Regular Article

Methyl- β -Cyclodextrin Impairs the Monocyte-Adhering Ability of Endothelial Cells by Down-Regulating Adhesion Molecules and Caveolae and Reorganizing the Actin Cytoskeleton

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Due to its powerful ability to deplete cholesterol from the plasma membrane of cells, methyl- β -cyclodextrin (M β CD) has been widely used as a putative research tool in cell biology. Recently, recruiting M β CD as an effective drug (*e.g.*, antitumor drugs) has been developed. However, it remains unclear whether M β CD, when it enters the blood circulation as a drug, influences the functions of the endothelium, *e.g.*, the adhesion of leukocytes to the endothelium. In this study, we found that M β CD can impair the adhesion of monocytes to the monolayer of endothelial cells by lowering the cell-surface adhesive force and expression of adhesion molecules and caveolae-related molecules on/in endothelial cells, and reorganizing the actin cytoskeleton of endothelial cells. The data imply that M β CD, when recruited as a drug, potentially helps to inhibit inflammation or initiation/progression of atherosclerosis since its important early step is the adhesion of circulating leukocytes (*e.g.*, monocytes) to the endothelium.

Key words methyl- β -cyclodextrin; monocyte; endothelial cell; adhesion molecule; caveola; actin cytoskeleton

Beta-cyclodextrin (β -CD) is a cyclic oligosaccharide consisting of seven α -(1,4)-linked glucopyranose subunits. Due to its relatively low water solubility (approximately 2% weight by weight),¹⁾ various derivatives of β -CD have been developed to significantly improve the water solubility, among which methyl- β -cyclodextrin (M β CD) is the one studied most intensively. M β CD is well known as a putative powerful cholesterol-depleting agent and commonly used to deplete plasma membrane cholesterol from cultured mammalian cells.^{2–4)} M β CD-cholesterol complexes also can efficiently replenish cells with cholesterol as cholesterol donors.^{3,4)}

Moreover, due to the structure of a lipophilic internal cavity surrounded by hydrophilic outer surface, M β CD was applied as a drug delivery system.⁵⁾ Recently, it was found that M β CD *per se* potentially has antitumor effects and has the possibility of being an effective antitumor drug.^{6–11)} However, it remains uncertain whether M β CD, as an anticancer drug, influences the functions of the endothelium, *e.g.* the adhesion of circulating leukocytes (*e.g.* monocytes) onto the endothelium which is an important step during inflammation or initiation/progression of atherosclerosis.

In this study, the effect of M β CD on the adhesion of monocytes onto the monolayer of endothelial cells was first evaluated. Then, the effects of M β CD on cell-surface adhesive force properties, expression/distribution of adhesion molecules and caveolae-related molecules, and actin cytoskeleton of endothelial cells were investigated to elucidate the potential underlying mechanisms.

MATERIALS AND METHODS

Cell Culture Human umbilical vein endothelial cells (HUVECs) were purchased from Xiangya Central Experiment Laboratory (Hunan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, U.S.A.) supplemented with 10% (w/v) fetal calf serum (Hyclone, South Logan, UT, U.S.A.), 100 U/mL penicillin, and 100 μ g/mL streptomycin. For all experiments, cell cultures had been passaged approximately 5 times.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay MTT assay was performed as reported previously.¹²⁾ Approximately 8×10^3 HUVECs were plated in each well of a 96-well plate and cultured at 37°C in a 5% CO₂ incubator for 24 h. After washing with phosphate buffered saline (PBS), cells in different wells were treated with different concentrations (0, 5, 10, 20 mM) of M β CD (Sigma, U.S.A.) for 30 min at 37°C. After PBS washes, 100 μ L of fresh medium and 20 μ L 5 mg/mL MTT were added to each well, and the cells were incubated for 4 h. After reaction termination and solution removal, 100 μ L of dimethyl sulfoxide (DMSO) was added to each well, and the plate was shaken gently at 37°C for 10 min. The absorbance (optical density) was measured at 578 nm with a microplate reader (Rayto).

