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A biotinylated perfringolysin O derivative: A new probe for detection of cell surface cholesterol

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Abstract

θ-Toxin is a cholesterol-binding, pore-forming cytolysin of *Clostridium perfringens*. To detect cell surface cholesterol, we prepared a θ-toxin derivative, BCθ by biotinylation of a protease-nicked θ-toxin, which has the same binding affinity for cholesterol as θ-toxin without cytolytic activity. Human erythrocytes, V79 cells and human umbilical vein endothelial cells (HUVEC), were stained with BCθ coupled with FITC-avidin, and then the cells were analyzed by either flow cytometry or laser confocal microscopy. The fluorescence intensity increased in both intact and briefly fixed cells when treated with BCθ. BCθ-treated V79 cells were stained by neither trypan blue nor propidium iodide, indicating that BCθ stained just the outer surface of the plasma membrane of vital cells. Treatment of the cells with digitonin, a cholesterol-sequestering reagent, decreased the fluorescence intensity to the background level, indicating that BCθ staining is specific for cholesterol. The fluorescence intensity of erythrocytes pre-permeabilized with a small amount of θ-toxin increased more than ten-fold, suggesting higher cholesterol contents in the inner layer of the plasma membrane. When cells were cultured with cholesterol-depleted medium, the fluorescence intensity stained by BCθ decreased remarkably in V79 cells, but did not change in HUVEC. This indicates that cell surface cholesterol may be provided in different ways with these two cell lines. These results suggest that BCθ can be a useful probe for visualizing cell surface cholesterol and for evaluating the effects of cellular events on the topology and distribution of cholesterol. © 1997 Elsevier Science B.V.

Keywords: Cholesterol; Cell surface; Fluorescence microscopy; Membrane bilayer; Cytolysin

1. Introduction

Cholesterol is one of the major constituents of biological membranes and lipoproteins. It affects the structure and function of biological membranes through determining the physico-chemical nature of membranes such as membrane fluidity. Cholesterol

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also influences the pathology of atherosclerosis and cholesterol-storage disorders such as type-C Niemann-Pick disease [1–3]. Recent and growing evidence suggests a role of cholesterol in the maintenance of integrity and function of membrane microdomain structures called caveolae [4–8]. In spite of the importance of cholesterol in membrane physiology and pathology, many questions remain to be answered concerning its organization and behavior in plasma membranes and intracellular organelles.

Cholesterol functions as a receptor for thiol-activated cytolysins [9–12], digitonin [13] and polyene antibiotics, such as filipin and amphotericin B [14,15]. θ-Toxin (perfringolysin O) is one of the thiol-activated cytolysins produced by *Clostridium perfringens*. This group of toxins binds to membrane cholesterol and causes membrane damage by forming pore-like structures in membranes [9–12]. These toxins are suggested to have the same underlying mechanism for membrane lysis because of close similarities in their primary structures [16–20] and other characteristics [21]. It has been reported that approximately 1.6 molecules of cholesterol neutralize the activity of one molecule of a thiol-activated cytolysin [22], suggesting that one molecule of the cytolysin binds one or two molecules of cholesterol.

In order to study the mechanism of hemolytic activity of θ-toxin, we have obtained two θ-toxin derivatives, Cθ and MCθ. Cθ is produced by limited proteolysis of θ-toxin with subtilisin Carlsberg and exists as a complex of 38 K and 15 K fragments [23]. MCθ is produced by reductive methylation of Cθ [24]. Cθ and MCθ have the same binding affinity to erythrocyte membranes as θ-toxin. They bind to cholesterol, but not to other membrane constituents including cholesterol esters and phospholipids [24,25]. They have no hemolytic activity at temperatures of 20°C or below in the case of Cθ, or even at 37°C in the case of MCθ [24,25]. Cθ and MCθ do not form oligomeric structures on membranes at temperatures where they do not cause hemolysis as judged by electron microscopy [24,25] and sucrose density gradient analysis [26,27].

Such modified toxins would be useful for visualizing cell surface cholesterol without membrane damage when used in combination with anti-(θ-toxin) antibody. In this report we prepared another modified θ-toxin, biotinylated Cθ (BCθ), which has similar characteristics to MCθ, as a tool for staining cellular cholesterol. When coupled with FITC-conjugated avidin, BCθ can stain cholesterol on the outer surface of plasma membranes of erythrocytes and cultured adherent cells in a procedure that (1) is easier than that using MCθ and (2) requires no antibody. In this paper, using such a θ-toxin derivative as a tool, we further demonstrate that BCθ can be used to envisage the effects of cellular events on the topology and distribution of cellular cholesterol.

