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TARGETING OF MACROPHAGE FOAM CELLS IN ATHEROSCLEROTIC PLAQUE USING OLIGONUCLEOTIDE- FUNCTIONALIZED NANOPARTICLES

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Abstract

Macrophage foam cells are key components of atherosclerotic plaque and play an important role in the progression of atherosclerosis leading to plaque rupture and thrombosis. Foam cells are emerging as attractive targets for therapeutic intervention and for imaging the progression of disease. Therefore, designing nanoparticles (NPs) targeted to macrophage foam cells in plaque is of considerable therapeutic significance. Here we report the construction of an oligonucleotide functionalized NP system with high affinity for foam cells. Nanoparticles functionalized with a 23-mer poly-Guanine (polyG) oligonucleotide are specifically recognized by the scavenger receptors on lipid-laden foam cells in vitro and ex vivo. The enhanced uptake of polyG-functionalized NPs by foam cells is inhibited in the presence of acetylated-LDL, a known ligand of scavenger receptors. Since polyG oligonucleotides are stable in serum and are unlikely to induce an immune response, their use for scavenger receptor-mediated targeting of macrophage foam cells provides a strategy for targeting atherosclerotic lesions.

Keywords

Atherosclerosis; Nanoparticles; polyG; foam cells; scavenger receptors

1. Introduction

Atherosclerosis is a major underlying cause of heart attack and stroke, events that claim more lives worldwide than any other disease.^{1, 2} Atherosclerosis is initiated by the recruitment of circulating monocytes into the vessel intima. These monocytes differentiate into activated macrophages that clear oxidized lipids via uptake from the blood. Eventually, this process causes macrophages to develop into lipid-laden foam cells that are unable to escape from the intima. Foam cells can become necrotic, causing their encapsulation by a thin fibrous cap that can rupture, resulting in thrombosis and vessel occlusion.^{3, 4}

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Early research established a strong association between the foam cell content of atherosclerotic plaque and the risk of plaque rupture, making the foam cell a viable target for therapeutic intervention.^{5, 6} Macrophages have been implicated in all stages of plaque development,⁵ are highly abundant within atherosclerotic plaques,⁷ and are also known to express the liver X receptors (LXR), a member of the nuclear receptor family of transcription factors that are important regulators of cholesterol, fatty acid, and glucose homeostasis and which when activated has been shown to elicit reverse cholesterol transport.⁸ These phenomena highlight the potential utility of macrophages as therapeutic targets in atherosclerosis.

Another potential advantage of targeting macrophages is that they are highly phagocytic and readily take up foreign particles, including synthetic nanoparticles (NPs).⁹ Thus, nanoparticles offer an attractive approach toward efficient delivery of drugs to foam cells. One way of targeting NPs to macrophages is to endow the NPs with a ligand that binds to specific receptors on the macrophage surface. An appealing candidate in this regard is the scavenger receptor (SR-AI), the principal receptor responsible for endocytosis of foreign objects and for mediating the influx of modified lipids into macrophages.¹⁰ SR-AI is a pattern recognition receptor that exhibits strong binding affinity towards quadruplex-pattern forming polyG oligonucleotides.¹¹ Based on this knowledge, one prior study examined binding of polyG-functionalized NPs to the macrophage surface.¹² However, this study failed to determine if the particles bound specifically to SR-AI, or to other surface components on the macrophage.

Here we extend the analysis of polyG-functionalized NPs to determine (a) if they bind to SR-AI on macrophages, (b) if macrophage activation has a role in the uptake of such particles, and (c) if the NPs can interact with macrophages residing in atherosclerotic plaques. Our results show that polyG-functionalized NPs bind better to macrophages than NPs functionalized with a control oligonucleotide. Binding and uptake of the polyG NPs is specifically mediated by SR-AI, as demonstrated by competition with acetylated low density lipoprotein (Ac-LDL), a physiologic SR-AI ligand.¹³ Using cholesterol-loaded macrophages as model foam cells, we verified that they express elevated levels of SR-AI. Furthermore, polyG-functionalized NPs are more readily recognized and internalized by these model foam cells, compared to both control NPs and normal macrophages. Finally, polyG-functionalized NPs can bind to macrophages in atherosclerotic plaques resected from the aortic wall of ApoE $-/-$ mice.

