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Research Article

Particle size influences fibronectin internalization and degradation by fibroblasts

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ABSTRACT

The application of nanotechnology for drug targeting underlines the importance of controlling the kinetics and cellular sites of delivery for optimal therapeutic outcomes. Here we examined the effect of particle size on internalization and degradation of surface-bound fibronectin by fibroblasts using polystyrene nanoparticles (NPs; 51 nm) and microparticles (MPs; 1 μm). Fibronectin was strongly bound by NPs and MPs as assessed by immuno-dot blot analysis ($5.1 \pm 0.4 \times 10^{-5}$ pg fibronectin per μm^2 of NP surface; $4.2 \pm \pm 0.3 \times 10^{-5}$ pg fibronectin per μm^2 of MP surface; $p > 0.2$). We estimated that ~193 fibronectin molecules bound to a MP compared with 0.6 fibronectin molecules per NP, indicating that ~40% of nanoparticles were not bound by fibronectin. One hour after incubation, fibronectin-coated NPs and MPs were rapidly internalized by Rat-2 fibroblasts. MPs and NPs were engulfed partly by receptor-mediated endocytosis as indicated by decreased uptake when incubated at 4 °C, or by depletion of ATP with sodium azide. Pulse-chase experiments showed minimal exocytosis of NPs and MPs. Internalization of NPs and MPs was inhibited by jasplakinolide, whereas internalization of MPs but not NPs was inhibited by latrunculin B and by integrin-blocking antibodies. Extraction of plasma membrane cholesterol with methyl β-cyclodextrin inhibited internalization of fibronectin-coated NPs but not MPs. Biotinylated fibronectin internalized by cells was extensively degraded on MPs but not NPs. Particle size affects actin and clathrin-dependent internalization mechanisms leading to fibronectin degradation on MPs but not NPs. Thus either prolonged, controlled release or an immediate delivery of drugs can be achieved by adjusting the particle size along with matrix proteins such as FN.

Introduction

In macrophage or neutrophil phagocytosis, particles are first opsonized by serum proteins, which enable cellular detection and engulfment. In addition to the major opsonins of serum such as immunoglobulins and complement factors, blood also contains cell attachment proteins including fibronectin (FN; [1,2]). The uptake and degradation of proteins by phagocytosis is not restricted to

Abbreviations: BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate; FN, fibronectin; mAb, monoclonal antibody; MP, microparticle; NP, nanoparticle; pAb, polyclonal antibody; PLL, poly-L-lysine; PBS, phosphate buffered saline; TBST, Tris-buffered-saline with 0.1% Tween-20

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professional phagocytic cells; other cell types exhibit phagocytosis as part of normal tissue homeostatic mechanisms [3]. Notably, fibroblast-mediated remodeling of the extracellular matrix involves intracellular degradation of extracellular matrix proteins like collagen and FN by the phagocytic pathway [4].

FN is a major cell adhesive glycoprotein of extracellular matrix that mediates adhesive interactions with cell surface adhesion receptors such as integrins, but also enables binding to other matrix proteins like collagen [5]. FN may provide important regulatory signals that augment macrophage phagocytic responses and can influence resolution of inflammation in connective tissues [6]. As FN participates in cell adhesion and can be readily phagocytosed by fibroblasts [7,8] it can facilitate internalization of other matrix proteins like collagen and, when bound to particles, could be used to facilitate gene or drug delivery into connective tissue cells [9,10].

