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A novel method for determining functional LDL receptor activity in familial hypercholesterolemia: Application of the CD3/CD28 assay in lymphocytes

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ABSTRACT

Background: The objective of this study was to develop a new and simple method for measuring low-density lipoprotein receptor (LDLR) activity using peripheral lymphocytes enabling us to clinically diagnose familial hypercholesterolemia (FH) and ascertain the involved mutations (such as K790X mutation), that might not be clearly detected in the conventional method.
Methods: Our method comprised the following 2 features: first, we used anti-CD3/CD28 beads to stimulate T-lymphocytes to obtain a uniform fraction of lymphocytes and maximum up-regulation of LDLR. Second, we excluded the possibility of overestimation of lymphocyte signals bound only to its surface, by adding heparin to the cultured lymphocytes used for the LDLR assay.
Results: Based on the genetic mutation, the FH subjects were divided into 2 groups, K790X, (n=20) and P664L, (n=5), and their LDLR activities was measured by this method, which was found to be 55.3±8.9% and 63.9±13.8%, respectively, of that of the control group (n=15). In comparison, the LDLR activity was 86.1±11.6% (K790X) and 73.3±6.3% (P664L) of that of the control group when measured by the conventional method, indicating that impairment of LDLR function in FH K790X subjects was much more clearly differentiated with our method than with the conventional method (paired t-test, $p<0.0001$). The levels of LDLR expression also showed similar tendencies, that is, 89.4±13.2% (K790X) and 76.9±17.4% (P664L) of that of the control group when measured by the conventional method, and 78.1±9.7% (K790X) and 70.3±26.5% (P664L) when measured by our new method. In addition, we confirmed that there was little influence of statin treatment on LDLR activity among the study subjects when our method was used.
Conclusion: These results demonstrate that our new method is applicable for measuring LDLR activity, even in subjects with an internally defective allele, and that T-lymphocytes of the FH K790X mutation possess characteristics of that allele.

1. Introduction

Familial hypercholesterolemia (FH), a genetic defect that causes marked elevation of plasma low-density lipoprotein cholesterol (LDL-C), tendinous xanthomas, and premature coronary artery disease, is a result of genetic abnormalities of the LDL receptor (LDLR), apolipoprotein B, and neural apoptosis-regulated convertase 1 (NARC-1) [1,2]. Although all of these abnormalities create disturbances in the metabolism of LDL, the LDLR defect is the most important and frequent cause of FH. The LDLR, which is located on the surfaces of hepatocytes and other organs, binds to LDL and facilitates both its uptake by receptor-mediated endocytosis and its delivery to lysosomes, where the LDL particle is degraded [3].

The LDLR gene comprises 18 exons that span 45 kb, and encodes a single-chain glycoprotein containing 839 amino acids in its mature form [4,5]. Currently, >800 different mutations have been identified worldwide [2]. These mutations can be divided into 5 classes, based on their phenotypic effects: 1) null alleles; 2) transport-defective alleles;

Abbreviations: DiI, 3,3"-dioctadecylindocarbocyanin; FH, familial hypercholesterolemia; NARC-1, neural apoptosis-regulated convertase 1; FITC, fluorescein isothiocyanate; LDLR, low-density lipoprotein receptor; LPDS, lipoprotein deficient serum; rIL-2, recombinant interleukin-2; MF, mean fluorescence; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; CV, coefficient of variation.

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Table 1
Plasma lipid concentrations in study subjects

Subjects	Gender (m/f)	Age (yr.)	BMI (kg/m^2)	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)
Normolipidemia (n=15)	6/9	67±16	23.5±3.0	184±29	53±17	114±28	89±30
FH heterozygous K790X (n=20)	10/10	52±16	23.6±2.8	342±59*	47±14	274±54*	95±50
FH heterozygous P664L (n=5)	4/1	51±23	21.3±3.0	366±50*	42±7	283±67*	114±47

Values are shown as mean±SD. Lipid concentrations were measured before any medication was prescribed.
FH, familial hypercholesterolemia; BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride;
*p<0.0001, data compared with normolipidemia.

3) binding-defective alleles; 4) internalization-defective alleles; and 5) recycling-defective alleles.

