methods

A method of direct measurement for the enzymatic determination of cholesteryl esters

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Abstract **A direct measurement method for the enzymatic determination of cholesteryl esters (CEs) without measuring total cholesterol (TC) and free cholesterol (FC) is described. In the first step, hydrogen peroxide generated by cholesterol oxidase from FC was decomposed by catalase. In the second step, CE was measured by enzymatic determination using a colorimetric method or a fluorometric method. The measurement sensitivity of the fluorometric method was more than 20 times that of the colorimetric method. Optimal conditions of the assay were determined, and examples of measured CE in human plasma, rat liver, and cultured cells are indicated. The method of directly measuring CE was simple and has exceptional reproducibility compared with the technique of subtracting FC from TC using each measured TC and FC.**—Mizoguchi, T., T. Edano, and T. Koshi. **A method of direct measurement for the enzymatic determination of cholesteryl esters.** ***J. Lipid Res.*** **2004.** 45: **396–401.**

Supplementary key words cholesterol assay • cholesterol oxidase catalase • plasma • liver • tissue culture

Free cholesterol (FC) has an important role as a component of cell membranes and a starting material for bile acid synthesis. However, cholesteryl ester (CE) is inactive when it is stored. In the progression of arteriosclerosis, CE accumulates in macrophages and smooth muscle cells and leads to the formation of foam cells. Determination of CE in cells or various tissues is of great importance in the fundamental research into atherosclerosis and the development of anti-atherosclerotic drugs.

Several methods of enzymatic determination for FC and total cholesterol (TC) have been published (1–5). Measurement of TC has measured FC resulting from the decomposition of CE by cholesterol esterase, and FC was contained in the native sample. To measure CE, individual TC and FC are measured, and FC is then subtracted from TC (indirect assay). Measuring CE by indirect assay is difficult in a sample solution with a low ratio of CE to FC.

This report describes a direct assay of CE by enzymatic determination without measuring TC and FC. In the first step, FC is oxidized by cholesterol oxidase to yield the corresponding cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide is decomposed into water and oxygen by catalase. At the second step, CE is measured by enzymatic determination using a colorimetric method or a fluorometric method. CE is hydrolyzed to FC by cholesterol esterase. FC is oxidized by cholesterol oxidase to yield the corresponding cholest-4-en-3-one and hydrogen peroxide. Thus, the enzymatic method for assaying FC is based on the measurement of hydrogen peroxide by way of peroxidase-coupled oxidation of hydrogen-sensitive probes. The hydrogen peroxide reacts with 4-aminoantipyrine or Amplex Red in the presence of peroxide to form a pigment or fluorescent products. The enzymatic reactions involved in the assay are as follows:

1. Decomposition of FC

$$FC + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-ene-3-one} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

2. Determination of CE by colorimetric method

$$CE \xrightarrow{\text{cholesterol esterase}} FC + \text{fatty acids}$$

$$FC + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-ene-3-one} + H_2O_2$$

$$\text{4-Aminoantipyrine} + \text{DAOS} + 2H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Quinone pigment}$$

3. Determination of CE by fluorometric method

$$CE \xrightarrow{\text{Cholesterol esterase}} FC + \text{fatty acids}$$

$$FC + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-ene-3-one} + H_2O_2$$

$$\text{Amplex red} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Resorufin (fluorescent)}$$

CE content in human plasma, rat liver, and cultured cells was measured using this method, and its usefulness was evaluated.

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MATERIALS AND METHODS

Materials

Cholesterol oxidase (*Pseudomonas* sp.), cholesterol esterase (*Pseudomonas* sp.), and MES were obtained from Wako Pure Chemical Industries (Osaka Japan). Cholesterol, cholesteryl oleate, catalase (bovine liver), 4-aminoantipyrine, and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS) were purchased from Sigma (St. Louis, MO). Horseradish peroxidase was from Boehringer Mannheim (Indianapolis, IN). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) was obtained from Molecular Probes (Eugene, OR).

Standards and CE sample preparations

FC, CE standards, and CE present in biological samples were diluted with 2-propanol containing 10% Triton X-100. The plasma samples were diluted with water. The amount of cholesteryl oleate was converted to the amount of FC, and a standard solution of CE was used. A rat liver was obtained from a 4-week-old Sprague-Dawley male rat. The liver was surgically removed under pentobarbital anesthesia. Lipids were extracted with chloroform-methanol (2:1) at 20 ml/g. Heparinized human plasma from healthy volunteers was prepared by centrifugation.

