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Genetic engineering of platelets to neutralize circulating tumor cells

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ABSTRACT

Mounting experimental evidence demonstrates that platelets support cancer metastasis. Within the circulatory system, platelets guard circulating tumor cells (CTCs) from immune elimination and promote their arrest at the endothelium, supporting CTC extravasation into secondary sites. Neutralization of CTCs in blood circulation can potentially attenuate metastases to distant organs. Therefore, extensive studies have explored the blockade of platelet–CTC interactions as an anti-metastatic strategy. Such an intervention approach, however, may cause bleeding disorders since the platelet–CTC interactions inherently rely on the blood coagulation cascade including platelet activation. On the other hand, platelets have been genetically engineered to correct inherited bleeding disorders in both animal models and human clinical trials. In this study, inspired by the physical association between platelets and CTCs, platelets were genetically modified to express surface-bound tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a cytokine known to induce apoptosis specifically in tumor cells. The TRAIL-expressing platelets were demonstrated to kill cancer cells *in vitro* and significantly reduce metastases in a mouse model of prostate cancer metastasis. Our results suggest that using platelets to produce and deliver cancer-specific therapeutics can provide a Trojan-horse strategy of neutralizing CTCs to attenuate metastasis.

1. Introduction

Metastasis contributes to more than 90% of cancer-associated mortality [1,2]. It occurs after primary tumors shed circulating tumor cells (CTCs) *via* hematogenous dissemination to distant organs [3,4]. Despite advancements in the understanding and detection of CTCs, effective neutralization of CTCs for the prevention of metastasis remains clinically challenging. Previous studies have demonstrated intrinsic tumor-tropic properties of bacteria and multiple types of stem cells including mesenchymal stem cells (MSCs), neural stem cells (NSCs) and endothelial precursor cells, making them attractive candidates for the targeted delivery of anticancer biological agents [5–8]. The mechanisms of tumor tropism were found to be multifactorial including, but not limited to, gradients of hypoxia, growth factors, and inflammatory cytokines generated within solid tumors [9,10]. The distinct transport system in the blood circulation, however, allows for rapid exchange of blood components within the vasculature. We reason that the gradients found in solid tumors are absent in circulation, which in turn makes existing cellular engineering approaches ineffective for targeting CTCs.

To explore alternative vectors for the delivery of cancer therapeutics in circulation, platelets were selected since they are capable of recognizing and interacting with CTCs immediately after the release of CTCs into circulation [11,12]. Platelets are anuclear cytoplasmic bodies released from megakaryocytes in the bone marrow. It is estimated that 1 L of blood contains about 400 billion circulating platelets [13]. In contrast to the long history of studies on the hemostatic function of platelets, their role in cancer metastasis has only recently become appreciated. It is generally believed that platelets interact with CTCs and promote metastasis *via* multiple mechanisms: 1. Platelet–CTC aggregates have a greater potential to become trapped in microvessels than individual CTCs. Such aggregation facilitates subsequent extravasation of cancer cells [14]; 2. Aggregation of platelets around CTCs protects against immune-mediated clearance of CTCs largely by natural killer (NK) cells [15]; 3. CTC evasion of NK cells is not merely attributed to physical shielding of platelets. The cytotoxic activity of NK cells is guided by the principles of "missing-self" and "induced-self". Cells lacking expression of MHC class I (missing-self) and/or a stress-induced expression of ligands for activating NK receptors (induced-self) are preferentially recognized and eliminated [16]. While CTCs are often associated with a lack of MHC class I ligands, platelets can disrupt "missing self" recognition of NK cells by grafting MHC class I onto CTCs [17].

In light of the harmful association between platelets and CTCs, a variety of anti-platelet drugs have been tested to block platelet–CTC interactions or inhibit platelet activation in preclinical mouse models [18–20]. Anti-platelet therapies, however, may inevitably impair the

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normal hemostatic function of platelets in the presence of bleeding [19,20]. In contrast to existing platelet intervention therapies, an alternative approach was explored in the current study by expressing the cancer cell-killing cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the surface of platelets while maintaining the platelets' normal hemostatic function and cancer cell adhesion ability. TRAIL was selected to be ectopically expressed in platelets for four reasons: (1) TRAIL is abundantly expressed on the surface of natural killer cells and cytotoxic T cells. It is responsible for the tumoricidal activity of these immune cells [21]. (2) TRAIL exerts a tumor cell-specific apoptotic effect by recognizing death receptors (DRs) highly expressed on the surface of cancer cells [22–24]. Previous studies have successfully modified mesenchymal stem cells (MSCs) and neural stem cells (NSCs) to express TRAIL to kill solid tumors in mice [7,25,26]. (3) The adhesion and aggregation of platelets to CTCs may facilitate the DR-mediated TRAIL apoptosis signaling by clustering DRs on cancer cells [27–29]. (4) Despite TRAIL resistances exhibited by certain cancer cells in solid tumors, it was found that these cells become more sensitive to TRAIL when they lose attachment to extracellular matrix [30]. The natural detachment of CTCs from a primary tumor likely increases their sensitivity to TRAIL.