2. Materials and methods

2.1. Preparation of θ-toxin and its derivatives

θ-Toxin was purified from a culture filtrate of *C. perfringens* type A strain PB6K N5, and subtilisin Carlsberg-digested θ-toxin (Cθ) and methylated Cθ (MCθ) were prepared as described previously [23,24].

Biotinylation of Cθ was performed with a biotinylation kit (RPN 28, Amersham, UK). The reaction was started by addition of 100 μl of biotinylation reagent to 1 mg Cθ in 1 ml of 50 mM sodium borate buffer pH 8.6. After 1 h stirring at 25°C, the reaction mixture was applied to a Sephadex G-25 column (9.1 ml) equilibrated with phosphate-buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 2.1 mM Na_2HPO_4 and 1.2 mM KH_2PO_4). The column was eluted with PBS and fractions containing biotinylated Cθ (BCθ) were collected and stored at 4°C after the addition of 1 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO).

Hemolytic activity, hemolysis inhibition activity and binding specificity of modified toxins were measured as described previously [26].

2.2. Erythrocytes and cells

Human and sheep blood were mixed with equal volumes of sterile Alsever solution and stored at 4°C. Erythrocytes were collected by centrifugation (900 × *g*, 5 min) and washed four times with PBS. Aliquots of washed erythrocytes were suspended in PBS containing 0.1% glutaraldehyde at a 1% hematocrit and kept in an ice bath for 15 min. The suspensions were washed four times with PBS and used as fixed erythrocytes.

V79 cells established from Chinese hamster lung were obtained from the Japanese Cancer Resources Bank. The cells were maintained in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, ICN Biomedical, CA). Human umbilical vein endothelial cells (HUVEC) were prepared and maintained as described previously [28]. In some experiments, medium containing 10% fetal bovine lipoprotein-deficient serum (LPDS, Sigma) was used.

2.3. Fluorescent staining of erythrocytes and cultured cells with BCθ

Washed or fixed erythrocytes were suspended in PBS containing 1 mg/ml BSA (PBS/BSA) or in PBS/BSA containing 5 μg/ml BCθ at a hematocrit of 0.3%. The suspensions were incubated at 25°C for 20 min, then washed three times with 10 volumes of PBS, and resuspended in 40 μg/ml fluorescein avidin D (Vector Laboratories, Burlingame, CA) diluted with PBS/BSA. After incubation for 20 min at 25°C, erythrocytes were washed with PBS and then analyzed for FITC fluorescence using an EPICS Elite flow cytometer (Coulter Electronics, Hialeah, FL) at a wavelength of 488 nm for excitation and 525 nm for emission. In some experiments, fixed erythrocytes were treated with 1.5 ng/ml θ-toxin for 10 min at 25°C, and then stained as described above.

V79 cells or HUVEC seeded in 24-well plates (Nunc, Roskilde, Denmark) were cultured to semi-confluence in MEM containing 10% FBS. The medium was changed to serum-free MEM and cells were incubated further for 2 h to eliminate the effects of serum cholesterol. Cells in the 24-well plates were put on ice and rinsed three times with ice-cold PBS. Then, 0.5 ml of 0.1% glutaraldehyde in PBS was added to each well and the plates were kept on ice for 15 min. Fixed cells were washed with PBS four times, and then incubated with 30 μg/ml BCθ for 20 min at room temperature. Control cells were incubated with PBS/BSA. After several washes with PBS, the cells were treated with 40 μg/ml fluorescein avidin D for 20 min at room temperature. The cells were rinsed with PBS, and then the FITC fluorescence was detected with an Olympus microscope IMT2-RFL. The cells were also analyzed with a fluorescence image analyzer-equipped confocal laser cytometer (ACAS 570, Meridian Instrument, Okemos, MI, USA).

To examine the specific binding of BCθ to membrane cholesterol, fixed cells were preincubated with 4 μM digitonin (Wako Pure Chemical Industries, Japan) for 10 min at 25°C. After the incubation, BCθ was added to the reaction mixture to give the final concentrations of 5 μg/ml for erythrocytes and 30 μg/ml for cultured cells. Following procedures for fluorescent staining were the same as described above.

