^{99m}Technetium-labeled low density lipoprotein: receptor recognition and intracellular sequestration of radiolabel

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Abstract ^{99m}Technetium-labeled low density lipoprotein (^{99m}Tc-labeled LDL) was developed to detect atherosclerosis by external imaging with the gamma scintillation camera (Lees, et al. *J. Nucl. Med.* 1985. **26:** 1056–1062; Lees, et al. *Arteriosclerosis.* 1988. **8:** 461–470). The present study examined high affinity LDL receptor recognition and intracellular sequestration of ^{99m}Tc-labeled LDL by fibroblasts. There were no significant differences between ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL in binding parameters or percent inhibition of accumulation, which indicated that ^{99m}Tc labeling did not alter receptor recognition of LDL. At 4°C the K_d (± SE) for ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL, respectively, was 1.52 ± 0.24 and 1.45 ± 0.14 μg/ml; B_{max} (± SE) was 5.45 ± 0.48 and 4.89 ± 0.25 ng/well, respectively. Binding was saturated at about 2 μg/ml. The complete linearity of ^{99m}Tc-labeled LDL accumulation from 0–6 h and the positive slope from 6–24 h indicated that radiolabel that entered cells as ^{99m}Tc-labeled LDL was sequestered; pulse-chase experiments, which measured residual cell-associated radioactivity out to 24 h, also showed that radiolabel was trapped. Because radiolabel sequestration was essentially complete, and because ^{99m}Tc-labeled LDL was recognized by the LDL receptor equally as well as ^{125}I-labeled LDL, it should be useful not only for imaging atherosclerosis, but also for quantitatively determining sites of utilization and degradation of LDL. —**Lees, A. M., and R. S. Lees.** ^{99m}Technetium-labeled low density lipoprotein: receptor recognition and intracellular sequestration of radiolabel. *J. Lipid Res.* 1991. **32:** 1–8.

Supplementary key words radiolabel trapping

^{99m}Tc-labeled LDL has been used successfully to localize atherosclerotic plaques in human subjects (1), and healing lesions in the balloon-catheter de-endothelialized rabbit aorta (2). The focal sequestration of ^{99m}Tc-labeled LDL that permits successful imaging of atherosclerosis depends on the composition of affected arterial tissue and on its level of metabolic activity (1–3). Arterial accumulation of ^{99m}Tc-labeled LDL is not dependent on high affinity LDL receptor binding in the healing rabbit aorta (4). This tissue accumulated over threefold more methylated ^{99m}Tc-labeled LDL, which is not recognized by any cell surface receptor, than native ^{99m}Tc-labeled LDL. However, the question of whether ^{99m}Tc-labeled LDL could be recognized by the receptor remained; the rabbit adrenal (which uses the LDL receptor to obtain cholesterol) accumulated threefold less methylated ^{99m}Tc-labeled LDL than native ^{99m}Tc-labeled LDL (4), which suggested that ^{99m}Tc-labeled LDL was recognized by the receptor.

Earlier studies had also suggested that once ^{99m}Tc-labeled LDL entered cells, its radiolabel remained sequestered there. Adrenal accumulation of ^{99m}Tc-labeled LDL radiolabel was tenfold greater than that of ^{125}I-labeled LDL (4), and in human studies, 24-h urine output of radiolabel from ^{99m}Tc-labeled LDL ranged from 4% to 12% of the injected dose (1).

The goals of the present study with normal human skin fibroblasts were to determine whether ^{99m}Tc-labeled LDL is recognized by the high affinity LDL receptor, whether ^{99m}Tc labeling alters receptor recognition of LDL, and whether radiolabel that enters the cell as ^{99m}Tc-labeled LDL is trapped intracellularly.

MATERIALS AND METHODS

Materials

Normal human skin fibroblasts were obtained from the American Type Culture Collection, Rockville, Maryland. Tissue culture supplies and reagents were from Grand

Abbreviations: LDL, low density lipoproteins; PBS, phosphate-buffered saline.

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Island Biological, Sigma Chemical, Corning Glass, and Becton-Dickenson Labware.