2. Materials and Methods

2.1 Preparation of oligonucleotide functionalized NPs

polyG- and polyC-functionalized NPs were prepared using standard one-step carbodiimide chemistry. Briefly, 0.75 mg of carboxylate-modified near-infrared dye loaded NPs (Spherotech; IL, USA) were washed and resuspended in MES, pH 6.0 buffer. 38.5 μ g of the amine-modified oligonucleotides (Microsynth; Switzerland) was added to the particles and allowed to mix for 15 min. To this, 25 μ l of freshly prepared EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride) (Thermo Scientific; IL, USA) solution in MES, pH 6.0 buffer was added to give a final EDC concentration of 100 mM and allowed to react at room temperature for 4 hrs with gentle mixing. The reaction was quenched by adding 40 mM glycine solution prepared in RNase/DNase free water. Particles were filtered through Bio-spin chromatography columns (Bio-rad Lab; CA, USA) to remove any unbound oligo and stored at 4°C until further use.

2.2 Macrophage cell culture

The RAW264.7 mouse macrophages cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). They were cultured in DMEM media containing L-glutamine and sodium pyruvate (Mediatech; Manassas, VA), supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin. The human THP-1 monocytic cell line, also obtained from ATCC, was cultured in RPMI 1640 medium supplemented with 10% FBS, HEPES buffer, 1 mM sodium pyruvate, 50 nM 2-mercaptoethanol and 100 IU/ml penicillin/streptomycin. To induce cell differentiation and activation, THP-1 cells were seeded in media containing 100nM phorbol 12-myristate 13-acetate (PMA) purchased from Sigma (St Louis, MO) for 24h.

For foam cell preparation, RAW264.7 macrophages were incubated with 100 µg/ml of AcLDL (Biomedical Technologies; MA, USA) for 72 hrs at 37°C.

2.3 Measurement of NP uptake and SR-AI expression with Flow Cytometry

Normal and AcLDL-loaded RAW264.7 or PMA-treated THP-1 cells were plated at 1×10^5 cells/well in 12-well tissue culture plates and allowed to attach and grow for 24 hrs (37°C, 5% CO₂, humidified). After 24 hrs the old media was removed, cells were twice washed with serum free media and NPs (50 µg/ml in serum free media) were added to the cells and incubated at 4°C or 37°C for 2–4 hrs. Unbound NPs were removed by repetitive washing with ice-cold PBS and the cells were collected by detaching them from the wells using 0.05% trypsin, fixed with 4% paraformaldehyde (PFA) in PBS for 5 min and stained with phalloidin Alexa:488 (Molecular Probes; OR, USA). After washing twice in PBS, the number of particles associated with cells was analyzed by flow cytometry with a FACSCanto (BD Biosciences). For each experiment, 10,000 Alexa:488-positive events were collected and analyzed using FACSDiVa software.

For SR-AI detection, normal and AcLDL-loaded RAW264.7 or PMA-treated THP-1 cells were plated at 2×10^5 cells/well in 12-well tissue culture plates and allowed to attach overnight (37°C, 5% CO₂, humidified). Next day cells were harvested and fixed as described above and stained with Rat anti-Mouse SR-AI antibody (Serotec; CA, USA) followed by Goat anti-Rat:Alexa 647 (Invitrogen; OR, USA). After washing twice in PBS, cells were analyzed by flow cytometry with a FACSCanto (BD Biosciences). For each experiment, 10,000 Alexa:647-positive events were collected and analyzed using FACSDiVa software.

