SPECT Imaging of Neovascularization With ^{99m}Tc -3PRGD2 for Evaluating Early Response to Endostar Involved Therapies on Pancreatic Cancer Xenografts in Vivo

Xiaona Jin

Peking Union Medical College Hospital

Chengyan Dong

Siemens Healthcare China

Kun Zheng

PUMCH: Peking Union Medical College Hospital

Ximin Shi

Peking Union Medical College Hospital

Yu Liu

PUMCH: Peking Union Medical College Hospital

Li Huo

Peking Union Medical College Hospital

Fan Wang

Medical Isotopes Research Center, Peking University

Fang Li (✉ lifang@pumch.cn)

Peking Union Medical College Hospital

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Abstract

Background

Molecular imaging targeting angiogenesis is warranted for specific monitoring of molecular changes as early therapeutic effects via antiangiogenesis. We explore the predictive value of ^{99m}Tc -PEG₄-E[PEG₄-c(RGDfK)]₂ (^{99m}Tc -3PRGD₂) as an integrin $\alpha v\beta 3$ targeting imaging agent for monitoring the efficacy of endostar antiangiogenic therapy and chemotherapy in an animal model, to find a specific pathway that could monitor early therapeutic effects.

Results

Tumor growth was significantly lower in treatment groups than in the control group ($P < 0.05$), and also in Endostar + Gemcitabine group than in Endostar group ($P = 0.034$) or Gemcitabine group ($P = 0.021$), and the differences was observed at 28 days after treatment. The difference of uptake of ^{99m}Tc -3PRGD₂ was observed between the control group and Endostar group ($P = 0.033$) or the combination therapy group ($P < 0.01$) at 7 days after treatment, and at 14 days after treatment between the control group and Gemcitabine group ($P < 0.01$). ^{99m}Tc -3PRGD₂ accumulation was significantly correlated with microvessel density ($r = 0.998$, $P = 0.002$).

Conclusion

With ^{99m}Tc -3PRGD₂ SPECT, the tumor response to antiangiogenic therapy, chemotherapy, and combination therapy can be assessed at a very early stage of treatment, much earlier than the tumor volume change, which provides new opportunities to develop individualized therapeutic approaches and optimized dosages for effective treatments.

Introduction

Inhibition of angiogenesis can provoke vascular regression, impeding delivery of oxygen and nutrients, and ultimately starving the tumor. Antiangiogenic therapy has been approved by many countries as an effective strategy to inhibit tumor growth, providing a novel treatment approach for cancer patients [1-2]. As a recombinant human endostatin—Endostar, was mainly used as an antiangiogenic agent for cancer treatment [3-4], which was approved by the China Food and Drug Administration (CFDA) in 2005 for treatment of lung cancer. Endostar exhibits antiangiogenic effects and has been used to treat numerous types of cancer, including non-small lung, breast, melanoma tumor, and gastric cancer [5-10]. Nevertheless, the benefits of Endostar on pancreatic cancer are currently poorly known. Endostar combined with temozolomide or dacarbazine + 5-FU was effective in the treatment of advanced pancreatic neuroendocrine tumors, and the combinations were well tolerated [11]. A preclinical study revealed an unfavorable proangiogenic side effect of cantharidin via targeting the pancreatic cancer angiogenic microenvironment in vivo. Antiangiogenic therapeutics or inhibitors of proangiogenic kinase pathways could antagonize the growth-promoting effect of cantharidin and present additive antitumor effects, exhibiting adequate efficacy. As an antiangiogenic agent, Endostar showed good safety profile and tolerance in previous studies, without toxicities commonly seen with other VEGF or VEGFR inhibitors, such as hypertension and proteinuria [12-16].

In past years, clinical trials of anti-angiogenic therapy with anti-VEGF (bevacizumab) or anti-VEGFR (sorafenib, Axitinib) for pancreatic cancer, have been carried out [17-21]. As is often observed in clinical trials, patient responses are variable, with only a subset of patients benefiting from the therapy [22-23]. Therefore, it is in great demand to develop an alternative approach to identify patients who most likely benefit from antiangiogenic treatment, to detect emerging resistance, and to monitor early therapeutic efficacy [24].

