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# Preparation and biodistribution of $^{99m}$technetium labelled oxidized LDL in man

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**Abstract**

Radiolabelled autologous low density lipoprotein (LDL) has previously been used to study in vivo distribution and metabolism of native-LDL. Non-invasive imaging of atherosclerotic lesions using $^{99m}$Tc-LDL was shown to be feasible in animal models and patients but the clinical utility remains to be assessed. Since recent reports suggest that oxidized LDL may play a major role in the pathogenesis of atherosclerosis, we developed a technique to oxidize autologous LDL and compared the biodistribution of oxidized-LDL with that of native-LDL in man. In addition, we evaluated the uptake in vivo of oxidized- and native-LDL by atherosclerotic plaques. LDL, obtained from human plasma was treated with various combinations of copper ions and $H_2O_2$ to induce oxidative modification by increasing the content of lipid peroxidation products and electrophoretic mobility. When LDL (0.3 mg/ml) was incubated with 100 $\mu$M $Cu^{2+}$ and 500 $\mu$M $H_2O_2$ oxidation occurred rapidly within 1 h, and was labelled with $^{99m}$Tc efficiently as native LDL. In vivo distribution studies revealed a faster plasma clearance of oxidized-LDL compared to native-LDL, and a higher uptake by the reticuloendothelial system. Tomographic scintigraphy of the neck in patients suffering from transient ischemic attacks, revealed accumulation of radiolabelled LDL preparations in the carotid artery affected by atherosclerotic lesions. We developed a technique to rapidly oxidize LDL using copper and $H_2O_2$. Biodistribution data demonstrate that oxidized-LDL is rapidly cleared from circulation, is taken up mostly by organs rich in macrophages, and can be detected at the level of carotid plaques.

*Keywords:* Lipoproteins; LDL-biodistribution; Oxidized-LDL; Imaging; Free radicals; Atherosclerosis

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## 1. Introduction

Radiolabelled LDL has previously been used as a tracer to study the in vivo distribution and metabolism of native-LDL [1]. It has also been shown that the in vivo administration of LDL labelled with radioiodine, $^{99m}$Tc or $^{111}$In in animal models allows the detection of atherosclerotic lesions [1–4]. Similarly, non-invasive imaging of atherosclerotic plaques in man using radiolabelled-LDL was shown to be feasible [4–6] but the clinical utility of this method remains elusive. The main disadvantage of radiolabelled native-LDL is that it clears slowly from circulation and, as a result, the target/background ratio is sub-optimal for non-invasive imaging studies. This encouraged the search for new radiopharmaceuticals for imaging atherosclerosis in man [7].

One of the most characteristic constituents of atherosclerotic lesions is the macrophage-derived foam cell [8–13]. However, cultured macrophages do not accumulate lipids when incubated with native-LDL [14]. Oxidative modification of LDL may be responsible for lipid loading within macrophages [15] through recognition and uptake via the scavenger receptor [16]. Oxidized-LDL may have higher affinity with the monocyte-macrophage in the atherosclerotic lesion and thus may play a greater role in the pathogenesis of atherosclerosis. Therefore, the use of $^{99m}$Tc-labelled-oxidized-LDL might be more useful than the use of $^{99m}$Tc-labelled-native-LDL for non-invasive imaging of atherosclerotic lesions in vivo.

The aim of this study was to develop a rapid and reproducible method to obtain ox-LDL suitable for radiolabelling with the versatile isotope $^{99m}$Tc. Thus, we studied the biodistribution and uptake by atherosclerotic plaques of both native- and oxidized-LDL in patients suffering from transient ischemic attacks.

## 2. Materials and methods

### 2.1. Materials

$CuSO_4$, $H_2O_2$ and tetraethoxypropane were purchased from Aldrich (Milwaukee, PA); bicinchoninic acid from Pierce (Rockford, IL); Paragon agarose gel precoated plates from Beckman (Fullerton, CA); Sephadex, sodium dithionite and trinitrobenzenesulfonic acid were from Sigma (St. Louis, MO). Portable technetium generators were purchased from Sorin Biomedica (Saluggia, Turin, Italy). All other reagents were of the highest grade available from Merck (Darmstadt, Germany). Water of high purity ( > 18 Mohms) was obtained by treating double distilled water in a Milli-Q purifying system (Millipore, Bedford, MA). Spectrophotometric measurements were made on a HP 8542A diode array instrument (Hewlett-Packard, Palo Alto, CA).