2.4 Animal model of atherosclerosis and ex-vivo experiments

ApoE null C57Bl/6 mice were fed with Western diet (20% fat and 0.15% cholesterol) beginning from the age of 8 weeks to induce atherogenesis. After 16 weeks of induction, mice were anesthetized with Avertin and perfused with ice-cold PBS via ventricular perfusion. Aortic arch was isolated and cut open longitudinally, atherosclerotic plaques were detached from the internal face of the wall under a dissecting microscope. Plaque fragments (2x2 mm²) were incubated in DMEM with 10% FCS and 50 µg/ml of functionalized NPs with and without 5 µM free polyG and polyC for 2 hrs at 37°C.

To determine whether AcLDL can inhibit the binding of polyG-functionalized NPs to plaque associated macrophage foam cells, 0, 10, 20, 50 or 100 µg/ml of AcLDL was added into corresponding incubation system at the beginning. Tissues were triple washed with DMEM with 10% FCS for 5 min at 37°C, fixed with 4% PFA, dehydrated with 20% glucose overnight at 4°C and frozen into block with OTC. Frozen tissues were sliced into 20 µm section using cryostat. Dried sections were rehydrated with PBS and directly mounted with DAPI containing mounting medium and imaged with a confocal microscope. For

macrophage staining, Rat anti-Mouse:CD68 (BD Bioscience; MD, USA) and Goat anti-Rat:Alexa 488 (Invitrogen; OR, USA) were used as primary and secondary antibodies respectively. Acquired images were quantified with Image pro plus 5.0 software. For each group, six sections were used for quantification and each experiment was repeated at least three times.

3. Results and Discussion

3.1 Binding and uptake of polyG-functionalized NPs by cultured macrophages depends on SR-AI expression

Fluorescent NPs functionalized with either a 23-mer polyG oligonucleotide or control 23-mer polyC oligonucleotide (henceforth referred to as NP-23pG and NP-23pC respectively) were prepared using standard carbodiimide chemistry (Materials and Methods). The polyC oligonucleotide is used as a negative control due to its inability to form the quadruplex structure required for binding to SR-AI.¹¹ Human THP-1 monocyte/macrophages were used as model cells to evaluate binding of functionalized NPs because they can differentiate into activated macrophages over-expressing SR-AI upon treatment with phorbol esters (PMA).¹⁴ The expression of SR-AI on THP-1 cells was measured by flow cytometry (Figure 1A). PMA-activated THP-1 cells exhibited a >1.5-fold increase in SR-AI expression (Fig. 1B), consistent with previous reports.¹⁴

Next, we determined the ability of NP-23pG and NP-23pC to bind to THP-1 cells. NPs were incubated with cells in serum-free media at 4°C or at 37°C so that binding could be distinguished from uptake.¹⁵ At 4°C there was little binding of either NP-23pG or NP-23pC to unstimulated macrophages. However, macrophages stimulated with PMA prior to the binding experiment exhibited enhanced binding of NP-23pG compared to NP-23pC (Fig. 1B). When the same experiment was performed at 37°C, uptake of NP-23pG was substantially higher than that of NP-23pC (Fig. 1D). Similar results were obtained for NPs incubated with RAW264.7 mouse macrophages expressing SR-AI (data not shown). Together these results indicate that polyG, but not polyC-functionalized NPs can bind to and be phagocytosed by activated macrophages.

3.2 Binding of polyG-functionalized NPs to macrophages is blocked by Acetylated LDL

To determine if the interaction of NP-23pG with macrophages is mediated by SR-AI, we tested binding in the presence of AcLDL, a known SR-AI ligand. AcLDL competitively reduced uptake of NP-23pG in a concentration-dependent manner. NP binding to macrophages was reduced by 70% in the presence of 75 ug/ml of AcLDL (Fig. 2).