Total cellular cholesterol was measured using the method of Richmond [29] after solubilization with detergent as described by Gamble et al. [30].

3. Results

3.1. Characterization of biotinylated Cθ (BCθ)

Hemolytic activity and hemolysis-inhibition activity of Cθ, MCθ, and biotinylated Cθ (BCθ) are shown in Table 1. Hemolytic activity (HD_{50}) was defined as the amount of toxin required to cause 50% lysis of sheep erythrocytes (0.5% suspension, 1 ml) in a 30 min incubation at 37°C. Cθ caused 50% hemolysis at a concentration of 0.57 ng/ml. However, no hemolysis was detected with MCθ or BCθ at concentrations of 68.8 and 164 μg/ml, respectively. In addition, when V79 cells were incubated with 10 μg/ml of BCθ for 30 min at room temperature, the ratio of dead cells stained with trypan blue was only 1.1%. In contrast, more than 95% of V79 cells incubated with 2.5 ng/ml of θ-toxin were stained with trypan blue. Therefore, biotinylation was as effective as methylation in decreasing hemolytic activity and cytotoxicity.

Binding activities of toxin derivatives were measured by their ability to inhibit θ-toxin-induced

Table 1
Hemolytic activity and binding activity of θ-toxin derivatives

	Hemolytic activity HD_{50} (ng/ml)	Binding activity ID_{50} (ng/ml)
θ	0.29	–
Cθ	0.57	36.5
MCθ	> 68 800	25.8
BCθ	> 164 000	25.5

hemolysis. Hemolysis caused by 30 ng/ml θ-toxin at 10°C was inhibited by addition of toxin derivatives to the reaction mixture. The amounts of the derivatives required to cause 50% inhibition of θ-toxin-induced hemolysis (ID_{50}) are listed in Table 1. The ID_{50} value of BCθ was close to those of Cθ and MCθ, whose binding affinities to erythrocytes were the same as that of intact θ-toxin, as measured by binding of ^{125}I-labeled toxin derivatives to erythrocytes [24,25].

For analysis of binding specificity to cholesterol, lipids extracted from human erythrocytes were developed on a TLC plate. The plate was incubated with BCθ, and then immunostaining was carried out using anti-(θ-toxin) serum and peroxidase-conjugated second antibody. Only one spot, corresponding to

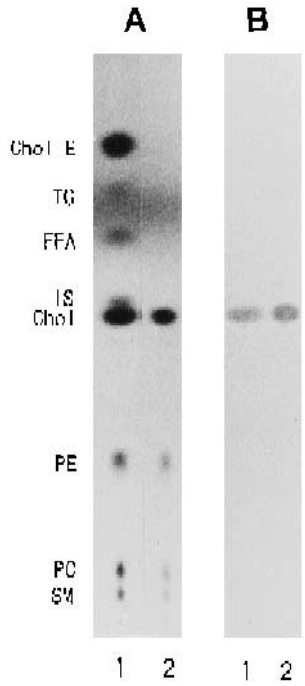

Fig. 1. Specific binding of BCθ to cholesterol. Aliquots of standard lipids (lane 1) and chloroform/methanol extract from human erythrocytes (lane 2) were applied on TLC plates (Polygram Sil G; Machery-Nagel, Duren) and developed with two solvent systems as described previously [25]. (A) One piece was soaked in a 3% cupric acetate/8% phosphoric acid solution and heated at 140°C for detection of lipids. (B) The other piece was treated with 10 μg/ml BCθ followed by 1/250 diluted anti-(θ-toxin) rabbit serum and then 1/500 diluted horseradish peroxidase-conjugated anti-(rabbit IgG) serum (Cappel, Organon Teknika, Belgium). After washing, the plate was developed with 4-chloro-1-naphthol and hydrogen peroxide. The standard lipids are indicated as follows: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Chol, cholesterol; IS, oleyl alcohol; FFA, oleic acid; TG, triolein; Chol E, cholesterol oleate.

