# 透析患者におけるマクロファージ・スカベンジャー受容体からみた動脈硬化症進展機序

（研究課題番号06671158）

平成７年度科学研究費補助金（一般研究（Ｃ））研究成果報告書

平成８年７月

研究代表者　佐中　孜
（東京女子医科大学医学部助教授）

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## はしがき

動脈硬化症は，動脈内膜の脂肪沈着を伴う線維性肥厚（アテローム硬化症），筋性動脈中膜における石灰化を特徴とするメンケベルグ型動脈硬化症，細小動脈内膜硝子様肥厚（細小動脈硬化症）などの３種類の異なった機序によって起きると考えられる病変を総称しているが、これらのうち，アテローム硬化症は，その病変にコレステロールエステルを蓄積した泡沫細胞が浸潤しているという特徴をもつ。この泡沫細胞は，マクロファージ由来であるといわれているが，通常のLDLは全く取り込まず，アセチル化や酸化，過酸化などの化学修飾を受けたものになって初めてスカベンジャー・レセプタを介して活発に取り込むようになると考えられている。

一方、透析患者では活性酸素が作られ易い状況にあるだけでなく，過酸化脂質や動脈硬化惹起性リポ蛋白の上昇が認められており、死因の約50％は動脈硬化症疾患であるなど、非腎不全患者と比較して、加齢現象が急速に進行しているという臨床的事実もある。

このような動脈硬化の進展機構には、脂質代謝異常、過酸化脂質産生亢進など多数の因子が関与していると推察されるが、従来から、慢性腎不全患者を対象としたこの分野での臨床研究の多くは、レムナントリポ蛋白、Lp(a)、過酸化脂質などの血中濃度と大動脈壁肥厚狭窄係数や心電図パラメーターなどのような動脈硬化指標との比較検討に留まっている。しかしながら、最近の分子生物学を取り入れた脂質研究の進歩は目ざましいものがあり、1980年代後半にいたり、マクロファージによる酸化LDLなどの修飾LDL取り込みが動脈硬化進展機構の中でも特に重要な位置を占めていることが実証された。更にまた、その脂質取り込みは、主として、マクロファージ上のスカベンジャー・リセプターによりコントロールされていることも解明された。

そこで、我々は、慢性腎不全患者における動脈硬化進展機構について、透析療法による生体反応、マクロファージによる脂質取り込み、マクロファージのレセプター機能の変化という観点から捉え、臨床材料を対象として、分子生物学的手法を用いることにより、全く新しい角度からの解明に取り組んで行きたいと考える。

研究組織

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# 目　次

# 第１章

# 背　景

# BACKBROUND

# 背 景 Background

Atherosclerosis is considered to be accelerated in dialysis patients and closely related to their morbidity and mortality. This could be due to several factors including uremic dyslipidemia which is a prominent finding in dialysis patients. Most studies on this subject have emphasized the presence of causative factors such as the accumulation of remnant lipoproteins, high levels of Lp (a), the alterations of components of lipoproteins and the production of modified low-density lipoprotein (LDL). However, these studies did not approach the specific mechanism of atherosclerosis in uremia. Recent advances in lipidology have provided us with some new clues to study accelerated atherogenesis in uremia at the cellular and molecular level.

The first step towards the progression of atherosclerotic lesion is thought to be the uptake of modified LDL through its scavenger receptor on the surface of macrophage following form cell formation. Many modified forms of LDL, such as acetylated LDL, oxidized LDL(OX-LDL), malondialdehide LDL, glycated LDL, and VLDL are known. Among these, OX-LDL was especially interesting, because it was demonstrated to occur in vivo. It is reported that anti-oxidant drugs can prevent progression of atherosclerosis and OX-LDL deposition at the site of sclerotic lesion of aorta are demonstrated by immunohistochemical staining.

Although the mechanism of oxidative modification of LDL or factors that affect oxidation of LDL are obscure, composition of fatty acid, anti-oxidants (including drugs) and oxidants in plasma are thought to be liable. High cardiovascular mortality is commonly observed in patients undergoing hemodialysis. In such patients certain pattern of lipid abnormalities were reported: hypertriglycerides,

decrease of high density lipoprotein (HDL), and almost normal low-density lipoprotein (LDL). Recently, not only such abnormalities in quantities, but also in qualities, such as abnormal apolipoprotein B or increased OX-LDL have been reported.

The aim of our study is to clarify a mechanism of accentuated arteriosclerosis due to increased plasma oxidized LDL and enhanced expression of scavenger receptor for oxidized LDL in hemodialysis patient.

# 第２章

## LDL吸着療法によって判明した動脈硬化症および高脂血症性腎疾患の発症進展における酸化LDLの重要な役割

## AN IMPORTANT ROLE OF OXIDATIVE LDL IN PROGRESSION OF ARTERIOSCLEROSIS AND HYPERLIPIDEMIC DISEASE EVIDENCED FROM LDL APHERESIS

# AN IMPORTANT ROLE OF OXIDATIVE LDL IN PROGRESSION OF ARTERIOSCLEROSIS AND HYPERLIPIDEMIC DISEASE EVIDENCED FROM LDL APHERESIS


〈Summary〉

Oxidatively modified low density lipoprotein (LDL) exhibits several potentially atherogenic properties, and inhibition of LDL oxidation in rabbits decreases the rate of the development of atherosclerotic lesions. Clinical studies using LDL apheresis have suggested that removal of oxidized LDL may lead to improvement of such clinical symptoms of due to arteriosclerosis obliterans as intermittent claudication and sensory disturbance.

We now reported that oxidized LDL are also found in patients with steroid-resistant nephrotic syndrome as well as in more advanced atherosclerotic disease. Of them four patients aged 32 to 66 years old was introduced to LDL apheresis. Two of them was succeeded in reduction of oxidized LDL following remission of severe proteinuria. However, the others, who did not show significant reduction of oxidative LDL, did not demonstrate any improvement in proteinuria. The more oxidatively modified, the more negatively charged and hard to remove with commercially

available LDL absorbent.

We suggest that it is important to reduce not only LDL but also oxidized LDL in cases of application for LDL aphersis therapy.

〈Introduction〉

It is well known that hyperlipidemia often accompanies renal diseases(1). Lipid-laden cells, similar to the foam cells in atherosclerotic lesions, have been observed in glomeruli in experimental nephrotic animals (2,3). Materials related to oxidized fatty acids were also detected in the glomerulosclerotic lesions (3), although the mechanism for foam cell formation in glomeruli has not been clarified.

Moreover, results of studies of animals fed lipogenic diets (4-19), of animal models of endogenous hyperlipidemia(10-12) and of hyperlipidemic animals treated with lipid-lowering drugs (13-16), all provide evidence for the adverse effects of hyperlipidemia on the progression of renal injury.

In particular, oxidatively modified low density lipoprotein (LDL) has been shown to be involved in the initiation and promotion of atherosclerosis (17-19). Formation of lipid-laden foam cells from macrophages has been demonstrated by incubating oxidized LDL

(OX-LDL) with macrophages in vitro (20, 21), while incubation with native LDL did not result in accumulation of lipid droplets. OX-LDL also exhibited a number of cell biological activities including enhancement of interaction of leukocytes and endothelial cells (22, 23), inhibition of endothelial cell migration (24), and induction of endothelin secretion from endothelial cells and macrophages (25, 26).

〈Evidence for Generation of Oxidized LDL〉

It has been known that in several animal species, extensively oxidized LDL is rapidly cleared from the circulation by sinusoidal endothelial cells in the liver, spleen, and bone marrow(27-29). Nevertheless, a number of investigators using a variety of techniques have obtained evidence that at least a modest degree of oxidative modification of LDL occurs in plasma. Schuh and coworkers demonstrated fragmentation of apoß in plasma LDL that could not be explained as oxidative artifact during isolation, and was taken as evidence for the presence of a modest degree of oxidation of plasma LDL in vivo(30). Other studies have shown that relatively low levels of lipid peroxide or thiobarbituric acid-reactive substances can be detected in association with plasma

LDL(31). Avogaro and coworkers reported the isolation of a modified LDL fraction in the plasma of Lp(a) negative normolipidemic healthy male subjects(32). This fraction was more electronegative than the bulk of plasma LDL, and could be isolated by ion exchange chromatography. It seems that the modified LDL is more heterogeneous in size, has a lower phospholipid content, a higher content of conjugated diene, and is internalized more rapidly by macrophages than the bulk of plasma LDL. Furthermore, Steinbrecher mentioned that if such electronegative LDL indeed exists in plasma(33), it must be a very minor fraction.

