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Anti-atherogenic effects of CD36-targeted epigallocatechin gallate-loaded nanoparticles

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Abstract

Intimal macrophages play a critical role in atherosclerotic lesion initiation and progression by taking up oxidized low-density lipoprotein (oxLDL) and promoting inflammatory process. 1-(Palmitoyl)-2-(5-keto-6-octene-diyl) phosphatidylcholine (KODiA-PC), a major type of oxidized phosphatidylcholines (PC) found on oxLDL, has a high binding affinity to the macrophage scavenger receptor CD36 and participates in CD36-mediated recognition and uptake of oxLDL by intimal macrophages. We successfully synthesized epigallocatechin gallate (EGCG)-loaded nanoparticles (Enano), which were composed of EGCG, PC, (+) alpha-tocopherol acetate, and surfactant. We also incorporated KODiA-PC on the surface of Enano to make ligand-coated Enano (L-Enano) to target intimal macrophages. The objectives of this study were to determine the anti-atherogenic effects of Enano and L-Enano in LDL receptor null (LDLr^{-/-}) mice.

Our *in vitro* data demonstrated that L-Enano had a higher binding affinity to mouse peritoneal macrophages than Enano. This high binding affinity was diminished by CD36 antibodies or knockdown of the CD36 receptor in mouse peritoneal macrophages, confirming the specific binding of L-Enano to the macrophage CD36 receptor. LDLr^{-/-} mice were randomly divided to six groups and received weekly tail vein injection with PBS, EGCG, void nanoparticles (Vnano), Enano, ligand-coated Vnano (L-Vnano), or L-Enano once per week for 22 weeks. The dose of EGCG was 25 mg per kg body weight. L-Enano at 20 µg/mL significantly decreased production of

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monocyte chemoattractant protein-1, tumor necrosis factor alpha, and interleukin-6 from mouse macrophages, while having no effect on their plasma levels compared to the PBS control. There were no significant differences in blood lipid profiles among six treatment groups. Mice treated with L-Enano also had significantly smaller lesion surface areas on aortic arches compared to the PBS control. Liver EGCG content was decreased by treatments in the order of EGCG>Enano>L-Enano. Native EGCG had inhibitory effects on liver and body fat accumulation. This molecular target approach signals an important step towards inhibiting atherosclerosis development via targeted delivery of bioactive compounds to intimal macrophages.

Graphical abstract

Illustration of composition, structure, and the targeting and delivery mechanism of CD36-targeted EGCG nanoparticles (L-Enano) to intimal macrophages. Representative images showing inhibitory effects of L-Enano on lesion formation in aortic arches of LDLr^{-/-} mice.

Keywords

Epigallocatechin gallate; nanoparticles; CD36; targeted delivery; macrophages; atherosclerosis

1. Introduction

Intimal macrophages are predominant leukocytes in atherosclerotic lesions and their accumulation positively correlates with atherosclerotic lesion size [1–3]. Intimal macrophages play an important role in atherosclerosis initiation, formation, progression, and atherothrombotic complications by amplifying inflammatory responses and facilitating cholesterol accumulation in the blood vessel wall [4, 5]. After inflammatory monocytes migrate into the arterial intima in response to monocyte chemoattractant protein 1 (MCP-1), these monocytes are differentiated into macrophages. Intimal macrophages release tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6), and other inflammatory factors to amplify inflammatory responses. Macrophage scavenger receptor 1 (MSR1) and CD36 are membrane proteins involved in the uptake of oxidized low-density lipoproteins (oxLDL) as cholesterol influx. Two important macrophage membrane proteins involved in cholesterol efflux are ATP-transporter cassette A1 (ABCA1) and scavenger receptor B class 1 (SR-B1). When cholesterol influx is greater than efflux in intimal macrophages, cholesterol homeostasis is disturbed and cholesteryl ester accumulates in cytoplasmic droplets of these macrophages. The subsequent lipid-laden macrophages are called foam cells, a hallmark of

early stage of atherosclerotic lesions [6]. Targeting intimal macrophages is an approach for atherosclerosis diagnosis, prevention, and treatment.

The CD36 receptor, an integral membrane protein, belongs to class B scavenger receptors, and is the most important receptor in regulating cholesterol influx. CD36 receptor expression correlates with atherosclerotic lesion severity [7–9]. CD36-null mice had a 70% decrease in aortic lesion size, and peritoneal macrophages isolated from the CD36-null mice had a 60–80% reduction in binding and taking up oxLDL [10]. This suggests that macrophage CD36 plays an important role in foam cell and lesion formation.

Oxidized phosphatidylcholines (PCs) on the surface of oxLDL are the ligands facilitating the binding of oxLDL to the macrophage CD36 receptor [11, 12]. Among all oxidized PCs, 1-(palmitoyl)-2-(5-keto-6-octene-diyl) PC (KODiA-PC) has a high binding affinity to the CD36 receptor and participates in uptake of oxLDL by intimal macrophages [11, 12]. We have incorporated KODiA-PC on the surface of liposome-like nanovesicles [13]. Our published *in vitro* data demonstrated that these KODiA-PC-incorporated nanovesicles had a high binding affinity to the macrophage CD36 receptor and increased uptake by both human and mouse macrophages [13]. Our *in vivo* data further validated that KODiA-PC-incorporated nanovesicles targeted aortic lesions in LDL receptor null (LDLR^{-/-}) mice and were co-localized with intimal macrophages and their CD36 receptor [13].

