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Tumor vasculature normalization by orally fed erlotinib to modulate the tumor microenvironment for enhanced cancer nanomedicine and immunotherapy

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ABSTRACT

The abnormal tumor vasculature is one of key reasons that lead to the limited tumor perfusion as well as hypoxic and immunosuppressive tumor microenvironment (TME). Herein, we uncover that by normalizing the tumor vasculature with erlotinib, a specific inhibitor of epidermal growth factor receptor (EGFR), the tumor perfusion and tumor oxygenation statuses in different types of tumors including murine breast tumors, colorectal tumors, and squamous cell carcinoma tumors, could be remarkably enhanced. As the results, the tumor uptake of drug-loaded nanoparticles as well as their interstitial penetration within the tumor would be greatly increased for mice pre-treated with erlotinib at the oral feeding dose of 50 mg/kg, leading to remarkably improved chemotherapeutic efficacy of nanomedicine. On the other hand, owing to the erlotinib-induced normalization of tumor vasculatures, the relieved hypoxic state in the three different types of tumors could alter the immunosuppressive TME into immunosupportive. Such an effect together with the increased tumor retention of anti-PDL1 antibody, a clinically approved checkpoint blockade agent, finally contributes to the greatly improved tumor inhibition effect in cancer immunotherapy. Therefore, our work presents a general yet effective strategy using a clinical drug to enhance the efficacies of cancer nanomedicine and immunotherapy by normalizing tumor vasculatures and modulating TME.

1. Introduction

The abnormal tumor vasculatures featured with heterogeneous distribution, tortuosity, dilation, and insufficient blood supply, is known to be closely associated with the increased interstitial fluid pressure (IFP), hypoxia, and reduced pH within the tumor microenvironment (TME) [1–5]. As the results of those specific characteristics of tumor blood vasculatures and high IFP within the TME, the diffusion of therapeutics, especially those with relatively large sizes (e.g. protein therapeutics, drug-loaded nanoparticles), may be largely limited within the solid tumors, hampering their therapeutic outcomes [6,7]. In the meanwhile, it has also been well recognized that the unique features of TME, such as hypoxia and reduced pH, would affect the recruitment, proliferation, and functions of immune cells within tumors, resulting in the immunosuppressive TME [8–10]. For instance, the hypoxic microenvironment may induce the polarization of tumor-associated macrophages (TAMs) from the immunosupportive phenotype, M1 type TAMs, to immunosuppressive M2 type TAMs, promoting tumor progression via inhibiting antitumor immunities [8,10,11]. In addition, the tumor hypoxia is also related to the up-regulation of many chemotactic factors and thus the recruitment of abundant regulatory T (Treg) cells, which are immunosuppressive lymphoid cells to compensate the functions of the immune systems in attacking tumor cells during cancer immunotherapy [12–14].

Considering the role of abnormal tumor vasculatures in regulating various features of the TME, in recent years, there have been

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many groups trying to normalize the tumor vasculature by various means so as to modify the TME [2,15–17]. It has been uncovered that treatment with anti-angiogenesis drugs at a moderate dose would be able to normalize tumor blood vessels by killing those small non-functional ones, leading to significantly improved accumulation and penetration of drugs and oxygen within solid tumors [2,16]. Moreover, since the thick extracellular matrix in the TME could compress tumor blood vessels and trigger the irregular vascular distribution inside tumors [18,19], decomposing the condensed extracellular matrix by various inhibitors or enzymes (e.g. hyaluronidase, cyclopamine, etc.) has recently been proposed to be another useful strategy to normalize tumor vasculatures and improve the efficacies of chemotherapy as well as photodynamic therapy [20,21]. Developing a convenient method, preferably by using clinically approved drugs, to normalize tumor vasculatures and modulate the hostile TME would thus have remarkable clinical values to optimize the therapeutic outcomes of existing cancer therapies.

