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Preparation and investigation of 99m technetium-labeled low-density lipoproteins in rabbits with experimentally induced hypercholesterolemia

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Abstract Low-density lipoproteins (LDL) were radiolabeled in atherosclerosis studies. The aim was to investigate the biodistribution and uptake of ^{99m}Tc-labeled LDL by atherosclerotic plaques in experimentally induced hyperlipidemia. Rabbits were fed a diet containing 2% cholesterol for 60 days to develop hyperlipidemia and atheromatous aortic plaques. A combination of preparative and analytical ultracentrifugation was used to investigate human LDL aliquots, to prepare radioactive-labeled lipoproteins and in rabbits with induced hyperlipidemia. Preparative density gradient centrifugation was applied for the simultaneous isolation of the major lipoprotein density classes, which form discrete bands of lipoproteins in the preparative tubes. The cholesterol and protein levels in the lipoprotein fractions were determined. LDL was subsequently dialysed against physiological solution and sterilized and apolipoprotein fragments and aggregates were eliminated by passage through a 0.22-micron filter. LDL was radiolabeled with ^{99m}Tc by using sodium dithionite as a reducing agent. Radiochemical purity and in vitro stability were controlled by paper chromatography in acetone. The labelling efficiency was 85–90% for human LDL. Two months after the start of cholesterol feeding, the total cholesterol in the blood serum had increased approximately 33-fold in comparison with the basal cholesterol content of hypercholesterolemic rabbits. Investigation of LDL was performed by Schlieren analysis after adjustment of the density of serum and underlayering by salt solution in a spinning ultracentrifugation capillary band-forming cell. Quantitative results were obtained by measuring the Schlieren areas between the sample curves and the reference baseline curve by means of computerized numerical and graphic techniques. In this manner we measured the concentrations of human LDL and analyzed rabbit LDL levels in induced hyperlipidemia. Gamma scintillation camera scanning of the rabbits was performed. Overnight fasted rabbits were injected in the marginal ear vein with ^{99m}Tc-labeled human LDL (4–10 mCi, 0.5–1.5 mg protein). The initial scintigram showing a typical blood-pool scan, gradually changing with time to an image of specific organ uptake of radioactivity by the liver, kidneys and brain and in the bladder. Gamma camera in vivo scintigraphy on rabbits revealed visible signals corresponding to atherosclerotic plaques in the aorta and carotid arteries. Our results show that ^{99m}Tc-LDL can be used to assess the organ distribution pattern of LDL in the rabbit, and to detect and localize areas of arterial atherosclerotic lesions.

Keywords Atherosclerosis · Hypercholesterolemia · Low-density lipoproteins · Technetium · Ultracentrifugation

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Introduction

Atherosclerosis and its main complication of infarctions frequently end in sudden death. A key problem of the disease is its silent stepwise progression over time towards its acute manifestation (Berliner et al. 1995). Atherosclerosis is usually clinically silent until the very late stages, when it causes life-threatening clinical symptoms. Low-density lipoproteins (LDL) are implicated in an atherosclerotic process in which the blood flow is restricted by cholesterol-related plaque in the vessels, which becomes a major component of atherosclerotic plaque lesions (Quinn et al. 1987). In hypercholesterolemia, the uptake of lipoproteins by the LDL receptors is lower due to downregulation, but they can be taken up by other receptors, whose expression is not regulated by the cell sterol content (Brown and Goldstein

1983; Endeman et al. 1993). The uptake of LDL by scavenger receptors may therefore be increased in cases of hypercholesterolemia. Once cholesterol enters the vessel wall it becomes modified by such processes as oxidation, which significantly accelerates the formation of atherosclerotic plaques (Rajavashisth et al. 1990). The accumulation of LDL in the arterial wall initiates monocyte and smooth muscle cell migration and transforms macrophages and smooth muscle cells into cholesterol-loaded foam cells, which are also the cell components found in the plaque (Penn and Chisolm 1994). Scavenger receptors can mediate the uptake and internalization of modified lipoproteins into the cell, which are expressed at the cell surface of macrophages. It is observed in advanced lesions that theexpression of high levels of scavenger receptors for oxidized LDL maymediate the extensive lipid accumulation in macrophage-derived foam cells (Griffith et al. 1988). The oxidized LDL and other forms of modified LDL, immunoglobulin glycation, aggregation, association with proteoglycans and incorporation into immune complexes are the major cause of injury to the endothelium and the underlying smooth muscle (Febbraio et al. 2001). Unfortunately, there is no easy or safe method with which to diagnose atherosclerosis in its early stages.

