Expression of Low Density Lipoprotein Receptors in Lymphoblasts Induced by Anti-CD3 Antibody in Patients with Hypercholesterolemia

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Familial hypercholesterolemia(FH) is a disease based on defects of low-density lipoprotein receptors(LDL-R). To interrupt and control the natural course of this disease, early identification of these patients is important. The routine lipid profile tests for hypercholesterolemia can not differentiate objectively FH from secondary hypercholesterolemia. The exact diagnosis of FH heterozygotes is especially essential because it is easier to develop premature coronary heart diseases compared with secondary hypercholesterolemia. A simplified rapid and precise method for the mass screening of FH patients and the differentiation between FH heterozygote and secondary hyperlipidemia was needed. For the test, lymphocytes were used as target cells in LDL-R assay. After a 5 day culture with anti-CD3 Ab as a mitogen, indirect immunofluorescence stain and flow cytometric analysis were applied. The results were as follows; $74 \pm 9\%$ of the stimulated lymphoblasts from normal controls expressed LDL-R activity. Cultured, but unstimulated, lymphocytes of normal controls showed $27 \pm 8\%$ positivity and total cultured lymphocytes showed positivity of $46 \pm 11\%$ positivity. Lymphoblasts, unstimulated lymphocytes, and total cultured lymphocytes from hypercholesterolemia without FH showed $74 \pm 10\%$, $25 \pm 10\%$ and $50 \pm 17\%$, respectively, which showed no significant differences from normal control groups. FH Heterozygotes showed LDL-R positivity, $21 \pm 11\%$ in lymphoblasts, $11 \pm 6\%$ in unstimulated lymphocytes and $18 \pm 7\%$ in total cultured lymphocytes. These data imply that adequately stimulated lymphocytes might be used for detecting defects in LDL-R and used to differentiate FH from secondary hypercholesterolemia.

Key Words: LDL receptor, Familial hypercholesterolemia, Flow cytometry

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INTRODUCTION

LDL cholesterol uptake to cells is usually regulated by LDL-R on coated pits in cell plasma membranes(Goldstein et al.,1979). LDL-R protein is composed of five domains and each of them is controlled

by gene level (Hobbs et al., 1990). Any defects in such LDL-R gene levels result in high blood cholesterol (Brown and Goldstein, 1979, 1986; Brown et al., 1981).

Accumulation of blood cholesterol causes premature coronary atherosclerosis and several associated symptoms and signs. Especially FH homozygotes usually die before age of 20 because of myocardial infarction. FH Heterozygotes have high blood cholesterol levels immediately after birth but signs and symptoms usually do not appear until adulthood and their severities are variable. Examination of family history, tendon xanthoma and blood cholesterol levels are effectively used for diagnosis of FH. However, 25 % of FH do not show tendon xanthoma and some of FH, probably do not have high blood cholesterol levels (usually above 280–300 mg/dL). And many other diseases rather than FH can elevate blood cholesterol levels. So, objective diagnostic aid is needed for confirmation of FH.

Methods using flow cytometry (FCM) for LDL-R binding capacity of blood lymphocytes have been developed (Hiramatsu et al., 1980; Traill et al., 1986; Benhamamouch et al., 1988; Suzuki et al., 1990). Peripheral blood lymphocyte is preferable to fibroblast for LDL-R assay because specimen collection is convenient and it is easy to acquire the log-phase of cell growth.

MATERIALS AND METHODS

Subjects

Venous blood was obtained after 14 hours fasting from fifty normal individuals (serum total cholesterol < 230 mg/dL and triglyceride < 200 mg/dL by enzymatic autoanalyzer method) who had no family history or symptoms of FH. Twenty heterozygous FH clinically diagnosed by family history, high blood cholesterol level (total cholesterol > 280 mg/dL) and tendon xanthomas or history of myocardial infarctions were tested. All of them had been administered 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors for variable periods. Twenty-three subjects with moderate hypercholesterolemia (total cholesterol between 230 mg/dL and 330 mg/dL) but without clinical signs or family history of FH were also tested. They were usually diagnosed as diabetes mellitus, nephritis or hepatitis.

Isolation and culture of lymphocytes

Peripheral blood lymphocytes were isolated under sterile conditions at room temperature using density gradient method with heparinized bloods (Cuthbert et al., 1986). 1×10^6 /mL viable lymphocytes were resuspended in lipoprotein deficient medium containing RPMI 1640 (Gibco, USA), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL) and supplemented with bovine serum albumin (10 g/L, Sigma Chemical, USA). Anti-CD3 Ab was used as mitogen. Phytohemagglutinin (PHA; 200 µg/mL) was also tested as mitogen to determine if it was adequate as a mitogen of lymphocytes in FCM analysis. After incubation at 37°C for 5 days, the cells were harvested by centrifugation and resuspended in ice cold working buffer and washed three times. Cell viability was assessed by eosin Y dye exclusion method and at least 90 % of the recovered cells were found to be viable.

