Indian Journal of Clinical Biochemistry, (1999), 14 (2), 176-183.

EARLY OXIDATIVE CHANGE IN LOW DENSITY LIPOPROTEINS DURING PROGRESSIVE CHRONIC RENAL FAILURE

M. Sasikala[1,2], C. Subramanyam[2]* and B. Sadasivudu[1]

[1]Vijaya Diagnostic Centre, Secunderabad - 500 003 and [2]Department of Biochemistry, Osmania University, Hyderabad - 500 007.

ABSTRACT

Modified low density lipoproteins (LDL), including their oxidized forms, have been widely implicated in the etiology of atherosclerosis and concomitant cardiovascular disease (CVD) in chronic renal failure (CRF). The nature of events that lead to oxidative changes in LDL proteins are not clearly understood. Thus, patients suffering from CRF were grouped into mild, moderate and severe categories based on their blood urea and serum creatinine levels. Progression of CRF was accompanied not only with gradual increase in serum malondialdehyde (MDA) but also parallel increase in conjugated diene and MDA levels in LDL fractions separated from serum. Serum superoxide dismutase (SOD) activity was concurrently found to decrease, along with a decrease in high-density lipoprotein (HDL) cholesterol, during the progression of CRF. Gradual increase in the appearance of LDL oxidation products seems to accompany progressive manifestation of CRF. The results presented suggest that determination of serum MDA and SOD levels may enhance the diagnostic significance of the study of lipid profile in determining the risk for cardio vascular disease in CRF.

KEY WORDS : Chronic renal failure; Malondialdehyde; Superoxide dismutase; Low density lipoproteins; Lipid peroxidation

INTRODUCTION

It is well known that the incidence of cardiovascular disease is the major cause of mortality in patients suffering from chronic renal failure (CRF) and that the process of atherosclerosis is accelerated in CRF (1). Increased amounts of modified low density lipoproteins (LDL), particularly the oxidised LDL, have been widely implicated in the etiology and progression of the atherosclerotic process accompanying CRF (2). Such alterations of LDL are supposed to occur within the vascular tissue, probably at the sites of endothelial dysfunction (3). Although a number of researchers, including Indians, have clearly established abnormal serum lipid patterns in CRF (4,5,6), factors that lead to LDL oxidation are yet to be deciphered completely. The increased activity of reactive oxygen species (ROS), which may contribute to the toxic state of uremic syndrome in renal failure, points also to their involvement in LDL oxidation. Further, increased levels of malondialdhyde (MDA), a

Author for correspondence :
Prof. C.Subramanayam at above address.

product of lipid peroxidation, has been reported in sera of CRF patients (7), indicating that alterations in LDL may be caused not only by oxidation but also by MDA. The lysine residue in the receptor binding site of LDL-ApoB protein has been found to be altered as a result of the oxidation of fatty acids of LDL (8) or by direct combination with MDA (9), leading to a decreased affinity of LDL to its receptor. Such an altered LDL is avidly taken up by macrophages through scavenger receptors. Thus, both the factors, i.e., LDL oxidation and modification by MDA, may accelerate the process of atherosclerosis occurring in CRF.

Increased activity of ROS may also be ascribed to depletion of antioxidants (such as glutathione) and to increased production of oxidants such as superoxide anion, which are disposed off by superoxide dismutase (SOD). A number of studies have also demonstrated changes in erythrocyte glutathione peroxidase and SOD activities in patients of CRF (10). Most of the secreted SOD has been found to be extracellular and extravascular, although a considerable amount of it is present in serum (11). Serum SOD is mostly bound to endothelial cells, protecting them from the damaging activity of oxygen free radicals (12). In order to elucidate the role of oxidative stress under conditions of CRF, we not only determined serum lipid profiles but also conducted a focussed study to correlate alterations in serum MDA and lipid peroxidation products (including conjugated dienes and MDA in separated LDL fractions) with serum SOD activity. Such a study, rather than a mere examination of lipid profiles, would explain the mechanisms involved in the occurrence of LDL oxidation that is more causally related to atherogenesis accompanying CRF.

