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Actin polymerization plays a significant role in asbestos-induced inflammasome activation in mesothelial cells *in vitro*

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Abstract

Asbestos exposure leads to malignant mesothelioma (MM), a deadly neoplasm of mesothelial cells of various locations. Although there is no doubt about the role of asbestos in MM tumorigenesis, mechanisms are still not well explored. Recently our group demonstrated that asbestos causes inflammasome priming and activation in mesothelial cells, which in part is dependent on oxidative stress. Our current study sheds light on yet another mechanism of inflammasome activation by asbestos. Here we show the role of actin polymerization in asbestos-induced activation of the nod like receptor pyrin domain containing protein 3 (NLRP3) inflammasome. Using human mesothelial cells we first demonstrate that asbestos and carbon nanotubes induced caspase-1 activation and high mobility group box 1 (HMGB1), interleukin 1 beta (IL-1 β) and interleukin 18 (IL-18) secretion was blocked by cytochalasin D (cyto D) an actin polymerization inhibitor. Next, to understand the mechanism, we assessed if phagocytosis of fibers by mesothelial cells is affected by actin polymerization inhibition. Transmission electron microscopy (TEM) showed the inhibition of fiber uptake by mesothelial cells in the presence of cyto D. Furthermore, localization of components of the inflammasome, apoptotic speck-like protein containing a CARD domain (ASC) and NLRP3, to the perinuclear space in mitochondria or endoplasmic reticulum (ER) in response to fiber exposure was also interrupted in the presence of cyto D. Taken together, our studies suggest that actin polymerization plays important roles in inflammasome activation by fibers via regulation of phagocytosis and/or spatial localization of inflammasome components.

Keywords

inflammasome; asbestos; mesothelial cells; actin polymerization

Introduction

Asbestos exposure is the main cause of mesothelial cell pathogenesis including malignant mesothelioma (MM). The mechanism(s) behind this pathogenesis is not well understood. We and others have demonstrated that asbestos can activate inflammasomes in mesothelial cells (Hillegass et al. 2013) as well as in macrophages (Dostert et al. 2008). Furthermore, the

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Conflict of interest

The authors wish to disclose that they have no conflicts of interest.

role of inflammasomes in asbestos-induced mesothelial to fibroblastic transition (unpublished data) as well as a therapeutic mediator for MM has also been depicted by our group (Westbom et al. 2015). It is becoming increasingly clear that inflammasomes may play essential roles in both the development and therapy of MM. With this knowledge it also becomes important to understand how inflammasomes are regulated by asbestos in mesothelial cells. Carbon nanotubes (CNT) are increasingly being used in different industries (Oberdorster et al. 2015) and resemble asbestos fibers in shape and size. It is important to study the effects of CNT on cell systems in parallel to asbestos to understand CNT toxicity and other cellular responses.

Among various known inflammasomes, nod like receptor pyrin domain containing protein 3 (NLRP3) is the most well studied inflammasome and the only inflammasome so far known to be activated by asbestos. The NLRP3 contains an N-terminal pyrin domain (PYD) followed by a central nucleotide-binding domain (NBD) and C-terminal leucine-rich repeats (LRR) (Dostert et al. 2008). It is a multicomplex cytosolic receptor, known to be regulated by many endogenous and exogenous activators. Activation of NLRP3 triggers oligomerization and recruitment of pro-caspase-1 by the adaptor molecule apoptotic speck-like protein containing a CARD domain (ASC) and this complex is responsible for IL-1 β and IL-18 processing. Several mechanisms, including reactive oxygen species (ROS), phagocytosis accompanied by lysosomal damage, potassium efflux and signaling molecules have been proposed as regulatory options for inflammasome activation in various cell types in response to different activators.

