Acid Cholesteryl Ester Hydrolase Activity of Mononuclear Leukocyte in Type 2 (Non-Insulin-Dependent) Diabetic Patients

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ONUMA, T., TSUTSUI, M., OCHIAI, S., BOKU, A., YANADA, A., HIRAI, Y., NAKAHATA, H. and TAKEBE, K. *Acid Cholesteryl Ester Hydrolase Activity of Mononuclear Leukocyte in Type 2 (Non-Insulin-Dependent) Diabetic Patients.* Tohoku J. Exp. Med., 1990, **160** (4), 375-381 — Acid cholesteryl ester hydrolase activity of mononuclear leukocytes was measured in 52 Type 2 (non-insulin-dependent) diabetic patients. Enzyme activity was significantly lower in the diabetic patients than in 14 age-matched control subjects (0.89 ± 0.08 (mean $\pm$ S.E.) vs. 2.20 ± 0.17 nmol/mg protein/hr, $p < 0.01$). In diabetic patients undergoing diet treatment only, the enzyme activity was significantly lower in poorly controlled patients than in well controlled patients (0.43 ± 0.03 vs. 1.15 ± 0.24 nmol/mg protein/hr, $p < 0.01$). In the diabetic patients, there was a significant negative correlation between the enzyme activity and serum total cholesterol or low density lipoprotein cholesterol level ($r = -0.361$, $p < 0.01$, $n = 52$ or $r = -0.630$, $p < 0.01$, $n = 28$). These results suggest that a low level of acid cholesteryl ester hydrolase activity in mononuclear leukocyte might play an important role in the progression of atherosclerosis in Type 2 diabetes. ——— cholesteryl ester hydrolase; mononuclear leukocyte; Type 2 (non-insulin-dependent) diabetes; atherosclerosis; low density lipoprotein cholesterol

It is well known that cholesteryl ester (CE) is accumulated in foam cells of atheromatous lesions, and accumulation is accelerated by a decreasing of acid CE hydrolase activity in lysosomes of the aortic wall (Small 1977). Also, this enzyme activity is considered to play an important role in the appearance and development of atherosclerosis in diabetes mellitus. It has been reported that this enzyme activity of the aortic wall in streptozotocin-induced diabetic rats was reduced and normalized by insulin treatment (Wolinsky et al. 1978). Further, it has been reported that the acid CE hydrolase activity in lysosomes of mononuclear leukocytes was markedly stimulated by insulin treatment in Type 2 (non-insulin-dependent) diabetic patients. However, it was not indicated whether the enzyme

activity was initially reduced in the patients when compared to healthy subjects (Henze and Chait 1981). Other reports in which acid CE hydrolase activity of mononuclear leukocytes was reduced (Yatsu 1981) or unchanged (Finegold and Coates 1984) compared to normal levels have disagreed. This study is designed to elucidate whether acid CE hydrolase activity of mononuclear leukocytes is reduced in Type 2 diabetic patients when compared to healthy subjects and to clarify whether change in the activity of this enzyme in diabetes mellitus is related to glycemic control of diabetes or the kind of treatment.

MATERIALS AND METHODS

Subject selection

Fifty-two Type 2 diabetic patients meeting the criteria for diagnosis of diabetes (National Diabetes Data Group 1979), and 14 normoglycemic healthy control subjects of matching ages were selected for the study. Fasting plasma glucose and HbA_{1c} levels in the patients ranged from 84 to 307 mg/100 ml (mean: 133 mg/100 ml) and 4.4 to 13.3% (mean: 6.4%), respectively. Duration of diabetes ranged from 0.3 to 23 years (mean: 8.8 years). Twenty-eight patients had evidence of background retinopathy and two had proliferative retinopathy. Eighteen patients had evidence of continuous proteinuria and six had coronary heart disease. There was no evidence of significant hepatic damage and infectious disorder.

Chemicals

Cholesteryl-[1-¹⁴C]oleate (specific activity 56 μ Ci/mmol) was obtained from the Japan Isotope Association, Tokyo. Egg yolk lecithin and cholesteryl oleate were obtained from Sigma Chemicals Co., St. Louis, Mo, USA. The purities of these preparations were confirmed by thin-layer chromatography.

