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Single-walled carbon nanotube exposure induces membrane rearrangement and suppression of receptor-mediated signalling pathways in model mast cells

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HIGHLIGHTS

- Carbon nanomaterials are emerging as important environmental toxicants.
- Mast cells reside at the body's interfaces with its environment, and promote inflammatory responses to environmental challenges.
- We examined model mast cell responses to SWCNT and C60-fullerenes, both of which negatively regulate antigen-mediated mast cell activation.
- Carbon nanomaterials act at both the surface or after internalization in mast cells and appear to interact with the clathrin pathway.

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ABSTRACT

Carbon nanotubes (CNT) are environmental challenges to the respiratory and gastrointestinal mucosa, and to the dermal immune system. Mast cells (MC) are pro-inflammatory immunocytes that reside at these interfaces with the environment. Mast cells are sources of pro-inflammatory mediators (histamine, serotonin, matrix-active proteases, eicosanoids, prostanoids, cytokines and chemokines), which are released in a calcium-dependent manner following immunological challenge or physico-chemical stimulation. Since C-60 fullerenes, which share geometry with CNT, are suppressive of mast cell-driven inflammatory responses, we explored the effects of unmodified SWCNT aggregates on mast cell signaling pathways, phenotype and pro-inflammatory function. We noted SWCNT suppression of antigen-induced signalling pathways and pro-inflammatory degranulation responses. Mast cells recognize unmodified SWCNT by remodeling the plasma membrane, disaggregating the cortical actin cytoskeleton and relocalizing clathrin. Clathrin was also identified as a component of an affinity-purified 'interactome' isolated from MC using an SWCNT affinity matrix for mast cell lysates. Together, these data are consistent with the ability of SWCNT to suppress mast cell pro-inflammatory function *via* a novel recognition mechanism.

1. Introduction

The interaction between biomolecules and nanoreagents has been identified as having the potential to yield new nanomaterials with unique properties (Ajayan et al., 1999; Battigelli et al., 2013; Bianco et al., 2005). Humans are beginning an era of unprecedented level of interaction with nanomaterials derived from elemental carbon (carbon nanotubes, CNT). Both the single-walled (SWCNT) and multiwalled (MWCNT) carbon nanotubes display unique mechanical, chemical and electrical properties that can be further modified or functionalized with additions of various side chain moieties (Battigelli et al., 2013; Bianco et al., 2005; Cui et al., 2012). The ability to modify CNTs have further increased their utility and made them compelling candidates for delivery of nano-therapeutics.

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While the applications for nanotechnology seem endless, the impact of their use environmentally and clinically has raised both safety concerns as well as a need for consistent methodologies to evaluate their safety (Ajayan et al., 1999; Firme and Bandaru, 2010; Stern and McNeil, 2008). Whether humans are exposed to CNTs directly as with drug delivery or indirectly through the use in the environment, these materials may represent novel challenges to the respiratory and GI mucosa, and to the dermal immune system (Ajayan et al., 1999; Firme and Bandaru, 2010; Stern and McNeil, 2008). The mast cell is a pluripotent, pro-inflammatory immunocyte that resides at these sites in the body. Mast cells release pro-inflammatory mediators (including histamine, serotonin, matrix-active proteases, bioactive eicosanoids and prostanoids, cytokines and chemokines) in response to immunological and physico-chemical stimuli (Galli and Tsai, 2010; Marshall, 2004; Moon et al., 2010; Sismanopoulos et al., 2012).

Mast cells respond to a wide array of stimuli, including challenges to adaptive and innate immunity, physical inputs (heat, pH) and biochemical stimuli (cytokines, growth factors, hormones and neurotransmitters) (Galli and Tsai, 2010; Marshall, 2004; Moon et al., 2010; Sismanopoulos et al., 2012). The ability to recognize simple repeated-pattern biomolecules is common to various immunocytes including mast cells, which bear innate receptors for collagens, and other extracellular matrix proteins. Pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) such as nucleic acids and DAMPs such as heparan and uric acid (Galli and Tsai, 2010; Koyasu and Moro, 2012; Sandig and Bulfone-Paus, 2012). Thus, mast cells couple the perception of structurally simple environmental challenges to inflammatory responses.

There is little extant literature on CNT and mast cells, with most studies having focused upon the phagocytic macrophage and various lymphocytes. However, a 2007 study examined the effect of functionalized polyhydroxy C-60 and *N*-ethyl-polyamino C-60CNTs on systemic and cutaneous anaphylactic responses, which are primarily mediated by histamines, vasoactive peptides and other pro-inflammatory mediators released from mast cells. These data suggest that fullerene compounds are markedly anti-inflammatory *in vivo*, *via* suppression of mast cell-mediated responses. These data create the need to first assess whether CNT, which share elements of the fullerene geometry, are similarly anti-inflammatory (Ryan et al., 2007). This is a possibility that would seem paradoxical since CNT are widely regarded as having the capacity to initiate macrophage-based inflammation and act pro-fibrotically (Ali-Boucetta et al., 2013; Firme and Bandaru, 2010; Murphy et al., 2012; Murray et al., 2009; Poland et al., 2008). Second, both CNT and fullerenes need to be assessed for their ability to directly interface with mast cells and identify any cognate receptors that are involved.

