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Serum biomarkers for personalization of nanotherapeutics-based therapy in different tumor and organ microenvironments

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ABSTRACT

Enhanced permeation and retention (EPR) effect, the mechanism by which nanotherapeutics accumulate in tumors, varies in patients based on differences in the tumor and organ microenvironment. Surrogate biomarkers for the EPR effect will aid in selecting patients who will accumulate higher amounts of nanotherapeutics and show better therapeutic efficacy. Our data suggest that the differences in the vascular permeability and pegylated liposomal doxorubicin (PLD) accumulation are tumor type as well as organ-specific and significantly correlated with the relative ratio of MMP-9 to TIMP-1 in the circulation, supporting development of these molecules as biomarkers for the personalization of nanoparticle-based therapy.

1. Introduction

Rapid advances in nanotechnology have enabled the incorporation of chemotherapeutics into nanoparticles to deliver large quantities of payload at target sites [1,2]. These nanotherapeutics include liposomes, albumin-based particles, polymer-drug composites, polymeric micelles, and dendrimers. Pegylated liposomal doxorubicin (PLD) is one of the most widely used nanotherapeutics approved for the treatment of various tumor types [3]. Although nanotherapeutics can be advantageous over treatment with free/unencapsulated drug/chemotherapeutics because of the preferential accumulation of nanoparticles in tumors due to the enhanced permeation and retention (EPR) effect, the relative benefit of this effect can vary from patient to patient based differences in the tumor and organ microenvironment [4–6]. Thus, the accumulation of nanotherapeutics in the tumor can be heterogeneous, and indeed, the increase in overall survival in patients treated with PLD has been modest [7–10]. For the most part, researchers have mainly focused on the molecular mechanisms of resistance to therapy in cancer cells and have overlooked that the accumulation of therapeutics in tumor sites may also determine therapeutic efficacy [11–13].

Personalized cancer therapy based on the levels of biomarkers, such as target proteins and specific genetic mutations in tumors, have recently emerged [14–16]. The assessment of these markers facilitates the pre-selection of patients who are likely to respond to the particular therapies. We hypothesized that the therapeutic efficacy of nanotherapeutics, including PLD, may also be improved by identifying molecules that could potentially serve as surrogate biomarkers to select patients who are likely to accumulate higher amounts of PLD in their tumors and thereby achieve a better therapeutic outcome. To the best of our knowledge, promising biomarkers for the personalization of nanoparticle-based therapy have not yet been identified.

The goal of the present study was to identify candidate serum markers that can be correlated with the EPR effect for the accumulation of PLD in various tumor and organ microenvironments to personalize cancer nanotherapy. For this purpose, we used 4T1 (breast cancer), 3LL (lung cancer), and CT26 (colon cancer) mouse tumor cells growing in the mammary fat pad (mfp), under the skin, or at the tumor's major metastatic sites, such as the brain or liver. We found that the differences in PLD accumulation in the tumors were statistically correlated with the relative ratio of Matrix metalloproteinase (MMP)-9 and its endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP)-1 in systemic circulation. To the best of our knowledge, this is the first study that describes

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candidate serum biomarkers to assess the vascular permeability to nanotherapeutics utilizing the EPR effect in tumors.

2. Materials and methods

2.1. Cell culture and in vitro cytotoxicity assay

4T1 murine breast cancer cells, 3LL murine lung cancer cells, and CT26 murine colon cancer cells were kindly provided by Dr. Isaiah J. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX) [17,18]. The cells were maintained in complete minimal essential medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin–streptomycin mixture (Flow Laboratories, Rockville, MD). The cells were validated by short tandem repeat DNA fingerprinting using the AmpFℓSTR Identifier kit (Applied Biosystems, Carlsbad, CA) [19]. For cytotoxicity assay *in vitro*, 4T1 or 3LL cells were seeded into 96-well plates and the cultures were fed with new medium (negative control) or medium containing different concentrations of PLD. After 3 days of the incubation, the number of metabolically active cells was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.

2.2. Mice

Female BALB/C, C57/BL6 and nude/nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The animal facilities at the University of Texas MD Anderson Cancer Center were approved by the American Association for Accreditation of Laboratory Animal Care and met all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institutes of Health. The 8–12 weeks old mice were used in accordance with institutional guidelines.

2.3. Establishment of experimental brain metastases

BALB/C and C57/BL6 mice were anesthetized with an intra-peritoneal (i.p.) injection of pentobarbital sodium. 4T1, 3LL or CT26 cells were injected slowly (1×10^5 cells/50 μl) into the right common carotid artery [17].

2.4. Establishment of experimental liver metastases, subcutaneous tumors, and primary breast tumors

BALB/C and C57/BL6 mice were anesthetized by inhaling isoflurane, and a small left abdominal flank incision was made. Then a tumor cell suspension (4T1, 3LL or CT26, 1×10^5 cells/50 μl) was injected into the spleen [20]. To generate primary breast tumors, 4T1 cells (1×10^5 cells/50 μl) were injected into the mammary fat pad (mfp) of BALB/C mice. To generate subcutaneous 3LL and 4T1 tumors, 1×10^5 -cells in 50 μl were injected into the subcutaneous space of nude/nude mice.

2.5. Therapy, blood sample collection, and necropsy

Ten days after the inoculation of cells, the mice were intravenously (i.v.) injected every 7 days with phosphate buffered saline (PBS) (control) or 6 mg/kg PLD (Doxoves™-Liposomal Doxorubicin HCl, FormuMax Scientific Inc., Palo Alto, CA), a total of 3 times ($n = 7$, each). Batimastat (EMD Millipore, Billerica, MA), a potent broad-spectrum inhibitor of MMPs, was resuspended with PBS to a concentration of 2.5 mg/ml [21]. The mice bearing 4T1 tumors in the mfp were i.p. injected once a day with batimastat (50 mg/kg) or diluent (control) for 3 days ($n = 6$, each). Six hours after the final injection, these mice were i.v. injected with PLD or PBS (control) and sacrificed 24 h later. Blood samples were collected from the tail vein (100 μl) before the injection of PLD. For survival experiments, the tumor bearing mice were sacrificed when they became moribund. For therapy experiments, the mice were sacrificed 24 h, 2 or 5 days after the i.v. injection of PLD.

