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Pre-instillation of tumor microparticles enhances intravesical chemotherapy of nonmuscle-invasive bladder cancer through a lysosomal pathway

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ABSTRACT

Nonmuscle-invasive bladder cancer (NMIBC) is treated with transurethral resection followed by intravesical chemotherapy. However, drug-resistant tumorigenic cells cannot be eliminated, leading to half of the treated cancers recur with increased stage and grade. Innovative approaches to enhance drug sensitivity and eradicate tumorigenic cells in NMIBC treatment are urgently needed. Here, we show that pre-instillation of tumor cell-derived microparticles (T-MP) as natural biomaterials markedly enhance the inhibitory effects of intravesical chemotherapy on growth and hematuria occurrence of orthotropic bladder cancer in mice. We provide evidence that T-MPs enter and increase the pH value of lysosomes from 4.6 to 5.6, leading to the migration of drug-loaded lysosomes along microtubule tracks toward the nucleus and discharging the drugs whereby for the entry of the nucleus. We propose that T-MPs may function as a potent sensitizer for augmenting NMIBC chemotherapy with unprecedented clinical benefits.

1. Introduction

Seventy percent of bladder cancer is initially found as nonmuscle-invasive superficial disease (stages Ta, T1 or CIS) [1,2]. Clinically, nonmuscle-invasive bladder cancer (NMIBC) is usually treated with transurethral resection of the bladder tumor followed by intravesical chemotherapy [3,4]. Unfortunately, about half of the treated cancers recur with increased stage and grade [5]. Intravesical chemotherapy, like intravenous injection of chemotherapeutic drugs, cannot eliminate cancer cells completely, especially for drug-resistant stem-like tumor-repopulating cells (TRCs) [6,7]. These remnant TRCs eventually lead to tumor recurrence and progression [8]. Besides chemotherapy, intravesical bacillus Calmette-Guérin (BCG) immunotherapy is also used for those patients [9,10]. However, BCG instillations are associated with more extent side effects and also seem to be inefficient in targeting TRCs [11]. Therefore, innovative approaches to enhance TRC drug sensitivity and eradicate TRCs in NMIBC treatment are highly desirable.

Cells are capable of releasing various microvesicles with different sizes. In response to stimuli, cells change their cytoskeleton, leading to encapsulation of cytosolic contents within cellular membrane to form vesicles that are subsequently released into

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extracellular spaces [12,13]. Such specialized subcellular vesicles with 0.1–1 μm sizes are called microparticles (MP). MPs as natural biomaterials appear to be capable of functioning as a carrier to deliver bioactive molecules to target cells [14]. We recently found that immune cell-derived MPs can effectively transfer parental antigens or enzymes to innate immune cells [15,16]. Meanwhile, tumor cell-derived MPs (T-MP) are capable of delivering chemotherapeutic drugs or oncolytic adenovirus to tumor cells [17,18]. Intriguingly, MPs selves can also function as messengers to trigger signaling pathways. For instance, T-MPs, once taken up by dendritic cells (DC) or macrophages, can activate cGAS-STING signaling pathway for the production of type I interferon [19]. Notably, tumor cells also have the ability to easily take up T-MPs. However, the biological significance of those eaten T-MPs on tumor cells is largely unknown. In this study, we try to address whether T-MPs taken up by bladder tumor cells influence the sensitivity of bladder cancer cells to chemotherapeutic drugs by altering intrinsic signals of cells, and if so, whether T-MPs can act as a chemosensitizer to improve intravesical chemotherapy of NMIBC.

2. Materials and methods

2.1. Cell lines and animals

Murine bladder carcinoma cell line MB49, human bladder carcinoma T24 and Biu-87 were purchased from Cell Resource Centre of Peking Union Medical College (Beijing, China). Doxorubicin-resistant Biu-87/ADR was purchased from KeyGEN Biotech (Nanjing, China). Cells were tested for mycoplasma detection, interspecies cross contamination and authenticated by isoenzyme and short tandem repeat (STR) analyses in Cell Resource Centre of Peking Union Medical College before the beginning of the study and spontaneously during the research. All cell lines were maintained in culture for a maximum of 20 passages (two months). Female C57BL/6, NOD-SCID mice, 8-wk-old, were purchased from Centre of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Used Committee of Tongji Medical College.

2.2. Reagents and antibodies

Doxorubicin (DOX), methotrexate (MTX), gemcitabine (GEM), mitomycin C (MMC), hydroxycamptothecine (HCPT), wheat germ agglutinin (WGA), verapamil and PKH26 Red Fluorescent Cell Linker Kit were purchased from Sigma. LysoTracker Green (L7526), LysoSensor Green (L7535) and Intracellular pH Calibration Buffer Kit (P35379) were purchased from Molecular Probes. SiRNAs for TFEB, dynein and rab7 were purchased from RIBOBIO (China). Neutral liposomal clodronate was purchased from FormuMax. The following primary antibodies: anti-LAMP2 (ab25631), anti-β-tubulin (ab15568) and anti-KIF5B antibody (ab167429) were purchased from Abcam. Anti-Rab 7 (sc-10767) and anti-dynein (sc-32191) were purchased from Santa Cruz. Anti-P-glycoprotein antibody (P7965) was purchased from Sigma. Anti-β-actin antibody (ANT009) was purchased from Ant Gene. Secondary antibodies: goat anti-mouse IgG FITC, goat anti-rabbit IgG Alexa Fluor 594, donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-goat IgG Alexa Fluor 594 and donkey anti-mouse IgG Alexa Fluor®555 were purchased from Abcam.