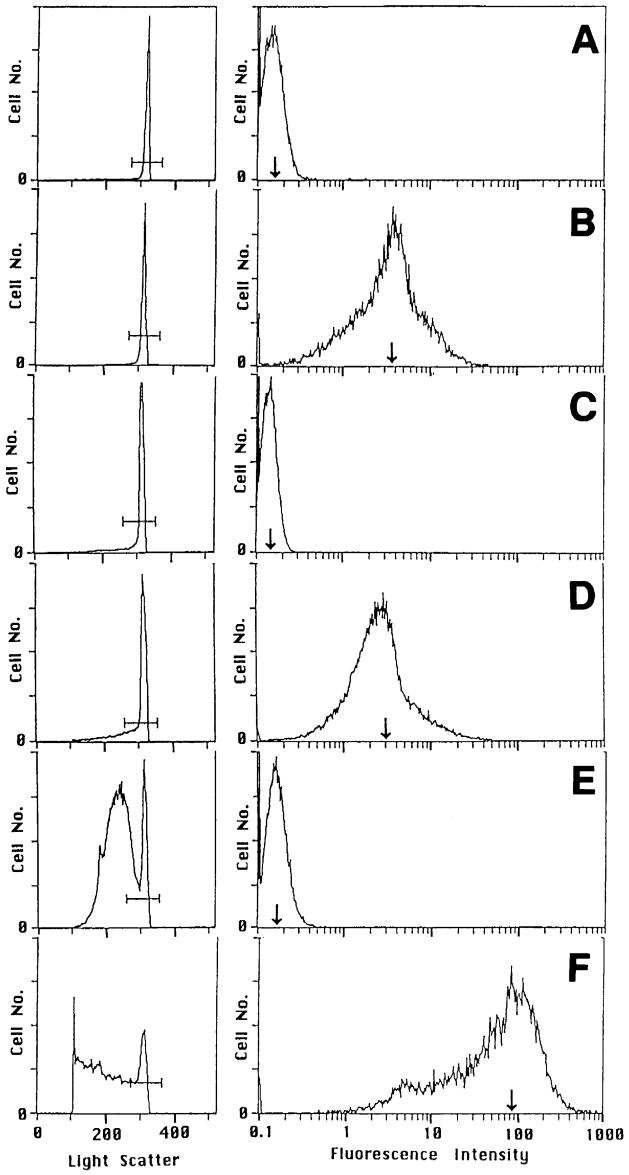

Fig. 2. Flow cytometric profiles of erythrocytes treated with BCθ and other reagents. Human erythrocytes were treated with BCθ and/or other reagents and then with 40 μg/ml fluorescein avidin D and analyzed by EPICS Elite. Control cells were treated with BSA instead of BCθ. Left: light scatter profiles which indicate particle size. Right: fluorescence intensity profiles of particles which have the size of intact erythrocyte indicated in the range of light scatter profiles. (A, B) intact erythrocytes; (C–F) fixed erythrocytes. (A, C) control; (B, D) 5 μg/ml BCθ; (E) 4 μM digitonin and 5 μg/ml BCθ; (F) 1.5 ng/ml θ-toxin treatment before 5 μg/ml BCθ addition.

cholesterol, was detected (Fig. 1). Other lipids such as phospholipids and neutral lipid standards including esterified cholesterol were not stained.

These results indicate that the binding affinity and specificity of BCθ to cholesterol is equivalent to that of θ-toxin.

3.2. Staining of erythrocytes with BCθ

Since BCθ has an ability to bind to cholesterol and no hemolytic activity, we tried to detect surface-bound BCθ by the fluorescence of FITC-conjugated avidin. Washed human erythrocytes were incubated with 5 μg/ml BCθ followed by 40 μg/ml fluorescein avidin D. The erythrocyte suspension was analyzed with a flow cytometer (EPICS), and fluorescence was measured only for particles whose sizes were the same as intact erythrocytes. As shown in Fig. 2, the fluorescence intensity of control erythrocytes treated with fluorescein avidin D alone stayed at a very low level (0.16 at peak, Fig. 2A), although the fluorescence intensity of BCθ-treated cells was obvious (3.8 at peak, Fig. 2B). The same results were obtained with erythrocytes fixed with glutaraldehyde: the peak fluorescence intensity was 0.15 for the control and 3.1 for BCθ (Fig. 2C and D). In addition,