Asbestos-induced NLRP3 activation in mesothelial cells has been shown to involve ROS and signaling molecule cAMP response element binding protein (CREB) (Thompson et al. 2014; Westbom et al. 2014). We have also reported that asbestos can cause oxidation of thioredoxin resulting in release of thioredoxin interacting protein (TXNIP) which can then activate NLRP3 in human mesothelial cells (Thompson et al. 2014). As mesothelial cells are capable of phagocytizing asbestos fibers and phagocytosis is one of the reported ways of NLRP3 activation, we hypothesized that phagocytosis of asbestos fibers by mesothelial cells plays an important role in inflammasome activation in these cells. By using an actin polymerization inhibitor, cytochalasin D (cyto D) that can inhibit the process of phagocytosis (Dostert et al. 2008), we first show that cyto D inhibits inflammasome activation by asbestos. To confirm, we studied uptake of fibers into cells in the presence and absence of cyto D by transmission electron microscopy (TEM). Finally, we studied the role of cyto D in recruitment of NLRP3 and/or ASC to mitochondria and/or endoplasmic reticulum (ER) in response to asbestos in mesothelial cells. Our findings demonstrate that inhibition of actin polymerization can lead to attenuated phagocytosis of fibers by mesothelial cells resulting in altered recruitment of NLRP3 and/or ASC to mitochondria and/or ER and decreased activation.

Materials and methods

Cell culture and fiber exposure

Human mesothelial cells (LP9) were purchased from Brigham and Women's Hospital, (Harvard University, Boston, MA). Cells were incubated at 37°C in 5% CO₂ and grown to

80-90% confluency as described previously (Hillegass et al. 2013). Well characterized National Institute of Environmental Health Sciences (NIEHS) reference sample of crocidolite asbestos was used (BeruBe et al. 1996). Glass beads (GB) used as negative control, demonstrated no significant effect on cell toxicity and inflammasome activation as previously reported (Hillegass et al. 2013). Multiwalled carbon nanotubes (Mitsui-7) (CNT) and carbon black (CB) were kind gifts from Dr. Gunter Oberdorster (University of Rochester, NY). After sterilization under UV light overnight, particulates were suspended in medium with 0.5% FBS, vortexed until in solution, sonicated for 15 min in a water bath sonicator and triturated 5 to 10 times through a 22-gauge needle. Cells were exposed to equal particle surface area concentrations of asbestos, CNT or CB. A final concentration of asbestos of $75 \times 10^6 \mu\text{m}^2/\text{cm}^2$ dish surface area was chosen as it is known to cause inflammasome activation, apoptosis, pyroptosis and compensatory proliferation of surrounding mesothelial cells and alveolar type II epithelial cells (Buder-Hoffmann et al. 2001; Goldberg et al. 1997; Hillegass et al. 2013; Thompson et al. 2014). CB at an equal particle surface area concentration was used as a negative particle control. Two doses of CNT (75 and $150 \times 10^6 \mu\text{m}^2/\text{cm}^2$) were studied as their effects on mesothelial cells have not been reported before. Cyto D was purchased from Sigma, (Saint Louis, MO) and Ca-074Me from Calbiochem, (San Diego, CA).

Cell viability assay

LP9 cells were grown to confluency in 35 mm dishes and after overnight replacement to reduced serum medium (0.5% FBS), were exposed to fibers/particles at equal surface area concentrations with and without cyto D pre-treatment (0.1 - $1.0 \mu\text{g}/\text{mL}$) for 1 hour. Twenty four hours later, cells were trypsinized and counted using a hemocytometer.

Western blot analyses

Cells were grown to confluency and then changed to reduced serum medium overnight. Cells were exposed to fibers with or without pre-treatment with cyto D ($0.1 \mu\text{g}/\text{mL}$) as mentioned above. Western blot analysis was performed on media supernatants after concentration. Equal volumes of media supernatants were concentrated using StrataClean resin beads (Agilent Technologies, Santa Clara, CA) as previously reported (Westbom et al. 2015). An equal volume of 4X sample buffer was added to beads after media had been aspirated and boiled for 5 min at 95°C . Thereafter 10 - $15 \mu\text{L}$ of each sample was resolved on a 15 % SDS PAGE for subsequent immunoblotting for inflammasome activation markers caspase-1p20 (Cell Signaling Technologies, Danvers MA) and the danger associated molecule HMGB1 (Abcam, Cambridge, MA).