MATERIALS AND METHODS

Subjects and Sampling

Patients suffering from CRF were randomly selected for the study. Based on serum creatinine levels the patients were categorized into three stages as per standard clinical notation (13). Stage I patients had serum creatinine levels ranging from 1.5 to 3.0 mg/dl; Stage II patients had serum creatinine levels in the range of 3.1 - 8.0 mg/dl and those patients with serum creatinine above 8.0 mg/dl were grouped under Stage III. Each group consisted of at least a minimum of 10-12 patients of the same age group (30-50 yr.). Simultaneous determinations were made in 12, age-matched, normal healthy individuals to serve as the controls. Blood samples were collected under fasting conditions and analyzed for serum levels of urea, creatinine, uric acid, calcium, phosphorous, alkaline phosphatase, amylase, electrolytes and lipid profiles. Blood samples were drawn in EDTA for isolation of LDL and for analysis of LDL oxidative products.

Methods

Lipid profile : Total cholesterol, triglycerides, HDL cholesterol were determined using commercial kits (Boerhinger Mannheim) employing a semi-autoanlyser (Eppendorf E com F 6124).

Malondialdehyde : Serum malon-dialdehyde (MDA) levels were estimated following the method described by Sadasivudu et. al. (14) which is a slightly modified procedure of the spectrophotometric assay as described by Belch et. al. (15). The method consisted of treating 0.5 ml of serum with 0.5 ml of 40% trichloroacetic acid followed by the addition of 1.0 ml of 0.67% of

thiobarbituric acid. The mixture was kept for 10 min in a boiling water bath and cooled immediately in ice cold water. The mixture was centrifuged at 6000 rpm for 15 min and then absorbance of the supernatant was determined at 530 nm. The MDA content was calculated using the molar extinction coefficient 1.56 x 105 and expressed as nmoles/ dl.

Determination of conjugated dienes in LDL fraction

a) Isolation of LDL and determination of LDL-cholesterol : Serum low-density lipoproteins (LDL) were isolated by a precipitation method (16). One ml samples of serum (containing 1 mg/ml EDTA) were brought to room temperature and treated with 7 ml of precipitation buffer (0.064 M trisodium citrate, pH 5.05, and 5,000 IU/L of heparin). After mixing the contents on a vortex mixer, the suspension was allowed to stand for 10 min. at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1000 x g for 10 min. The pellet was resuspended in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.9% NaCl. Aliquots (10 ml) of the above suspension were used for the estimation of LDL-cholesterol using the cholesterol reagent supplied in the Boerhinger Mannheim kit.

b) Determination of LDL oxidation products-conjugated dienes : LDL oxidation was estimated by determining the baseline diene conjugates in lipid fraction of the LDL (LDL - BDC). LDL samples (100 ml) isolated as above were treated with 0.5 ml of chloroform/methanol (2:1) to extract lipids and dried under nitrogen. The lipids were redissolved in cyclohexane and analyzed spectrometrically at 234 nm. Absorbance units were converted to molar units using the molar extinction coefficient 2.95 x 104 M-1 cm -1. The results were expressed as mmol/dl to have an estimate of the actual level of oxidized LDL in circulation.

c) Determination of malondialdehyde in LDL fraction : MDA contents in LDL fractions (isolated as described above) were estimated by taking an aliquot of LDL fraction in phosphate buffer and treating with thiobarbituric acid as described above.

Superoxide dismutase: Superoxide dismutase (SOD) was assayed employing the modified spectrophotometric method described by Kakkar et. al. (17). The assay mixture consisted of 1.2 ml of 0.052 M sodium pyro-phosphate buffer pH 8.3, 0.1 ml of 186 mM phenazine methosulphate, 0.3 ml of 300 mM nitrobluetetrazolium, 0.2 ml of 780 mM NADH and 100 µl serum. The reaction was initiated by the addition of NADH and terminated by adding 1 ml of glacial acetic acid after 90 seconds at room temperature. The reaction mixtures were stirred vigorously and extracted with 4 ml of n-butanol. After 10 minutes, the mixture was centrifuged to separate the butanol layer whose colour intensity was measured at 560 nm employing butanol as blank. One unit of the enzyme activity is defined as enzyme concentration required to cause 50% inhibition of the reduction of nitrobluetetrazolium in one minute under the assay conditions. The employed method was validated by comparing it with results obtained by adopting another method involving inhibition of nitrobluetetrazolium reduction and employing xanthine - xanthine oxidase system as the superoxide generator (18). The results of both the methods were identical and comparable.

