Lipoprotein-Immune Complexes and Diabetic Vascular Complications

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In earlier studies, we showed that incubation of HMM with LDL IC led to cellular CE accumulation and to the transformation of macrophages into foam cells. This study demonstrates that the stimulation of macrophages with RBC-LDL-IC also increases the uptake of native LDL, most likely because of an increased LDL receptor number, as shown by Scatchard plot analysis (*x*-axis intercept 1267 vs. 352 ng LDL/mg protein in control cells). To determine whether the increase in LDL-receptor activity was secondary to a decrease in the macrophage free (nonassociated) cholesterol content, we measured the T-UC and the UC associated with intracellular intact LDL and demonstrated that 50% of the T-UC is associated with intact LDL. UC not associated with LDL (free cholesterol) was lower in LDL-IC–stimulated cells than in control cells. These results suggest that UC associated with nondegraded intracellular LDL is nonregulatory, a conclusion that was also supported by finding increased sterol synthesis (192.8 ± 22.9 pmol/mg protein vs. 94.8 ± 11.8) in RBC-LDL-IC–stimulated macrophages. In conclusion, the uptake of RBC-LDL-IC by macrophages led to increased intracellular accumulation of CE and UC, to a decrease in the cell regulatory pool of free cholesterol, and to an increase in LDL-receptor activity. *Diabetes* 41 (Suppl. 2):92–96, 1992

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LDL, low-density lipoprotein; IC, immune complexes; CE, cholesteryl ester; UC, unesterified cholesterol; RBC, red blood cell; IDDM, insulin-dependent diabetes; PBS, phosphate-buffered saline; IgG, immunoglobulin G; HMM, human monocyte-derived macrophages; RBC-Tet-IC, red blood cell–adsorbed tetanus immune complex; RBC-LDL-IC, red blood cell–adsorbed low-density-lipoprotein–containing immune complex; apoB, apolipoprotein B; T-UC, total cellular unesterified cholesterol; FC, unesterified cholesterol not associated with low-density lipoprotein.

Diabetic vascular complications are responsible for the increased morbidity and mortality in both IDDM and non-insulin-dependent diabetic subjects (1–4). Therefore, considerable effort has been put in identifying factors responsible for diabetic vascular complications. Among these factors, IC are believed to play a role in the pathogenesis of diabetic macro- and microangiopathy. Several facts seem to support this hypothesis: Vascular lesions present in people with immune complex disease share morphological characteristics with those observed in people with diabetes (5–7), diabetic-like vascular lesions can be experimentally induced by immune mechanisms (8–10), and circulating IC were described in diabetic subjects (11,12). Recently, it was shown that modification of lipoproteins, such as glycation and oxidation, renders lipoproteins immunogenic (13,14) and triggers antibody and immune-complex formation. Previous studies performed in our laboratory showed that incubation of HMM with insoluble IC as well as with soluble lipoprotein IC led to CE accumulation (15,16) and to the transformation of these cells into foam cells, one of the most striking features of early atherosclerotic lesions. Furthermore, we also demonstrated that exposure of HMM to insoluble LDL-ICs led to an increase in LDL-receptor activity. This study investigated whether incubation of HMM with RBC-LDL-IC can also induce an increase in LDL receptor activity.

RESEARCH DESIGN AND METHODS

Preparation of red cell suspensions, isolation of monocytes, and macrophage transformation. RBCs were isolated from the blood of type O Rh^+ healthy volunteers after buffy coat separation by sedimentation in dextran and were washed and suspended in PBS, pH 7.35, as described earlier (16). Monocytes were isolated

from leukapheresis specimens of healthy normolipemic human volunteers by countercurrent centrifugal elutriation as previously described (15). Maturation of monocytes into macrophages was performed with a well-defined Iscove's modified Dulbecco's medium containing 30% whole human serum. After maturation of monocytes into macrophages, the same medium, without whole human serum and without added cholesterol, was used to perform all the experiments.

Lipoprotein isolation and labeling. LDLs were isolated from plasma of fasting normolipemic healthy volunteers by sequential ultracentrifugation as previously described (16). A sample of LDL was radiolabeled with ^{125}I by the McFarlane procedure as modified by Bratzler et al. (17).