### 2.2. Preparation of LDL

LDL was obtained from human plasma (d 1.025–1.050 g/ml) by sequential flotation ultracentrifugation [17], in the presence of EDTA to minimize oxidation. The purity of LDL was evaluated by agarose gel electrophoresis. Prior to oxidation purified lipoproteins were first desalted by extensive dialysis at 4°C in physiological saline or by ultrafiltration in a Spectrum apparatus (Spectrum, Haverton). Protein concentration of LDL, determined by the bicinchoninic acid method [18], was about 4 mg protein/ml. Following isolation from plasma, LDL was used for about 5 days for in vitro experiments.

### 2.3. LDL oxidation

Oxidation was carried out by exposing LDL (0.3 mg/ml) to $Cu^{2+}$ (12.5–200 $\mu$M), in the presence or absence of $H_2O_2$ (0.1, 0.5 or 1 mM), in 2 mM phosphate buffer, pH 7.35, 37°C for 1 h. At the end of incubation the extent of lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction as previously described [19]. Briefly, 100 $\mu$l of LDL subjected to oxidation was added to 15 $\mu$l butylated hydroxytoluene (2% in ethanol, freshly prepared) and 1 ml TBA test solution (1% w/v TBA in 50 mM NaOH, 20% w/v trichloroacetic acid, 1:9). The solution was incubated in boiling water for 15 min and after cooling the color formation was read at 532 nm. The entity of oxidation was expressed as malondialde-

hyde (MDA) equivalents using an extinction coefficient of the TBA-MDA adduct of 149 000 $M^{-1}$ $cm^{-1}$ [20]. The change in electric charge of the protein following oxidation was evaluated by agarose gel electrophoresis. Immediately after oxidation, LDL was washed from copper ions and residual $H_2O_2$ by rapid ultrafiltration with excess saline, and concentrated for $^{99m}$Tc labelling.

### 2.4. LDL derivatization

MDA was obtained by acid hydrolysis of tetraethoxypropane. Fifty millilitres of tetraethoxypropane solution (7%, w/v in 0.2 N HCl) were kept at 60°C for 5 min and stirred. At the end of incubation HEPES (60 mg) was added to the solution, which was cooled and titrated to pH 6.5. Ten microlitres of the solution were added to 5 ml 0.5 N NaOH to measure MDA concentration by a spectrophotometer assuming an $\epsilon_{267}$ of 34 000 $M^{-1}$ $cm^{-1}$ [20]. Derivatization was obtained by exposing LDL (2 mg/ml) to 200 mM MDA in 5 mM HEPES pH 6.5, for 60 min at 37°C.

### 2.5. Analysis of lysine residues

Free amino groups in LDL were measured using the lysine-specific reagent trinitrobenzenesulfonic acid [21]. 0.5 ml (0.2 mg/ml) were mixed with 50 $\mu$l of 100 mM EDTA (pH 8.5), 1 ml of trinitrobenzenesulfonic acid solution (0.5 mg/ml in 0.2 M $NaHCO_3$, pH 8.5), and incubated 2 h in the dark at room temperature. At the end of the incubation the solution was mixed with 1 ml of ice-cold Tris-HCl buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to stop the reaction. Samples were dialyzed by ultrafiltration in Centricon (Amicon, Danvers, MA) centrifugal microconcentrators and read in the spectrophotometer.

### 2.6. LDL electrophoresis

Lipoprotein electrophoresis was performed in barbiturate buffer (50 mM 5,5-diethylbarbituric acid sodium salt, pH 8.6) on Paragon agarose gel blotters and Sudan Black B stain.

### 2.7. Radiolabelling of native and modified LDL with $^{99m}$Tc

Native and oxidized-LDL preparations were radiolabelled with $^{99m}$Tc following reduction of technetium atoms with sodium dithionite [1,2]. Typically, 1–2 mg of LDL (0.5 ml) was mixed with 40–50 mCi of $^{99m}$Tc-pertechnetate solution (0.5 ml). Then, 0.1 ml of 0.5 M glycine buffer, pH 10, containing 10 mg of sodium dithionite was immediately added. The mixture was gently mixed and incubated for 30 min at room temperature. At the end of incubation time, $^{99m}$Tc-LDL was separated from free $^{99m}$Tc by gel filtration chromatography using Sephadex G-50 and 0.1 M sodium bicarbonate in saline (pH 8). Both native- and modified-LDL preparations, following $^{99m}$Tc labelling, were eluted in the void volume while the free $^{99m}$Tc was eluted from the column with the total volume. We have previously shown that $^{99m}$Tc-LDL preparation was quite stable under in vitro conditions for several hours following chromatography [2]. For biodistribution studies the labelled LDL solution was sterilized by filtration through a 0.22 $\mu$m Millipore filter.