The available non-invasive imaging techniques such as angiography, ultrasonography and computer tomography are much better at defining the extent of more advanced atherosclerotic lesions than at discovering earlier changes in the vascular wall. There has been growing interest in radioisotopic atheroscerotic imaging for early lesion detection, when the lesions are metabolically most active and therapeutic interventions could be more beneficial (Lees et al. 1985). Radiolabeled LDL offers a promising approach to the identification the local metabolic fate of these compounds and to the study of LDL accumulation in vascular tissue, because LDL acts as a trapped ligand in vivo and should be a good tracer for scintigraphic studies of atherosclerosis (Lees et al. 1988; Vallabhajosula and Goldsmith 1990). The rabbit rapidly develops severe hypercholesterolemia, leading to premature atherosclerosis, in response to dietary manipulation by supplementation of the diet with cholesterol (Bocan et al. 1993; Back et al. 1995). Plasma levels of cholesterol correlate closely with the extent of lesion development (Restori et al. 1990). We have developed rapid and reproducible ultracentrifugation methods with which to obtain lipoprotein aliquots for radiolabeling and to evaluate lipoprotein labeling techniques suitable for the scintigraphic delineation of experimental atherosclerotic lesions.

Materials and methods

Serum samples

The blood samples were taken after overnight fasting from the vein of normal and hyperlipidemic (HLP) subjects. Blood samples were drawn from the marginal vein of fasted rabbits(12 h). The blood was allowed to clot for 40 min at room temperature and the serum was then separated by centrifugation at 1200×*g* and 20 °C for 30 min. The serum was stored at 4 °C at the presence of 0.01 g/L EDTA salt.

Preparative density gradient ultracentrifugation; determination of lipid levels

The preparative isolation of serum lipoproteins was carried out in a fixed angle rotor on an ultracentrifuge (type 3180, Hungarian Optical Works, Budapest). The conditions of the gradient formation and lipoprotein separation were as follows.

Serum (3 mL) was adjusted to a density of 1.3 g/mL with solid KBr (0.49 g/mL), placed in the bottom of a cellulose acetate centrifuge tube and overlayered with 8.5 mL of 1.006 g/mL NaCl solution containing 0.01 g/L EDTA salt made to a volume of 11.5 mL.

Tubes were loaded into a P50 angular 20° rotor for 130 min at 50,000 rpm. In the rotor, the *g* force at the top of the tube was $g = 95{,}030$, while at the bottom it was $g = 187{,}265$. At the end of the run, the tubes were removed from the rotor and 23 fractions were collected. The tubes were removed according to the localization of lipoprotein bands: lipoprotein-free serum protein (1.5 mL), high-density lipoproteins (4 mL), low-density lipoproteins (2.5 mL), intermediate-density lipoproteins (1.5 mL) and very low-density lipoproteins (2 mL), respectively, and the LDLs in these aliquots were radioactively labeled. Analyses for the principal lipid constituents were performed on the whole serum and the isolated lipoprotein fractions. Serum cholesterol and triglycerides in the serum and lipoproteins were determined by enzymatic assays as specifiedby the manufacturer (Chung et al. 1980; Cooper et al. 1991).