However, despite of several negative opinions as above, Itabe and I found an evidence for generation of oxidized LDL(34). The LDL oxidation level in patients with hyperlipidemic nephrotic syndrome and patients who had been receiving hemodialysis treatment was increased more than eightfold over that of normal subjects(Table 1). Therefore, we suggested that LDL in human plasma is oxidatively modified under certain conditions and this method for measurement of OX-LDL could be used to study the relationship between in vivo oxidation reaction and various pathological conditions.

The present new method capable of measuring the very low concentrations of oxidized low density lipoprotein (OX-LDL) has been established by Itabe. In his previous study, we obtained a

novel murine monoclonal antibody against oxidized lipoproteins (35). According to his reports, the epitope of this antibody resides in oxidized products of phosphatidylcholine that can form complexes with polypeptides, including apolipoprotein B. When the monoclonal antibody was precoated onto microtiter wells prior to carrying out a sandwich ELISA using an anti-human apolipoprotein B antibody, it was possible to detect 0.5 ng protein of copper-induced OX-LDL. The detection of OX-LDL was dependent on the presence of monoclonal antibody and was blocked by oxidized phosphatidylcholine (OxPC). Under the same sandwich ELISA condition, native LDL showed a dose-dependent increase of absorbance that was inhibited by complex of OxPC with BSA. These results suggested the possible occurrence of oxidative modification of human plasma LDL which is recognized by the antibody against OxPC. The level of LDL oxidation of normal human subjects was found to be $0.52 \pm 0.35$ units per 5 $\mu$g protein of LDL, where one unit was defined as the reactivity corresponding to 1 ng of copper-induced OX-LDL by this assay.

1) Plasma Concentrations of Oxidized LDL in Hemodialysis Patients

The subject is 123 hemodialysis patients ($53.2 \pm 12.9$ years old, 65 Male and 58 Female).

Clinically obvious amyloidosis, orthopedic disease, diabetic

neuropathy was excluded from the subject. Severity of arteriosclerosis obliterans of the subject was assessed in accordance with Fountain s classification criteria.

Of all, a symptom of arteriosclerosis obliterans(ASO) was recognized in 41 (33.3%) patients. Of them, 11 (8.9%) patients demonstrated such symptom as numbness of the lower leg which belongs to the first degree of Fountain s classification. 12 (9.8%) patients showed the symptom which belongs to the second degree of Fountain s classification including intermittent claudication. 18 (14.6%) patients which was complained such the third degree of a symptom of Fountain s classification as leg-pain at the rest position. Their duration of hemodialysis were 13.4 ± 8.8 years.

Plasma oxidized LDL levels of hemodialysis patients were 59.5 ± 33.8 $\mu$ g/mg LDL, and significantly higher than that of healthy control (28.1±8.4 $\mu$g/mgLDL). In the hemodialysis patients who were associated with ASO plasma oxidized LDL levels were 60.8±33.8 $\mu$g/mgLDL, whereas in the dialysis patients who did not complicate arteriosclerosis obliterans was 44.8±19.6 $\mu$g/mgLDL ($p<0.05$).

Incidence of other complications related to the arteriosclerosis was obviously higher in a group of patients with ASO compared to without ASO.

Statistical analyses;

All data are presented as the mean ± SD. Statistical analyses were performed using the Student's t test. Statistical significance was accepted if a P value was less than 0.05.

2) Effect of LDL Aphresis Therapy to ASO

Of all subjects, 3 patients (57.3±2.3 years old, 1 male and 2 females), who were complicated with intermittent claudication caused by arteriosclerosis obliterans, were selected to clarify a therapeutic effect of LDL apheresis.

LDL apheresis therapy using a dextran sulfate column was carried out to these patients. Plasma of 3000 ~ 4000ml was processed by LDL apheresis.

It was examined about a correlation between a symptom due to the arteriosclerosis obliterans and a plasma level of oxidized LDL before and after the LDL apheresis.

A plasma oxidized LDL level of the subjected patient was 34.7 ± 21.6 $\mu$ g/mgLDL. It was significantly high compared to healthy control. A therapeutic effect of LDL apheresis therapy on

a symptom of ASO was expressed as an improvement rate of gate disturbance. An improvement ratio of gate disturbance calculated by the equation formula as below.

Improvement rate of gate disturbance = walk-able distance without leg pain (after LDL apheresis) / walk-able distance without leg-pain (before LDL aphasia)

Effect of LDL apheresis on improvement rate of gait disturbance were variable. Each case showed different response. A improvement rate of a gait disorder was 1.0～3.0. An adsorption rate of a LDL was maintained within almost same range to be 84～93% at the each LDL apheresis(Fig. 1).

However, an adsorption rate of oxidized LDL was variable, a wide variation was observed by 4.4～94.4% (67.1±29.8%) depending on cases (Fig. 1).

There was not any correlation between an adsorption rate LDL and an improvement rate of a gait disorder. A significant correlation was observed between plasma concentration of oxidized LDL and its adsorption rate.

LDL apheresis reduced plasma LDL by approximately 90 %. However, it was noticed that a plasma LDL level and a symptom do not correlate. On the contrary, reduction of oxidized LDL by

LDL apheresis brought about an improvement of clinical symptom in the patients with ASO.

3) Participation of Oxidized LDL in the Progression of Nephrotic Syndrome with Hyperlipidemia

(1) Influence of Oxidized LDL to Cytokine Net-Work in Renal Disease

Oxidized LDL leads to marked increase of secretion of LTC and PGE2 from macrophage and mesangial cells(36). The effects of oxidized LDL on prostaglandin synthesis were found to be specific since neither native LDL nor acetylated LDL altered prostaglandin synthesis(36, 37). It has been reported that oxidized LDL is more active than native LDL in promoting platelet aggregation(38). Oxidized LDL caused alterations in platelet membrane fluidity and stimulated the production of thromboxane B2 in the glomerular capillaries. The effect on platelet aggregation was not inhibited by indomethacin, and hence it was concluded that most of the effect on platelet aggregation was attributable to changes in membrane fluidity, rather than increased prostanoid production.

Recent studies by Rajavashlsth and coworkers showed that LDL that had been oxidized (probably to an extent much less than that

required to produce altered uptake in macrophages) stimulated the production of several growth factors by cultured endothelial cells, including GM-CSF, M-CSF, and G-CSF(39). It is likely that oxidized LDL stimulated production of specific monocyte chemotactic protein (MCP-1) by cultured human endothelial cells and mesangial cells.

Oxidized LDL can modulate the production of growth factors, prostaglandin, thromboxan, and inflammatory chemotactic factors by cultured mesangial and endothelial cells as in shown.

(2) Clinical Effects of Native LDL and Oxidized LDL-Aphresis on Steroid-Resistant Nephrotic Syndrome with Hyperlipidemia

Representative Case:

The patient was a 38 year old female, who was first admitted in 1939 with massive proteinuria and severe edema. The first renal biopsy showed minor glomerular abnormality. Steroid therapy with methylpredonin-pulse was started without successful effects. As a result, urinary protein excretion was reduced, but 2 years later, relapse of nephrotic syndrome occurred in spite of administration of high doses of steroid combined with anticoagulants and immunosuppressive agent. The second biopsy was planned with tentative diagnosis of focal glomerular sclerosis (FGS), but not performed because of severe malnutrition state. A large amount of prodding administration was continued with poor effectiveness.

Finally, we decided to perform LDL-apheresis. The patients showed an incomplete but notable reduction of urinary protein excretion by the Aphresis therapy to native LDL and oxidative LDL(Fig. 2).

LDL-apheresis using the specific sorbing of apoprotein B-containing lipoprotein, Liposorber LA-40, was perfumed 2 times for 3 months. 1 time LDL-apheresis consists of 3 sessions. The volume of plasma exchanged during each session was set at about 5L.

(3) Relationship between Adsorption Rate of LDL and Reduction Rate of Urinary Protein Excretion

Fig. 3, 4 and 5 demonstrate reduction rate of urinary protein excretion, adsorption rate of LDL, and adsorption rate of oxidized LDL, respectively. In Case 1 an 4, urinary protein excretion did not reveal any kinds of improvement at all, following less adsorption of oxidized LDL than that of case 2 and 3, although adsorption rate of LDL was 68% and 72%, respectively. Case 2 and 3 was succeeded in reduction of oxidized LDL (61% and 51%, respectively), following remission of severe proteinuria(65% and 96% of reduction rate, respectively). The more oxidatively modified, the more negatively charged and hard to remove with commercially available LDL absorbent.