Cell culture

Conditions were similar to those described previously (5). Fibroblasts were maintained in a monolayer at 37°C with 5% CO_2 in basal Eagle's medium supplemented with 10% fetal bovine serum; 1% nonessential amino acid mix; 0.12 mM $NaHCO_3$; penicillin, 100 units/ml plus streptomycin, 100 μg/ml; gentamycin, 5 μg/ml; and fungizone, 0.5 μg/ml (standard growth medium). Stock cultures of cells were grown in 75-cm^2 culture flasks. For experiments at 37°C, cells between the seventh and fifteenth passages were resuspended with 0.25% trypsin; cells, at an average concentration of 4×10^4 cells/ml, were seeded in 3.5-cm diameter wells, and grown for 11–12 days to ensure confluence. For studies of binding at 4°C, nonconfluent cells were prepared by seeding 55,000 cells/3.5-cm well and growing them for 4 days. In most experiments, 24 h before the start of each experiment, standard growth medium was replaced with 1 ml of supplemented basal Eagle's medium in which 10% fetal bovine serum was replaced by 10% lipoprotein-deficient human serum (LPDS), prepared as described previously (6), in order to induce maximal expression of LDL receptors (5). Noninduced cells were not treated with LPDS.

Radiolabeled low density lipoproteins

LDL (d 1.025–1.050 g/ml) was prepared from normal human plasma by sequential ultracentrifugation (7) and labeled with ^{99m}Tc, using a procedure previously described (2, 4). Briefly, LDL, dialyzed against 0.18 M NaCl and 1 mM disodium EDTA, pH 8.6, and $^{99m}TcO_4^-$ from a technetium generator were reacted with sodium dithionite, which was dissolved just before use in 0.5 M glycine buffer, pH 9.8. Reacting 1.5–2.5 mg of LDL and 30–40 mCi of ^{99m}Tc with 10 mg of dithionite dissolved in 0.1 ml of glycine buffer for 30 min gave a specific activity for freshly prepared ^{99m}Tc-labeled LDL ranging from 2 to 3×10^4 cpm/ng, after chromatography on Sephadex G25 to remove unbound ^{99m}Tc. Labeling efficiency was between 65% and 75%. One hundred to 200 μCi of ^{99m}Tc-labeled LDL was used per well. This was enough radioactivity so that with the 6 h half-life of ^{99m}Tc, there was never a problem with clearly distinguishing sample counts from background, even for pulse chase experiments in which the chase period extended to 24 h. An average of 8% (range 3–11%) of the radioactivity was incorporated into lipid. On paper electrophoresis (7), ^{99m}Tc-labeled LDL gave a sharp, Oil Red O-staining peak of radioactivity with the same migration as native LDL.

^{125}I-labeled LDL was prepared as described previously (6). ^{125}I was obtained from the New England Nuclear division of DuPont and Co.

Time-dependent accumulation

Induced cells were incubated with 10 μg/ml of ^{99m}Tc-labeled LDL at 37°C for 0–10 h in one set of experiments, and 0–24 h in another set. Both sets of experiments included measurements at 2 h and 4 h, so data for 20 and 24 h could be related to the 0–10 h data to give an integrated series of values for 0–24 h. After incubation, the cells were washed three times briefly with 3 ml of PBS containing 0.2% bovine serum albumin (PBS/albumin); this was followed by two 10-min washes with 3 ml of PBS/albumin and a brief wash with 3 ml of PBS alone. All washes were performed at 4°C. Then the cells were washed with 2 ml of PBS containing 10 mg/ml of heparin (PBS/heparin) for 1 h at 4°C. The PBS/heparin washes were collected and counted. A brief wash with 3 ml of PBS alone preceded digestion of cells with 2 ml of 1 N NaOH overnight at room temperature, with gentle stirring. The washing and digestion conditions were similar to those used previously (5). Solutions of digested cells were counted, and aliquots were assayed for protein content (8). The percent of cell counts in lipid was also measured. It did not exceed the amount in ^{99m}Tc-labeled LDL preparations, and did not change significantly over 24 h, which indicated that radiolabeled lipid entered cells only as part of LDL, not independently.

Noninduced cells were incubated in standard growth medium with 10 μg/ml of ^{99m}Tc-labeled LDL at 37°C for up to 5 h. Following incubation, noninduced cells were treated in the same way as induced cells.

Rates of accumulation with time were obtained from the slopes for accumulation between the indicated time points, as calculated by linear regression. In graphing the data for time-dependent accumulation of noninduced cells, a straight line was drawn from 0–5 h because the correlation coefficient for those data was 0.998. In graphing the data for time-dependent accumulation of induced cells, a straight line was drawn from 0–6 h because the correlation coefficient was 1.000. A polynomial fit for the data from 6–24 h was statistically indistinguishable from a straight line with a slope that was significantly less ($P < 0.02$) than that from 0–6 h. Therefore, a straight line with the appropriate slope was drawn from 6–24 h.

Concentration-dependent cell accumulation

An aliquot of medium was removed from each well that was equal to the volume of LDL to be added; cells were incubated at 37°C for 4 h with concentrations of LDL ranging from 10 to 400 μg/ml. The washing procedure for

the concentration experiments was the same as that described above, except that the three initial PBS/albumin washes each lasted about 7.5 min, and no additional PBS/albumin washes were carried out.