RESULTS AND DISCUSSION

The present study was conducted to ascertain oxidative changes in serum LDL fractions and to correlate them with the progression of CRF. Thus, alterations in MDA and conjugated dienes associated with LDL fractions of serum were correlated with serum MDA levels and SOD activity. Results obtained in this regard clearly demonstrated that advancement of CRF from stage I to stage III is indeed accompanied by progressive increase in total MDA levels in serum as well as MDA associated with LDL fractions and in the levels of conjugated dienes associated with LDL fractions. These changes were significant during progression of CRF from stage II to stage III and were accompanied by a decrease in serum SOD activity, which was evident from stage I itself. Simultaneously, gradual increases in serum triglyceride levels and decrease in HDL cholesterol contents were also observed during the progression of CRF. While the increase in triglyceride levels was significant during progression from stage II to stage III, the decrease in HDL cholesterol was significant during progression from stage I to stage III of CRF. However this decrease was unaccompanied with changes in total cholesterol. The results are summarised in Table 1.

The gradual increase in serum creatinine levels, observed from stage I to stage III, indicates a progressive deterioration in renal function. Correspondingly, serum MDA levels were found to increase from stage I to stage III indicating a progressive increase in the activity of ROS either due to increased production of ROS or due to decrease in the antioxidant functions and other protective mechanisms. The generation of ROS may be more in CRF due to increased oxidant stress caused by increased production of cytokines such as tissue necrosis factor. Several other studies have also clearly indicated a decrease in the content of glutathione accompanied by a decrease in the activity of glutathione peroxidase and SOD in blood cells (10).

Oxidised LDL has been very much implicated by several workers towards the initiation and progression of atherosclerosis. The oxidized lipid moiety of the LDL brings about an alteration in apo B. Even though the oxidized LDL has not been demonstrated in serum, it is possible that such oxidative products of lipid moiety may indeed occur as an evidence for the presence of oxidized LDL in serum (16). It may be noted that oxidation and / or alteration of apo B 100 is supposed to be occurring within the vascular tissue. Such an oxidized moiety of LDL is avidly taken up by macrophages, without any feedback suppression of endogenous cholesterol production, bringing about lipid loading of macrophages (19). Oxidised LDL is also shown to possess chemotactic and cytotoxic effects (20). Interestingly, the earliest change observed in LDL in the process of its oxidation is the depletion of β-carotene and vitamin E, followed by rise in conjugated dienes and culminating in a rise of MDA contents (21).

In stage I CRF we observed a slight (12%) rise in conjugated dienes in LDL, but the increase in MDA (15%) was negligible. This probably indicates that increase in oxidized LDL occurs prior to increase in MDA in CRF, which is in agreement with the above sequence of changes. Although β-carotene and vitamin E have not been

Table 1. Serum parameters during different stages of chronic renal failure

Parameter	Control	Chronic Renal Failure		
		Stage I	Stage II	Stage III
Urea (mg/dl)	21.0 ± 3.2	59.7 ± 8.7	112.4 ± 40.0	171.0 ± 38.5
Creatinine (mg/dl)	1.0 ± 0.086	2.31 ± 0.37	6.6 ± 0.88	9.47± 1.15
Triglycerides (mg/dl)	102.0 ± 16.4	114.6 ± 29.3	*133.4 ± 27.4	*160.71± 33.92
MDA (nmoles/dl)	149.8 ± 10.5	*251.8 ± 31.46	*329.86 ± 51.66	*372.12 ± 68.77
Cholesterol (mg/dl)	158.6 ± 9.69	166.1 ± 9.6	162.7 ± 24.0	163.87 ± 22.4
HDL-Cholesterol (mg/dl)	41.3 ± 1.5	*35.8 ± 2.89	*33.1 ± 2.76	* 31.75 ± 3.73
LDL-Cholesterol (mg/dl)	96.88 ± 9.11	107.0 ± 6.9	101.6 ± 27.4	*113.0 ± 16.6
LDL -conjugated dienes (mmoles/dl)	10.16 ± 0.67	11.34 ± 1.2	*12.93 ± 1.07	*17.45 ± 1.66
LDL-MDA (nmoles/dl)	79.47 ± 10.5	83.5 ± 19.88	*112.0 ± 25.58	*212.12± 10.19
SOD (Units/dl)	1210 ± 12	*510 ± 26	*479 ± 19	*463 ±28