To enable platelet-specific TRAIL expression, a platelet-targeted lentiviral transgene approach was utilized in this work through genetically engineering of hematopoietic stem and progenitor cells (HSPCs) followed by bone marrow transplantation (BMT). Such action has been successfully applied to correct genetic bleeding disorders in mice, dogs and certain human clinical trials while also meeting desired safety requirements [31–35]. The self-renewal ability of HSPCs would allow for continuous presence of TRAIL-expressing platelets upon maturation of megakaryocytes and release of platelets into the circulation. This approach can potentially enable long-term patrolling and neutralization of CTCs in circulation for the prevention or reduction of metastases.

2. Materials and methods

2.1. Construction of lentiviral vectors

A self-inactivating lentiviral vector pFUWG developed by Dr. David Baltimore's group was utilized in the study [36]. The human ubiquitin-C (Ubc) promoter was replaced by megakaryocyte-specific human integrin αIIβ promoter *via* BamHI and XbaI (NEB, Cambridge, MA, USA). Green fluorescent protein GFP and TRAIL were cloned separately downstream of αIIβ promoter *via* XbaI and EcoRI (NEB). All constructs were verified by DNA sequencing.

2.2. Cell lines and mice

MDA-MB-231, PC3, 293T, Dami and MEG-01 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 and 293T cells were cultured in DMEM (Invitrogen, Grand Island, NY, USA) with 10% FBS. PC3 were maintained in RPMI (Invitrogen) with 10% FBS. Dami and MEG-01 were expanded in IMDM (Invitrogen) with 10% FBS. Six to eight week old NOD SCID gamma (NSG) and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a SPF barrier animal facility at Cornell University.

2.3. Chemicals and antibodies

The following chemicals or kits were used for assaying cell proliferation and apoptosis: MTT (AMRESCO, Solon, OH, USA) and TACS® Annexin V-FITC Kit (Gaithersburg, MD, USA). Reagents for TEM were obtained from Electron Microscopy Sciences (Hatfield, PA, USA): glutaraldehyde, osmium tetroxide and uranyl acetate. APC-conjugated antibodies for human and mouse CD41 and PE-conjugated antibodies for human TRAIL were purchased from Biolegend (San Diego, CA, USA). Primary antibodies for human TRAIL and β-actin were obtained from PeproTech (Rocky Hill, NJ, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA). HRP-conjugated anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotech.

2.4. Flow cytometry

Cells were detached with enzyme-free Gibco® Cell Dissociation Buffer (Invitrogen) and suspended at a concentration of 5×10^5 cells in 100 μL cold PBS/1% bovine serum albumin (BSA). Fluorescent primary antibodies or isotype control were incubated with cells for 30 min on ice. Following two washes with 1 mL of PBS, fluorescence measurements were collected using a Guava easyCyte™ Flow Cytometry (Millipore, Billerica, MA, USA). Data were analyzed using the Flow Express software (De Novo Software, Los Angeles, CA, USA).

2.5. Western blotting

Western blotting was performed as previously described [37]. Briefly, whole cell lysates were prepared and separated using 10% SDS-PAGE. Membranes were incubated with primary antibodies and secondary antibodies diluted at 1:1000. Immobilized proteins were detected by using a chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

2.6. Cell proliferation assay

Cell proliferation was assayed by measuring mitochondrial dehydrogenase activity using MTT as the substrate. After treatment, cells were incubated with MTT at a concentration of 0.5 mg/mL, at 37 °C for 3 h. The purple MTT product was solubilized with DMSO and measured at 570 nm using a BioTek plate reader (Winooski, VT, USA).

2.7. Isolation, lentiviral transduction and in vitro differentiation of HSPCs

To isolate mouse HSPCs (Lin^-), bone marrow mononuclear cells (MNCs) were extracted from femurs and tibias of 6–8 week old mice. Following RBC lysis, unwanted cells were targeted with biotinylated antibodies directed against non-hematopoietic stem cells and non-progenitor cells (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119) followed by removal with streptavidin-coated magnetic particles (STEMCELL TECHNOLOGIES INC, Vancouver, BC, Canada). For lentiviral transduction, mouse Lin^- cells were cultured for 24-h prestimulation in serum-free X-VIVO-10 (Lonza, Allendale, NJ, USA) containing 100 ng/mL mouse SCF, TPO, and flt3 ligand (Prospecbio, East Brunswick, NJ, USA). Cells were then transduced twice within 24 h with lentivirus particles at a MOI of 50–100 in retronectin-coated plates. For *in vitro* differentiation, cells were differentiated in X-VIVO-10, 10% FBS, and 50 ng/mL mouse TPO for 10 days.

2.8. Bone marrow transplantation (BMT)

All mice were handled according to the Guide for the Care and Use of Laboratory Animals in compliance with US- and UK-based guidelines. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Cornell University (Protocol No. 2012–0113). For bone marrow transplantation, 6–8 week old mice were irradiated at a dosage of 2.5 Gy. Within 24 h of irradiation, each mouse received 1 million transduced HSPCs *via* retro-orbital injection.

2.9. Experimental metastasis mouse model with intra cardiac injection

4 weeks after BMT, NSG mice received 10^4 cancer cells expressing firefly luciferase through intra cardiac injection *via* left ventricle. Prior to the injection, mice were anesthetized by 2.5% isoflurane. Anesthetized animals were injected with 1×10^4 cancer cells suspended