Binding assays of ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL

Measurements of lipoprotein binding to cells were carried out as previously described (9, 10). Briefly, nonconfluent cells, pre-chilled at 4°C for 30 min, were incubated at 4°C with a range of radiolabeled lipoprotein concentrations, in the absence or presence of excess unlabeled LDL; the concentration range covered a 100-fold span, from 0.2 μg/ml to 20 μg/ml, so that receptor occupancy varied from few to saturation. Cells were washed as described in the previous paragraph. PBS/heparin washes were used to measure total and nonspecific cell-surface binding. Specific binding was calculated by subtraction.

Analysis of binding data

Binding parameters were calculated by nonlinear least squares analysis using a modified version of the nonlinear least squares program, LIGAND (Elsevier-BIOSOFT, Cambridge UK), as previously described (10). Because data for specific binding were analyzed, a term for nonspecific binding was unnecessary; thus, the binding curves were fitted to the data using the following equation:

$$[B] = B_{max}[L]/K_d + [L]$$

where [B] is the concentration of bound ligand; [L] is the concentration of free ligand; K_d is the dissociation constant of the ligand-receptor interaction; and B_{max} is the amount of ligand bound at saturating concentration (10). For each curve, the mean square was calculated; the mean square is the sum of the squares of the residuals/degrees of freedom, and correlates inversely with the goodness of fit (10, 11).

Residual intracellular ^{99m}Tc-labeled LDL measured by pulse-chase

Cells were incubated at 37°C with 10 μg/ml of ^{99m}Tc-labeled LDL. After 4 h, the medium was removed and the cells were washed briefly at 4°C three times with 3 ml of PBS/albumin, and then once with PBS. Immediately after the last wash, 1 ml of fresh growth medium, containing 10 μg/ml of unlabeled LDL and 10% lipoprotein-deficient serum (chase medium), was added to each well. Cells were incubated again at 37°C. Zero to 24 h later, cells were washed with PBS, digested with NaOH, counted, and assayed for protein as described above. Chase medium was also counted.

RESULTS

Cellular metabolism of ^{125}I-labeled LDL is commonly determined by measuring the degradation product, ^{125}I covalently bound to tyrosine, after trichloroacetic acid precipitation of unmetabolized lipoprotein (9, 12). However, metabolism of ^{99m}Tc-labeled LDL was determined by measuring accumulation of radiolabel because although some ^{99m}Tc binding to LDL may be by covalent linkage, a major portion appears to be by chelation. When ^{99m}Tc-labeled LDL was treated with 10% trichloroacetic acid, which does not affect covalent bonds, but does dissociate many chelates, 50% of the radioactivity on ^{99m}Tc-labeled LDL precipitated, and 50% remained in solution, even though a single peak, with the molecular weight of LDL, was isolated by gel chromatography of ^{99m}Tc-labeled LDL (2), and in human studies (A. M. Lees and R. S. Lees, unpublished results) at physiologic pH, there was no evidence of in vivo dissociation of ^{99m}Tc from LDL after up to 21 h circulation in plasma.

Time-dependent accumulation of ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL radiolabel in fibroblasts was compared (**Fig. 1A**). Accumulation of ^{99m}Tc-labeled LDL, at an LDL concentration in the medium of 10 μg/ml, was linear for 6 h (r = 1.000), indicating that no radiolabel left cells during that time (as explained in the Discussion); accumulation continued to increase more slowly between 6 and 24 h. In contrast, ^{125}I-labeled LDL accumulation reached a maximum by 2 h and did not increase thereafter, which indicated that an equilibrium was reached at 2 h between the amount of radiolabel entering and leaving the cells. As illustrated in Fig. 1A, the rate of ^{99m}Tc-labeled LDL accumulation by induced cells slowed after the first 6 h. As derived from linear regression analysis, the rates were: 98 ng/mg of cell protein per h for 0–6 h; 42 ng/mg per h for 6–10 h; 38 ng/mg per h for 10–20 h; and 25 ng/mg per h for 20–24 h. Although accumulation of radiolabel could have started to decrease at 6 h because ^{99m}Tc began to leave the cells, a more likely explanation was that accumulation decreased because the induced cells' need for cholesterol decreased after 6 h in the presence of LDL, leading to down-regulation of receptors (12). To test this hypothesis, accumulation of ^{99m}Tc-labeled LDL by noninduced cells was measured (Fig. 1B). This was also linear (r = 0.998), and had a rate of 27 ng/mg per h, the same as that of induced cells after 20 to 24 h of incubation with LDL. These results indicated that receptor down-regulation did explain the decreased rate of ^{99m}Tc-labeled LDL accumulation by induced cells after several hours of incubation.