Epigallocatechin gallate (EGCG) accounts for 25 to 55% of total green tea catechins [14]. Apolipoprotein E null mice receiving intraperitoneal injection of 10 mg per kg body weight per day of native EGCG for 21 days had a 55% reduction in cuff-induced evolving lesion size [15]. Apolipoprotein E null mice drinking water supplemented with green tea extract (0.8 g/L), around 37 mg EGCG per kg body weight for 14 weeks had 23% and 27% lower atheromatous area and aortic cholesterol content, respectively, compared to the control apolipoprotein E null mice [16]. Oral administration of 100 mg per day of nanoencapsulated compared to native EGCG in New Zealand white rabbits for 35 days reduced lipid deposition on aortic walls by 2.5 fold [17]. It is well known that EGCG is anti-oxidant and anti-inflammatory agent. Besides that, EGCG interact with plasma membrane phospholipids and protein to regulate intracellular signaling pathways. Meanwhile, EGCG is further transported to intracellular compartments, cytosol, nucleus, lysosome, mitochondria to exert additional biological actions such as transcription factors, DNA methylation, mitochondrial function, signal transduction, and autophagy [18, 19]. EGCG is also suggested to potentially prevent human cardiovascular disease. However, its effectiveness has not been consistently supported by the studies thus far [20, 21]. The primary reasons might be that EGCG is not very stable in water and physiological solutions, has low levels of bioavailability and target specificity, and is easily metabolized by enzymes in liver, kidneys and other tissues. Nanotechnology has emerged to be an ideal solution to all these issues and thus may increase the applicability of EGCG to the atherosclerotic prevention and treatment [22, 23].

Our previous study demonstrated that chitosan-coated nanostructured lipid carriers (CSNLC) loaded with EGCG (CSNLCE) increased EGCG stability. Treating THP-1-derived macrophages with CSNLCE resulted in increased cellular EGCG content, decreased cellular cholesterol accumulation, and reduced monocyte chemoattractant protein-1 (MCP-1)

expression [24]. Since CSNLC and CSNLCE contained a large amount of chitosan (carbohydrate) and triglyceride, administration of these nanocarriers to humans or research animals might increase blood glucose and triglyceride concentrations. Therefore, we removed chitosan, replaced triglyceride with (+) α (α)-tocopherol acetate, and added KODiA-PC as a CD36-targeted ligand in the new formula to make ligand EGCG nanoparticles (L-Enano) for targeted delivery of EGCG to intimal macrophages. We have already demonstrated CD36-targeted L-Enano had significantly higher binding affinity to, and uptake by, human THP-1-derived macrophages than non-targeted Enano [25]. Additionally, CD36-targeted L-Enano dramatically improved EGCG stability, increased EGCG content in THP-1-derived macrophages, decreased mRNA levels and protein secretion of MCP-1 in THP-1 derived macrophages, but did not significantly change cholesterol content in these macrophages [25]. Following the above *in vitro* study, we further moved to *in vivo* counterpart. In the current study, we hypothesized that L-Enano specifically would target macrophages via binding to their CD36 receptor, decrease cellular cholesterol content and inhibit production of inflammatory factors in mouse peritoneal macrophages; L-Enano compared to native EGCG and Enano would reduce atherosclerotic lesion surface area, and decrease circulating concentrations of inflammatory factors in LDLr^{-/-} mice, a commonly used atherosclerosis animal model.

2. Material and methods

2.1. Chemicals and reagents

EGCG (>95%) and KODiA-PC were from Cayman Chemical (Ann Arbor, MI, USA). (+)- α -tocopherol acetate, *E. coli* lipopolysaccharide (LPS), ethylenediaminetetraacetic acid disodium salt (EDTA- Na_2), and protease inhibitor cocktail were all from Sigma-Aldrich Chemical (St. Louis, MO, USA). Surfactant Kolliphor® HS15 was obtained as a gift from BASF Chemical (Florham Park, NJ, USA). Soy PC (purity, >95%) and 7-nitro-2-1, 3-benzoxadiazol-4-yl-phosphatidylcholine (NBD-PC) were from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Nanoparticle preparation and characteristics

Vnano, L-Vnano, Enano, and L-Enano were synthesized, and the formulation screening was conducted to optimize L-Enano components as we previously reported [25]. The optimal formulation by the weight ratio of PC/Kolliphor® HS15/(+)- α -tocopherol acetate was 1:1.25:0.25. Enano was synthesized using 73 mg lipid mixture containing 10% EGCG, 36.2% soy PC, 45% surfactant Kolliphor® HS15, and 8.8% (+)- α -tocopherol acetate (all in weight %). The lipid mixture in ethanol was dried under nitrogen to form a lipid film. Enano was produced by reconstituting the dried lipid film in 10 mL deionized water followed by homogenization (PowerGen 125, Fisher Scientific, Pittsburgh, PA) for 2 minutes and sonication for additional 2 minutes using a Branson Sonifier S-450. L-Enano was made by replacing 30 mol% soy PC with KODiA-PC. Vnano and L-Vnano were synthesized by the same procedures and components without adding EGCG. We measured particle size and polydispersity index of nanoparticles using a BI-MAS particle size analyzer, and their zeta potential using a ZetaPALS analyzer (Brookhaven Corporation, Holtsville, NY, USA). For primary mouse peritoneal macrophage binding and uptake experiments, fluorescence NBD-

PC was incorporated into nanoparticles by replacing 2 mol% total PC to make NBD-labeled nanoparticles.