Flow cytometric application and analysis

Lymphocyte binding assays were carried out in an ice-bath to avoid interference due to endocytosis. 1×10^6 cells were used for binding test in a final volume of 100 µL. LDL-R Ab (Oncogene Science, USA) was added to lymphocytes at a final concentration of 5 µg/mL. Isotypic IgG_{2a} (Becton Dickinson, USA) was also used as antibody for negative control. Incubation was performed for 45 minutes. Each tube was centrifuged at 400 g, 4°C for 5 min. The washing step was repeated three times. The cells were then incubated at 4°C for 45 min with fluorescein isothiocyanate-labelled anti-mouse rabbit Ab (Carppele, Organon Teknika, USA). After incubation, the cells were washed three times with Phosphate buffered saline (PBS) and the pellet was fixed by resuspension in 500 µL of 1 % paraformaldehyde and immediately used for Facscan (Becton-Dickinson, USA).

Forward angle light scatter and axial light scatter were used to discriminate lymphocytes, lymphoblasts population and to gate out debris and dead cells. The results were expressed as the percentage of lymphocytes with a fluorescence intensity above the threshold (5–6 % positivity of negative control).

Table 1. Results of lipid profiles

subject	mean age (yrs)	total cholesterol (mg/dL)	triglyceride (mg/dL)	HDL-cholesterol (mg/dL)	LDL-cholesterol (mg/dL)
normal(n=50)	30±11	172±26	102±49	64±15	87±23
HC(n=23)	47±13	252±21	207±99	56±14	155±23
FH*(n=20)	50±9	322±47	147±92	45±12	246±59

Values ; expressed as mean±SD.

Abbreviations ; HC : hypercholesterolemia other than FH. FH : familial hypercholesterolemia.

* Two of FH were tested their lipid profiles after myocardial infraction.

RESULTS

1. Results of lipid profiles

Results of lipid profiles are shown in Table 1. In the normal control group consisted of 21 men and 29 women, total cholesterol was 172 ± 26 mg/dL, triglyceride 102 ± 49 mg/dL and HDL-cholesterol 64 ± 15 mg/dL. LDL-cholesterol was calculated as 87 ± 23 mg/dL. In the group of hypercholesterolemia other than FH consisted of 13 men and 10 women, total cholesterol was 252 ± 21 mg/dL, triglyceride 207 ± 99 mg/dL, HDL-cholesterol 56 ± 14 mg/dL and LDL-cholesterol 155 ± 23 mg/dL. In the FH group consisted of 8 men and 12 women, total cholesterol was 322 ± 47 mg/dL, triglyceride 246 ± 59 mg/dL, HDL-cholesterol 45 ± 12 mg/dL and LDL-cholesterol 246 ± 59 mg/dL. Student-t tests revealed that there were no significant differences in their blood lipids levels between sexes.

2. Pattern of lymphoblastogenesis by anti-CD3 Ab

Lymphocytes were cultured with anti-CD3 Ab for 5 days in lipoprotein deficient culture media. Most lymphocytes were transformed to lymphoblasts on light microscopic observation. Gating was possible according to areas of total lymphoid cell, lymphoblasts and unstimulated lymphocytes in FCM analysis(Fig. 1).

3. Pattern of lymphoblastogenesis by PHA stimulation

Lymphocytes were cultured with PHA for 5 days in lipoprotein deficient culture media. Most lymphocytes were transformed to lymphoblasts on light microscopic observation but lots of them were severely clustered. A widely scattered pattern was observed in FCM analysis, so gating of lymphocytes was almost impossible(Fig. 2).

Fig. 1. Cytofluorometric scatter plot of peripheral blood mononuclear cells using FCM after 5 days culturing with anti CD3 Ab stimulation. The ordinate is forward light scattered and the abscissa is right angle scattered. R1 represents unstimulated lymphocyte area and R2 represents stimulated lymphoblast area. Total lymphoid cell group is R1 adding R2.

Fig. 2. Cytofluorometric scatter plot of peripheral blood mononuclear cells using FCM after 5 days culturing with PHA(200 ug/mL) stimulation. The ordinate is forward light scattered and the abscissa is right angle scattered. Mononuclear cells were widely scattered and could not be gated.

