Published in final edited form as:

Inhal Toxicol. 2012 July ; 24(8): 476–485. doi:10.3109/08958378.2012.689782.

Functional expression of system x_c^- is upregulated by asbestos but not crystalline silica in murine macrophages

Jean C. Pfau¹, Todd Seib², Jason J. Overocker¹, Jeremy Roe¹, and Aaron S. Ferro¹

¹Department of Biological Sciences, Idaho State University, Pocatello ID, USA

²Department of Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, MT, USA

Abstract

Context—Inhalation of asbestos or silica is associated with chronic and progressive diseases, including fibrosis, cancer, and increased risk of systemic autoimmunity. Because there is a need for treatment options for these diseases, a better understanding of their mechanistic etiologies is essential. While oxidative stress in macrophages is an early consequence of these exposures, it may also serve as a signaling mechanism involved in downstream immune dysregulation. The system x_c^- exchange protein is induced by oxidative stress, and exchanges equimolar levels of extracellular cystine for intracellular glutamate. Cystine is subsequently reduced to cysteine, the rate-limiting precursor for glutathione synthesis.

Objective—As the primary transporter responsible for cystine/glutamate exchange on macrophages, system x_c^- was hypothesized to be inducible in response to asbestos and silica, and to increase viability through protection from oxidative stress.

Results—When challenged with amphibole asbestos, but not crystalline silica, RAW 264.7 macrophages increased expression of xCT and the rate of cystine/glutamate exchange in sodium-free conditions. This upregulation was prevented with *N*-acetylcysteine, implicating oxidative stress. Cystine protected the macrophages from asbestos-induced oxidative stress and cell death, supporting the hypothesis that imported cystine was used for synthesis of cellular antioxidants. System x_c^- inhibitors, glutamate and *S*-4-carboxyphenylglycine ((S)-4-CPG), significantly increased oxidative stress and cell death of asbestos-treated macrophages.

Conclusion—System x_c^- plays a critical role in survival of macrophages exposed to asbestos, but not silica. These data demonstrate a very early difference in the cellular response to these silicates that may have important downstream implications in the pathologic outcome of exposure.

Keywords

Glutamate; cystine; glutathione; asbestos; silica

Introduction

Macrophages are key in orchestrating immune responses to inhaled material, using signals sent to lymphocytes that help tailor an appropriate response dependent on their recognition

© 2012 Informa Healthcare USA, Inc.

Address for Correspondence: Jean C. Pfau, Department of Biological Sciences, Idaho State University, 921 South 8th Ave, Stop 8007, Pocatello, ID, 83209, USA Tel: +1-(208) 282-3914. Fax: 208-282-4570. pfaujean@isu.edu.

Declaration of Interest

The Authors report no declarations of interest.

of various types of pathogen through specialized receptors. In the case of inhaled dusts such as silica and asbestos, however, there remain questions about (a) how the macrophage recognizes and distinguishes these materials and (b) what the subtle signals are that lead to the distinct disease outcomes following exposure. While much is known about macrophage-derived signals that influence immune function, there are many subtle regulatory steps that are still not understood. Interaction of macrophages with asbestos and silica causes production of reactive oxygen species (ROS) (Blake et al., 2007; Shen et al., 2001; Shukla et al., 2003). Some of the macrophages simply die from the oxidative damage (Blake et al., 2007; Shen et al., 2001), but some survive and impact ultimate immune/inflammatory outcomes. Macrophages play several roles in activating the immune system, primarily through the production of response-tailoring cytokines and lipid mediators, and these activities are sensitive to redox signals (Murata et al., 2002; Peterson et al., 1998; Pfau et al., 2004; Schneider et al., 2005).

System x_c^- is an amino acid transport system expressed on many cell types. It appears to be the major glutamate/cystine antiporter expressed on murine macrophages (Watanabe & Bannai, 1987), although another glutamate transport system (system x_{AG}^-) has also been reported on human macrophages (Rimaniol et al., 2000). Transport via system x_c^- is sodium-independent, unlike other anionic amino acid transporters such as system x_{AG}^- , but is dependent on chloride (Waniewski & Martin, 1984). It consists of two subunits xCT, the light chain; and a heavy chain, 4F2 (CD98), forming a functional heterodimer (Sato et al., 1999) (Figure 7). Although transport is reversible (dependent on amino acid concentration gradients), in macrophages undergoing oxidative stress, System x_c^- exchanges intracellular glutamate for extracellular cystine. Following import, cystine is reduced to cysteine, which may serve not only as an antioxidant itself (Banjac et al., 2008), but also acts as the rate-limiting precursor for the synthesis of glutathione, the predominant cellular thiol-antioxidant (Meister & Anderson, 1983).

Early experiments on system x_c^- illustrated that transport activity for cystine is induced by oxygen (Bannai et al., 1989). Accordingly, xCT expression decreases when cells are cultured under low oxygen conditions (Sato et al., 2001). Thus, oxygen affects system x_c^- transporter expression and activity, and it follows logically that particles that cause oxidative stress in macrophages, such as silica and asbestos (Vallyathan et al., 1992) would likewise induce its activity.

The ultimate pathologies resulting from exposure to amphibole asbestos are unique when compared to those caused by crystalline silica (Maeda et al., 2010; Mossman & Churg, 1998), despite the fact that both are inhaled (impacting macrophages early on), both cause oxidative stress in macrophages, both elicit a variety of pro-inflammatory mediators, and both can induce apoptosis in macrophages (Table 1). Initially, we expected that silica and asbestos would cause similar upregulation of system x_c^- due to these commonalities. The data presented here demonstrate that while silica did not upregulate system x_c^- , asbestos treatment resulted in increased functional expression of the transporter. Additionally, a GSH-precursor, *N*-acetylcysteine (NAC), was able to decrease ROS, prevent the increase in system x_c^- , and protect RAW macrophages from cell death. These data suggest that the oxidative conditions produced by asbestos exposure are able to enhance survival of macrophages through the upregulation of system x_c^- . In this way, system x_c^- may serve as an initial response that could ultimately be responsible for a whole host of downstream signaling cascades as a result of asbestos exposure, as well as represent an attractive therapeutic target for early intervention in asbestos-induced disease.

