Journal of Controlled Release 248 (2017) 1–9

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Surface engineering tumor cells with adjuvant-loaded particles for use as cancer vaccines

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ARTICLE INFO

Article history:
Received 22 August 2016
Accepted 29 December 2016
Available online 3 January 2017

Keywords:
Cell surface engineering
Cancer vaccines
Streptavidin-biotin cross-linking
PLGA particles

ABSTRACT

Cell surface engineering is an expanding field and whilst extensive research has been performed decorating cell surfaces with biomolecules, the engineering of cell surfaces with particles has been a largely unexploited area. This study reports on the assembly of cell-particle hybrids where irradiated tumor cells were surface engineered with adjuvant-loaded, biodegradable, biocompatible, polymeric particles, with the aim of generating a construct capable of functioning as a therapeutic cancer vaccine. Successfully assembled cell-particle hybrids presented here comprised either melanoma cells or prostate cancer cells stably adorned with Toll-like receptor-9 ligand-loaded particles using streptavidin–biotin cross-linking. Both cell-particle assemblies were tested in vivo for their potential as therapeutic cancer vaccines yielding promising therapeutic results for the prostate cancer model. The ramifications of results obtained for both tumor models are openly discussed.

1. Introduction

The use of cells as therapeutic agents is well established, examples of which include adoptive T cell therapy for the eradication of cancers and the use of multipotent stem cells for tissue regeneration [1–3]. Most cell therapies have involved the use of viable cells, capable of proliferation, differentiation, or performing an inherent functional task. However, the use of irradiated, and therefore dying, whole tumor cells is a cancer vaccine strategy that has shown promising outcomes in preclinical and clinical settings [4,5]. The aim of such a strategy is to generate effective adaptive immune responses to a wide range of tumor-associated, and tumor-specific, antigens and therefore reduce the possibility of immune evasion by tumor cells as a result of antigen loss variants [6]. Cancer accounts for 1 in 4 deaths in the United States [7]. A number of these malignancies, which are often resistant to conventional therapies, have shown promise in terms of responses to immune therapies including irradiated whole tumor cell vaccines which can act as a source of tumor antigens [8]. Examples of such malignancies include melanoma, prostate and pancreatic cancer [9–11]. Irradiation is used to ensure such cell-based vaccines are incapable of proliferation, however, sometimes irradiation has the additional benefit of increasing the antigenicity of tumor cells by promoting an immunogenic form of apoptosis [12,13]. Whilst genetically modified versions of these cells, often involving transfection with GM-CSF, have delivered encouraging results in clinical trials, it is still apparent that further enhancement of the immunogenicity of these vaccines is required [14]. Cell surface engineering has been the focus of many studies where various cell types have been surface engineered with different macromolecules or nanomaterials for a variety of applications [15–20]. Cell surface engineering of tumor cells with an immune adjuvant can enhance the immunogenicity of the vaccine compared to providing the adjuvant in soluble form. The importance of co-delivering tumor antigen and adjuvant to the same dendritic cell has been illustrated in vitro [21] and has been further confirmed in vivo using a whole cell tumor vaccine where vaccination with the Toll-like receptor 9 ligand, CpG oligodinucleotide (CpG ODN), chemically linked to apoptotic tumor cells resulted in slower tumor growth of established TRAMP C-1 tumors [22]. A limitation of direct chemical linking of immune adjuvants is that one is restricted to using only those adjuvants that are not functionally compromised by the chemical coupling process. By packaging adjuvants into polymeric particles and chemically linking *the particles* to cells it is possible to circumvent the potential problem of adjuvant inactivation. The use of synthetic particles to tune immune responses is well established [23,24]. However, there have been very few reports in the literature of attempts to engineer cell surfaces with discrete particles [25–27]. These studies have involved either chemical modification of cell surface residues or simple adsorption of particles to cells. The work presented here focuses on the design of a cancer vaccine formulation where the immune adjuvant is loaded into poly(lactic-*co*-glycolic acid) (PLGA) particles that are then anchored to the tumor cell surface. The particles were anchored onto the cell surface using the streptavidin-biotin cross link that is commonly applied in tissue engineering [28–30]. The method expounded upon here is relatively simple, resulting in a product that should be capable of clinical translation into therapy due to the already established track records of the constituents. For instance, PLGA has FDA approval for

