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Re-assessing the enhanced permeability and retention effect in peripheral arterial disease using radiolabeled long circulating nanoparticles

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ABSTRACT

As peripheral arterial disease (PAD) results in muscle ischemia and neovascularization, it has been claimed that nanoparticles can passively accumulate in ischemic tissues through the enhanced permeability and retention (EPR) effect. At this time, a quantitative evaluation of the passive targeting capabilities of nanoparticles has not been reported in PAD. Using a murine model of hindlimb ischemia, we quantitatively assessed the passive targeting capabilities of ^{64}Cu-labeled PEGylated reduced graphene oxide – iron oxide nanoparticles (^{64}Cu-RGO-IONP-PEG) through the EPR effect using positron emission tomography (PET) imaging. Serial laser Doppler imaging was performed to monitor changes in blood perfusion upon surgical induction of ischemia. Nanoparticle accumulation was assessed at 3, 10, and 17 days post-surgery and found to be highest at 3 days post-surgery, with the ischemic hindlimb displaying an accumulation of 14.7 ± 0.5% injected dose per gram (%ID/g). Accumulation of ^{64}Cu-RGO-IONP-PEG was lowest at 17 days post-surgery, with the ischemic hindlimb displaying only 5.1 ± 0.5%ID/g. Furthermore, nanoparticle accumulation was confirmed by photoacoustic imaging (PA). The combination of PET and serial Doppler imaging showed that nanoparticle accumulation in the ischemic hindlimb negatively correlated with blood perfusion. Thus, we quantitatively confirmed that ^{64}Cu-RGO-IONP-PEG passively accumulated in ischemic tissue via the EPR effect, which is reduced as the perfusion normalizes. As ^{64}Cu-RGO-IONP-PEG displayed substantial accumulation in the ischemic tissue, this nanoparticle platform may function as a future theranostic agent, providing both imaging and therapeutic applications.

1. Introduction

Development of multifaceted theranostic nanoparticles has become increasingly popular, as researchers strive to produce simplified strategies for disease treatment and imaging. In particular, reduced graphene oxide (RGO)-based nanocomposites are widely investigated for several imaging and therapeutic applications. Previously, we described the preparation and functionalization of RGO-nanoparticles for drug loading [1]. In addition to the therapeutic potential of RGO-based nanoparticles, this nanoplatform may function as a multimodality imaging agent. Specifically, RGO-iron oxide nanoparticles (RGO-IONPs) display physical and chemical characteristics suitable for multimodality imaging, including an NIR absorbance required for photoacoustic imaging,

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T2-relaxivity properties needed for magnetic resonance imaging (MRI), and RGO-IONPs may be easily functionalized for other imaging modalities. For example, Hong *et al.* utilized positron emission tomography (PET) to map the distribution of ^{64}Cu-labeled RGO in a murine breast cancer xenograft model [2].

Peripheral artery disease (PAD) is an ailment prevalent in the elderly that arises from arterial stenosis in the extremities, including legs and feet [3]. As the vasculature fails to provide vital oxygen to the tissues, patients experience slower healing times, intermittent claudication, and possible gangrene [4]. PAD has become a global health concern affecting approximately 12 million individuals in the United States alone [5]. Furthermore, people with PAD are at a higher risk of developing coronary artery disease and cerebrovascular disease in comparison to the normal population, both of which may lead to stroke or heart attack [6]. The most severe form of obstructive PAD, known as critical limb ischemia (CLI), is the leading cause of non-injury amputation [7]. Current treatments for CLI aim to normalize blood perfusion and include surgical intervention to bypass blocked arteries, proangiogenic growth factors, and anti-platelet medications [8]. Surgical intervention to treat PAD has several limitations. First, bypass surgery is an invasive surgery with serious adverse effects, such as heart attack, stroke or infection. Second, bypass surgery only targets the macrovascular system, so an immediate response may not be sufficient for CLI patients. To enhance the circulation through the microvascular system, proangiogenic growth factor treatment has been assessed and shown efficacy in preclinical studies [9,10]. However, proangiogenic growth factor treatment is limited by low delivery efficiency and systemic off-target effects and has failed to show benefits in several clinical trials [8]. In this regard, nanoparticle-based proangiogenic gene or growth factor delivery has been proposed. Multiple preclinical studies using nanoparticle-based growth factor treatment showed improvement of blood perfusion [11]. However, accumulation of nanoparticles has not been quantitatively assessed, even though the high accumulation of nanoparticle in the ischemic site is a prerequisite to overcome the limitation of conventional proangiogenic gene or growth factor treatment.

