Biochimica et Biophysica Acta 1861 (2017) 58–67

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Quantitative analysis of the role of fiber length on phagocytosis and inflammatory response by alveolar macrophages

Trudy Padmore [a], Carahline Stark [a], Leonid A. Turkevich [b], Julie A. Champion [a,*]

[a] Chemical & Biomolecular Engineering, Georgia Institute of Technology, United States
[b] CEMB/DART/NIOSH/CDC, United States

ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form 16 July 2016
Accepted 20 September 2016
Available online 23 October 2016

Keywords:
Macrophage
Glass fibers
Length
Frustrated phagocytosis
TNF-α: Tumor necrosis factor-α

ABSTRACT

Background: In the lung, macrophages attempt to engulf inhaled high aspect ratio pathogenic materials, secreting inflammatory molecules in the process. The inability of macrophages to remove these materials leads to chronic inflammation and disease. How the biophysical and biochemical mechanisms of these effects are influenced by fiber length remains undetermined. This study evaluates the role of fiber length on phagocytosis and molecular inflammatory responses to non-cytotoxic fibers, enabling development of quantitative length-based models.
Methods: Murine alveolar macrophages were exposed to short and long populations of JM-100 glass fibers, produced by successive sedimentation and repeated crushing, respectively. Interactions between fibers and macrophages were observed using time-lapse video microscopy, and quantified by flow cytometry. Inflammatory biomolecules (TNF-α, IL-1α, COX-2, PGE_2) were measured.
Results: Uptake of short fibers occurred more readily than for long, but long fibers were more potent stimulators of inflammatory molecules. Stimulation resulted in dose-dependent secretion of inflammatory biomolecules but no cytotoxicity or strong ROS production. Linear cytokine dose-response curves evaluated with length-dependent potency models, using measured fiber length distributions, resulted in identification of critical fiber lengths that cause frustrated phagocytosis and increased inflammatory biomolecule production.
Conclusion: Short fibers played a minor role in the inflammatory response compared to long fibers. The critical lengths at which frustrated phagocytosis occurs can be quantified by fitting dose-response curves to fiber distribution data.
General significance: The single physical parameter of length can be used to directly assess the contributions of length against other physicochemical fiber properties to disease endpoints.
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1. Introduction

Phagocytosis by macrophages is critical in the degradation and clearance of pathogenic materials in the body [1]. High aspect ratio materials such as asbestos fibers or carbon nanotubes can be cleared by phagocytosis or persist and induce frustrated phagocytic interactions leading to chronic inflammation, oxidative stress, direct cell injury, and chromosomal abnormalities [2–5]. This evasion can lead to diseases such as fibrosis, asbestosis, lung cancer, mesothelioma for asbestos-like materials, and carbon nanotubes have recently been shown to induce asbestos-like chronic inflammation. In this work we define frustrated phagocytosis as the failure to engulf after attaching, spreading and manipulation of fibers by macrophages. The extent to which high aspect ratio materials evade clearance is strongly length-dependent, both *in vivo* and *in vitro*[6–17]. However, there is no consensus about a critical length beyond which materials persist, as these studies are confounded by other material physicochemical properties such as diameter and surface chemistry, or by cell type and location within the body.

For asbestos specifically, comparative study of length-based contributions between different types remains challenging since they possess different physicochemical properties and lead to varied disease endpoints and health outcomes, ranging from cancerous lesions to genotoxicity [18–21]. *In vivo* study of the role of fiber length on disease endpoints is further complicated by other length-dependent processes, which can obscure any correlation of residual fibers with the disease endpoint. *In situ* fiber breakage reduces the population of long fibers and increases the population of short fibers [22]. *In situ* dissolution reduces fiber diameter, which may then lead to additional breakage [23, 24]. Phagocytosis removes shorter fibers, changing the length distribution over time. Translocation reduces the fiber population at the deposition site and, together with all clearance mechanisms, may have an efficiency that depends on fiber length [25]. It is thus difficult to

* Corresponding author.
E-mail address: julie.champion@chbe.gatech.edu (J.A. Champion).

http://dx.doi.org/10.1016/j.bbagen.2016.09.031
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associate unambiguously fibers recovered in pathology with those that have induced disease. In *in vitro* experiments, after an induction time, macrophages will successfully engulf short fibers, and we lose the information of whether the short-fiber/cell interaction differs from the long-fiber/cell interaction. Therefore, we need to monitor cell-fiber interactions on short timescales following initial contact. An *in vitro* model that quantitatively captures all length-based contributions to the cellular response is critical to understanding pathogenic mechanisms.

A challenge in studying the effect of fiber properties in biological systems is the difficulty in obtaining fiber samples with well-controlled physical properties. The Baron dielectrophoretic classifier enabled earlier studies with length-separated glass fibers [15,16], while the use of JM-100 model glass fibers decouples fiber length from surface chemistry. Blake et al. [15] and Ye et al. [16] revealed a length-dependent cytotoxicity and induction of inflammatory cytokines after exposure of alveolar macrophages to glass fibers of varied lengths *in vitro*. However, while the Baron classifier can prepare short fibers with a narrow distribution of lengths, the long fibers are inherently broad in their length distribution. Without characterization of long fiber length distributions, it is not possible to attribute a critical fiber length to the cellular responses reported.

