Uptake of Remnant Like Particles (RLP) in Diabetic Patients from Mouse Peritoneal Macrophages

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To investigate whether the remnant like particles (RLP), separated from serum by an immunoaffinity gel mixture of anti-apo B-100 and apo A-I monoclonal antibodies, are relevant to the initiation or progression of atherosclerosis, the incorporation of RLP into mouse macrophages was studied using histochemical and biochemical techniques. Remnant lipoproteins such as RLP are reported to contain a large quantity of chylomycin and very low density lipoprotein (VLDL) remnants, especially in diabetic patients. The RLP separated from the sera of 32 diabetic patients were found to be predominantly taken up into macrophages harvested from mouse abdominal cavities by the staining method applying oil red O. Furthermore, using 14C-oleate to prove the uptake of lipoproteins by macrophages, the uptake of RLP-VLDL, a VLDL fraction of RLP by ultracentrifugation, was the next highest to that of the oxidized LDL, which suggests that RLP-VLDL is also aggressively taken up by macrophages. The degree of uptake of RLP-VLDL by macrophages was positively correlated with HbA1c of these diabetic patients (r = 0.556, p < 0.01), irrespective of the ways of the treatment of diabetes. In conclusion, RLP can contribute to the foaming of macrophages, which in turn may explain the acceleration of atherosclerosis in diabetic patients.


Key words: Diabetes mellitus, Macrophage, Foam cell, Remnant-like particles (RLP)

Fatty streaks are regarded as the first gross lesion of atherosclerosis. In these fatty streaks, there exist a number of foam cells derived from macrophages (1, 2). The principle component in foam cells is believed to be cholesteryl esters coming from circulating lipoproteins. Goldstein et al. (3) have reported that the native low density lipoprotein (LDL) itself is not taken up by macrophages, but that acetylated LDL is absorbed by macrophages. However, because of the lesser possibility of acetylation occurring in vivo, the question of the origin of the chemically modified LDL arises. Steinberg et al. (4) reported that oxidized LDL cultured with arterial endothelial cells is accumulated by macrophages. On the other hand, chylomicron remnants and VLDL remnants may also be accumulated in the macrophages (5). It has been difficult to separate remnants satisfactorily because of the difficulty of their handling. However, based on a method developed by Japan Immunoresearch Laboratories, the remnant-like particles (RLP) containing a large quantity of chylomicron and VLDL remnants can be easily separated for further analyses (6, 7). In fact, these RLP have often been found abundant in diabetic patients by this technique (8). Thus, RLP could be one of the factors in the acceleration of atherosclerosis in diabetic patients (9), since it is generally known that the circulating remnants often increase in diabetic patients.

In this study, the mouse peritoneal macrophages were cultured with the RLP separated from diabetic patients, to study the possible role of RLP in the foaming of macro-
Subjects and Methods

Subjects
The serum samples for this study were taken from 32 diabetic patients attending our out-patient clinic. Table 1 shows their clinical features (19 males and 13 females). These subjects were selected from the patients whose cholesterol level in RLP (RLP-C) was higher than 5 mg/dl for this study. By the present method, subnormal RLP were detected in normal control subjects and in some diabetic patients who were well controlled. The range of RLP-C in normal control subjects was considered to be less than 5 mg/dl (10). The patients who have diseases other than diabetes were excluded. Control serum used for the uptake study was always taken from a certain healthy volunteer whose clinical and laboratory data were confirmed to be within normal limits.

Separation of RLP-VLDL
RLP were routinely prepared by the method already reported (7). Briefly, 1 ml of an immunoaffinity gel mixture consisting of anti-human apo A-I and anti-human apo B-100 monoclonal antibodies coupled to CNBr-activated sepharose was put into a test tube, and mixed with 250 μl of serum for 1 hour at room temperature on the shaking bath, and then the unbound fraction was recovered as RLP, applying 0.1 ml PBS (phosphate buffered saline; 0.01 M with 2.9% NaCl, SG 1.006). The total capacity of immunoaffinity gel to bound Apo A-I and B-100 was usually more than 5 times higher than the concentrations of those in serum (7).

