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PDGFR-β inhibitor slows tumor growth but increases metastasis in combined radiotherapy and Endostar therapy

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ABSTRACT

Background: Pericytes are pivotal mural cells of blood vessels and play an essential role in coordinating the function of endothelial cells. Previous studies demonstrated that Endostar, a novel endostatin targeting endothelial cells, can enhance the effect of radiotherapy (RT). The present study addressed whether inhibiting pericytes could potentially improve the efficacy of combined RT and Endostar therapy.

Methods: Platelet-derived growth factor beta-receptor inhibitor (CP673451) was chosen to inhibit pericytes and RT (12 Gy) was delivered. Lewis lung carcinoma-bearing C57BL/6 mice were randomized into 3 groups: RT, RT + Endo, and RT + Endo + CP673451. Subsequently, tumor microvessel density (MVD), pericyte coverage, tumor hypoxia, and lung metastasis were monitored at different time points following different therapies.

Results: Compared to the other two groups, RT + Endo + CP673451 treatment markedly inhibited tumor growth with no improvement in the overall survival. Further analyses clarified that in comparison to RT alone, RT + Endo significantly reduced the tumor MVD, with a greater decrease noted in the RT + Endo + CP673451 group. However, additional CP673451 accentuated tumor hypoxia and enhanced the pulmonary metastasis in the combined RT and Endostar treatment.

Conclusions: Tumor growth can be further suppressed by pericyte inhibitor; however, metastases are potentially enhanced. More in-depth studies are warranted to confirm the potential benefits and risks of anti-pericyte therapy.

1. Introduction

Radiotherapy (RT) is a commonly used treatment strategy that plays a vital role in curative and palliative settings. With the aim of improving the efficiency of RT and minimizing side effects, RT, and combined biological or pharmaceutical therapies are currently being investigated as potential modalities, showing excellent antitumor activity [1,2].

As one of the potent endogenous vascular inhibitors, endostatin is under intensive focus. Endostar is a novel recombinant human endostatin synthesized in China, and in 2005, Endostar was approved by the State Food and Drug Administration. We previously found that Endostar enhanced the effect of RT within the vasculature-remodeling period by promoting apoptosis of endothelial cells and changing proangiogenic factors [3,4].

Pericytes are structural cells located outside of the vasculature, coordinating the function of endothelial cells [5]. During vascular morphogenesis, new tubes formed by endothelial cells require the recruitment of pericytes to provide a physical and chemical support to the blood vessels. Within the tumor microenvironment, the role of pericytes is yet controversial. Pericyte-targeting tends to be a promising therapy due to the ability of disturbing the vasculature that feeds the tumors [6]. Nevertheless, some studies have argued that low pericyte coverage is related to a decreased survival of patients and pericyte loss may promote cancer metastasis [7,8].

The crucial role of platelet-derived growth factor beta-receptor (PDGFR-β) signaling in pericyte recruitment with respect to tumor vascularization has been well-documented [9,10]. Radiation-induced autocrine and paracrine PDGF signaling plays an essential role in promoting the proliferation of endothelial cells [11]. Furthermore,

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emerging studies have revealed that activating PDGF signaling can protect the endothelial cells from apoptotic stimuli, resulting in pericyte-mediated resistance to antiangiogenic therapies [12,13]. PDGFR-β inhibition can sensitize the endothelial cells to antiangiogenic therapy, and dual targeting of endothelial cells and pericytes may be efficacious in postponing the growth of tumor [14,15]. Additionally, Garcia-Barros et al. [16] have shown that endothelial apoptosis regulates tumor response to radiation since apoptosis-resistant tumors are resistant to radiation compared to wild-type tumors. In this regard, PDGF-β inhibition may be benifitial for enhancing the efficacy of RT.

Due to the endothelial-supporting role of pericytes and the potential synergies between pericytes-targeting and RT, we hypothesize that dual endothelial cell and pericyte inhibition coupled with RT might maximize the efficacy of RT. Herein, based on our previous studies of Endostar and RT, we chose CP673451, a potent inhibitor of PDGFR-β, for examining the role of pericytes in combined RT and Endostar therapy. CP673451 is > 450-fold selective towards PDGFR-β as compared to other angiogenic receptors [17]. We established a subcutaneous tumor model, evaluated the therapeutic effect, and dissected the mechanisms underlying the combined radiation and anti-angiogenesis therapy with/without anti-pericyte treatment.

2. Methods

2.1. Cell culture

The Lewis lung carcinoma (LLC) cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Hoffmann-La Roche Ltd., Basel, Switzerland) and 1% penicillin/streptomycin (Hyclone), followed by incubation at 37 °C in 5% CO_2.