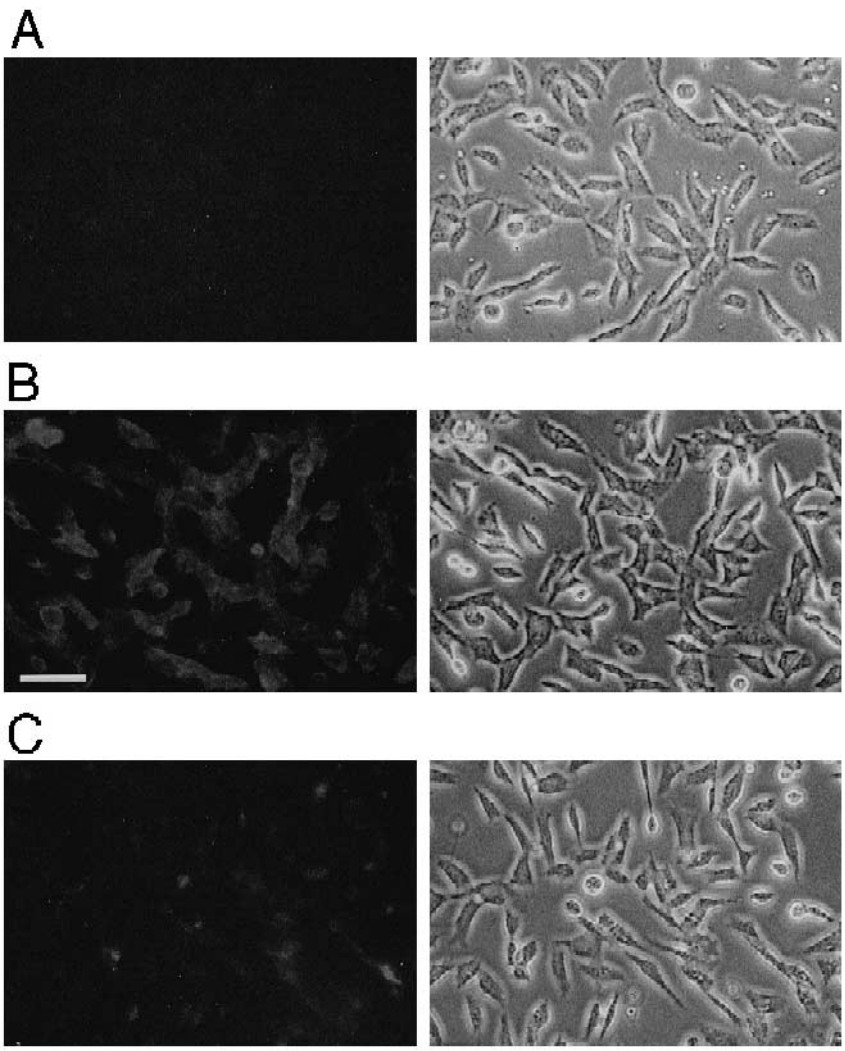

Fig. 3. Surface cholesterol staining of V79 cells with BCθ. Fluorescence (left) and phase contrast (right) observations of V79 cells treated with or without BCθ in BSA-containing buffer and then with fluorescein avidin D. (A) control; (B) 30 μg/ml BCθ; (C) 4 μM digitonin and 30 μg/ml BCθ. Bar = 50 μm.

the treatment of fixed erythrocytes with 4 μM digitonin erased the fluorescence intensity by BCθ (0.17 at peak, Fig. 2E). The decrease in fluorescence by the addition of digitonin, a cholesterol-sequestering agent, indicates that BCθ binding to erythrocytes is specific for cholesterol.

This fluorescence by BCθ seems to originate from cell surface cholesterol because BCθ does not lyse cellular membranes. In order to confirm this, fixed erythrocytes were treated with θ-toxin at a low concentration (1.5 ng/ml) before staining with BCθ and fluorescein avidin D. The peak of fluorescence intensity of θ-toxin-treated erythrocytes was 83 (Fig. 2F), over 20 times greater than that of BCθ-treated erythrocytes. This increase in fluorescence intensity seems to be due to the increase in the amount of BCθ that could pass through the plasma membrane pores made with θ-toxin oligomers [11,26,27] and then bind to cholesterol in the inner leaflet.