Concentration-dependent accumulation of ^{99m}Tc-labeled LDL radiolabel increased in a nonlinear fashion as LDL concentration in the medium increased (**Fig. 2A**). The slope of the concentration curve was greater at

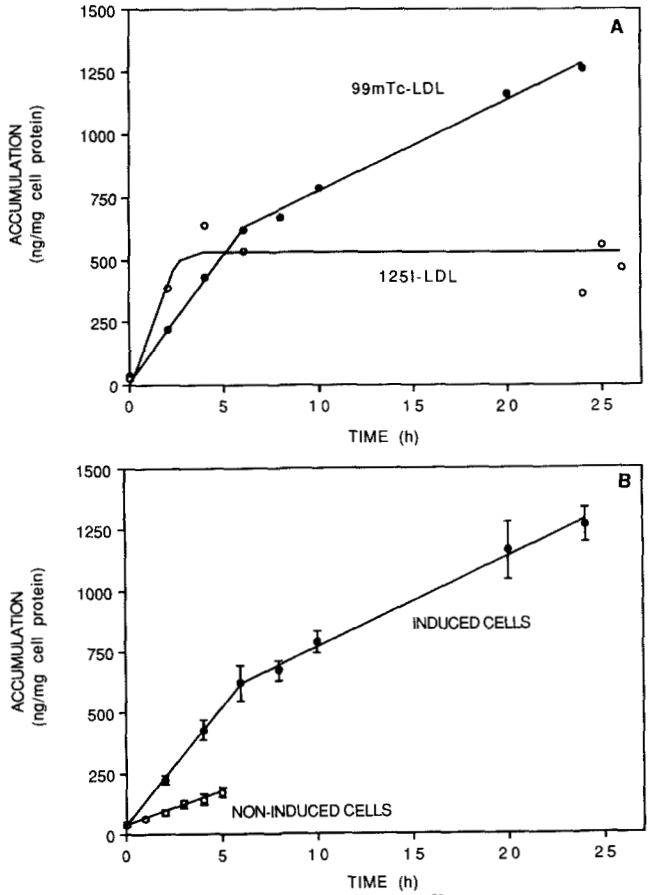

Fig. 1. (A) Comparison of accumulation of ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL by fibroblasts. Cells (induced to express maximal numbers of high affinity LDL receptors by growth in lipoprotein-deficient serum) were incubated at 37°C for the indicated times with 10 μg/ml of either ^{99m}Tc-labeled LDL (solid circles) or ^{125}I-labeled LDL (open circles). Data for ^{125}I-labeled LDL were from a single experiment done in duplicate. ^{99m}Tc-labeled LDL results were the mean (± SEM) of five experiments (0–10 h) and three experiments (20–24 h), each done in duplicate. The set of five experiments included time points at 0, 2, 4, 6, 8, and 10 h and the set of three included points at 0, 1, 2, 4, 20, and 24 h. The overlapping results at 2 and 4 h were used to normalize the values for 20 and 24 h to the values in the 0–10 h experiments. (B) Comparison of accumulation of ^{99m}Tc-labeled LDL by induced (solid circles) and noninduced (open circles) cells. Results from induced cells shown in (A) were compared with results from noninduced cells that had been maintained in standard growth medium and incubated with 10 μg/ml of ^{99m}Tc-labeled LDL. Results for induced and noninduced cells were the mean (± SEM) of five experiments.

lower concentrations than at higher concentrations, consistent with a high affinity, low capacity receptor-mediated binding process. The accumulation of ^{99m}Tc-labeled LDL was competitively inhibited by unlabeled LDL (Fig. 2B). With a 20- to 40-fold excess of unlabeled LDL, accumulation of ^{99m}Tc-labeled LDL decreased by 75%. The percent inhibition of ^{125}I-labeled LDL accumulation was the same as that of ^{99m}Tc-labeled LDL (Fig. 2B), consistent with the hypothesis that ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL were recognized equally well by the LDL receptor.

To compare binding and binding constants for ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL, equilibrium binding studies at 4°C were performed. Direct measurements of binding (**Fig. 3A**), showed that fibroblast binding of both forms of radiolabeled LDL was saturated at a lipoprotein concentration of about 2 μg/ml. Nonlinear least squares analysis with the LIGAND program (13) showed similar binding parameters for both LDLs. The K_d and B_{max} (with approximate standard errors) for ^{99m}Tc-labeled LDL were 1.52 ± 0.24 μg/ml and 5.45 ± 0.48 ng/well, respectively. For ^{125}I-labeled LDL, they were 1.45 ± 0.14 μg/ml and 4.89 ± 0.25 ng/well. The differences between the pairs of parameters were not statistically significant. For the pooled data for both LDLs, K_d and B_{max} were 1.48 ± 0.14 μg/ml and 5.16 ± 0.27 ng/well, respectively.