ELISA for IL-1 β and IL-18

Media supernatants from *in vitro* experiments were concentrated in Amicon centrifugal filtration units with a molecular weight limit of 10 kDa (Millipore, Billerica MA) as described previously (Hillegass et al. 2013). The levels of IL-1 β and IL-18 secreted in response to asbestos exposure were then measured using the Human Quantikine IL-1 β /IL-1f2 Immunoassay (R&D Systems, Minneapolis, MN) and Human IL-18 ELISA kits (MBL, Woburn, MA) respectively following the manufacturer's directions. Values are expressed as pg (IL-1 β or IL-18)/mL of total culture supernatant initially collected.

Assessment of cathepsin B inhibitor (Ca-074Me) effect on inflammasome activation

To assess if phagocytosis of asbestos-fibers results in lysosomal disruption, release of cathepsin B and inflammasome activation, we used cathepsin B inhibitor Ca-074Me. Cells were pre-treated with Ca-074Me (12.5 μ M) for 1 hour before treating them with asbestos for 24 hours. Cell culture medium was analyzed for caspase-1p20.

TEM imaging of asbestos exposed LP9 cells with and without cyto D

Cells were grown in 12 well plates on 22 mm diameter Thermanox plastic coverslips (Nunc, Naperville, IL) until confluent. After addition of asbestos for 4 hours, with and without pre-treatment of cyto D, cells were fixed for 45 minutes in Karnovsky's fixative at 4°C (2.5% glutaraldehyde, 1% paraformaldehyde in 0.1M cacodylate buffer). They were then rinsed in 0.1M cacodylate buffer and post fixed in 1% OsO₄ in 0.1M cacodylate buffer for 30 minutes at 4°C, followed by another rinsing in 0.1M cacodylate buffer. Cells were then dehydrated through a series of graded ethanols and then coverslips were embedded in 100% Spurr's resin. Blocks were sectioned and specimens on grids were imaged using a JEOL 1400 TEM (JEOL, Peabody, MA).

Quantitation of asbestos fibers in LP9 Cells

A total of 25 randomized cell containing fields on TEM grids were imaged for asbestos exposed and asbestos + cyto D exposed LP9 cells. Using MetaMorph image analysis software, the area of one imaged cell per field as well as the total area of asbestos fibers (black) within the cell was calculated. Mean % $\pm$ SEM of asbestos area per cell was determined as an index of asbestos prevalence in each group.

Assessment of NLRP3/ASC recruitment to mitochondria and/or ER

Cells were grown to near confluency in four well culture slides (Becton Dickinson, Franklin Lakes, NJ) and exposed to either asbestos or CNT for 24 hours with or without pre-treatment of cyto D. Cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes followed by permeabilization with 0.1% Triton-X for 10 minutes at room temperature (RT). Auto fluorescence was blocked with 0.3M glycine and non-specific antibody binding was blocked with 20% goat serum in 1x PBS for 1 hour. Cells were incubated with either anti-rabbit ASC or NLRP3 (Novus Biologicals, Littleton, CO) combined with either mitochondrial marker anti-mouse PRDX3 (AB Nova, Walnut, CA) or ER marker anti-mouse calnexin (Novus Biologicals) diluted 1:50 in 1% BSA/PBS overnight at 4°C in a humidified chamber. The following day, a mixture of secondary antibodies Alexa Fluor® 647 goat anti-rabbit and Alexa Fluor® 568 goat anti-mouse (Life Technologies, Grand Island, NY) diluted 1:400 in 1xPBS were applied to cells for 1 hour at RT. The nuclear stain DAPI (Life Technologies) was diluted 1:200 and added to cells for 20 minutes at RT. Slides were then mounted and coverslipped with Aqua-Poly/Mount (Polysciences Inc., Warrington, PA) and stored at 4°C. Confocal images were acquired using a Zeiss 510 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

Statistical analysis

All experiments were performed in duplicate or triplicate and repeated at least twice. A one-way analysis of variance (ANOVA) followed by a Newman-Keuls procedure for adjustment of multiple pairwise comparisons or the Student's t-test was applied to all data points to establish the significance of observed differences between the various experimental groups. A p value ≤ 0.05 was considered to be significant. All statistical analyses were performed using the GraphPad v 6.0 software program.