Preparation of enzyme

Peripheral mononuclear leukocyte were isolated from freshly drawn, heparinized, venous blood obtained at 8:00 a.m. after an overnight fast by the method of Böyum (1968). The isolated cells were washed with Hanks balanced salt solution (pH 7.2) 3 times. Cell pellets ($2-3 \times 10^6$) were dissolved in 1 ml of 0.9% NaCl and frozen at -70°C until assayed within the next two weeks. Prior to the assay, the preparation was thawed and sonicated on ice for 30 sec using Ultrasonic Disruptor Model UR-200P with a microtip at a power setting of 40 watts (Tomy Seiko Co. Ltd., Tokyo).

Measurement of enzyme activity

Acid CE hydrolase activity was determined by a modification of the radioisotopic method of Brecher et al. (1977).

Preparation of vesicles. All vesicle preparations were made in order that the final mixture would contain 1% lecithin. 60 mg portion of egg yolk lecithin dissolved in chloroform-methanol 2:1 was mixed with 8.0 μ Ci of cholesteryl-[1-¹⁴C]oleate. Appropriate amounts of unlabeled cholesteryl oleate were then added so that the molar ratio of lecithin to the corresponding lipid was 66:1. The organic solvents were evaporated under nitrogen, the samples lyophilized, and the lipids resuspended in 6.0 ml of 0.15 M NaCl, 0.02% sodium azide, and 0.01 M Tris buffer (pH 7.4). A suspension was made by stirring the preparation on a vortex-type mixer, and the suspension was sonicated within a temperature range of 40–50°C for 10 min using a Ultrasonic Disruptor Model UR-200P at a power setting of 90 watts (Tomy Seiko Co. Ltd., Tokyo). The resulting suspension was centrifuged at $150,000 \times g$ for 30 min to sediment small fragments of titanium from the

disruptor horn as well as trace amounts of aggregated lipid. The final concentrations of lipids in the vesicle preparations were 12.1 mM egg yolk lecithin, 0.18 mM cholesteryl oleate.

Enzyme assay. Incubation tubes routinely contained 100 μ l of 0.15 M acetate buffer pH 4.5, 200 μ l of the vesicle preparation, and 100 μ l of enzyme solution. The final volume was adjusted to 450 μ l with 0.01 M Tris buffer. The resulting final pH was not different from the expected pH of 4.5. Incubations were routinely performed at 37°C for 60 min. The reaction was terminated by the addition of 3.0 ml of benzene-chloroform-methanol 1.0 : 0.5 : 1.2 (by vol.). A 0.6 ml portion of 0.3 M NaOH was then added; the mixture was stirred for 15 sec and then centrifuged at $1000 \times g$ for 10 min. A 0.2 ml portion of the upper phase was added to a Triton-toluene scintillation cocktail and the radioactivity was determined. Enzyme activity of samples in all subjects was measured on the same day. The intra-assay coefficients of variation of this assay were varified to be 3–4%. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per mg of protein per hour.

Other methods

Serum total cholesterol was measured enzymatically with cholesteryl ester hydrolase, cholesterol oxidase and peroxydase (Allain 1974). Triglyceride was assayed enzymatically with lipoprotein lipase, glycerol dehydrogenase and diaphorase (Tiffany 1974). High density lipoprotein (HDL)-cholesterol was estimated after lipoproteins containing apolipoprotein B were precipitated with heparin-MnCl (Burstein et al. 1970). Low density lipoprotein (LDL)-cholesterol was calculated using the method of Friedewald et al. (1972). Protein concentration was estimated by the method of Lowry et al. (1951).

Statistical analysis

All results are represented as mean $\pm$ s.e. Comparisons of values were made by analysis of variance (ANOVA) and least significant difference (LSD) methods. Correlation analysis was performed by Spearman's Rank Order Correlation method.

RESULTS

Acid CE hydrolase activity of mononuclear leukocyte was significantly lower in Type 2 diabetic patients than in control subjects (Table 1). Serum total cholesterol level was slightly higher in Type 2 diabetic patients than in control subjects, though the difference was not significant. Sex ratio, age and body mass index were similar in both groups.