Analytical density gradient ultracentrifugation

The runs were carried out in an A65-2 analytical rotor operating in refractometric Schlieren mode at 546 nm. We applied band-forming, capillary underlayering-type single sector centerpieces furnished with special holes. The 4° band-forming and 12-mm optical path length centerpieces were assembled with negative-angle wedge windows (−1°40') on the bottom of the cell. The setting of the optical system was selected at a constant Philpot angle of 20°. Solid KBr was used to adjust the density of sera or other preisolated lipoprotein fractions. The samples were adjusted to a density of 1.3 g/mL (0.49 g KBr/mL serum) and 100-μL volumes were injected into the holes of the centerpiece from the upper window with a microsyringe. The cell was assembled and 0.5 mL NaCl solution ($\rho = 1.006$ g/mL) containing 0.1 g/L EDTA was placed into the sector of the centerpiece. Runs were performed at 50,000 rpm and 20 °C. When the rotor was accelerated to 2000–4000 rpm, the increasing hydrostatic pressure forced the adjusted-density sample from the holes of the centerpiece through capillaries under the physiological solution in the sector. The maximum speed was reached after 5 min. Photographs of the Schlieren pattern were taken 80 min after attaining full speed. The coordinates of the Schlieren distribution curves of the samples were obtained from the photograph, input into the computer, interpolated using piecewise cubic polynomials and displayed. The interpolation of the Schlieren distribution curves of the gradient baseline was made in the same way, the curves were subtracted and the result was integrated. The lipoprotein particle concentrations were calculated from the area under the integral of the Gaussian curve, using a calibration data constant (Lindgren et al. 1969; Medgyessy 1977; Dixin 1981).

Animals

Ten male New Zealand White rabbits (LATI, Hungary) weighing 3–3.5 kg were housed in separate cages at room temperature and

submitted to a 12-h light/dark cycle. Rabbit chow and water were available ad libitum to all rabbits. The rabbits were fed on a diet of normal rabbit chow (Biofarm, Hungary) enriched with cholesterol (Sigma) to a content of 2% for 60 days. The serum cholesterol and triglyceride levels of the NC and HC rabbits were determined by enzymatic methods at the beginning of the experiment (normocholesterolemia), during feeding and after 60 days (hypercholesterolaemia). Full care was provided for the animals in accordance with national and international standards, and the experimental protocol was accepted by the local Animal Experimental Ethics Committee.

LDL labeling with ^{99m}Tc

Lipoprotein aliquots obtained by preparative centrifugation (a pool of normolipidemic and type II HLP sera was centrifuged) were used for the radiolabeling of lipoproteins with ^{99m}Tc. Briefly, LDL containing 1–3 mg in a volume of 1–3 mL was mixed with 10–30 mCi [^{99m}Tc]pertechnetate (TcO_4^-) and 10 mg sodium dithionite, which was dissolved just before use in 0.5 M glycine buffer, pH 9.8, and the mixture was incubated for 30 min. Protein content was determined in an assay of the complex with the dye Coomassie Brilliant Blue. Radiochemical purity and in vitro stability were controlled by paper chromatography in acetone.

^{99m}Tc-LDLs were separated from free ^{99m}Tc by Sephadex G25 chromatography. The purified ^{99m}Tc-LDLs were dialyzed against 150 mM NaCl and sterilized through a 0.22-μm filter. The labeled lipoproteins were used for experiments immediately.

Histological examination of arteries

The exsanguinated rabbits were autopsied. The whole lengths of the aorta and carotid arteries were isolated. Segments of the aorta and carotid containing atherosclerotic lesions were fixedin formaldehyde (10%) and embedded in paraffin. Serial 8- to10-μm-thick sections were stained for lipid by using hematoxylin-eosin and Oil-red O.