As a prognostic indicator of progression, histopathologic evaluation of microvessel density (MVD) is not practical for routinely evaluating tumor angiogenesis because of its invasive nature of the procedure [25]. Noninvasive imaging technologies such as dynamic contrast-enhanced (DCE) magnetic resonance imaging (MRI) or computed tomography (CT), used to provide evidence on tumor blood flow, permeability, and volume, are technically challenging and cannot directly and effectively quantify the changes of post-treatment tumoral vascularity [26–28]. Positron emission tomography (PET) using ^{18}F -FDG (2-deoxy-2- ^{18}F -fluoro-D-glucose) also was used to monitor antiangiogenic therapy by determining glucose metabolism changes, but it may not be an ideal modality because it is not a tumor-specific radiotracer. Therefore, molecular imaging targeting specific pathways involved in angiogenesis is

warranted for specific monitoring of some molecular changes as early therapeutic effects via antiangiogenesis, with the benefit that it allows repetitive noninvasive follow-ups during the course of therapy.

Imaging integrin $\alpha_v\beta_3$ may provide new opportunities to document tumor angiogenesis and monitor response to antiangiogenesis treatment [29]. The Arg-Gly-Asp (RGD) sequence has been known to bind with the integrin $\alpha_v\beta_3$ that is expressed on the surface of angiogenic blood vessels or tumor cells [30]. Thus, various radiolabeled derivatives of RGD peptides have been developed for angiogenesis imaging by positron emission tomography (PET) imaging, such as ^{18}F -FPRGD₂ and ^{68}Ga -NOTA-PRGD₂, and single-photon emission computed tomography (SPECT) imaging for the diagnosis of cancers, such as $^{99\text{m}}\text{Tc}$ -3PRGD₂ [31-35].

In our previous studies, high uptake of $^{99\text{m}}\text{Tc}$ -3PRGD₂ was observed in tumors of human pancreatic cancer xenograft model PANC-1 and patients with pancreatic cancer, $^{99\text{m}}\text{Tc}$ -3PRGD₂ SPECT showed the value of the diagnosis of pancreatic cancer [36]. And now we try to investigate $^{99\text{m}}\text{Tc}$ -3PRGD₂ as an integrin $\alpha_v\beta_3$ targeting imaging agent for monitoring the efficacy of endostar antiangiogenic therapy and chemotherapy in an animal model, comparing with MVD, to find a specific pathway which could early monitor therapeutic effects and is applicable for follow-ups during therapy.

Methods

Radiopharmaceutical Preparation

Synthesis of the labeling precursor, kit preparation, and subsequent $^{99\text{m}}\text{Tc}$ -labeling were performed as previously described [35]. Briefly, the kit for the preparation of $^{99\text{m}}\text{Tc}$ -3PRGD₂ was formulated by combining 20 mg of hydrazinonicotinamide-3PRGD₂, 5 mg of trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS), 6.5 mg of tricine, 40 mg of mannitol, 38.5 mg of disodium succinate hexahydrate, and 12.7 mg of succinic acid. For $^{99\text{m}}\text{Tc}$ radiolabeling, to the kit vial was added 1 mL of 1,110–1,850 MBq (30–50 mCi) of $^{99\text{m}}\text{TcO}_4^-$ saline solution, and then the vial was water-bathed at 100°C for 15–20 min. The resulting solution was analyzed by instant thin-layer chromatography using Gelman Sciences silica-gel paper strips and a 1:1 mixture of acetone and saline as eluant. The radiochemical purity was always greater than 95%. The reaction mixture was then diluted to approximately 370 MBq/mL (10 mCi/mL) with saline and was filtered with a 0.20-mm Millex-LG filter (EMD Millipore). Each animal was injected with 7.4–11.1 MBq (0.2–0.3 mCi) of $^{99\text{m}}\text{Tc}$ -3PRGD₂ per mice. The resulting solution was analyzed by instant thin-layer chromatography using paper strips and acetone as eluent. The radiochemical purity was >95%.

Animal Model Establishment.

Female BALB/c mice (5 weeks of age) were purchased from Vital River Lab Animal Technology Co., Ltd. PANC-1 mice model was established by subcutaneous injection of 2×10^6 PANC-1 cells into the right rear legs of mice. Once the tumor diameter reached 5–7 mm, the mice were initiated with treatment (2 weeks after inoculation of PANC-1 cells).

Treatment Protocol.