Confirmation of the diagnosis of FH requires either documentation of an LDLR gene mutation, or demonstration of a decrease in LDLR activity [1]. However, clinically diagnosed FH is genetically more heterogeneous than conventionally expected [6], and approximately 40% of those diagnosed in the Japanese population do not exhibit these defects by genetic analysis [7]. This proportion is similar in other countries [8]. Therefore, a method that can estimate LDLR activity accurately and is complementary to genetic analysis is needed. Evaluation of LDLR activity using measurement of 3,3''-dioctadecylindocarbocyanin (DiI)-labeled LDL uptake in peripheral blood lymphocytes is conventionally used in the clinical setting [9]. Although highly sensitive, some studies have shown that FH can be distinguished from hypercholesterolemic non-FH by stimulation of T-lymphocytes [10,11]. Unfortunately, conventional methods currently available overestimate the LDLR activity of some types of defective LDLR, such as FH internalization-defective alleles, not because of the internalization but rather due to binding of DiI-LDL to the surface of lymphocytes [12]. The K790X mutation, which belongs to the class of internalization-defective alleles [13], is one of the most common mutations among Japanese FH subjects with a frequency of occurrence is nearly 19.5% [7]. Therefore, it is essential to develop a new and simple method to detect FH with this type of defective LDLR. If we can measure functional LDLR activity accurately, it would be easy to differentiate carriers of apolipoprotein B and NARC-1 mutations from carriers of LDLR mutations among clinically diagnosed FH subjects, as LDLR activity of the former would be in the normal range.

On the other hand, P664L mutation, which is classified as a transport-defective allele [14], is also one of the common mutations among Japanese FH subjects, and its frequency of occurrence is 6% [7]. Binding, internalization, and degradation were measured in the presence of sulfated glycosaminoglycans, such as heparin, to remove LDL from the surface of LDLR when skin fibroblasts were used [15]. Suzuki et al. used rIL-2 and anti-CD3 monoclonal antibodies to stimulate lymphocytes for proliferation [16]. Recently, an anti-CD3/CD28 monoclonal antibody with functional properties that allow easy stimulation of T-lymphocytes with high specificity became available [17]. In this study, we have developed a novel and simple method for detecting internalization-defective LDLR activity, especially in FH with an internalization-defective allele, by application of anti-CD3/CD28 beads stimulation and heparin-mediated assay of lymphocytes.

2. Materials and methods

2.1. Subjects

The study subjects were 25 genetically determined heterozygous FH subjects and 15 normal controls. The FH subjects were divided into 2 groups, based on their LDLR gene mutation: K790X (n=20) and P664L (n=5) (Table 1). Informed consent was obtained from all the subjects.

2.2. Lipid measurements

Fasting blood samples were drawn for assays. Concentrations of serum total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C)

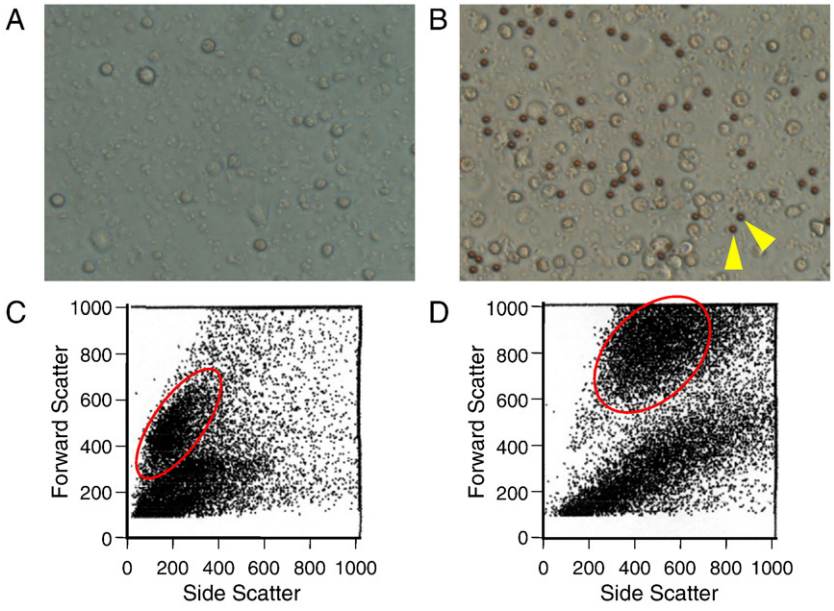

Fig. 1. Pattern of lymphoblastogenesis by LPDS and anti-CD3/CD28 beads A: Light microscopic findings of lymphoblastogenesis cultured with LPDS for 72 h. Magnification 400×. B: Light microscopic findings of lymphoblastogenesis cultured with anti-CD3/CD28 beads for 72 h. Beads are round (indicated by yellow arrows) while lymphocytes have a distorted shape. Magnification 400×. C: Forward-scatter (FSC) versus side-scatter (SSC) plots for lymphoblastgenesis cultured with LPDS for 72 h. D: FSC versus SSC plots for culture with anti-CD3/CD28 beads for 72 h. Stimulated lymphoblasts can be distinguished from unstimulated lymphoblasts according to their areas (surrounded by red circles).