Materials and methods

Chemicals, particulates and fibers

Libby amphibole asbestos, collected and characterized by the US Geological Survey, and purified crocidolite were kindly provided by the Center for Environmental Health Sciences (CEHS), University of Montana. Libby MT asbestos (6-Mix) has been called “6-Mix”, because it was collected from six sites around the mine area near Libby. It is amphibole asbestos a mixture of tremolite, amosite, richterite and other amphiboles. Crystalline silica and wollastonite were obtained from US Silica and the National Institutes of Standard and Technology (NIST), respectively, by the CEHS and kindly provided for these experiments. Fibers or silica were made up as stock suspensions at 1 mg/mL in sterile phosphate buffered saline (PBS) or medium and then sonicated in a water-bath sonicator (Branson Ultrasonics, Danbury, CT, USA) for at least 1 min before adding the suspension to the cell cultures on an equal mass basis. Most experiments were performed at 35 $\mu\text{g}/\text{cm}^2$, which was shown not to induce significant cell death by silica or asbestos, and most treatments were for 24 h, unless otherwise indicated.

System x_c^- inhibitor, *S*-4-carboxyphenyl-glycine ((*S*)-4-CPG), was purchased from Tocris Bioscience (Ellisville, MO, USA). Lipopolysaccharide (LPS, from *Salmonella minnesota*), L-cystine, L-glutamic acid, *N*-acetylcysteine (NAC) and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Cells and cell culture

RAW 264.7 macrophages were obtained from *American type culture collection* (ATCC, Rockville, MD, USA) and maintained at 37°C in Dulbecco’s modified eagle’s medium (DMEM) (CellGro Mediatech, Manassas, VA, USA) containing L-glutamine, 100 U/mL penicillin/streptomycin, and 5% fetal calf serum (FBS, Hyclone) in a humidified CO₂ incubator. For experiments, the cells were switched to serum free DMEM without cystine, cysteine or glutamate (Gibco/Invitrogen, Carlsbad, CA, USA) to which amino acids could be added individually at known concentrations. Sodium-free buffer consisted of 1.1 mM CaCl₂, 5.36 mM KCl, 0.77 mM KH₂PO₄, 0.71 mM MgSO₄, 137 mM choline chloride, 11 mM D-glucose, and 1 mM HEPES.

Flow cytometry

RAW 264.7 cells were harvested and counted, then blocked in PBS with 3% bovine serum albumin and Fc Block (anti-CD16/32, BD Biosciences, San Jose, CA, USA) for 10 min at room temperature. Approximately 1×10^6 cells per sample were stained in 100 μL of blocking buffer with antibodies to xCT (Santa Cruz Biotech, clone Q-18) and CD98 (BD Biosciences). Secondary antibodies were anti-goat IgG conjugated with fluorescein isothiocyanate (FITC) and anti-rat conjugated with Phycoerythrin (PE), respectively (Molecular Probes/Invitrogen). Stained cells were washed thoroughly with cold PBS and analyzed immediately or fixed with 1% paraformaldehyde overnight. Staining was analyzed on a BD Biosciences (San Jose, CA, USA) FACS Calibur flow cytometer, with gates set on live cells, and a positive/negative cut-off gate was based on cells stained with secondary antibody alone. Data analysis was performed using Cell Quest software (BD Biosciences), with data expressed as percent positive, or median fluorescence intensity (MFI) of the entire population.

System x_c^- activity (L-Glu transport assay)

RAW 264.7 cells were plated at 5×10^5 cells/well in 12-well plates containing RPMI 1640 growth media supplemented with 5% FBS. Cultures were maintained at 37°C and 5% CO₂

for 24-h post-plating until cells were 80–90% confluent. The cultures were then treated with indicated amounts of asbestos, silica, and LPS for 12, 24, or 48 h. System x_c^- activity was assayed using a protocol previously described with a few modifications (Warren et al., 2004). Briefly, on the day of the assay, plates were removed from the incubator, growth media (RPMI with 5% FBS) was removed from the 12-well culture plates and replaced with a Na^+ -free buffer (mM), $CaCl_2$ (1.1), KCl (5.36), KH_2PO_4 (0.77), $MgSO_4$ (0.71), choline chloride (137.5), D-glucose (11), HEPES (10), pH 7.4. Following 5 min pre-incubation in Na^+ -free buffer (30°C), the buffer was aspirated and replaced with buffer containing 100 μM [3H]-L-glutamate (New England Nuclear). Following 5-min incubation at 30°C, the assays were terminated by three sequential 1-mL washes with ice-cold buffer and then the cells were dissolved in 1 mL of 0.4 M NaOH for 24 h. An aliquot (200 μL) was transferred into a 5-mL glass scintillation vial and neutralized with 5 μL glacial acetic acid, followed by the addition of 3.5 mL Liquiscint scintillation fluid (National Diagnostics) to each sample. Incorporation of [3H]-L-glutamate was quantified by liquid scintillation counting (LSC, Beckman LS 6500). All transport activity rates were normalized for protein determined via the BCA method (Pierce). Values are reported as mean pmol/min/mg protein $\pm$ SEM and are corrected for nonspecific uptake (e.g. leakage and binding) by subtracting the amount of [3H]-L-glutamate accumulated at 4°C.

Viability assay

Viability was assessed using the CellTiter Blue reagent from Promega. RAW 264.7 cells were plated at 1×10^6 cells/mL in 96-well cell culture plates (100 μL /well) in media lacking cystine, cysteine and glutamate, unless supplemented, and challenged with indicated amounts ($\mu g/cm^2$) of particles or fibers, in the presence or absence of amino acids, NAC, or (S)-4-CPG for 24 h. Triton-X-100 at 1% was used as a positive control for cell death. Following the challenge, 20 μL /well of CellTiter Blue dye was added to each well and the plate was tapped gently to mix. The plate was incubated at 37°C for 1 h and read on a Synergy HT microtiter plate reader (BioTek Instruments, Winooski, VT, USA) at 570 nm.

