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Nanoparticles for Tumor-specific Intracellular Drug Delivery

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Abstract—**While intraperitoneal (IP) therapy of ovarian cancer is a theoretically promising treatment option, it is not clinically well accepted due to the several challenges in IP drug delivery. Nanoparticles are promising drug carriers, which may alleviate the difficulties in IP chemotherapy. However, currently available nanoparticles need to be further improved to fulfill the following requirements: (i) they must remain non-interactive with normal cells and prevent the payload from premature leaking; (ii) once the drug carriers reach the tumor, they should enter the cells efficiently and release the drug in the cells to effectively kill the targeted cells. Our recent observation indicates that a popular nanoparticle system fails these expectations by large margin. For safe and effective IP chemotherapy, new types of carriers and/or surface modification strategies are urgently needed.**

I. Introduction

OVARIAN cancer accounts for 3% of all cancers among women, and the frequency ranks second among gynecologic cancers [1]. The 5-year survival rate has increased from 30% to 44% over the last 30 years, but it is still the most lethal gynecologic cancer [1, 2]. Current treatment for patients diagnosed with ovarian cancer relies on surgical debulking of the tumor followed by intravenous (IV) administration of a combination of taxanes and platinum analogues [3]. On the other hand, there are a growing number of preclinical and clinical studies promoting intraperitoneal (IP) chemotherapy as an alternative post-operative therapy [4-6].

Given that ovarian tumors are largely confined to the peritoneum cavity, IP chemotherapy is a theoretically appealing treatment option for ovarian cancer [7, 8]. However, IP chemotherapy has not become a widespread practice [9], because of the controversial clinical outcomes [10-12] and significant toxic side effects [13]. Potential challenges in IP chemotherapy are: First, drugs in the peritoneum are rapidly absorbed to the systemic circulation; therefore, the residence time of drugs in the peritoneum is relatively short. Second, IP anti-cancer drugs may have pan-peritoneal toxicity, unless they are delivered specifically to the tumors. Third, even if a high level of drugs can reach the tumors, it is not guaranteed that enough drug molecules will be taken up by the target cancer cells and remain in the cells until they are completely eradicated [14]. This is particularly problematic for treatment of multidrug-resistant tumors.

Central to overcoming these challenges is to develop an efficient drug carrier, which will deliver an anti-cancer drug specifically to tumor cells and provide a high level of intracellular drugs until they can eradicate the tumor cells completely. To this end, ideal drug carriers for IP chemotherapy of ovarian cancer should satisfy following requirements: (i) A drug carrier should remain non-interactive with normal cells but readily enter the target tumor cells. (ii) The carrier should not leach out the payload outside target tissues but readily release the drug in the cells to provide an intracellular reservoir of cytotoxic drugs to completion of cell death.

While a number of polymeric nanoparticle (NP) systems are currently investigated as a potential carrier, few systems meet all these requirements. According to our recent observations, despite the popularity and some indirect evidence indicating efficient cellular uptake, NPs made of poly(lactic-co-glycolic acid) (PLGA) are not readily taken up by cells. Instead, they randomly deliver the payload to cells by extracellular drug release and/or direct drug transfer to contacting cells [15]. Recent studies also suggest that instability of other nanocarriers and non-specific drug leakage may be more prevalent problems than thought [16, 17].

Here, we review our recent report indicating the inefficiency of PLGA NPs as an intracellular drug delivery system [15], and discuss new strategies that may be useful for overcoming the limitations of existing systems.

II. Methods

A. Preparation and Characterization of PLGA NPs

PLGA NPs physically encapsulating Nile red (NR/NP) were prepared using the single emulsion method. PLGA and Nile red were dissolved in dichloromethane or a mixture of dichloromethane and acetone. The polymer solution was directly added to 5% polyvinyl alcohol. The mixture was then homogenized using a probe sonicator to generate an oil-in-water (o/w) emulsion. The formed emulsion was added to distilled water and stirred for 1 hour at room temperature. After removing the remaining solvents by evaporation, the NPs were collected by centrifugation, and further purified by ultrafiltration or by multiple washing with distilled water.

PLGA NPs chemically modified with a fluorescent dye (fl-NP) were prepared using a mixture of plain PLGA and PLGA conjugated to fluoresceinamine (FA-PLGA). FA-PLGA was prepared by conjugating fluoresceinamine to PLGA via carbodiimide chemistry.

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B. Confocal Microscopy

For confocal microscopy of real-time uptake of NR/NPs or fl-NPs, overnight-grown mesothelial cells were incubated with NR/NPs or fl-NPs up to 3 hours. The medium was quickly replaced with a particle-free fresh medium, and the cells were imaged with confocal laser scanning fluorescence microscopy.