3.3. Staining of adherent cells with BCθ

As the surface cholesterol of erythrocytes was stained with BCθ, we next tried to stain cultured adherent cells. V79 cells fixed with glutaraldehyde were treated with 30 μg/ml BCθ, and then 40 μg/ml fluorescein avidin D. Control cells treated with fluorescein avidin D alone were not stained. The BCθ-treated cells were clearly stained and the fluorescence was abrogated by the addition of digitonin (Fig. 3). To investigate cellular membrane damage, BCθ-treated cells were incubated with 5 μg/ml propidium iodide for more than 20 min. Almost none of the cells stained with BCθ had stained nuclei; the

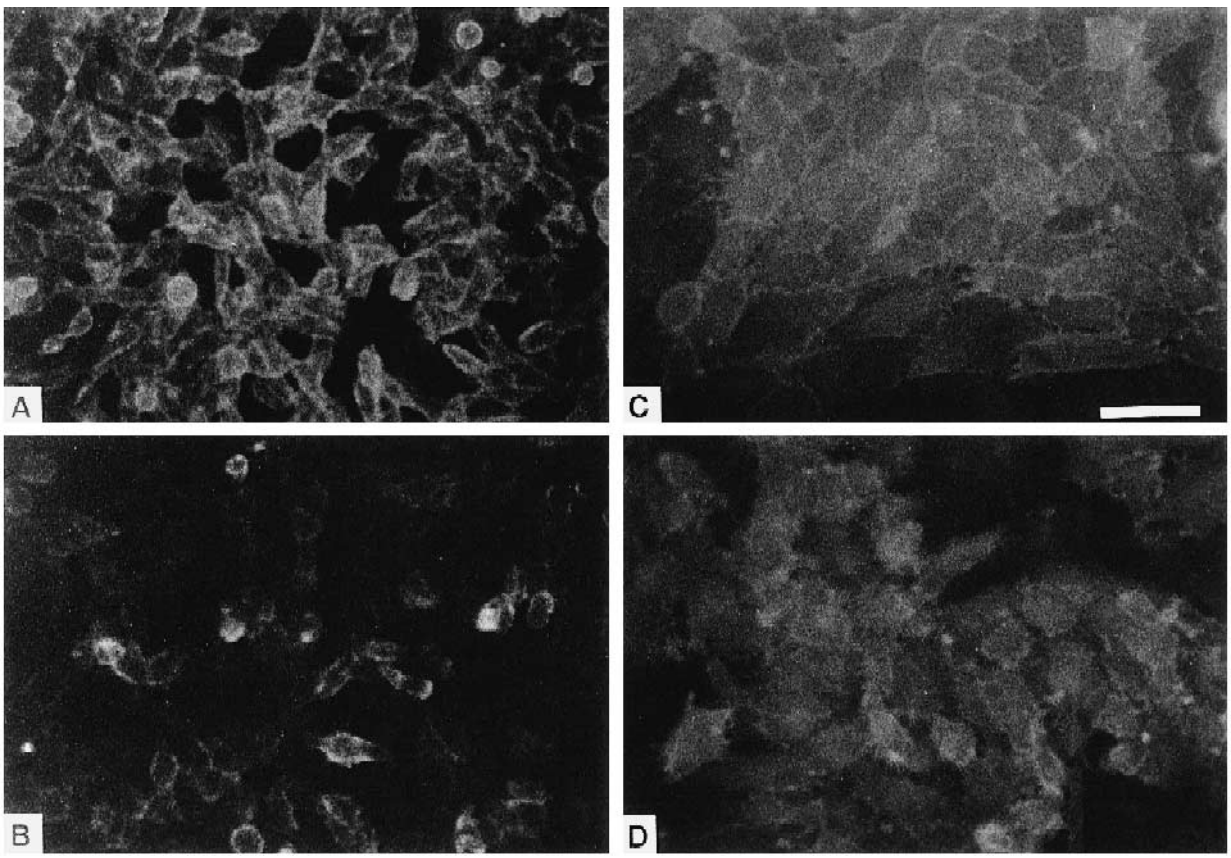

Fig. 4. Decrease in fluorescence intensity of V79 cells cultured in lipoprotein-deficient serum. Fluorescence observations of V79 cells (A, B) and HUVEC (C, D) treated with BCθ in BSA-containing buffer and then with fluorescein avidin D. (A, C) Cells cultured in MEM containing 10% FBS. (B, D) Cells cultured in MEM containing 10% LPDS. Bar = 50 μm.

exceptions were a few dead cells which were detaching from the wells (data not shown). These results indicate that BCθ can detect cell surface cholesterol without cellular membrane injury. Other cells, such as PC12, 3T3, WI-38 and HeLa cells, were also stained with BCθ (data not shown).