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http://dx.doi.org/10.1016/j.jconrel.2016.12.036
0168-3659/

clinical use, whilst CpG ODN has been shown to have a good safety profile in clinical trials [31]. The engineered cell-particle assemblies reported here impart a level of versatility to the vaccine formulation where the polymeric particles can be loaded with different immune adjuvants, or even a combination of immune adjuvants as required, and can be tailored to different tumor types.

2. Materials and methods

2.1. Cell lines

The murine melanoma cell line, B16.F10, was obtained from ATCC (Manassas, VA). The murine prostate cancer cell line, RM11, was a generous donation from Professor David Lubaroff, (University of Iowa, Iowa city, IA). Cells were maintained in DMEM complete media supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM Glutamax™, and 50 ng/ml gentamicin sulfate. Cells were incubated in a 5% CO_2 humidified incubator at 37 °C. For vaccinations, a B16.F10 GM-CSF clone expressing 220 ng GM-CSF/10^6 cells/day was derived by transducing B16.F10 cells with a lentiviral vector encoding murine GM-CSF (AMSBIO, Cambridge, MA).

2.2. Fabrication and characterization of streptavidin-coated particles

Particles were prepared using a double emulsion solvent evaporation technique [32]. In brief, 50 μl 1% polyvinyl alcohol (PVA) solution (water phase 1) was emulsified in 1.25 mL dichloromethane (DCM) containing 100 mg PLGA (75:25 m.wt 68 kD, with uncapped carboxyl end groups) (oil phase) using a sonic probe for 30 s at 40% amplitude, generating a primary emulsion. This was then emulsified in 8 mL 2.5% PVA (MW:67 kD) in 0.1 M MES buffer (2-(N-morphino) ethanesulfonic acid) (water phase 2) using the same probe settings, generating a secondary emulsion. The secondary emulsion was then rapidly poured into 20 mL 1% PVA in 0.1 M MES buffer and stirred in fume hood. EDC and NHS, dissolved in MES buffer, were added sequentially at a ratio of 2 mg EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 3 mg NHS (N-hydroxysuccinimide) per 1 mg PLGA polymer (the amounts of EDC/NHS were empirically determined). The particle suspension was then stirred in a fume hood for 2 h to allow DCM evaporation and carboxyl end group activation. Particles were collected by sequential centrifugation to ensure narrow size distribution where particle suspension was first centrifuged at 115 $\times g$ for 5 min and the pellet was discarded. Particles used in the study were collected from the supernatant of the previous step by centrifugation at 10,000 $\times g$ for 10 min and washed 3 times using nanopure water. Particle suspension was frozen and lyophilized overnight. CpG loaded particles were prepared using 2 mg CpG ODN 1826 in the 50 μL 1% PVA-water phase 1. Rhodamine B-loaded particles were prepared by dissolving 1 mg rhodamine B in the PLGA polymer/DCM solution. Particles were characterized for size and zeta potential using a Zetasizer Nano ZS (Malvern). For rhodamine B-loaded particles (which could not be measured using a Zetasizer), size was measured from SEM images using ImageJ software ($n = 100$). This size measurement method was validated by measuring the sizes of blank particles using ImageJ software and comparing results to their sizes determined using the Zetasizer Nano ZS. To estimate CpG loading, 5–10 mg of particles were degraded in 1 ml 0.3 N NaOH until a clear solution was obtained. This solution was neutralized with 1 N HCl. The CpG ODN concentration in the neutralized solution was estimated using an OliGreen® assay kit according to product instructions.