Monocyte Adhesion Assay Celltracker C34552 (Invitrogen, U.S.A.) was utilized to stain THP-1 cells for *ca.* 30 min at 37°C according to the manufacturer's instruction. The cells were washed twice with serum-free medium to remove excess dyes. Then the cells were added onto the confluent monolayer of HUVECs pretreated with or without 1, 5, and 10 mM M β CD for 30 min at 37°C and incubated for 2 h at 37°C. After washing twice with PBS to remove the unattached cells, the samples were observed under a confocal microscope. The

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excitation and emission wavelengths for Celltracker C34552 were 577 and 602 nm, respectively. The number of attached fluorescently-stained THP-1 cells in each field was counted.

Adhesive Force Mapping by Atomic Force Microscopy (AFM) The living HUVECs treated with or without 10 mM M β CD for 30 min at 37°C were immediately detected by Agilent series 5500 AFM in contact mode. The adhesive force mapping of an area of 30×30 μ m on the cell surface was performed. The length and width of the AFM cantilevers were 200 and 28 μ m, respectively, with a resonant frequency of 77 kHz and a spring constant of *ca.* 0.04 N/m.

Confocal Microscopy and Flow Cytometry For fluorescence imaging, an LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss inverted microscope and a Zeiss Plan-Neofluar objective (40×/0.75) was recruited. Flow cytometric acquisition and analysis were performed in a FACSCalibur flow cytometer (BD Biosciences, U.S.A.).

Fluorescence Staining of Adhesion Molecules, Caveolin-1, and Phosphatidylinositol 4,5-Bisphosphate (PIP₂)

The fluorescence staining of each molecule was performed according to the manufactory provided protocol. Briefly, the cells pre-treated with or without M β CD for 30 min at 37°C were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS (for adhesion molecules and caveolin-1) or Tris-buffered saline (TBS); for PIP₂) two times, and then subjected to fluorescence staining.

For the surface staining of intercellular adhesion molecule 1 (ICAM-1), the fixed cells were incubated with phycoerythrin (PE)-conjugated anti-human CD54 (eBioscience, CA, U.S.A.) at a final concentration of 5 μ g/mL in PBS for 1 h at 37°C. After washing with PBS three times, the samples were subjected to flow cytometry.

For caveolin-1 staining, the fixed cells were incubated with 5 μ g/mL rabbit anti-caveolin-1 (Abcam, London, U.K.) in PBS plus 0.05% saponin for 1 h at 37°C and then with Pacific blue-conjugated goat anti-rabbit immunoglobulin G (IgG) (Life Technologies, U.S.A.) in PBS (1:1000) for 30 min at 37°C. After washing with PBS three times, the samples were subjected to confocal microscopy and flow cytometry.

For PIP₂, the intracellular staining was performed as reported previously.¹³⁾ The fixed cells were permeabilized with 0.5% saponin for 15 min at room temperature, washed three times with TBS, and blocked with 1% bovine serum albumin (BSA) in TBS over night at 4°C. Then, the cells were stained with biotinylated mouse anti-PIP₂ IgM (Echelon Biosciences, UT, U.S.A.) at a final concentration of 10 μ g/mL in TBS for 1 h at 37°C and subsequently with streptavidin-conjugated Alexa Fluor 555 (Life Technologies, U.S.A.) in TBS for 30 min at 37°C. After washing thoroughly with distilled water, the cells were subject to confocal microscopy and flow cytometry.