Values depicted are mean ± SD (n=12). *p values are <0.01as compared to controls.

estimated in the present study, it is interesting to observe that the sequential changes in conjugated dienes and MDA tend to provide some indication that the process of LDL oxidation has set in and accelerated making the patients more susceptible to atherosclerosis and to the clinical sequences thereof, under conditions of CRF.

Another important obsérvation made in the present study relates to SOD. Most of this enzyme in the serum is reportedly anchored to endothelial cells through heparan sulphate (22). Such a localization of SOD on the endothelial cells would protect them besides protecting serum LDL from the damaging effects of ROS. The decrease in the activity of SOD in the present study was very significant even in stage I when compared to controls and such a decrease persisted through

stage II & III, accounting for the continued oxidation of LDL in CRF. However, the early profound decrease in SOD activity observed even in stage I may probably be the contributing factor for oxidation of LDL by ROS in patients of renal failure. Although the mechanism involved in the decreased activity of serum SOD is not clearly understood in CRF, it may be probably due to increased presence of ROS such as H_2O_2 which is known to suppress SOD activity (23).

Although hyperlipidemia is one of the predisposing causative factors in aethero-sclerosis and the same is known to be associated with CRF, the results in the present study do not indicate hypercholesterolemia in our patients. Hyperlipidemia without the presence of altered levels of LDL may not be contributing to the process of atherosclerosis since it has been shown in cell culture experiments that unmodified LDL does not accumulate in the cells (21). However a decrease in HDL cholesterol and a progressive increase in serum triglycerides from stage I to III may also be contributing to the progress of atherosclerosis. Decrease in HDL may also be contributing to the increased oxidation of LDL in CRF (24). Since the important event in the causation of athero-sclerosis is oxidation of LDL, the rise in conjugated dienes and MDA in LDL fraction observed in the present study clearly indicate such a process.

In conclusion, our studies indicate that conditions to promote LDL oxidation seem to exist from a very early stage in renal failure. However, a clear manifestation of LDL oxidation becomes evident during subsequent progression of the disease. These changes in LDL oxidation may occur probably as a result of decrease in serum SOD and HDL-cholesterol, which may contribute towards accelerated atherosclerosis in chronic renal failure.

Acknowledgements : Our grateful thanks are due to Dr. K. S. Nayak, Nephrologist, CDR Hospitals, Hyderabad who provided us with the samples.

REFERENCES

1. Degoulet, P., Legarin, M., Reach, A.F., Devris, C., Rojas, P. and Jacobs, C. (1982) Mortality risk factors in patients treated by chronic hemodialysis. Nephron. 31, 103-110.

2. Steinberg, D., Parthsarathy, S., Carew, T.E., Khooj, C. and Witzum, J. L. (1989) Beyond cholesterol, modification of LDL that increases its atherogenesity. New Eng. J. Med. 320, 915-924.

3. Gibbsons, G. H. and Dzam, V. J. (1996) Molecular therapies for vascular diseases. Science, 272, 689-693.

4. Begdade, J.D., Porte, D.J.R. and Bierman, E.L. (1968) Hypertriglyceridemia a metabolic consequence of chronic renal failure. New Eng. J. Med. 264, 181-185.

5. Senti, M., Ramero, R., Pedro-Botet, J., Pellegro, A., Nogues, Z and Rubies-Prat, J. (1992) Lipoprotein abnormalities in hyperlipidemic and normolipidemic men on hemodialysis with chronic renal failure. Kidney Int. 41, 1394-1399.