Preparation of soluble and RBC-LDL-IC. Immune complexes were prepared using human LDL and the IgG fraction of a rabbit anti-LDL antiserum as previously described (16). RBC-LDL-ICs were prepared by incubating, at 37°C for 30 min and under constant mixing, 1×10^8 RBC with soluble LDL-ICs. The cells were washed afterwards to remove the IC, LDL, and IgG not bound to RBCs. Washed RBC pellets were resuspended in PBS and sterilized by γ-radiation before addition to HMM cultures. The amount of IC adsorbed to RBCs was determined in experiments in which IC were prepared with ^{125}I-labeled LDL or [^{125}I]IgG. The preparations had, on average, 6 μg of LDL and 15 μg of IgG bound per 10^7 RBCs.

Measurement of sterol synthesis. Sterol synthesis was determined by measuring the incorporation of [2-^{14}C]acetate into nonsaponifiable sterols as described previously (18). The yields of products were calculated from the specific activities of the substrates, with the assumption that all radiolabel was incorporated into C_{27} sterols, which contained 15 labeled positions derived from [2-^{14}C]acetate.

Degradation and accumulation experiments. The proteolytic degradation of ^{125}I-labeled proteins (either LDL or IgG) by human HMM was measured by assaying the amount of [^{125}I]trichloroacetic acid–soluble (noniodide) material formed by the cells and excreted into the culture medium, as previously described (16). Corrections were made for the small amounts of ^{125}I-labeled acid-soluble material that was found in parallel incubations without cells. Accumulation was determined as previously described (16).

Stimulation experiments. Macrophages were incubated at 37°C with medium containing RBC-LDL-ICs or RBCs suspended in PBS. After a 20-h incubation, the medium was removed, the cells were extensively washed, and medium containing 20 nM (10 μg/ml) [^{125}I]-labeled native LDL (as protein) with or without a 25-fold excess of unlabeled LDL was added to the cells. To determine total specific and nonspecific accumulation and degradation of native [^{125}I]LDL in stimulated and nonstimulated cells, the second incubation was carried out at 37°C for 20 h. To determine [^{125}I]LDL binding, the cells were cooled to 4°C before addition of [^{125}I]LDL and unlabeled LDL, and the second incubation was performed at 4°C for 4 h. Saturation-binding isotherm studies were performed by incubating the cells with medium containing increasing concentrations of [^{125}I]LDL with or without the addition of a 25-fold excess of unlabeled LDL. Scatchard analysis was performed on the saturation-binding data as described (16).

Experiments measuring simultaneously LDL accumulation, cholesterol mass, and sterol synthesis. To perform these experiments, HMMs were incubated for 20 h with medium containing either ^{125}I-labeled RBC-LDL-IC (RBC:HMM ratio 50:1) or native [^{125}I]LDL (at a concentration similar to that in the LDL-ICs). [^{14}C]acetate was added to the wells used for measurement of sterol synthesis. After the incubation, the cells were extensively washed, and they were either solubilized with sodium hydroxyde to determine LDL accumulation or extracted with hexane/isopropanol, as described (19), to measure free and esterified cholesterol by gas chromatography or the rate of sterol synthesis (wells containing [^{14}C]acetate). The amount of UC associated with the intact LDL that accumulated intracellularly was calculated on the basis of the LDL composition. That value was subtracted from the T-UC level measured by gas chromatography, and the difference was considered a more relevant measurement of the regulatory pool of free cholesterol.

Statistics. Results are reported as mean ± SE. The nonparametric Wilcoxon signed rank-sum test for unpaired data was used for statistical comparisons. Significance was defined as $P < 0.05$.