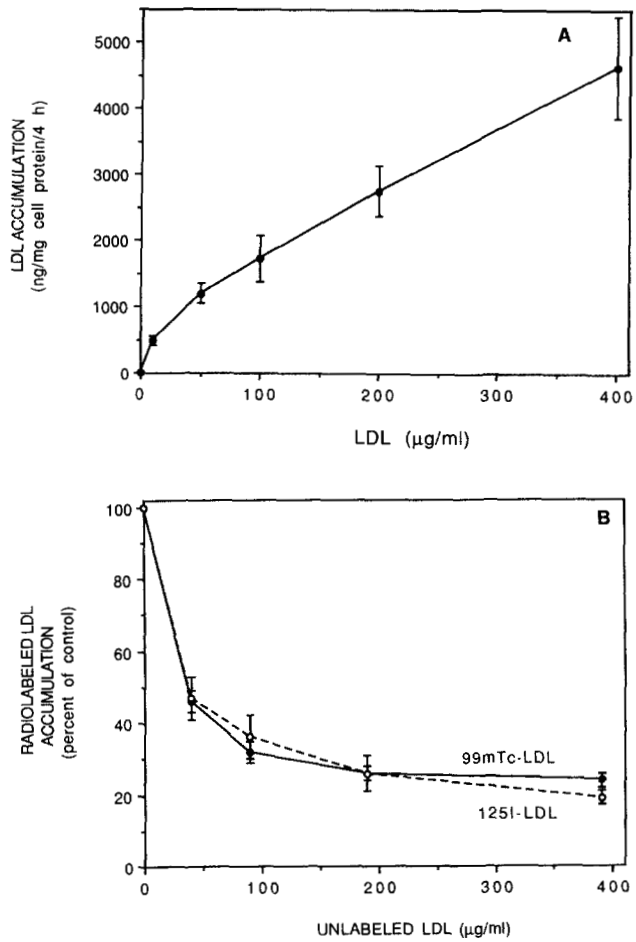

Fig. 2. (A) Concentration-dependent accumulation of ^{99m}Tc-labeled LDL by induced fibroblasts. Confluent, induced cells were incubated for 4 h at 37°C with increasing concentrations of LDL, obtained by adding unlabeled LDL to 10 μg/ml of ^{99m}Tc-labeled LDL, (mean ± SEM of five experiments, in duplicate). (B) Competitive inhibition of radiolabeled LDL accumulation by unlabeled LDL. To determine the percent inhibition of radiolabeled ^{99m}Tc-labeled LDL (solid circles) accumulation by increasing concentrations of unlabeled LDL, the results shown in 2A were replotted. For the inhibition of ^{125}I-labeled LDL (open circles) accumulation, the experiments were repeated with iodinated LDL (n = 4).