### 2.8. Patients for in vivo biodistribution studies with native-LDL and ox-LDL

We studied 7 consecutive patients (2 females and 5 males, age 40–60; 14 carotids) who in the previous 6 months suffered from transient ischemic attacks, defined as an acute disturbance of focal neurological function with symptoms lasting less than 24 h. Each patient underwent ultrasound Doppler ecotomography and 4 out of 7 patients were also studied by angiography to evaluate the presence of carotid stenosis. Three patients had monolateral and 4 patients had bilateral carotid stenosis. Control were carotid arteries of patients without detectable atherosclerosis lesions. Thus, according to the degree of stenosis, carotid arteries were divided in 2 groups: no stenosis (control, $n = 3$) and stenosis $\geq$ 40% (affected carotid arteries, $n = 11$). Patients did not take any antioxidant medication, such as vitamin E, in the month preceding the study. They gave informed consent to participate in the study, which was

approved by the Ethical Committee of the University of Rome 'La Sapienza'.

### 2.9. Gamma camera imaging studies in patients

Each patient was positioned supine under the collimator of an Elscint SP4 gamma camera (Elscint, Israel) and 10 mCi of $^{99m}$Tc-LDL or 7–10 mCi of $^{99m}$Tc-ox-LDL, were injected i.v. in bolus. Dynamic gamma camera images of the chest and upper abdomen were acquired every 20 s for 30 min and stored in a 64 × 64 matrix. At the end of the study regions of interest (ROI) were drawn over the heart, liver and spleen and TAC were generated. For comparison, heart TAC were normalized by % of maximum counts and spleen and liver TAC were normalized by the injected dose. For $^{99m}$Tc-LDL studies, a planar antero-posterior image and tomographic images (SPET) of the neck were acquired at 6 h post-injection and a planar antero-posterior image was also acquired after 20 h. For $^{99m}$Tc-ox-LDL studies, a planar antero-posterior image and tomographic images (SPET) of the neck were acquired at 1 h post-injection and a planar antero-posterior image was also acquired after 6 h. Planar images (500 kcounts) were stored in a 256 × 256 matrix in zoom mode and analyzed qualitatively by two independent observers. SPET images were acquired for 15 s (or 20 s for ox-LDL) every 6° for 360° with a medium resolution collimator. After reconstruction, 8 consecutive trans-axial sections of the neck (2 cm thickness) were obtained, starting from the carotid origin. For quantitative analysis of radioactivity uptake by carotid plaques, a small circular ROI was drawn in each section on each carotid and counts in ROIs of sections 3 and 4 (corresponding to the carotid bifurcation where plaques were located) were added and divided by the sum of counts in ROIs of sections 1 and 2 (considered as regional blood pool background). A positive score was then assigned to those carotids having a target/background ratio (T/B) higher than the mean of control carotids +2 S.D. (> 95° centile).

## 3. Results

### 3.1. LDL oxidation

No lipid peroxidation products were measured in LDL freshly prepared from the donors participating in this study. In the preliminary phase of the study it was shown that copper, in a broad concentration range (5–200 $\mu$M) was ineffective in inducing changes in TBA reactive material and electrophoretic mobility of human LDL. This could be attributed to the protection provided by antioxidants associated to the LDL molecule [22]. In fact, LDL could be modified, if before oxidation it was stored for several days in the refrigerator, in agreement with consumption of antioxidant molecules (data not shown).

