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The transport pathways of polymer nanoparticles in MDCK epithelial cells

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ABSTRACT

Epithelial cell membranes as the typical biological barrier constitute the prime obstacle for the transport of therapeutic agents including nanomedicines. The previous studies on the interaction between nanomedicines and cells are mostly emphasized on cellular uptake and intracellular trafficking, but seldom on epithelial cells, although more and more oral nanomedicines are available now. In an attempt to clarify the transport pathways of nanomedicines in epithelial cells, the different molecular mechanisms among endocytosis, exocytosis and transcytosis processes were carefully studied and compared here using a kind of polymer nanoparticles (PNs) and MDCK epithelial cells as models. As the result, their similarity and difference were demonstrated. The similarities among all the three processes included the mediation of lipid rafts, the involvement of some protein kinases such as protein tyrosine kinase (PTK), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), and the existence of multiple pathways. However, the difference among these processes was very significant, including different pathways, and especially the disparate effects of lipid rafts and protein kinases for different processes. The endocytosis involved both lipid raft and clathrin mechanisms but no macropinocytosis, via the invagination of membrane but no pore formation, the exocytosis contained ER/Golgi and Golgi/PM pathways, and transcytosis included AEE/CE/BSE and Golgi/BSE pathways. The roles of lipid rafts on endocytosis were positive but that on exocytosis and transcytosis was negative. The impacts of PTK and PKC on endocytosis were positive, while the influences of PTK, PKC and P13K on AEE/CE/BSE, as well as PTK and P13K on Golgi/BSE transcytosis pathways were negative. Moreover, the discrepancy between inward and outward transport of PNs elucidated an interesting fact that the endocytosis was rather easy and outward transport including exocytosis and transcytosis was rather difficult. Finally, it was indicated by comparison with previous reports that the molecular mechanisms between PNs and macromolecules such as proteins were also dissimilar.

1. Introduction

Biological barrier, mainly represented by epithelial tissue, controls the absorption of exogenous substances and protects against the invasion of pathogenic microorganisms. The prime characteristic of epithelial cells is the polarity. Because of diversities on lipid composition and protein distribution, the plasma membranes (PM) of epithelial cells are distinguished as apical and basolateral membrane respectively, selectively controlling the transportation of different substances [1]. Furthermore, the existence of tight junction makes epithelial cell membranes into a compact barrier [2]. In clinic, most medicines with different administration routes suffer the hindrance from epithelial cell monolayer because of the throughout distribution of epithelial cells in our body. Especially for the drugs with poor water-solubility or membrane permeability, their oral bioavailability and therapeutic efficacy are often reduced significantly due to the block effect of epithelial cells on the drug absorption in gastrointestinal tract [3]. How to conquer the epithelium barrier and accelerate the transport of drugs through are the great challenges for pharmaceutical science for a long time.

Over the last couple of decades, the application of nanotechnology has been one of the main approaches to overcome the epithelial barrier [4]. Compared with free drug, polymer nanoparticles (PNs) alter the pathways of cell uptake and promote the transport of medicines loaded in PNs through epithelial cell monolayer [5]. As a representative of PNs, polylactic-co-glycolic acid

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(PLGA) nanoparticles are frequently reported as the drug delivery systems because of its biocompatibility and biodegradability [6]. Via different fabrication methods, this kind of PNs can be obtained with desired particle size from 50 nm to 300 nm [7]. Drugs with different solubility characteristics, water-soluble or water-insoluble, can all be loaded into this kind of nanoparticles by different techniques to improve their bioavailability [8,9]. Various surface and structure modifications are also reported to facilitate the uptake and transport efficiency of PLGA nanoparticles in cells [10].

