Journal of Controlled Release 183 (2014) 146–153

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Targeting and depletion of circulating leukocytes and cancer cells by lipophilic antibody-modified erythrocytes

Rajesh Mukthavaram [a,b,1], Guixin Shi [a,1], Santosh Kesari [b,c], Dmitri Simberg [d,*]

[a] Moores UCSD Cancer Center, UC San Diego, 3855 Health Sciences Drive, La Jolla 92093, USA
[b] Neuro-Oncology Program and Translational Neuro-Oncology Laboratories, Moores UCSD Cancer Center, UC San Diego, 3855 Health Sciences Drive, La Jolla 92093-0819, USA
[c] Department of Neurosciences, UC San Diego, La Jolla, USA
[d] The Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus, 12850 E. Montview Blvd., Aurora 80045, USA

ARTICLE INFO

Article history:
Received 24 October 2013
Accepted 20 March 2014
Available online 28 March 2014

Keywords:
Targeting
RBC
Lymphoma
Rituximab
Depletion

ABSTRACT

There is a great interest in targeting and selective ablation of populations of circulating cells for research or therapeutic purposes. Red blood cells (RBCs) are readily available and fully biocompatible long-circulating intravascular carriers (natural life is 120 days) that are amenable to chemical modifications, drug loading and reinjection. Here we demonstrate that using our previously described lipophilic ligand painting strategy, red blood cells (RBCs) could be in one step converted into targeted entities that selectively seek and bind various cells *in vitro* and *in vivo*. *In vitro*, RBCs modified with lipophilic anti-EpCAM or anti-CD45 antibodies efficiently bound to cancer cells and leukocytes, forming characteristic rosettes. *In vivo*, intravenously injected RBCs painted with anti-CD45 antibody immediately associated with CD45 positive cells in blood, forming RBC-leukocyte rosettes. Moreover, anti-CD45-modified RBCs, but not the same amount of anti-CD45 antibody or anti-CD45-lipid conjugate (1–2 μg/mouse), depleted over 50% of CD45 + leukocytes from circulation, with main clearance organs of leukocytes being liver and spleen with no visible deposition in kidneys and lungs. Anti-CD20 (Rituximab)-painted RBCs efficiently (over 90%) depleted CD19 +/CD20 +/CD45 + human lymphoma cells in mantle cell lymphoma (MCL) JeKo-1 model, while the same amount of rituximab–lipid (2 μg/mouse) was much less efficient in lymphoma cell depletion. Treatment of MCL mice with rituximab-modified RBCs carrying only 2 μg of the antibody resulted in a significant prolongation of survival as compared to the same amount of antibody–lipid control. Lipophilic ligand-painted RBCs is a novel tool that can be utilized for targeting blood borne cells for experimental immunology and drug delivery applications.

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1. Introduction

Selective ablation of cell populations in living subjects is one of the most useful tools in experimental immunology and clinical medicine. The existing strategy for cell depletion is based on injection of a specific antibody against cell surface markers intravenously or intraperitoneally [1]. The binding of antibody triggers the clearance of cells through enhanced macrophage recognition via Fcγ receptors, complement lysis and cytotoxic T-cell response [2]. One of the great examples of clinical use of antibodies for cell depletion is rituximab, a monoclonal antibody against CD20 antigen on the surface of B-lymphoma cells [2]. However, antibody-mediated depletion has several disadvantages, including systemic toxicity and high cost of treatment. In some cases, antibody therapy fails to reduce the counts of tumor cells in patients. Thus, some proportion of patients with multiple myeloma, mantle lymphoma and chronic lymphocytic leukemia develops resistance to Rituximab [3], possibly due to the expression of complement inhibitors and/or activation of antiapoptotic pathways [4,5]. In addition, use of systemically injected antibodies is problematic when only the circulating pool of cells needs to be targeted. For instance, antibody against epithelial cell adhesion molecule (EpCAM), albeit effective against circulating metastatic cells in mice [6], showed significant systemic toxicity in clinical trials [7].