Results

Cyto D decreases asbestos and CNT-induced cell death

Asbestos fibers or CNT exposure for 24 hours cause significant death of human mesothelial cells. Pre-treatment with actin polymerization inhibitor cyto D (0.1 $\mu\text{g/mL}$) prevents this death (Fig. 1a) possibly by inhibiting the uptake of fibers and inflammasome activation. CB at equal particle surface concentration had no effect on cell viability or inflammasome activation (data not shown), therefore, it was not included in further experiments. As CNT at higher dose (150) showed similar effects on viability as asbestos dose of 75, in some experiments we used only CNT 150.

Cyto D attenuates asbestos and CNT-induced inflammasome activation

Asbestos or CNT exposure to human mesothelial cells at the same particle surface area concentration dose caused activation of the inflammasome as measured by caspase-1 p20, HMGB1, IL-1 β and IL-18 release in medium (Fig. 1b-h). Pre-treatment with cyto D resulted in significant attenuation of fiber-induced levels of the caspase-1, HMGB1, IL-1 β and IL-18 levels (Fig. 1b-h) and thereby activation of the NLRP3 inflammasome.

Cyto D inhibits fiber uptake by mesothelial cells as assessed by TEM

As cyto D can inhibit actin polymerization resulting in attenuated phagocytosis, we next tested if cyto D can inhibit asbestos fiber uptake by mesothelial cells. LP9 cells were exposed to asbestos (4 hours) with and without cyto D pre-treatment. TEM images show unexposed control cells (Fig. 2b), uptake of asbestos fibers by cells (Fig. 2c), with other fibers shown lying in the cytoplasm near nucleus. Pre-treatment with cyto D before exposure to asbestos inhibited the uptake of fibers with fewer fibers detected inside the cell (Fig. 2d). A quantitative assessment of asbestos fibers within cells revealed that without pretreatment with cyto D, an average of 1.0% of the cell area contained asbestos. However, pretreatment with cyto D reduced this to only 0.2%, an 80% decrease in fiber uptake (Fig. 2a). These results suggest that uptake of fibers by mesothelial cells is attenuated by inhibition of actin polymerization by cyto D.

Asbestos-fiber uptake by mesothelial cell does not involve lysosome disruption

It is reported that particles after being taken up by cells, enter into lysosomes and disrupt it, resulting in the release of cathepsin B, which is a known activator of the inflammasome in some cell types. Asbestos fibers accumulating into lysosomes were not observed by TEM (Fig. 2c), but still to confirm whether cathepsin B is involved in inflammasome activation or

not, cells were pre-treated with cathepsin B inhibitor (Ca-074 Me) and asbestos-induced inflammasome activation was measured. No effect of Ca-074 Me on inflammasome priming or activation as measured by NLRP3 protein levels or caspase-1 p20 release was observed (data not shown) suggesting no significant role of lysosomal disruption in asbestos-induced inflammasome activation.

Asbestos-induced recruitment of ASC and NLRP3 to mitochondria is dependent on actin polymerization

Activation of the NLRP3 inflammasome requires complex formation with ASC and the process requires their strategic localization in the perinuclear space with ER and mitochondria. By using multiple fluorescent markers (blue-DAPI for nucleus; green- peroxiredoxin3 (PRDX3), mitochondrial marker and red-ASC/NLRP3) here we show that asbestos exposure causes a robust localization of ASC in mitochondria (yellow color Fig. 3a, upper panel). CNT exposure also caused localization of ASC to mitochondria, with more remarkable results obtained at higher particle surface area concentration (150) (Fig. 3a, upper panel). Pre-treatment with cyto D before fiber exposure caused a significant reduction in yellow color, suggesting decreased recruitment of ASC to mitochondria (Fig. 3a, lower panel). Unstimulated or resting cells also show some yellow which is a normal phenomenon. Similarly, Fig. 3b, upper panel shows recruitment of NLRP3 to mitochondria in response to asbestos and CNT. Perinuclear yellow color depicts NLRP3 in mitochondria in response to asbestos or CNT treatment. This localization was not as robust as that of ASC (Fig. 3a) but certainly more than untreated control. Again like ASC, CNT at higher concentration showed more remarkable localization. Inhibition of actin polymerization by cyto D inhibited fiber-induced NLRP3 localization to mitochondria (Fig 3b, lower panel).