Generally, to improve the transport of nanoparticles across the epithelial cell membrane, it is vital to comprehend the mechanisms related, which has caused increasing interesting in recent years. Currently, studies on the transport mechanism are mostly focused on the uptake pathway and partially on intracellular trafficking of nanoparticles in different cells, but mostly tumor cells. In fact, during the transport across the epithelial barrier, the nanoparticles need to undergo the whole process from the apical membrane to the basolateral side. Namely, the transcytosis of PNs may include their entry into cytoplasma via the endocytosis, transport intracellularly, and final exit from apical and basolateral membrane via exocytosis. So, the comprehensive understanding of the whole trans-cellular process is desperately needed. On the other hand, the roles and pathways in different steps of whole process may be different. Therefore, the careful comparison among the endocytosis, exocytosis and transcytosis of PNs should be significant for the further comprehension of the molecular mechanisms related during the transport of nanoparticles across epithelial cells.

In order to study the whole trans-cellular process of PNs and the mechanism diversity among endocytosis, exocytosis and transcytosis, MDCK cell line is chosen as the model of epithelial barrier and PLGA nanoparticles as the representative of PNs in this work. Due to the polarity and tight junctions, MDCK epithelial cells are often utilized as the simulation of gastrointestinal tract [11], and their similarity with Caco-2 cells is already demonstrated [12]. Various models of endocytosis (uptake in cells in 12-well plate), exocytosis (exit upward from cells in 12-well plate) and transcytosis (across cell monolayer on porous polycarbonate membrane) were established here. Namely, exocytosis model only involved the exit from apical side in order to simply the situation. Plenty of techniques or systems were applied here, including confocal laser scanning microscope (CLSM) with different scanning models, near-infrared fluorescence (NIRF) imaging system, flow cytometry system (FCS), quantitative colocalization and so on. Regulators of protein kinases and transport pathways were also utilized to illustrate the molecular mechanisms related.

2. Materials and methods

2.1. Materials

MDCK cell lines were obtained from National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Poly (D, L-lactic-co-glycolic acid) (PLGA, 50:50, Av. MW 15000) was from Shandong Daigang Institute of Medical Instrument (Jinan, Shandong, China). LysoTracker® Red DND-99 dye, ER-Tracker™ Red dye and Rhodamine-phalloidine were all supplied by Invitrogen (Eugene, Oregon, USA). Rabbit anti-Rab5 and mouse anti-Rab7 antibodys were obtained from Abcam (Cambridge, MA, USA). Coumarin-6, poloxamer 188 (Pluronic F68), methyl-β-cyclodextran (MβCD), fillipin, nystatin, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and genistein were purchased from Sigma–Aldrich (St. Louis, MO, USA). Wortmannin, LY294002, staurosporine, brefeldinA, monensin, phorbol 12-myristate 13-acetate (PMA), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide (DiR) and hoechst 33258 were obtained from Beyotime (Haimen, Jiangsu, China). Texas Red-conjugated goat anti-rabbit IgG, Texas Red-conjugated goat anti-mouse IgG, DMEM (4.5 g/L glucose) culture solution, penicillin, streptomycin, trypsin, EDTA, phosphate buffered saline (PBS) and sucrose were supplied by Mcgene Co (Beijing, China). All other chemical reagents were analytical grade or better.

2.2. Preparation and characterization of PNs

PNs were prepared by a modified emulsion solvent-evaporation method [13]. In brief, 5 mg PLGA was dissolved in 2 ml acetone. Under the 2000rpm stirring at the room temperature, the organic phase was slowly poured into 5 ml water phase with 2 mg F68. After stirring for 15 min, the initial emulsion was transferred into a 50 ml flask followed by evaporation for 20 min at 60 °C. The nanoparticle dispersion was then centrifuged at 10,000 g for 20 min, and the obtained pellets were dispersed again in certain mediums. The preparation of C6-PNs was similar and the only exception was the addition of 5 μg coumarin-6 in acetone. The size and surface charge of PNs dispersed in different mediums were measured by a dynamic light scattering (DLS) analyzer (Malvern, Zetasizer Nano ZS, UK). In addition, PNs were also detected by transmission electron microscopy (TEM). After negatively stained by phosphotungstic acid, 10 μg/ml nanoparticle water dispersion was dropped on the copper grid and examined by a transmission electron microscope (JEM1230, JEOL, Japan).