2.3. *In vitro* binding and uptake

2.3.1. Cell culture and CD36 knockdown—Peritoneal macrophages were elicited and isolated from male C57BL/6J mice (The Jackson's Lab, Bar Harbor, Maine, USA) as previously described [26, 27]. Briefly, on the fifth day before C57BL/6 mice were sacrificed, they were given an intraperitoneal injection of 1.0 mL Brewer thioglycollate broth (4.05g/100mL), to induce an inflammatory reaction and recruit macrophages to the peritoneal cavity. The mice were sacrificed by CO₂ and skin is sterilized with 70% ethanol. Mouse macrophages are collected by peritoneal lavage with 10 mL of cold Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution four times from the peritoneal cavity.

Macrophages were transfected with CD36 siRNA or scramble siRNA using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies) for 48 hours as previously described [13]. The CD36 mRNA expression was measured using real-time PCR. For measuring CD36 protein expression, macrophages were washed with PBS, fixed with ice cold methanol for 10 minutes, incubated with 1% bovine serum albumin (BSA) for 1 hour at room temperature, and stained with RPE-conjugated rat anti-mouse CD36 antibody (1:200) overnight at 4°C in the dark. Cell nuclei were stained with DAPI. After washing, cells were visualized under an EvoS Auto fluorescence microscope and fluorescence intensity was analyzed using the NIH image J software.

2.3.2. Binding and uptake of Vnano and L-Vnano by macrophages—Mouse peritoneal macrophages (5×10^5 /well) in 24-well plates were treated with NBD-labeled Vnano or L-Vnano at 4°C or 37°C for 2 hours for formal binding and uptake assay. Moreover, for competitive binding assay with mouse CD36 antibody, mouse peritoneal macrophages were treated with RPE-labeled anti-CD36 antibody (MCA2748PE, AbD Serotec, Raleigh, NC, USA) in combination with NBD-labeled Vnano or L-Vnano at 4°C for 2 hours.

After incubation, macrophages were washed three times with cold PBS (pH 7.4) and fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 minutes at room temperature in the dark. After washing cells with cold PBS (pH 7.4) three times, cells were treated with ProLong® Gold Antifade Mountant with DAPI at room temperature in the dark. Cells were visualized under an EvoS Auto fluorescence microscope using the same parameters to allow equal comparison of the images as previously described [13].

For binding and uptake assay in CD36 knockdown macrophages, cells were treated with CD36 siRNA, or scrambled siRNA, or none at 37°C for 48 hours to knockdown their CD36. After that, cells were incubated with NBD-labeled Vnano or L-Vnano at 37°C for 2 hours, followed by washing and imaging as mentioned above.

After incubation, macrophages were washed three times with cold PBS (pH 7.4) and fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 minutes at room temperature in the dark. After washing cells with cold PBS (pH 7.4) three times, cells were treated with ProLong®

Gold Antifade Mountant with DAPI at room temperature in the dark. Cells were visualized under an EvoS Auto fluorescence microscope using the same parameters to allow equal comparison of the images as previously described [13].

2.4. Secretion of inflammatory factors from mouse macrophages

Mouse peritoneal macrophages in 24-well plates were pretreated with PBS, native EGCG, Vnano, Enano, L-Vnano and L-Enano in RPMI1640 containing 10% FBS at 37°C for 2 hours and then co-incubated with 100 ng/mL of *E. coli* LPS (Sigma-Aldrich) for additional 16 hours. Stock solution of native EGCG and nanoparticle were dissolved and diluted in PBS. EGCG concentrations were 10 and 20 µg/mL. MCP-1, TNFα, IL-6 concentrations in the culture medium were determined using DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), and total cell protein was measured by a bicinchoninic acid assay (BCA) kit. MCP-1, TNFα, IL-6 concentrations were expressed as ng per mg of cell protein.

2.5. Animal study

Forty-four male 6-week old LDLr^{-/-} mice (Jackson's laboratory) were placed in individual cage at 22–24°C, 45% relative humidity and a daily 12/12 light/dark cycle with the light period from 07:00 to 19:00. Food and water were given *ad libitum*. Food intake was measured by food given minus food left. After 1-week of acclimatization, mice were weighed and randomly assigned into one of the following six treatment groups: PBS (n=11), native EGCG (n=7), Vnano (n=5), Enano (n=7), L-Vnano (n=7) and L-Enano (n=7). The treatments at a dose of 25 mg EGCG per kg body weight or equivalent amount of void nanoparticle or saline controls were given to mice via tail vein injection once per week for 22 weeks. Meanwhile, they were fed with an atherogenic diet (Harlan Teklad, TD.88137) for 22 weeks. Body weight and food intake were recorded weekly. Body composition was measured at week 0, 10 and 22 using an Echo-MRI system (EchoMRI LLC, Houston, TX) according to manufacturer's instructions. Weights of the liver, white adipose tissue (WAT) depots, interscapular brown adipose tissue (BAT) were recorded. The animal protocol was approved by the Animal Care and Use Committee of Texas Tech University.