2.2. Mouse model

All animal experiments were approved by the Institutional Animal Care and Use Committee (NoSYXK2007-008) of West China Hospital. Six-week-old female C57BL/6 mice (18 ± 2 g) were purchased from Beijing HuaFukang Biological Technology Co. Ltd. (HFK Bioscience, Beijing, China) and maintained in specific pathogen-free laminar flow frame and constant temperature (20–26 °C) and humidity (50–65%). To establish tumor models, 5×10^5 LLC cells were inoculated subcutaneously into the right proximal hind legs of mice.

2.3. Tumor therapy

Tumor growth was monitored by measuring the length and width of the tumor using a caliper every alternate day, and the tumor volume (V) was calculated as V = length × width × width × 1/2.

In order to determine the optimal CP673451 dosage for combining with RT and Endostar, when the tumor volume reached 150-200 mm^3, the mice were randomly allocated into different dosage groups (0, 2.5, 5, 10, and 20 mg/kg). Various dosages of CP673451 (Selleck Chemicals, Houston, TX, USA) were administered intraperitoneally for 5 days (starting from the 6th day after grouping).

Endostar (Simcere Pharmaceutical Group, Shandong, China) was administered by caudal vein injection at 20 mg/kg dose for 10 consecutive days after grouping, as specified by our previous study [3]. Our previous animal study showed that tumor hypoxia was significantly reduced 5 days after Endostar administration, and radiation efficacy was maximal when radiation (12Gy) was administered on day 5 [4].

In our study, corresponding sham treatment (vehicle-control) was conducted in the non-treatment groups: when the experimental group mice were administered with CP673451 intraperitoneally, non-treatment mice received intraperitoneally vehicle (10% 1-methyl-2-pyrrolidinone and 90% polyethylene glycol 300) and when the experimental group mice were administered with Endostar by caudal vein injection, the mice as negative control received caudal vein injections of 0.9% saline.

Before radiation treatment, each mouse was anesthetized by 3.5% chloral hydrate and shielded by a lead box such that only the tumor was exposed. Radiation was delivered using a Varian Clinac 600C X-ray unit at 1.37 Gy/min (distance from the X-ray source to the target was 50 cm). Each mouse underwent focal tumor treatment with a single 12 Gy radiation dose.

The cancer treatment was commenced after the optimal dose of CP673451 was determined as outlined above. Tumor-bearing mice (150-200 mm^3 tumor size) were randomly divided into 3 groups as follows: radiotherapy (RT), radiotherapy + Endostar (RT + Endo), and radiotherapy + Endostar + P673451 (RT + Endo + CP673451). Subsequently, the treatment experiment was repeated. Four mice from each group were sacrificed at 11, 13, 15, 17, 19 and 21 days after treatment initiation for harvesting the tumor tissues as well as 17, 21 and 25 days for harvesting the lung tissues.

2.4. Western blot (WB)

Tumors from each group were homogenized and extracted using RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were cleared of debris by centrifugation, followed by measuring the protein concentrations using bicinchoninic acid (BCA) assay. Protein extracts were resolved on 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h, the membranes were probed with primary antibodies: anti-PDGFR-β (1:200, Affymetrix eBioscience, San Diego, CA, USA) or anti-α-smooth muscle actin (α-SMA) (1:500, Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-Bio Co., Fuzhou, China) at 1:5000 in blocking solution for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence kit (Millipore, USA). β-actin (1:1000, ZSGB-Bio) was used as the loading control.

2.5. Immunofluorescence (IF)

The tumor-excised tissues from mice were immediately snap frozen in liquid nitrogen and stored at −80 °C. For IF, 10 central cross sections with a thickness of 5 μm were sliced per tumor. Then, the slides were air dried, fixed in 4% ice-cold paraformaldehyde, and rehydrated with PBS. After serum-blocking for 1 h at room temperature, the slides were incubated with rat anti-mouse platelet/endothelial cell adhesion molecule (CD31) monoclonal antibody (1:300, BD Biosciences, San Jose, CA, USA) and rabbit anti-mouse α-SMA polyclonal antibody (1:200, Abcam) overnight at 4 °C. Subsequently, the slides were incubated for 60 min with secondary antibodies (1:800, Invitrogen, USA). The control slides were treated similarly, albeit without primary antibodies. The sections were observed under a Zeiss Axioplan fluorescence microscope. Images were captured with an Axiocom MR Camera using Axiovision 3.1 software, and further analyzed by ImageJ. The quantification of the images was conducted as described previously [18].

2.6. Flow cytometry

Pimonidazole hydrochloride (PIM, Chemicon International, Temecula, CA, USA) was solubilized in 0.9% saline at a final concentration of 10 mg/mL. 1 h before execution, the mice were injected intraperitoneally with 60 mg/kg PIM. Tumor tissues were extracted into single cell suspensions, which were incubated with Fluorescein isothiocyanate (FITC)-conjugated anti-pimonidazole antibody (1:100 dilution) for 45 min at 4 °C. Then, the cells were washed and analyzed by flow cytometry (BD FACSAria, USA).