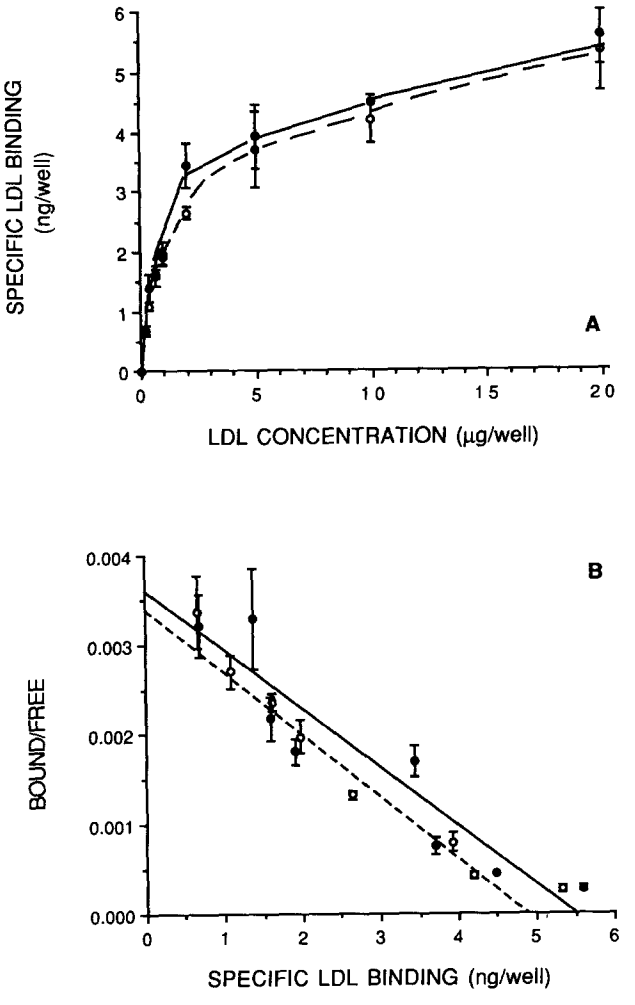

Fig. 3. (A) Specific binding of ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL. Nonconfluent, induced cells were incubated in duplicate at 4°C for 2 h with increasing concentrations of either ^{99m}Tc-labeled LDL (solid circles) or ^{125}I-labeled LDL (open circles), in the absence and presence of excess unlabeled LDL. After washing, counts released by heparin were measured to determine total and nonspecific cell-surface binding. Specific binding (mean ± SEM) was calculated by subtraction. (B) Scatchard plots of specific binding data. The data shown in 3A were analyzed using a modified nonlinear least squares program, LIGAND, which showed that they were best fitted to a one-site model. See text for binding constants.

There was no improvement in the fit for either form of LDL when the results were analyzed for two binding sites rather than one. This indicated that the curves for both LDLs were best fitted to a one-site model; Scatchard plots for each LDL, using a one-site model, are shown in Fig. 3B.

To test further the possibility of intracellular radiolabel trapping, residual intracellular accumulation of ^{99m}Tc-labeled LDL was measured in pulse-chase experiments (**Fig. 4**). Because cells were seeded at the same density for each experiment and incubated until confluent, it was possible to express accumulation in terms of ng accumulated/culture well. When this was done, there was an 11% loss of cell-associated radiolabel in the first 4 h, with no loss between 4 and 24 h (Fig. 4A). When the results were expressed in the usual way, as ng accumulated per mg of cell protein (Fig. 4B), there was a 28% loss of radiolabel from the cells in the first 4 h of the chase period. Again, between 4 and 24 h, intracellular radiolabel remained steady, averaging 31% less than at the start