After the separation of RLP from the serum of fasted patients using an immunoaffinity mixed gel as mentioned above, collected RLP were further ultracentrifuged at 114,000 g for 16 hours to separate RLP-VLDL using an RPS 40TRROTOR (Hitachi Co. Ltd., Tokyo).

Separation of VLDL and LDL
The VLDL fraction (total VLDL) was also obtained from the patient’s whole serum by the ultracentrifugation method (d=1.006 of specific gravity separation fluid at 114,000 g for 16 hours), as stated above. At the same time, the fasted serum from a healthy individual (one person throughout the study) was ultracentrifuged using the same method stated above for further 20 hours and the LDL fraction was isolated (N-LDL) (d=1.019–1.063). Cupric sulfate acid was added to prepare oxidized LDL (O-LDL), according to the method by Steinbrecher et al. (11). In brief, the obtained LDL was dialyzed in 0.15 M NaCl for 6 hours, 3 times, and 150 μg of treated LDL was incubated at 37°C for 24 hours in a CO2 incubator. The samples were then prepared by filtration by a 0.45 μM filter (Millipore Co. Ltd., USA).

Preparation of mouse macrophages
Female BALB/c mice (24-30 g) were used for the preparation of abdominal macrophages. After the injection of 8 ml of tissue culture medium (RPMI-1640 Sigma, USA) into the abdominal cavity, the peritoneal cells were harvested. These cells were then distributed on 24-well culture plates (Cell Wells 25820, Corning, USA), in the concentration of 2 × 10⁶ cells/plate, and incubated at 37°C, in the atmosphere of 5% CO₂ for two hours. Macrophages adhered onto the plates were used for further study.

Histochemical examination of the uptake of lipoproteins by macrophages
After 24 hours of co-culture of macrophages with RLP and N-LDL (final concentration of cholesterol was 10 mg/dl), cells were fixed with 10% formalin, and then stained with Oil Red O in 60% isopropylalcohol (final concentration of Oil Red O was 0.18%). Nuclei were also stained by Meyer’s hematoxylin solution.

Incorporation of ¹⁴C-oleic acid into macrophages
The incorporation of [¹⁴C]-oleic acid (25.9 GBq/mm, Daiichi Kagaku, Co Ltd., Japan) into macrophages was measured according to the method described by Gianturo et al. (12). Each of RLP-VLDL, total VLDL, N-LDL, O-LDL, or PBS samples, along with ¹⁴C-oleic acid was added to the plates where macrophages had adhered.
The concentrations of cholesterol in these samples in media were matched with each other in the experiment. Namely, adhered cells were mixed in 1 ml of RPMI-1640 containing 5% LPDS (Lipoprotein deficient serum) with ^14_C-oleic acid (37 kBq) and various lipoproteins, the cholesterol content of which was 5  \mu g/dl. Then, they were incubated at 37°C, in an atmosphere of 5% CO\textsubscript{2} for 24 hours. After the incubation, the culture medium was discarded, followed by washing of the plates with PBS, and the fat inside the cells was extracted with 2 ml of hexane : isopropanol (3 : 2). The extracted samples were spread onto a thin layer plate (Kieselgel 60, Merck, USA), and the estersection was stained with iodine gas, cut off and analyzed with a liquid scintillation counter. Each sample was assayed in 4 wells and the average of 4 readings was used.

The data were expressed as mean values SD. The statistical analysis was conducted using the Student’s paired t test. A probability of p<0.05 was used as the level of statistical significance.

Results

Histochemical staining of macrophages

Figure 1 shows the pictures of macrophages after the incubation with RLP or N-LDL followed by the oil red O staining. Oil droplets were clearly observed as red spots in macrophages after incubation with RLP, but there were no oil droplets in the macrophages in the case of N-LDL.