Glutamate assay

The amount of glutamate that was transported out of the cells was measured using the Amplex®-Red Glutamic Acid/Glutamate Oxidase assay kit (Molecular Probes/Invitrogen). The assay is based on the oxidation of glutamate by glutamate oxidase, producing alpha-ketoglutarate, NH_3 , and H_2O_2 . The H_2O_2 reacts with the Amplex®-Red reagent to produce the fluorescent product, risorufin. A standard curve from 0 to 20 μM was prepared using the kit's glutamate stock. The positive control was 10 μM H_2O_2 in reaction buffer. Macrophages were cultured in 24-well plates, in media lacking glutamate, but containing cystine as the substrate for glutamate transport. Media from the challenged RAW 264.7 cells was diluted in the kit's reaction buffer and placed in the wells of an opaque 96-well plate, 50 μL /well. 50 μL of Amplex®-Red working solution was added to each well, and the plate was incubated at 37°C for 30 min to 1 hour. Fluorescence was detected at 590 nm using the Synergy plate reader. Concentrations of L-glutamate were calculated from the standard curve.

DCFDA assay

Dichlorofluorescein diacetate (DCFDA, Molecular Probes/Invitrogen) was used as a measure of general oxidative stress. The cells were plated in media lacking cystine, cysteine or glutamate, and challenged in 96-well opaque cell culture plates as described above, with or without supplemented NAC, cystine, or glutamate. Following the challenge period, the media was removed and 50 μL of 10 μM DCFDA was added to each well. Wells containing no cells served as a negative control, and cells treated with 10 μM hydrogen peroxide served

as the positive control. The plate was incubated for designated times at 37°C, and then read at 520 nm on the microtiter plate reader.

Glutathione assay

Cellular GSH was measured using the GSH-Glo™ Glutathione assay from Promega following the manufacturer's instructions. Briefly, after challenge with asbestos in media containing cystine as the substrate for system x_c^- , the media was removed from the macrophages, and the Luciferin-NT and glutathione *S*-transferase (GST) were added to the reaction buffer and the wells. After 30-min incubation, the Luciferin Detection Reagent (containing luciferase) was added to the plate, incubated for an additional 15 min and then read on the luminescence setting of the microtiter plate reader. In the presence of GSH, the Luciferin-NT is reduced by GST to Luciferin, which then fluoresces in the presence of ATP and Luciferase.

Statistical analyses

All experiments were repeated at least three times, and representative data is shown. Bar graphs represent mean values in each group, and error bars are standard error of the mean (SEM). Statistical analyses were performed using Excel or Kaleidograph software (Synergy Software, Reading PA, USA). Unpaired two-tailed *t*-tests were used wherever appropriate, for numerical measurements of a mean against a single control mean. One-way ANOVA was used to compare treatment groups with one independent variable, with a Tukey *post-hoc* test. Statistical significance was assigned as $p < 0.05$, with significance at $p < 0.01$ indicated with double asterisk or letters as indicated in the legends.

Results

Increased expression of xCT subunit by 6-Mix asbestos

Exposure to 6-Mix asbestos or to lipopolysaccharide (LPS) increased the expression of xCT on RAW cells as determined by flow cytometry (Figure 1A). Treatment with silica or wollastonite (a non-fibrogenic control fiber) did not significantly upregulate the constitutive level of expression. LPS was used as a positive control, since it has been shown to increase system x_c^- activity (Sato et al., 1995). CD98 was expressed at a high level on over 90% of RAW cells regardless of treatment, indicating that its expression is constitutive on these cells (data not shown). A representative flow cytometry histogram demonstrating upregulation of xCT following 6-Mix treatment on RAW macrophages is shown in Figure 1B.

Increased system x_c^- transport activity following 6-Mix exposure

The increased expression of the xCT subunit induced by 6-Mix also led to increased Na⁺-independent [³H]-L-Glu transport, indicating that the upregulated expression yields a functional system x_c^- (Figure 2A). [³H]-L-Glu transport activity increased in a dose-dependent manner at both 12 and 24 h, in response to asbestos. However, no increase in transport was seen with silica exposure (Figure 2B), consistent with the lack of surface expression upregulation from control values. In fact, there appears to be a decrease in the transporter activity at the higher concentrations (Figure 2B). We hypothesized that this might be due to increased stress and/or cell death, which are addressed below.

Because the cystine transport model assay used for Figure 2 forces glutamate import (due to application of high extracellular glutamate), we also tested the physiologically relevant efflux of glutamate out of the macrophages using the Amplex®-Red assay. Glutamate measured in the macrophage media following exposure to 6-Mix was significantly

increased, and this effect was inhibited by addition of (S)-4-CPG, the selective system x_c^- inhibitor (Figure 3).

NAC prevents asbestos-induced ROS and system x_c^- expression

To demonstrate that the upregulation of system x_c^- was due to ROS following 6-Mix exposure, RAW cells exposed to 6-Mix were first assayed for oxidative stress using the ROS-indicator dye, dichlorodihydrofluorescein diacetate (DCFDA). Indeed, as shown in Figure 4A, the fluorescence of cells loaded with 6-Mix asbestos and DCFDA increased above controls, indicating that ROS production is linked to asbestos exposure, consistent with earlier studies (Blake et al., 2007). This increase in fluorescence was prevented by providing *N*-acetylcysteine (NAC, 2.5 mM). NAC pretreatment also prevented the increase in system x_c^- expression (Figure 4B). These data demonstrate (albeit indirectly) that system x_c^- induction is linked to ROS generation following asbestos exposure. This is consistent with reports that xCT expression is regulated, at least in part, by redox signaling (Bannai et al., 1989; Sato et al., 2001).

