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Cellular attachment and internalization of cationic liposomes containing mycobacterial cell wall

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ABSTRACT: The cell wall derived from *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is a potent immunopotentiator and has recently been suggested as an alternative treatment for in situ bladder carcinoma. In contrast to the live BCG, the loss of infectivity and the negatively charged nature of BCG cell wall physically inhibit its attachment and subsequent internalization to the urothelial bladder cells. As part of our research involving the delivery of macromolecules to target cells, we developed cationic liposomes that anchor arginine octamers on the liposome surface. In this study, we used cationic liposomes as a delivery tool to facilitate the attachment and internalization of the BCG cell wall. Using confocal scanning microscopy, we verified that cationic liposomes incorporated with BCG cell wall could attach to the cellular membrane of murine bladder tumour (MBT-2) cells and become internalized. Cationic liposomes containing BCG cell wall were taken up by MBT-2 cells mainly via clathrin-mediated endocytosis. These results would be useful to understand the mechanism of action of BCG cell wall against bladder tumour cells as well as to develop an immunotherapeutic agent for clinical use.

KEYWORDS: cationic liposomes, internalization, bladder tumour cells, *Mycobacterium bovis*

INTRODUCTION

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is a live attenuated strain that was originally produced in 1921 to help prevent tuberculosis. BCG is also well-known as a potent immunotherapeutic agent[1]. Morales *et al*. successfully used intravesical BCG for a therapy of superficial bladder cancer[2]. Intravesical immunotherapy with BCG is clinically well established and accepted worldwide[1,3,4]. The mechanism of action of intravesical BCG is not completely understood[3]. After instillation of the live BCG into the bladder, the bacteria is thought to bind to the urothelial lining. This binding is dependent on the interaction of a fibronectin attachment protein (FAP) on the surface of BCG with fibronectin, a matrix protein, presenting onto the surface of bladder epithelial cells[5]. Subsequently, BCG is taken up by bladder epithelial cells and BCG-derived antigens are then processed and presented on cell surface for immune recognition. The production of cytokines and chemokines by activated immune cells and infected bladder epithelial cells would then induce the innate and cell-mediated immune responses, and lead to an eradication of residual bladder cancer[3,6,7].

Although instillation of live BCG has proven to be a successful immunotherapy for superficial bladder cancer, many patients experience undesirable side effects, such as fever, granulomatous infection, sepsis and even death[8]. An alternative therapy devoid of side effects associated with live BCG instillation is therefore required. Cell walls derived from *M. bovis* or *M. phlei* are well known modulators of immune activation and have been investigated as an alternative therapy for *in situ* carcinoma of the bladder[9,10]. However, the anti-tumour effect of BCG cell wall is generally less pronounced than that of live BCG, which can be ascribed partly to the loss of infectivity of BCG cell wall[11]. In addition, the high negative surface charge and high molecular weight of BCG cell wall physically inhibit the attachment to the urothelial cell surface.

Liposomes have been used as a delivery vehicle of pharmaceutical active agents and more recently they have been applied as a vector for macromolecules, such as DNA, RNA, and protein[12]. In general, conventional liposomes offer a limited cell binding and intracellular delivery. Thus a modification of liposomes to allow molecular interactions with the plasma membrane is carried out to enhance cellular association and to trigger cell transport processes.

We recently reported the development of a BCG cell wall delivery system using cationic liposomes which could overcome the unfavourable physicochemical properties and also facilitate the cellular uptake of BCG cell wall[13]. The liposome surface was modified by attaching octaarginine (RRRRRRRR; R8), a well-known cationic cell-penetrating peptide, which is rapidly and efficiently bound to the cellular membrane[14]. The positive charge of cationic liposomes would interact with the negatively charged cell membrane and trigger the endocytotic uptake of BCG cell wall.

The cellular association and uptake of cationic liposomes containing BCG cell wall is important for exploring the mechanisms by which an artificial BCG mediates antitumour activity. This study demonstrated the internalization mechanisms of cationic liposomes containing BCG cell wall by murine bladder cells. We showed the results for the first time that cationic liposomes incorporating BCG cell wall efficiently attach and internalize to the surface of bladder tumour cells. The uptake mechanism of cationic liposomes containing BCG cell wall occurs through an energy-dependent process mainly *via* clathrin-mediated endocytosis.