Cell preparation was performed by the following method. THP-1 cells (human monocytic leukemia cell line) were cultured in RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM 2-mercaptoethanol in a humidified CO_2 incubator (5%) at 37°C. THP-1 cells were suspended in RPMI 1640 containing 10% FBS and phorbol 12-myristate 13-acetate (PMA) followed by plating at a density of 1×10^6 cells/ml. The cells were cultured for 3 days in the same medium containing PMA. PMA-treated THP-1 cells (1×10^5 cells/well) in a 96-well plate were incubated with 50 μg/ml acetylated LDL (Ac-LDL) for 48 h. Lipids were extracted from the cells with hexane-2-propanol (3:2, v/v). The CE content was determined by the fluorometric method. The remaining cellular protein was dissolved in 0.1 N NaOH for protein determination. Protein was determined by the BCA method using a kit obtained from Pierce.

Method of direct measurement of CE

The composition of the reagent solution is given in **Table 1**. Measurement of CE by the colorimetric method was performed in the 96-well microplate. FC decomposition reagent (150 μl) was added to 25 μl of standard or samples and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the colorimetric method was added to the solution and allowed to react at 37°C for 15 min. The amount of CE was determined by measuring the increase in absorbance at 600 nm using a microplate reader (Labsystems Multiskan MS-UV).

Measurement of CE by the fluorometric method was performed in the 96-well microplate. FC decomposition reagent (150 μl) was added to 25 μl of standard or samples and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the fluorometric method was added to the solution and allowed to react at 37°C for 15 min. The fluorescence intensities were measured using a multi-well plate reader equipped with a filter set for excitation and emission at 530 and 580 nm, respectively (PE Biosystems CytoFlour).

Method of indirect measurement of CE

TC and FC contents were assayed by the colorimetric method with Cholesterol E-test wako and Free Cholesterol E-test wako (Wako Pure Chemical Industries). TC and FC contents were assayed by the fluorometric method with the Amplex Red Cholesterol Assay Kit (Molecular Probes). To measure CE, FC was subtracted from TC.

TABLE 1. Composition of reagents for measuring CE directly

Reagent	Composition
FC decomposition reagent	
MES buffer, pH 6.1	0.05 M
Cholesterol oxidase	1 U/ml
Catalase	45 U/ml
CE measurement reagent for the colorimetric method	
MES buffer, pH 6.1	0.05 M
Cholesterol oxidase	0.5 U/ml
Cholesterol esterase	4 U/ml
4-Aminoantipyrine	0.2 mM
N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt	1 mM
Horseradish peroxidase	4 U/ml
CE measurement reagent for the fluorometric method	
MES buffer, pH 6.1	0.05 M
Cholesterol oxidase	0.5 U/ml
Cholesterol esterase	4 U/ml
Amplex Red	20 μg/ml
Horseradish peroxidase	4 U/ml

CE, cholesteryl ester; FC, free cholesterol.

RESULTS

Optimization of conditions for a method of direct measurement of CE

The concentration of cholesterol oxidase in FC decomposition solution was examined using the FC standard solution (200 μg/ml). FC was completely decomposed when the concentration of cholesterol oxidase was at least 0.5 U/ml. Based on this result, the concentration of cholesterol oxidase was fixed as 1 U/ml (**Fig. 1A**). The concentration of catalase in FC decomposition solution was examined using the FC standard solution (200 μg/ml). FC was completely decomposed by catalase at a concentration of 7 U/ml (Fig. 1B). Moreover, the concentration of catalase in FC decomposition reagent was examined. In the colorimetric method, the absorbance values when 45 and 450 U/ml were used for the concentration of catalase were 95% and 80%, respectively (**Fig. 2A**). In the fluorometric method, the slopes when 45 and 450 U/ml were used for the concentration of catalase were almost unchanged. However, the intercept decreased depending on the concentration of catalase (Fig. 2B). The catalase concentration in FC decomposition reagent was 45 U/ml, which has little influence on the absorbance or fluorescence. The amount of decomposition of FC by direct assay of CE was examined. In the colorimetric method, the concentration at which FC is completely decomposed by this measurement method was 800 μg/ml (**Fig. 3A**). In the fluorometric method, the concentration at which FC is completely decomposed by this method was 80 μg/ml (Fig. 3B). CE standard solution was measured according to the direct assay using the colorimetric or fluorometric method. The linear relationship between CE concentration and absorbance or fluorescence is shown in **Fig. 4**. The measure-