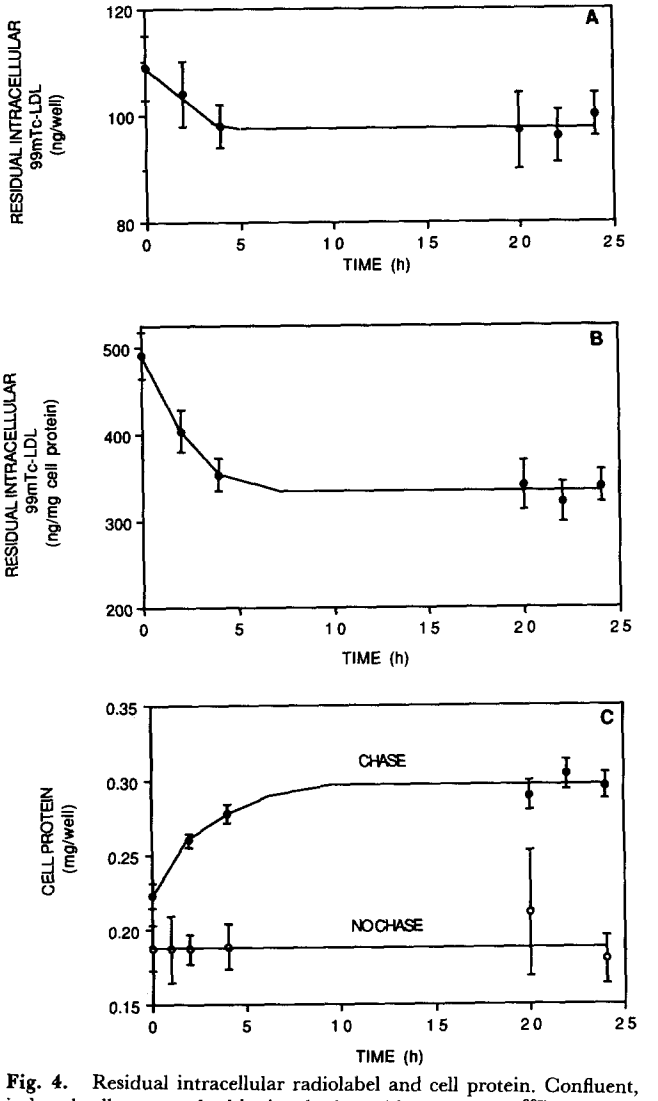

Fig. 4. Residual intracellular radiolabel and cell protein. Confluent, induced cells were pulsed by incubation with 10 μg/ml of ^{99m}Tc-labeled LDL for 4 h at 37°C. After removal of radiolabel by washing with PBS/albumin, cells were incubated with unlabeled LDL for up to 24 h. When residual intracellular radiolabel was expressed as ng accumulated/well (A), the results were not affected by an increase in cell-associated protein (see below) which occurred in the first 4 h of the chase period, and so gave a better estimate of the extent of radiolabel trapping. The 11% loss of radiolabel in the first 4 h, which was independent of the increase in protein, was the result of transfer of loosely bound radiolabel to the medium (see text). Chase results expressed as ng/mg cell protein (B) were affected by the increase in cell-associated protein (C, solid circles). The protein increase did not occur in cells incubated with ^{99m}Tc-labeled LDL for the same time periods when the cells were washed and dissolved in NaOH immediately following the incubation (C, open circles).

of the chase period. However, the apparent loss of radiolabel was accompanied by matching increases in cell-associated protein of 25% during the first 4 h of the chase period, and 31% between 4 and 24 h of the chase (Fig. 4C). Cells that had no chase period, but were incubated with ^{99m}Tc-labeled LDL for up to 24 h, had no increase in cell-associated protein (Fig. 4C).

The limited loss of radiolabel that was independent of the increase in cell protein was explained by the fact that the post-pulse washes did not contain heparin, which is known to remove LDL bound to the cell surface (5). Heparin washes were not used because their use was associated with a 250–300% increase in cell protein in the chase period (data not shown), instead of the 31% increase that occurred with PBS/albumin buffer. To estimate whether the 11% protein-independent loss of radiolabel in the first 4 h of the chase could be attributed to surface-bound LDL, confluent cells, which had been seeded into wells at the same time from the same stock flask, were incubated with ^{99m}Tc-labeled LDL, and then divided into two groups. The first group of cells was washed exactly as the cells in the time- and concentration-dependent experiments were washed, including a 1-h wash with PBS/heparin. The second group was washed only with PBS/albumin, as was done in the pulse-chase experiments. Cells washed with PBS/heparin accumulated 11% less ^{99m}Tc-labeled LDL than cells washed with PBS/albumin. Thus, the cell protein-independent 11% loss in the first 4 h of the chase could be attributed to loss of surface-bound, rather than intracellular, ^{99m}Tc.

The increase in cell protein seen during the chase period was investigated to determine whether there was a deleterious effect of ^{99m}Tc-labeled LDL on cells or whether, instead, the increase was associated with the PBS/albumin washing done between the pulse and the chase. Again, confluent cells, which had been seeded into wells at the same time from the same stock flask, were divided into two groups. One group was first washed with PBS/albumin before being incubated at 37°C with fresh medium containing unlabeled LDL, while control cells had labeled LDL added to the medium without any prior washing. Cell protein was measured at 0, 2, and 4 h. In complementary experiments, the washing was reversed, so that cells receiving labeled LDL were first washed, and cells receiving unlabeled LDL had no wash before incubation. In other experiments, both groups of cells were washed, or not washed, before incubation with labeled or unlabeled lipoprotein. Whether incubated with labeled or unlabeled LDL, there was an increase in protein only when cells were first washed with PBS/albumin. Cells that were not washed showed no increase in protein. The results indicated that ^{99m}Tc-labeled LDL did not have an adverse effect on cells.

DISCUSSION

The results of the present study indicate that ^{99m}Tc-labeled LDL is recognized by the high affinity LDL receptor, and its radiolabel is sequestered intracellularly. Competitive inhibition of ^{99m}Tc-labeled LDL accumulation by excess unlabeled LDL was the same as that seen for ^{125}I-labeled LDL. [Although inhibition of accumulation for both forms of radiolabeled LDL reached a maximum of only 75%, maximal inhibition of ^{125}I-labeled LDL degradation was 90 to 95%, as would be expected (results not shown)]. Cell surface binding of ^{99m}Tc-labeled LDL at 4°C was also the same as that for ^{125}I-labeled LDL; both were saturated at an LDL concentration in the medium of 2.5 μg/ml, in agreement with an earlier finding for ^{125}I-labeled LDL (5). These results demonstrate that ^{99m}Tc labeling of LDL did not alter receptor recognition of the lipoprotein. LDL receptor recognition of ^{99m}Tc-labeled LDL is not surprising since the metabolism of ^{99m}Tc-labeled LDL has been shown previously to be similar to that of native LDL by several criteria. The biexponential clearance kinetics of ^{99m}Tc-labeled LDL in the rabbit (4) are similar to those of ^{125}I-labeled LDL (13). For both compounds, approximately 65% of the initial dose remained in plasma at 4 h, and about 25% at 24 h; for both, the major component of the die-away curve had a half-time of about 20 h. The biodistributions of ^{99m}Tc-labeled LDL and ^{125}I-labeled LDL 18–21 h after injection were also qualitatively similar (2).