3.3 Binding of polyG-functionalized NPs is enhanced in cholesterol-loaded macrophages

Studies were conducted to assess the binding of NP-23pG to cholesterol-loaded macrophages, which model the foam cells found in atherosclerotic plaques. These studies were conducted with RAW264.7 macrophages which have been used extensively as model foam cells.^{16–19} RAW264.7 macrophages were loaded with cholesterol by incubation with AcLDL for 72 hrs at 37°C. Flow cytometry revealed that cholesterol loading caused a ~ 2-fold increase in expression of SR-AI (Fig. 3A). This increase in expression of SR-AI as a result of cholesterol loading produced a 2.5-fold increase in binding of NP-23pG (Fig. 3B). This enhanced SR-AI expression and corresponding increase in NP-23pG binding to AcLDL-loaded macrophages provides additional support for the idea that SR-AI is a viable receptor for NPs designed to target macrophage foam cells.

3.4 polyG-functionalized NPs interact with foam cells in atherosclerotic plaques

To investigate whether polyG-functionalized NPs interact with foam cells in atherosclerotic plaques, we incubated NP-23pG and NP-23pC with atherosclerotic plaques excised from the aorta of ApoE null mice. These plaque tissues were subsequently detached from the aorta wall in order to facilitate NP penetration inside the tissue. This was done since plaques in most mouse model of atherosclerosis, including the ApoE null model, lacks intimal blood vessels²⁰ through which intravenously injected NPs typically permeate inside tissues. Human atherosclerotic plaques, however, are well vascularized²⁰ thereby providing the mode of entry for NPs. The association of functionalized NPs with macrophages in the plaque was assessed by fluorescence co-localization using antibodies against the macrophage marker CD68. NP-23pG (Fig. 4A), but not NP-23pC (Fig. 4B), were retained by atherosclerotic plaques. This accumulation of NP-23pG by plaques was competitively blocked by Ac-LDL in a concentration-dependent manner (Fig. 4C), strongly suggesting that accumulation in plaques is mediated by macrophage SR-AI. This idea is substantiated by the fact that NP-23pG is co-localized with CD68 in atherosclerotic plaques (Fig. 4D–F). These results provide strong support for the conclusion that polyG-functionalized NPs can bind to SR-AI on macrophage foam cells in atherosclerotic plaques.

4. Discussion

The lipid-laden macrophage foam cell has for long been considered a promising target for therapeutic intervention in atherosclerosis.^{21, 22} In the present study we demonstrate the feasibility of scavenger receptor (SR-AI) mediated targeting of foam cells using functionalized NPs. The salient conclusions of this study are: i) NPs functionalized with polyG oligonucleotide are readily bound and internalized by cultured macrophages, ii) NP uptake is mediated by SR-AI expressed on the surface of macrophages, iii) Macrophage foam cells over-express SR-AI when compared to normal macrophages, consistent with previous reports in the literature, and this increased SR-AI expression leads to increased NP binding, and iv) polyG-functionalized NPs can interact with foam cells in plaque tissue.

The SR-AI/polyG receptor/ligand combination we used for targeting NPs to foam cells in plaque has several advantages. SR-AI is the principal receptor involved in the endocytosis of modified LDL and is over-expressed on plaque macrophages.²³ SR-AI mediates rapid internalization of bound ligands,^{23, 24} is repeatedly recycled by the macrophage, and is not down-regulated by ligand binding.^{23, 25} SR-AI has been employed to deliver photosensitizers for thermal ablation of macrophages *in vitro*,^{26, 27} in intimal hyperplasia *in vivo*²⁸ and for targeting of liposomes to macrophages in the organs of body's reticuloendothelial system (RES) such as liver and spleen.²⁹ SR-AI is a pattern recognition receptor for a variety of polyanionic substances, including modified lipids and oligonucleotides such as polyI and polyG that form a quadruplex secondary structure.^{11, 13} PolyG can be easily synthesized, is non-immunogenic,^{30, 31} is stable in serum,³² and is clinically relevant for use as a targeting element. PolyG-mediated targeting of SR-AI, therefore, offers a facile mechanism for delivery of therapeutic agents to macrophage foam cells.