6. Bhagwat, R., Joshi, S. P., Salgia, P and Sepaha, A. (1997) Lipid abnormalities in chronic renal failure. Ind. J. Clin. Biochem. 12, 81-85.

7. Mahfouz, M. O., Hariprasad, I. A., Shaffe and Sadasivudu, B. (1986) Serum MDA levels in myocardial infarction and chronic renal failure. IRCS Med. Sci. 14, 1110-1111.

8. Jackson, P., Loughrey, F.M., Lightbody, J.H., McNamee, P.T. and Young, I.S. (1995) Effect of hemodialysis on total antioxidant capacity and serum antioxidants in patients with chronic renal failure. Clin.Chem. 41, 1135-1138.

9. Holvoet, P., Perez, G., Zhao, Z., Brouwers, E., Bernar, H. and Collen, D. (1995) Malondialdehyde - modified low density lipoproteins in patients with atherosclerotic disease. J. Clin. Invest. 95, 2611-2619.

10. Chauhan, D.P., Gupta, P.H., Nampoothiri, M.R.N., Singhal, P.C., Chugh, K.S. and Nair, C.R. (1982) Determination of erythrocyte superoxide dismutase, catalase, glucose 6 phosphate dehydrogenase, reduced glutathione and malondialdehyde in chronic renal failure. Clin. Chim. Acta. 123, 153-159.

11. Fridovich, I. (1995) Superoxide radical and superoxide dismutases. Ann. Rev. Biochem. 64, 97-112.

12. Karlson, K. and Marklund, S.L. (1989) Binding of human extracellular superoxide dismutase to cultured cell lines and to blood cells. J. Lab. Invest. 60, 659-666.

13. Whelton, A., Watson, A.J. and Robert, C.R. (1994) Nitrogen metabolites and renal function. In: Teitz Text book of clinical chemistry, Eds. Burtis, C.A. and Ashwood, E.R. W.B. Saunders Co. USA, 2nd edition. p. 1513-1568.

14. Sadasivudu, B., Sashikala, M., Sailaja, V. and Surender Reddy, S. (1997) Serum malondialdehyde, insulin, glucose and lipid profile in hypertension. Med. Sci. Res. 25, 631-633.

15. Belch, J.J.F., Bridges, A.B., Scott, N. and Chopra, M. (1991) Oxygen free radicals and congestive heart failure. Brit. Heart J. 65, 215-218.

16. Ahotupa, M., Ruutu, M. and Mantyla, E. (1996) Simple methods for quantifying oxidation products and antioxidant potential of low density lipoporteins. Clin. Biochem. 29, 139-145.

17. Kakkar, P., Das, B. and Viswanathan, P. N. (1984) A modified spectrophotometric assay of superoxide dismutase. Ind. J. Biochem. Biophys. 21, 130-132.

18. Oberley, L.W. and Li,Y. (1988) Simple method for clinical assay of superoxide dismutase. Clin. Chem. 34/3, 501-504.

19. Stainberg, D. and Witztum, J.L. (1990) Lipoproteins and atherogenesis. J. Amer. Med. Assoc. 264, 3047-3052.

20. Morel, D. W. and Chisolm, G.M. (1989) Antioxidant treatment of diabetic rats inhibits lipoprotein oxidation and cytotoxicity. J. Lipid Res. 30, 1827-1834.

21. Witzum, J.L and Steiburg, D. (1991) Role of oxidized LDL in atherogenesis. J. Clin. Invest. 88, 1785-1792.

22. Karlson, K. and Marklund, S.L. (1987) Heparin induced release of extracellular SOD to human blood plasma. Biochem. J. 242, 55-59.

23. Kono, Y. and Fridovich, I. (1982) Superoxide radical inhibits catalase. J. Biol. Chem. 257, 5751-5754.

24. Huang, J., Huang, Z. and Zhu, W. (1998) Mechanism of high density lipoprotein subfractions inhibiting copper catalysed oxidation of low density lipoproteins. Clin. Biochem. 31, 537-543.