Red blood cells (RBCs) are readily available and fully biocompatible long-circulating carriers (natural life is 120 days) that are amenable to chemical modifications and drug loading [8,9]. Previously, biomolecules and immunoconjugates were successfully coupled to RBCs [10–12], and efficient targeting to collagen surfaces *in vitro* and to endothelial cells *in vivo* was demonstrated [11–13]. Our group previously demonstrated a versatile approach for painting RBC membrane with antibodies and small ligands via distearoyl anchors [14]. This painting strategy allows very fast (15–30 min incubation) and efficient (up to 30,000 ligand molecules per RBC) incorporation. Depending on the amount of surface antibody, ligand painted RBCs can circulate in blood for several days

* Corresponding author.
E-mail address: dmitri.simberg@ucdenver.edu (D. Simberg).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.jconrel.2014.03.038
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[14]. We wondered whether RBCs painted with targeting antibodies would bind and deplete blood borne cells, akin to previously described capture of circulating pathogens by antibody modified RBCs [15–17]. Here we prepared and tested antibody painted RBCs targeted to blood borne cells following injection *in vivo*. We demonstrate that antibody painted RBCs efficiently and specifically bind to target cells *in vitro* and *in vivo*, deplete cells from circulation and induce therapeutic effect in the mouse model of lymphoma. The data have direct implications for drug delivery and therapy of blood-borne diseases using RBCs as drug carriers.

2. Results

In order to prepare lipophilic antibodies for incorporation into the RBC membrane, thiolated IgG (see Materials and methods) was conjugated at 1:1 ratio to DSPE-PEG_{3400}-maleimide as described before [14].

Two different strategies were used to immobilize a targeting antibody on the RBC surface. Whenever the targeting antibody was readily available, it was directly conjugated to the lipid anchor (Fig. 1A). DSPE-PEG_{3400}-IgG construct was then incubated with washed mouse RBCs for 30 min at 37 °C, resulting in incorporation of ~2 μg IgG per 5×10^8 RBCs (approximately 16,000 IgG/RBC). In cases when the targeting antibody was not available in large quantities and therefore not easily amenable to lipid conjugation, we synthesized DSPE-PEG_{3400}-anti-Fc IgG construct, incorporated it in the RBC membrane, and then immobilized the targeting antibody (Fig. 1A). Modification of RBCs with this moderate amount of lipid–antibody construct did not result in a significant hemoglobin release or aggregation (Supplemental Fig. S1). The presence of the antibody was detected with a secondary fluorescent IgG (Fig. 1B). In addition to antibodies, lipophilic cyanine dye DiI was incorporated into the RBC membrane in order to enable independent tracking of RBCs *in vivo* [14]. Two-antibody capture approach often resulted in a more efficient incorporation of the targeting antibodies in the RBC membrane as determined with flow cytometry (Fig. 1C). Stability of the lipid construct in the membrane as well as clearance of RBCs in circulation was determined as described by us before [14]. Following intravenous injection of DSPE-PEG_{3400}-IgG/DiI-RBCs (thereafter IgG/DiI-RBCs), double-stained RBC population was detectable in blood for at least 48 h (Fig. 2A).

DSPE-PEG_{3400}-IgG construct gradually disappeared from the RBC surface with a terminal half-life of 610 min (Fig. 2B), while RBCs painted with ~16,000 copies of IgG (one antibody) exhibited an elimination half-life of 670 min (Fig. 2C). Previously we found that the concentration of DSPE-PEG_{3400}-IgG in the membrane affects the RBC half life (possibly due to the presence of PEG [18]); empirically, 16,000 IgG molecules per RBCs is a trade-off between RBC circulation half-life and the avidity for the target cells.

2.1. Targeting of ligand-painted RBCs to cells in vitro

First, we tested the ability of IgG/DiI-RBCs to bind to cells *in vitro*. Since RBCs are mainly constrained to the blood compartment, we used antibodies against intravascular targets. Thus, advanced stages of metastatic growth are characterized by large numbers of EpCAM-positive circulating tumor cells (**CTC**) in blood [19] and disseminated tumor cells (**DTC**) in bone marrow [20]. In order to target RBCs to EpCAM+ cancer cells, we prepared anti-mouse EpCAM/DiI-RBCs using two-antibody painting approach (Fig. 1A). 4T1 mouse breast carcinoma cells (suspended in cell medium) were mixed with EpCAM-targeted RBCs (RBC/tumor cell ratio of 10:1) for 60 min. 4T1 cells formed characteristic rosettes with anti-EpCAM/DiI-RBCs, but not with control

