Supporting Information

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SI Materials and Methods

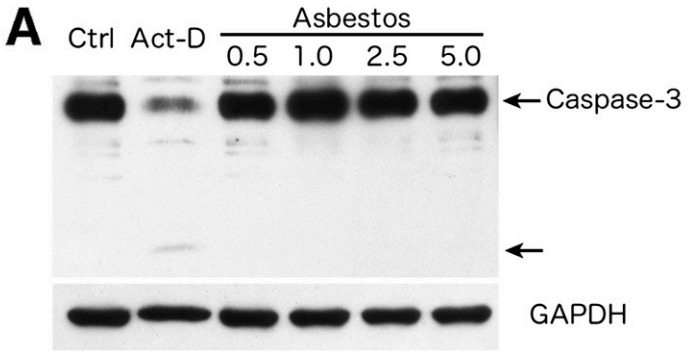
Cohort Studied for Serological Levels of HMGB1: Characterization of Exposure. The sera of asbestos-exposed individuals that we tested for HMGB1 (collected under an IRB–approved protocol to H.I.P.) were randomly chosen from a cohort of asbestos-exposed individuals that was described in detail in a previous publication (1). As stated in that publication, asbestos-exposed individuals have previously worked in an asbestos-related trade. Both exposure to asbestos and the duration of exposure were documented with the use of the American Thoracic Society Division of Lung Diseases adult questionnaire. More information regarding the exposure time and the occupation of these individuals has been previously published (1). As reported by Pass et al. (1), radiographic evidence of fibrosis was found in 33% and pleural plaques were found in 72% of the individuals in this cohort. None of these individuals had mesothelioma or other type of malignancy. The characteristics of the cohort of heavy smokers are shown in Table S1. (These individuals participated in an IRB-approved chemoprevention trial led by H.I.P.) Before treatment, all of these individuals had had bronchoscopies with bronchoscopic biopsies revealing mild to severe dysplasia. Serum was collected before treatment.

RNA Preparation and Quantitative Real-Time PCR. HM were exposed to crocidolite asbestos (5 μg/cm^2) for 6, 12, and 24 h. Total RNA was isolated using RNeasy kit (Qiagen) and treated with RNase-free DNase. Quantitative real-time PCR was performed as described elsewhere (2). The following primers were used: HMGB1, 5′-CCCAAAAGCGTGAGCTTAAAAT-3′ and 5′-AGTCTCGTTTCCTGAGCAGTCC-3′; and 18S, 5′-TGATTAAGTCCCTGCCCTTTGT-3′ and 5′-TCAAGTTCGACCGTCTTCTCAG-3′. Relative HMGB1 mRNA expression levels for each sample were normalized to the internal control 18S rRNA. Relative PARP-1 mRNA expression levels for each sample were normalized to the housekeeping gene ACTB. PARP-1 and ACTB primers were purchased from Qiagen.

Immunohistochemistry. Immunohistochemistry was performed in both mouse and hamster tissues with rabbit polyclonal IgG purified anti-HMGB1 and anti–TNF-α antibodies (Abcam), both diluted 1:200 (Fig. 5 and Fig. S4). The inflammatory infiltrate around asbestos deposits was characterized in mice using the following antibodies: antimacrophage markers used: F4/80 (MCA497, Serotec), rat IgG2b (diluted 1:400), rabbit polyclonal pancytokeratin antibody (Santa Cruz) was used to stain mesothelial cells. Matching rat IgG isotype control and rabbit IgG isotype control were also used to rule out nonspecific staining (Fig. S4). CD4, CD8, and CD20 antibodies were also used to further identify the cells around asbestos deposits. Immunohistochemistry was independently performed by the investigators at the University of Hawaii and the University of Chicago, and the results were similar.

1. Pass HI, et al. (2005) Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. *N Engl J Med* 353:1564–1573.

2. Yang H, et al. (2006) TNF-alpha inhibits asbestos-induced cytotoxicity via a NF-kappaB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc Natl Acad Sci USA* 103:10397–10402.