In the Type 2 diabetic patients, the enzyme activity was lower in the group under diet only or with oral hypoglycemic agent (OHA), Glibenclamide treatment than in the group with insulin treatment, though the differences were not

TABLE 1. *Acid CE hydrolase activity of mononuclear leukocyte in Type 2 diabetic patients and control subjects*

	Number	Sex ratio (male : female)	Age (years)	BMI (kg/m ²)	Serum TC (mg/100 ml)	Acid CE hydrolase activity (nmol/mg protein/hr)
Diabetics	52	22 : 30	55.3 $\pm$ 1.9	22.3 $\pm$ 0.4	218 $\pm$ 52**	0.89 $\pm$ 0.08††
Controls	14	6 : 8	53.4 $\pm$ 3.2	22.1 $\pm$ 0.7	192 $\pm$ 21	2.20 $\pm$ 0.17

Values are given as means $\pm$ s.e. ** $p < 0.1$, †† $p < 0.01$ from Controls; BMI, body mass index; TC, total cholesterol.

TABLE 2. *Acid CE hydrolase activity of mononuclear leukocyte in Type 2 diabetic patients — Relation to kind of treatment and level of glycemic control*

Kind of treatment and level of glycemic control	Number of patients	Acid CE hydrolase activity (nmol/mg protein/hr)
Diet only	20	0.81 ± 0.13
Good control	9	1.15 ± 0.24
Poor control	11	$0.43 \pm 0.03^{\dagger\dagger}$
Oral hypoglycemic agent	15	0.83 ± 0.15
Good control	8	0.59 ± 0.28
Poor control	7	1.05 ± 0.13
Insulin	17	1.01 ± 0.14
Good control	5	1.23 ± 0.27
Poor control	12	0.93 ± 0.17

Values are given as means $\pm$ s.e. $^{\dagger\dagger}p < 0.01$ from Good control with diet only; Good control, less than 6.5% of HbA1c; Poor control, more than 6.5% of HbA1c.

Fig. 1. Correlation between acid CE hydrolase activity of mononuclear leukocyte and serum total cholesterol level or low density lipoprotein (LDL) cholesterol level in Type 2 diabetic patients. The correlation coefficients for the scattergram were calculated to be -0.361 ($p < 0.01$) and -0.630 ($p < 0.01$), respectively.

significant. In the Type 2 diabetic patients being treated with diet only, the enzyme activity was significantly lower in the poorly controlled (HbA_{1c} level more than 6.5%) group than in the well controlled (less than 6.5%) one (Table 2). The enzyme activity was similar in both groups with OHA or insulin treatment.

In the Type 2 diabetic patients, there was a significantly negative correlation between the enzyme activity and serum total cholesterol level (Fig. 1). HDL-cholesterol levels were determined and LDL-cholesterol levels calculated for twenty-eight Type 2 diabetic patients of all. In these patients, there was also a significant negative correlation between the enzyme activity and LDL-cholesterol level.

DISCUSSION

Both acid and neutral CE hydrolase activities have been reported in arterial tissue and in cultured arterial smooth muscle cells (Small 1977). Changes in acid CE hydrolase activity are considered to play an important role in appearance and development of atherosclerosis in Type 2 diabetic patients. Henze and Chait (1981) have reported that acid CE hydrolase activity of mononuclear leukocytes in Type 2 diabetic patients was markedly stimulated by insulin therapy, although they did not indicate whether enzyme activity was initially low in diabetics and restored to normal levels by insulin therapy, or whether it was normal and increased to supranormal levels. They did not report normal levels of acid CE hydrolase activity in their laboratory. Finegold and Coates (1984) have reported that acid lipase activity of mononuclear leukocytes in Type 2 diabetic patients was not different from that in control subjects and that it was unchanged following insulin treatment. However, Yatsu (1981) has briefly reported that acid CE hydrolase activity of mononuclear leukocytes was lower in diabetic patients than in control subjects. In this study, acid CE hydrolase activity of mononuclear leukocytes in Type 2 diabetic patients was reduced in comparison with age-matched control subjects. In order to clarify the reason why this enzyme activity was reduced in Type 2 diabetic patients, the relationship between this enzyme activity and insulin treatment was studied. This enzyme activity in the insulin-treated group was slightly high, but not significantly so when compared with two other groups treated with diet only and OHA. Furthermore, enzyme activity in the insulin-treated group was lower than that in age-matched control subjects. These data suggest that insulin treatment might cause a slight recovery of enzyme activity in Type 2 diabetic patients, even though the level is not restore to the normal range. Some kinds of insulin with short and intermediate acting have been used in this study. It has been unclear whether the enzyme activity was changed by these kinds of insulin. It is needed to clear this problem. In Type 2 diabetic patients, the relationship between activity of enzyme and glycemic control of diabetes was studied in each of the three groups (treatment with diet only, OHA or insulin). Enzyme activity was lower in poorly controlled patients

