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Macrophage mediated biomimetic delivery system for the treatment of lung metastasis of breast cancer

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ABSTRACT

The biomimetic delivery system (BDS) based on special types of endogenous cells like macrophages and T cells, has been emerging as a novel strategy for cancer therapy, due to its tumor homing property and biocompatibility. However, its development is impeded by complicated construction, low drug loading or negative effect on the cell bioactivity. The present report constructed a BDS by loading doxorubicin (DOX) into a mouse macrophage-like cell line (RAW264.7). It was found that therapeutically meaningful amount of DOX could be loaded into the RAW264.7 cells by simply incubation, without significantly affecting the viability of the cells. Drug could release from the BDS and maintain its activity. RAW264.7 cells exhibited obvious tumor-tropic capacity towards 4T1 mouse breast cancer cells both in vitro and in vivo, and drug loading did not alter this tendency. Importantly, the DOX loaded macrophage system showed promising anti-cancer efficacy in terms of tumor suppression, life span prolongation and metastasis inhibition, with reduced toxicity. In conclusion, it is demonstrated that the BDS developed here seems to overcome some of the main issues related to a BDS. The DOX loaded macrophages might be a potential BDS for targeted cancer therapy.

1. Introduction

Despite great advances in medicine, breast cancer including its lung metastasis remains a great threat to women. Of all the limiting factors in varied treatments, the inadequate amount of drugs accumulating in tumor tissues and the destroying effects of cytotoxic compounds to healthy tissues account a lot. As a result, cancer targeted drug delivery system is urgently needed.

Recently, the biomimetic delivery system (BDS) has been emerging as a novel strategy to actively carry payloads to the tumor sites. The BDS shows its superiority over other cancer targeted drug delivery systems in the aspects of inherent tumor-homing tendency and biocompatibility [1]. Generally, isolating macrophages, T cells or mesenchymal stem cells (MSCs) from peripheral blood or tumor samples, modifying genetically or loading with treating agents in vitro, are infused back to patients to perform anti-cancer effects. The therapy is mainly based on chemotaxis of these special cells. Namely, cytokines secreted by tumor cells are able to attract immunocytes as well as MSC, and enable them to accumulate in tumor sites. The phenomenon is also named as tumor homing property.

Numbers of approaches have been developed to endue carrier cells with tumor curing effects. Enabling the cellular vehicles to express anti-cancer molecules by gene technologies is most often employed. One strategy engineered MSC to express TRAIL and Interferon-β separately to treat glioma and melanoma [2–4]. However, transduction of the cells may lead to unwanted outcomes, such as transformation, increasing the risk of secondary malignancies. Instead, some investigators suggested combining cell mediated BDS with photothermal therapy [5–10]. The disadvantages associated with this method lie in its inconvenience to operate, as precise instruments are required. Other reports tried to load chemotherapeutic agents containing nanomaterials into cellular vehicles [11–13]. However, the drug loading content seems too low to be clinically meaningful in most cases. To reduce the harmfulness of treating agents to the cellular vehicles themselves, some studies anchored drug containing nanomaterials at the surface of cells by chemical reactions [14–17], while others developed a BDS by linking prodrug to the related enzyme expressing macrophages [18,19]. These systems exhibited minimal toxicity to the cellular vehicles and healthy tissues, but perhaps the complicated operating procedures resist their possible clinical application.

Concerning all the advantages and disadvantages of the above explorations, the purpose of this study is to create a new BDS, carrying therapeutically meaningful amount of drugs by a simple method without impacting the tumor homing property of cell carriers, and displaying encouraging anti-cancer efficacy. For the proof of concept, RAW264.7 cells, a kind of mouse macrophage-like cell line with similar functions to primary macrophage cells, were used here as the cell carriers. RAW264.7 cells are often used as cellular vehicles by other

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investigators [5,18–20] and also employed as the model of tumor-associated macrophages [20]. Then, for the first time, doxorubicin (DOX) loaded RAW264.7 cell delivery system was constructed and related studies in vitro and in vivo including its tumor homing ability, anti-cancer efficacy and toxicity to main organs in a mouse model of lung metastasis of breast cancer, were conducted.

2. Materials and methods

2.1. Materials

Doxorubicin (DOX) was purchased from Meilun Biology Technology Co., Ltd (Dalian, China). Triton X-100, bovine serum albumin (BSA), RNase A, propidium iodide and MTT were obtained from Solarbio Biology Technology Co., Ltd (Beijing, China). 4% paraformaldehyde solution and Hoechst 33258 were produced by M&C Gene Technology (Beijing, China). Anti-mouse P-gp antibody (rabbit, KG22525) and Alexa® Fluor 647 labelled anti-rabbit secondary antibody (goat, ab150079) were purchased from Keygen Biotech (China) and Abcam (Britain) separately. The near infrared fluorescent dye DIR (KGMP0026) was supplied by Keygen Biotech (China). Luciferin potassium was purchased from Cellcyto Biology Technology Co., Ltd (Shanghai, China). Firefly-luciferase coding adenovirus was constructed by Hanbio Biology Technology Co., Ltd (Shanghai, China). H&E staining reagents were supplied by Leagene Biology Technology Co., Ltd (Beijing, China). RPMI-1640, Dulbecco's Modified Eagle Media (DMEM), penicillin/streptomycin and trypsin were purchased from M&C Gene Technology (Beijing, China). Fetal bovine serum (FBS) was the product of Gibco, Invitrogen Corp. (Carlsbad, CA, USA). All other solvents and reagents were either of analytical or of chromatographic grade.