2.5.1. Quantitation of atherosclerotic lesion of aortic arches—After overnight fast, mice were euthanized with CO₂, and fasting blood was collected from the abdominal vein into EDTA-Na₂ coated tubes. Hearts were perfused *in situ* with 60 mL of diethylpyrocarbonate-treated PBS containing a protease inhibitor cocktail through a cannula inserted into the left ventricle. The protease inhibitor cocktail contains 104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM, 2 mM leupeptin, and 1.5 mM pepstatin. After removing surrounding fat and connective tissues, aortas were isolated from the aortic root to the iliac bifurcation and opened longitudinally under a stereoscopic microscope. The images of pinned aortas were taken under a stereoscopic microscope. The lesion areas on the surface of aortic arches were analyzed using a NIH image J software.

2.5.2. Plasma lipid profile—Blood was collected as mentioned above and plasma was isolated by centrifugation at 1,500 × g at 4°C for 25 minutes. Plasma triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) concentrations were measured by enzymatic methods using an Olympus AU400 analyzer (Olympus, Center

Valley, USA) as previously described [27]. Non HDL-C was calculated as difference between TC and HDL-C, and the TC/HDL-C ratio was also calculated.

2.5.3. Plasma concentrations of inflammatory factors—Plasma TNF α , IL-6 and MCP-1 concentrations were measured using Quantikine® ELISA kits (R&D Systems, Minneapolis, MN) [27].

2.5.4. Liver EGCG extraction—Liver EGCG was extracted as previously described [28]. Briefly, liver (around 500 mg) was homogenized in 2 mL saline. Ethyl gallate (internal standard, 100 ng/mL, 10 μ L) and ascorbic acid-EDTA solution (20% ascorbic acid, 0.1% EDTA, pH3.6), 250 units of β -glucuronidase and 20 units of sulfatase were added to the 200 μ L homogenates. The mixture was incubated at 37°C for 45 minutes to convert EGCG derivatives to native EGCG. After 0.5 mL methylene chloride and 0.3 mL water were added to the mixture, aqueous phase (supernatant) was transferred to a new tube. This step was repeated twice. EGCG was extracted from the aqueous phase using ethyl acetate twice. After centrifugation, organic ethyl acetate was transferred into a new tube, and dried under nitrogen. The extract was reconstituted with water and acetonitrile (95/5, v/v) containing 0.1% formic acid. After centrifugation, the supernatant (50 μ L) was injected into a liquid chromatography–mass spectrometry (LC-MS, Shimadzu instruments, Columbia, MD, USA) [29].

LC-MS was equipped with SHIMAZU C18 reversed-phase column (50 $\times$ 4.6 mm, 5 μ m size). The mobile phase A was composed of water and tetrahydrofuran (995/5, v/v) containing 0.4% formic acid. The mobile phase B was composed of tetrahydrofuran and acetonitrile (10/990, v/v) containing 0.4% formic acid. The gradient elution was based on mobile phase B: 8–15% of B for 0–2.5 minute, 15–50% of B for 2.5–3 minute. The probe voltage was +4.5 kV (ESI Positive mode) and –3.5kV (ESI Negative mode); nebulizing gas flow was 1.5 L/minute; drying gas flow was 20 L/minute; gas temperature was 250°C.

2.5.5. Liver α -tocopherol extraction—Liver tissue (200 mg) was homogenized, followed by saponification by saturated potassium hydroxide. Liver tocopherols were extracted by hexane [30]. The dried samples were reconstituted into methanol followed by injection to a high-performance liquid chromatography (HPLC) system (Waters Co, Milford, MA, USA). HPLC was equipped with a C18 reverse-phase column (150 $\times$ 4.6 mm, 5 μ m size) and fluorescence detector with wavelengths of excitation 295 nm and emission 325 nm. The mobile phase was composed of methanol and water (98/2, v/v) and the flow rate was 0.8 mL/minute.

2.5.6. Complete necropsies and histological evaluation of tissues—Mice were assigned for complete necropsies on the sacrifice day. This included full gross and microscopic evaluation of all organ-systems by a certified veterinarian.

For histological staining and analysis, brain, liver, kidney and muscle tissues were fixed with 10% neutral formalin. After dehydrating, the fixed tissues were embedded in paraffin wax and sectioned in 4 μ m slices, and then placed on clean glass slides and dehydrated in an oven at 60°C for 30 minutes. Prior to staining, the tissue slides were deparaffinized, rehydrated,

and then stained with Masson's trichrome. The tissue slides were mounted with mounting media after air-drying for 24 hours and subsequently processed for histopathological examination under an EVOS® microscope camera system.

2.6. Statistical analysis

Data analysis was conducted using Statistical Package for the Social Sciences (SPSS). In order to compare multiple group means, we conducted one-way ANOVA followed by Tukey HSD test if equal variances assumed or Dunnett's C Post Hoc test if equal variances not assumed. Differences were considered statistically significant at $p < 0.05$. Data in figures and tables are expressed as means $\pm$ standard deviation (S.D.). Bars without a common superscript differ, $p < 0.05$.

3. Results

3.1. Nanoparticle characteristics

The loading capacity of EGCG in Enano and L-Enano was 10% (EGCG/nanoparticle weight, wt%) and encapsulation efficiency was around 95% (encapsulated EGCG/total EGCG, wt%). Nanoparticles were spherical and around 104 nm in diameter. The Zeta potentials were between -20 and -25 mV. Polydispersity index of all nanoparticles was less than 0.2 (Table 1).