Imaging studies

After overnight fasting, rabbits were anesthetized with 10 mg/kg of ketamine (SBH-Ketamin, SBH, Hungary) and 5 mg/kg of xylazine (Primazin, Alfasan, The Netherlands), given intramuscularly. A 20G cannula (Medicor, Hungary) was placed into the marginal ear vein, and approximately 500 MBq of labeled ^{99m}Tc-LDL was injected into the animals. Images were acquired with a large field-of-view gamma camera (Nucline X-Ring, Mediso, Hungary), with a low-energy high-resolution collimator. Dynamic studies were run in either 30-s or 1-min frames for 15 min immediately after injection, in a left lateral view. Static images were acquired 30 min and 1, 2, 4 and 24 h post-injection at a 256×256×8 matrix size until 600,000 counts was reached. For each time point, ventrodorsal, left lateral zoom and ventrodorsal view whole-body static images were taken. Images were processed with dedicated software (InterView 1.83.2, Mediso, Hungary).

Isolation of lipoprotein fragments for analytical ultracentrifugation

The components of the aorta were extracted in physiological solution after homogenization and centrifugation of the cells for 30 min. The extracted components in the supernatant fraction were investigated by analytical ultracentrifugation.

Results and discussion

Assessment of human LDL for radiolabeling by analytical ultracentrifugation

The Schlieren optics in the band centrifugation experiment clearly illustrate the processes taking place in the cell, yielding a quantity that is directly related to the concentration gradient function in the cell. Flotation distribution curves produce a visual display of partially to completely separated lipoproteins for further computed-assisted analyses. The appearance of the peak in the centre region of Schlieren distribution curves indicates the flotation of LDLs in the ultracentrifugal cell. The Schlieren pattern obtained for serum pools from healthy subjects is shown in Fig. 1A. The Schlieren pattern obtained from type II serum is shown in Fig. 1B. The Schlieren patterns obtained from normocholesterolemic rabbit are shown in Fig. 2A, and from hypercholesterolemic rabbits in Fig. 2B. The hypercholesterolemic rabbits were autopsied. The whole length of aorta was isolated. A segment of the aorta containing an atherosclerotic lesion is shown in Fig. 3. Deposition of lipids in the aorta can be seen in Fig. 4. We obtained lipoprotein fragments isolated from plaque tissue shown in Fig. 5. Gamma camera in vivo scintigraphy of hypercholesterolemic rabbits is shown in Fig. 6A–C.

Lipid analysis

Human samples

We determined VLDL, LDL and HDL cholesterol in normal sera ($n = 5$) and in sera from patients with type II

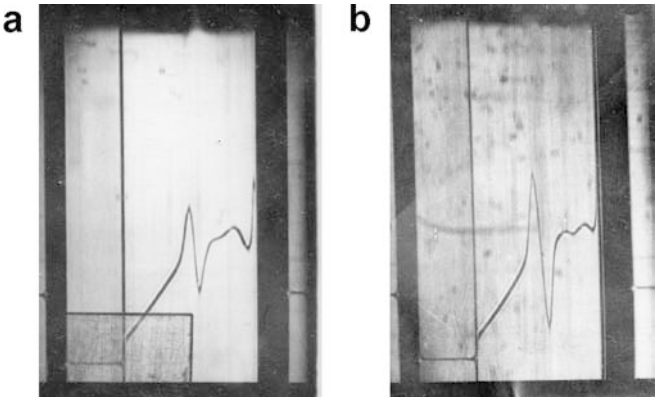

Fig. 1 **A** LDL in normolipidemic sera. The photograph of the Schlieren pattern was taken 80 min after attainment of full speed (50,000 rpm). Schlieren diagrams of LDL in normolipidemic sera containing 120 mg/dL LDL cholesterol. **B** LDL in type II HLP serum. The photograph of the Schlieren pattern was taken 80 min after attainment of full speed (50,000 rpm). Schlieren diagrams were produced of LDL in type II HLP sera containing 205 mg/dL LDL cholesterol