Table 2. Positive rates of LDL-R expression on lymphocytes in normal control, hypercholesterolemia other than FH and FH groups.

	total cultured lymphoid cells	unstimulated lymphoid cells	lymphoblast cells
Control(n=50)	46±11 % ^a	27±9 % ^b	74±9 % ^c
male(n=21)	46±9 %	26±8 %	76±7 %
female(n=29)	47±12 %	28±8 %	72±9 %
HC(n=23)	50±17 % ^a	25±10 % ^b	74±10 % ^c
FH(n=20)	18±7 % ^a	11±6 % ^b	21±11 % ^c

Values ; expressed as mean± SD.

Abbreviations ; same as Table 1.

* Significant differences between a, b, c-a⁺, b⁺, c⁺ groups and a⁺, b⁺, c⁺ group, respectively(student-t test, p<0.0001).

4. LDL receptor quantification

Positive expression rates of LDL-R on the cell surface were classified according to the groups of total lymphoid cells, lymphocytes and lymphoblasts (Table 2). In the normal control, positive rates of LDL-R in the groups of total lymphoid cells, lymphocytes and lymphoblasts were 46±11 % 27±9 % and 74±9 % respectively. No statistical difference was found between sexes in student-t test. Histogram patterns of LDL-R expression in FCM were as in Fig. 3. In hypercholesterolemia other than FH, positive rates of LDL-R were 50±17 % 25±10 % and 74±10 % respectively. Histogram patterns were similar to those of normal control group. No statistical difference was found between normal controls and hypercholesterolemia other than FH. In FH heterozygotes,

positive rates of LDL-R in the groups of total lymphoid cells, lymphocytes and lymphoblasts were 18±7 % 11±6 % and 21±11 % respectively. There was significant difference in LDL-R positivity between normal control and FH heterozygotes(p<0.0001). Histogram patterns are shown in Fig. 4.

DISCUSSION

Previously, ¹²⁵I-LDL binding or immunoblotting method have been used for the assay of LDL-R on the surface of fibroblasts or lymphocytes(Ho et al., 1976). FCM analysis in lymphocytes allows better estimation of LDL-R activity because the intensity of the cellular fluorescence is measured independently for each viable cell by relatively easy procedures and

Fig. 3. LDL receptor expression from one of the normal controls on total cultured lymphocytes(A), cultured but unstimulated lymphocytes(B), stimulated lymphoblasts(C). Isotypic IgG_{2a} was used as primary control antibody and LDL-R antibody as primary test antibody.

Fig. 4. LDL receptor expression from one of the heterozygotes of FH on total cultured lymphocytes(A), cultured, but unstimulated lymphocytes(B), stimulated lymphoblasts(C).

acquisition of specimens for lymphocyte is much easier than for fibroblasts.

It has been known that freshly isolated lymphocytes rarely expressed LDL-R activity but LDL-R activity was increased after 3 days with culturing in lipoprotein deficient media(Ho et al.,1976 ; Bilheimer et al.,1978). But full expression of LDL-R was not acquired only by culturing in lipoprotein deficient media. Blastogenesis induced by mitogenic stimulation was an essential step for full expression of LDL-R activity, and the patterns of blastogenesis by PHA were very different from those by anti-CD3 Ab. Both mitogens showed strong blastogenic effects but FCM analysis was only possible in lymphocytes group using anti-CD3 Ab as mitogen. Lymphocytes stimulated by PHA were variously clustered and accurate gating of the lymphoid cell group was impossible. FCM analysis using PHA as mitogen has been previously reported(Hiramatsu et al., 1980 ; Benhamamouch et al.,1988), but Suzuki et al.(1990) suggested a problem similar to our study.

Lymphoblasts induced by anti-CD3 Ab were relatively well separated(Fig. 2) and even some clustered cells were easily separated by manual pipetting, so enumeration and gating by FCM were suitable (Fig. 1).

The lymphoblasts group was the most favorable to quantify the expression rates of LDL-R. Because not all lymphocytes were able to be transformed to lymphoblasts by mitogenic stimulation, such groups expressed relatively lower LDL-R activity. 20-40 % of lymphocytes also still remained as lymphocytes in

spite of mitogenic stimulation in our study. The lymphoblast group expressed the LDL-R activity fully enough to differentiate the control group and FH group. The margin between lymphoblasts and unstimulated lymphocytes was not clear cut on cytogram but we were able to discriminate these areas as seen in Fig. 1.

It was better that the decision about cut-off values(threshold) of negative controls should be made by experience at each laboratory. The cut-off value of LDL-R activity using FCM analysis has not been clearly defined. Because expression of LDL-R on lymphocytes largely depended on degree of blastogenesis, cut-off values could not be clearly defined. We decided the threshold value as 5-6 % of positivity of negative control.