MATERIALS AND METHODS

Reagents and cell culture

Egg phosphatidylcholine (EPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). Sulphorhodamine B, fluorescein-5-thio-semicarbazide (FTSC) were obtained from Molecular Probes (Eugene, OR). Stearylated-octaarginine (STR-R8) was synthesized and purified as described previously[14]. Amiloride and methyl-β-cyclodextrin (mβCD) were obtained from Sigma (St. Louis, MO). Heparin, sodium azide, sodium fluoride, antimycin A, sucrose and nystatin were purchased from Wako Chemicals (Osaka, Japan). All other chemicals were of analytical grade and used as received without further purification. Murine bladder tumour cells (MBT-2) derived from C3H/HeN mice were kindly obtained from Prof. H. Akaza (University of Tsukuba, Ibaraki, Japan). The cells were cultured in RPMI-1640 medium (Sigma, St.Louis, MO), supplement with 10% foetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml).

M. bovis culture and cell wall isolation

M. bovis BCG Tokyo 172 (ATCC 35737) cells were grown on Sauton's synthetic liquid medium at 37 °C for 8 days. After heat inactivation at 121 °C for 15 min, the bacterial culture was centrifuged and the wet cells were then suspended in deionized water at a concentration of 1 g/ml. The intact cells were disrupted using a French Pressure Cell (5501-MF, Ohtake Works, Japan) at 180 MPa for three times. The disrupted mycobacterial suspension was then fractionated by centrifugation at 6,500 *g* for 20 min at 4 °C to remove the intact cells and undisrupted cell debris. The supernatant was further centrifuged at 18,000 *g* for 60 min to obtain a cell wall fraction. Finally the isolated cell wall was lyophilized and kept at room temperature until used.

Fluorescent labelling of BCG cell wall

To facilitate the analysis of cellular association and uptake of BCG cell wall, carbohydrate moieties exposed onto the surface of the cell wall were labelled with carbazide marker as described previously[13]. Briefly, 400 μg of BCG cell wall was suspended in 0.1 M sodium acetate, pH 5.5 containing 1 mM sodium periodate. Following a 30-min incubation at 4 °C with gentle rotation, 0.1 mM glycerol was added to stop the reaction. The BCG cell wall was then washed twice with HBS (5.0 mM Hepes, 0.15 M NaCl, pH 7.4) and resuspended in HBS containing 1 mM fluorescein-5-thiosemicarbazide (FTSC). Following an overnight incubation at 4 °C, the FTSC-labelled BCG cell wall was washed twice with HBS to remove free FTSC. The FTSC-labelled BCG cell wall was then lyophilized and kept in a desiccator at room temperature until used.

Preparation and characterization of cationic liposomes containing BCG cell wall

Liposomes were composed of EPC and Chol (7:3 molar ratio) and stearylated octaarginine (STR-R8) was incorporated at 5 mole% of the total lipid. Cell wall derived from *M. bovis* BCG was incorporated into liposomes as described previously[13]. Briefly, a lipid film composed of EPC, Chol, STR-R8 and 400 μg of BCG cell wall pre-labelled with FTSC was prepared by solvent evaporation with a rotary evaporator under reduced pressure. Hydration of the lipid film containing BCG cell wall was achieved by adding 1 ml of sulphorhodamine solution (5 mM in HBS) and gently shaking for 20 min at 65 °C. The fluorescent labelled liposomes were then sequentially extruded through polycarbonate membrane filters with pore sizes varying from 0.8 to 0.1 μm using a hand extruder (Avanti Polar Lipids, AL, USA). The non-incorporated components were then removed from liposomal formulations by dialysis against several changes of HBS at room temperature.

The particle size and morphology of the prepared

liposomes were analysed by transmission electron microscopy. The formulations were adsorbed onto carbon-stabilized formvar-coated grids, negatively stained using 2.0% potassium phosphotungstate (pH 5.2) and analysed using a transmission electron microscope HD-2000 (Hitachi, Tokyo, Japan) at an operating voltage of 200 kV. The zeta potential of the liposomes was measured by using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan). The lipid content was determined by measuring cholesterol concentrations using Chol-E-test kit (Wako Chemicals, Osaka, Japan).

Confocal laser scanning microscopy

To investigate the cellular association and uptake of liposomes with BCG cell wall incorporated, MBT-2 cells were pulsed with 0.1 mM double labelled cationic liposomes (FTSC-labelled BCG cell wall and sulphorhodamine-labelled aqueous phase) in serum-free medium at 37 °C for 1 h. The cells were then washed three times with ice-cold PBS (10 mM Na_2HPO_4, 0.15 M NaCl) and kept on ice until analysed by a confocal laser scanning microscope (LSM510 meta, Carl Zeiss, Oberkochen, Germany).