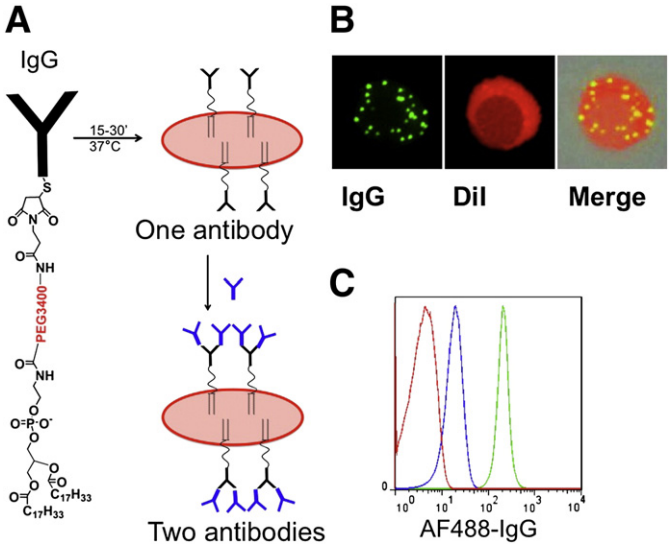

Fig. 1. Synthesis of lipophilic ligand painted erythrocytes. A, Thiolated IgG was conjugated to DSPE-PEG_{3400}-maleimide as described in Materials and methods. DSPE-PEG_{3400}-IgG was incorporated in the membrane of washed mouse RBCs after incubation at 37 °C. Targeting antibody was either directly conjugated to the lipid or immobilized via anti-Fc antibody; B, A fluorescent image of a RBC labeled with DiI and DSPE-PEG_{3400}-IgG (detected via secondary antibody). Note that antibody localization is punctate, whereas DiI is homogenously distributed; C, Flow cytometry histogram shows that amount of IgG on RBCs (detected via secondary Alexa 488 labeled antibody) is higher for two-step painting method (green trace) than for one-step method (blue trace). Red traces are non-labeled RBCs.

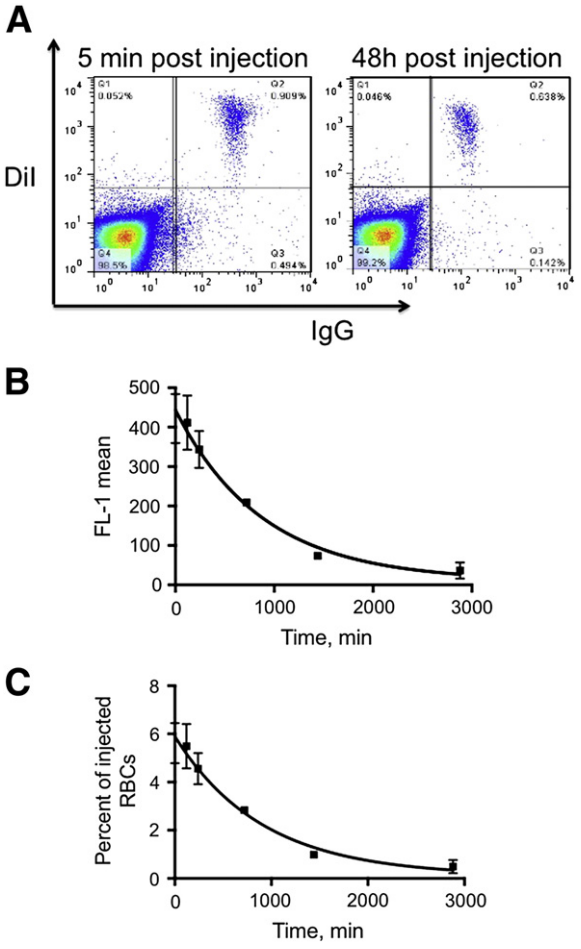

Fig. 2. Circulation properties of modified RBCs. A, Following i.v. injection, IgG/DiI-RBCs formed a distinct double labeled population in the upper right corner that persisted over 48 h; B, Retention of lipophilic IgG on RBC surface over time monitored with flow cytometry; C, RBC half-life in blood as monitored with flow cytometry. Data shown as means of n = 3 and SD.

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