Visualization and Quantification of Actin Cytoskeleton HUVEC Cells were plated in petri dishes and cultured in the incubator (37°C, 5% CO₂) for 24 h to reach a 70% confluence. After treated with or without 10 mM M β CD for 30 min at 37°C, the cells were fixed by 4% paraformaldehyde for 20 min, washed three times with PBS, and stained by anti-paxillin monoclonal antibody (mAb) (overnight at 4°C; Abcam) plus Alexa fluor 488 goat anti-mouse IgG (for 2 h at 37°C) and tetramethylrhodamine (TRITC)-Phalloidin (for 1 h at room temperature; Sigma) as usual. After washing twice with

Fig. 1. MTT Assay Detects the Effects of M β CD at Different Concentrations on the Viability of HUVECs

PBS, the cells were observed under the confocal microscope. The excitation/emission wavelengths for Alexa fluor 488 and TRITC were 488 nm/(493–630 nm) and 561 nm/(570–670 nm), respectively. The length of each fluorescently labeled actin filament was measured using the Zeiss LSM 710 Zen software.

Cell Spreading Assay Spread area-based spreading assay was performed as reported previously.^{14,15)} Briefly, HUVECs at 70% confluence were treated with or without 10 mM M β CD for 30 min at 37°C. The cells were then fixed by 4% paraformaldehyde, rinsed with PBS, and imaged in PBS by the confocal microscope. The spread area of each cell was extracted by planimetry via the Zeiss LSM 710 Zen software.

Statistical Analysis All graphs were made using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, U.S.A.) on which all data were expressed as the mean $\pm$ standard deviation (S.D.) from at least three independent experiments. Statistical analysis was performed using Student's *t*-test. A difference was regarded as significant when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

RESULTS AND DISCUSSION

The effects of M β CD on the membrane alternation, cytoskeletal reorganization, proliferation, and apoptosis of endothelial cells have been intensively studied.^{16–18)} However, it remains unclear whether and how M β CD affects the adhesion of leukocytes (*e.g.* monocytes) onto the endothelium which is the first step of leukocyte *trans*-endothelial migration.

Firstly, we studied the effects of M β CD at different concentrations on the viability of HUVECs using MTT assay (Fig. 1). The data shows that the 20 mM M β CD treatment for 30 min induced significant death of HUVECs whereas ≤ 10 mM M β CD treatments for 30 min caused no statistically significant, although slight, decrease in cell viability. Therefore, in the following experiments we used ≤ 10 mM M β CD to treat HUVECs for 30 min.

To test whether M β CD influence the adhesion of monocytes onto endothelial cells, THP-1 cells (a putative monocyte cell line) were fluorescently stained with Celltracker, co-cultured with the monolayer of HUVEC cells (a widely used endothelial cell line) pretreated with or without 1, 5, and 10 mM

M β CD, respectively and imaged by confocal microscopy. The confocal fluorescence images clearly show that compared with untreated HUVECs there were less THP-1 cells on the monolayer of the HUVECs treated by 5 or 10 mM M β CD (Fig. 2A). It was further confirmed by the statistical analysis (Fig. 2B), implying that M β CD significantly inhibited the adhesion of THP-1 cells onto HUVECs.

Since the biomechanical properties of cell surfaces may be involved in cell-cell adhesion, the cell-surface adhesive force of HUVECs treated with or without 10 mM M β CD was then detected by the force measurement function of AFM. The adhesive force mapping on a cell surface region of 30 $\times$ 30 μ m shows that M β CD can impair the cell-surface adhesive force of HUVECs (Fig. 3).

It is well known that adhesion molecules on cell surfaces are the major determinants of the adhesion of leukocytes onto the endothelium. Therefore, we then investigated the effects of M β CD on the expression of ICAM-1 on HUVECs by using flow cytometry (Fig. 4). The data shows that the mean fluores-

cence intensity (MFI) of ICAM-1 on HUVECs significantly decreased after treated with 5 and 10 mM M β CD. The data implies that M β CD can inhibit the expression of adhesion molecules on HUVECs in a dose-dependent manner.

Caveolae, a special type of cholesterol-enriched lipid raft, are highly abundant in endothelial cells and play multiple roles in cell adhesion and signal transduction.¹⁹⁾ Another important molecule PIP₂ which plays a string-puller role in membrane-cytoskeleton coupling^{20,21)} is also enriched in caveolae.^{22,23)} Therefore, we tried to evaluate the effects of M β CD on caveolae and PIP₂. The data from both confocal microscopy and flow cytometry indicate that M β CD also inhibited the expressions of caveolin-1 (Fig. 5), a structural protein of caveolae, and PIP₂ (Fig. 6).