System x_c^- inhibition increases asbestos-induced ROS and GSH depletion

The synthesis of intracellular GSH levels in macrophages is highly dependent upon the transport of cystine via system x_c^- (Sato et al., 2001). Inhibition of cystine transport by the non-transportable inhibitor (S)-4-CPG increased the asbestos-induced ROS (Figure 5A). As expected, inhibition of system x_c^- also led to a dramatic depletion in the intracellular levels of GSH (Figure 5B). Thus, through its link to GSH production, system x_c^- limits the production of ROS in macrophages exposed to asbestos and, when inhibited, leads to increased ROS.

System x_c^- activity helps protect macrophages from asbestos-induced cell death

To better understand the influence of system x_c^- on macrophage viability in response to ROS, asbestos exposure both with and without GSH-precursors and transport-inhibitors was examined. Exposure to 6-Mix asbestos in the absence of cystine resulted in a loss of macrophage viability in a dose-dependent fashion starting at 25 $\mu\text{g}/\text{cm}^2$ (Figure 6A, solid line with diamonds). Pretreatment with NAC (2.5 mM) resulted in the highest level of protection of RAW cells from asbestos-induced cell death, and cystine (2.5 mM) protected up to 50 $\mu\text{g}/\text{cm}^2$ asbestos. Conversely, extracellular glutamate increased the toxicity of asbestos (Figure 6A). We hypothesize that this is due to inhibition of the system x_c^- transporter, since glutamate is a substrate inhibitor of cystine transport (Patel et al., 2004). Although addition of NAC helped to protect the macrophages from silica-induced cell death, cystine did not, providing further evidence that macrophages do not use system x_c^- to protect from silica-induced cytotoxicity (Figure 6B), and thus do not use this pathway to replenish intracellular cystine. Figure 6C shows that while supplemental cystine was protective for asbestos-induced cell death, blocking system x_c^- with a specific inhibitor led to cell death even in the presence of cystine.

Discussion

This study reports a novel function for the system x_c^- glutamate/cystine transporter in protecting macrophages from oxidative stress caused by amphibole asbestos. System x_c^- is an amino acid antiporter that exchanges intracellular glutamate for extracellular cystine in macrophages (Figure 7). Import of cystine is critical for maintenance of redox status in many cell types. Cystine is reduced intracellularly to cysteine, which can serve as a redox modifier itself (Banjac et al., 2008), or as a precursor to glutathione. Glutathione (GSH), a major intracellular antioxidant, is composed of glutamate, glycine and cysteine, with the

bioavailability of cysteine being the limiting factor for glutathione synthesis (Townsend et al., 2003). Transport by system x_c^- is sodium-independent, unlike other glutamate transporters such as system x_{AG}^- , but is dependent on chloride (Waniewski & Martin, 1984). It is also ATP-independent, driven by concentration gradients of the amino acids. This study shows that RAW 264.7 cells express the heavy chain of system x_c^- , 4F2 (CD98) constitutively, and that xCT, which is the actual transporter, is inducible on these cells by amphibole asbestos (6-Mix) and LPS. The complete heterodimer is required for transport function (Sato et al., 1999). Since outside the central nervous system the normal gradients for cystine and glutamate strongly drive the system in the direction of cystine import, protein expression of xCT appears to be the limiting factor for the rate of transport activity. Early experiments on the system x_c^- illustrated that transport activity for cystine is induced by oxygen (Bannai et al., 1989), and Sato et al., (2001) have shown that under low oxygen conditions, xCT expression is reduced compared to normal cell culture oxygen conditions. Thus, oxygen affects system x_c^- transporter expression and activity, and it follows logically that particles that cause oxidative stress in macrophages, such as silica and asbestos (Vallyathan et al., 1992), would likewise induce its activity. Indeed, amphibole asbestos (Libby MT 6-Mix) increased protein expression of xCT, and caused induction of system x_c^- activity in a dose-dependent manner. Because the increased expression was inhibited by addition of cell-permeable cysteine (*N*-acetylcysteine, NAC), which can serve as an antioxidant (Voisin et al., 1987), this induction appears to be related to oxidative stress.

Unexpectedly, silica did not induce increased expression and transport activity, despite strong evidence of oxidative stress and glutathione depletion by silica (Hamilton et al., 2008; Hu et al., 2006). It is possible that the activation of cell death signaling pathways predominated with silica under these conditions, since it has been shown that cytokine release and cytotoxicity following silica exposure are independently regulated, and depend on time and dose considerations (Claudio et al., 1995). Our data show that starting around 50 $\mu\text{g}/\text{cm}^2$, silica did reduce cell viability in a dose-dependent fashion in the absence of cystine. However, the cells were not protected by supplementation by cystine or cell death exacerbated by glutamate, ruling out a role for system x_c^- . It is possible, therefore, that silica induces cell death through specific receptors (Claudio et al., 1995; Hamilton et al., 2008; Hamilton et al., 2006) which then lead to upregulation of other antioxidant pathways since pharmacological mimics for catalase and superoxide dismutase (SOD) blocked silica-induced cytokine production (Premasekharan et al., 2011). In fact, restoration of glutathione levels in macrophages can occur through a variety of anti-oxidant mechanisms. *In vivo*, the profile of antioxidant enzyme expression is different for silica versus amphibole asbestos exposure, suggesting different lung defense mechanisms to these dusts (Janssen et al., 1992). All of this is consistent with our data showing that NAC protected the macrophages from silica-induced cytotoxicity at concentrations above 50 $\mu\text{g}/\text{cm}^2$. However, apparently maintenance of cellular GSH in response to silica and subsequent ROS does not include uptake of cystine, since supplementation with cystine along with silica challenge did not protect the cells from silica cytotoxicity as it did for asbestos. Therefore, the system x_c^- is not induced by silica exposure, and in fact amino acid transport appeared to be somewhat inhibited in silica-treated macrophages (Figure 2). It is very unlikely that silica led to cystine uptake by another transporter for three reasons: (a) cystine was not protective for silica cytotoxicity in our system, (b) system x_c^- has been shown to transport the vast majority of cystine needed by murine macrophages (Watanabe & Bannai, 1987), and (c) our activity assays were performed in sodium-free buffer. In fact, the other glutamate transport system shown to be expressed on macrophages, system x_{AG}^- does not transport cystine and is sodium-dependent (Rimaniol et al., 2000, 2001). Therefore, our data showing that silica-exposed macrophages do not use the system x_c^- as an antioxidant system, unlike asbestos-exposed macrophages, are novel and interesting in view of differences in the antioxidant

responses and ultimate outcomes of these two exposures. Table 1, while not intended to be a complete review of the literature, summarizes some of the similarities and differences between responses to crystalline silica and amphibole asbestos.