In attempting to overcome the resistance of freshly isolated LDL to oxidation, we used $H_2O_2$ as amplifier. In contrast to $Cu^{2+}$ alone, $Cu^{2+}$ plus $H_2O_2$ caused a concentration dependent increase in lipid peroxidation and $Cu^{2+}$ concentrations below 75 $\mu$M were without any effect notwithstanding the presence of $H_2O_2$ (Fig. 1). At the given concentration of $H_2O_2$ the maximum extent of lipid peroxidation was reached with 100 $\mu$M copper. The appearance of lipid peroxidation products during $Cu^{2+}/H_2O_2$ treatment was associated with a change in electrophoretic mobility, that was concentration dependent with respect to $H_2O_2$ (Fig. 2) and resembled that of MDA-derivatized LDL (Fig. 3). The change in electrophoretic

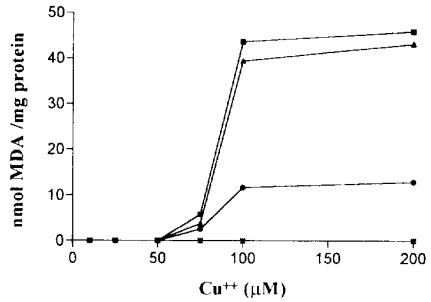


Fig. 1. Oxidation of LDL, at 37°C for 1 h under air, as a function of $Cu^{2+}$ and $H_2O_2$ concentration. (*) $Cu^{2+}$ alone; (●) $Cu^{2+}$ plus 0.1 mM $H_2O_2$; (▲) $Cu^{2+}$ plus 0.5 mM $H_2O_2$; (■) $Cu^{2+}$ plus 1.0 mM $H_2O_2$.

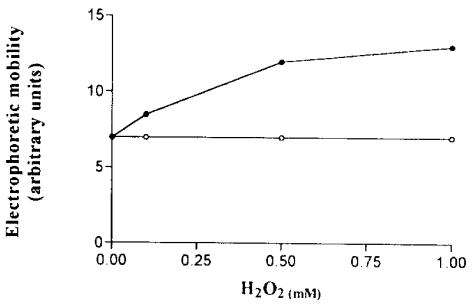


Fig. 2. Change in electrophoretic mobility of LDL upon oxidation with $Cu^{2+}$ plus $H_2O_2$. The extent of electronegativity of LDL was measured after 1 h incubation at 37°C with increasing concentration of $H_2O_2$. Results are shown for 25, 50 or 75 $\mu$M $Cu^{2+}$ (o) and for 100 or 200 $\mu$M $Cu^{2+}$ (●).

mobility was observed only at 100 and 200 $\mu$M $Cu^{2+}$. At the given $H_2O_2$ concentration there was no significant change in electrophoretic mobility at 100 and 200 $\mu$M $Cu^{2+}$. Copper concentrations of 25, 50 and 75 $\mu$M were not able to induce any electrophoretic change, independent of the amount of $H_2O_2$ used. The change in electrophoretic mobility was associated with a loss of lysine residues titratable by the trinitrobenzenesulfonic acid reaction (Fig. 4), suggesting that a lipid peroxidation product generated by $Cu^{2+}/H_2O_2$ reacted with lysine residues of LDL.

Experiments reported above indicated a favourite oxidizing concentration of copper and $H_2O_2$ of 100 and 500 $\mu$M respectively. To find the best copper/protein ratio, the LDL concentration was varied in the reaction mixture. As shown in

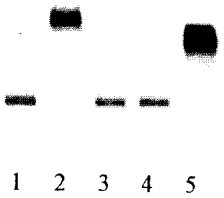


Fig. 3. Agarose gel electrophoresis of native and chemically treated LDL. 1: no additions; 2: 200 mM MDA; 3: 0.1 mM $Cu^{2+}$; 4: 0.5 mM $H_2O_2$; 5: 0.1 mM $Cu^{2+}$ plus 0.5 mM $H_2O_2$. Incubation conditions as in Fig. 1.

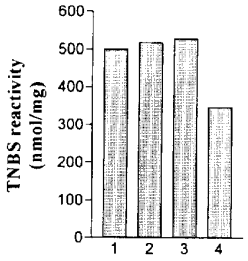


Fig. 4. Free lysine groups of LDL incubated for 1 h at 37°C with vehicle (1); 0.1 mM $Cu^{2+}$ (2); 0.5 mM $H_2O_2$ (3); 0.1 mM $Cu^{2+}$ plus 0.5 mM $H_2O_2$ (4).

Fig. 5, by increasing the $Cu^{2+}$/protein ratio from 0.1 to 1.3 $\mu$mol/mg protein a progressive increase in both the absolute amounts of lipid peroxidation and extent of electrophoretic mobility is observed. Therefore, the preparation of ox-LDL for $^{99m}Tc$ labelling was arbitrary based on 300 $\mu$g/ml LDL, 100 $\mu$M $Cu^{2+}$ and 500 $\mu$M $H_2O_2$. This protocol was routinely applied for oxidative modification of human LDL, that were used for in vivo studies.

