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Activatable fluorescence imaging of macrophages in atherosclerotic plaques using iron oxide nanoparticles conjugated with indocyanine green

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ABSTRACT

Background and aims: Macrophages are key factors in the formation of unstable atherosclerotic plaques, which may be identified through macrophage imaging. We tested whether activatable fluorescence probes of iron oxide nanoparticles (IONPs) conjugated with indocyanine green (ICG) (IONP-ICG), consisting of biocompatible reagents, can visualize macrophages present in atherosclerotic plaques.
Methods: IONP-based probes conjugated with different numbers of ICG molecules were synthesized. Six-week-old spontaneously hyperlipidemic (SHL) mice were fed either a Western or normal diet for 14 weeks, and were intravenously injected with IONP-ICG (55.8 mg Fe/kg). Aortas were harvested 48 h later, and aortas containing atherosclerotic plaques were imaged.
Results: Phantom imaging studies using IONP-ICG solution demonstrated that the addition of surfactants to IONP-ICG solutions yielded fluorescence activation. Incubation of macrophages with IONP-ICG led to internalization of IONP-ICG and near infrared fluorescence (NIRF) activation. In NIRF imaging studies, intense fluorescence signals were clearly visible primarily at the margins of atherosclerotic plaques, and relatively weak signals were evident inside the plaques, demonstrating the feasibility of detection of NIRF signals at atherosclerotic plaques. In the quantitative evaluation of NIRF, administration of a probe conjugated with more ICG molecules led to a significant increase in the NIRF signal, indicating that probes with greater numbers of ICG molecules are effective for sensitive NIRF detection. SHL mice given a low-cholesterol normal diet showed a significantly lower NIRF signal compared with mice given the Western diet. Histologically, NIRF signals in atherosclerotic plaques strongly correlated with the location of macrophages, suggesting the possibility of NIRF macrophage imaging using IONP-ICG.
Conclusions: Localization of macrophages in atherosclerotic plaques may be achieved using the activatable NIRF probe, IONP-ICG.

1. Introduction

Macrophages play important roles in atherosclerotic plaque formation [1,2] and are known to be involved in the destabilization of atherosclerotic plaques [3,4]. Detection of macrophage-rich regions in blood vessels may therefore lead to the identification of unstable atherosclerotic plaques. Recently, near-infrared fluorescence (NIRF) imaging, which can detect sensitively with relatively good tissue permeability and minimal effects from autofluorescence, has been attracting attention for the detection of unstable atherosclerotic plaques [5–7].

Indocyanine green (ICG), an NIRF molecule approved for clinical use by the Food and Drug Administration (FDA) in the United States, is highly biocompatible [8], and several imaging studies using ICG

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conjugated to various carrier molecules to detect tumor cells have been reported [9–13]. In the first place, ICG is the only NIRF imaging probe targeting inflamed atherosclerotic plaques that can be used clinically [14,15]. The circulating ICG binds rapidly to low-density and high-density lipoproteins in the blood, due to its lipophilic properties, and then the complexes of lipoproteins bound to ICG are taken up by macrophages of atherosclerotic plaques [14]. In this way, the uptake of ICG by macrophages is an indirect passage; but the direct uptake seems to be more effective for accurately visualizing macrophages. After injection, circulating ICG is rapidly taken up by liver and then released with an elimination half-life of 2–4 min [16], suggesting that almost all the injected ICG can't reach macrophages. For these reasons, imaging probes, which are directly taken up by macrophages and have a long circulating half-life are considered to be more effective than ICG. The fluorescent signals of ICG molecules can be quenched via a self-quenching mechanism in which interactions between ICG molecules occur when multiple fluorescent molecules are in close proximity [17]. Furthermore, the auto-quenching mechanism can contribute to fluorescent quenching of ICG where ICG molecules interact with carrier molecules [12,13]. Based on these mechanisms, the fluorescent signals of activatable ICG-labeled probes are active only when taken up by the target cells followed by lysosomal degradation, enabling the suppression of background signals from non-target tissues and more specific visualization of the target cells [18,19]. For these reasons, in comparison to other agents such as activatable NIRF probes and the Cy5.5/Cy7-labeled macrophage mannose receptors tracer, which can detect inflamed atherosclerotic plaques [5,20,21], activatable ICG-labeled probes with high background ratio are biocompatible NIRF imaging probes, and this allows their clinical application.