2.3. Preparation of T-MPs

Preparing, counting and staining of T-MPs were described as previously [18]. Briefly, tumor cells were exposed to ultraviolet irradiation (UVB, 300 J/m^2) for 1 h [20]. 16 h later, the cells suspension was first centrifuged for 8 min at 1300 rpm to get rid of tumor cells and then centrifuged for 10 min at 5000 rpm to wipe off debris. Finally, T-MP suspension was centrifuged for 1 h at 14000 g to pellet T-MPs. The T-MPs was re-suspended with PBS or cultured medium for the experiments. T-MPs were counted with a flow cytometry-based method as described [18]. For T-MP labeling, 10^7 T-MPs were collected and re-suspended with 100 μl dilute C. 1 μl PKH26 staining solution was added into the suspension. 20 min later, 100 μl FBS was added. The PKH26 labeled T-MPs were collected for further study.

2.4. Animal model and MPs treatment protocol

The orthotopic bladder cancer model was established as described previously [21]. Briefly, 2×10^6 MB49 bladder cancer cells (100 μl) were intravesically instilled into the bladders of C57BL/6 mice after 20 min pretreatment with Poly-L-Lysine (0.1 mg/mL). Tumor cell suspension was kept in bladder for 60 min with venous indwelling catheter. On day 4, 1×10^6 T-MPs were intravesically instilled into the bladder by catheter and kept for 1 h 12 h later, 100 μl chemotherapeutic drugs were intravesically instilled into the bladder. On day 12, 6 mice in each group (PBS, free drugs, T-MPs combined with drugs) were sacrificed and bladders were harvested for further studies.

2.5. Apoptosis detection

Cells were collected and stained with FITC-Annexin V and 7-ADD viability staining solution for apoptosis detection with a C6 flow cytometer (BD).

2.6. Real-time PCR

Real-time PCR analyses were performed with 2 μg of cDNA as a template, using a SYBR Green mix (Applied Bioscience) and an Agilent Technologies Stratagene Mx3500P real-time PCR system. The primers for all genes tested, including internal control GAPDH, were synthesized by Sangon Biothech: GAPDH 5′- GGAGCGAGATCCCTCCAAAAT-3′(sense) and 5′-GGCTGTTGTCATACTTCT CATGG-3′(antisense), ABCB1 5′-TTGCTGCTTACATTCAGGTTTCA-3′(sense) and 5′- AGCCTATCTCCTGTCGCATTA-3′(antisense), TFEB 5′-ACCTGTCCGA GACCTATGGG-3′(sense) and 5′-CGTCCAGACGCAAATGTTG TC-3′(antisense), Rab7 5′-CTCATTATCGTCGGAGCCATTG-3′(sense) and 5′-AGTGTGGTCTG GTATTCCTCATA-3′(antisense), ATP6V_0E 5′-GTCCTAACCGGGGAGTAT CA-3′(sense) and 5′-AAAGAGAGGGTTGAG TTGGGC-3′(antisense), ATP6V_1A 5′- GGGTGCAGCCATGTATGAG-3′(sense) and 5′-TGCGAAGTACAGGATC TCCAA-3′(antisense), ATP 6V_1B2 5′- AGTCAGTCGGAACTACCTCTC -3′(sense) and 5′- CATC CGGTAAGGTCAAATGGAC-3′(antisense), ATP6V_1C1 5′-GAGT TCTGG CTTATATCTGCTCC -3′(sense) and 5′-GTGCCAACCTTTAAGTCA GGA AT-3′(antisense), ATP6V_1E1 5′-AACATAGAGAAAGGTCGGCTTG -3′(sense) and 5′- GACTTTGAGTCTCGCTTGATTCA -3′(antisense), ATP6V_1F1 5′-CTCATCGCAGTGATCGGAGAC-3′(sense) and 5′-CGGTT CTTGTTAAGCT CCCCTAT-3′(antisense), ATP6V_1G1 5′-CTAGTCAGTCT-CAGGGGATTCA -3′(sense) and 5′-GTTCTGCCGGAAGTATGTCTG-3′(antisense), ATP6V_1H 5′-GCAAAGAACAGACCGTTCAGT-3′(sense) and 5′-ATTGGCAGAAAGTAG GGCCAC-3′(antisense) Analysis of the results was performed using Mxpro software (Stratagene) and relative quantification was performed using the ΔΔCt method with GAPDH as a reference. The entire procedure was repeated in at least three biologically independent samples.

2.7. HPLC analysis

The concentration of Dox efflux in medium was measured by