C. Fluorescence Activated Cell Sorting (FACS)

Overnight-grown mesothelial cells were incubated with NR/NPs equivalent to 0.5 μM Nile red. Fresh medium was used as a negative control. After up to 3 hours of incubation in the presence of NPs, cells were washed with fresh medium twice, then with phosphate-buffered saline (PBS), and trypsinized for FACS analysis. Another set of cells were treated and washed in the same way, and then further incubated in the particle-free fresh medium for 3 hours, prior to FACS analysis.

D. Release of fluorescence probes in the presence of serum or lipid

NR/NPs or fl-NPs were incubated in PBS, PBS with serum protein, or PBS with liposomes at 37˚C. At regular intervals, supernatants were collected to determine their fluorescence levels.

III. Results and Discussion

PLGA NPs physically encapsulating Nile red (NR/NPs) were prepared in two sizes (99.2 nm and 311.0 nm). At pH 7.4, both particles showed negative zeta potential. To observe uptake of NR/NPs, mesothelial cells were incubated in the presence of NR/NPs for 1, 30, or 180 min and imaged with confocal microscopy immediately after replacing the medium with fresh particle-free medium. Irrespective of the particle size, red fluorescence was seen inside the cells, especially in the perinuclear region, as early as 1 min after incubation with the NR/NPs. Fluorescence intensity in the cells increased with incubation time. The increase in fluorescence intensity of the cells was also observed with FACS. Interestingly, irrespective of particle size and duration of exposure, the cellular fluorescence decreased significantly when the cells were exposed to particle-free medium.

A series of experiments were performed to explain the loss of cellular fluorescence. Cytocompatibility of PLGA NPs and stability of Nile red fluorescence allowed us to exclude cell death or concentration-/pH-dependent fluorescence quenching. Notably, the dye lost from the cells was recovered from the particle-free medium, in which the particle-fed cells were maintained. This observation indicated that intracellular Nile red was "exocytosed" from the cells, although it was unclear at the moment whether it was still an encapsulated form or a free dye.

On the other hand, several pieces of evidence indicate that NPs were rarely taken up by the cells, and it was rather the extracellularly released Nile red that entered the cells and was subsequently exocytosed. First, while NR/NPs did not immediately release the dye in PBS, they released a significant amount of Nile red in less than 3 hours in the presence of relatively hydrophobic components like serum proteins or liposomes (i.e., lipid bilayer). Second, unlike NR/NPs, fl-NPs that contained chemically conjugated dyes to PLGA were rarely seen inside the cells even after 3 hours of incubation. The lack of cellular uptake of PLGA NPs was also confirmed using Coherent Anti-Stokes Raman Scattering (CARS) microscopy [18], which did not require fluorescence labeling of the object. These results consistently suggest that PLGA NPs are not readily taken up by cells, and the fluorescence signal observed in cells incubated with NR/NPs is likely to be an artifact due to the extracellular release and transfer of the lipophilic dye to the adjacent cells.

Although our observation is limited to small molecular-weight and hydrophobic molecules, it provides an alternative view that challenges the widespread optimism for PLGA NPs as an intracellular drug delivery system. According to our observation, there are at least two main disadvantages to PLGA NPs. First, PLGA NPs can leach out the payload non-specifically and prematurely, especially when in contact with relatively hydrophobic components including cell membrane. Second, due to the lack of cellular uptake, their utility would be very much limited in treatment of multidrug-resistant tumors, for which maintenance of high level of intracellular drug is desirable.

To address these limitations, our laboratory is developing a new surface modification strategy, which will prevent a nanocarrier from interacting with normal cells but allow for the carrier to enter the target cells in a tumor-specific manner. One such effort is to create chitosan derivatives amidized with carboxylic acid anhydrides, which assume positive charges at relatively acidic pH and negative charges at higher pH. Their net charges become zero at a certain pH (isoelectric pH), which varies with the choice of acid anhydrides and the degree of amidization. Coating a cationic nanocarrier with the chitosan derivatives may allow for the carrier to remain negatively charged at physiological pH but to reveal the cationic surface at weakly acidic tumoral pH, enabling effective cellular uptake of the carriers.

In a related context, a number of new strategies are recently introduced to achieve both "stealth" effect and improved cellular uptake with a nanocarrier [19]. These strategies explore the unique tumoral extracellular environment such as a weakly acidic pH [20] or a high level of proteinases [21] as a means to trigger tumor-specific transformation of the nanocarrier. An example is a micelle system made of a blend of two block copolymers: polyhistidine-b-poly(ethylene glycol) (polyHis-b-PEG) and polylactide-b-PEG-b-polyHis-biotin [22] (or polylactide-b-PEG-b-polyHis-TAT [23]). At pH 7.4, the micelle maintains a PEG shell, and biotin (or TAT peptide) termini are hidden from the surface. When the micelle is exposed to <pH 7.2, ionization of polyHis prompts biotin (or TAT) termini to emerge and interact with cells.

Although at the early stage, these strategies have good potential to reduce non-specific release of anti-cancer drugs, facilitate tumor-specific cellular uptake of the drug carriers, and enhance IP chemotherapy of ovarian cancer and related malignancies.

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