The amount of glutamate found in media from untreated cells (Figure 3) is consistent with the moderate level of system x_c^- expression in cultured untreated macrophages (Figure 1). Watanabe and Bannai (1987) have shown that simply culturing cells in a typical cell culture incubator (with near atmospheric oxygen partial pressure as opposed to physiological levels *in vivo*) induces expression of the transporter. Because even the moderate level of glutamate in the cell-conditioned media from untreated cells was inhibited by pre-treatment with (S)-4-CPG (a selective non-substrate inhibitor) (Figure 3), system x_c^- clearly helps protect macrophages from the oxygen levels in culture by providing adequate cystine for maintenance of redox balance. Further evidence of protection from oxidative stress by system x_c^- activity comes from pretreatment with (S)-4-CPG, which led to significantly increased ROS and depleted GSH in the presence of amphibole asbestos (Figure 5).

The pharmacology of system x_c^- continues to be defined. Because glutamate acts at a number of receptors and transporters in the CNS and elsewhere, a certain degree of cross-reactivity is associated with all pharmacological agents developed thus far (Bridges et al., 2012). (S)-4-CPG, while originally developed as a group I metabotropic receptor antagonist (Bedingfield et al., 1995), is now one of the most potent system x_c^- inhibitors characterized to date (Ye et al., 1999; Patel et al., 2004). Unlike many of the other known substrate inhibitors of system x_c^- , (S)-4-CPG is ideal for inhibition studies as it essentially acts as a non-substrate (very weak substrate), allowing for inhibition of the cystine/glutamate exchange without leading to increased glutamate efflux (Patel et al., 2004). In this study, used purely as a pharmacological agent to block cystine or glutamate entry over brief periods, (S)-4-CPG allowed us to examine the short-term effects of near complete transporter inhibition at acute time points (Patel et al., 2004; Seib et al., 2011).

The ability of system x_c^- to protect macrophages from asbestos cytotoxicity was examined by culturing the cells with asbestos in the presence or absence of NAC, cystine, glutamate, or (S)-4-CPG (Figure 6). NAC maintained viability up to very high concentrations of asbestos, but cystine was protective (relative to cells in the absence of any cystine/cysteine) only up to 50 $\mu\text{g}/\text{cm}^2$. There are several possibilities for the loss of protection above that asbestos level. First, other cell death pathways or simple membrane disruption may be activated at higher levels of asbestos exposure in culture. Second, the amount of ROS generated by the asbestos may exceed the rate of cystine transport in these conditions. It has been shown that L-glutamine is also required for maximal activity of system x_c^- , as it is the primary source of intracellular glutamate (Piani and Fontana, 1994). In our experimental media, no L-glutamine was provided, mostly because we did not want extracellular conversion of glutamine to glutamate to confound glutamate measurements. However, this likely led to the cell death at higher concentrations of asbestos despite the presence of cystine. Third, assuming the cells did have adequate intracellular glutamate, export of that glutamate into the culture medium would eventually diminish the gradient driving the transporter and thus inhibit the ability to import cystine. Also, supplementing the cells with glutamate increased the cytotoxicity of asbestos at all concentrations, again likely due to its role as a transport inhibitor. Addition of (S)-4-CPG prevented cystine supplementation from protecting the cells, although NAC was able to do so even in the presence of the inhibitor (Figure 6c). NAC is cell permeable and does not require a transporter, and is not affected by blocking system x_c^- . These data demonstrate the critical role of cystine/cysteine in protection of cells from asbestos toxicity and emphasizes the importance of system x_c^- in that protection.

The fact that both LPS and asbestos upregulate system x_c^- may provide clues to the signaling involved. While it is possible that the 6-Mix asbestos used in this study was contaminated LPS, it was not detectable by PyroGene® Recombinant Factor C endotoxin assay (Cambrex, Walkersville, MD, USA), which is sensitive to 0.01 EU/ mL (data not shown). LPS signals through a complex that includes the toll-like receptor 4 (TLR-4), and also through non-receptor mechanisms. Both silica and asbestos are putative “ligands” for pattern recognition receptors such as scavenger receptors and TLRs (Hamilton et al., 2008; Resnick et al., 1993). While crystalline silica has been shown to signal through scavenger receptors such as MARCO (Hamilton et al., 2006), amphibole asbestos may signal through TLR4 (Pfau, unpublished data). Interestingly, others have reported effects that are similar for amphibole asbestos and LPS, but different for silica, such as induction of iNOS and of superoxide anion production through protein kinase C by asbestos and/or LPS but not silica (Lim et al., 1997; Quinlan et al., 1998), and induction of tyrosine phosphorylation by silica (Holian et al., 1994). These differences remain to be confirmed and clarified, and the current study provides a new pathway where differences in the effects of silica and asbestos may lead to a much better understanding of the molecular mechanisms that govern the ultimate outcomes of exposure.

Conclusions

In conclusion, system x_c^- is functionally upregulated in cultured macrophages by amphibole asbestos exposure, apparently in response to oxidative stress. The uptake of cystine is associated with decreased oxidative stress and increased survival of the macrophages. Silica exposure did not induce system x_c^- , suggesting that silica-exposed cells may use alternate strategies for dealing with the oxidative stress of the exposure. Therefore, system x_c^- may play an integral role in regulating immune outcomes following asbestos exposure by enhancing macrophage survival. More research is needed to determine the specific downstream events impacted by system x_c^- , particularly cytokine expression that might dysregulate the overall immune response. Use of siRNA to selectively down-regulate xCT is an approach being explored for this purpose.