### 3.2. Imaging studies in patients

Biodistribution data in patients showed that autologous ox-LDL is cleared from circulation more rapidly than autologous native-LDL (Fig. 6). Plasma half-life of ox-LDL, calculated by bi-exponential fitting of heart time-activity curves (first 30 min), was significantly shorter than na-

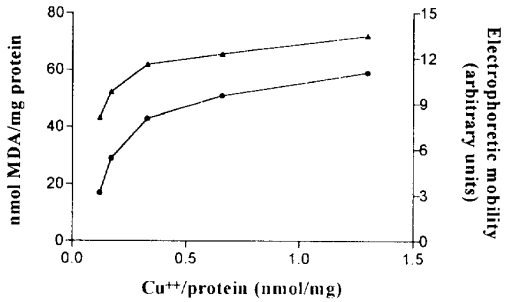


Fig. 5. Effect of LDL concentration on the yield of lipid peroxidation (●) and entity of electronegativity (▲) induced by 0.1 mM $Cu^{2+}$ plus 0.5 mM $H_2O_2$.

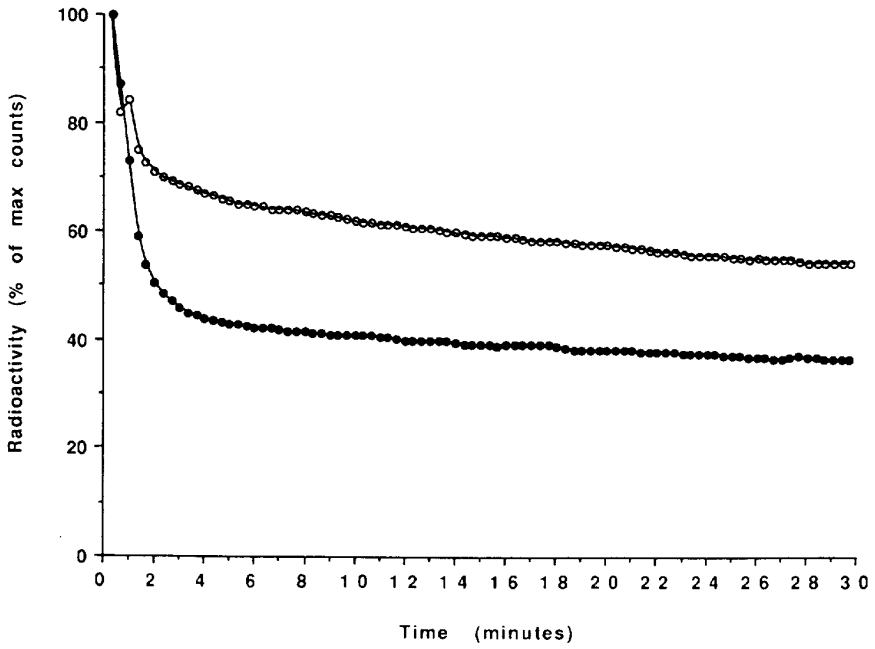


Fig. 6. Heart time activity curves in a patient suffering from transient ischemic attacks, after i.v. administration of $^{99m}$Tc labeled native-LDL (o) or ox-LDL (●). A faster blood clearance of ox-LDL in respect to native-LDL can be observed.

tive-LDL ($t_{1/2}$ = 85.8 ± 22.1 min vs. 124 ± 37.2 min, $P < 0.001$). The time of maximum liver and spleen uptake of ox-LDL was within 15 min in all patients, whereas it was after 30 min for LDL (Fig. 7). At 1 and 6 h post-injection the liver uptake of ox-LDL was significantly higher than native-LDL: at 1 h, 16.3% ± 3.2 vs. 8.9% ± 2.2 (% of injected dose), $P < 0.0001$; at 6 h, 17.4% ± 4.4 vs. 10.9 ± 2.8, $P < 0.003$.