Uptake mechanism of cationic liposomes containing BCG cell wall

To determine the mechanism of internalization, 1 x 10^6 MBT-2 cells were incubated in 1 ml serum-free RPMI 1640 medium in the absence and presence of a mixture of metabolic inhibitors (0.1% sodium azide, 10 mM sodium fluoride, and 1 µg/ml antimycin A) for 30 min, 0.4 M sucrose for 30 min, 5 mM amiloride for 10 min, 25 µg/ml nystatin for 30 min, or 10 mM methyl-β-cyclodextrin (mβCD) for 30 min. After treatment cells with various biochemical inhibitors, double-labelled cationic liposomes were then added (final concentration 0.1 mM lipids) and the cells were further incubated for 1 h in the presence or the absence of the inhibitors. The cells were washed three times with ice-cold PBS containing heparin 20 U/ml. Then the cells were suspended in 0.5 ml PBS and analysed by flow cytometry using a FACSort (Becton Dickinson) and Cell Quest software (BD Biosciences).

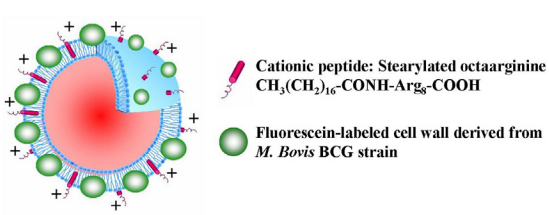

Fig. 1 Schematic structure of cationic liposomes with BCG cell wall incorporated. The lipid moieties of BCG cell wall intercalate into the lipid bilayer. The liposomal surface was modified by coupling with stearylated-octaarginine, a cationic peptide.

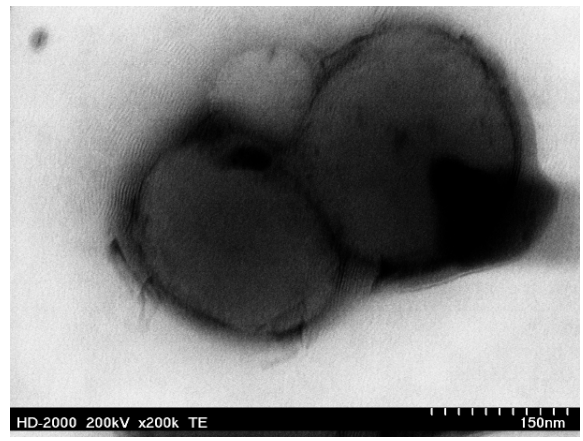

Fig. 2 Negative-staining electron microscopy study of cationic liposomes containing BCG cell wall.

RESULTS

Preparation and characterization of cationic liposomes containing BCG cell wall

Liposome containing BCG cell wall was prepared by hydration of thin lipid films in aqueous solution followed by size extrusion to obtain lipid vesicles with a homogeneous size distribution. The structure of liposomes prepared is illustrated in Fig. 1. In this preparation, STR-R8 was incorporated onto the surface of liposomes. The stearyl moiety acts as an anchor to the lipid surface leaving the arginine octamers freely attached to the surface. In addition, cell wall derived from the *M. bovis* BCG strain was also incorporated onto the surface of liposomes. The zeta potential of cationic liposomes was highly positive (42.6 ± 8 mV), whereas the zeta potential of control liposomes (without arginine octamers) was negative (–12 ± 5 mV). The electron microscopic image of cationic liposomes with BCG cell wall incorporated showed that the particles had a spherical structure with an average size of 200–250 nm (Fig. 2). To investigate the cellular association and uptake of liposomes, the carbohydrate moieties exposed onto the surface of cell walls were tagged with fluorescein-5-thiosemicarbazide (FTSC), which showed a green fluorescent signal under a confocal laser scanning microscope (CLSM). The

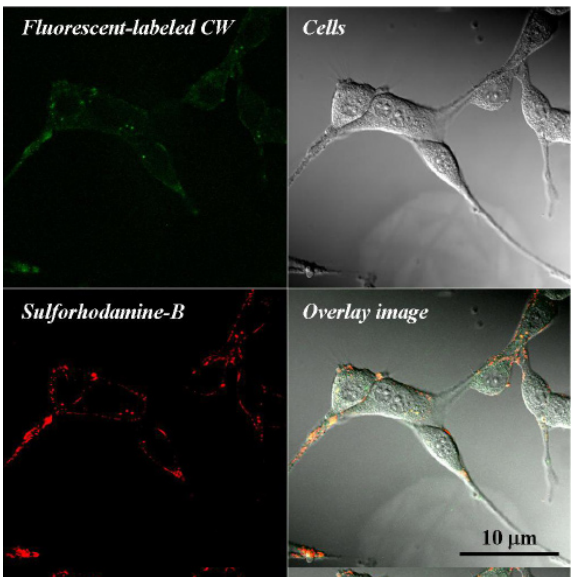

Fig. 3 Cellular association and uptake of double-labelled cationic liposomes containing BCG cell wall. Representative images of confocal laser scanning micrographs after incubation cells with double-labelled cationic liposomes (rhodamine-labelled aqueous phase and FTSC-labelled BCG cell wall) for 1 h at 37 °C.