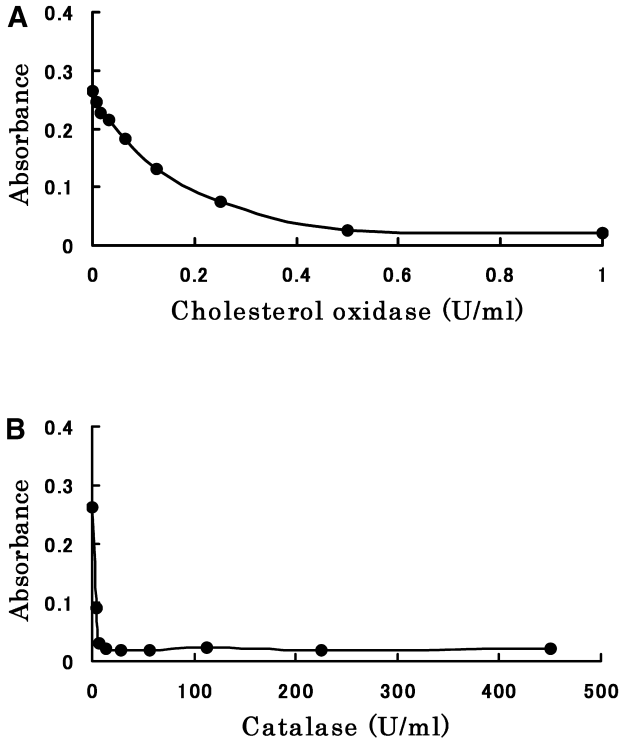

Fig. 1. Examination of cholesterol oxidase and catalase concentration in cholesterol decomposition reagent. A: Free cholesterol (FC) solution (200 μg/ml) was reacted with various amounts of cholesterol oxidase and 45 U/ml of catalase. Then, cholesterol was measured by the colorimetric method. B: FC solution (200 μg/ml) was reacted with 1 U/ml of cholesterol oxidase and various amounts of catalase. Then, cholesterol was measured by the colorimetric method.

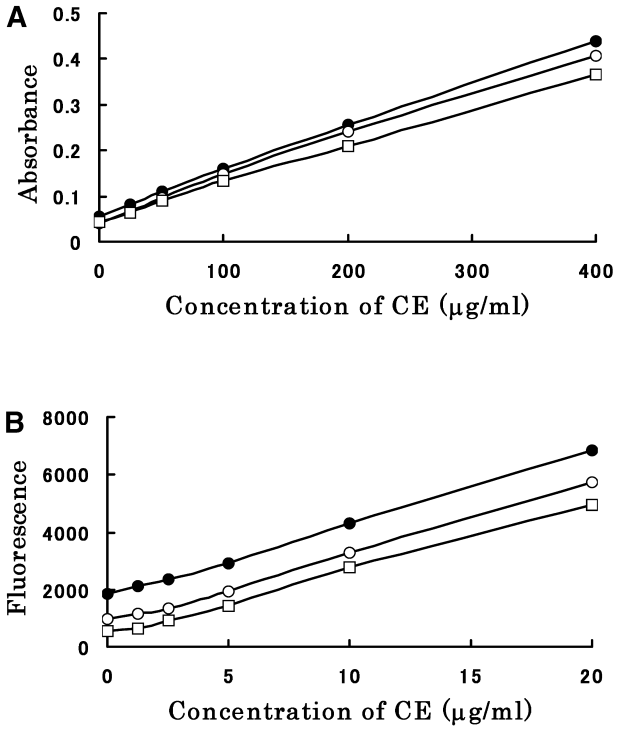

Fig. 2. Influence of catalase in FC decomposition reagent on the absorbance or the fluorescence of cholesteryl ester (CE). CE was measured using cholesterol decomposition reagent containing catalase (0, 45, or 450 U/ml) and cholesterol oxidase (1 U/ml) by the colorimetric method (A) or the fluorometric method (B). Open circles, 0 U/ml catalase; closed circles, 45 U/ml catalase; open squares, 450 U/ml catalase.

ment sensitivity of the fluorometric method was more than 20 times that of the colorimetric method.