A schematic representation of the method used for quantification of radioactivity uptake in the carotid plaque is shown in Fig. 8. Due to different plasma half-life of the 2 LDL preparations, analysis of plaque uptake was performed at 1 h for ox-LDL and at 6 h for native-LDL. Fig. 9 shows a transaxial section obtained in a patient 1 h after injection of $^{99m}$Tc-ox-LDL, whereas Fig. 10 shows the antero-posterior gamma-camera image of the neck of the same patient. Results from quantitative analysis are reported in Table 1. The uptake of both native- and oxidized-LDL by carotid plaques was significantly higher compared to normal carotids ($P = 0.03$ for native-LDL; $P = 0.02$ for ox-LDL). However, while an increased uptake of native-LDL was observed in 6 out of 11 carotid plaques (54.5%, confidence limits 23.4–83.3), a positive score of oxidized-LDL was observed in 10 out of 11 (91%, confidence limits 58.7–99.8). There was no correlation between the degree of stenosis and the target to background ratio. No changes in blood routine analysis were observed after infusion of radiolabeled LDL or ox-LDL (data not shown).

## 4. Discussion

Previous studies have demonstrated the feasibility of detecting atherosclerotic lesions in vivo by gamma camera imaging. Radiolabelled-LDL is a specific tracer sequestered by experimental arterial lesions [5] and human atherosclerotic plaques [4]. Thus, Lees et al. [4] reported significant accumulation of $^{99m}$Tc-LDL by atherosclerotic plaques in

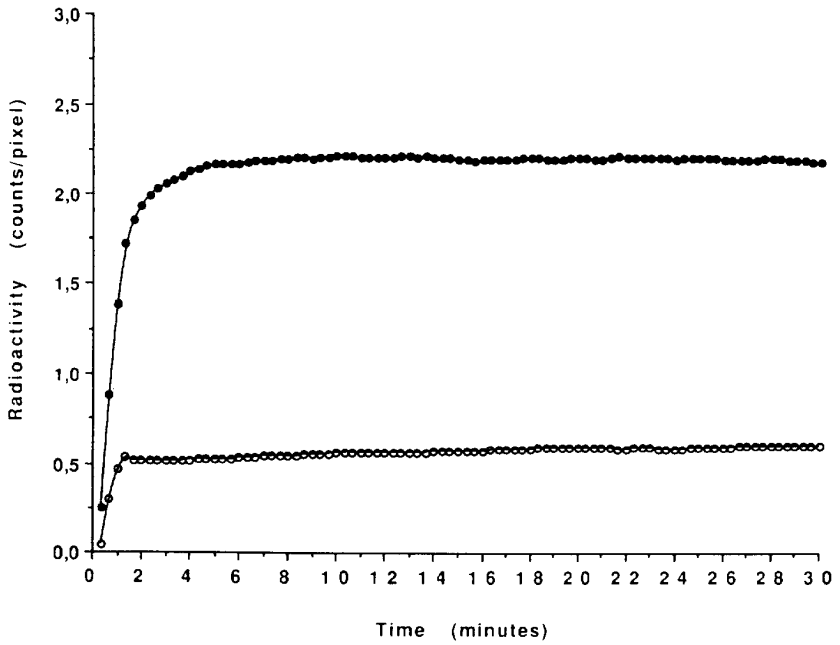


Fig. 7. Liver time activity curves of the same patient as in Fig. 9 after i.v. administration of $^{99m}$Tc labeled native-LDL (○) or ox-LDL (●). A rapid uptake of ox-LDL occurs within the first 15 min after injection.

vivo to allow their scintigraphic detection in 7 out of 17 patients (40%) with atherosclerotic disease. We obtained similar results using radiolabelled native-LDL since focal accumulation was observed in 54% of carotid plaques. Alternative approaches to image atherosclerotic plaques, have been made by using radiolabelled polyclonal human immunoglobulin G [23], monocytes [24] or platelets [25] but none significantly improved the rate of detection in comparison to LDL. This is due to both the low level of plaque uptake of these radiopharmaceuticals and to their plasma clearance that is slower than the physical half-life of the isotope. These two factors lead to a low target/background ratio and poor diagnostic accuracy. An ideal tracer should have a high and specific binding to atherosclerotic lesions and a fast plasma clearance. Recently, Shih et al. [7] have reported the results of a study using an apo B-based synthetic peptide labelled with $^{99m}$Tc, for in vivo detection of atherosclerotic lesions. This radiopharmaceutical has a short plasma half-life and was a good candidate for in vivo plaque detection despite the nature of its binding to plaques was proven not to be receptor mediated. Our strategy to image atherosclerotic plaques using a receptor specific radiopharmaceutical was to prepare in vitro oxidized-LDL.