3.4. Effects of cholesterol turnover perturbation on cholesterol staining with BCθ

When V79 cells were cultured in the medium with 10% lipoprotein-deficient serum (LPDS) for 24 h, total cellular cholesterol decreased to 1.31 $\mu g/10^6$ cells, which is 65% of the level in the control cells (2.02 $\mu g/10^6$ cells) cultured in the medium with 10% FBS. At the same time, cell surface cholesterol was detected by BCθ-derived fluorescence (Fig. 4). The fluorescence intensity of V79 cells cultured in LPDS medium decreased to $15.18 \pm 2.35\%$ of that in the control cells. In contrast, HUVEC cultured with LPDS had the same fluorescence intensity as the control cells. The fluorescence intensity of HUVEC was calculated by the ACAS 570 equipped with a computer. The intensities were $10.7 \pm 1.22 \times 10^6$/cell for the control and $9.66 \pm 0.63 \times 10^6$/cell for LPDS (Fig. 5). However, the treatment of HUVEC with normal medium containing 1000 ng/ml of simvastatin, an HMG-CoA reductase inhibitor, caused a decrease in the fluorescence intensity to 42% of that in the control cells. Moreover, when simvastatin was added to the LPDS medium, the fluorescence intensity was reduced in a dose-dependent manner (Fig. 5). Addition of 100 ng/ml simvastatin to HUVEC cultured with LPDS medium reduced the fluorescence intensity to less than 10% of that in the control culture. Although we were unable to obtain a sufficiently large number of HUVEC for analysis of cellular cholesterol contents, these results suggest that de novo cholesterol synthesis is induced in HUVEC cultured with LPDS, and that inhibition of cholesterol synthesis with simvastatin diminishes cell surface cholesterol.

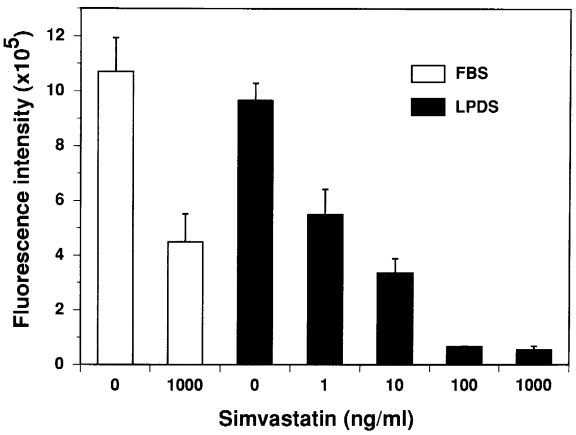

Fig. 5. Decrease in fluorescence intensity by treatment with simvastatin. HUVEC were cultured in MEM containing 10% FBS or 10% LPDS for 2 days and then the medium was replaced with medium containing simvastatin at the indicated concentrations. After 2 days culture in the presence of simvastatin, cells were stained with BCθ and fluorescein avidin D and analyzed as described in Section 2.

4. Discussion

Thiol-activated cytolysins specifically bind to membrane cholesterol resulting in membrane lysis. Although Pendleton et al. [31] attempted to analyze cholesterol distribution in membrane with some thiol-activated cytolysins (streptolysin O and cereolysin) and their ferritin-labeled antibody, the native localization of cholesterol was not clear because of the cytolytic activity of the cytolysins. Using θ-toxin derivatives, cell surface cholesterol can be stained without membrane damage. We successfully stained cells using MCθ, anti-(θ-toxin) antiserum and FITC-conjugated second antibody (data not shown). However, BCθ makes the procedure much easier and requires no anti-(θ-toxin) antibody to give similar results.

In the flow cytometric analysis of erythrocytes, both intact cells and cells fixed with 0.1% glutaraldehyde were well stained with BCθ at the same intensity. These results suggest that BCθ binding did not cause membrane lysis even in intact cells, and that the fixing condition was mild enough for BCθ to bind to cell surface cholesterol as well as it did in intact cells. In contrast, digitonin and filipin are difficult to use without membrane damage. Digitonin and polyene antibiotics such as filipin form specific complexes with 3-β-hydroxy sterols which can be detected by electron microscopy [32,33]. The subcellular distribution of cholesterol was detected with a

relatively low dose (10^{-5} M) of filipin by fluorescence microscopy using ultraviolet excitation [34]. Although these methods are useful for analyzing cholesterol distribution in fixed cells, they are hardly applicable to viable cells. Since BCθ binds cell surface cholesterol at a low concentration (30 μg/ml; 10^{-7} M) without membrane impairment, BCθ serves as a more useful probe for the analysis of cell surface cholesterol.