RESULTS

To determine the effect of the incubation of HMM with RBC-LDL-ICs on the uptake and degradation of ^{125}I-labeled native LDL, we incubated human macrophages for 20 h, before the addition of 20 nM (10 μg/ml) of [^{125}I]LDL, with RBC-LDL-IC, RBCs suspended in PBS (RBC-HMM ratio 50:1), or insoluble LDL-ICs. Incubation of human macrophages with RBCs suspended in PBS served as negative control and incubation with insoluble LDL-ICs (250 μg/10^6 HMMs) as positive control. The RBC-HMM ratio was chosen based on previous experiments (16) showing that this ratio of RBCs to HMMs would deliver 100 to 200 μg of LDL-ICs to the cells. Under these conditions, we observed a marked increase in the total accumulation of native [^{125}I]LDL by cells preincubated with RBC-LDL-ICs or insoluble LDL-ICs when compared with cells incubated with RBCs suspended in PBS (Fig. 1). In contrast, although preincubation with insoluble LDL-ICs also increased the amount of LDL degraded by the cells, no difference was observed in the amount of LDL degraded by cells preincubated with RBC-LDL-IC and RBCs suspended in PBS (Fig. 1). Preincubation of macrophages with RBC-LDL-ICs at a lower concentration (RBC-HMM ratio 5:1), although able to promote marked intracellular accumulation of CE, as previously reported (16), did not induce an increase in native LDL accumulation (data not shown).

To determine whether the enhanced LDL uptake by cells incubated with high concentrations of RBC-LDL-IC was due to an increased affinity or an increased number of LDL receptors, we performed isotherm-binding experiments in cells preincubated, for 20 h, with RBC-LDL-IC

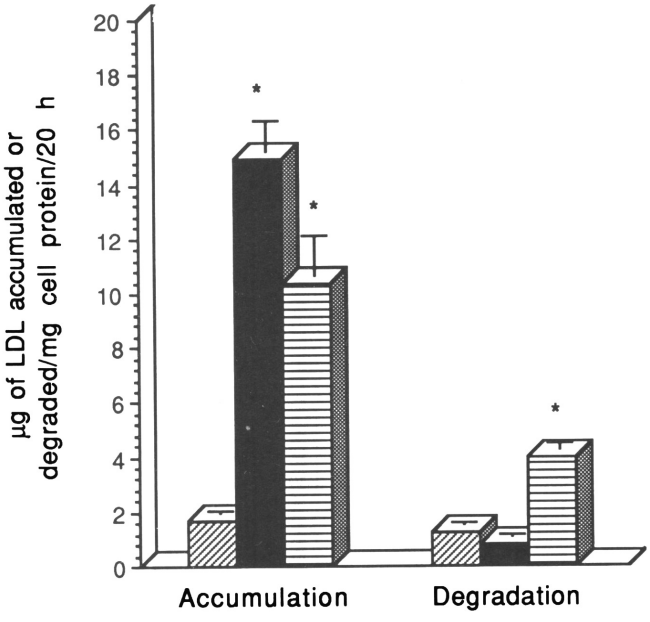

FIG. 1. Accumulation and degradation of native [^{125}I]LDL (μg/mg cell protein) by HMMs preincubated for 20 h with RBCs suspended in PBS (*diagonally hatched bars*), RBC-LDL-IC (*solid bars*) (RBC-HMM ratio = 100:1), or insoluble LDL-IC (*horizontally hatched bars*) (250 μg/ml). After preincubation, the cells were washed 4 times with PBS and then incubated for another 20 h with [^{125}I]LDL (10 μg/ml). Values are mean ± SE of 3 experiments run in triplicate. Control experiments with soluble LDL-ICs not adsorbed to RBCs, RBC-adsorbed LDL, RBC-adsorbed IgG (anti-LDL), and medium alone were also performed, and results obtained were not significantly different from those observed in cultures incubated with RBCs suspended in PBS. *P < 0.01 (Wilcoxon's signed-rank test for paired data).