Most LDL modification methods are based on oxidative treatment with copper, in a free radical mediated process [22]. Copper is a very efficient LDL modifier in cell-free systems, at micromolar concentrations. Also in the cell-mediated LDL modification the presence of copper appears to be necessary. Cell-mediated LDL modification does not occur in culture media lacking this redox metal [27,28]. Therefore, we used copper in an attempt to oxidize LDL for $^{99m}$Tc labelling. Unfortunately, copper alone, even in a wide range of concentrations, did not induce appreciable LDL modification, at least on the time scale needed for in vivo studies. Thus, we looked for an alternative means of achieving rapid LDL oxidation. We reasoned that copper could work in a redox cy-

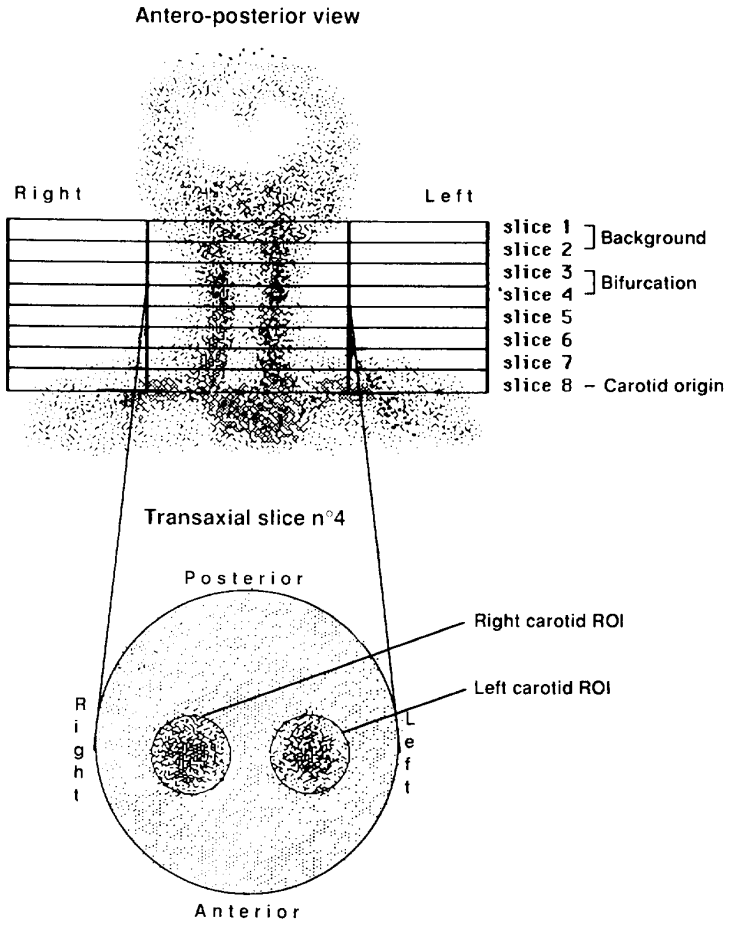


Fig. 8. Schematic representation of the method for selecting the transaxial sections of the neck, after tomographic scintigraphy, for quantitative assessment of T/B radioactivity ratios in carotid plaques (see Section 2 for details).

cling according to a Fenton reaction [29]. If this was true, the addition of $H_2O_2$ would amplify the reaction [30]. $H_2O_2$ successfully triggered LDL oxidation in the presence of copper, whereas it was without effect in the absence of copper in solution. For these experiments we selected a priori an incubation time of 1 h to render the method rapid and potentially suitable for human studies. In contrast, MDA-LDL were not taken into account for human studies because incomplete hydrolysis products are formed during the preparation of malondialdehyde from acid hydrolysis of tetraethoxypropane. One such product, $\beta$-methoxyacrolein, is mutagenic and difficult to separate from malondialdehyde [26]. Native- and oxidized-LDL were labelled with $^{99m}$Tc with the same efficiency. No side effects were observed after injection of modified LDL in man. Dynamic gamma camera imaging revealed a faster plasma clearance of oxidized-LDL than native-LDL. The rapid blood clearance of oxidized-LDL resulted as a consequence of more rapid uptake by the liver,