〈Conclusion〉

We suggested that it is important to aim reduction of not only LDL but also oxidized LDL in case of application for LDL aphersis therapy.

It became obvious that oxidized LDL get deeply involved in the progress of arteriosclerosis in hemodialysis patients and progression of hyperlipidemic nephrotic syndrome.

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Table 1; Plasma LDL oxidation levels in normal, hyperlipidemic, and hemodialytic subjects

| | Normal | Hemodialysis | Nephrotic Syndrome |
|---|---|---|---|
| | (n=9) | (n=16) | (n=4) |
| Age (yr.) | 29.3±4.9 | 57.7±12.2 | 40.3±17.8 |
| LDL oxidation level, (units/5㎍ LDL protein) | 0.52±0.35 | 4.19±2.59 | 5.6±0.93 |
| Total plasma cholesterol, (mg/dl) | 165±34 | 165±61 | 356±9.5 |

LDL oxidation levels and total cholesterol of normal and hemodialytic patients plasma were determined as described in above. Briefly, after the LDL fraction was separated from each plasma, LDL(5 ㎍/well) was added to microtiter wells precoated with the monoclonal antibody or murine IgM. One unit of LDL oxidation level represents the reactivity corresponding to 1ng of standard OX-LDL. Values are mean ± standard deviations.

Figure 1; Effect of LDL Apheresis on Improvement Rate of Gait Disturbance

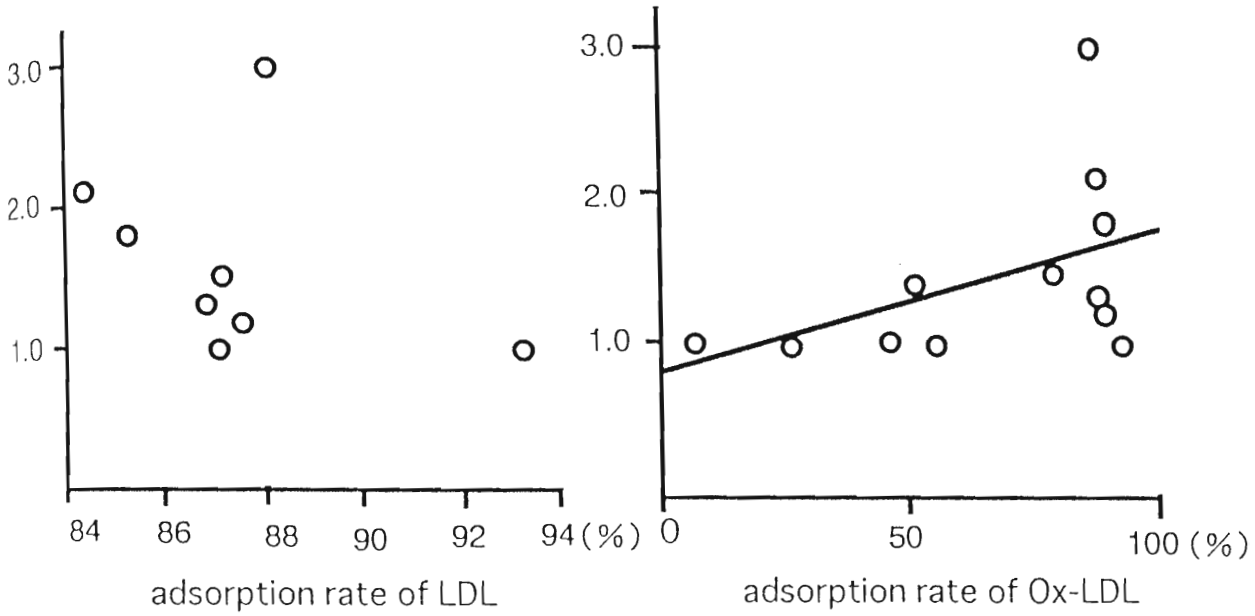


Figure 2; Clinical Effects of Native LDL and Oxidized LDL-Aphresis on Steroid-Resistant Nephrotic Syndrome with Hyperlipidemia

