Measurement of Aortic Cell Fluid-Phase Pinocytosis in vivo by Flow Cytometry

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Keywords

Fluid-phase pinocytosis · Macropinocytosis · Micropinocytosis · Macrophage · Flow cytometry · Atherosclerosis · Aorta

Abstract

Objective: Fluid-phase pinocytosis is a receptor-independent mechanism of endocytosis that occurs in all mammalian cells and may be a mechanism for the uptake of LDL by macrophages. As there are currently no methods for the measurement of fluid-phase pinocytosis by individual aortic cells in vivo, we sought to identify a suitable method. **Methods:** ApoE^{-/-} mice were retro-orbitally injected with AngioSPARK fluorescent nanoparticles specifically designed to not interact with cells. After 24 h, mice were sacrificed, and the aortas were isolated and then digested to analyze aortic cell uptake of AngioSPARK by flow cytometry. **Results:** CD11b-expressing aortic macrophages from mice injected with AngioSPARK showed high levels of fluid-phase pinocytosis compared to aortic cells not expressing CD11b (4,393.7 vs. 408.3 mean fluorescence intensity [MFI], respectively). **Conclusion:** This new technique allows for the measurement of fluid-phase pinocytosis by aortic cells in vivo, making it possible to examine the cell-signaling molecules and drugs that affect this process.

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Introduction

Endocytosis is an essential process in mammalian cells that can occur by receptor-mediated endocytosis or, independently of receptors, by fluid-phase pinocytosis. Delivery of cargo into cells by receptor-mediated endocytosis occurs by solutes binding to cell-surface receptors whereas fluid-phase pinocytosis occurs independently of receptors by the nonspecific uptake of solutes [1]. Fluid-phase pinocytosis is an important mechanism for the delivery of foreign molecules into antigen-presenting cells and for pathogen access inside the cell for survival [2, 3]. In addition to these processes, we recently identified fluid-phase pinocytosis as an important mechanism of LDL uptake by macrophages, a process that likely contributes to cholesterol accumulation in atherosclerotic plaques [4–9].

There are currently no suitable methods to quantitatively measure the fluid-phase pinocytosis of individual cells in vivo. Although fluorescent dextrans have been extensively used as fluid-phase pinocytosis tracers in vitro and in vivo, these molecules can bind to mannose receptors and be endocytosed by receptor-mediated endocytosis [10]. We recently utilized fluorescent nanoparticles, specifically designed not to interact with cell-surface molecules, as tracers to measure the fluid-phase pinocytosis

of whole aortas of atherosclerosis-prone mice [6]. While we were able to measure fluorescent nanoparticle accumulation in tissue by near-infrared fluorescence imaging, this method does not allow for the measurement of fluid-phase pinocytosis by individual cells.

Seeking to identify drug targets that inhibit the macrophage fluid-phase pinocytosis of LDL in vivo [11], we developed an in vivo technique to measure the fluid-phase pinocytosis of individual aortic macrophages by injecting atherosclerotic ApoE^{-/-} mice with AngioSPARK fluorescent nanoparticles. AngioSPARK nanoparticles are 20- to 50-nm nanoparticles, similar in size to LDL (22 nm), that are specifically designed for the in vivo imaging of mice. Due to pegylation, AngioSPARK nanoparticles display minimal interaction with cell-surface molecules and have an extended half-life in vivo (14 h) [12]. Using AngioSPARK nanoparticles and the macrophage marker CD11b, we describe a new flow cytometry technique that will allow for the in vivo measurement of fluid-phase pinocytosis by individual aortic macrophages and be useful for identifying the cellular mediators of fluid-phase pinocytosis.

Methods

AngioSPARK Injection of Mice

Ten-month-old apoE^{-/-} male mice were weighed, and then the volume of AngioSPARK 680 (PerkinElmer) to be injected was calculated. Each mouse was anesthetized with isoflurane and then injected retro-orbitally with 0.125 nmol AngioSPARK/kg of body weight (BW), or an equivalent volume of vehicle (PBS) using a 28-gauge insulin syringe. After 24 h, mice were anesthetized with 2.5% Avertin (0.015 mL/g BW, i.p.) and then euthanized by exsanguination. All animal procedures were performed according to a research protocol approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (NHLBI).