Measurement of CE content in human plasma, rat liver, and cultured cells

The result of measuring CE in human plasma by the colorimetric method is shown in **Table 2**. The mean value and standard deviation of CE as measured by indirect and direct assay were 110 ± 6.5 mg/dl and 113 ± 1.9 mg/dl, respectively. The result of measuring CE in human plasma by the fluorometric method is shown in **Table 3**. The mean value and standard deviation of CE as measured by indirect and direct assay were 120 ± 9.4 mg/dl and 116 ± 2.8 mg/dl, respectively. The result of measuring CE in rat liver by the colorimetric method is shown in **Table 4**. The mean value and standard deviation of CE as measured by indirect and direct assay were 0.355 ± 0.088 mg/g and 0.390 ± 0.023 mg/g, respectively. The result of measuring CE in rat liver by the fluorometric method is shown in **Table 5**. The mean value and standard deviation of CE as measured by indirect and direct assay were 0.351 ± 0.060 mg/g and 0.342 ± 0.013 mg/g, respectively. There was no significant difference in the mean value of CE measured according to the indirect and direct assay. However, measurement error was significantly smaller for the indirect assay than for the direct assay. The effect on the CE accumulation in PMA-treated THP-1 cells (1×10^5 cells/well) incubated with Ac-LDL in a 96-well plate was examined. The result of measuring the amount of CE is shown in **Fig. 5**. The increase in CE in cells was confirmed by Ac-LDL.

DISCUSSION

Although various methods of measuring cholesterol by enzymatic determination have been reported, they are methods of measuring TC or FC. To measure CE, the technique of subtracting FC from TC is performed after measuring TC and FC. The method of measuring CE directly by HPLC has been described (6). This method requires only lipid extraction and hydrolysis of triglycerides and is of sufficient sensitivity to allow measurement in cultured cells. The HPLC method has the advantage of allowing quantification of individual CE species. However, the HPLC method is unsuitable for measuring total CE.

This research examined a direct assay of CE by enzymatic determination without measuring TC and FC. In the first step, FC was oxidized by cholesterol oxidase to yield the corresponding cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide was decomposed into water

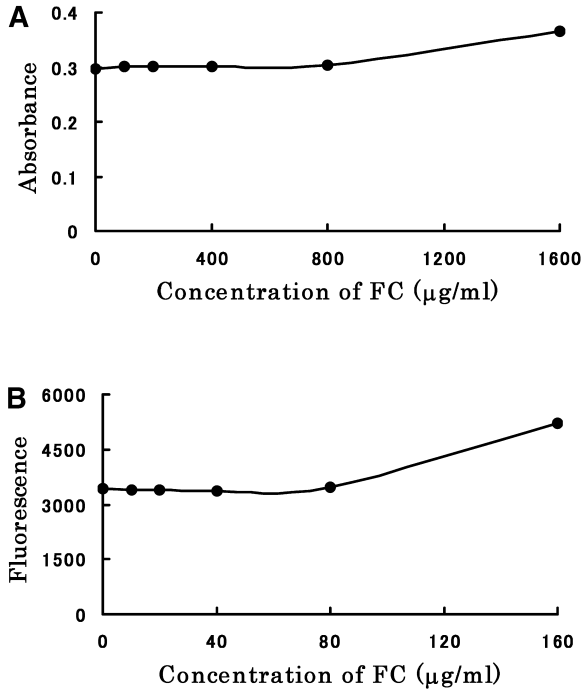

Fig. 3. Examination of the amount of decomposition of FC in the sample solution. A: Various amounts of FC were added to CE solution (200 μg/ml) and measured by the colorimetric method. B: Various amounts of FC were added to CE solution (10 μg/ml) and measured by the fluorometric method.

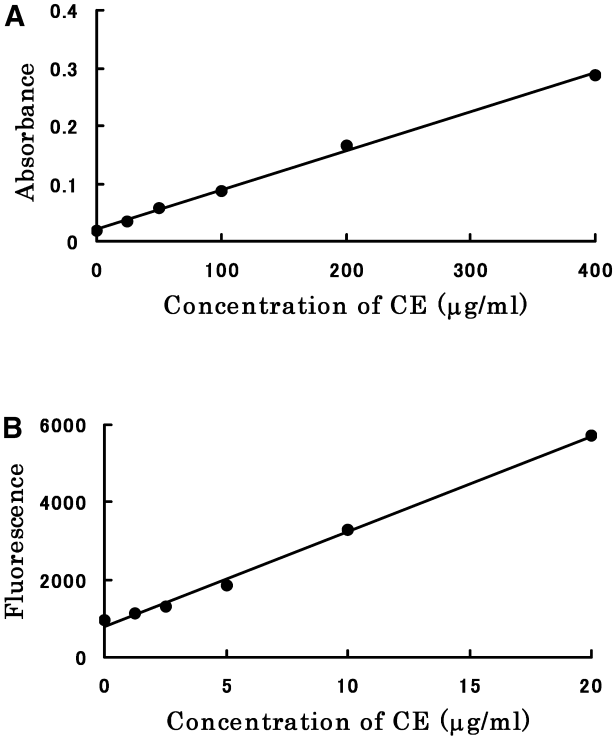

Fig. 4. Standard curves for the determination of CE. Various amounts of CE were measured by the colorimetric method (A) or the fluorometric method (B).