2.6. Protein array analysis and measurement of MMP-9 and TIMP-1 protein levels

A Proteome Profiler™ Array Mouse Angiogenesis Array Kit (R&D Systems, Minneapolis, MN) or ELISA kits (R&D Systems) were used according to the manufacturer's instructions to analyze the protein expression profile of the cells *in vitro* or measure the MMP-9 and TIMP-1 levels in the serum samples of the tumor bearing mice, respectively.

2.7. Immunohistochemical analysis to detect MMP-9, TIMP-1 and Vascular Endothelial cell Growth Factor (VEGF) in tumors and organs

Paraffin-embedded tumor sections were deparaffinized, and endogenous peroxidase was blocked with 3% hydrogen peroxide. Samples were incubated with an antibody to MMP-9 (EMD Millipore, Billerica, MA), TIMP-1 (R&D Systems) or VEGF (Santa-Cruz Biotechnology, Inc., Dallas, TX). After incubation with a peroxidase-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA), protein-antibody complexes were detected by exposure to 3,3′-Diaminobenzidine (Sigma–Aldrich Corp. St. Louis, MO).

2.8. Immunofluorescent imaging of endothelial cells (CD31), basement membrane (type IV collagen), proliferating cells (Ki67), p-glycoprotein (p-GP), macrophages (CD204), and tumor tissue perfusion

The frozen sections of the tumor tissue were immunofluorescently stained using antibodies to CD31 (BD Biosciences, San Jose, CA), type IV collagen (Abcam, Cambridge, MA), Ki67 (Abcam), p-GP (GeneTex Inc., Irvine, CA) or CD204 (AbD Serotec, Raleigh, NC). Sections were then incubated with corresponding secondary antibodies (Jackson Immunoresearch). The area of tumor tissue perfused by blood was evaluated by imaging of a lysine-fixable 70 kDa fluorescein dextran tracer (Molecular Probes, Inc. Eugene, OR) 1 min after i.v. injection. The images were captured using a laser scanning confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) and analyzed using the built-in image analysis software [19]. The ratio of pixels in the whole image that has higher fluorescence intensity over the threshold (background) was shown as area fraction [22,23]. The data were shown as the average ± SD from representative sections of more than 5 images of tumors or uninvolved organs. The coverage of endothelial cells was expressed as the fractional area of endothelial cells (pseudo color in red) co-localized with basement membrane (pseudo color in green), which is indicated by the emission of yellow fluorescence, relative to the total area of endothelial cells in five randomly selected tumors.

2.9. Immunofluorescence imaging of PLD in tumors

The red fluorescence of anthracyclines enables direct visualization of doxorubicin in tissue by using confocal laser scanning microscopy. The excitation wavelength was set to 488 nm, and the doxorubicin emission was collected using a 590 nm filter [24,25].

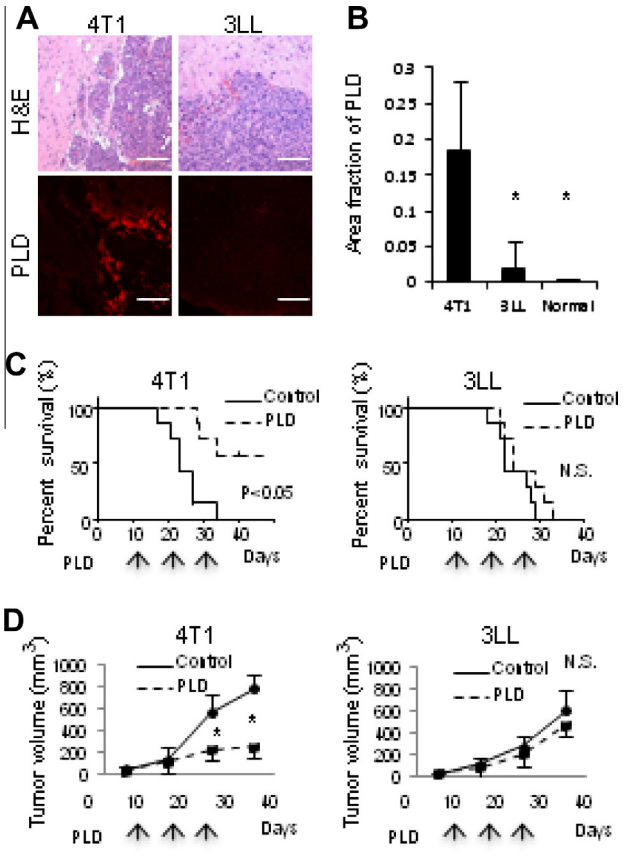

Fig. 1. PLD accumulation and therapeutic efficacy. (A) Hematoxylin and eosin (H&E) staining and fluorescence imaging of accumulated PLD in 4T1 and 3LL tumors grown in brain. Bar indicates 100 μm. (B) Quantification of PLD accumulation in tumors. * Indicates $p < 0.05$ versus 4T1. The therapeutic efficacy of PLD was assessed by analyzing the survival of mice bearing tumors in the brain (C), or by measuring subcutaneous tumor growth (D). The mice were treated three times with PLD or PBS (control) at the times indicated by arrows. N.S. indicates no significant difference in the survival or tumor size. * Indicates $p < 0.05$ versus control.