The study flowchart is provided in Fig. 1. PANC-1 tumor-bearing BALB/c mice with a tumor size of 5–7 mm were randomly assigned to four groups (n = 7 mice per group). The mice were randomly assigned to four major groups. The first group was injected 10 mg/kg of Endostar, the second one with 10 mg/kg of Gemcitabine, the third one with 10 mg/kg of Endostar and 10 mg/kg of Gemcitabine at the same time, and the control group with 0.9% saline. The treatments were performed daily for 28 days continuously. Tumor dimensions were measured every day with digital calipers, and the tumor volume was calculated using the formula (volume = $1/2 \times \text{length} \times \text{width} \times \text{width}$). To assess potential toxicity, body weight was measured daily. All mice were euthanized and the tumor tissues were harvested for further immunohistochemical staining when the treatment was complete.

Imaging Protocol

The scanner was dual-head gamma cameras, using low-energy high-resolution collimators and a 20% energy window centered on 140 keV. After intravenous injection of $^{99\text{m}}\text{Tc}$ -3PRGD₂, static planar scans of the mice were obtained at 1.5 h, the highest time point of tumor uptake. The acquisition count was 3×10^5 . The matrix is 256×256 , and the magnification is 1.33. The region of interest

(ROI) of the tumor (T) and contralateral corresponding site (NT) were delineated, and the ratio of radioactivity (T/NT) was calculated. The study flowchart is provided in Fig. 1.

Immunofluorescence staining

Vascular endothelium was labeled using immunohistochemical staining with isolectin B4. The fluorescence staining study was carried out as previously described [37]. Slides were incubated with the fluorescein-labeled Griffonia Simplicifolia Lectin I (GSLI) Isolectin B4 (FL-1201, 1: 50, Vector Laboratories, USA) overnight at 4°C and sealed with DAPI Fluoromount-G® mounting medium (Southern Biotech, USA). Microvessel density was counted on the isolectin B4-stained slides in three fields in a blinded way using a fluorescence microscope in three fields under 40×magnification and the results were averaged.

Statistical Analysis

Quantitative and semiquantitative data were expressed as the mean $\pm$ SD and analyzed using SPSS version 17.0 (IBM, Chicago, IL, USA). Mean values were compared using one-way analysis of variance (ANOVA) or Student's t-test. Two-way repeated-measures analysis of variance (ANOVA) was used to evaluate the differences between different treatment groups.

Results

Effect of Treatments on Tumor Growth

The antiangiogenic therapy with Endostar, Gemcitabine, Endostar, and Gemcitabine were carried out in PANC-1 tumor-bearing mice. No significant tumor growth inhibition was observed in Endostar or Gemcitabine group as compared with the control group before day 21 post-treatment ($p > 0.05$). At the end of the therapeutic study (day 28 post-treatment), the tumor growth of the control group was rapid with the tumor sizes reaching over $1881 \pm 523 \text{ mm}^3$, but $1160 \pm 212 \text{ mm}^3$ in the Endostar group, $1171 \pm 496 \text{ mm}^3$ in the gemcitabine group, and $801 \pm 399 \text{ mm}^3$ ($P < 0.05$), demonstrating the tumor growth inhibition effect of treatments. For the therapeutic effect evaluated by tumor volume, two-way repeated-measures ANOVA was statistically significant for differences between treatment groups and the control group shown in Table1 Fig.2A. Tumor growth was significantly faster in controls than in all other groups ($P < 0.05$) and in Endostar or Gemcitabine alone vs. Endostar + Gemcitabine ($P = 0.034$ for Endostar and $P = 0.021$ for Gemcitabine). Treatment was the only regimen that resulted in slowing tumor growth.

Monitoring the Efficacy of Antiangiogenic Therapy by SPECT.

For monitoring the efficacy of antiangiogenic therapy, SPECT imaging was performed by using $^{99\text{m}}\text{Tc}$ -3PRGD₂ on before treatment and days 7, 14, 21, and 28 post-treatment, respectively. At baseline, the tumor uptake values (T/NT) of $^{99\text{m}}\text{Tc}$ -3PRGD₂ were $1.50 \pm 0.08, 1.50 \pm 0.17, 1.52 \pm 0.11, 1.55 \pm 0.19$, and T/NT in treatment groups at this time was no significant difference compared to the control group. 7days after treatment, T/NT in the Endostar group was significantly lower than the control group (1.67 ± 0.16 vs 1.87 ± 1.15 , $P = 0.033$), and the difference lasted until the end of treatment. The difference was also observed between the control group and the both of Endostar and Gemcitabine group. But there was no difference between the Gemcitabine group and the control group until 14 days after treatment. For the therapeutic effect evaluated by T/NT, two-way repeated-measures ANOVA was statistically significant for differences between treatment groups and the control group shown in Table2. T/NT raise was significantly faster in controls than in all other groups ($P < 0.05$) and in Endostar or Gemcitabine alone vs. Endostar + Gemcitabine ($P = 0.034$ for Endostar and $P = 0.021$ for Gemcitabine), treatment was the only regimen that resulted in slowing the growth of the T/NT Fig.2B