RLP-VLDL uptake by macrophages

The readings of radioactivity incorporated into ester in macrophages after the incubation with PBS, N-LDL, N-VLDL, O-LDL or RLP concomitant with ^14_C-oleate were 8169 731 dpm, 8498 1142 dpm, 7228 1935 dpm, 10492 2356 dpm and 9468 1964 dpm, respectively. Although there were no differences among readings of PBS, N-LDL and N-VLDL, that of O-LDL or RLP-VLDL was significantly higher than that of PBS, N-LDL and N-VLDL (p<0.05, respectively, Fig. 2). Therefore, applying the level of ^14_C-oleate incorporation in the case of N-LDL as a basal standard, the level of the uptake into macrophages in the case of RLP-VLDL were expressed by percentage against that in N-LDL for each patient. In 28 of 32 diabetic cases, they were over 100%. A significant relationship was seen between the levels of RLP-VLDL uptake and the glycemic control of diabetes i.e., HbA\textsubscript{1c} (r=0.556, p<0.01), as shown in Fig. 3. However, we could not find any relationships between the RLP uptake and TC, TG, each of apoproteins, RLP-C or RLP-TG.

Fig. 1. The macrophages stained by oil red O after incubation with the remnant like particles (RLP) and the normal low density lipoproteins (N-LDL). For the RLP-added macrophages culture, there were stained oil drops seen, but there were no oil drops seen in the macrophages with added N-LDL.
The uptake of lipoproteins and subsequent foaming of macrophages can be seen as the first step towards atherosclerosis. Chemically modified acetylated LDL and oxidized LDL has been shown to be absorbed by macrophages, while native LDL existing in the blood are not (13). However, there is no evidence that either acetylated LDL or oxidized LDL exists in circulating blood. On the other hand, remnant lipoproteins exist in the blood and are assumed to be one of the factors in the progression of atherosclerosis. The RLP used in this study is also found in the human blood in vivo and RLP-C has been reported to be increased in patients with diabetes (13) and ischemic heart disease (14) as well. It may contribute to the acceleration of atherosclerosis in diabetic patients. The RLP are rich in triglycerides and apo E and contains many remnants derived from chylomicrons and VLDL (6, 7). An electron microscopic study has revealed irregularities of the RLP and they were over 40 nm in diameter, just the same size as VLDL and chylomicron (10). The RLP-C level in non-diabetic and normolipidemic persons (total serum cholesterol below 220 mg/dl, and triglycerides below 150 mg/dl) was 2.1±1.6 mg/dl (mean±SD), and using a non-parametric method, over 5.0 mg/dl is considered as abnormal (10). When diabetic and non-diabetic patients were compared, a significant number of diabetic patients had abnormal RLP readings, i.e., 42% as opposed to 12% (8). Therefore, even in patients with equivalent serum lipid levels, the diabetic patients will still have a higher rate of abnormal RLP compared with the non-diabetic patients (8).

In this study, we demonstrated that macrophages harvested from mice can predominantly absorb RLP by the oil red O staining. Additionally, since the incorporation of \(^{14}C\)-oleate into esters in macrophages incubated with N-LDL did not differ from the control level using PBS, the associated uptake of \(^{14}C\)-oleate with total VLDL, RLP-VLDL and the O-LDL from diabetic patients was significant. The uptake of RLP-VLDL was the next highest to that of the O-LDL in average, which suggested that RLP-VLDL can be aggressively taken up by macrophages. Since the concentration of cholesterol in these samples in the medium were in agreement with each other, the present findings are considered to be accurate. Macrophages have many kinds of receptors, including scavenger receptors which could contribute to the foaming of macrophages (15). O-LDL are taken up by the macrophages with the help of the O-LDL receptor (16), but the actual process of RLP uptaken is not clear, and more investigations are needed. Interestingly, when we examined the relationship between the degree of uptake of RLP-VLDL by macrophages and glycemic control, a significant positive trend was seen; that is as the HbA\(_{1c}\) levels increased the RLP-VLDL/N-LDL ratio became high. One factor may be that the more RLP that undergo
glycation, the easier they are to be absorbed by macrophages. Glycation of RLP may lead to its further oxidation (17).

In conclusion, we suggest that RLP modified by glucose can be predominantly taken up by macrophages, and that this may explain the exasperated macroangiopathy i.e. atherosclerosis in diabetic patients.

**Conclusion**

We have found that RLP separated from the sera of diabetic patients is predominantly taken up by macrophages from mouse abdominal cavities by the oil red O staining. RLP-VLDL was also suggested to contribute to the cholesteryl oleate production for the foaming of macrophages, and this could be influenced by the patient's glycemic control. Therefore, RLP in turn may be relevant to the acceleration of atherosclerosis in diabetic patients.

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