aqueous phase of liposomes was also labelled using a polar phase marker, sulphorhodamine B, exhibiting a red fluorescence under CLSM.

Cellular association and uptake

The cellular association and uptake of cationic liposomes containing BCG cell wall were investigated by CLSM. Fixation was not used in this experiment to avoid any possible detrimental effects of the fixatives. As shown in Fig. 3, the FTSC-tagged BCG cell wall was mainly co-localized with sulphorhodamine-labelled liposomes, indicating the presence of intact liposomes upon binding the cell surface and internalization. The double fluorescent labels of cationic liposomes were localized at the plasma membranes and intracellular vesicles in the cells. Control liposomes containing BCG cell wall (without cationic peptides modification) could not be detected using the same photomultiplier settings.

Uptake mechanism

MBT-2 cells were incubated with fluorescently labelled cationic liposomes containing BCG cell wall, and the level of cell association was quantified using flow cytometry. A highly significant level of liposomal-cell association was seen after just 1 h, which was in agreement with the CLSM study. The role of endocytosis was assessed by pretreating cells with the endocytosis inhibitors (sodium azide, sodium fluoride, and antimycin A), which inhibit glycolysis and oxidative metabolism required for most endocytic processes[16]. As shown in Fig. 4, the inhibition of all endocytic pathways by the mixture of metabolic inhibitors caused a strong inhibition (70%) of the uptake of liposomes, suggesting the uptake is highly energy dependent. The contribution of different endocytotic pathways was further investigated using various biochemical inhibitors. We examined the effects of the following inhibitors: a hypertonic medium (0.4 M sucrose) to specifically inhibit clathrin-mediated endocytosis (CME), amiloride and methyl-β-cyclodextrin (mβCD) to inhibit macropinocytosis, and nystatin to inhibit the caveolar uptake pathway[16]. The use of a hypertonic medium strongly inhibited the uptake of liposomes by ~62%. In the presence of the amiloride and mβCD macropinocytosis inhibitors, 25% and 35% of liposome particles could not be taken up by the cells, respectively. The caveolar inhibitor, nystatin, inhibited the uptake of liposomes only slightly (15%), indicating the minor contribution of caveolae in the uptake process.

DISCUSSION

Cell walls derived from BCG have a low ability to attach and traverse the plasma membrane of urothelial cells. Since the cellular association and internalization of BCG components are necessary events for the activation of immunity to BCG and for the induction of antitumour effect[5,11]. Through this present work, we have demonstrated the cellular attachment and uptake of BCG cell wall in murine bladder tumour cells by using cationic liposomes as a novel BCG cell wall delivery system. Previously, Rosenkrands *et al.* have demonstrated the immunostimulatory capacity of a lipid extract of BCG mycobacteria, which was delivered in cationic liposomes based on dimethyl dioctadecyl ammonium

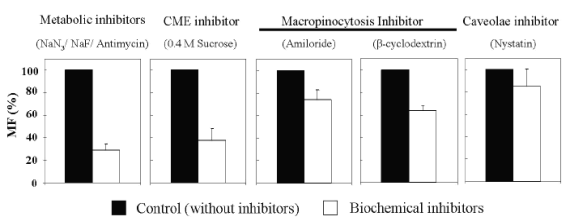

Fig. 4 Effect of biochemical inhibitors on the uptake of cationic liposomes containing BCG cell wall. Data represent the mean and standard deviation of three different experiments performed in duplicate.

bromide (DDAB)[17]. They found that the administration of lipids derived from BCG in DDAB-based cationic liposomes induced a powerful Th1 response. In line with these studies, we have reported recently the immunological activation of dendritic cells was greatly enhanced by cationic liposomes containing BCG cell wall[13]. By using CLSM we found that an artificial BCG, cationic liposomes with BCG cell wall incorporated, could attach and internalize to murine bladder tumour cells efficiently (Fig. 3). The enhanced intracellular localization could be attributed to the decoration of liposomes with cationic arginine octamers. Small cationic peptides, such as poly-L-arginine and transportan, that mediate the internalization of macromolecules across lipid bilayers, have been reported as a very useful tool for high efficiency protein and DNA delivery[14,18,19]. The direct physical interaction of cationic arginine-rich peptides with negatively charged cell membrane has been proposed as the major mechanism which dramatically enhances the cellular binding and uptake of macromolecules linked to the peptides[18,19].