than in well controlled ones in the group under diet only treatment, and was similar in both poorly and well controlled patients in the groups under OHA and insulin treatment. These results suggest that activity of this enzyme might be reduced in poor glycemic control of diabetes, and might be also modified by OHA or insulin treatment.

It has been considered that serum total cholesterol and LDL-cholesterol levels could be regulated by a function of the LDL pathway in Type 2 diabetic patients (Chait et al. 1979). In this study, serum total cholesterol level in Type 2 diabetic patients was slightly higher than that in age-matched healthy subjects. It is an interesting problem whether changes in the activity of this enzyme are closely related to the function of the LDL pathway. The results of this study show that the activity of this enzyme is negatively correlated with serum total cholesterol or LDL-cholesterol. These results suggest that acid CE hydrolase of mononuclear leukocytes in diabetic patients might be a rate-limiting step affecting cholesterol metabolism and that acid CE hydrolase activity in the aortic wall might be reduced and stimulate cellular cholesterol accumulation over the long term, secondarily increasing the risk of atherosclerosis. Further investigation of this problem should be needed.

References

- 1) Allain, C. (1974) Enzymatic determination of total serum cholesterol. *Clin. Chem.*, **20**, 470-475.
- 2) Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest.*, **97**, Suppl. 21, 77-89.
- 3) Brecher, P., Pyum, H.Y. & Chobanian, A.V. (1977) Effect of atherosclerosis on lysosomal cholesterol esterase activity in rabbit aorta. *J. Lipid Res.*, **18**, 154-163.
- 4) Burstein, M., Scholnick, M.R. & Morfin, R. (1970) Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.*, **11**, 583-592.
- 5) Chait, A., Bierman, E.L. & Albers, J.J. (1979) Low-density lipoprotein receptor activity in cultured skin fibroblasts: Mechanism of insulin-induced stimulation. *J. Clin. Invest.*, **64**, 1309-1319.
- 6) Finegold, D.N. & Coates, P.M. (1984) Effect of diabetes and insulin therapy on human mononuclear leukocyte lysosomal acid lipase activity. *Metabolism*, **33**, 85-89.
- 7) Friedewald, W.T., Levy, R. & Fredrickson, D.S. (1972) Estimation of the concentration of low density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.*, **18**, 499-502.
- 8) Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
- 9) Henze, K. & Chait, A. (1981) Lysosomal enzyme activities and low density lipoprotein receptors in circulating mononuclear cells. *Diabetologia*, **20**, 625-629.
- 10) National Diabetes Data Group (1979) Classification and diagnosis of diabetes and other categories of glucose intolerance. *Diabetes*, **28**, 1039-1057.
- 11) Small, D.M. (1977) Cellular mechanisms for lipid deposition in atherosclerosis. *N. Engl. J. Med.*, **297**, 873-877.

- 12) Tiffany, K.O. (1974) Clinical evaluation of kinetic enzymatic fixed time. *Clin. Chem.*, **20**, 476-481.
 - 13) Wolinsky, H., Goldfischer, S., Capron, L., Capron, F., Coltoff-Schiller, B. & Kasak, L. (1978) Hydrolase activities in the rat aorta. *Circ. Res.*, **42**, 821-831.
 - 14) Yatsu, F. (1981) Lysosomal cholesterol ester hydrolase activity in mononuclear cells with atherosclerosis risk: Hyperlipidemia and diabetes mellitus (abstract). *Ann. Neurol.*, **10**, 104.
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