3.2. L-Vnano were bound to and taken up by mouse macrophages via their CD36 receptor

NBD-labeled L-Vnano compared to Vnano had higher macrophage binding affinity at both 37°C (Figure 1A) and 4°C (Figure 1B) and higher uptake by macrophages at 37°C (Figure 1A). More Vnano and L-Vnano were bound to and taken up by macrophages at 37°C than at 4°C (Figure 1A&B). For the competitive assay, the binding affinity of L-Vnano to macrophages was significantly decreased when macrophages were co-incubated with CD36 antibody at 4°C (Figure 1C). After CD36 siRNA transfection for 48 hours, mouse peritoneal macrophages had a 82% reduction in CD36 mRNA expression [13] and a 63% decrease in CD36 protein expression (Figure 1D). Scramble siRNA transfection as a negative control did not change CD36 expression (Figure 1D). Control and CD36 negative control macrophages had similar binding and uptake of L-Vnano (Figure 1E). However, CD36 knockdown macrophages bound and took up less NBD-labeled L-Vnano than control or CD36 negative control macrophages (Figure 1E).

3.3. Secretion of inflammatory factors

Enano and L-Enano resulted in the least MCP-1, TNF α , and IL-6 secretion from mouse peritoneal macrophages among all treatments (Figure 2). As compared to native EGCG, Enano and L-Enano at 20 $\mu\text{g/mL}$ of EGCG concentrations significantly decreased IL-6 secretion from mouse peritoneal macrophages (Figure 2). However, there was no significant difference in mRNA levels of MCP-1 and IL-6 among all treatments. Even though Enano and L-Enano compared to the PBS control increased TNF α mRNA levels, but no significant differences were achieved when compared them to native EGCG (Figure S1).

3.4. Cholesterol content in peritoneal macrophages elicited from mice

There was no significant difference in TC, FC and CE content in peritoneal macrophages in mice of all treatment groups (Figure S2). Even though macrophages treated with L-Enano had the lowest TC and FC content, the difference did not reach statistical significance (Figure S2).

There were no significant differences in mRNA levels of CD36, SR-B1, ABCA1 in these peritoneal macrophages among all treatment groups. Even though Vnano and L- Vnano compared to PBS and native EGCG increased MSR1 mRNA levels, but no significant differences were found when compared them to Enano and L-Enano, respectively (Figure S3).

3.5. Food intake, body weight, body composition and tissue weights

After LDLr $-/-$ mice were treated with PBS, EGCG, Vnano, Enano, L-Vnano, or L- Enano, there was no significant difference in food intake among six treatment groups through the 22-week treatment period (Figure 3A). Body weight and body composition were similar among six treatment groups at the baseline (week 0). Compared to the PBS control group, native EGCG and L-Enano groups had significantly lower body weight and body fat percentage at week 10; native EGCG and Enano groups had significantly lower body weight and body fat percentage at week 22 (Figure 3B&C). Furthermore, compared to the PBS control, EGCG and L-Enano groups had significantly higher lean mass percentage at week 10; EGCG and Vnano had significantly higher lean mass percentage at week 22 (Figure 3D). Weights of liver, gonadal WAT, retroperitoneal WAT, perirenal WAT, inguinal WAT, and interscapular BAT did not significantly differ among six treatment groups (Table S1).

3.6. Quantitation of lesion surface areas on aortic arches

The representative inner surface images of aortic arches were shown in Figure 4A [31]. The lesion surface areas of aortic arches were used to quantify atherosclerosis. The average lesion surface area on aortic arches in the PBS control group was 48 mm². The average lesion surface areas on aorta arches in the native EGCG, Vnano, L-Vnano, Enano and L-Enano groups were 44, 42, 40, 38, and 33 mm², respectively. Compared to the PBS control group, only L-Enano-induced decrease reached statistically difference ($p=0.033$) (Figure 4B&C).

3.7. Plasma lipid profile and inflammatory factors

Plasma concentrations of TC, HDL-C, non HDL-C, TG, and the ratios of TC to HDL-C were not significantly different among six treatment groups (Figure 5A).

L-Vnano-treated mice had the lowest plasma MCP-1 and TNF α among six treatment groups. There was no significant difference in plasma IL-6 concentrations among six treatment groups (Figure 5B).

3.8. Liver EGCG and α -tocopherol content

Mice treated with Enano or L-Enano had significantly lower liver EGCG content than mice treated with native EGCG (Figure 6). Even though mice treated with L-Enano had lower

liver EGCG content than mice treated with Enano, but the difference did not reach statistical significance. Since α -tocopherol acetate is a component of all nanoparticles, mice treated with Enano or L-Enano had significantly higher liver α -tocopherol content, but there was no significant difference between these two groups.

3.9. Liver lipid content

The complete necropsies indicated that all mice had hepatosteatosis, and thus we further measured their liver lipid content. Liver fat and TG content did not statistically differ among six treatment groups (Figure S4A&B). Even though EGCG-treated mice had the lowest liver fat and TG content among six treatment groups, no statistical significance was reached compared to any other groups. Enano-treated mice had significantly lower liver cholesterol content than EGCG- and L-Enano-treated mice (Figure S4C).

3.10. Complete necropsies and histological evaluation of tissues

To investigate the safety of EGCG-encapsulated nanoparticles treatments, complete necropsies and histological analysis on brain, liver, kidney and muscle tissues were performed. Besides hepatosteatosis, no other abnormality was found during the complete necropsies. Furthermore, no abnormal histopathological gross lesions were observed in brain, liver, kidneys and skeletal muscle among all treatment groups (Figure 7).