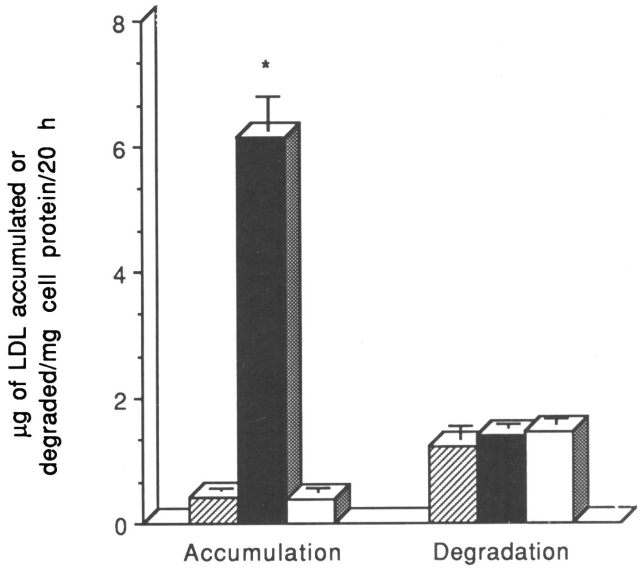

FIG. 2. Receptor-mediated accumulation and degradation of [^{125}I]LDL (10 μg/ml) by HMM preincubated with RBC-adsorbed tetanus–antitetanus-IC (*open bars*), RBC-adsorbed LDL-IC (*solid bars*), or RBC suspended in PBS (*horizontally hatched bars*) (RBC-HMM = 50:1). Values are mean ± SE of 3 experiments run in triplicate. Control experiments using RBC-adsorbed Tet-ICs, RBC-adsorbed tetanus toxoid, and RBC-adsorbed tetanus antibody at RBC-HMM ratios of 5:1, 30:1, 50:1, and 100:1 were also carried out. Results were not significantly different from those observed when HMM were preincubated with RBC suspended in PBS. *P < 0.01 (Wilcoxon's signed-rank test for paired data).

or RBCs suspended in PBS (RBC-HMM ratio = 50:1). [^{125}I]LDL was added to the prestimulated cells, at concentrations from 10–60 nM (5 to 30 μg/ml) of medium. Scatchard plot analysis of the binding data shows that the specific binding of native [^{125}I]LDL by macrophages stimulated with RBC-LDL-IC had a slope of −9.0 with an extrapolated x-intercept of 1267 ng/mg cell protein. Similar analysis of the data obtained from nonstimulated macrophages reveals an approximately similar slope of −12.4 and an extrapolated x-intercept of 351.8 ng/mg cell protein. The data suggest an increased number of LDL receptors in HMMs stimulated with RBC-LDL-ICs.

To determine whether the increased uptake of native LDL by cells stimulated by RBC-LDL-ICs was due to a specific effect of LDL-IC or was secondary to stimulation of the cell by any type of immune complex, we performed experiments using RBC-Tet-ICs. Figure 2 shows that preincubation with RBC-LDL-ICs but not with RBC-Tet-ICs affects the metabolism of native LDL by HMMs.

As previously mentioned, our earlier study (16) showed marked intracellular accumulation of unesterified and esterified cholesterol in HMM incubated with RBC-LDL-ICs. Furthermore, the experiments investigating the metabolism by the same cells of both moieties of the soluble LDL-IC (apoB and anti-apoB IgG) showed a markedly delayed degradation of the apoB moiety (16). Therefore, we decided to determine how much of the increase in UC observed in macrophages after incubation with RBC-LDL-ICs was due to intracellular accumulation of intact LDL. To accomplish that, we performed several experiments in which [^{125}I]LDL-IC accumulation (incubation with RBC-[^{125}I]LDL-IC), free cell cholesterol content, and sterol synthesis were simultaneously measured (Table 1). Similar measurements were performed in cells incubated with corresponding concentrations of native [^{125}I]LDL. As seen in Table 1, the content of UC not associated with intact LDL was reduced by approximately 50% in cells stimulated with RBC-LDL-ICs compared with cells incubated with native LDL. In contrast, endogenous sterol synthesis was increased by approximately 100%.

DISCUSSION

Although there is a general consensus that IC circulate in the sera of diabetic patients (10–12,20–23), little is known about their specific nature. Studies performed in this laboratory previously showed that insulin-antiinsulin–soluble IC form in a large proportion of patients treated with heterologous insulins (24,25). The lack of agreement between nonspecific tests for IC and the specific assay for insulin-antiinsulin IC (26) suggests that other antigen-antibody systems may also be involved.