No previous study has specifically explored the role of system x_c^- in macrophages exposed to asbestos, nor compared this with responses to silica. Therefore, this novel data reveals a mechanism through which cells trigger survival mechanisms and downstream signaling cascades that can affect ultimate outcomes of exposure. Because oxidative stress is an important component of the pathology resulting from asbestos exposure in humans, system x_c^- may be an attractive therapeutic target for early intervention in asbestos-induced disease.

Acknowledgments

The Authors gratefully acknowledge the Center for Structural and Functional Neuroscience (P20 RR015583 (CoBRE) and the Center for Environmental Health Sciences P20 NCRR 017670 (CoBRE) at the University of Montana for their support of this project. The work was also supported by these grants from NIH: INBRE P20 RR016454, R15 ES018986-01.

Literature Cited

- Arakawa H, Honma K, Saito Y, Shida H, Morikubo H, Suganuma N, Fujioka M. Pleural disease in silicosis: pleural thickening, effusion, and invagination. *Radiology*. 2005; 236:685–693. [PubMed: 16040925]
- Banjac A, Perisic T, Sato H, Seiler A, Bannai S, Weiss N, Kölle P, Tschoep K, Issels RD, Daniel PT, Conrad M, Bornkamm GW. The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death. *Oncogene*. 2008; 27:1618–1628. [PubMed: 17828297]

- Bannai S, Sato H, Ishii T, Sugita Y. Induction of cystine transport activity in human fibroblasts by oxygen. *J Biol Chem.* 1989; 264:18480–18484. [PubMed: 2808385]
- Bedingfield JS, Kemp MC, Jane DE, Tse HW, Roberts PJ, Watkins JC. Structure-activity relationships for a series of phenylglycine derivatives acting at metabotropic glutamate receptors (mGluRs). *Br J Pharmacol.* 1995; 116:3323–3329. [PubMed: 8719814]
- Biswas R, Bunderson-Schelvan M, Holian A. Potential role of the inflammasome-derived inflammatory cytokines in pulmonary fibrosis. *Pulm Med.* 2011; 2011:105707. [PubMed: 21660282]
- Blake DJ, Bolin CM, Cox DP, Cardozo-Pelaez F, Pfau JC. Internalization of Libby amphibole asbestos and induction of oxidative stress in murine macrophages. *Toxicol Sci.* 2007; 99:277–288. [PubMed: 17578862]
- Blake DJ, Wetzel SA, Pfau JC. Autoantibodies from mice exposed to Libby amphibole asbestos bind SSA/Ro52-enriched apoptotic blebs of murine macrophages. *Toxicology.* 2008; 246:172–179. [PubMed: 18295955]
- Bridges RJ, Natale NR, Patel SA. System xc⁻ cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *Br J Pharmacol.* 2012; 165:20–34. [PubMed: 21564084]
- Claudio E, Segade F, Wróbel K, Ramos S, Lazo PS. Activation of murine macrophages by silica particles *in vitro* is a process independent of silica-induced cell death. *Am J Respir Cell Mol Biol.* 1995; 13:547–554. [PubMed: 7576690]
- Cooper GS, Gilbert KM, Greidinger EL, James JA, Pfau JC, Reinlib L, Richardson BC, Rose NR. Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease. *Environ Health Perspect.* 2008; 116:695–702. [PubMed: 18560522]
- Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science.* 2008; 320:674–677. [PubMed: 18403674]
- Elshazley M, Shibata E, Hisanaga N, Ichihara G, Ewis AA, Kamijima M, Ichihara S, Sakai K, Sato M, Kondo M, Hasegawa Y. Pleural plaque profiles on the chest radiographs and CT scans of asbestos-exposed Japanese construction workers. *Ind Health.* 2011; 49:626–633. [PubMed: 21828957]
- Hamilton RF Jr, Thakur SA, Holian A. Silica binding and toxicity in alveolar macrophages. *Free Radic Biol Med.* 2008; 44:1246–1258. [PubMed: 18226603]
- Hamilton RF Jr, Thakur SA, Mayfair JK, Holian A. MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice. *J Biol Chem.* 2006; 281:34218–34226. [PubMed: 16984918]
- Holian A, Kelley K, Hamilton RF Jr. Mechanisms associated with human alveolar macrophage stimulation by particulates. *Environ Health Perspect.* 1994; 102(Suppl 10):69–74. [PubMed: 7705310]
- Holley JA, Janssen YM, Mossman BT, Taatjes DJ. Increased manganese superoxide dismutase protein in type II epithelial cells of rat lungs after inhalation of crocidolite asbestos or cristobalite silica. *Am J Pathol.* 1992; 141:475–485. [PubMed: 1323223]
- Hu S, Zhao H, Al-Humadi NH, Yin XJ, Ma JK. Silica-induced apoptosis in alveolar macrophages: evidence of *in vivo* thiol depletion and the activation of mitochondrial pathway. *J Toxicol Environ Health Part A.* 2006; 69:1261–1284. [PubMed: 16754540]
- Janssen YM, Marsh JP, Absher MP, Hemenway D, Vacek PM, Leslie KO, Borm PJ, Mossman BT. Expression of antioxidant enzymes in rat lungs after inhalation of asbestos or silica. *J Biol Chem.* 1992; 267:10625–10630. [PubMed: 1316905]
- Lim Y, Kim SH, Kim KA, Oh MW, Lee KH. Involvement of protein kinase C, phospholipase C, and protein tyrosine kinase pathways in oxygen radical generation by asbestos-stimulated alveolar macrophage. *Environ Health Perspect.* 1997; 105(Suppl 5):1325–1327. [PubMed: 9400746]
- Maeda M, Nishimura Y, Kumagai N, Hayashi H, Hatayama T, Katoh M, Miyahara N, Yamamoto S, Hirastuka J, Otsuki T. Dysregulation of the immune system caused by silica and asbestos. *J Immunotoxicol.* 2010; 7:268–278. [PubMed: 20849352]
- Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 1983; 52:711–760. [PubMed: 6137189]
- Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med.* 1998; 157:1666–1680. [PubMed: 9603153]