Finally, we sought to investigate the effects of M β CD on actin cytoskeleton which is associated with plasma membrane generally *via* caveolae/PIP₂. The data show that M β CD induced significant decrease in the average length of individual actin filaments causing the decrease in the average spreading

Fig. 2. M β CD Impairs the Adhesion of Monocytes onto HUVECs

Celltracker C34552 was utilized to stain THP-1 cells. (A) Fluorescently stained THP-1 cells on the monolayer of HUVECs treated with 0, 1, 5, and 10 mM M β CD for 30 min, respectively. (B) Average numbers of THP-1 cells adhered on the monolayer of HUVECs treated with or without M β CD in a field with the same size.

Fig. 3. M β CD Lowers the Cell-Surface Adhesive Force of HUVECs

(A) Representative adhesive force mapping on an untreated cell detected by AFM. (B) Representative adhesive force mapping on a M β CD (10 mM)-treated cell detected by AFM. Scan size: 30 μ m $\times$ 30 μ m.

Fig. 4. MβCD Inhibits the Expression of Adhesion Molecules on HUVECs

Left: representative flow cytometric data showing the effects of MβCD on the mean fluorescence intensity (MFI) of fluorescently stained ICAM-1 on HUVECs. Right: quantification and statistical analyses of MβCD-induced changes in MFI of ICAM-1.

Fig. 5. MβCD Inhibits the Expression of Caveolin-1 in HUVECs

(A) The representative (left) and statistical analyses (right) of flow cytometric data show that the MFI of caveolin-1 in MβCD-treated cells was significantly lower than that on untreated cells. (B) Confocal microscopy shows that compared with untreated cells (upper panel) there were fewer caveolin-1 molecules in the treated cells by 10mM MβCD (lower panel). From left to right: DIC image, fluorescence image, and merged image, respectively.

Fig. 6. MβCD Inhibits the Expression of PIP₂ in HUVECs

Confocal microscopy shows that compared with untreated cells (upper panel) there were fewer PIP₂ molecules in the treated cells by 10 mM MβCD (middle panel). From left to right: DIC image, fluorescence image, and merged image, respectively. The representative (left) and statistical analyses (right) of flow cytometric data (bottom panel) show that the MFI of PIP₂ on MβCD-treated cells was significantly lower than that on untreated cells.

Fig. 7. MβCD Causes Cytoskeletal Reorganization/Shortening

Left panel: a representative fluorescence confocal image showing actin filaments and focal adhesions at both ends of filaments by fluorescently staining F-actin (red) and paxillin (a main focal adhesion molecule; green), respectively. Middle panel: the average length of individual actin filaments in HUVECs treated with or without MβCD. Right panel: the average spreading area of individual HUVECs treated with or without 10 mM MβCD.

area of individual HUVECs (Fig. 7). Therefore, MβCD also caused cytoskeletal reorganization/shortening in HUVECs probably due to the MβCD-induced weakening of the caveolae/PIP₂ which mediates the membrane-cytoskeleton coupling.

Taken together, in a concentration-dependent manner MβCD can inhibit the adhesion of monocytes onto endothelial

cells by down-regulating the cell-surface adhesive force property and the expression of adhesion molecules on endothelial cells which is probably correlated with caveolae (or lipid rafts) and cytoskeletal reorganization although it still remains controversial whether adhesion molecules (*e.g.* ICAM-1) are present in caveolae.^{24–28} It implies that MβCD, when recruited

as an anticancer drug, will impair the adhesion of circulating leukocytes (e.g. monocytes) onto the endothelium and therefore potentially help to inhibit inflammation or initiation/progression of atherosclerosis.

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Conflict of Interest The authors declare no conflict of interest.

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