Inhibition of actin polymerization attenuates the fiber-induced recruitment of NLRP3 and ASC at ER

Under resting or unstimulated conditions ASC and NLRP3 are present in the cytosol and ER and upon activation with fibers they accumulate in ER around the perinuclear region. By using multiple fluorescent markers (blue-DAPI for nucleus; green-calnexin for ER and red-for ASC/NLRP3) here we show that asbestos exposure causes increased localization of ASC and NLRP3 to ER (yellow color), a process that is inhibited in presence of actin polymerization inhibitor cyto D (Fig. 4a,b). CNT exposure had no significant effect on localization of ASC to ER at any dose (Fig. 4a). On the other hand, NLRP3 localized to ER upon stimulation with CNT (yellow color) and this recruitment was attenuated in the presence of cyto D (Fig. 4b).

Discussion

Asbestos is well known to cause mesothelial cell pathogenesis and one of the mechanisms could be the activation of the inflammasome in these cells resulting in secretion of pro-inflammatory markers or fibrin and fibrinolysis inhibitors (Hillegass et al. 2013). It has been reported previously that asbestos exposure to mesothelial cells results in cell death followed by compensatory proliferation (Goldberg et al. 1997). A fraction (17%) of total cell death by asbestos in mesothelial cells is contributed by pyroptosis, inflammatory cell death, caused by

inflammasome activation (Thompson et al. 2014). Inflammasomes can be activated by multiple mechanisms and phagocytosis is one of many. Our group has studied the activation of inflammasomes in mesothelial cells by asbestos and have demonstrated the role of reactive oxygen species (ROS), thioredoxin interacting protein (TXNIP) and cAMP response element binding protein (CREB) in the process (Thompson et al. 2014; Westbom et al. 2015). In addition, potassium efflux and ATP receptors had no significant effect on asbestos-induced inflammasome activation in mesothelial cells (unpublished data). As mesothelial cells are known to actively phagocytize fibers and phagocytosis is a process known to activate inflammasomes, (Dostert et al. 2008; Dowling and O'Neill 2012; Hornung et al. 2008), we focused our attention to the process of phagocytosis in inflammasome activation in mesothelial cells. It has been shown by Dostert et al. (2008) that in asbestos exposed macrophages, phagocytosis of fibers results in ROS generation by NADPH oxidase (NOX). In mesothelial cells we have shown that asbestos can cause oxidation of thioredoxin resulting in release of TXNIP and NLRP3 activation (Thompson et al. 2014). N-acetyl cysteine (NAC), a precursor for glutathione synthesis has also been demonstrated to attenuate asbestos-induced inflammasome activation in both macrophages and mesothelial cells (Dostert et al. 2008; Thompson et al. 2014). Furthermore in support of our findings, the role of ROS is well documented in inflammasome activation in various cell types (Dowling and O'Neill 2012). The role of TXNIP in NLRP3 activation by direct interaction has also been demonstrated and this is also a ROS driven process (Bauernfeind et al. 2011; Zhou et al. 2010).

Phagocytosis has been identified as a process by which macrophages (and many other cells) uptake particles, which can then activate NLRP3 (Dostert et al. 2008; Hornung et al. 2008). This process can be inhibited by actin-destabilization by cytochalasin D (Bauernfeind et al. 2011). It has been reported by various research groups that phagocytosis of particles by macrophages results in lysosome destabilization and release of cathepsin B enzyme. This enzyme is known to activate NLRP3 as measured by caspase-1 p20 release, by unknown mechanisms (Bauernfeind et al. 2011). In our study, TEM images show phagocytosis of asbestos fibers by mesothelial cells, however, we don't see them in lysosomes. We find fibers lying in cytosol close to the nucleus. Furthermore, the use of cathepsin B inhibitor (Ca074 Me) had no significant inhibitory effect on asbestos-induced NLRP3 activation as measured by caspase-1p20 release in the medium (data not shown). This suggests that unlike other particles and crystals, asbestos-induced inflammasome activation is dependent on phagocytosis but may not involve lysosomal rupture and cathepsin B release.