- Murata Y, Shimamura T, Hamuro J. The polarization of T(h)1/ T(h)2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production. *Int Immunol*. 2002; 14:201–212. [PubMed: 11809739]
- Patel SA, Warren BA, Rhoderick JF, Bridges RJ. Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine. *Neuropharmacology*. 2004; 46:273–284. [PubMed: 14680765]
- Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA*. 1998; 95:3071–3076. [PubMed: 9501217]
- Pfau JC, Schneider JC, Archer AJ, Sentissi J, Leyva FJ, Cramton J. Environmental oxygen tension affects phenotype in cultured bone marrow-derived macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2004; 286:L354–L362. [PubMed: 14527932]
- Pfau JC, Sentissi JJ, Weller G, Putnam EA. Assessment of autoimmune responses associated with asbestos exposure in Libby, Montana, USA. *Environ Health Perspect*. 2005; 113:25–30. [PubMed: 15626643]
- Piani D, Fontana A. Involvement of the cystine transport system xc- in the macrophage-induced glutamate-dependent cytotoxicity to neurons. *J Immunol*. 1994; 152:3578–3585. [PubMed: 8144936]
- Premasekharan G, Nguyen K, Contreras J, Ramon V, Leppert VJ, Forman HJ. Iron-mediated lipid peroxidation and lipid raft disruption in low-dose silica-induced macrophage cytokine production. *Free Radic Biol Med*. 2011; 51:1184–1194. [PubMed: 21741475]
- Quinlan TR, Berube KA, Hacker MP, Taatjes DJ, Timblin CR, Goldberg J, Kimberley P, O'Shaughnessy P, Hemenway D, Torino J, Jimenez LA, Mossman BT. Mechanisms of asbestos-induced nitric oxide production by rat alveolar macrophages in inhalation and *in vitro* models. *Free Radic Biol Med*. 1998; 24:778–788. [PubMed: 9586808]
- Resnick D, Freedman NJ, Xu S, Krieger M. Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos. *J Biol Chem*. 1993; 268:3538–3545. [PubMed: 8381434]
- Rimaniol AC, Haïk S, Martin M, Le Grand R, Boussin FD, Dereuddre-Bosquet N, Gras G, Dormont D. Na⁺-dependent high-affinity glutamate transport in macrophages. *J Immunol*. 2000; 164:5430–5438. [PubMed: 10799909]
- Rimaniol AC, Mialocq P, Clayette P, Dormont D, Gras G. Role of glutamate transporters in the regulation of glutathione levels in human macrophages. *Am J Physiol, Cell Physiol*. 2001; 281:C1964–C1970. [PubMed: 11698255]
- Sato H, Fujiwara K, Sagara J, Bannai S. Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide. *Biochem J*. 1995; 310 (Pt 2):547–551. [PubMed: 7654193]
- Sato H, Kuriyama-Matsumura K, Hashimoto T, Sasaki H, Wang H, Ishii T, Mann GE, Bannai S. Effect of oxygen on induction of the cystine transporter by bacterial lipopolysaccharide in mouse peritoneal macrophages. *J Biol Chem*. 2001; 276:10407–10412. [PubMed: 11136724]
- Sato H, Tamba M, Ishii T, Bannai S. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J Biol Chem*. 1999; 274:11455–11458. [PubMed: 10206947]
- Schneider JC, Card GL, Pfau JC, Holian A. Air pollution particulate SRM 1648 causes oxidative stress in RAW 264.7 macrophages leading to production of prostaglandin E2, a potential Th2 mediator. *Inhal Toxicol*. 2005; 17:871–877. [PubMed: 16282164]
- Seib TM, Patel SA, Bridges RJ. Regulation of the system x(c)-cystine/glutamate exchanger by intracellular glutathione levels in rat astrocyte primary cultures. *Glia*. 2011; 59:1387–1401. [PubMed: 21590811]
- Shen HM, Zhang Z, Zhang QF, Ong CN. Reactive oxygen species and caspase activation mediate silica-induced apoptosis in alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2001; 280:L10–L17. [PubMed: 11133490]
- Shukla A, Stern M, Lounsbury KM, Flanders T, Mossman BT. Asbestos-induced apoptosis is protein kinase C delta-dependent. *Am J Respir Cell Mol Biol*. 2003; 29:198–205. [PubMed: 12626342]

- Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother.* 2003; 57:145–155. [PubMed: 12818476]
- Vallyathan V, Mega JF, Shi X, Dalal NS. Enhanced generation of free radicals from phagocytes induced by mineral dusts. *Am J Respir Cell Mol Biol.* 1992; 6:404–413. [PubMed: 1312851]
- Voisin C, Aerts C, Wallaert B. Prevention of *in vitro* oxidant-mediated alveolar macrophage injury by cellular glutathione and precursors. *Bull Eur Physiopathol Respir.* 1987; 23:309–313. [PubMed: 2825864]
- Waniewski RA, Martin DL. Characterization of L-glutamic acid transport by glioma cells in culture: evidence for sodium-independent, chloride-dependent high affinity influx. *J Neurosci.* 1984; 4:2237–2246. [PubMed: 6207276]
- Warren BA, Patel SA, Nunn PB, Bridges RJ. The Lathyrus excitotoxin beta-N-oxalyl-L-alpha, beta-diaminopropionic acid is a substrate of the L-cystine/L-glutamate exchanger system xc-. *Toxicol Appl Pharmacol.* 2004; 200:83–92. [PubMed: 15476861]
- Watanabe H, Bannai S. Induction of cystine transport activity in mouse peritoneal macrophages. *J Exp Med.* 1987; 165:628–640. [PubMed: 2880923]
- Ye ZC, Rothstein JD, Sontheimer H. Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. *J Neurosci.* 1999; 19:10767–10777. [PubMed: 10594060]
- Zaghi G, Koga F, Nisihara RM, Skare TL, Handar A, Rosa Utiyama SR, Silva MB. Autoantibodies in silicosis patients and in silica-exposed individuals. *Rheumatol Int.* 2010; 30:1071–1075. [PubMed: 19705119]

Figure 1.