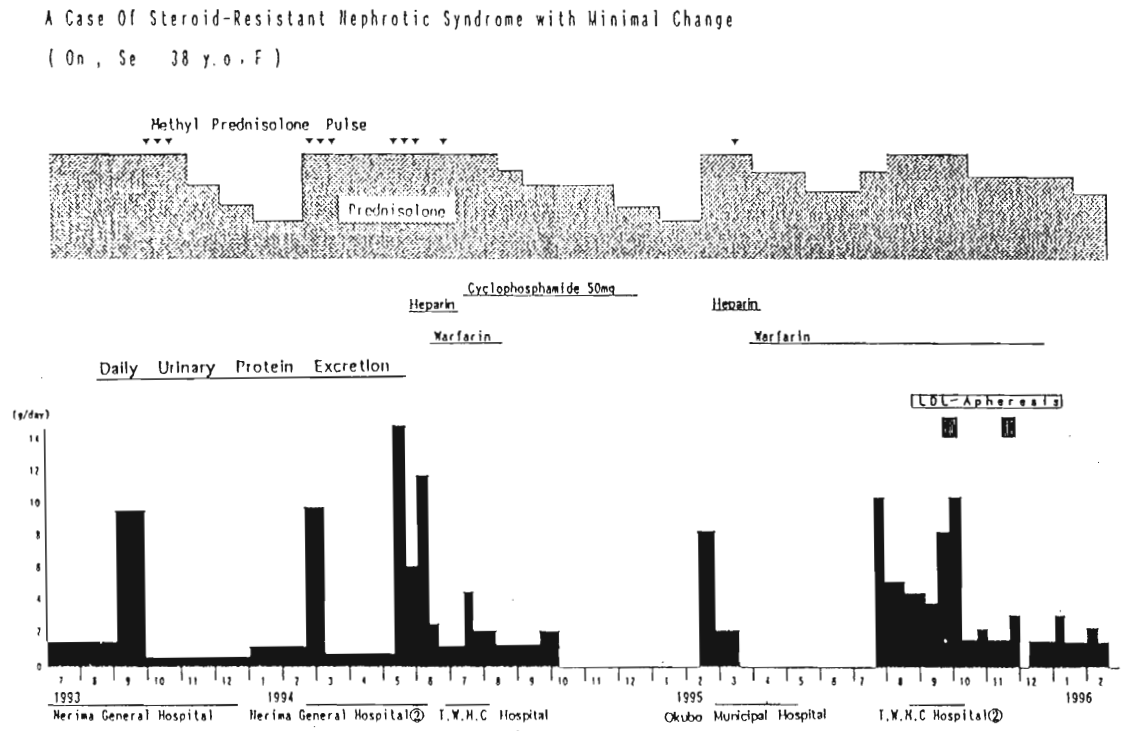


Figure 3; Urinary Protein Excretion Rate

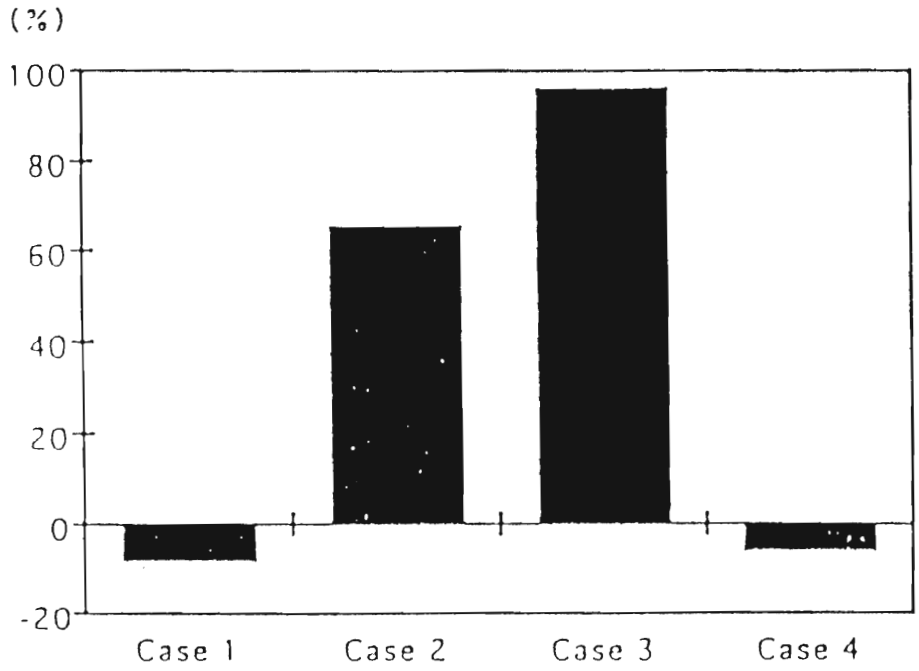


Figure 4; Adsorption Rate of LDL

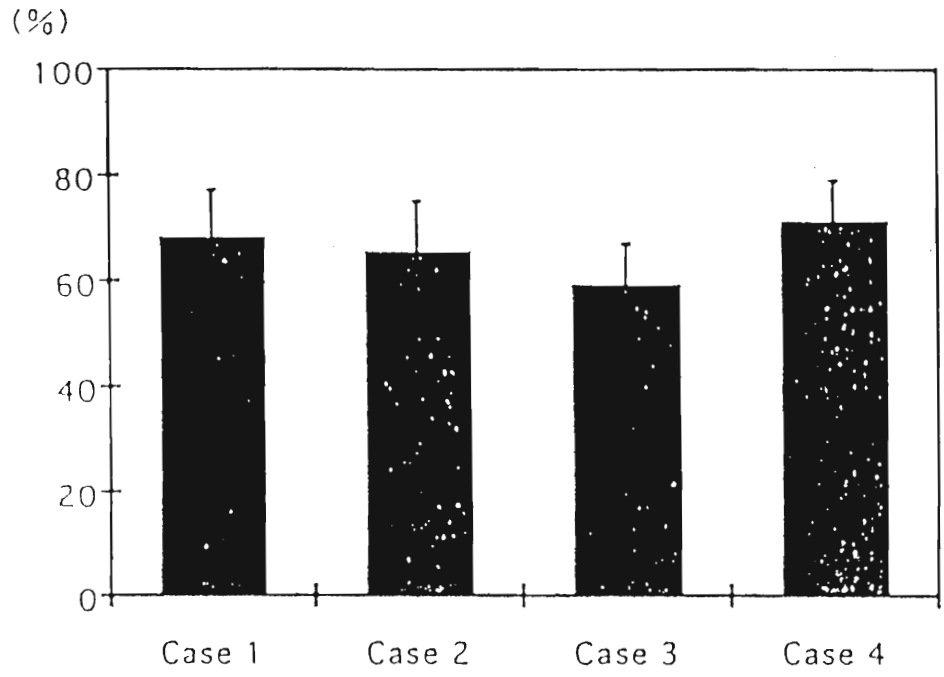
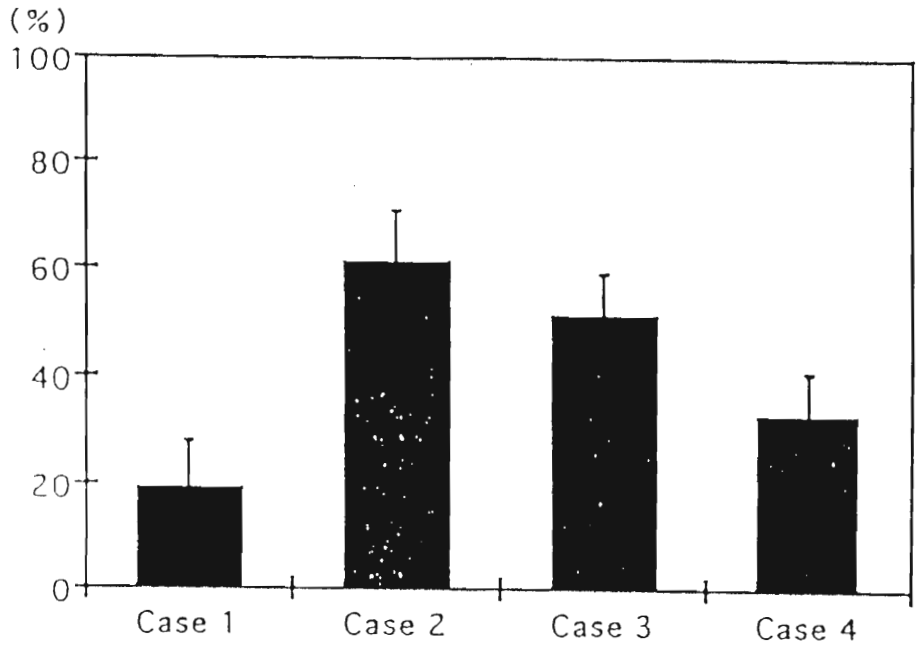


Figure 5; Adsorption Rate of Oxidized LDL



# 第３章

# ビタミンＥ被覆ダイアライザによる酸化LDL除去と臨床効果

# REDUCTION OF OXIDIZED LDL AND ITS CLINICAL EFFECTS USING THE DIALYZER EMPLOYING WITH VITAMIN E-BOUND MEMBRANE

# REDUCTION OF OXIDIZED LDL AND ITS CLINICAL EFFECTS USING THE DIALYZER EMPLOYING WITH VITAMIN E-BOUND MEMBRANE


<Summary>

As the chronic hemodialysis population has been increasing, the incidence of dialysis complications in a chronic hemodialysis patient increases as the period for which the patient has been maintained on HD increases. It has been suggested that the increasing incidence of dialysis complications may partly be assigned to the dialysis membranes used, whose biocompatibility has been evaluated from various angles.

In the present study, we evaluated the biocompatibility of the CL-ES 15 dialyzer, which has vitamin E bonded on the modified surface of a cellulose membrane, focusing on neutrophils and lipids peroxide. The use of the CL-ES15 resulted in the increased production of adhesion molecules (CD11b) in neutrophils, though there was no significant difference statistically in the production between the CL-ES 15, the CL-SS, and the PMMA membrane dialyzer. The increased H202 production by neutrophils also resulted from the use of the CL-ES15, with no significant difference observed between the three models of dialyzers. MPO concentration in blood at the start of the last of the three HD sessions was lower for the CL-ES15 than for the other two dialyzers, and the increase in MPO

concentration during the session was smaller for the CL-ES15 than for the others.

The use of the CL-ES15 obviously reduced the rate of increase in MPO and OX-LDL levels in blood, while the CL-ES15 did not influence the expression of CD11b and production of $H_2O_2$ in neutrophils compared to the other dialyzers.

<Introduction>

The biocompatibility of hemodialysis (HD) therapy has been a subject of study for many years, and HD membranes with improved biocompatibility have been developed year after year, however there is still much room for further improvement. Our earlier studies revealed that HD membranes stimulate leukocytes to produce increased amounts of adhesion molecules, active oxygen, myeloperoxidase (MPO), etc., with the result that the plasma concentration of lipids peroxide in the patients increases(1). In the present study, we evaluated the biocompatibility of a dialyzer (the CL-ES15) employing a surface-modified cellulose hollow-fiber membrane with vitamin E bonded to its inner lumen, focusing on the effect on leukocytes and the subsequent increase in the plasma concentration of lipids peroxide in the patients.

〈Subjects and Methods〉

Eight HD patients with chronic renal failure (four males and four females, 52.6 ± 10.6 years of age and maintained on HD for 11.9 ± 6.11 years) received three HD sessions in succession using the CL-ES 15 dialyzer within a week. During each session, sample blood was collected at intervals at the inlet and outlet of the dialyzer.

Blood samples were tested for the adhesion molecules (CD11b) and $H_2O_2$ produced in neutrophils. Sample blood was also collected at intervals during the last of the three HD sessions for MPO and oxidized LDL (Ox-LDL). The determination of CD11b and $H_2O_2$ production was performed by flow cytometry using the anti-human CD11b monoclonal antibody and 2', 7'-dichlorofluorescin diacetate (DCFH-DA), and the percentage of each outlet-side value thus obtained was calculated with the corresponding inlet-side value as 100 percent. MPO concentration was determined using an RIA kit from Pharmacia, and OX-LDL was by ELISA using a monoclonal antibody (DLH3). The values determined during the HD sessions using the CL-ES 15 dialyzer were compared to values determined with sessions using a conventional high-flux cellulose membrane (the CL-SS

dialyzer) or a polymethylmethacrylate (PMMA) membrane.

〈Statistical analyses〉

All data are presented as the mean ± SD. Statistical analyses were performed using the Student's t test. Statistical significance was accepted if P value was less than 0.05.