Epidermal growth factor receptor (EGFR), which plays an important role in promoting cancer cell proliferation, invasion and metastasis, is over-expressed on many types of solid tumors [22,23]. Over expression of EGFR on tumor cells would lead to increased secretion of vascular endothelial growth factor (VEGF) [24–26], which together with other proangiogenic factors are able to promote the rapid formation of irregular blood vessels within tumors and result in abnormal tumor vasculatures [27,28]. Herein, we uncover that erlotinib, a clinically used EGFR inhibitor to treat different types of cancers under the trade name Tarceva®, is able to normalize tumor vasculatures for three different mouse tumor models including 4T1 breast tumors, CT26 colorectal tumors, and SCC7 carcinoma tumors, by inhibiting EGFR and down-regulating VEGF (Fig. 1). As observed in three different types of tumor models, the normalized tumor vasculature after oral feeding of erlotinib would lead to obviously improved tumor perfusion, which in turn would greatly increase not only the tumor oxygenation, but also the tumoral accumulation and penetration of nanoscale therapeutic agents by normalizing the tumor vasculature. By using human serum albumin (HSA)-bound paclitaxel (PTX) as the model nanomedicine drug, it is found that erlotinib treatment could greatly enhance the efficacy of nanoparticle-based chemotherapy. On the other hand, the efficacy of programmed death-ligand 1 (PD-L1) checkpoint blockade therapy is also remarkably enhanced by erlotinib on the colorectal cancer model, not only by increasing the tumor retention of anti-PD-L1 antibody, but also by altering the TME from immunosuppressive to immunosupportive (e.g. polarizing TAMs from M2-like to M1-like phenotype) via relieving tumor hypoxia. Our study presents a promising way of tumor vasculature normalization with a clinically existing drug, which is able to effectively modulate the TME, improve the tumor uptake of nanoscale particles or proteins, and relieve tumor hypoxia, offering tremendous therapeutic benefits for nanomedicine-based chemotherapy as well as emerging cancer immunotherapy.

2. Materials and methods

2.1. Materials

Erlotinib was purchased from Selleck.cn. Human serum albumin (HSA) was obtained from Sigma-Aldrich. Paclitaxel (PTX) was purchased from J&C chemical CO. Pimonidazole hydrochloride and anti-pimonidazole antibody were obtained from Hypoxyprobe Inc. Anti- HIF-1α antibody and the Alex 488-conjugated goat anti-mouse secondary antibody were purchased from Jackson Inc. Anti-PDL1 used in vivo was obtained from Bioxcell. Antibodies against cell surface markers for flow cytometry (FACS) assay were purchased from eBioscience. The microbubble ultrasound contrast agent was obtained from Fujifilm.

2.2. Synthesis of HSA-PTX and HSA-PTX-Cy5.5 nanoparticles

HSA-PTX nanoparticles were synthesized according to the protocol reported previously [29]. To synthesize HSA-PTX-Cy5.5, HSA was firstly labeled with Cy5.5 at the molar ratio of 1:1 to form HSA-Cy5.5. Then, HSA-PTX-Cy5.5 nanoparticles were prepared by the same procedure except that HSA-Cy5.5 was used to replace HSA.

2.3. Synthesis of liposome-Did nanoparticles

Liposome-Did nanoparticles were prepared according to previous study [30]. The mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, PEG-5000 conjugated 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-mPEG5k) and 1,1-Dioctadecyl-3,3,3,3- Tetramethylindodicarbocyanine Perchlorate (Did) at a molar ratio of 6: 4: 0.5: 0.5 was dissolved in chloroform and then dried under a rotary evaporator. Afterwards, the dried lipid film was hydrated with phosphate buffered saline (PBS) and stirred at 45 °C for 30 min, followed by extruding through a 200 nm polycarbonate filters at 45 °C for 20 times.

2.4. Cell lines

Murine breast cancer 4T1, colorectal cancer CT26, and squamous cell carcinoma SCC7 cell lines were originally obtained from American Type Culture Collection (ATCC), and cultured at 37 °C under 5% CO_2 within RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

2.5. Tumor models

Female BALB/c mice and nude mice (6–8 weeks) were purchased from Nanjing Peng Sheng Biological Technology Co Ltd and used under protocols approved by Soochow University Laboratory Animal Center. Tumors were established by subcutaneous injection of 4T1 cells, CT26 cells or SCC7 cells (1 × 106) on the right flank of each mouse. Studies were carried out when tumor volumes reached about ~100 mm^3.

2.6. Erlotinib treatment

To normalize tumor vasculatures, erlotinib (50 mg/kg), an EGFR inhibitor, was administered by oral gavage (dissolved in 6% Captisol) 5 days, 3 days and 1 day before other analyses. The dosage of erlotinib used in our experiment was based on that used in a previous study [31]. To study the EGFR and VEGF expressions in different tumor models, 4T1, CT26 and SCC7 tumors were harvested from mice with or without erlotinib pre-treatment. Tumor tissues were cut into small pieces and put into a glass homogenizer containing phosphate buffered saline (PBS). Then, the tumor lysates were transferred into 1.5 ml Eppendorf tubes and centrifuged. Supernatants were transferred to fresh tubes and the protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay kit. For Western blotting, each sample with the equal amount of total protein was mixed with an equal volume of 2 × Laemmli buffer and boiled at 95 °C for 5 min before loading onto the gel. After completion of gel electrophoresis, proteins were transferred to a Hybond nitrocellulose membrane for 2 h using a blotting apparatus. Then, anti-EGFR antibody at a dilution of 1:1000, and anti-β-actin antibody (Abcam, ab8226) at a 1:10,000 dilution were used as primary antibodies. The secondary antibody used for these blots was a goat anti-mouse antibody (Epizyme, PAB001). The VEGF