Recently, data supporting the possibility that anti-LDL antibodies may be formed in vivo has been accumulating. Witztum et al. demonstrated autoantibodies to glycated LDL in diabetic patients (14). Circulating antibodies to oxidized LDL were also described in the sera of normal individuals, diabetic subjects, and patients with coronary heart disease by us (unpublished observations)

TABLE 1
Sterol synthesis and free cholesterol in HMMs incubated with LDL and RBC-LDL-ICs*

	LDL-UC	T-UC	FC	Sterol synthesis
LDL	0.06 ± 0.004†	39.96 ± 4.4†	39.90†	94.8 ± 11.8‡
RBC-LDL-IC	20.89 ± 0.05	40.02 ± 0.9	19.13	192.8 ± 22.9

*HMM were incubated for 20 h with RBC–^{125}I-labeled–LDL-ICs (RBC-HMM ratio 50:1) and medium containing matched amounts of native [^{125}I]LDL. After incubation, intracellular [^{125}I]LDL accumulation was determined, and T-UC was measured by gas chromatography. Amount of LDL-UC accumulated intracellularly was calculated from known LDL composition (apoB mass–unesterified cholesterol mass ratio = 2:1). FC was obtained by subtracting from T-UC amount of FC. Sterol synthesis was measured as [2-^{14}C]acetate incorporated into digitonin-precipitable sterols as described in detail in METHODS. Data are mean ± SE of triplicates of 1 representative experiment from total of 3 experiments.
†μg of cholesterol/mg of cell protein/20 h.
‡pmol of sterols/mg of cell protein/20 h.

and others (13). Glycation of proteins is one of the important biochemical abnormalities in diabetes, and we demonstrated that the extent of glycation of LDL from diabetic patients closely reflects their glycemic control (18).

LDL-ICs, prepared in vitro, lead to intracellular CE accumulation in HMM and to their transformation into foam cells, as we have demonstrated previously (15,16). Our earlier studies (15,27) also indicated that macrophages stimulated with insoluble LDL-ICs paradoxically showed an increase in LDL-receptor–mediated uptake of native LDL.

Our study shows that, like insoluble LDL-ICs, high concentrations of soluble LDL-ICs adsorbed to RBCs also induce increased LDL-receptor activity in human macrophages. This effect seems to be specific for LDL-ICs, because incubations with non-lipoprotein-containing ICs, such as tetanus toxoid–antitetanus toxoid, had no effect on LDL-receptor activity. Scatchard plot analysis of the binding data suggests that the increase in receptor-mediated uptake of native LDL is due to an increased number of LDL receptors in the stimulated cells.

Because LDL-receptor activity is negatively regulated by the cellular free cholesterol content, and our previous studies showed not only that the cells incubated with RBC-LDL-ICs had a high content of UC but also that they poorly degraded the native LDL ingested as part of RBC-LDL-ICs, we decided to determine whether the majority of the UC in the stimulated cells was not free but associated with intact (nondegraded) LDL.

Series of experiments measuring simultaneously the T-UC content in RBC-LDL-IC–stimulated macrophages and the UC associated with the intact (nondegraded) LDL accumulated inside the same cells showed that most of the UC in these cells was indeed associated with intact LDL. Because the UC contained in intact LDL is unlikely to be regulatory, the above experiments suggest that the regulatory pool of free cholesterol in RBC-LDL-IC–stimulated cells is markedly decreased. This decrease may explain the increased LDL-receptor activity observed in the stimulated cells. Also in support of a decrease in the regulatory free cholesterol pool during stimulation of the cells with RBC-LDL-ICs is the increased sterol synthesis observed in the same cells in parallel experiments. Our data do not, however, permit the definitive conclusion that the regulatory free cholesterol pool is decreased in RBC-LDL-IC–stimulated cells. That dissociation between the degradation of the lipid and protein moieties of LDL did not occur needs to be proved before a final conclusion can be made. Also, the distribution of UC between the membrane pool and the intracellular regulatory pool needs to be determined. In conclusion, although our data suggest that the increase in LDL-receptor activity observed in RBC-LDL-IC–stimulated cells is due to a decrease in the intracellular regulatory pool, the precise mechanism still remains to be determined.

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