〈Results〉

1) CD11b expression in neutrophils

Regardless of the type of HD membrane used, there was a tendency for CD11b production to be greater at the dialyzer outlet than at the inlet during HD. In the sessions using the CL-ES15 dialyzer, this tendency was marked as compared to the case with the CL-SS dialyzer or the PMMA membrane dialyzer, though no difference of statistical significance was noticed between the three types of membrane(Fig. 1).

2) $H_2O_2$ production in neutrophils

During HD using the CL-ES15 or the PMMA membrane dialyzer, there was a tendency for $H_2O_2$ production to be greater at the dialyzer outlet, though no such tendency was noticed with the CL-SS. At any sampling time point, the differences between the three dialyzers were not significant (Fig. 2).

3) MPO concentration in blood

The sample blood collected in the last of the three HD sessions performed using the same model of dialyzer, was tested for MPO and OX-LDL. At the start of the last session using the CL-SS dialyzer, MPO concentration in blood was high with 1 ,288.5±905.0 μg/l, and increased to 1,896.8± 2,1170 μ g/l after 15 minutes of HD. In the last session using the PMMA membrane、 we observed a tendency for MPO concentration to increase with time, from the initial value of 284.5±155.8 μg/l to 739.0± 244.8 μg/l after 240 minutes. The last session using the CL-ES15 dialyzer provided some increase in MPO concentration from the initial value as low as 201 .5 ± 104.6 μ g/l to a maximum of 298.3±67.8 μg/L after 30 minutes. The increase was smaller than the increase observed with

the other compared membrane dialyzers(Fig. 3).

4) OX-LDL content in blood

With the same eight patients we determined OX-LDL content in blood at the start of the last of the three HD sessions performed using the same model of dialyzers (Fig. 4-a). The dialyzers used were the CL-ES 15 and the conventionally used dialyzers of CTA and PS membranes. While the normal content of OX-LDL in our blood is 1.3±0.3ng per μg of LDL, we determined OX-LDL content in blood at the start of the last session was high with 4.6±1.2ng per μg of LDL for the conventional dialyzers. For the CL-ES 15, OX-LDL content at the start of the last session was 2.9± 2.9ng per μg of LDL, lower than the value determined for the conventional dialyzers.

We also determined variations of OX-LDL content during HD in four of the eight patients (Fig. 4-b). Regardless of the dialyzer used, we observed a tendency for OX-LDL content to increase during HD, though the increase was smaller for the CL-ES 15 than for the compared dialyzers.

〈Discussion〉

As the chronic hemodialysis population has been increasing, the incidence of dialysis complications in a chronic hemodialysis patient increases as the period for which the patient has been maintained on HD increases. It has been suggested that the increasing incidence of dialysis complications may partly be assigned to the dialysis membranes used, whose biocompatibility has been evaluated from various angles. In our previous study, we found that dialysis membranes stimulate neutrophils to enhance an expression of adhesion molecules and to produce more active oxygen. And according to a recent report, amyloidosis which is a major complication of chronic HD patients is caused by AGE- $\beta$ 2-microglobulin(2). It is suggested that oxygen radicals take a part in the formation of AGE-$\beta$2-microglobulin. Therefore, we recognized that active oxygen is an important factor influencing the biocompatibility of dialysis membranes.

On the other hand, it has been reported that vitamin E concentration decreases in the blood(3) and in the membrane of such cells as red cells and monocytes4) of HD patients as suggesting that their antioxidative capability has failed. In the present study, we evaluated the biocompatibility of the CL-ES15 dialyzer, which has vitamin E bonded on the modified surface of a cellulose

membrane, focusing on neutrophils and lipids peroxide.

The use of the CL-ES15 resulted in the increased production of adhesion molecules (CD11b) in neutrophils, though there was no significant difference statistically in the production between the CL-ES 15, the CL-SS, and the PMMA membrane dialyzer. The increased $H_2O_2$ production by neutrophils also resulted from the use of the CL-ES15, with no significant difference observed between the three models of dialyzers. MPO concentration in blood at the start of the last of the three HD sessions was lower for the CL-ES15 than for the other two dialyzers, and the increase in MPO concentration during the session was smaller for the CL-ES15 than for the others. Vitamin E is reported to accelerate $O_2^-$ production in neutrophils but reduces the release of $H_2O_2$ from them(5). Though our measurements of $H_2O_2$ production using DCFH-DA showed no significant difference in the increase in H202 production in neutrophils between the three models of dialyzers, the increase in MPO concentration in blood was much lower for the CL-ES15 than for the other two dialyzers.

Our explanation of this is that vitamin E worked to reduce the active oxygen released from neutrophils, resulting in the reduced rate of increase in MPO concentration in the blood. Lipids are peroxidized through radical reactions, and the lipids peroxide are known to participate largely in the acceleration of

atherosclerosis(6). HD patients suffer from marked acceleration of atherosclerosis and the increasing incidence of atherosclerotic complications such as cardiac infarction and cerebrovascular diseases in these patients has become a problem(7). For OX-LDL content, HD patients showed higher values as compared with healthy individuals, which agrees with the data obtained by Maggi et al(8). Though OX-LDL content increased with time during HD, the value at the start of the last session was lower and the increase during the session was smaller for the CL-ES15 than for the conventional dialyzers. This may be ascribed to the effect of vitamin E; that is, vitamin E seems to have quenched the lipids radicals formed through the contact of blood with the dialysis membranes.

In the present study, differences were observed between the CL-ES 15 and the conventional dialyzers. However, the differences were not significant because only three HD sessions were performed. The differences would be greater in longer-term HD therapy showing the advantage of the CL-ES15 dialyzer over the conventional dialyzers in reducing the rate of increase in the production of lipids peroxide.

## 〈Conclusion〉

The biocompatibility of the CL-ES15 dialyzer having a vitamin E-modified cellulose membrane was evaluated in comparison with conventional dialyzers. The use of the CL-ES15 obviously reduced the rate of increase in MPO and OX-LDL Levels in blood, while the CL-ES 15 did not influence the expression of CD11b and production of $H_2O_2$ in neutrophils compared to the other dialyzers.

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Figure 1; CD11b Expression in Neutrophils