Here we present a quantitative assessment of phagocytic and inflammatory responses of MH-S murine alveolar macrophages to long and short populations of JM-100 glass fibers with well-characterized fiber distributions. Parameterization of the length distributions enabled the development of models that propose critical lengths for varied phagocytic interactions between fibers and cells. These length-dependent interactions were captured by time-lapse microscopy and flow cytometry. Production of inflammatory biomolecules, tumor necrosis factor α (TNF-α), Interleukin-1 α (IL-1α), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE_2), was quantified after macrophage exposure to short and long glass fiber populations. These pathological hallmarks are evidence of macrophage activation and fiber-induced inflammatory signaling [17]. We used the dose-response curves of the directly-stimulated cytokines (TNF-α, IL-1α) to identify critical fiber lengths that increase inflammatory biomolecule production in macrophages during frustrated phagocytosis of long fibers.

2. Materials and Methods

2.1. Fiber sample preparation

Fibers were prepared from a Pall glass fiber depth filter sheet, type AE binder free (Pall Life Sciences, Ann Arbor, MI, available as SKC no. 225-7-07, SKC Inc., Eighty Four, PA). This media consisted of entangled uncoated borosilicate glass fibers (of nominal diameters 0.1 μm < d < 10 μm), designed to retain 1-μm particles on liquid filtration. Batches of 24 sheets were cut out to fit into a 1¼″ die cavity and crushed with a lab press for 60 s. Short fibers were obtained by crushing at 10 tons and re-crushed at 15 tons, while long fibers were crushed at 2 tons. Each batch yields ~1.3 g of fiber [26].

Individual fibers were liberated from the residual fibrous mat after crushing through suspension in 500 mL of DI water and sonication (Fisher Scientific Sonic Dismembrator Model 500 with ½″ horn), at 50% amplitude (*i.e.* tip amplitude ~76 μm), for 30 min (1 s on, 1 s off). This procedure was followed to prepare the stock suspension of short fibers (no sedimentation step). The long fiber samples were allowed to gravitationally settle for 20 min, with the resulting supernatant decanted. The sediment was re-suspended in 500 mL and sonicated as described above. This sonication, settling, decanting, re-suspension procedure was iterated 10 times; the 10th sediment constituted the long fiber sample. All samples were prepared for diameter measurement by vacuum filtering 1 mL of a 1000:1 dilution through a 0.8 μm nitrocellulose filter (Millipore AAWP 02500); deposition is nominally ~1 $\mu g/cm^2$.

2.2. Fiber length measurement

All fiber samples were subjected to a final filtration through a 35 μm mesh to separate entangled fibers before length measurement and exposure to macrophages. Fibers were imaged (see Time-Lapse Video Microscopy section) on an incubation stage of an Axio Observer Z1 inverted light microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) and observed using differential interference contrast at 100× magnification. This imaging differs from the typical phase contrast microscopy analysis of fibers collected on acetone cleared MCE filters [27,28].

The length of the fibers was measured, using the line tool from Motic Images Plus 2.0 ML (Motic Group, Richmond, BC, Canada); faint fibers were identified with the aid of the magnification tool at 200% magnification. Only fibers entirely contained within the field of view were included for length measurement; this restriction actually biases the measured length distribution against the longer fibers, but since the dimensions of the field of view (220 μm × 170 μm) are quite large compared to almost all of the measured fibers, this distortion was neglected (Turkevich, unpublished). At this magnification, 1 μm represents the image resolution limit.

2.3. Fiber diameter measurement

Fibers were imaged with scanning electron microscopy (SEM) to analyze fiber diameter. The nitrocellulose filters were mounted on 25 mm planchettes or stubs, using colloidal graphite adhesive, and were sputter coated with gold, to prevent charging by the electron beam. The samples were analyzed using a Hitachi S3000N scanning electron microscope. Secondary electron images were obtained at an accelerating voltage of 25 keV. Images were taken at 800× and 4000×. The length and diameter of the fibers were again measured using the line tool from Motic Images Plus 2.0 ML. At 800×, fiber diameter quantitation was not possible below 0.25 μm; at 4000×, fiber diameter quantitation was not possible below 0.15 μm.

2.4. Fiber count

Serial dilutions of suspended fibers were counted using a haemocytometer mounted on a light microscope at 40× magnification; fiber counts were accepted when the difference in count among serial dilutions was <5%. Short fiber counts were verified by an Accuri C6 flow cytometer (Becton Dickinson). Dose-response experiments were reported as a function of optically detected (40× mag) fibers/cell.

2.5. Fiber labeling with fluorescent probe

Approximately 1.5 mg of glass fibers were suspended in 1 mL of 1 M KOH by sonication (pulse mode; 4 s on, 2 s off; 30% amplitude; 2 min total process time) and incubated for one hour. Fibers were washed with 1 mL deionized water (18.3 MΩ·cm at 25 °C) followed by a wash with 1 mL ethanol. Washes consisted of centrifuging the fibers at 125 ×*g* for 5 min, 2400 ×*g* for 10 min, and 21,000 ×*g* for 1 min. To maximize fiber retention while minimizing breakage of fibers, the fiber pellet was retained after each centrifugation step and only the supernatant was centrifuged in the next step. Fibers were dried in an oven at 37 °C for 1.5 h. Fibers were then incubated for 2 min in a solution of 1 mL toluene and 33 μL 3-mercaptopropyl trimethoxysilane, washed with excess toluene to remove unconjugated silane, and suspended in 1 mL of 20 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic (TES) sodium salt buffer solution. 15 μl of 20 mM of 5-iodoacetamidofluorescein (5-IAF) in dimethyl formamide (DMF) was added. The reaction was allowed to proceed in the dark for 2 h at 4 °C under constant stirring. Fibers were washed twice in deionized water by centrifugation at 125 ×*g* for 5 min, 2400 ×*g* for 10 min, and 21,000 ×*g* for 1 min to remove unreacted reagents before exposure to cells.

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