Immunohistochemical Findings

Twenty-eight specimens were stained with isolectin B4 to correlate with the imaging findings. The microvessel density MVD were $10.5 \pm 1.7, 15.3 \pm 2.5, 9.7 \pm 1.4, 23.1 \pm 2.7$ in each microscopic in the Endostar group, Gemcitabine group, both of Endostar and Gemcitabine group, the control group (Fig.3). $^{99\text{m}}\text{Tc}$ -3PRGD₂ accumulation was significantly correlated with MVD counted on the isolectin B4-stained slides ($r = 0.998, P = 0.002$). MVD in the treatment groups were was significantly lower than the control group ($P < 0.05$). The difference was observed between the Endostar group and the Gemcitabine group but there was no difference between the Endostar group and the both of Endostar and Gemcitabine.

Discussion

Pancreatic cancer remains one of the deadliest malignancies, responsible for substantial morbidity and mortality worldwide. The 5-year survival rate at the time of diagnosis is 10% in the USA and about 7% in China, as approximately 80–85% of patients present with either unresectable or metastatic disease [38-39]. Systemic chemotherapy combinations including FOLFIRINOX (5-fluorouracil, folinic acid [leucovorin], irinotecan, and oxaliplatin) and gemcitabine plus nab-paclitaxel remain the mainstay of treatment for patients with advanced disease [40]. Therefore, new effective therapeutic schemes and sensitive evaluation of curative effect by noninvasive imaging methods become very important. In addition to conventional chemotherapy combinations, multiple trials of anti-angiogenic therapy with anti-VEGF (bevacizumab), anti-VEGFR (sorafenib, Axitinib) or for pancreatic cancer, have been carried out with different results, some producing additional benefits [41-43] and others didn't [16-21] with different therapies. In our study, Endostar was chosen as an antiangiogenic drug for the treatment of pancreatic tumors on mouse pancreatic cancer xenografts alone or combined with Gemcitabine. And ^{99m}Tc -3PRGD₂ SPECT was used to evaluate the therapeutic effect targeting neovascularization.

The results of two-way repeated-measures ANOVA showed that the three therapeutic schemes were effective in inhibiting tumor growth. At the end of the 14 days, Endostar or Gemcitabine treatment didn't induce a significant reduction in the slope of tumor growth, as compared to controls. But the combination of Endostar and Gemcitabine the tumor volume of all the treated mice was significantly smaller than that of the control group. Until the end of 28 days of treatment—in monotherapy groups—the tumor growth was observed to be lower than that in the control group. In the Endostar-treated group, these results are explained by the mechanism of action of Endostar, which blocks the VEGF/VEGFR signaling pathway, it hampers tumor growth by mediating the regression of existing tumor vasculature and preventing regrowth over time [44-45]. But this agent is not able to eradicate the tumor all by itself [46]. In the Gemcitabine-treated group, chemotherapy created cytotoxic lesions resulting in an aberrant DNA repair leading to cell apoptosis. The combination of those two agents appeared to be more efficient thanks to their synergic action on two different pathways that promote tumor growth. It is now becoming increasingly clear that vascular normalization is associated with decreased tumor metastasis [47-48]. Jain and Lin and Sessa proposed the theories of “vascular normalization” and “window” successively [49-50]. They reported that following administration of the antiangiogenic agents, a unique “window” occurred, where irregular vessels inside the tumor were normalized. In recent years, it has been reported that Endostar can not only inhibit neovascularization, but also induce pathological vascular normalization which has shown great clinical potential for enhancing effective drug delivery, improving local immunosuppressive microenvironment, and reducing distant metastasis [51-52]. Therefore, Endostar may be a potential drug for anti-angiogenic therapy of pancreatic cancer.