In this study, we explored the effects of unmodified SWCNT aggregates on mast cell signaling pathways, phenotype and pro-inflammatory function. We noted suppression of antigen-induced signalling pathways and pro-inflammatory degranulation responses when exposed to unmodified SWCNT. In addition to looking at effects on function, we tested the ability of mast cells to recognize the basic structures formed by unmodified SWCNT. Confocal microscopy was used to create three-dimensional reconstructions of the remodeled plasma membrane around SWCNT aggregates. Consistent with this plasma membrane remodeling, staining of cortical actin revealed disruption of the cytoskeleton in the contact zone between the plasma membrane and SWCNT. Identification of possible interactions between SWCNT and mast cell proteins were identified by analysis of the proteins purified when unmodified SWCNT were used as an affinity matrix. When compared with controls, several components of the clathrin internalization complex were over-represented. Additional staining revealed clustering of clathrin at or near the contact point of the plasma membrane and SWCNT. Together, these data are consistent with the ability SWCNT to suppress mast cell pro-inflammatory function *via* a previously uncharacterized recognition mechanism.

2. Materials and methods

2.1. Cell culture

RBL2H3 (Passante and Frankish, 2009) were grown at 37 °C, 5% CO_2, in 95% humidity in DMEM (containing ~10 μg/ml Fe)/10% heat-inactivated FBS/2 mM glutamine.

2.2. Chemicals, antibodies

General chemicals were from VWR (West Chester, PA) and Sigma (St. Louis, MO). Phorbol 12,13-myristate acetate (PMA) and ionomycin were from Calbiochem (Gibbstown, NJ). Antibodies were sourced as follows: phospho-p72syk, phospho-ERK1/2, phospho-AKT (Ser473) were from Cell Signaling Technologies (Danvers, MA). Anti-phospho-phospholipase C gamma 1 was from BD Biosciences (San Jose, CA). Anti-phospho-NFATC1 (Ser54) and anti-clathrin were from Abcam (Cambridge, MA). Anti-Grb2 was from Upstate Biotechnologies (Billerica, MA). Alexa-conjugated wheat germ agglutinin (WGA), Alexa- and HRP-conjugated secondary antibodies were from Invitrogen (Temecula, CA) and Amersham (Piscataway, NJ).

Water-soluble hydroxylated fullerene derivatives ($C_{60}(OH)_n$, polyhydroxy C-60 modified with 18–22 hydroxyls) were obtained from Bucky USA (Houston, TX). SWCNT were produced by CVD at >95% purity and <5% Fe contamination (Nano-Labs, Detroit, MI). Note that DMEM contains ~0.0001 g/l $FeNO_3 \cdot 9H_2O$. TEM shows a SWCNT width range of 10–21 nm and a mixture of agglomerated and single SWCNT with lengths ranging from <2 to >10 microns. Measured aggregate sizes interacting with cells ranged from ~100 nm to >1 micron diameter. Doses of SWCNT from 10 to 100 μg/ml were calculated to equate to 2.8–28 μg/cm^2 in microscopy applications.

2.3. Affinity purification and MALDI-MS

Single walled carbon nanotubes (SWCNT, 10 μg) were incubated with Tx-100 lysates (see below) from 10 million RBL2H3 in triplicate. Lysis buffer alone and an irrelevant matrix (marine collagen) were used in parallel. Affinity purifications proceeded at 4 °C with rotation for 2 h, and then the matrices were washed 6 times in lysis buffer. Tryptase digestion was performed on the matrix, without elution, and multidimensional protein identification technology (MuDPIT) using 2D LC coupled to MS/MS was performed at the Keck Biotechnology Resource Laboratory, Yale University.

2.3.1. Cell stimulations

FcεRI stimulation used 0.1 μg/ml IgE anti-dinitrophenol (DNP, 16 h/37 °C) (Sigma) followed by three washes and the addition of 250 ng/ml keyhole limpet hemocyanin (KLH) conjugated-dinitrophenol (DNP, Calbiochem) for indicated times. Phorbol 12,13 myristate acetate (PMA) and ionomycin were both used at 500 nM.

2.3.2. Cell lysis and western blot

10^7 cells were lysed (ice/30 min) in 350 μl of lysis buffer (50 mM Hepes pH 7.4, 250 mM or 75 mM (high and low salt, respectively) NaCl, 20 mM NaF, 10 mM iodoacetamide, 0.5% (w/v) Triton X100, 1 mM PMSF (phenylmethylsulfonylfluoride), 500 μg/ml aprotinin, 1.0 mg/ml leupeptin and 2.0 mg/ml chymostatin).