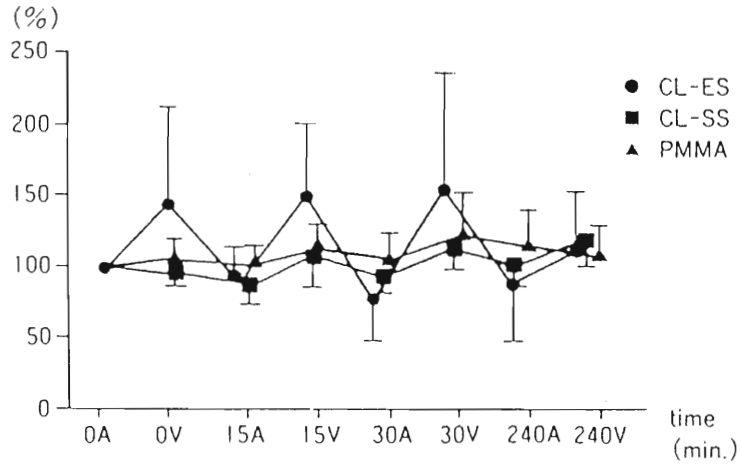


Figure 2; $H_2O_2$ production in Neutrophils

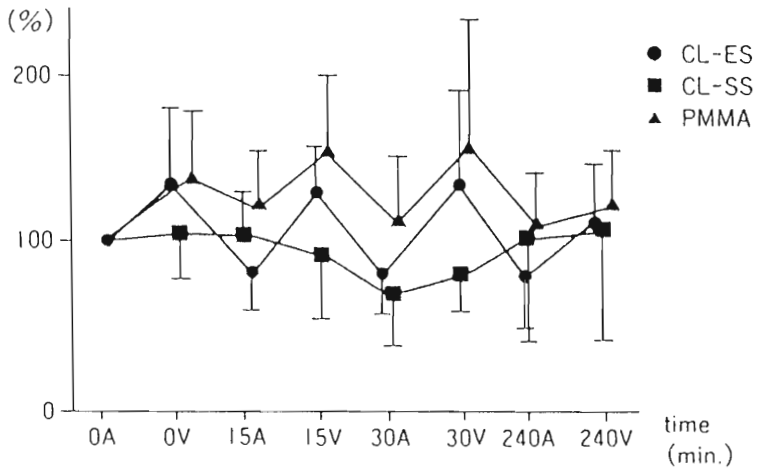


Figure 3; MPO Concentration in Blood

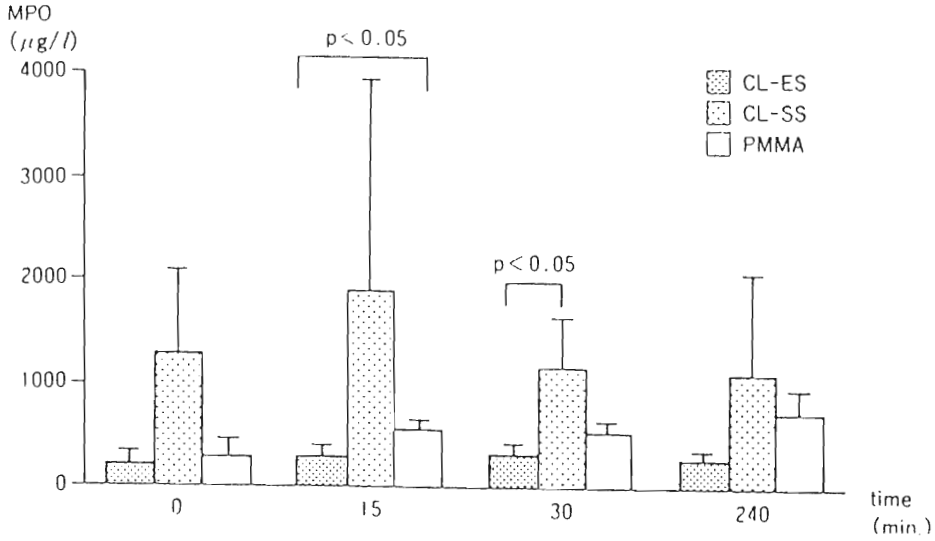
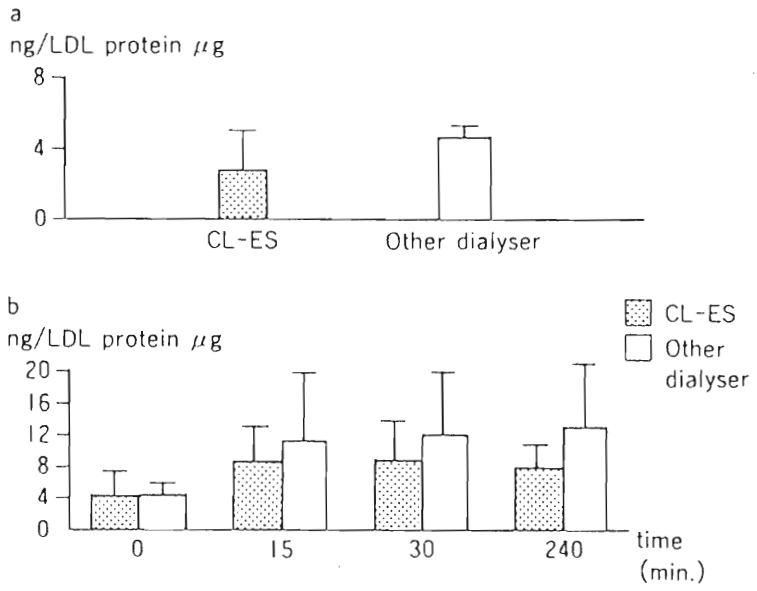


Figure 4; OX-LDL Content in Blood



# 第４章

# 透析患者における単球由来マクロファージの酸化LDLに対するスカベンジャ受容体の発現亢進

# ENHANCED EXPRESSION OF SCAVENGER RECEPTOR ON MONOCYTE-MACROPHAGES TO OXIDIZED LDL IN DIALYSIS PATIENTS

# ENHANCED EXPRESSION OF SCAVENGER RECEPTOR ON MONOCYTE-MACROPHAGES TO OXIDIZED LDL IN DIALYSIS PATIENTS


〈Summary〉

Enhanced scavenger receptor expression in monocyte-macrophages in Hemodialysis patients. The macrophage scavenger receptor (SR), which has two isoforms named type I and II, plays a leading role in the atherosclerotic process. To elucidate the mechanism of atherosclerosis in dialysis patients, SR expression was studied in monocyte-macrophages from thirteen dialysis patients and eight healthy controls. SR mRNA expression was examined in four hemodialysis patients and four controls matched for age and sex. Monocytes were allowed to differentiate into macrophages in in vitro cultures for seven days and SR type I and 11 mRNA expression were analyzed with the reverse transcription-polymerase chain reaction and quantitated using radiation densities of Southern blots. Only SR type I expression increased during differentiation and was accelerated by one or two days and enhanced after five days in patients, as compared to controls.

In conclusion, SR expression is enhanced in macrophages from dialysis patients, probably by up-regulation of type I, which may contribute to the development of atherosclerosis in these patients.

<Introduction>

Since Goldstein et al (1) proposed the "modified LDL and scavenger receptor (SR) hypothesis," a considerable number of investigations have been performed on this subject. SRS are trimeric membrane glycoprotein implicated in the pathologic deposition of cholesterol in arterial walls during atherogenesis (2). Studies of the pathology of atherosclerosis indicate that the key cellular components of atheromatous plaque are foam cells derived from peripheral monocyte-macrophages (3). The endocytosis of plasma lipoprotein cholesterol by monocyte-macrophages is carried out by SRS on macrophages forming these foam cells (4). SRS recognize LDL which has been chemically or biologically altered (such as acetylated, malondialdehyded or oxidized), and SR expression is not regulated by the cellular cholesterol levels (5). In vivo studies strongly support the notion that uptake of modified LDL by SRS on macrophages is involved in the development of atherosclerosis (6,7).

Two types of complementary DNA (cDNA) for SR of human macrophages have been cloned (8). The type I and type II receptors are generated from a single gene by alternative splicing (9) and both of them can be detected in lipid-laden macrophages in human atherosclerotic lesions (2, 8). SR type I and type 11

are capable of binding and internalizing modified LDL (10, 11), but it remains unknown whether there are fine functional differences between the two types.

Maggi et al (12) demonstrated that LDL from uremic patients is more susceptible to in vitro and in vivo oxidation than LDL from control subjects, which could lead to the accelerated atherosclerosis in uremia. The mechanisms of oxidation seem to be implicated in development of uremia (13, 14).

In contrast, there are no reports referring to the relationship between uremia and SR, which must be associated with the oxidized LDL (OX-LDL) metabolism in uremia. The present study was designed to examine the atherogenesis in dialysis patients with regard to the SR-mediated mechanism. In this work, it was assessed whether SR mRNA and protein expression is enhanced in hemodialysis (HD) patients and, if so, which type of SR is involved.

〈Subjects〉

Eight non-diabetic HD patients (4 female, 4 male, mean age 40 ±8.3 years) were subjected. Eight healthy controls (4 female, 4 male, mean age 41 ±7.6 years) were recruited from the staff of our hospital, and served as controls. Four HD patients and four

healthy subjects matched for age and sex were selected for SR mRNA analysis. Peripheral blood from the patients was obtained at the beginning of dialysis, before the administration of anticoagulants in HD patients, and all blood analyses were determined by standard methods in our clinical chemistry laboratory.

〈Method〉

1) Culture of human monocyte-macrophages

Forty milliliters of venous peripheral blood were collected into heparinized tubes (5 U/ml) from each patient and control. Mononuclear cells were isolated by centrifugation on Lymphoprep (Nycomed, Oslo, Norway) and were washed twice with phosphate-buffered saline (PBS). The washed cells were dispersed into 24×16 well plastic plates (Costar, USA) at 3 × 106 cells/ml in RPMI 1640 (Gibco, USA). After incubation of the cells for two hours at 37℃ in an atmosphere of 5% $CO_2$, the nonadherent cells were removed. The adhesion cells were gently washed with performed PBS (the final cell density was approximately 1×106 cells/ml) and cultured with RPMI 1640 supplemented with 20% autologous serum which had been filtered through a 0.45 $\mu$m filter, 20 mM Hepes buffer, 10% fetal

calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM L-glutamate. Cell viability, determined by trypan blue exclusion, was > 95% in all experiments. Cells of each patient-control pair were cultured in the same plate for a maximum period of seven days.

2) RNA extraction from macrophages

Total RNA was extracted from the cultured cells on days 4. 6. and 7, using the acid-guanidinium thiocyanate-phenol-chloform method ( 15 ) . RNA concentration and purity were determined by spectrophotometer (Beckman Instruments, USA) at 260 and 280 nm.