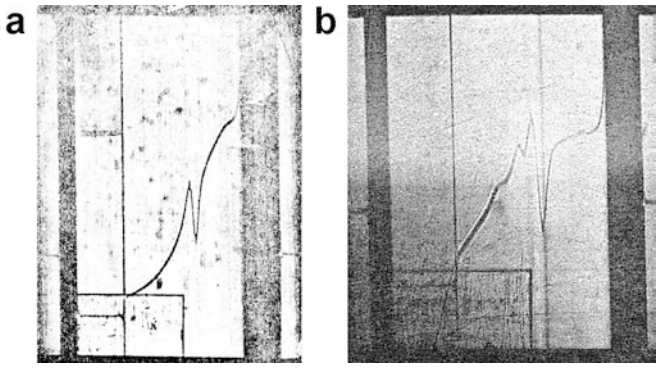

Fig. 2 **A** Assessment of rabbit LDL before cholesterol diet by analytical ultracentrifugation. The photograph of the Schlieren pattern was taken 80 min after attainment of full speed (50,000 rpm). Schlieren diagrams were produced of LDL in normolipidemic rabbits containing 35 mg/dL LDL cholesterol. **B** Assessment of rabbit LDL after cholesterol diet by analytical ultracentrifugation. The photograph of the Schlieren pattern was taken 80 min after attainment of full speed (50,000 rpm). Schlieren diagrams were produced of LDL in hyperlipidemic rabbits containing 190 mg/dL LDL cholesterol

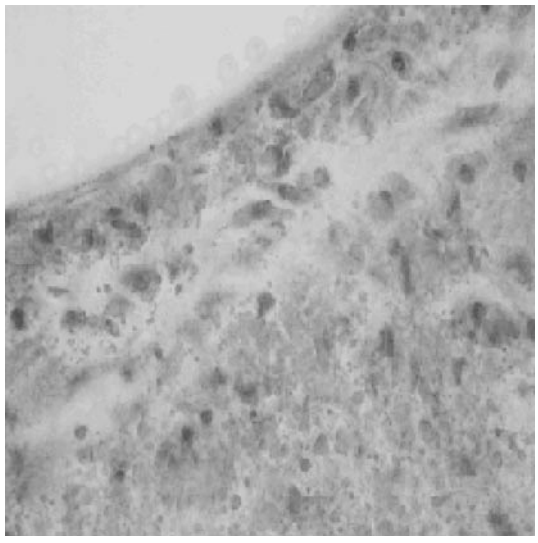

Fig. 4 Deposition of lipids in the aorta. Staining of aortic sections for lipid with Oil Red O revealed that cells in the atherosclerotic lesions contained a high content of lipid

Fig. 3 Segment of the aorta containing an atherosclerotic lesion. Autopsy revealed the formation of atheromatous plaques on the inner aortic surfaces of rabbits in induced hyperlipidemia

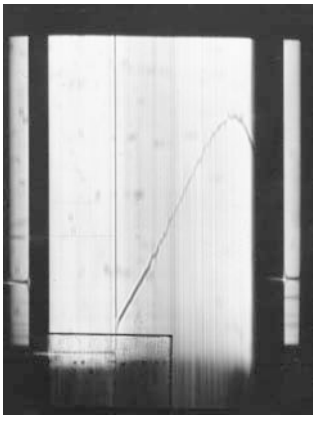

Fig. 5 Analytical ultracentrifugation of lipoprotein fragments isolated from plaque tissue. The photograph of the Schlieren pattern was taken after 80 min when the gradient baseline was well visible after attainment of speed (50,000 rpm). Density gradient ultracentrifugation revealed LDL-like particles in the plaque tissue. This Schlieren picture shows that particles accumulate in the arterial wall, partly in the form of lipoprotein-like particles, contributing to plaque formation

B HLP ($n = 5$). Normolipidemic samples with 210–220 mg/dL (5.42–5.68 mmol/L) content were chosen as the upper limit for serum cholesterol. VLDL cholesterol was isolated from samples that contained < 40 mg/dL (1.03 mmol/L). LDL-cholesterol was up to 160 mg/dL (4.13 mmol/L) and HDL-cholesterol was in the range up to 60 mg/mL (1.55 mmol/L).

In the series of type II B HLP sera, the isolated VLDL-cholesterol was > 90 mg/mL (2.3 mmol/L), LDL cholesterol was > 200 mg/dL (5.16 mmol/L) and HDL-cholesterol was in the range up to 60 mg/mL (1.55 mmol/L).

