Journal of Controlled Release 263 (2017) 57–67

Contents lists available at ScienceDirect

Journal of Controlled Release

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

A quantitative method for screening and identifying molecular targets for nanomedicine

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A R T I C L E I N F O

Keywords:
Cancer target
Nanomedicine
Metastatic melanoma
Immunoliposome
ICAM-1
Drug delivery

A B S T R A C T

Identifying a molecular target is essential for tumor-targeted nanomedicine. Current cancer nanomedicines commonly suffer from poor tumor specificity, "off-target" toxicity, and limited clinical efficacy. Here, we report a method to screen and identify new molecular targets for tumor-targeted nanomedicine based on a quantitative analysis. In our proof-of-principle study, we used comparative flow cytometric screening to identify ICAM-1 as a potential target for metastatic melanoma (MM). We further evaluated ICAM-1 as a MM targeting moiety by characterizing its (1) tumor specificity, (2) expression level, (3) cellular internalization, (4) therapeutic function, and (5) potential clinical impact. Quantitation of ICAM-1 protein expression on cells and validation by immunohistochemistry on human tissue specimens justified the synthesis of antibody-functionalized drug delivery vehicles, which were benchmarked against appropriate controls. We engineered ICAM-1 antibody conjugated, doxorubicin encapsulating immunoliposomes (ICAM-Dox-LPs) to selectively recognize and deliver doxorubicin to MM cells and simultaneously neutralize ICAM-1 signaling via an antibody blockade, demonstrating significant and simultaneous inhibitory effects on MM cell proliferation and migration. This paper describes a novel, quantitative metric system that identifies and evaluates new cancer targets for tumor-targeting nanomedicine.

1. Introduction

Tumor-targeted nanomedicines have the potential to mitigate the spatial and temporal challenges of therapeutic delivery to primary tumors and metastatic lesions via tumor recognition [1–4]. For example, MM-302 (human epidermal growth factor receptor 2 (HER2) antibody-conjugated liposomal doxorubicin), a receptor targeting immunoliposome, has recently demonstrated clinical benefits for HER2-positive metastatic breast cancer patients by significantly improving median progression free survival by 7.6 months and demonstrating an overall patient response rate of 11% [5–9]. However, other aggressive cancers, such as metastatic melanoma (MM) and pancreatic cancer, have no or limited clinically effective targets [10–14]. On these cancer cell membranes, hundreds of different proteins are upregulated or downregulated to promote cancer development and progression. Choosing an appropriate target is a critical parameter, therefore, in the development of tumor-targeted nanotherapeutics, which impacts therapeutic biodistribution, efficacy, and safety. To date, there remains a lack of quantitative studies to systematically identify, evaluate, and validate cancer targets for nanotherapeutics.

The goal of our study is to develop an unbiased method to screen and identify molecular targets that may be useful for tumor-targeting. A flowchart of the procedures for this method is shown in Fig. 1. First, we applied comparative flow cytometric screening to profile the expression of cancer-related cell surface antigens on cancer cells and their noncancerous (control) counterparts. Candidates were ranked and selected based on the level of overexpression. Second, the screened candidate was evaluated as a molecular target for tumor-targeted nanomedicine by characterizing its (1) tumor specificity, (2) expression level, (3) cellular internalization, (4) therapeutic function and (5) potential

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http://dx.doi.org/10.1016/j.jconrel.2017.03.030
Received 14 December 2016; Received in revised form 10 March 2017; Accepted 17 March 2017
Available online 22 March 2017
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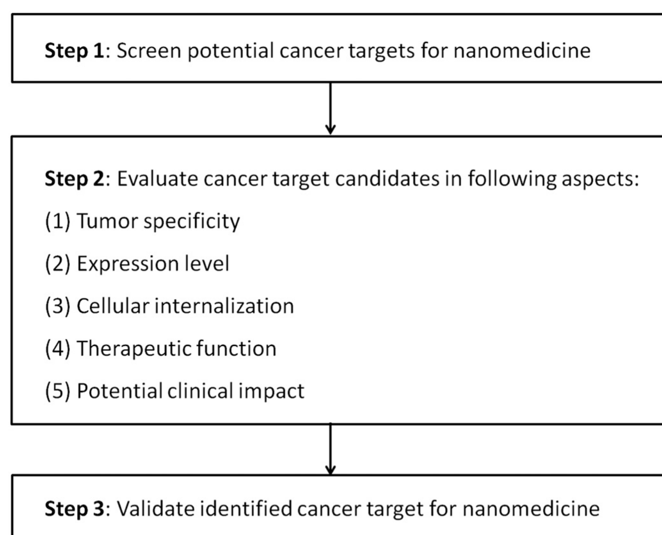

Fig. 1. Workflow of procedures to screen and identify cancer targets for nanomedicine.

clinical impact. Third, we validated the effectiveness of the identified target by constructing tumor-targeted immunoliposomes conjugated with antibodies recognizing the target and measuring their tumor-specific affinity and half maximal inhibitory concentration (IC50).

In this study, we focused our attention on MM, the most fatal skin cancer [15–18]. The methodology presented here identifies parameters necessary for evaluating a potential therapeutic target to disrupt cancer pathogenesis, and offers the opportunity to systematically and quantitatively, in an unbiased way, discover molecular targets for nanomedicine applications.

