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Inducing enhanced immunogenic cell death with nanocarrier-based drug delivery systems for pancreatic cancer therapy

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ABSTRACT

Immunogenic cell death (ICD) occurs when apoptotic tumor cell elicits a specific immune response, which may trigger an anti-tumor effect, via the release of immunostimulatory damage-associated molecular patterns (DAMPs). Hypothesizing that nanomedicines may impact ICD due to their proven advantages in delivery of chemotherapeutics, we encapsulated oxaliplatin (OXA) or gemcitabine (GEM), an ICD and a non-ICD inducer respectively, into the amphiphilic diblock copolymer nanoparticles. Neither GEM nor nanoparticle-encapsulated GEM (NP-GEM) induced ICD, while both OXA and nanoparticle-encapsulated OXA (NP-OXA) induced ICD. Interestingly, NP-OXA treated tumor cells released more DAMPs and induced stronger immune responses of dendritic cells and T lymphocytes than OXA treatment *in vitro*. Furthermore, OXA and NP-OXA exhibited stronger therapeutic effects in immunocompetent mice than in immunodeficient mice, and the enhancement of therapeutic efficacy was significantly higher in the NP-OXA group than the OXA group. Moreover, NP-OXA treatment induced a higher proportion of tumor infiltrating activated cytotoxic T-lymphocytes than OXA treatment. This general trend of enhanced ICD by nanoparticle delivery was corroborated in evaluating another pair of ICD inducer and non-ICD inducer, doxorubicin and 5-fluorouracil. In conclusion, although nanoparticle encapsulation did not endow a non-ICD inducer with ICD-mediated anti-tumor capacity, treatment with a nanoparticle-encapsulated ICD inducer led to significantly enhanced ICD and consequently improved anti-tumor effects than the free ICD inducer. The proposed nanomedicine approach may impact cancer immunotherapy via the novel cell death mechanism of ICD.

1. Introduction

The design and synthesis of nanomaterials to encapsulate and deliver drugs for tumor treatment is a burgeoning area in nanomedicine [1,2]. Nanoparticle-encapsulated drugs (nanocarrier-drug delivery systems) comprise the therapeutic entities, such as chemotherapeutic drugs and therapeutic siRNA [3–6], and the nanocarriers formed by biocompatible materials, such as polymers and liposomes [7–11]. It has been widely believed that the improved anti-tumor effects of nanomedicine are mainly mediated by enhanced direct cytotoxicity of chemotherapeutics (such as inducing apoptosis), due to elevated drug concentrations in tumor tissue via passive- and/or tumor-targeting and favorable pharmacokinetics [12,13]. Although immune parameters significantly affect the anti-tumor efficacy of chemotherapeutics, to date there are no reports on nanomedicines' contributions to immunogenic cell death (ICD) and the associated indirect long-term anti-tumor effects.

Over the past decades, apoptosis, identified as a programmed cell death, as opposed to necrosis, is believed not to provoke

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immune responses [14–16]. However, there is accumulating evidence to support that some apoptotic cells can also produce immunostimulatory, damage-associated molecular patterns (DAMPs), which can be engulfed and presented by dendritic cells (DCs) to elicit T lymphocyte-mediated immunologic cytotoxicity [17–21]. This functionally peculiar type of cell death is termed "immunogenic cell death" (ICD), mainly in the context of anti-tumor therapy of chemotherapeutics for their profound clinical implications [17,18,21]. ICD implies that dying cancer cells under the "first-hit" by ICD inducers (some chemotherapeutic agents) would operate as a tumor vaccine that stimulates a tumor-specific immune response, which can give a "second-hit" to the residual tumor tissue, possibly providing patients a long-term clinical benefit indirectly from the initial drug cytotoxicity [22,23]. During the ICD process, the composition of tumor-infiltrating immune cells changes, which can be crucial for the chemotherapeutic responses of cancer patients. For example, the increased proportion of tumor infiltrating cytotoxic $CD3^+CD8^+$ T lymphocytes (CTLs) predicts apropensity of breast and colorectal cancer patients to benefit from anthracyclines and oxaliplatin treatment, respectively [24–27]. In addition, loss-of-function alleles of toll-like receptor 4 (TLR4) and purinergic receptor P2RX7, which are required in DCs for the reception of ICD signals, indicate poor outcomes in patients treated with anthracyclines or oxaliplatin [28,29]. These studies show that ICD plays an important role in cancer chemotherapy. However, the induction of tumor cell apoptosis by chemotherapy is widely considered immunologically silent [14,15]. In addition, the National Cancer Institute guidelines for drug screening for anti-tumor therapy demand testing in immunodeficient mice with human tumor xenografts [30]. Therefore, the role of ICD in chemotherapy has not been fully explored [31].