Aorta Isolation and Antibody Labeling of Cells

The inferior vena cava was cut and the vasculature was then immediately perfused with 30 mL of PBS using a 25-gauge needle injected into the left ventricle. The aortic adventitia was gently removed using a stereomicroscope, and then the aorta (from the heart to the iliac arteries) was removed and incubated at 37°C for 1 h in PBS with Ca²⁺ and Mg²⁺ and 20 mM HEPES containing 125 U/mL collagenase XI, 450 U/mL collagenase I, 60 U/mL DNase I (all from Sigma), 60 U/mL hyaluronidase, 100 U/mL elastase, and 1 mg/mL soybean trypsin inhibitor (all from Worthington). Each sample was triturated 4 separate times with a 1-mL pipette during the 1-h incubation. The digested aortas were then mashed through a 30-µm (pore-size) filter and centrifuged for 10 min at 200 g in 1.5-mL microfuge tubes. Supernatants were removed and the cell pellets were reconstituted with 1 µL of Fc block (AbD Serotec) and 89 µL of PBS containing 0.1% BSA and 2 mM EDTA for 10 min on ice. One million cells were placed into separate tubes

Table 1. AngioSPARK uptake by aortic cells

Cell type	Percentage positive		MFI	
	vehicle	AngioSPARK	vehicle	AngioSPARK
CD11b ⁻	0.8±0.7	7.7±0.3 ^a	52.8±1.6	408.3±115.6 ^b
CD11b ⁺	4.2±1.8	47.8±6.0 ^a	347.3±54.4	4,393.7±1,322.3 ^b

Data are presented as mean ± SEM of 3 independent experiments. Paired one-tailed *t* tests were performed to determine statistical significance values. ^a *p* = 0.01. ^b *p* = 0.05.

and incubated with mouse anti-human CD11b or isotype control for anti-CD11b. After a 30-min incubation at 4°C, samples were centrifuged for 10 min at 200 g and 4°C. Cell pellets were reconstituted in 0.5 mL PBS and then analyzed by flow cytometry.

Flow Cytometry

Flow cytometry was performed with a Becton Dickinson LSR II using the 488-nm laser for CD11b and the 633-nm laser for AngioSPARK. Compensation was performed using unstained and stained cells during data acquisition. A final compensation matrix was calculated after data acquisition using FlowJo software v8.8 (Tree Star, Inc., OR, USA). Gating of forward- and side-scatter was used to eliminate small debris from the analysis, CD11b⁺ and CD11b⁻ cells were identified, and AngioSPARK uptake was then assessed for CD11b⁺ and CD11b⁻ cells. Isotype control antibodies and control vehicle injections were used to set gates to define positive immunofluorescence. Data was analyzed using FlowJo v8.8.

Results

We first assessed the percentage of CD11b⁺ cells in the aortas of apoE^{-/-} mice. CD11b⁺ cells represented 10.4% of all aortic cells (Fig. 1a). We next assessed aortic-cell fluid-phase pinocytosis in 3 independent experiments by injecting apoE^{-/-} mice with AngioSPARK nanoparticles. CD11b⁺ cells from the aortas of AngioSPARK-injected mice showed a high level of AngioSPARK uptake as measured by mean fluorescence intensity (MFI) compared with the vehicle-injected mice (4393.7 vs. 347.3 MFI, respectively) (Table 1; Fig. 1c). CD11b⁻ cells also showed an increase in AngioSPARK uptake (408.3 vs. 52.8 MFI, respectively), although the absolute level of AngioSPARK uptake by CD11b⁻ cells was substantially less than in CD11b⁺ cells (Table 1; Fig. 1b). AngioSPARK uptake was not examined in wild-type mice because atherosclerosis does not occur [13] and aortic CD11b⁺ cells are not present in the intima [14] in these mice. The data show that CD11b⁺ cells from the aortas of atherosclerosis-prone

Fig. 1. AngioSPARK uptake by aortic cells. **a** Aortic cells were examined for CD11b expression by flow cytometry. CD11b⁻ (**b**) and CD11b⁺ (**c**) cells were gated from (**a**) and AngioSPARK fluorescence was measured for apoE^{-/-} mice injected with vehicle (PBS) or AngioSPARK. Results are representative of 3 independent experiments.

mice have high levels of fluid-phase pinocytosis when compared with CD11b⁻ cells, consistent with the high levels of LDL uptake by macrophages that is observed in atherosclerotic plaques [15].