The normal control group showed 27 ± 8 % positivity in the unstimulated lymphocyte group but 74 ± 9 % positivity in the lymphoblasts group. Total cultured lymphocytes(unstimulated lymphocytes plus lymphoblasts) represented 46 ± 11 % positivity. These results were rather lower positive rates than those of other studies(Benhamamouch et al., 1988 ; Suzuki et al., 1990). However, because threshold levels were not definitely represented in other studies and positive rates are altered according to change of threshold level, it seemed not to be an important factor. There were no overlapping values among study groups.

LDL-R positivity in hypercholesterolemia other than FH was 74 ± 10 % 25 ± 10 % 50 ± 17 % in lymphoblasts, unstimulated lymphocytes and total cultured lymphocytes groups, respectively. The results could

be expected but were meaningful as objective verification.

Clinically diagnosed heterozygotes of FH represented $18 \pm 7\%$, $11 \pm 6\%$ and $21 \pm 11\%$ positivity in total cultured lymphocytes, unstimulated lymphocytes and lymphoblasts groups, respectively. This could guide differentiation between FH and hypercholesterolemia other than FH objectively. Because all of these FH patients have been administered one of HMG-CoA reductase inhibitors, Lovastatin, it was proven that these drugs did not influence the expression of LDL-R on lymphocytes in this study. The results were probably that the culture method of our study represented the maximum expression rates of LDL-R on stimulated lymphocytes regardless of the pharmacological principle of HMG-CoA reductase inhibitors (increase of LDL-R expression). These findings made the diagnosis of FH brief and objective regardless of administration of drugs.

A study on FH homozygotes was not done because these are probably very rare in the Korean population and we could not find any. We need more studies about oxidized LDL (Witztum and Steinberg, 1991; Salonen et al., 1992), and Lp(a) etc, because FH only attributed about 5% of etiology of myocardial infarctions and ages which represent clinical symptoms are variable though the patients are diagnosed as FH.

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REFERENCES

- Benhamamouch S, Kuznierz JP, Agnani G, Marzin D, Leoerf JM, Fruchart JC, Clavy C. Determination of the LDL receptor binding capacity of human lymphocytes by immunocytofluorimetric assay. *Biochim Biophys Acta* 1988; 1002: 45-53.
- Bilheimer DW, Ho YK, Brown SM, Richard GW. Genetics of the low density lipoprotein receptor. *J Clin Invest* 1978; 61: 678-96.
- Brown MS, Goldstein JL. Receptor mediated endocytosis insights from the lipoprotein receptor system. *Proc Natl Acad Sci USA* 1979; 76: 3330-7.
- Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986; 232: 34-47.
- Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. *Science* 1981; 212: 628-35.
- Cuthbert JA, East CA, Bilheimer DW, Lipsky PE. Detection of familial hypercholesterolemia by assaying functional low-density lipoprotein receptors on lymphocytes. *N Engl J Med* 1986; 314: 879-83.
- Goldstein JL, Anderson RGW, Brown MS. Coated pits, coated vesicles and receptor mediated endocytosis. *Nature* 1979; 279: 679-85.
- Hiramatsu K, Sakai H, Endoh M, Arimori S. Surface properties of LDL-binding lymphocytes in human peripheral blood. *Immunology* 1980; 39: 311-16.
- Ho YK, Brown MS, Bilheimer DW, Goldstein JL. Regulation of low density lipoprotein receptor activity in freshly isolated human lymphocytes. *J Clin Invest* 1976; 58: 1465-74.
- Hobbs HH, Russel DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: Mutation analysis of a membrane protein. *Annu Rev Genet* 1990; 24: 133-70.
- Salonen JT, Yla-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssonen K, Palinski W, Witztum JL. Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet* 1992; 339: 883-87.
- Suzuki K, Hara M, Kitani A, Harigai M, Norioka K, Kondo K, Hirata F, Skita N, Kawakami M, Mitsuhiro K, Nakamura H. Augmentation of LDL receptor activities by interleukin-2 and anti-CD3 antibody: A flow cytometric analysis. *Biochim Biophys Acta* 1990; 1042: 352-58.
- Traill KN, Bock G, Winter U, Hilchenbach M, Jurgens G, Wick G. Simple method for comparing large numbers of flow cytometry histograms exemplified by analysis of the CD4(T4) antigen and LDL receptor on human peripheral blood lymphocytes. *J Histochem Cytochem* 1986; 34: 1217-21.
- Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991; 88: 1785-92.