3) Oligonucleotide primers and probes

All oligonucleotides for analysis of SR and glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized with DNA synthesizer Model 380 B (Applied Biosystem, USA). The primer sets for PCR and cDNA probes for Southern blot analysis are listed in Table 2. Nucleotide position of the primers and the probes is indicated by the nucleotide number of cDNA sequences described in earlier studies (2, 16) . The specific probe for the Southern blot of SR corresponds to a sequence in the collagen-like domain of SR

which is common to both types GAPDH was used as an internal control for evaluating PCI efficiency and the amount of template RNA.

4) RT-PCR

Five hundred nanograms of total RNA was used as a template for RT-PCR. The RT reaction was set up in a 20 $\mu$ l mixture containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl2, 0.5 mM of each dNTPs, 2.5 /nM random hexamers, 20 U RNAsin, and 50 U of M-MLV reverse transcriptase (Perkin-Elmer Cetus, USA). Incubations were performed in a DNA Thermal Cycler (Techne Corporation, Cambridge, UK) for 10 minutes at 23℃, followed by 40 minutes at 42℃ and five minutes at 99℃. After cDNA synthesis by RT, the incubation mixture was split into two 10 $\mu$ l aliquots for separate amplification of the SR cDNA and the GAPDH cDNA (17). For PCR, the total incubation volume was adjusted to 100 $\mu$ l by adding premixed components, which were overlaid with 50 $\mu$ l mineral oil. The final concentrations of the PCR reaction mixture were 0.3 $\mu$M (for SR) or 0.15 $\mu$M (for GAPDH) of 5' primer, 0.15 $\mu$M of each 3' primer, 200 $\mu$M of each dNTPs, 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus), 2 mM MgCl2, 50 mM KCl, and 10 mM Tris/HCl. After incubation for two minutes at 94℃, 40 cycles were performed with

30 seconds at 94℃, 60 seconds at 55℃, and 60 seconds at 72℃. The PCR products were analyzed on a 2% agarose gel in Tris borate/EDTA buffer, visualized by staining with 1 $\mu$ g/ml ethidium bromide and documented on Polaroid film.

5) Southern blot analysis and quantitative evaluation

Thirty microliters of each PCR reaction was subjected to electrophoresis in a 2% agarose gel. Nucleic acids were transferred to a nylon membrane (Hybond-C Extra; Amersham, UK), using the Southern analysis method (18). The nylon membranes were probed with 32P labeled oligonucleotides. The DNA probes were labeled using random oligonucleotides primers (18). Membranes were irradiated by UV stratalinker for one minute at 1,200 J, prehybridized in 5 x Denhardt's solution, 6 % SSC, 0.1% SDS and 5% dextransulfate at 55℃, hybridized with at least I x 106 cpm／ml of the labeled probe in the same solution as prehybridization at 50℃ for four hours (for the SR probe) or overnight (for the GAPDH probe) and then washed twice for 10 minutes at 42℃ in 2 % SSC and 0.1% SDS. The blots were exposed to imaging plates (Fuji film, Japan) for one hour. Radiation densities of SR and GAPDH blots on the imaging plates were evaluated as the amount of radiolabeled

material by the programmed correction rule, using Bio-Imaging Analyzer BAS 2000 (Fuji film, Japan) and Image File Manager 3.0 software. Relative radiation density (the ratio of SR to GAPDH) was calculated for each sample to adjust for the differences in RNA mass between templates and was used for the quantitative comparisons.

6) Immunofluorescent analysis of scavenger receptor on the surface of monocyte/macrophage lineage cells

In order to detect SR protein expression, we planned to evaluate uptake of fluorescently labeled oxidized LDL was by flow cytometry.

Scavenger receptors on the surface of monocyte/macrophage lineage cells of patient's peripheral blood were determined by indirect immunofluorescence with a FACS flow cytometer. Two color analysis was done using rabbit anti-human scavenger receptor antibody (kindly offered by Dr. Kodama), FITC- conjugated gout anti- rabbit immunoglobulin antibody, and PE-conjugated anti-human CD14. Increased expression of scavenger receptors on the surface of monocyte/macrophage lineage was seen in CAPD patients with hyperlipidemia.

〈Statistical analyses〉

All data are presented as the mean ± SD. Statistical analyses were performed using the Student's t test. Statistical significance was accepted if a P value was less than 0.05.

〈Results〉

1) RT-PCR analysis for detection of SR type I and type II

The RT-PCR analysis simultaneously demonstrated two types of SRS of 451 bp and 295 bp on the gel electrophoresis (Fig. 1). Each band corresponded to the expected size of type I and type II cDNA sequences, amplified with the specific primer sets. The identity of the RT-PCR products was confirmed by dideoxy DNA sequencing. Also, Southern blot hybridization with the specific probe, corresponding to the collagen-like domain of SR, confirmed that the two bands were SR fragments (Fig. 1).

2) Kinetics of expression of SR type I and type Ⅱ mRNA

SR type 11 was expressed simultaneously or 1 to 2 days earlier than SR type I in both patients and controls. Using monocyte-macrophages from healthy individuals, SR type 11 was detected also in the monocyte state (that is, on day 0 in culture), whereas type I was absent in the early stages of cell differentiation. Appearance of SR type I expression was variable among the individuals, in particular among the controls; for example, type I expression was accelerated in controls 3 and 4 who were older female subjects, as compared to controls 1 and 2.

When comparing patient-control pairs who were matched for age and sex, Southern blot analysis showed that type I mRNA expression was accelerated in the patients: type I was expressed by day 4 (patients 3 and 4) or by day 5 (patients 1 and 2) in the patients, whereas it was expressed by day 5 (controls 3 and 4), day 6 (control 2), and day 7 (control 1) in the controls (Fig. 1).

3) Intensity of SR mRNA expression

SR type I expression was significantly enhanced on days 5, 6, and 7 in the patient group, as compared to the control group. Type I expression increased in parallel to days in culture (that is,

cell differentiation) in the patient group (Fig. 2A). SR type II expression fluctuated between the number of culture-days both in patients and in controls, and consequently the difference between days in culture or between the two subject groups was not significant (Fig. 2B).

4) Immunofluorescent analysis of scavenger receptor on the surface of monocyte/macrophage lineage cells

It was elucidated that the mean fluorescence intensity of labeled cells was significantly higher in the hemodialysis patients with hyperlipidemic ASO than in controls, although the number of SR-positive cells remained constant. SR type I and II expression increased during differentiation and was accelerated by one or two days, as compared to controls(Fig. 3). SR expression is enhanced in macrophages from hyperlipidemic patients.

〈Discussion〉

Atherosclerosis in dialysis patients is a serious complication since it is closely involved in their morbidity and

mortality. The mechanisms underlying development of the disease are still unclear. We have addressed this problem from the aspect of SR-mediated uptake of cholesterol. Our results show that SR expression and activity is enhanced in in vitro cultured monocyte-macrophages from dialysis patients.

To elucidate the process of atherogenesis, the expression of SR type I and type 11 mRNA in HD patients was analyzed. For this purpose, specific oligonucleotides primers were used. The two types of SRS are generated by alternative splicing of a single gene in the same cell (9, 19), but the mechanisms responsible for promoting transcription and splicing of the SR gene are incompletely understood. In vitro studies showed that SR expression is dependent on cell density in the culture plate (20, 21) and on the state of cell differentiation which is mainly regulated by protein kinase C pathways (22, 23). However, mRNA expression of this receptor under physiological conditions is not well documented, and it is unknown even whether there are gender or age differences in SR mRNA expression. In our study using macrophage from the HD patients, only type I mRNA expression was increased during cell differentiation and was significantly enhanced in the patients after day 5 in culture, as compared to the controls. In contrast, type II mRNA expression appeared to be constitutive from the early stage of cell differentiation and was not enhanced during

maturation.

The regulation of SR activity by serum components has clinical significance since this receptor could be crucial in the genesis of atherosclerosis. Further studies are in progress in our laboratory to isolate and characterize the possible factor in uremic serum responsible for SR activity. There is an inverse relationship between SR activity and LDL receptor activity in the same macrophage. In THP-1 cells stimulated with phorbol ester, an activator of protein kinase C, LDL receptor activity is diminished while SR is induced (24). A similar phenomenon is observed during culture of freshly isolated human monocyte-macrophages (20). Furthermore, LDL receptor mRNA expression is suppressed in atherosclerotic lesions where SR mRNA expression is detectable (8, 20). Portman et al (25) revealed that LDL receptor mRNA expression is significantly decreased in monocyte from uremic patients including HD and CAPD patients, as compared to healthy controls, using a ribonuclease protection assay. These findings could indirectly support the presence of enhanced SR expression and activity in monocyte-macrophages from HD patients.