and oxygen by catalase. In the second step, CE was measured by enzymatic determination that used a colorimetric or a fluorometric method. The measurement sensitivity of the fluorometric method was more than 20 times that of the colorimetric method. The same FC decomposition reagent was used in the colorimetric and fluorometric methods. FC concentrations in the sample solution, which did not affect the measurement of CE by the colorimetric or fluorometric method, were 800 and 80 μg/ml, respectively. The difference could be attributable to differences of sensitivity. In the colorimetric method, the catalase in FC decomposition reagent hardly influenced the slope and intercept of the CE standard curve. In the fluorometric method, although the slope did not change, the intercept decreased depending on the concentration of catalase. The increased amount of catalase may decompose the few oxides contained in 2-propanol-containing 10% Triton X-100 used for sample dilution. The intercept did not change in the colorimetric method because of low sensitivity. The slope for the standard curve did not change because the coupling velocity with peroxidase is much faster than the decomposition velocity with catalase. Direct assay of CE by the fluorometric method is an effective means of measuring CE in minute quantities in a sample.

CE in human plasma and rat liver was measured by indirect and direct assay. In either case, there was no significant difference in the mean value of CE measured by indirect or direct assay. However, the direct assay has exceptional reproducibility compared with the indirect assay. When the ratio of CE present is small compared with FC, the measurement error with the indirect assay becomes substantial. The cause of the large error was the small difference in the two measured values, FC and TC. In contrast,

TABLE 2. Measurement of CE in human plasma by the colorimetric method

	Indirect Assay			Direct Assay
Exp. No.	TC[a]	FC[b]	CE[c]	CE[d]
			mg/dl	
1	142	36	106	112
2	146	36	110	112
3	150	36	114	114
4	150	36	114	112
5	147	36	111	112
6	156	37	119	112
7	153	36	117	113
8	136	36	100	112
9	141	40	101	116
10	142	36	106	117
Mean	146	37	110	113
SD	6.1	1.3	6.5	1.9

[a] Total cholesterol (TC) was assayed with Cholesterol E-test wako.
[b] FC was assayed with Free Cholesterol E-test wako.
[c] CE was calculated by subtracting FC from TC.
[d] CE was measured using the direct assay method. Human plasma was diluted with water (1:5) and used as a sample solution. FC decomposition reagent (150 μl) was added to 25 μl of sample and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the colorimetric method was added to the solution and allowed to react at 37°C for 15 min. The amount of CE was determined by measuring the absorbance at 600 nm.

TABLE 3. Measurement of CE in human plasma by the fluorometric method

Exp. No.	Indirect Assay TC[a]	Indirect Assay FC[b]	Indirect Assay CE[c]	Direct Assay CE[d]
	mg/dl			
1	156	45	111	112
2	169	41	128	119
3	163	40	123	113
4	173	42	131	116
5	172	40	132	111
6	161	45	116	116
7	151	47	104	118
8	169	46	123	117
9	157	46	111	118
10	162	46	116	118
Mean	163	44	120	116
SD	7.3	2.7	9.4	2.8

[a] TC was assayed with the Amplex Red Cholesterol Assay Kit.
[b] FC was assayed with the Amplex Red Cholesterol Assay Kit.
[c] CE was calculated by subtracting FC from TC.
[d] CE was measured using the direct assay method. Human plasma was diluted with water (1:100) and used as a sample solution. FC decomposition reagent (150 μl) was added to 25 μl of sample and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the fluorometric method was added to the solution and allowed to react at 37°C for 15 min. The fluorescence intensities were measured with a filter set for excitation and emission at 530 and 580 nm, respectively.