Discussion

We have described a technique to measure in vivo fluid-phase pinocytosis of individual cells by flow cytometry. The AngioSPARK nanoparticles utilized in this study were pegylated to prevent interaction with the components of the cell surface, and were thus unable to enter cells by receptor-mediated endocytosis [6].

Our data show high levels of AngioSPARK uptake by mononuclear phagocytic cells within the aortas in vivo, similar to the LDL uptake by aortic mononuclear phagocytic cells [7–9, 15]. We demonstrate that CD11b⁺ cells from murine atherosclerotic aortas showed high levels of AngioSPARK uptake compared with CD11b⁻ cells. As CD11b is a widely utilized marker of macrophages [16] and AngioSPARK nanoparticles are taken up by murine macrophage fluid-phase pinocytosis in vitro [6], these data indicate that most AngioSPARK fluid-phase pinocytotic

uptake by aortic cells in vivo occurs in the macrophages, but not in other cells of the mouse aorta (which is mainly composed of smooth muscle cells).

Monocyte-derived arterial cells express markers associated with macrophages and dendritic cells. Almost all monocyte-derived arterial foam cells in the mouse express both CD11b and a commonly used dendritic marker, CD11c [17]. We previously showed that CD11c is expressed on cultured GM-CSF-differentiated murine bone marrow-derived macrophages that showed an adherent, vesicular phenotype and an accumulation of high levels of LDL-derived cholesterol by fluid-phase pinocytosis, all features associated with a macrophage phenotype [7]. It is unclear if myeloid-derived arterial cells are a mixture of macrophages and dendritic cells or are the same population of cells; this is due to the inability to distinguish these 2 cell types using these markers [18]. The commonly used macrophage markers CD68 and Moma-2 are intracellular markers that require cell permeabilization, which we previously found causes AngioSPARK nanoparticles to leak out of cells (unpubl. observ.). F4/80 is another commonly used macrophage marker, but it is only expressed on some CD11b-expressing cells [19]. We were therefore unable to use other commonly used macrophage markers.

Fluid-phase pinocytosis can occur by the formation of micropinosomes that are <100 nm in diameter and macropinosomes that are usually >200 nm in diameter [20, 21]. As AngioSPARK nanoparticles are 20–50 nm in size, they can be taken up by both micropinocytosis and macropinocytosis. Thus, cellular uptake of AngioSPARK represents total fluid-phase pinocytosis levels (i.e., both micropinocytosis and macropinocytosis). There are currently no fluid-phase pinocytosis fluorescent probes that do not interact with cells and are large enough to be taken up only by fluid-phase macropinocytosis. High-molecular-weight fluorescently-labeled dextrans are commonly used as fluid-phase macropinocytosis tracers. However, these molecules can interact with cell-surface receptors and are not large enough to be excluded from fluid-phase micropinocytosis [10, 22].

Even though we did not examine AngioSPARK nanoparticle transmigration into the intimal layer, previous studies show that most LDL enters the intima by endothelial-cell fluid-phase transcytosis [23, 24]. Due to the size similarity of LDL and AngioSPARK and the inability of AngioSPARK to bind to cellular components, AngioSPARK nanoparticles likely enter the intima primarily through endothelial cell transcytosis, similar to LDL. Entry of AngioSPARK nanoparticles into the intima is un-

likely to occur between endothelial cells because particles of this size cannot diffuse through the tight junctions.

Our data demonstrate a novel method to measure fluid-phase pinocytosis by individual aortic cells in vivo. This technique can also be adapted for cells from other tissues that were not assessed in this study, as long as single-cell suspensions can be generated for interrogation by flow cytometry. As fluid-phase pinocytosis is an important mechanism for LDL uptake [25], the entry of pathogenic organisms [26], and the uptake of nutrients by cancer cells [27], this method will allow for in vivo examination of fluid-phase pinocytosis by relevant cells and drug targeting of these cells to limit fluid-phase pinocytosis.

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Disclosure Statement

The authors declare no conflicts of interest.

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