2. Experimental section

2.1. Materials

Dulbecco's phosphate buffered saline (PBS), 4′,6-diamidino-2-phenylindole (DAPI), 0.25% trypsin/2.6 mM ethylenediaminetetraacetic acid (EDTA) solution, Gibco® Dulbecco's Modified Eagle Medium (DMEM), Gibco®DMEM/F12(1:1) were purchased from Invitrogen (Carlsbad, CA, USA). Quantum Simply Cellular microbeads were purchased from Bangs Laboratory (Fishers, IN, USA). Mouse anti-human ICAM-1 monoclonal antibody, immunoglobulin G (IgG) isotype controls were purchased from R & D Systems (Minneapolis, MN, USA). For antibodies used in flow cytometric analysis, Phycoerythrin (PE)-conjugated mouse anti-human VEGFR1, VEGFR2, and FLOR1 antibodies were purchased from R & D Systems, and all other PE-conjugated antibodies and PE-conjugated mouse or rat IgG isotype controls were purchased from BioLegend (San Diego, CA, USA). Human melanoma tissue (ME2080b, T382a and T386) and normal tissue (BN00011 and BN1002a) arrays were purchased from US Biomax (Rockville, MD, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Slide-A-Lyzer™ Dialysis Cassette (MWCO 20 kDa), Corning Costar Transwell Permeable Supports and Lab-Tek II Chamber Slide System were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Fluorogel with tris buffer was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). RNeasy mini kit was purchased from Qiagen (Valencia, CA, USA).

2.2. Cell culture

A375SM human metastatic melanoma was obtained from Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX, USA) [19,20]. C32 human metastatic melanoma was obtained from American Type Culture Collection (ATCC, Manasses, VA, USA) [21–23]. Adult human epidermal melanocytes from lightly-pigmented skin (HEMa-LP, normal human melanocytes) were purchased from Cascade Biologics (Invitrogen). A375SM and C32 cells were cultured in DMEM, normal human melanocytes in MGM™-4 melanocyte growth medium (Lonza, Portsmouth, NH, USA), with all recommended supplements, respectively. All cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.3. Flow cytometry measurement

Cell membrane expression of molecular target candidate was evaluated by a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) as described previously [24]. Quantification of the molecular target density on the cell surface was determined with reference to Quantum Simply Cellular microbeads, using the protocol as provided by the manufacturer. Briefly, 10^6 cells were collected and rinsed twice through suspension-spin cycles. Cells were blocked by 1% bovine serum albumin (BSA) in PBS for 30 min in an ice bath. After BSA blocking, cells were incubated with PE-conjugated antibodies for 1 h at RT. Cells were rinsed with 1% BSA in PBS three times, resuspended in PBS, and evaluated by flow cytometry.

Quantitative analysis of MM cell binding and uptake of synthesized liposomes was studied by flow cytometry analysis. 10^6 cells were placed in each well of a 6-well cell culture plate and incubated for 2–4 h at 37 °C with (1) rhodamine-dextran (RD)-encapsulating, nonspecific IgG conjugated liposome (IgG-RD-LP), (2) RD-encapsulating ICAM-1 antibody conjugated liposome (ICAM-RD-LP) at a final concentration of 1 μM lipids per 10^6 cells. All liposome-treated cells were washed with PBS, harvested using a 0.25% trypsin/2.6 mM EDTA solution, and washed with PBS three times. Binding data were acquired using a BD FACSCalibur flow cytometer and analyzed using FlowJo software. The specific cell uptake of ICAM-RD-LP with reference to non-specific IgG-RD-LPs was calculated by dividing the mean fluorescence intensity of ICAM-RD-LP stained cells by that of the IgG-RD-LP stained cells.

2.4. Immunohistochemistry

162 human melanoma tissue samples and 178 normal human tissue samples from 20 different organs were stained and evaluated for ICAM-1 expression level and tumor specificity. Immunohistochemical staining was performed on paraffin-embedded human melanoma tissue microarrays (ME2080b, T382a and T386) and normal tissue microarrays (BN00011 and BN1002a). The individual tissue cores in the microarrays were scored by an independent pathologist, with no knowledge of sample identity, for no staining (0), weak staining (1 +), moderate staining (2 +), or strong staining (3 +). Photomicrographs were taken on an Olympus BX41 microscope by using an Olympus Q-Color5 digital camera (Olympus America, Center Valley, PA, USA).

2.5. Immunofluorescent staining

A375SM, C32 or human melanocytes (2×10^5 cells) were seeded in a Lab−Tek II Chamber Slide System with 2 mL media overnight at 37 °C. After media was removed, cells were rinsed with PBS three times and fixed with 4% formaldehyde in PBS at RT for 10 min, followed by three rinses with PBS. Samples were blocked with 1% BSA in PBS for 30 min in an ice bath. After BSA blocking, samples were stained with PE-conjugated ICAM-1 antibodies or PE-conjugated control IgG for 1 h and rinsed with PBS. DAPI was used to stain the cell nuclei. Immunofluorescent stained samples were dried overnight in the dark and examined under a Leica TCS SP5 confocal fluorescent microscope (Leica Microsystems, Buffalo Grove, IL, USA). Digital images were captured with AxioVision digital image processing software.

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