Measurement of triglycerides

Triglycerides determined in normolipidemic sera ($n = 5$) comprised < 100 mg/mL (1.14 mmol/L). Triglycerides in subjects with mildly elevated VLDL were up to 120 mg/mL (1.37 mmol/L), while those in subjects with severe hypertriglyceridemia exceeded 250 mg/mL (2.85 mmol/L) VLDL triglyceride in type II HLP ($n = 5$).

Rabbit samples

The rabbits fed normal chow had an average serum cholesterol of 72.4 ± 36.2 mg/dL (1.55 mmol/L) (mean $\pm$ SD, $n = 5$) and average serum triglycerides of 60.5 ± 25.3 mg/dL (mean $\pm$ SD, $n = 5$). After 30 days of cholesterol feeding,the average plasma cholesterol level was 1850 ± 361 mg/dL (47.7 mmol/L) (mean $\pm$ SD, $n = 5$), 25-fold that of normal-fed rabbits. Two months

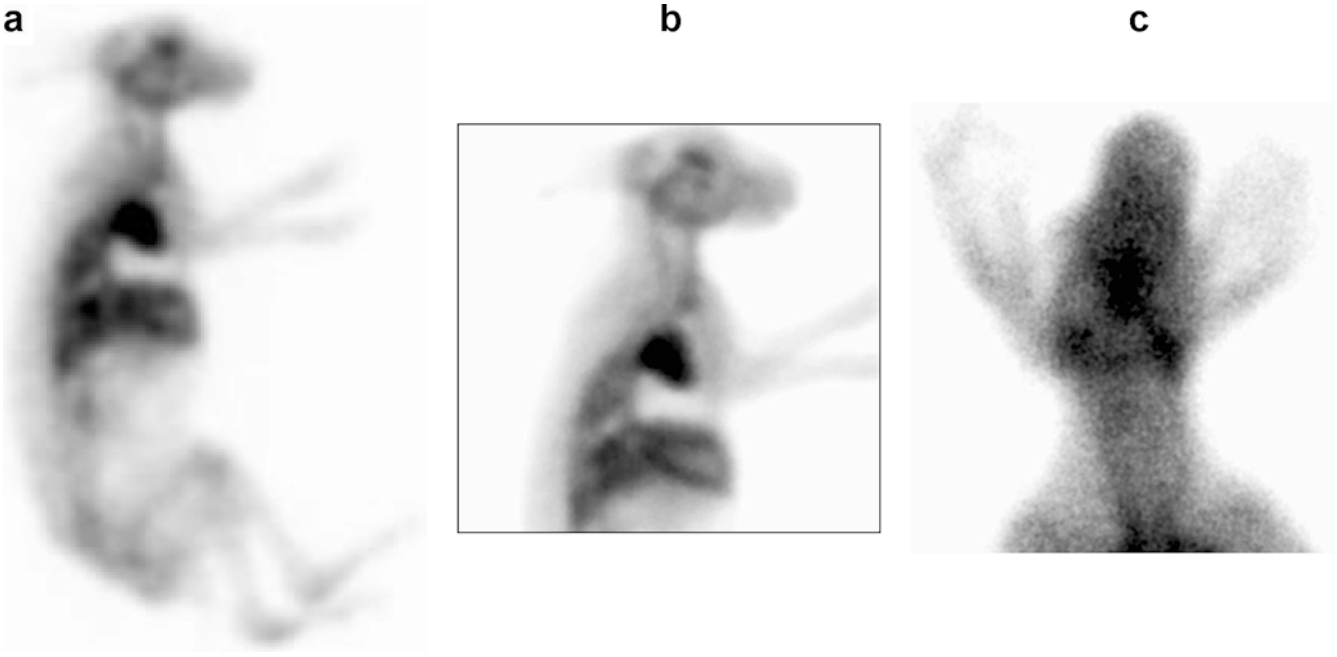

Fig. 6 **A** Gamma camera in vivo scintigraphy of hypercholesterolemic rabbit. Dynamic gamma camera imaging was performed in the first 30 min post-injection of 10 mCi (370 MBq) ^{99m}Tc-LDL. The radiolabeling efficiency was >85%. This scintigram shows a typical blood pool scan; it gradually changed with time to an image of specific organ uptake of radioactivity (right lateral view of the whole body acquisition). **B** Gamma camera in vivo scintigraphy of hypercholesterolemic rabbit. Lateral view of rabbit at 1 h post-injection. Uptake of radiolabelled LDL can be observed in the heart, liver, lungs and carotid arteries. **C** Gamma camera in vivo scintigraphy of hypercholesterolemic rabbit. Ventrodorsal view of a rabbit at 1 h post-injection. Uptake of radiolabelled LDL can be observed in the brain, aortic arch and the common carotid arteries

after the beginning of cholesterol feeding, the total cholesterol level in the serum of the HC rabbits had increased approximately 33-fold, compared with the NC rabbits, to 2430 ± 423 mg/dL (62.6 mmol/L) (mean ± SD, $n = 5$). Triglyceride concentration in the blood serum was also increased by the cholesterol-rich diet to 1130 ± 122mg/dL (12.8 mmol/L) (mean ± SD, $n = 5$) in the HC rabbits, an increase of approximately 18-fold compared with that of the NC rabbits.

More than 85% of the total cholesterol was found in the serum lipoprotein fraction of the HC rabbits, with a density $d < 1.019$ g/mL including chylomicrons, VLDL and IDL.

Conclusions