Evidence of intracellular sequestration of radiolabel came from both time and pulse-chase experiments. Two aspects of the accumulation versus time data supported trapping, the linearity of accumulation in the first 6 h, and the positive slope for accumulation from 6–24 h. The complete linearity ($r \approx 1.000$) of ^{99m}Tc-labeled LDL accumulation for 6 h (Fig. 1) indicated that the rate of accumulation was constant over that time period; this could only occur if there were no efflux of radiolabel. While it is possible to observe apparently linear accumulation for a short period time despite first order release of material back into the medium, as illustrated by ^{125}I-labeled LDL accumulation in the first 2 h (Fig. 1A), the apparent linearity cannot continue indefinitely. A graph of constant influx accompanied by detectable first order efflux will be curvilinear, with an initial positive slope which decreases to zero as equilibrium is reached between influx and efflux. This can be explained as follows. When the LDL concentration in the medium is kept constant, influx is constant. Efflux cannot be constant because there is no intracellular radioactivity present at zero time to efflux from the cell. By definition, if efflux is first order, it must increase with increasing accumulation. It will continue to

increase until efflux equals influx. When that happens, the slope of accumulation becomes zero (^{125}I-labeled LDL, Fig. 1A). If the rate of efflux were very slow, the flattening of the curve might appear later than it does for ^{125}I-labeled LDL, but with constant influx and increasing efflux, the slope would eventually have to reach zero. The ^{99m}Tc-labeled LDL accumulation curve with time is different from that ^{125}I-labeled LDL; it never reaches a slope of zero, even out to 24 h. The slope does not change until 6 h, and then becomes lower, but still clearly positive, from 6–24 h. If measurable efflux were occurring, it is improbable that it would not be detected by 24 h. The change in slope from 6–24 h is best explained by down-regulation of LDL receptors, since the slope for accumulation from 20–24 h by induced cells is the same as that for cells that have never been induced. Thus, the ^{99m}Tc-labeled LDL time curve shown in Fig. 1 is incompatible with significant cellular efflux. The pulse-chase results provided additional, independent support for the conclusion that radiolabel introduced into cells as ^{99m}Tc-labeled LDL is sequestered. Since the two types of experiments both demonstrated trapping of radiolabel, we concluded that no radiolabel was lost from cells in 24 h. Sequestration very likely occurs because the radiolabel bound to LDL is reduced intracellularly to an insoluble compound, such as $^{99m}TcO_2$.

Although the factors responsible for the increase in cell-associated protein seen during the chase period were not isolated, the increase is unlikely to be an experimental artifact. It occurred only with cells that had been washed with PBS/albumin (or PBS/heparin) prior to a chase period with unlabeled LDL, and did not occur with cells that had not been washed. The only difference between the chase and non-chase experiments was the inclusion or omission of the wash; all analytical procedures were identical. Removal of ^{99m}Tc-labeled LDL from the medium also did not explain the protein increase; cells incubated with unlabeled LDL demonstrated an increase in cell-associated protein too, when they were first washed with PBS/albumin. The increase in cell protein may have reflected an increase in protein production by cells, an increase in cell number, or both. Since the cells were confluent, the former is more likely. Wight et al. (14) have reported that heparin produced a marked stimulation of proteoglycan production by cultured fibroblasts. The effect on protein production seen with the albumin wash may be explained by the fact that many components of plasma adhere to albumin and one or more of these could stimulate cell proliferation or extracellular matrix formation by fibroblasts.

The use of an intracellularly trapped radiolabeled derivative of LDL was first demonstrated by Pittman et al. (15), who developed ^{125}I-labeled tyramine-cellobiose LDL for use in measuring the amount of LDL degraded by various tissues. The innovative derivative was shown to be very useful, and others have made similar labels for proteins, such as ^{125}I-labeled dilactitol tyramine (16) and inulin-^{125}I-labeled tyramine (17). However, the synthesis of these residualizing labels is quite complex, and their radioiodinated degradation products slowly diffuse out of cells (15–17). LDL can be labeled with ^{99m}Tc easily and rapidly; the radiolabel itself, rather than a nonmetabolizable substrate, is trapped intracellularly. Because the trapping is essentially complete, and because ^{99m}Tc-labeled LDL is recognized by the LDL receptor equally as well as ^{125}I-labeled LDL, it should prove useful not only for imaging atherosclerosis but also for determining sites of utilization and degradation of LDL (18).

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