Loading was calculated as follows:

$$\text{Loading (μg CpG per mg particles)} = \frac{\text{CpG concentration (μg per ml) in neutralized solution} \times \text{neutralized solution volume (ml)}}{\text{weight of degraded particles (mg)}}.$$

Lyophilized particles were coated with streptavidin by incubating surface activated particles with streptavidin in phosphate buffered saline (PBS), pH 7.4 at a ratio of 8 μg streptavidin per 1 mg particles for 30 min at room temperature. Excess streptavidin was removed by washing particles with PBS 3 times. To confirm successful particle coating, as proof of principle, fluorescently labeled streptavidin (streptavidin-PE) was used and particles were analyzed by flow cytometry (FACScan, Becton-Dickinson) ($n = 3$). Streptavidin-coated particles (unlabeled) were incubated with fluorescently labeled biotin to ensure availability of surface streptavidin for biotin binding. Samples were analyzed using flow cytometry ($n = 3$).

2.3. Surface engineering tumor cells with PLGA particles

B16.F10 or RM11 tumor cells were coated with biotin using biotinylated antibody targeting the β1 integrin (*anti*-mouse/rat CD29). Surface expression of CD29 by tumor cells and successful tumor cell biotinylation were confirmed by an immunofluorescent assay using streptavidin-PE and samples were analyzed by flow cytometry ($n = 3$). To surface functionalize tumor cells with particles, biotinylated tumor cells were incubated with streptavidin-coated particles at a ratio of 1 mg particles per 5×10^5 cells in complete cell culture media for 15 min on ice followed by 15 min at 37 °C. Successful cell surface engineering was confirmed using flow cytometry and microscopic imaging. Initially, the following two negative controls were developed to ensure cell-particle hybrids were formed as a result of streptavidin-biotin chemistry: biotinylated cells incubated with uncoated particles and non-biotinylated cells incubated with streptavidin-coated particles. As both controls showed similar results (see Suppl. Fig. 1), only non-biotinylated cells + streptavidin-coated particles were used as the negative control for all upcoming experiments. For scanning electron microscopy (SEM) imaging, cell-particle mixtures were fixed in glutaraldehyde and mounted on poly-L-lysine treated silica wafers. Samples were stained with osmium tetraoxide and gradually dehydrated with ethanol and hexamethyldisilazane (HMDS). Samples were sputter coated with gold/palladium for 3 min prior to imaging on SEM at 2 kV accelerating voltage (Hitachi S-4800). For laser scanning confocal microscopy, cell-particle hybrids were assembled using rhodamine loaded particles. Cell-particle mixtures were fixed in 4% paraformaldehyde and deposited on slides using a cytospin centrifuge at 700 rpm for 7 min. Cover slides were then mounted using Vectashield mounting media with DAPI. Samples were imaged using differential interference contrast (DIC)/fluorescence mode (Zeiss 710 confocal microscope).

To estimate the percentage of particles binding to the cell surface, an indirect assay was developed (see Suppl. Fig. 2). Briefly, cell-particle hybrids were assembled as described above. Aliquots were added to wells of a 96 well tray at a cell density of 1×10^5 cells/well and incubated with biotin-linked alkaline phosphatase (7.5 μg/ml) for 30 min. Unbound particles and enzyme were simultaneously removed through washing prior to adding an enzyme substrate (p-nitrophenyl phosphate) in Tris buffer. The plate was centrifuged at 230 $\times g$ for 5 min and an aliquot of the supernatant was transferred to a new plate to record the absorbance at 450 nm using SpectraMax® Plus384 microplate reader. Blanks involved hybrid samples where the p-nitrophenyl phosphate was added to samples without prior addition of biotinylated enzyme (see Suppl. Fig. 2B). A calibration curve was generated by serially diluting known amounts of streptavidin-coated particles that were incubated with biotinylated enzyme prior to mixing with cells and subsequently adding the enzyme substrate. Blanks for the calibration curve involved mixing known amounts of particles with cells without the incorporation of the enzyme. All samples were carried out in triplicate. Assays were performed independently for cell-particle hybrids assembled using the B16.F10 and the RM11 tumor cells.