4. Discussion

Intimal macrophages play a central role in the development of atherosclerosis, a pathological change involving inflammation and lipid accumulation in the artery walls [32]. In this study, we successfully employed CD36-targeted nanoparticles to deliver EGCG to intimal macrophages for inhibition of lesion development.

Macrophages express phagocytic macrophage scavenger receptors (MSRs) including MSR1/CD204 and CD36, phosphatidylserine receptors, and others [33]. The following ligands targeting macrophages have been used: 1) peptides targeting MSR1 [34] or peptides that have the function of apolipoprotein A-1 in HDL-C [35]; 2) antibodies against CD204 [36], CD36 [6], and oxLDL [37]; 3) phosphatidylserine that recognizes macrophage phosphatidylserine receptors [38]. However, peptides and antibody ligands may increase immune responses, and peptides are also easily degraded by enzymes in the body [39–41]. To overcome the above problems, we used oxidized PC found in oxLDL as natural ligands to target the macrophage CD36 receptor in this study. Different from macrophages in other tissues and organs, intimal macrophages highly express the CD36 scavenger receptor, which increases their recognition and binding capability for oxLDL [42]. Macrophages take up non-targeted nanoparticles by engulfing them as foreign substances, and bind and take up more targeted than non-targeted nanoparticles by enhanced binding affinity and recognition [32].

Since the macrophage CD36 receptor can recognize and bind to oxLDL, it has been hypothesized that one or more components on oxLDL must be ligand(s) for the CD36 receptor [43, 44]. Given this, all types of lipids from oxLDL have been extracted and reconstituted into microemulsions, using which the authors showed that oxidized PC-

reconstituted microemulsions effectively competed with intact oxLDL to bind macrophages [43, 44]. Oxidized PC on the surface of the plasma membrane of apoptotic cells could facilitate phagocytosis of those cells through binding to the phagocyte CD36 receptor [45]. Oxidized PC is enriched in atherosclerotic lesions of animals [12, 46], and it is the major ligand for oxLDL to bind the macrophage CD36 receptor. KODiA-PC as a keto acid analog is a type of oxidized PC found in oxLDL [12]. Compared to other oxidized PC, KODiA-PC has higher binding affinity to the CD36 receptor and it is the major contributor to oxLDL recognition and uptake by macrophages [11, 12].

In this study, CD36-targeted L-Vnano had a higher binding affinity to mouse peritoneal macrophages than non-targeted Vnano, and its binding affinity was significantly reduced by knocking down macrophage CD36 or co-incubating with CD36 antibody, which indicated that L-Vnano bound to macrophages via their CD36 receptor. These results are consistent with our previous *in vitro* study showing that L-Vnano compared to Vnano had higher binding affinity to human THP-1-derived macrophages, and L-Vnano competed with oxLDL to bind to these macrophages [25]. These results have also confirmed our findings in a previous *in vivo* study that after intravenous injection, KODiA-PC-incorporated nanovesicles accumulated in the lesions and were co-localized with intimal macrophages and their CD36 receptor in the cross-sections of aortas isolated from LDLr^{-/-} mice [13]. In the *ex vivo* experiments of the same study, we found that KODiA-PC-incorporated nanovesicles compared to the control nanovesicles had higher binding affinity to and accumulation in the human atherosclerotic lesions collected from patients undergoing vascular surgery [13]. Together, these results convincingly suggest that KODiA-PC enhanced nanoparticles' target specificity to intimal macrophages via binding to their CD36 receptor. Since more L-Enano target intimal macrophages, their accumulation in the liver was decreased that was indicated by a low EGCG content in the liver in the L-Enano-treated mice.

Some investigators have established macrophage-targeted nanoparticles by loading with several agents to enhance their efficacy in prevention or treatment of atherosclerosis. These agents include statins that decrease blood TC and LDL-C concentrations via inhibiting cholesterol biosynthesis [47], corticoids as anti-inflammatory drugs [48], small interfering RNA (siRNA) that silence inflammation genes such as the C-C chemokine receptor type 2 (CCR2)/monocyte-recruiting receptor [49], and TNF α [50]. Intimal macrophages promote the atherosclerotic pathogenesis from initiation to progression to advanced lesions. In advanced lesions, depletion of macrophages cannot reverse advanced atherosclerosis indicating that macrophages may not be necessary in lesion maintenance of atherosclerosis [51]. Therefore, targeting macrophages may be effective only for atherosclerosis prevention and early treatment. To our knowledge, the present study is the first to show that atherosclerosis formation may be prevented by targeted delivery of EGCG to intimal macrophages using biocompatible and biodegradable L-Enano.