The use of polyG to target NPs differs from many other strategies that primarily target the surface of atherosclerotic plaques.^{33–35} With the ability to deliver particles to macrophage foam cells inside the plaque, one can develop entirely new approaches for treating atherosclerosis. For example, NPs loaded with anti-atherogenic drugs could be used to induce reverse cholesterol transport (RCT) in foam cells as a means of reducing or eradicating plaque.³⁶ This could be accomplished by foam cell-specific delivery of synthetic LXR agonists to promote RCT via LXR activation.^{8, 37} Specific targeting to foam cells would reduce serious side effects such as liver steatosis and hypertriglyceridemia that are associated with systemic administration of such agonists.³⁸ Another strategy might be

reduction of lipid accumulation in foam cells by inhibition of plaque SR-AI activity.³⁹ Inflammatory properties of macrophages could also be reduced by delivery of an anti-inflammatory compound such as dexamethasone.⁴⁰ In addition, because of the long lifespan of macrophages, it may be possible to use MRI to track atherosclerosis progression using NPs loaded with contrast agents²

Our findings conclusively prove the concept that an oligonucleotide ligand for SR-AI can be used to target plaque macrophages. We are mindful, however, that deploying this approach *in vivo* poses additional challenges. In particular, resident RES macrophages also express SR-AI and could therefore clear polyG-functionalized NPs from the circulation. In addition, SR-AI-targeted NPs will have to compete with physiologic ligands for this receptor, such as OxLDL. We anticipate that these hurdles may be overcome by optimizing the affinity of the polyG ligand for SR-AI, and possibly by cloaking this ligand so that it only becomes exposed in the vicinity of foam cells.

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Fig. 1. The levels of scavenger receptor (SR-AI) correlate with the extent of nanoparticle binding and uptake by THP-1 macrophages

A) Analysis of cell surface SR-AI expression using FACS in human THP-1 cells with or without activation by 100 nM PMA; B) Quantification of SR-AI expression following PMA-induced activation. Jurkat cells were used as a negative control. C) Functionalized nanoparticles were incubated with either inactivated or PMA-activated macrophages in vitro at C) 4°C and at D) 37°C. Significance levels at $P < 0.01$ (*); Student *t* test;

Fig. 2. NP-23pG binds specifically to SR-AI on macrophage surface

Macrophages were incubated with 50 $\mu\text{g/mL}$ of NP-23pG and increasing concentrations of AcLDL for 4 hrs at 4°C. AcLDL blocks the binding of NP-23pG to macrophages in a concentration-dependent manner, with 70% inhibition achieved at approximately 75 $\mu\text{g/mL}$.

Fig. 3. polyG-functionalized NPs show enhanced binding to cholesterol-loaded macrophages
 (A) Increase of SR-AI expression on macrophages by AcLDL, as detected by FACS. RAW264.7 mouse macrophages were incubated with AcLDL for 72 hrs at 37°C to develop into model foams cells. APC-labeled SR-AI antibody was used for detection of cell surface SR-AI. Peak A, incubated without AcLDL and stained with APC-labeled mouse IgG. Peak B, incubated without AcLDL and stained with APC-labeled SR-AI antibody. Peak C, incubated with 100 µg/mL AcLDL and stained with APC-labeled SR-AI antibody; (B) The increase in SR-AI expression leads to >2-fold increase in NP-23pG binding to AcLDL-loaded macrophages.

Fig. 4. polyG-functionalized NPs binds to macrophage foam cells in tissue sections of atherosclerotic plaque (ex-vivo)

Confocal images of tissue sections from atherosclerotic plaques incubated with nanoparticles (red). (A) NP-23pG; (B) NP-23pC (C) Acetylated LDL blocks the binding of polyG-NPs to atherosclerotic plaque tissues in a concentration dependent manner. Quantitative analysis is based on six atherosclerotic sections in each group; (D) NP-23pG colocalization with CD68-positive macrophages (NPs are shown in red, macrophages are shown in green). Scale bar = 10 μ m.