Hypercholesterolemia is an important risk factor for the development of the atherosclerotic process. Radiolabeled LDL was tested in atherosclerosis. ^{99m}Tc-LDL can be used as a radiotracer because it acts as an intracellularly trapped ligand, allowing a scintigraphic measurement of lipoprotein uptake by tissue. The present study provides data on the distribution and atherosclerotic lesion uptake of ^{99m}Tc-LDL in cholesterol-fed rabbits. The HC rabbits displayed the extensive presence of atherosclerotic lesions in the aorta and a detectable accumulation of ^{99m}Tc LDL in carotid arteries. The atherosclerotic lesions of the aorta arteries in the cholesterol-fed rabbits accumulated more ^{99m}Tc-LDL, mainly in the aortic arch, than that in the control rabbits. Rapid preparative isolation of serum lipoproteins was carried out by single-spin density-gradient ultracentrifugation. Analytical ultracentrifugation proved useful for a clear and immediate visual presentation of the concentrations of LDL. Atherosclerosis was induced in rabbits with a cholesterol diet. Two months after the start of cholesterol feeding, atherosclerosis had developed in the rabbits. The plasma cholesterol level increased from a normal baseline of about 70 mg/dL to reach peak levels of about 1800–2400 mg/dL by 4–8 weeks. ApoB-containing particles, migrating as LDL, IDL and VLDL, were the most abundant classes of lipoproteins. On the basis of analytical ultracentrifugation, the LDL flotation peaks were investigated before and after cholesterol feeding of rabbits. We determined the concentration of LDL without the need for the preparative isolation of that density class. Modified forms of LDL, especially with characteristics of serum LDL, were detected in atheroma and the plasma of rabbits with experimentally induced atherosclerosis. Histological examination showed the formation of atheromatous plaques on the inner aortic surfaces of rabbits with induced hyperlipidemia. Radiolabeled LDL were produced with ^{99m}Tc using sodium dithionite as reducing agent. The radiolabeling efficiency was >85%. Our preliminary results showed that gamma camera in vivo scintigraphy of rabbits revealed visible signals corresponding to atherosclerotic plaques in the aorta and carotid arteries. In the future we should like to

investigate which radiolabeled lipoprotein fractions are better for the early lesion detection of atherosclerosis. The in vivo imaging studies in rabbits suggest that the non-invasive imaging of atherosclerotic plaques with radiolabeled LDL may be feasible in humans.

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