Under resting or unstimulated conditions, NLRP3 localizes to cytoplasm and ER, whereas active inflammasome complex formation requires recruitment of NLRP3 and its adaptor, ASC to mitochondria and ER, the perinuclear space and the process is driven by ROS (Hayashi et al. 2009; Zhou et al. 2011). To demonstrate that co-localization of NLRP3 and ASC to the perinuclear space happens upon stimulation with asbestos or CNTs, cells were co-stained with different fluorescent markers after asbestos/CNT exposure. We observed that ASC and NLRP3 which are present in cytoplasm and ER in resting condition accumulate in mitochondria and ER in the perinuclear space upon stimulation with asbestos or CNT. We also demonstrate that inhibition of actin polymerization (cyto D) completely or partially inhibited this event. It is clear from our studies presented here that asbestos-induced

inflammasome activation requires actin polymerization. However, whether actin polymerization is required for phagocytosis/ROS generation and subsequent recruitment of NLRP3/ASC, or for direct recruitment of inflammasome components to the perinuclear space, needs to be investigated further. The role of ROS in the process of asbestos-induced inflammasome redistribution/activation has been previously demonstrated (Dostert et al. 2008; Thompson et al. 2014; Zhou et al. 2011). In our study, mostly we observed common responses between asbestos and CNT, however, we don't see ASC localization to ER with CNT at any dose. Also, mitochondrial localization of ASC and NLRP3 in response to a low concentration of CNT (75) was less robust as compared to a similar particle surface area concentration of asbestos.

In conclusion, asbestos-induced inflammasome activation in mesothelial cells is in part dependent on actin polymerization. We show here that phagocytosis of fibers causes ROS generation which in turn causes recruitment of inflammasome components to the perinuclear space (mitochondria and ER) resulting in complex formation and activation (Fig. 5). Inhibition of actin polymerization by cyto D attenuates inflammasome activation by inhibiting phagocytosis of fibers and subsequent release of ROS. Alternatively, inhibition of actin polymerization can directly affect the spatial recruitment of ASC and NLRP3 to mitochondria or ER in the perinuclear space, which is required for activation.

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Fig. 1.

Asbestos and CNT-induced toxicity and inflammasome activation in mesothelial cells is inhibited by cytochalasin D. Asbestos and CNT-induced **a** viability **b,c** caspase-1, **d,e** HMGB1, **f,g** IL-1 β and **h** IL-18 for 24 h with and without cyto D (1 μ g/mL **b,d,f**). Data is represented as mean $\pm$ SEM. * p < 0.05 compared to control (no Asb/CNT) group. † p < 0.05 compared to no cyto D of same Asb/CNT group

Fig. 2.

Asbestos fiber phagocytosis by mesothelial cells is inhibited by actin polymerization inhibitor cyto D. **a** Quantitation of asbestos fiber areas within LP9 cells with and without pretreatment with cyto D. * $p < 0.05$ compared to asbestos group. TEM images showing **b** unexposed cells and **c** uptake of asbestos fibers by LP9 cells at 4 h, arrows showing fibers or aggregates of fibers inside the cell. **d** In the presence of cyto D (0.1 μg/mL) uptake of fibers is inhibited (Magnification bar = 2 μm).

Fig. 3.

Asbestos-induced mitochondrial localization of ASC and NLRP3 is inhibited by cyto D. Cells were grown in chamber slides and exposed to either asbestos or CNT for 24 h with or without pre-treatment with cyto D (0.1 μg/mL). At the termination of the experiment cells were fixed and stained with DAPI (blue), **a** ASC or **b** NLRP3 (red) and PRDX3 (green) a mitochondrial marker. Yellow is the color of localization of ASC or NLRP3 to mitochondria (Magnification bar = 50 μm)

Fig. 4.

Asbestos-induced ER localization of ASC and NLRP3 is inhibited by cyto D. Cells were grown in chamber slides and exposed to either asbestos or CNT for 24 h with or without pre-treatment with cyto D (0.1µg/mL). At the termination of the experiment cells were fixed and stained with DAPI (blue), **a** ASC or **b** NLRP3 (red) and calnexin (green) an ER marker. Yellow is the color of localization of ASC or NLRP3 to ER (Magnification bar = 50µm)

Fig. 5.
Schematic representation of events of asbestos uptake by mesothelial cells and inflammasome activation