In contrast, iron oxide nanoparticles (IONPs), which are biocompatible and biodegradable molecules with low toxicity, have been used in a wide range of biomedical applications [22] and have been approved as an intravenous magnetic resonance (MR) contrast agent (Resovist®; Bayer Healthcare, Berlin, Germany) since 2001 in the European market [23]. IONPs are MR imaging probes that can locally accumulate at inflammatory sites where macrophages are present and can also change the magnetic field through nonspecific receptor-mediated endocytosis, especially scavenger receptors by macrophages. By exploiting these properties, the presence of macrophages in atherosclerotic plaques can be detected [7,24,25]. Therefore, we deduced that NIRF probes synthesized using the highly biocompatible fluorescent molecule ICG, and the carrier molecule IONP will be activatable fluorescent probes and may have the potential for clinical application to detect the macrophages associated with inflammation in atherosclerotic plaques.

Furthermore, since the *in vivo* kinetics of the carrier molecule can potentially be affected when conjugated with a large number of fluorescent molecules [9,11,26], we synthesized and compared probes with different numbers of ICG molecules per IONP molecule with regard to the *in vivo* biodistribution. The present study tested the hypothesis that IONPs conjugated with ICG as a biocompatible and activatable fluorescent probe would be capable of visualizing the macrophages present in atherosclerotic plaques.

2. Materials and methods

Details of the Materials and methods section are presented in the Supplementary Data.

2.1. Reagents

IONPs coated with dextran (nanomag®-D-spio; diameter, 20 nm; mean molecular weight, 3500 kDa, amino groups on particle surface) were purchased from Corefront Co. (Tokyo, Japan). ICG-EG4-Sulfo-OSu was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Methoxy polyethylene glycol (PEG) succinate N-hydroxysuccinimide (NHS) (PEG-NHS ester; molecular weight: 2 kDa, SUNBRIGHT ME-020CS) was purchased from NOF America Co. (White Plains, NY).

2.2. Preparation of IONPs conjugated with ICG

We synthesized IONPs conjugated with ICG probes (IONP-ICG; Supplemental Fig. 1). IONP-ICG synthesized at IONP:ICG molecular ratios of 1:5, 1:10, and 1:20 was denoted as IONP-ICG5, IONP-ICG10, and IONP-ICG20, respectively, according to the number of ICG molecules mixed with IONP.

2.3. Cell culture and phagocytotic activity of macrophage cells in vitro

NR8383 (rat alveolar macrophage cell line; ATCC CRL-2192) was purchased from the American Type Culture Collection (ATCC) center (Manassas, VA). Cells were incubated for 1, 8, 24, and 48 h followed by washing once with phosphate-buffered saline (PBS), and fluorescence microscopy was performed. Subsequently, iron staining was performed using a Berlin blue staining set (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Animals

All animal experiments were performed in accordance with institutional guidelines and were approved by the Kyoto University Animal Care Committee. Six-week-old male spontaneously hyperlipidemic (SHL) mice (BALB/c. KOR/Stm Slc-Apoe^{shl}) with a disrupted apolipoprotein E (apoE) gene were purchased from Japan SLC (Shizuoka, Japan). These SHL mice were fed a diet high in fat and cholesterol (Western diet; containing 16.5% fat and 1.25% cholesterol; Western diet group) or a normal chow diet (normal diet group) for 14 weeks. Corresponding wild-type 6-week-old male BALB/cCrSlc mice (control group; Japan SLC, Shizuoka, Japan), as negative controls, were fed with a normal chow diet for 14 weeks. The 20-week-old mice were intravenously injected with IONP-ICG (55.8 mg Fe/kg, 1 mmol Fe/kg (33.2 nmol IONP-ICG/kg), 200 μl) via the tail vein. IONP-ICG5 and IONP-ICG10 were injected into mice in the Western diet group (n = 5), respectively, and IONP-ICG20 was injected into mice in the Western diet group (n = 5), normal diet group (n = 5), and control group (n = 5) (Supplemental Table 1).

2.5. Biodistribution study

To quantify the concentration of each type of IONP-ICG in the blood (percentage injected dose per gram of tissue (%ID/g)), mouse blood (2 μl) was collected at 5, 15, and 30 min and 1, 3, 6, 12, 24, and 48 h after each IONP-ICG injection. The half-life of the IONP-ICG in the blood was calculated using GraphPad Prism software (GraphPad Prism Software, La Jolla, CA). At 48 h after administration, mice were deeply anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital and perfused transcardially with 4% paraformaldehyde after 30 μl of blood was collected from the heart to measure the fluorescence intensity. Fluorescence images of tissues except the aorta were acquired using the IVIS Imaging System 200.

2.6. NIRF imaging and fluorescence quantitative analysis of arteriosclerotic plaque

The excised aorta was imaged ex vivo using a Nuance EX