To examine the applicability of BCθ in delineating the cholesterol topology, fixed erythrocytes were treated with a small amount of θ-toxin and then stained with BCθ. θ-Toxin was used to form large pores on the membrane [10,11] so that BCθ could get inside the cells. As the dose of θ-toxin used in this experiment (1.5 ng/ml, 1/3000 of BCθ) was very small, BCθ binding was not practically affected. After the treatment, the fluorescence intensity increased more than 10-fold (Fig. 2F). Thus, in θ-toxin-treated cells, BCθ can bind cholesterol located on both the outer and inner leaflets of the membrane bilayer. The result shown in Fig. 2F indicates that the amount of BCθ bound to cholesterol in the inner leaflet is greater than that bound to cholesterol in the outer surface of the cell membrane. One explanation is that the majority of cholesterol might exist in the inner leaflet of erythrocyte membrane. The asymmetric distribution of phospholipids in the plasma membrane is well known [35,36], and an asymmetric distribution of cholesterol was also reported in erythrocytes [37,38], fibroblasts [39], tumor cells [40] and synaptic plasma membranes [41]. In these reports, the cholesterol content of the inner leaflet was in the range of 75–88%, which means that only 1/4–1/10 of the total cholesterol exists on the cell surface. Our observation is consistent with the reported results.

In the second experiment, we employed BCθ staining for elucidating cholesterol metabolism in the cultured adherent cells. We have shown that total cellular cholesterol decreased to 65% of the control in V79 cells cultured with LPDS medium. This result suggests that in V79 cells, de novo synthesis of cholesterol does not efficiently occur under lipoprotein-deficient conditions. It should be noted that the fluorescence intensity of LPDS-treated V79 cells decreased to less than 20% of the control cells (Fig. 4A and B), suggesting that cholesterol in the cell surface is mainly supplied by extracellular lipoproteins. This remarkable decrease in fluorescence intensity might also imply that the decrease in cellular cholesterol primarily affects the cholesterol located in the outer leaflet of the plasma membrane.

Another type of cell, HUVEC gave a different result when treated with LPDS. The fluorescence intensity of LPDS-treated HUVEC was apparently the same as that of control cells (Fig. 4C and D). However, when simvastatin, an HMG-CoA reductase inhibitor, was added to the LPDS medium, the fluorescence intensity of HUVEC decreased dramatically and dose dependently (Fig. 5), indicating that the main origin of the cholesterol in the cell surface was de novo synthesis in LPDS medium. On the other hand, treatment of HUVEC with simvastatin in FBS medium decreased the fluorescence intensity to about 42% of that in the control cells, suggesting that cell surface cholesterol supplied from serum lipoproteins as well. These results suggest that the origin of cell surface cholesterol is different among cell lines. BCθ seems to be a useful tool for analyzing cellular cholesterol metabolism.

Recent evidence in literature indicates that cholesterol affects the organization and function of glycophosphatidyl inositol (GPI)-anchored proteins and caveolae, which are non clathrin-coated pits. It has been reported that the clustered organization of the folate receptor, a GPI-anchored protein, and the integrity of caveolae are disrupted when MA104 cells are deprived of cholesterol by addition of cholesterol-sequestering agents, such as filipin and nystatin [6,42]. Metabolical deprivation of cholesterol also affects the clustered organization and transport function of the folate receptor [7]. Cholesterol has been reported to regulate the cell surface expression of GPI-anchored CD14 on human monocytes [43]. The cholesterol-staining method described here seems to be useful for analyzing the cholesterol dynamics in membranes. In fact, we have demonstrated that FITC-labels concentrate to caveola-rich areas during the incubation of living keratinocytes treated with BCθ [44,45].

In the present study, a new probe BCθ was successfully employed to analyze membrane cholesterol distribution and dynamic changes when the cholesterol metabolism was modulated. This probe would have further potential if applied to other detection

systems using enzyme-, ferritin- or gold-conjugated avidin.

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