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Fibrin degradation by rtPA enhances the delivery of nanotherapeutics to A549 tumors in nude mice

Bo Zhang [a], Ting Jiang [a], Xiaojian She [b], Shun Shen [b], Sheng Wang [b], Jun Deng [a], Wei Shi [a], Heng Mei [a, c], Yu Hu [a, c, *], Zhiqing Pang [b, **], Xinguo Jiang [b]

[a] Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, Hubei, 430022, PR China
[b] School of Pharmacy, Fudan University, Key Laboratory of Smart Drug Delivery, Ministry of Education, 826 Zhangheng Road, Shanghai, 201203, PR China
[c] Collaborative Innovation Center of Hematology, Huazhong University of Science and Technology, PR China

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ABSTRACT

Effective drug delivery to a tumor depends on favorable blood perfusion within the tumor. As an important component of tumor extracellular matrix, fibrin is abundant near tumor vessels. Inspired by the distinct distribution pattern and vessel-dependent production of fibrin, we hypothesized that fibrin depletion in tumors decompresses tumor vessels to improve tumor blood perfusion and accordingly enhance drug delivery to tumors rich in vessels. In the present study, we attempted to employ a clinically used thrombolytic drug, recombinant tissue plasminogen activator (rtPA), to modulate fibrin deposition in tumors. We then combined this drug with a nanoparticle drug delivery system for tumor therapy. RtPA treatment (25 mg/kg/d i. p. administration for two weeks) successfully depleted fibrin deposition and enhanced blood perfusion within A549 tumor xenografts. Furthermore, rtPA treatment also improved the *in vivo* delivery of 115-nm nanoparticles to tumor tissues. Finally, rtPA combined with therapeutic agent-loaded nanoparticles resulted in the most effective shrinkage of A549 tumor xenografts compared with the control groups. Overall, the present study provides a new strategy to enhance the delivery of nanotherapeutics to tumors rich in vessels.

1. Introduction

Blood perfusion is consistently and prominently lower in tumors than in normal tissues because of the leakiness and/or mechanical compression of tumor vessels [1,2]. Poor perfusion compromises the *in vivo* delivery of systemically administered nanomedicine, which has become the main strategy for tumor drug delivery [3]. Strategies have been developed to improve tumor perfusion for various tumor types, including tumor vessel normalization to reduce vascular permeability and physical force alleviation to decompress tumor vessels [4]. Specifically, the judicious application of anti-angiogenic agents has been employed to normalize tumor vessels in order to improve tumor perfusion for tumors rich in vessels, such as gliomas, lung cancers et al. [1,5]. This strategy preferentially benefits the *in vivo* delivery of free drugs or small nanomedicines (10–40 nm) [6,7]. Alternatively, physical force alleviation, including depletion of tumor stroma cells and disruption of tumor extracellular matrix (ECM) components such as collagen and hyaluronan, has been used to decompress tumor vessels in order to improve blood perfusion and accordingly the delivery of chemotherapeutics to tumors characterized by an abundance of ECM, such as pancreatic carcinoma [8,9]. To the best of our knowledge, ECM disruption-related strategies capable of improving nanomedicine delivery to tumors rich in vessels have not yet been reported, partly because the expression levels of common ECM components, including collagen and hyaluronan, are generally low in these tumors [1].

The leakage of coagulation factors from the circulation system into tumor interstitial and the high level of coagulation initiator tissue factor (TF) on tumor cells jointly contribute to the local coagulation response in tumor tissues [10,11]. As the end product of coagulation response, fibrin is also mostly covalently cross-linked in tumor tissues and functions as an important component of the tumor ECM [12,13] that is primarily located near tumor vessels [14].

* Corresponding author. Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, Hubei, 430022, PR China.
** Corresponding author.
E-mail addresses: dr_huyu@126.com (Y. Hu), zqpang@fudan.edu.cn (Z. Pang).

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This distinct distribution pattern differs from that of other matrix components, such as collagen and hyaluronan, which always extensively distributes throughout the tumor ECM.

The vessel-dependent production and unique distribution pattern of fibrin prompted us to hypothesize that high fibrin deposition near the vessels of vessel-rich tumors might compress tumor vessels, reduce blood perfusion and compromise the accumulation of nanomedicine in tumor tissues. Accordingly, a strategy capable of removing fibrin might decompress tumor vessels, improve tumor perfusion and thus enhance drug delivery to tumor tissues. To the best of our knowledge, this was the first study that tried to modulate fibrin in tumor tissues in order to improve drug delivery to the tumor.

In the present study, lung cancer with abundant vessels [1] which allowed effective leakage of coagulation factors into tumor interstitial was selected as the tumor model and TF highly-expressed [15] human-derived lung cancer cells A549 with the ability to induce coagulation response and produce a large amount of fibrin in tumor interstitial was used as the model cell line. A widely used thrombolytic drug, recombinant tissue plasminogen activator (rtPA) [16], was selected as the model drug to modulate fibrin deposition in tumor tissues and enhance the delivery of nanoparticles (NPs) drug delivery system consisting of biodegradable poly (ethylene glycol)-poly (lactic acid) (PEG-PLA) [17] and paclitaxel (PTX) for A549 tumors. The effect of rtPA on fibrin deposition in tumor tissues was assessed using immunofluorescence staining and enzyme-linked immunosorbent assay (ELISA). Furthermore, the effect of rtPA treatment on tumor perfusion was evaluated by a lectin-labeling experiment and an erythrocyte retention experiment. The delivery of NPs to tumor tissues was assessed by *in vivo* imaging and frozen tumor slices. Finally, antitumor efficacy study was also performed to investigate therapeutic benefits of rtPA in combination with the NPs drug delivery system. Moreover, the potential adverse effect of rtPA was also assessed because of the bleeding risk associated with this drug. As rtPA is a clinically approved thrombolytic drug, our findings may be potentially translated to the clinic to improve tumor treatment.