Monitoring the success of therapeutic intervention remains critical for improving patient survival, yet there are few reliable noninvasive imaging techniques for PAD patients. Duplex Doppler ultrasound imaging can evaluate the location and extent of the disease and arterial hemodynamics, yet the scans are time-consuming and calcification stenosis can limit the evaluation [12]. Besides Doppler imaging, magnetic resonance angiography (MRA) and computed tomography angiography (CTA) are two additional modalities utilized for noninvasive imaging of PAD [13]. MRA can effectively assess the location and degree of stenosis; yet is limited by high cost, motion artifacts, and decreased signal caused by metal clips or stents. While CTA is less sensitive than MRA, this imaging modality has fast acquisition times. Both CTA and MRA could cause renal toxicity in patients with renal insufficiency, which is a relatively common status in patients with PAD [14]. Nanoparticles have also been used to image PAD in preclinical studies. Previously, Kim *et al.* developed fluorescently-labeled PEGylated silica nanoparticles for optical imaging of PAD using the hindlimb ischemia model [15]. Similarly Zhang *et al.* investigated fluorescently-labeled gelatin nanoparticles for imaging of PAD [16]. Using optical imaging techniques, they showed a significant difference in nanoparticle uptake at 4 h and 24 h post-injection. Nanoparticle accumulation was highest in the ischemic hindlimb at 4 h and significantly decreased at 24 h. While optical imaging showed a statistically significant difference in fluorescence signal between the ischemic and non-ischemic hindlimb, optical imaging lacks the sensitivity and penetration depth needed for accurate quantification. The high sensitivity of PET is well suited for quantitative evaluation of serial responses to medical intervention in patients with PAD by providing information regarding physiological changes in response to various therapies; yet currently, there is no established PET tracer to image PAD [17]. Also, multimodality imaging of PAD will allow for simultaneous assessment of the anatomy and physiology of the disease, guiding physicians in developing patient-specific treatment protocols [12].

While passive targeting via the enhanced permeability and retention (EPR) effect has been extensively examined in solid tumors, few studies have examined this phenomenon in other disease models, including PAD [15,18]. Similar to solid tumors, several angiogenic factors are upregulated in ischemic tissue that may activate and mobilize endothelial cells to form new leaky vessels, resulting in EPR-attributed nanoparticle localization [15]. However, the degree and the time course of the EPR effect have not been evaluated quantitatively in PAD. The EPR effect of nanoparticles depends on several factors, including the stability and circulation half-life [19]. The long circulation half-life of PEGylated RGO-IONPs *in vivo* provides adequate time for the EPR effect to cause nanoparticle localization in the ischemic hindlimb. Additionally, RGO-IONPs have shown excellent theranostic properties for disease imaging and treatment, including its optical absorbance properties, ability to deliver large drug payloads, and passive targeting capabilities [1].

Herein, we investigated ^{64}Cu-RGO-IONP-PEG for the noninvasive multimodal imaging of PAD using a murine model of hindlimb ischemia. A surgical procedure recreated the conditions found in PAD patients, and as the ischemic hindlimb healed (15–20 days), blood flow was restored to normal in the diseased hindlimb [20]. At 3, 10, and 17 days post-surgery, mice received an intravenous injection of ^{64}Cu-RGO-IONP-PEG and imaged for 72 h with PET. We could observe and quantify the accumulation of nanoparticles in the ischemic hindlimb. Also, we found that fewer nanoparticles localized in the ischemic tissue as the vasculature normalized, which might be explained by the lessened EPR effect. Accumulation of ^{64}Cu-RGO-IONP-PEG in the ischemic hindlimb confirmed the influence of the EPR effect, while also showing that ^{64}Cu-RGO-IONP-PEG is a suitable nanoplatform for the noninvasive multimodality imaging of PAD.

2. Materials and methods

2.1. Synthesis and surface functionalization of RGO-IONP

Synthesis of RGO-IONP-PEG was previously reported [21]. RGO-IONP was synthesized using a modification of the Hammer method. Briefly, GO (20 mg), $FeCl_3 \cdot 6H_2O$ (270 mg), sodium acrylate (750 mg), and sodium acetate (750 mg) were dissolved in a mixture of ethylene glycol (0.5 mL) and diethylene glycol (9.5 mL). The solution was heated at 200 °C for 10 h and resulting RGO-IONP was washed with ethanol and deionized water. Synthesis of the RGO-IONP nanocomposites was accomplished through a hydrothermal reaction between the RGO and iron chloride hexahydrate. To ensure biocompatibility and increased blood circulation *in vivo*, RGO-IONP (1 mg) were functionalized with 10 mg of poly(maleicanhydride-alt-1-octadecene) (PEG) (Sigma-Aldrich, Madison, WI, USA). The solution was sonicated for 30 min and centrifuged at 4000 rpm for 5 min to remove unstable aggregates before the supernatant was washed through a 100-nm filter membrane to remove unbound PEG. On the RGO-IONP-PEG, we conjugated additional PEG for a longer circulation time and NOTA for radiolabeling. Before the addition of second PEG, nanoparticles were conjugated to p-SCN-Bn-NOTA (NOTA, Macrocyclics, Inc., Dallas, TX, USA) with 1:4 M ratio of the nanoparticle to the chelator. The pH of the solution was