Currently, the identification of ICD inducers mainly relies on detecting exposed DAMPs after treatment, such as surface exposure of calreticulin (CRT), extracellularly secreted adenosine triphosphate (ATP), and passively released chromatin-binding protein high mobility group B1 (HMGB1) *in vitro* [18]. *In vivo*, ICD must satisfy two requirements: first, subcutaneous injection with dying mouse cancer cells, which is succumbed to candidate ICD inducers *in vitro*, protects immunocompetent mice against a subsequent re-challenge with the live tumor cells; second, the tumor inhibition effect of ICD inducers on established tumor is stronger in immunocompetent than in immunodeficient mice [17,18,21]. Using these criteria, several chemotherapeutics have been demonstrated to be ICD inducers. These include anthracyclines, mitoxantrone, oxaliplatin, cyclophosphamide and bortezomib [32–37]. However, there are no reports on the roles of ICD in treatment with nanomedicines, although nanocarriers for chemotherapeutics largely improve the targeted tumor delivery and tumor accumulation of chemotherapeutic drugs. In the present study, we selected monomethoxy-poly(ethylene glycol)-poly(D,L-lactide-*co*-glycolide) (mPEG-PLGA) polymeric nanoparticles as a model nanocarrier to encapsulate oxaliplatin (OXA, an ICD inducer) or gemcitabine (GEM, a non-ICD inducer) [34,38], both of which are used clinically as first-line chemotherapy regimens for pancreatic cancer treatment [39]. We focused on the comparative effects of the respective drug preparations as well as the levels of ICD effects provoked by nano-drug formulations and the possible mechanisms, to reveal the potential of ICD in nanomedicines.

2. Materials and methods

2.1. Preparation and characterization of nanoparticles

mPEG-PLGA nanoparticles were prepared by the double emulsion (W/O/W) method to improve the stability of the resulting nanoparticles and drug loading capacity, compared to single emulsion method [4]. Briefly, 20 mg of diblock copolymer mPEG-PLGA (molecular weight: 5000–15,000) was dissolved in 1 mL of methylene chloride and 0.2 mL of water (containing OXA or GEM if required), and transferred to a centrifuge tube. The mixture was emulsified by sonication for 4 min. The emulsion was added to 2 mL of 2% polyvinyl alcohol (PVA) and emulsified by sonication for 5 min. The emulsion was then slowly dropped in 10 mL of 0.6% PVA and stirred for 10 min at room temperature. The solvent was removed by vacuum evaporation. TEM measurement for nanoparticles morphology and DLS measurement for the size distribution were performed as previously described [3,4].

2.2. Encapsulation efficiency calculation

The drug encapsulation efficiency was calculated by the following equation: Encapsulation efficiency $= (A-B)/A \times 100\%$, where A is the initial amount of drug added in the system and B represents the amount of drug obtained in the supernatant after centrifugation determined by HPLC for the absorption spectra. HPLC measurements for OXA and GEM were performed as previously described [3,5].

2.3. Cell culture and drug administration

The human pancreatic cancer cell line Panc-1 and murine pancreatic cancer cell line Pan02 were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. Panc-1 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and the Pan02 cells were a gift from Prof. Yang SY (H. Lee Moffitt Cancer Center and Research Institute, USA). For *in vitro* treatment, the concentrations of OXA, GEM, 5-Fu and DOX are 4, 16, 4 and 3 μM, respectively.

2.4. Cell apoptosis

Cancer cells were seeded into 6-well plates at a density of 100,000 cells per well. After 48 h treatment with the different drug formulations (the concentrations of OXA and GEM were 4 and 16 μM, respectively), the cells were stained by Annexin V-FITC Apoptosis Detection Kit (BD, USA). Briefly, the cells were collected and washed once by cold PBS. Then the cells were stained by 5 μl Annexin V-FITC reagent for 5 min at room temperature, followed by 5 μl PI reagent for 5 min at room temperature before detection using flow cytometry. According to the staining, the cells can be divided into three groups: normal (Annexin V and PI negative), early apoptosis (Annexin V positive and PI negative), and end stage apoptosis (Annexin V and PI positive). In this study, the apoptotic cells were designated as all of Annexin V positive cells. In addition, the cells were stained by CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Life technologies, USA). Briefly, the cells were collected and washed once by cold PBS. Then the cells were stained by 500 nM Caspase-3/7 Green Detection Reagent for 60 min, followed by 1 μM AADvance dead cell stain solution for 5 min. The cells were then analyzed using flow cytometry without washing, and the apoptotic cells were designated as Caspase 3/7 positive and AADvance negative cells.

2.5. HMGB1 and ATP release assay

Cancer cells were seeded into 6-well plates at a density of 100,000 cells per well. After 48 h treatment with the different drug formulations (the concentrations of OXA and GEM were 4 and