Intimal macrophages and foam cells secrete pro-inflammatory factors, which amplify local inflammatory responses. Macrophage-dense inflammation can make the lesion fibrous cap thin leading to lesion rupture and thrombus formation, which can cause stroke and myocardial infarction [52–55]. Inflammatory factors such as MCP-1, TNF α and IL-6 play a central role in atherosclerotic lesion formation, progression, and its complications. MCP-1

as the most important chemokine binds to its receptor CCR2 on monocytes to recruit and direct

monocyte migration into the artery intima layer where monocytes differentiate into macrophages [32]. Moreover, by secreting more MCP-1 and other inflammatory factors macrophages residing in atherosclerotic lesions recruit more monocytes into the evolving intimal lesion [32]. MCP-1 overexpression is positively associated with intimal monocyte accumulation [56]. In addition, circulating MCP-1 is suggested to be a biomarker of atherosclerosis [57]. TNF α and IL-6 are major pro-inflammatory factors secreted by macrophages/monocytes, adipocytes, endothelial cells, T cells, and other types of cells [58]. They can also stimulate secretion of other inflammatory factors. Circulating TNF α and IL-6 concentrations are positively related with risk of atherosclerosis [58]. Studies have demonstrated that EGCG inhibits inflammatory factor expression and secretion from different types of cells through upregulation of peroxisome proliferator-activated receptor gamma and downregulation of p38 mitogen-activated protein kinases and nuclear factor-kappa B [59–62]. In this study, we found that Enano and L-Enano decreased secretion of MCP-1, TNF α and IL-6 from mouse peritoneal macrophages. There was no significant difference between Enano and L-Enano. It might be due to the high EGCG dose (>20 μ M), which is higher than the physiological circulating EGCG concentration (<10 μ M) [63–65]. Although not as potent as L-Enano and Enano, Vnano and L-Vnano also reduced secretion of MCP-1, TNF α and IL-6 to a certain extent. Since nanoparticles contain (+)- α -tocopherol acetate, which has anti-inflammatory capacity [66], this might be the possible reason for inhibitory effects of Vnano and L-Vnano on inflammation. The extent of inhibition was increased by the treatments in the order of Vnano, L-Vnano, native EGCG<Enano<L-Enano, except for IL-6 at 10 μ g/mL treatment, in which L-Enano<Enano. In our previous study, L-Enano enhanced EGCG delivery to human THP-1 derived macrophages and significantly decreased MCP-1 secretion [25]. Some studies demonstrated that EGCG administration decreased circulating inflammatory factors in LDLr $^{-/-}$ mice [67] and the other animal models [68]. However, in this *in vivo* study, there was no significant difference in plasma concentrations of MCP-1, TNF α and IL-6 among six treatment groups. This might be due to dermatitis developed in some LDLr $^{-/-}$ mice that induces their inflammatory response thus masking the differences. Such a problem has been reported by other investigators [69, 70].

EGCG has a potential to prevent atherosclerotic lesion development. However, the evidence is inconclusive about effectiveness of EGCG treatment in humans [21, 71]. The major problems are its low levels of stability, bioavailability, and target specificity. Our previous studies have indicated that nanoencapsulation can increase EGCG stability and cellular uptake. Several studies in mice and rabbits demonstrated that native EGCG and nanoencapsulated EGCG had anti-atherogenic activities [15–17]. But these studies did not target atherosclerotic lesions. In this study, compared to PBS, only L-Enano significantly decreased lesion surface areas of aortic arches by about 30%. The Enano-treated mice had the second lowest lesion areas on aortic arches but it did not reach a significant difference compared to the PBS control. The results indicated that L-Enano might prevent atherosclerotic lesion formation via targeted delivery of EGCG to intimal macrophages.

Liver is the major organ to accumulate and metabolize EGCG via methylation, sulfation, and glucuronidation [72]. Oral EGCG is mainly absorbed by the small intestine, metabolized by the liver, and excreted into urine by kidneys and feces by the colon in animal and human studies [29, 72]. After single oral administration of 50–2,000 mg EGCG per kg body weight in mice, liver EGCG absorption after 50 or 180 minutes has a linear-dose relationship without plateau, achieving 0.09–18.3 μg EGCG per g liver [73]. After single intravenous administration of 25 mg radioactive EGCG per kg body weight to dogs, the liver had the highest EGCG concentrations among all tissues [74]. Similarly, in our unpublished work, the liver had the strongest fluorescence signal after fluorescence dye-labeled nanoparticles were given to LDLr^{-/-} mice by intravenous injection. In this study, the native EGCG-treated mice had the 3-fold higher liver EGCG content than Enano- and L-Enano- treated mice, which may be due to sustained release of low doses of EGCG from Enano and L-Enano, prolonged circulation time, and targeted delivery of L-Enano to intimal macrophages. In our previously published paper, we demonstrated that L-Enano had sustained release of EGCG for 20 hours, but native EGCG was released completely from dialysis bags within 1 hour *in vitro* [24, 25, 75]. The surfactant Kolliphor® HS15 contains 30% of free polyethylene glycol (PEG), which may help Enano and L-Enano avoid rapid clearance by the liver and other tissues to achieve prolonged circulation time [76]. Even though Enano and L-Enano had a long circulation time and prolonged release, majority of Enano and L-Enano were still accumulated in the liver. Since L-Enano targeted intimal macrophages, L-Enano compared to native EGCG and Enano had less uptake by the liver.

Obesity is characterized by pathologically excessive adipose tissue mass and expansion. Adipose tissue is a critical energy storage and endocrine organ, which is very important to energy balance and homeostasis of metabolism and inflammation. Both cellular and animal studies showed beneficial effects of EGCG and green tea extract for combating obesity [77]. Most of animal studies used oral administration of EGCG at a dose range 2–160 mg per kg body weight daily, which is a common route in rodents. Intravenous administration is challenging, especially long-term injection. Our Enano and L-Enano in the gastrointestinal tract might be completely digested and degraded. In order to ensure the intact nanoparticles in the circulation system, we chose intravenous administration. In this study, we demonstrated that native EGCG significantly lowered body weight and percentage of body fat of LDLr^{-/-} mice. In addition, native EGCG-treated mice compared to the mice treated with PBS, Enano or L-Enano had lower weights of gonadal, perirenal, inguinal white adipose tissue. Native EGCG in the adipose tissue can decrease adipogenesis and lipogenesis and inflammation, subsequently result in body weight and fat loss [77]. As compared to Enano- and L-Enano-treated mice, native EGCG-treated mice had the highest liver EGCG content, which was associated with the lowest hepatic fat and TG content. The reasons may be that native EGCG can be quickly taken up by and accumulate in the liver to inhibit lipogenesis.