TABLE 5. Measurement of CE in rat liver by the fluorometric method

Exp. No.	Indirect Assay TC[a]	Indirect Assay FC[b]	Indirect Assay CE[c]	Direct Assay CE[d]
	mg/g			
1	2.377	1.987	0.390	0.335
2	2.230	2.026	0.204	0.357
3	2.332	2.022	0.310	0.365
4	2.350	2.005	0.345	0.343
5	2.403	2.014	0.389	0.330
6	2.355	1.974	0.381	0.355
7	2.452	2.060	0.392	0.326
8	2.347	1.974	0.373	0.344
9	2.395	2.064	0.331	0.326
10	2.372	1.973	0.399	0.343
Mean	2.361	2.010	0.351	0.342
SD	0.058	0.034	0.060	0.013

[a] TC was assayed with the Amplex Red Cholesterol Assay Kit.
[b] FC was assayed with the Amplex Red Cholesterol Assay Kit.
[c] CE was calculated by subtracting FC from TC.
[d] CE was measured using the direct assay method. Rat liver was extracted with 20 ml/g chloroform-methanol (2:1). The extract was dissolved in 30 ml/g 2-propanol-containing 10% Triton X-100 and used as a sample solution. FC decomposition reagent (150 μl) was added to 25 μl of sample and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the fluorometric method was added to the solution and allowed to react at 37°C for 15 min. The fluorescence intensities were measured with a filter set for excitation and emission at 530 and 580 nm, respectively.

the error in the direct assay was slight because there was only one type of measurement.

Because CE in cultured cells is very slight compared with FC, there is a large measurement error in the indirect assay. Moreover, because there was little CE content in cells, numerous cells were required to measure CE. The effect on CE accumulation in PMA-treated THP-1 cells incubated with Ac-LDL was examined. As a result of measuring the amount of CE in 1×10^5 cells in a 96-well cultivation plate by the direct assay, the increase in CE in cells was confirmed by Ac-LDL. Recently, HMG-CoA reductase and ACAT have attracted attention as therapeutic agents for atherosclerosis. This direct assay method should be-

TABLE 4. Measurement of CE in rat liver by the colorimetric method

Exp. No.	Indirect Assay TC[a]	Indirect Assay FC[b]	Indirect Assay CE[c]	Direct Assay CE[d]
	mg/g			
1	1.960	1.826	0.134	0.376
2	1.963	1.634	0.329	0.385
3	1.927	1.540	0.387	0.387
4	1.962	1.570	0.392	0.452
5	1.933	1.585	0.348	0.380
6	1.948	1.596	0.352	0.394
7	1.952	1.552	0.400	0.393
8	1.990	1.541	0.449	0.370
9	1.933	1.608	0.325	0.385
10	1.994	1.557	0.437	0.378
Mean	1.956	1.601	0.355	0.390
SD	0.023	0.085	0.088	0.023

[a] TC was assayed with Cholesterol E-test wako.
[b] FC was assayed with Free Cholesterol E-test wako.
[c] CE was calculated by subtracting FC from TC.
[d] CE was measured using the direct assay method. Rat liver was extracted with 20 ml/g chloroform-methanol (2:1). The extract was dissolved in 2 ml/g 2-propanol-containing 10% Triton X-100 and used as a sample solution. FC decomposition reagent (150 μl) was added to 25 μl of sample and allowed to react at 37°C for 15 min. CE measurement reagent (75 μl) for the colorimetric method was added to the solution and allowed to react at 37°C for 15 min. The amount of CE was determined by measuring the absorbance at 600 nm.

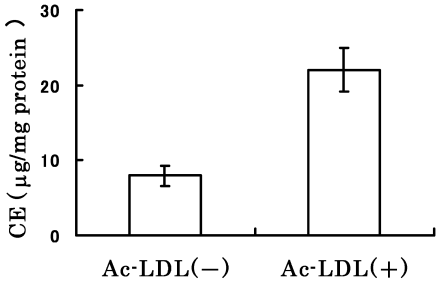

Fig. 5. Effect on CE accumulation in phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells of incubation with acetylated LDL (Ac-LDL). Each value represents the mean ± SD (n = 6). PMA-treated THP-1 cells (1×10^5 cells/well) in a 96-well plate were incubated with 50 μg/ml Ac-LDL for 48 h. The cells were washed two times with 200 μl of 0.9% NaCl. Lipids were extracted from the cells with 200 μl of hexane-2-propanol (3:2, v/v). The extract was dissolved in 25 μl of 2-propanol-containing 10% Triton X-100 and was used as a sample solution. The CE content was determined by the fluorometric method. The remaining cellular protein was dissolved in 0.1 N NaOH for protein determination.

come an effective means of evaluating medicine in a cultured cell line and investigating changes in CE in cells in particular.

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