2. Materials and methods

RtPA was obtained from Boehringer Ingelheim (Germany). DyLight® 488-labeled tomato lectin (Lycopersicon esculentum) (DyLight® 488-lectin) was purchased from Vector (USA). Methoxy-PEG (MPEG, MW 3000 Da) was purchased from NOF (Tokyo, Japan), and D, L-lactide (purity: 99.5%) was ordered from PURAC (Arkelsedijk, Holland). Methoxy-poly (ethylene glycol)-poly (lactic acid) (MPEG–PLA, Mw 33000 Da) block copolymers were synthesized by the ring-opening polymerization of D, L-lactide using MPEG as the initiator as described previously [18]. Fibrinogen rabbit polyclonal primary antibody and erythroid lineage (TER-199) rat monoclonal antibody were obtained from Santa Cruz biotechnology (USA), CD31 goat polyclonal primary antibody was obtained from R&D (USA). Cy™ 3-conjugated Affinipure donkey anti-goat secondary antibody, Alexa Fluor® 488-conjugated donkey anti-rabbit secondary antibody and Alexa Fluor® 488-conjugated donkey anti-rat secondary antibody were obtained from Jackson Laboratories (USA). Mouse D-Dimer ELISA kit was purchased from ElabScience (Wuhan, China). Fluorescence trackers, including 1,1′-Dioctadecyl-3,3,3′,3'-tetramethylindo-tricarbocyanineiodide (DiR), a near-infrared dye, were obtained from Biotium (Invitrogen, USA), and coumarin-6 was purchased from Sigma (USA). Hoechst 33342 was ordered from Beyotime® Biotechnology Co., Ltd (Nantong, China). Sodium cholate was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Fetal bovine serum (FBS), trypsin-EDTA (0.25%), penicillin-streptomycin and cell culture medium were obtained from Gibco (CA). Deionized water from Millipore Simplicity System (Millipore, Bedford, MA) was used in all experiments. All other reagents were of analytical reagent grade and ordered from Sinopharm Chemical Reagent (Shanghai, China).

2.1. Cells and animal models

The A549 cell line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Male Balb/c nude mice (approximately 20 g) were purchased from the Shanghai Slac Lab Animal Ltd. (Shanghai, China). All protocols were reviewed and approved by the ethics committee of Fudan University. A549 tumor xenografts were prepared by subcutaneously injecting an A549 tumor-cell suspension (5×10^6 cells/100 μl) into nude mice. The tumor volume was calculated according to the following formula: $V = 0.5 \times d_{max} \times d_{min}^2$, where V represented the tumor volume, $d_{\max}$ was the maximum perpendicular diameter and $d_{\min}$ was the minimum perpendicular diameter. Tumor xenografts with a diameter of 4–5 mm were selected for subsequent study.

2.2. RtPA treatment and safety evaluation

When the tumor reached 5 mm in diameter, mouse models ($n = 5$) received i. p. administration of rtPA at a dose of 25 mg/kg/day for two weeks. To identify the potential adverse effects of rtPA, the abdominal skin of the injection site, tumor size and body weight of the mice were carefully monitored every other day throughout rtPA treatment. Blood samples were obtained 24 h after the treatment ended, and coagulation function-related indicators, such as pro-thrombin time (PT), activated partial thromboplastin time (APTT) and the plasma concentration of fibrinogen (Fbg) were assessed.

2.3. Effects of rtPA on tumor microenvironment

For fibrin distribution investigation, the mice models were sacrificed at the end of rtPA treatment, and tumor xenografts were perfused with 4% paraformaldehyde to prepare histological tumor sections. The tumor sections were first stained with primary rabbit polyclone fibrin (ogen) antibody (1:100) and primary goat polyclone CD 31 antibody (1:100), followed by staining with Cy™ 3-conjugated Affinipure donkey anti-goat secondary antibody and Alexa Fluor® 488-conjugated donkey anti-rabbit secondary antibody to label both fibrin and blood vessels. Finally, the sections were stained with Hoechst 33342 (1 μg/ml) for 10 min at room temperature. The fibrin deposition in the tumor sections was analyzed under a confocal microscope (ZEISS, 710, LSM, Germany). In addition, as the specific degradation product of fibrin, the level of D-Dimer [19] in tumor tissues was also evaluated by ELISA according to the manufacturer's protocol.

For lectin-labeling experiment, six tumor xenograft-bearing mice were randomly assigned into rtPA treatment group and control group and treated with DyLight® 488-lectin (5 mg/kg) 1 h before tumor perfusion with 4% paraformaldehyde [20]. Then frozen tumor slice were prepared and subjected to CD31 immunofluorescence staining. The co-localization of the CD31 and DyLight® 488-lectin signals in the tumor sections were captured in six randomly-assigned regions in each tumor slice ($n = 3$) with a laser-scanning confocal microscope at 200 × magnification (ZEISS, 710, LSM, Germany) and further analyzed using the Image J software. CD31-positive blood vessels that co-localized with DyLight® 488-lectin were identified as well perfused blood vessels, and tumor perfusion was indicated as the percentage of well perfused vessels ($CD31^+$DyLight®488-lectin$^+$) in all blood vessels ($CD31^+$).

For erythrocytes retention evaluation, erythrocytes and tumor