5. Conclusions

Taken together, L-Enano targeted macrophages via their CD36 receptor, lowered secretion of inflammatory factors in mouse peritoneal macrophages, and decreased the lesion surface areas of aortic arches in LDLr^{-/-} mice. CD36-targeted L-Enano had more potent anti-

atherogenic activities than native EGCG and Enano. Native EGCG compared to Enano and L-Enano had a higher accumulation in liver and stronger inhibitory effects on liver and body fat accumulation. This molecular target approach signals an important step towards inhibiting atherosclerosis development via targeted delivery of bioactive compounds to intimal macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Targeted delivery of epigallocatechin gallate (EGCG) to intimal macrophages can prevent atherosclerosis.
- The targeting ligand is a type of natural oxidized phosphatidylcholines found on oxidized low density lipoprotein (LDL).
- Targeted nanoparticles have a high binding affinity to the macrophage scavenger receptor CD36.
- Atherosclerotic lesions were significantly decreased after animals given intravenous injection of targeted nanoparticles.

Figure 1.

The binding and uptake of NBD-labeled Vnano and L-Vnano by mouse peritoneal macrophages via their CD36 receptor. A, Mouse peritoneal macrophages were treated with non-targeted Vnano or targeted L-Vnano at 37°C; B, Mouse peritoneal macrophages were treated with Vnano or L-Vnano at 4°C; C, Mouse peritoneal macrophages were treated with red RPE-labeled anti-mouse CD36 antibody (λ of excitation is 496 nm, λ of emission is 578 nm) in combination with Vnano or L-Vnano at 4°C; D, Mouse peritoneal macrophages were transfected without CD36 siRNA (control) or with scramble siRNA (negative control) or with CD36 siRNA (CD36 knockdown). CD36 siRNA transfection decreased CD36 protein

expression in mouse peritoneal macrophages; E, The binding and uptake of L-Vnano by control, CD36 negative control or CD36 knockdown mouse peritoneal macrophages. Bar length was 200 μm . NBD-labeled Vnano and L-Vnano were green (λ of excitation is 460 nm; λ of emission is 535 nm). Cell nuclei were stained by DAPI (λ of excitation is 358 nm, λ of emission is 461 nm) (blue color). Images were representatives of three independent experiments.

Figure 2.

Secretion of inflammatory factors from mouse peritoneal macrophages. Mouse peritoneal macrophages were pretreated with PBS, native EGCG, Vnano, Enano, L-Vnano or L-Enano at EGCG dose of 10 μg/mL and 20 μg/mL at 37°C for 2 hours and then co- incubated with LPS for additional 16 hours. Values are means ± S.D. Bars without a common superscript differ, $p < 0.05$. At least, three independent experiments were conducted.

Figure 3.

Food intake, body weight and body composition of mice. A, Food intake through the 22-week period; B, Body weight at week 0, 10 and 22; C, Fat %: Body fat mass/body weight at week 0, 10 and 22; D, Lean mass %: lean mass/body weight. Values are means $\pm$ S.D. Bars without a common superscript differ, $p < 0.05$. N=5–11.

Figure 4.

A, Representative imaging of aortas from PBS, EGCG and L-Enano groups. White areas denote atherosclerotic lesions; B, Average lesion surface area of aortic arches isolated from LDLr^{-/-} mice fed with the atherogenic diet and treated with PBS, EGCG, Vnano, Enano, L-Vnano and L-Enano at EGCG dose of 25 mg per kg body weight once per week for 22 weeks via intravenous injection. Values are means $\pm$ S.D. Bars without a common superscript differ, $p < 0.05$; C, Each black dot denotes lesion surface areas of each mouse and red lines denoted the average lesion areas with standard deviation. N=5-11.

Figure 5. Plasma lipid profiles (A) and plasma concentrations of inflammatory factors (B) in LDLr^{-/-} mice. Values are means $\pm$ S.D. Bars without a common superscript differ, $p < 0.05$. N=5–11.

Figure 6. EGCG (A) and α -tocopherol (B) content in the liver isolated from LDLr^{-/-} mice. Values are means $\pm$ S.D. Bars without a common superscript differ, $p < 0.05$. N=5–11.

Figure 7.

The representative Masson's trichrome histological images of the brain, liver, kidney and muscle isolated from LDLr^{-/-} mice. N=5-11.

Table 1.

Particle size, zeta potential, and polydispersity index of nanoparticles.

Name	Vnano	Enano	L-Vnano	L-Enano
Particle size (nm)	103±7.0	101±9.1	104±2.7	106±2.5
Zeta potential (mV)	-24.90±3.00	-23.01±4.49	-20.64±0.76	-20.60±0.42
Polydispersity	0.16±0.10	0.19±0.01	0.16±0.07	0.19±0.01

Values are means ± S.D. Three independent experiments were conducted.