2.3. In vitro leakage of coumarin-6 from C6-PNs

Briefly, 2 mg C6-PNs were dispersed in 2 ml PBS or serum free medium (SFM) with pH 7.2 and pH 5.5, respectively. Then the nanoparticle dispersion was located in a dialysis bag (MW cut off 12kD) and dialyzed against 48 ml release medium same as the dispersion solution within the dialysis bag. Under the 100rpm at 37 °C, 0.5 ml release medium was taken out and detected by a high performance liquid chromatography (HPLC) system (Shimadzu, Japan) equipped with a fluorescence detector (Model RF-10AXL) and a pump (Model LC-10AT). The excitation wavelength was set at 467 nm and that of emission at 502 nm. Under the column temperature of 35°C, 20 μl release medium was injected into the HPLC system and eluted via the mobile phase consist of methanol and water (95:5, *v*/v) at a flow rate of 1 ml/min. The peak area of C6 was recorded at the retention time of 5 min.

2.4. MDCK cell culture

For endocytosis and exocytosis studies, Madin–Darby canine kidney (MDCK) cells were grown in culture flasks containing Dulbecco modified Eagle's minimal essential medium (DMEM, 25 mM glucose) supplemented with 10% (*v*/v) fetal bovine serum, 1% (*v*/v) L-glutamine, 100UI/ml penicillin and 100 μg/ml streptomycin. After culture at 37 °C with 5% CO_2 for 6 days, MDCK cells were digested with 0.25% trypsin/0.02% EDTA and seeded in 12-well sterile plate at 1×10^5/ml.

For transcytosis studies, MDCK cells were cultured on a polycarbonate membrane (Transwell®, 12 wells, CORNING) with pores of 3 μm in diameter. 500 μl DMEM was added into the upper compartment of transwell insert and the medium volume in basolateral side was set as 1.5 ml. During the culture of 7 days, mediums in both upper and basilar compartments were changed every other day and the transepithelial electrical resistances (TEER) were measured by an epithelial volt-Ω m (Millicell® ERS-2, Millipore). At the end of one week culture, the cell monolayers with TEER value above 180 Ω/cm^2 were selected for the subsequent studies.

2.5. Endocytosis characterization of C6-PNs by MDCK cells

2.5.1. Lactate dehydrogenase (LDH) release assay

MDCK cells were seeded into 96-well plate at 5×10^4/ml in 200 μl culture medium until 80% cells confluence. Prior to the detection, cells were incubated with 1 mg/ml PNs in SFM dispersion at 37 °C for 1 h. After the incubation, 20 μl lactate dehydrogenase (LDH) release solution was added into each well for subsequent 1 h incubation at 37 °C. Then, 120 μl obtained cell supernatant was aspirated and transferred into another plate according to the instruction. Finally, the absorbance of supernatant was recorded at 490 nm by a multiskan FC (Thermo Scientific, USA).

2.5.2. Confocal image series of cellular uptake of C6-PNs

The internalization of C6-PNs by MDCK cells was monitored by a confocal laser scanning microscope (CLSM, LEICA TCS SP5, Germany). When MDCK cells cultured in sterile cover slips met the requirement of 80% confluence, 1 mg/ml SFM dispersion of C6-PNs was added and incubated for 5 min, 10 min, 30 min and 60 min, respectively. In the end of incubation, cells were rinsed with cold PBS, fixed by 3.7% paraformaldehyde and sealed with glycine/PBS (*v*/*v* = 1:1). Finally, the obtained cover slips containing cell monolayer were detected by CLSM under 488 nm excitation condition.

Real-time imaging of MDCK cells in endocytosis study was also conducted via CLSM (LEICA TCS SP2, Germany). MDCK cells were cultured in a sterile glass bottom dish and incubated with 1 μM DiI at 37 °C for 1 h to label the lipid membranes. After rinsed by warming PBS, the living MDCK cells were incubated with 1 mg/ml SFM dispersion of C6-PNs for 1 h at 37 °C. The excitation wavelength for C6-PNs detection was set at 488 nm and adjusted to 561 nm for lipid membrane. The merged images between C6-PNs and lipid membrane were obtained by image acquisition software of LEICA.