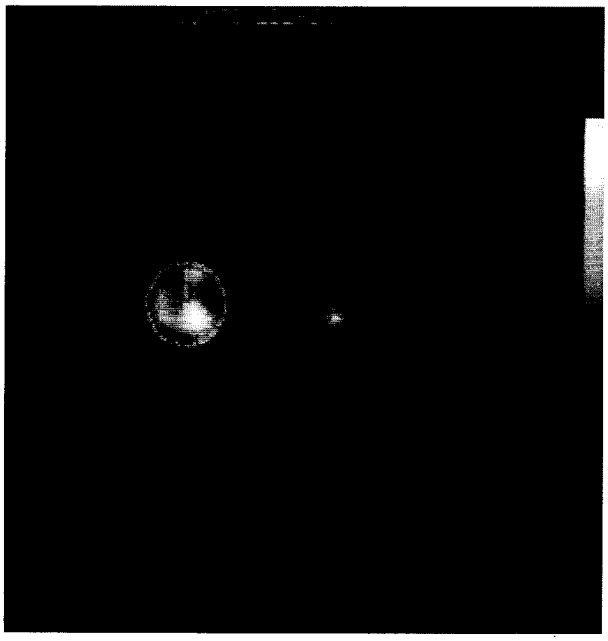

Fig. 9. Transaxial section of the neck, obtained by SPET acquisition of radioactivity, in a patient with 80% stenosis of the right carotid 1 h after injection of $^{99m}$Tc-ox-LDL. The uptake in the plaque is detectable in sections 3 and 4.

Table 1
Target/background (T/B) radioactivity ratios calculated for carotid plaques after administration of either $^{99m}$Tc-native-LDL (n-LDL) or $^{99m}$Tc-oxidized-LDL (ox-LDL)

| Carotid arteries | % Stenosis | T/B ratio (6 h after n-LDL) | T/B ratio (1 h after ox-LDL) |
|---|---|---|---|
| Control ($n$ = 3) | 0 | 1.019 ± 0.08 | 1.053 ± 0.059 |
| | | (0.95–1.107) | (0.992–1.059) |
| Affected ($n$ = 11) | 75 ± 16 | 1.278 ± 0.20 | 1.495 ± 0.32 |
| | 40–95 | (1.076–1.687) | (1.026–2.055) |

spleen and bone marrow, which are macrophage rich tissues. Similarly, a faster plasma clearance and high uptake in the bone marrow and in the liver has also been demonstrated for acetoacetylated, methylated and oxidized-LDL in rats [31], rabbits [32] and guinea pigs [33], respectively. In the present study, radiolabelled LDL were also detected in the carotid sites of atherosclerotic lesions. More ox-LDL than n-LDL accumulated in the affected carotids, even if we were not able to observe a statistical difference probably for the small number of carotids examined.

In conclusion, this is the first report of a new, rapid and simple method to obtain ox-LDL, having both lipid oxidation products and changed electronegativity. The use of naturally occurring copper and $H_2O_2$ render this method suitable for human studies and we present for the first time the in vivo kinetic and preliminary data of uptake by the atherosclerotic plaque of ox-LDL in man.

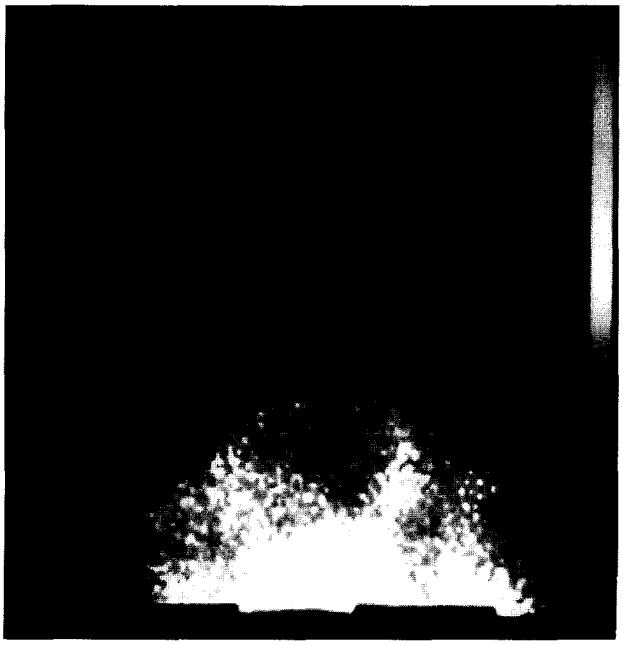

Fig. 10. Antero-posterior gamma-camera image acquired 1 h after injection of $^{99m}$Tc-ox-LDL in the same patient as in Fig. 9. Radioactive uptake in the right carotid plaque is indicated by the arrow.

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