Macromolecules are internalized to cells by a variety of mechanisms, and their intracellular fate is usually linked to the entry mechanism[15]. Therefore the systematic and successful design of an artificial BCG requires an understanding of cellular interaction between the delivery system and target cells. Once the live BCG or its cell wall were taken up by cells, they would be processed and presented onto the surface of the cells. For example, glycolipids, which are abundant constituents of the cell wall mycobacteria, such as lipoarabinomannan, phosphatidylinositol manoside, and mycolic acids can be recognized by T-cells in conjunction with CD1 molecules[20,21]. The recognition of the glycolipids-CD1 complex would lead to T-cells activation, production of Th1 and Th2 cytokines, and eventually eliminate the bystander tumour cells. Unlike live mycobacteria, which has been clearly demonstrated to actively enter and infect the urothelial cells[22], no studies have examined the internalization mechanism of cationic liposomes containing BCG cell wall.

Endocytosis refers to the cellular uptake of external materials into membrane bound vesicles. Endocytosis can be classified into two main categories, phagocytosis and pinocytosis[23]. Phagocytosis is typically restricted to specialized mammalian cells including macrophages, dendritic cells and monocytes, whereas pinocytosis occurs in all cells by at least four basic mechanisms, namely, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin caveolae-independent endocytosis[23]. Ikramy *et al.* have recently reported that the uptake of octaarginine-modified liposomes was highly energy dependent[24]. We also found that the uptake of an artificial BCG was markedly inhibited in the presence of a mixture of metabolic inhibitors (antimycin A, sodium azide, and sodium fluoride), a potent inhibitor of all kinds of endocytosis through ATP depletion (Fig. 4)[16].

It has been reported that the caveolae-mediated pathways can be selectively blocked by depletion of cholesterol in the plasma membrane[25]. Nystatin, which disrupts caveolae by sequestration of cholesterol[25], had no effect on the cellular uptake of artificial BCG[16]. The relatively small size of caveolae (~50–100 nm) obviously places limitations on the endocytosis of cationic liposomes containing BCG cell wall (ranging in size between 200–250 nm)[23]. Clathrin-mediated endocytosis (CME) is by far the major and best characterized endocytic pathway[23,25]. The uptake *via* CME can be specifically inhibited by potassium depletion, cytosol acidification, or hypertonic treatment[16]. The latter was used in this study because it is an efficient and non-invasive method. It was suggested that the presence of a hypertonic medium (0.4 M sucrose) removed the membrane-associated lattice of the clathrin coated pits, which is essential for the formation of intracellular clathrin-coated vesicles[16]. The uptake of cationic liposomes containing BCG cell wall was markedly inhibited in the presence of a hypertonic medium. In addition, the size of cationic liposomes with BCG cell wall incorporated coincided more with the size of clathrin-coated vesicles (150–200 nm) than that of the caveolae[16,23].

Recently, several authors reported that macropinocytosis was a major uptake pathway of cationic polyarginine peptides[24,26–28]. Macropinocytosis refers to the formation of large endocytic vesicles (macropinosomes). Due to their large size, it allows cells to take up large amounts of cell surface bound particles and extracellular fluids[23]. Amiloride is a specific inhibitor of macropinocytosis through inhibition of the Na^+/H^+ exchange, which is required for the ruffling of the plasma membrane[28]. In contrast to previous studies, we found that amiloride showed little effect on the uptake of octaarginine-modified liposomes containing BCG cell wall. To deplete cell surface associated cholesterol, which is required to form the lipid rafts of macropinosomes, the cells were pretreated with methyl-β-cyclodextrin (mβCD)[16]. We found that mβCD also showed no substantial inhibition of cellular uptake of cationic liposomes. It is likely that the BCG cell wall incorporated into liposomes could possibly

affect the internalization mechanism of liposomes modified with poly-L arginine peptides.

In summary, we have reported that cationic liposome with BCG cell wall incorporated could be attached and internalized mainly *via* CME to murine bladder tumour cells. The results would be useful for further mechanistic study of artificial BCG against bladder tumours. The biodistribution and cellular uptake of BCG cell wall after in *vivo* instillation of artificial BCG would be a subject worthy of further study.

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