(A) Expression of xCT on RAW 264.7 cells by flow cytometry, showing percent positive (above secondary antibody alone). Cells were treated with 35 $\mu\text{g}/\text{cm}^2$ of 6-Mix asbestos, silica, wollastonite, or 1 $\mu\text{g}/\text{mL}$ LPS for 24 h. * $p < 0.05$ compared to no treatment, $n = 3$ in each group. (B) Representative histogram shows untreated cells (gray) at 14.2% positive and 6-Mix asbestos (black line) is 67.7% positive. The M1 gate is set on background staining with the secondary antibody only.

Figure 2.

(A) Asbestos increased [^3H]-L-glutamate transport in a dose dependent manner (10, 50 100 $\mu\text{g}/\text{cm}^2$), using a radiolabeled glutamate uptake assay, as described in “Materials and Methods”. (B) Using the same technique as in A, silica time course/dose response for [^3H]-L-glutamate transport. * $p < 0.05$, ** $p < 0.01$, using t -test compared to control, or one-way ANOVA, $n = 3$ in each group.

Figure 3.

Asbestos increased L-glutamate in the media of RAW 264.7 cells. The RAW cells were cultured in media free of glutamate for 2 days in the absence or presence of 35 $\mu\text{g}/\text{cm}^2$ asbestos, 1 $\mu\text{g}/\text{mL}$ LPS, and (S)-4-CPG (500 μM). The media was collected, centrifuged to remove any cells or debris, and assayed for L-glutamic acid using the Amplex®-Red glutamic acid assay. L-Glutamate was quantified against a standard curve using a fluorescence microtiter plate reader, ex 530 nm/em 590 nm. * $p < 0.05$ compared to no treatment, $n = 5$ in each group; ** $p < 0.01$ compared to no CPG with same treatment. CPG, carboxyphenylglycine.

Figure 4. RAW 264.7 cells treated for 24 h with 35 $\mu\text{g}/\text{cm}^2$ 6-Mix increased ROS, as measured by DCFDA fluorescence. (A) 6-Mix asbestos increased ROS in RAW cells, which was inhibited by addition of NAC (2.5 mM) or cystine (100 μM). $n = 6$ in each group, ^a $p < 0.05$ compared to no treatment, ^b $p < 0.05$ compared to 6-Mix asbestos only. (B) Percent positive for xCT expression with asbestos treatment, in the presence or absence of NAC (2.5 mM). $n = 4$ in each group, * $p < 0.05$ compared to no treatment. ROS, reactive oxygen species; DCFDA, dichlorofluorescein diacetate; NAC, *N*-acetylcysteine.

Figure 5.

(A) ROS production increased with addition of system x_c^- inhibitor (S)-4-CPG (500 μ M) in the presence of 6-Mix asbestos. (B) Glutathione was measured following 24 h treatment with 35 μ g/cm² 6-Mix, in the presence or absence of NAC or (S)-4-CPG. $n = 4$ in each group, ^a $p < 0.05$ compared to no treatment, ^b $p < 0.05$ compared to asbestos treatment. ROS, reactive oxygen species; NAC, *N*-acetylcysteine; (S)-4-CPG, *S*-4-carboxyphenylglycine.

Figure 6.

(A) Supplementing with cystine protected the cells from asbestos toxicity (measured by CellTiter Blue) up to 50 $\mu\text{g}/\text{cm}^2$. RAW 264.7 cells were plated in media containing no cystine or glutamate, unless otherwise indicated. Pretreatment with *N*-acetylcysteine protected the cells at all concentrations, relative to cells treated with asbestos or silica only. (B) Cystine or glutamate had no effect on silica-induced cytotoxicity. (C) In a separate experiment, cystine is again shown to be protective from asbestos cytotoxicity, and this protection is lost by addition of (S)-4-CPG. For A, B, $n = 8$, $*p < 0.05$ compared to asbestos (or silica) only at the same concentration of asbestos or silica. For C, $n = 3$, $^ap < 0.01$ compared to no treatment, $^bp < 0.01$ compared to black bars (no antioxidant added) by unpaired *t*-test. (S)-4-CPG, *S*-4-carboxyphenylglycine.

Figure 7.

A simplified representation of the system x_c^- transporter and exchange of amino acids for the purpose of cystine import and protection from oxidative stress. System x_c^- is a heterodimer of the proteins xCT and CD98, which is the constitutively expressed heavy chain (4F2).

Table 1

Summary of effects of crystalline silica and amphibole asbestos.

	Amphibole asbestos	Crystalline silica	References
Pulmonary inflammation	Yes	Yes	Holley et al. (1992); Mossman and Churg (1998)
ROS production by macrophages	Yes	Yes	Blake et al. (2007); Vallyathan et al. (1992); Hu et al. (2006)
Apoptosis in macrophages	Yes	Yes	Hamilton et al. (2008); Shen et al. (2001); Blake et al. (2008); Shukla et al. (2003)
Superoxide production by macrophages	Yes, via PLC/PKC pathway	Mixed results	Holian et al. (1994); Premasekharan et al. (2011); Lim et al. (1997); Quinlan et al. (1998)
Lung Mn Superoxide Dismutase (MnSOD) expression & activity	Yes	No (some mixed results)	Holley et al. (1992); Janssen et al. (1992)
NALP3 Inflammasome activation	Yes	Yes	Dostert et al. (2008); Biswas et al. (2011)
Pro-fibrotic	Yes, asbestosis	Yes, silicosis	Mossman and Churg (1998) (Reviewed)
Radiography	Bilateral diffuse interstitial fibrosis, lower zones	Silicotic nodules, upper zones	Mossman and Churg (1998) (Reviewed)
Pleural disease	Common	Less common; found in advanced disease	Mossman and Churg (1998); Arakawa et al. (2005); Elshazley et al. (2011)
Carcinogenic	Yes, with mesothelioma	Yes, without mesothelioma	Maeda et al. (2010)
Anti-nuclear antibodies	Yes	Yes (some mixed results)	Pfau et al. (2005); Cooper et al. (2008); Zaghi et al. (2010)