In conclusion, SR expression is enhanced in in vitro cultured macrophages from dialysis patients, probably by up-regulation of SR type I, which may contribute to the development of atherosclerosis in these patients.

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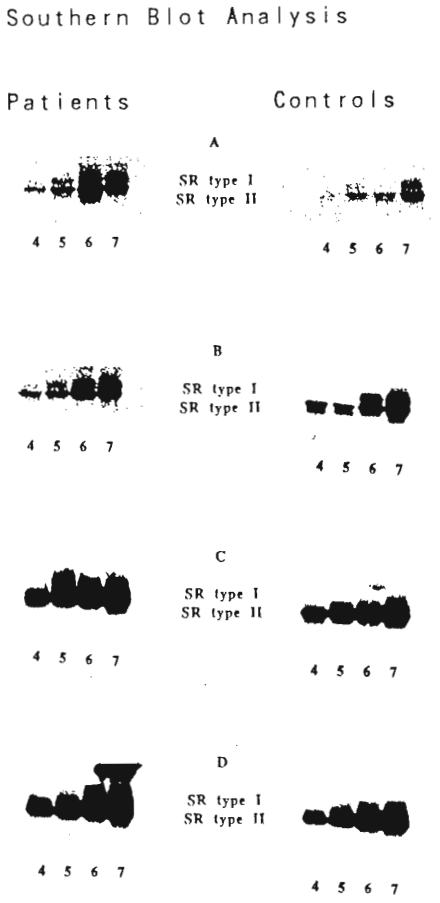
Figure 1; RT-PCR Analysis for Detection of SR type I and type II



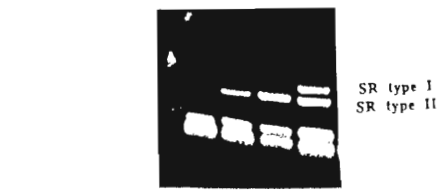
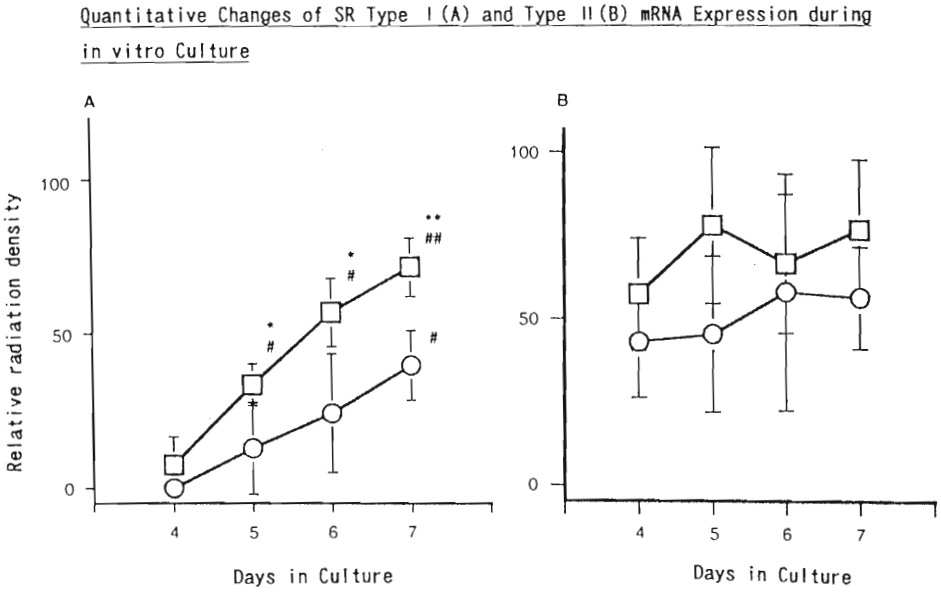



Figure 2; Intensity of SR mRNA expression

Fig. 2A; SR type I and Fig. 2B; SR type II



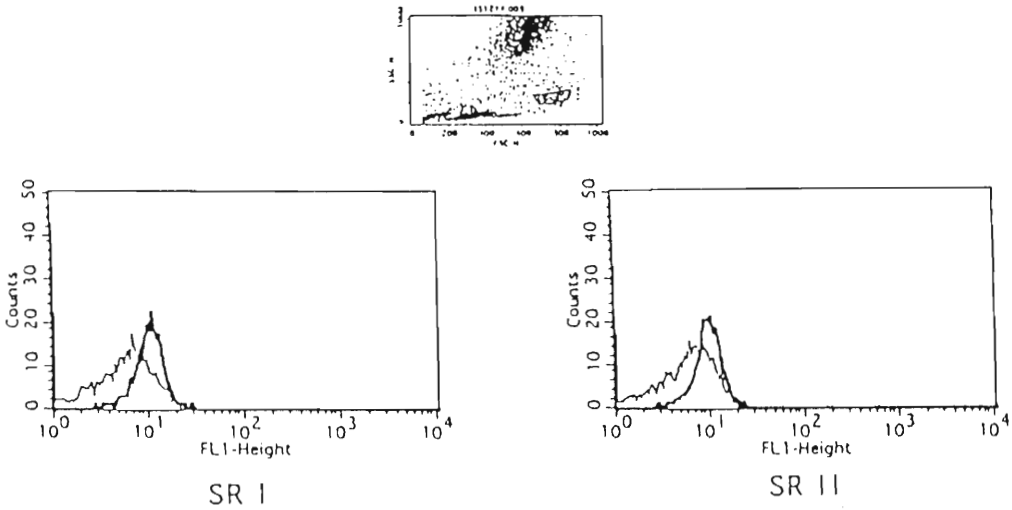

The relative radiation density(SR/GAPDHratio) of the Southern blot was used for the quantitative evaluation. Details are described in Methods.

Symbols are (□)HD patients(N=4), and (○)healthy controls(N=4).

Data are presented as the mean±SD.

Significant diferences are marked ‡P<0.05 versus controt, ‡‡P < 0.01versus control; #P<0.05 versus day4, and ##P < 0.01 versus day4.

Figure 3; Immunofluorescent Analysis of Scavenger Receptor on Surface of Monocyte/Macrophage Lineage Cells



# 第５章

# 総　括

# CONCLUSIONS

# 総　括　Conclusions

腎疾患患者血液中の過酸化脂質と動脈硬化進展機序の相関性を解明する目的で、酸化LDL抗体を作成し、透析患者およびLDL吸着療法実施患者の血漿について酸化LDLをはじめとする脂質代謝関連指標を測定した。

その結果、次のことが判明した。

1)　血液透析における酸化LDL値は、健常者と比較して明らかに高値を示す。

2)　これらの対象患者においては、LDLｺﾚｽﾃﾛｰﾙ値が正常範囲にあるにも関わらず、LDL当たりの酸化LDL値が高値を示す患者が多数存在する。

3)　血液透析患者の動脈硬化と酸化LDLの関連について慢性閉塞性動脈硬化症(+)群と(-)群を比較した結果、両群の酸化LDL値に有意な差が認められる。

4)　このような慢性閉塞性動脈硬化症患者は，LDL吸着療法による酸化LDLの除去率が高いほど症状の改善傾向が得られることから、酸化LDLはこれらの発症進展に重要な役割を演じていると推察された。なお､高脂血症を有し､ステロイド剤抵抗性ネフローゼ症候群もLDL吸着療法によって酸化LDLが除去される場合においてのみ､LDLｽｶﾍﾞﾝｼﾞｬｰ受容体が染色され､蛋白尿が軽減する傾向にあった。

5)　血液透析患者の末梢血から採取されたマクロファージは，酸化LDLなどに対するスカベンジャー受容体，なかでもtype IのmRNAの発現亢進が認められた。

以上、酸化LDLは、血液透析患者の動脈硬化進展における臨床的意義を考える上でも極めて重要な物質であると判断された。今後とも、疾患別に症例を重ね、酸化LDLと腎疾患との関連性についても解明を推進するとともに、単球由来マクロファージにおけるスカンベンジャー受容体の発現亢進の意義について更に解析する必要があると結論される。