this difference was not statistically significant. In the control group no differences between the two labeling methods were observed (Table 2). For the borohydride method, uptake of radioactivity was statis-
veloped Tc-labeling technique for LDL with borohy-

tically higher in the atherosclerotic thoracic aortas compared with control aortas.

**Discussion**

Until now, scintigraphic detection of atherosclerotic lesions within the arterial wall has been complicated by high blood pool radioactivity, which makes it difficult to discern focal accumulation from background. The use of LDL as a marker in the scintigraphic detection of atherosclerosis has yielded promising results, although the slow plasma clearance of the labeled LDL particle leads to a high background radioactivity, resulting in decreased lesion-to-background ratios. Our newly developed \(^{99m}\)Tc-labeling technique for LDL with borohydride and stannous chloride results in an accelerated clearance of the \(^{99m}\)Tc-LDL in normal and hypercholesterolemic rabbits compared with LDL radiolabeled by the iodine monochloride method and the dithionite method described by Lees et al.\(^9\)

Since this more rapid clearance may yield higher lesion-to-background ratios, we decided to study whether the \(^{99m}\)Tc-LDL labeled by our method has a better potential to localize atherosclerotic plaques in rabbits. We injected the LDL radiolabeled by the borohydride method into hypercholesterolemic and normal NZW rabbits. For comparison, the same \(^{99m}\)Tc-LDL labeled by the dithionite method was also studied. Labeling efficiencies and specific activities of the resulting \(^{99m}\)Tc-LDL were comparable for both methods. After injection, both \(^{99m}\)Tc-LDL preparations lost some of their label in the first hour, but little additional loss of label was observed afterward.

The entire length of thoracic and abdominal aortas in both the athero and control group was visualized in the images obtained with \(^{99m}\)Tc-LDL labeled by either method. The decrease of radioactivity in the abdominal aorta in the hypercholesterolemic group was slower than in the control group. When LDL labeled by the borohydride method was used, the radioactivity in the abdominal aorta in the athero group was significantly higher than in the control group 4 hours after injection of the \(^{99m}\)Tc-LDL. When LDL labeled by the dithionite method was used, significant differences were reached only after 16 hours. Although the decrease in blood pool

**Table 1. Distribution of Radioactivity in Fractions of Plasma Samples**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Radioactive fraction (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VLDL</td>
<td>LDL</td>
</tr>
<tr>
<td>8 Hours</td>
<td>1</td>
<td>88</td>
</tr>
<tr>
<td>4 Hours</td>
<td>2</td>
<td>74</td>
</tr>
<tr>
<td>8 Hours</td>
<td>2</td>
<td>72</td>
</tr>
<tr>
<td>16 Hours</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td>24 Hours</td>
<td>1</td>
<td>68</td>
</tr>
</tbody>
</table>

**Table 2. Biodistribution of \(^{99m}\)Tc-Low Density Lipoprotein Labeled by Two Different Methods in Normal and Hypercholesterolemic Rabbits**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Borohydride method</th>
<th>Dithionite method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Atherosclerotic</td>
</tr>
<tr>
<td>Blood</td>
<td>0.02±0.01</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>0.02±0.01</td>
<td>0.10±0.05*</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>0.02±0.01</td>
<td>0.07±0.05</td>
</tr>
<tr>
<td>Liver</td>
<td>0.80±0.22</td>
<td>0.38±0.13*</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.92±0.28</td>
<td>0.35±0.08*</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>1.87±0.76</td>
<td>0.28±0.06*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.44±0.11</td>
<td>0.42±0.10</td>
</tr>
<tr>
<td>Lung</td>
<td>0.02±0.004</td>
<td>0.03±0.01</td>
</tr>
</tbody>
</table>

Values are expressed as a percentage of the injected dose per gram wet organ weight (mean±SEM, n=3). Statistically significant differences between normal and atherosclerotic animals are marked by *(p<0.05).*
In our model, \(^{99m}\)Tc-LDL labeled by the borohydride method was taken up by the hypercholesterolemic aortas to the same extent as \(^{99m}\)Tc-LDL labeled by the dithionite method. At the same time, it was cleared from the circulation much faster, resulting in a lower background radioactivity. In control animals, aortic radioactivity decreased quickly when the borohydride method was used, resulting in a significant difference with atherosclerotic aortas only 4 hours after injection, whereas this was 16 hours when the dithionite method was used. On the basis of our results we conclude that \(^{99m}\)Tc-LDL labeled by the borohydride method may have advantages over \(^{99m}\)Tc-LDL labeled by the dithionite method.

References


Potential of 99mTc-LDLs labeled by two different methods for scintigraphic detection of experimental atherosclerosis in rabbits.
D E Atsma, R I Feitsma, J Camps, F M van't Hooft, E E van der Wall, W Nieuwenhuizen and E K Pauwels

doi: 10.1161/01.ATV.13.1.78
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/1/78