The mouse macrophage cell line RAW264.7 and the mouse breast cancer cell line 4T1 were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS, Beijing, China).

Female Balb/c mice (6 weeks old) were purchased from the Vital Laboratory Animal Center (Beijing, China) and kept under SPF conditions for 1 week before the study, with free access to standard food and water. All of the studies comply with the Principles of Care and Use of Laboratory Animals set by the Institutional Animal Care and Use Committee of Peking University Health Science Center.

2.2. Cell culture

RAW264.7 cells were cultured in DMEM containing 10% fetal bovine serum. 4T1 cells were cultured in RPMI 1640 media containing 10% fetal bovine serum. Both of the cells were placed in a 37 °C humidified incubator with 5% carbon dioxide.

2.3. Loading of DOX into RAW264.7 cells and drug release

The RAW264.7 cells were lifted by scrapping, washed by PBS and then counted. After being spun at 2000 rpm for 5 min, 1 million cells were resuspended in 1.8 ml DOX solution (PBS, 400 μg/ml) for 10 s. Then the cells were separated from the DOX solution by centrifugation and resuspended in 1.5 ml DMEM containing 10% fetal bovine serum. The tube containing the cells was placed in a 37 °C swing bed at 100 rpm. At 1 h, 1.5 h, 2 h and 2.5 h, drug concentration in the media was determined by HPLC, and the media was completely replaced by a fresh media. DOX solution before and after incubating with cells was also analyzed to get the total amount of drug loading. The experiment was carried out three times. To visualize drug loading into RAW264.7 cells, DOX solution (400 μg/ml) was added to the cells with 70% confluency and incubated for 10 min. After removing the DOX solution and washing the cells by PBS, the DOX loaded RAW264.7 cells were observed under confocal microscopy (TCS SP5, Leica).

2.4. Impact of DOX encapsulation on RAW264.7 cells

Twenty thousand RAW264.7 cells were added into each well of 96 well plate. The volume of the culture media was 200 μl. There were five groups, and each included 3 wells. After adherence at about 12 h, the media of group 5 was replaced by 100 μl DOX solution (400 μg/ml) and incubated for 10 min. Then the DOX solution was removed and the cells were washed by PBS. At last, 200 μl fresh media was added to each well. From then on, at 1 d and 2 d, cells of group 4 and 3 were treated as above. At 3 d, the appearance of the cells in different groups was pictured to show the toxicity of the drug to the cellular vehicles and MTT assay was used to quantify the toxicity. Groups 1 and 2 were used as blank and control.

Cell cycle assay was employed to clarify the resistant mechanisms of RAW264.7 cells to DOX. RAW264.7 cells were lifted by scrapping and were fixed by 70% ethanol solution at 4 °C. After being washed by PBS, the cells were incubated in 50 μg/ml RNase A solution at 37 °C for 0.5 h. Then a drop of propidium iodide solution was added to make the concentration of propidium iodide about 40 μg/ml. After incubating for about 5 min, the cell cycle distribution of the cells was assayed by flow cytometer (Vantage TM, Becton Dickinson).

2.5. Expression of P-gp in RAW264.7 cells

The expression of P-gp in RAW264.7 cells was examined by confocal microscopy. The cells were seeded 12 h before the following experiments. The cells were fixed by 4% paraformaldehyde solution and washed by PBS three times. Then 0.1% Triton X-100 solution was added to permeabilize the cells for 10 min. After washing three times by PBS, the cells were incubated in 5% BSA solution for 1 h to block unspecific sites. Then the cells were incubated in P-gp antibody solution (1: 50) at 4 °C overnight. After washing three times by PBS, Alexa® Fluor 647 labelled secondary antibody solution (1: 200) was added for 2 h at room temperature. After removing antibody solution and washing three times by PBS, the sample was scanned by confocal microscopy at 633/670 nm.

2.6. Cytotoxicity of DOX released from RAW264.7 cells against 4T1 tumor cells

The RAW264.7 cells were lifted by scrapping, washed by PBS and then counted. After being spun at 2000 rpm for 5 min, 1 million cells were resuspended in 1.8 ml DOX solution (PBS, 400 μg/ml) for 10 s. Then the cells were separated from DOX solution by centrifugation and resuspended in 12 ml DMEM containing 10% fetal bovine serum. The cells were cultured for another 6 h. The conditioned media was measured by HPLC to determine drug concentration and, then was diluted with the fresh media by different times. Media of 4T1 tumor cells in 96 well plate prepared one day before were replaced by the varied conditioned media to generate the drug concentration of 0, 0.8, 1.2, 1.6, 2, 2.4, 2.8, 3.2, 3.6 and 4 μg/ml, respectively. 4T1 tumor cells were cultured for another 24 h and, the cytotoxicity was assayed by MTT. The cytotoxicity of the diluted conditioned media with drug concentration of 4 μg/ml to 4T1 cells for 1 d, 2 d and 3 d was also assayed by similar method.

2.7. The chemotaxis of RAW264.7 cells towards 4T1 tumor cells in vitro

The tumor tropic ability of RAW264.7 cells towards 4T1 tumor cells in vitro was examined by transwell migration test. The transwells (8 μm, Corning) were first soaked in sterile 1% BSA solution (PBS) at 37 °C for 1 h. Then the solution was removed and 1 ml fresh DMEM or conditioned media of 4T1 tumor cells was added to the subnatant wells according to Table 1. Thirty thousand RAW264.7 cells with or without DOX loading in 200 μl fresh DMEM were added to the supernatant wells. The cells were cultured for another 36 h. Then all the media