Various physical and chemical properties of particles such as size [11–14], shape [15,16], surface charge [17,18] and surface functionalization [19] influence how synthetic materials interact with cells and ultimately determine their fate. Particle size is considered to be one of the critical parameters that govern the nature of the endocytic pathway and the kinetics of particle internalization [13,20]. In theory, interactions between particles and cell surface receptors are optimal when particles are 30–50 nm in diameter, and when the concentration of ligand on the particle surface and receptor abundance on the cell surface are not limiting factors [21]. However, because different cell types can express varying levels of target receptor and can utilize different internalization pathways, the optimal size of particles for internalization may depend on the cell type being assayed [22]. For example, as measured by particle number and total mass, gastrointestinal epithelial cells preferentially uptake 100 nm diameter polylactic polyglycolic acid-based particles compared with 0.5–10 μm particles [12,23]. In murine melanoma B16 cells, the endocytic pathway used for particle internalization is size-dependent: particles 200 nm are internalized by clathrin-mediated endocytosis while particles >500 nm are internalized by the caveolae-mediated pathway [11], which suggested an approach by which the utilization of discrete particle sizes for protein delivery may enable specific engagement of different endocytic pathways. Larger sized particles (>1 μm) are preferentially internalized by the phagocytic pathway of endocytosis [24]. Although the optimum size may vary considerably, larger particles (~250 nm to 3 μm) are more rapidly internalized than smaller diameter particles (<250 nm in diameter) [25]. In view of our current lack of definitive understanding of the uptake and processing of nanoparticles and their surface proteins by fibroblasts, we examined the dynamics, mechanisms of FN uptake and FN degradation patterns exhibited by Rat-2 fibroblasts when FN was coated on nanoparticles (NPs; ~50 nm diameter) or on much larger microparticles (MPs; 1 μm diameter).

Materials and methods

Reagents

Carboxylate-coated fluorescent polystyrene MPs (1 μm diameter; yellow–green, FITC) and crimson-red (CRM) MPs were purchased from Polysciences (Warrington, PA) and Molecular Probes (Eugene, OR), respectively. Fluorescent polystyrene NPs (51 nm diameter; FITC or CRM) were purchased from Bangs Laboratories (Fishers, IN). Bovine serum albumin (BSA) was from Miles Diagnostics (Kankakee, IL). Bovine plasma FN, tetramethyl rhodamine isothiocyanate phalloidin, Genistein, Chlorpromazine hydrochloride as well as mouse mAb and rabbit pAb to fibronectin were purchased from Sigma-Aldrich (Oakville, ON). Blocking antibody to mouse fibronectin receptor (α5β1) and mouse mAb to GAPDH were from Millipore (Billerica, MA). Latrunculin B and jasplakinolide were obtained from Calbiochem (La Jolla, CA).

Cells

Rat-2 fibroblasts and NIH 3T3 fibroblasts were cultured at 37 °C in complete Dulbecco's Modified Eagle's medium containing 5% fetal bovine serum and antibiotics (0.17% w/v penicillin V, 0.1% gentamycin sulfate, and 0.01% μg/ml amphotericin). Cells were maintained in a humidified incubator gassed with 95% air and 5% CO_2, and were passaged with 0.05% trypsin with 0.53 mM EDTA (Invitrogen, Burlington, ON).

Fibronectin and BSA particle coating

BSA-coated particles were used to study non-specific uptake of the particles as a control for the FN-coated particles. NPs and MPs were coated with BSA or FN for 1 h at 37 °C with shaking as described previously [26]. Briefly, following thorough dispersion by vortexing, 10–100 μl aliquots of MPs or NPs were incubated with 1 ml of FN in PBS (10 μg/ml) or with 1% (w/v) BSA. FN or BSA-coated particles were then sedimented by centrifugation (8160*g* for 3–min or 110,000*g* for 15 min for MPs and NPs, respectively), re-suspended in PBS and sonicated to ensure even distribution of the particles in solution prior to use for incubation with the cells.

Biotinylated fibronectin preparation

Aliquots of Sulfo-NHS-LC-Biotin (50–100 μL of 2 mg/mL stock dissolved fresh in DMSO) were mixed by magnetic stirring with FN that was diluted in sodium phosphate buffer (pH 8.2) every hour for 5 h, at 4 °C. Following addition of the final aliquot, the solution was left stirring at 4 °C overnight. The volume of buffer was adjusted so that the final biotinylated-FN concentration was 100 μg/mL. NPs and MPs were coated with biotinylated-FN solution as described above.

Particle characterization

Dynamic light scattering (DLS) and zeta potential (ζ) analyses of FN-coated NPs and MPs were performed using a ZetaSizer Nano ZS (Malvern Instruments) in the laboratory of Dr. W. Chan (IBBME, University of Toronto) using his previously described methods [27]. Particle diameters were also assessed by negative staining with uranyl acetate and electron microscopy of particles incubated on formvar grids.

Dot blot analysis

We estimated the amount of FN binding to particles by first eluting FN from particles by boiling for 5 min in Laemmli sample buffer. Aliquots (2 μl) of the eluates were dotted onto nitrocellulose membranes along with known amounts of FN as separate