At the end of the second week, a reduction in ^{99m}Tc -3PRGD₂ tumor uptake (T/NT) was observed in the mice treated with Endostar alone or combined with Gemcitabine, compared with control, in agreement with a reduction in tumor growth. At the end of the 14 days T/NT in the Gemcitabine group was significantly lower than the control group. The results of two-way repeated-measures ANOVA made sure that the treatment was the regimen that resulted in slowing the growth of the T/NT. Compared with the tumor volume, the difference of the T/NT between treatment groups and the control group was observed earlier. And also, the difference of the T/NT in treatment groups including Endostar appeared earlier than in the Gemcitabine group. The possible reason may be that Endostar mediates the reduction of neovascularization earlier than Gemcitabine.

Endostar inhibited the newborn vascular endothelial cells, causing a decrease of integrin expression, which led to the decrease of integrin-targeted imaging probe uptake in the tumor. The reduction of neovascularization may also occur in the Gemcitabine. The findings were confirmed by immunofluorescence staining isolectin B4. MVD in all the treatment groups was significantly lower than the control group. MVD in the Gemcitabine group was higher than in the Endostar group and the combination of those two agents group, but no difference between the Endostar group and the combination of those two agents group—that maybe because of Endostar reducing neovascularization more obviously than Gemcitabine. T/NT was significantly correlated with MVD. ^{99m}Tc -3PRGD₂ SPECT could be a noninvasive method for evaluating MVD.

There were some limitations in the study, The tumor is not very small for the convenience of imaging—so a difference between the treatment groups and the control group came late. “vascular normalization” and “window” had not been studied, because the focus of our research is imaging to monitor therapeutic effects.

Conclusions

With ^{99m}Tc -3PRGD₂ SPECT, the tumor response to antiangiogenic therapy, chemotherapy and can be assessed at a very early stage of treatment, much earlier than the anatomical structure change by monitoring the tumor volume, which provides new opportunities to develop individualized therapeutic approaches and establish optimized dosages and dose intervals for an effective treatment that benefit patients.

Declarations

Ethics approval and consent to participate:

This study had been approved by the Institute Review Board of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College.

Consent for publication:

Not applicable.

Availability of data and material:

The datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests:

There is no conflict of interest.

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Authors' contributions

LF, WF designed the study. LY, DC were responsible for radiosynthesis. ZK, SX were responsible for animal studies. HL helped in study supervision. The manuscript was drafted by JX and DC. All authors read and approved the final manuscript.

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Author details

1.Department of Nuclear Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, China .2.GE Healthcare China, Beijing, 100176, China.3.Medical Isotopes Research Center, Peking University, Beijing 100191, China;

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Tables

Table 1 Unpaired t-test(treatment groups vs control group)										
Time	Day 0		Day 7		Day 14		Day 21		Day 28	
Groups	Tumor	T/NT	Tumor	T/NT	Tumor	T/NT	Tumor	T/NT	Tumor	T/NT
	Volume		Volume		Volume		Volume		Volume	
Endostar vs Control	P=0.953	P=0.573	P=0.696	P=0.033	P=0.143	P=0.01	P=0.07	P=0.015	P=0.01	P=0.01
Gemcitabine vs Control	P=0.917	P=0.630	P=0.441	P=0.108	P=0.258	P=0.01	P=0.191	P=0.041	P=0.023	P=0.044
Endostar + Gemcitabine vs Control	P=0.959	P=0.769	P=0.136	P=0.01	P=0.01	P=0.01	P=0.01	P=0.01	P=0.001	P=0.01

Table 2 Repeated measurement AVONA					
Groups	AVONA	Tumor Volume		T/NT	
		F	P	F	P
Endostar vs Control		5.660	0.035	12.981	0.004
Gemcitabine vs Control		4.899	0.047	6.913	0.022
Endostar + Gemcitabine vs Control		11.873	0.005	16.133	0.002
Gemcitabine vs Endostar + Gemcitabine		7.086	0.021	11.838	0.005
Endostar vs Gemcitabine		0.047	0.832	4.955	0.051
Endostar vs Endostar + Gemcitabine		5.735	0.034	0.302	0.593

Figures

Figure 1

Therapy and imaging protocol

Figure 2

99mTc-3PRGD2 SPECT in Endostar, Gemcitabine, the combination of those two agents and the control group. PANC-1 tumor bearing mice at days 0, 7, 14, 21 and 28 post-treatment.

Figure 3

Tumor growth profiles of control group and treatment groups (7 mice per group). PANC-1 tumors bearing mice were treated with via intraperitoneal injection. Saline-treated animals served as controls. ^{99m}Tc -3PRGD2 tumor uptake (T/N) in the groups.

Figure 4

MVD in Endostar(A) Gemcitabine(B) the combination of those two agents(C) and the control group(D).