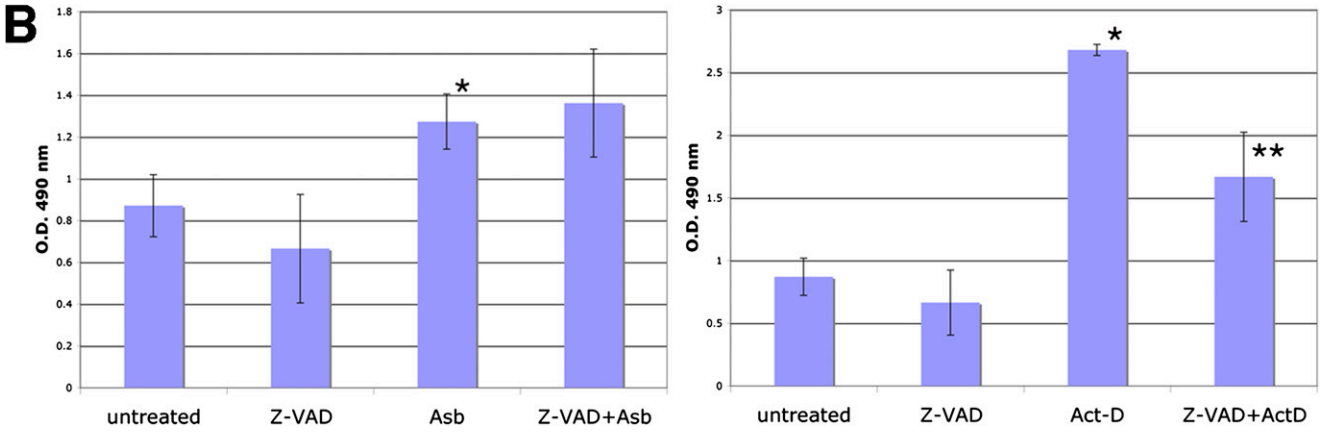


Fig. S1. (*A*) Caspase-3 cleavage is not detected in HM undergoing asbestos-induced death. HM were exposed to increasing amounts of crocidolite (0.5, 1.0, 2.5, and 5.0 μg/cm^2) or to actinomycin D (positive control for apoptosis, 0.1 μM) for 24 h. Cell extracts were analyzed by Western blot using caspase-3 antibody. GAPDH was used as a loading control. Actinomycin D caused caspase-3 cleavage, a marker of apoptotic cell death. No caspase-3 and PARP cleavage (Fig. 1) were detected in HM exposed to asbestos. (*B*) z-VAD-fmK does not inhibit asbestos-induced cytotoxicity. HM were incubated with or without caspase pan-inhibitor z-VAD-fmk (100 μM) for 1 h, followed by addition of actinomycin D (0.05 μM) or crocidolite asbestos (representative results showing average of three separate experiments at a dose of 10.0 μg/cm^2) for 24 h. Cytotoxicity was calculated by measuring the amount of LDH released from the cytosol of damaged cells into the supernatant. Results show that the z-VAD-fmk does not inhibit asbestos-induced cytotoxicity (left), although it does inhibit the actinomycin D-induced cell death (right). *Significantly different compared with untreated cells ($P < 0.05$); **significantly different compared with cells without z-VAD-fmk pretreatment ($P < 0.05$).

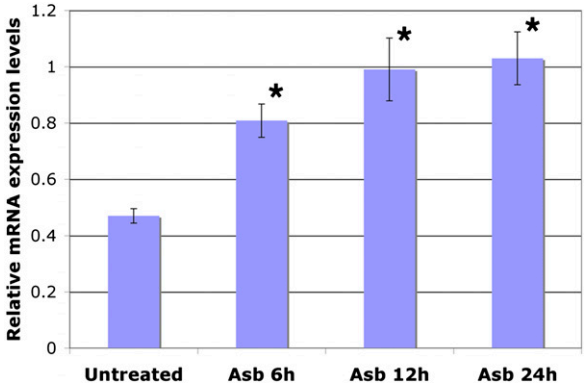


Fig. S2. Quantitative RT-PCR. Transcripts of PARP1 are increased by asbestos exposure (5 μg/cm^2). *Significantly different compared with untreated HM ($P < 0.05$).

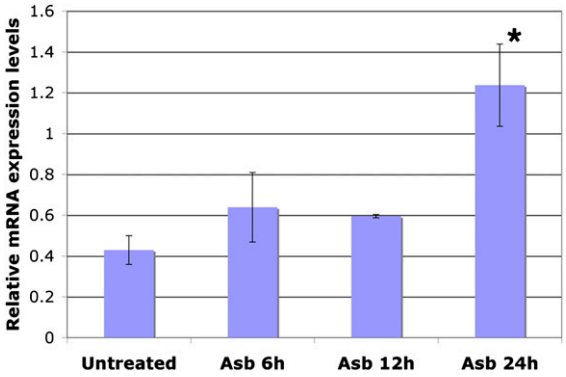


Fig. S3. Quantitative RT-PCR. Transcripts of HMGB1 are increased by asbestos exposure (5 μg/cm^2). *Significantly different compared with untreated HM ($P < 0.05$).

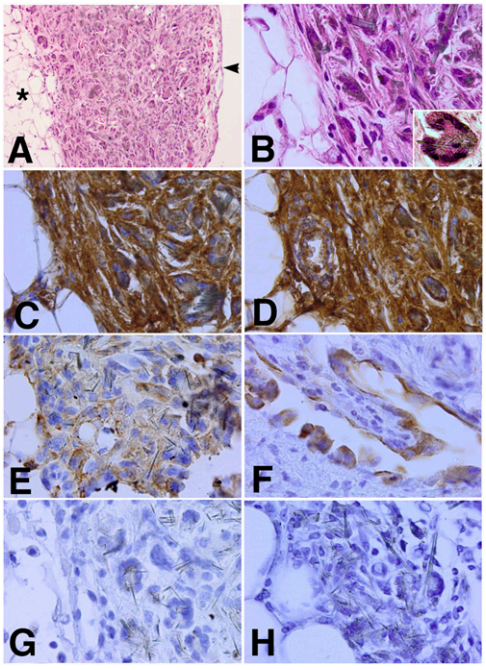


Fig. S4. Immunohistochemical analyses in murine specimens. (*A*) H&E staining shows a nodular area of chronic inflammation. (Original magnification: 100×.) Asterisk indicates adipose tissue; arrow pointing indicates mesothelial cells. Space between single layer of mesothelial cells (peritoneum) and underneath adipose tissue is expanded by a nodule of inflammatory reaction in the areas of asbestos deposits containing mostly macrophages. (*B*) H&E staining. (Original magnification: 400×.) Asbestos fibers are surrounded by cells with morphology consistent with macrophages. Several of these cells contained asbestos fibers or part of these fibers in their cytoplasm. Right corner: giant multinucleated cell containing asbestos fibers in cytoplasm. (Original magnification: 1,000×). (*C*) HMGB1 staining. (Original magnification: 400×.) (*D*) TNF-α staining. (Original magnification: 400×.) (*E*) Macrophage marker F4/80 staining. (Original magnification: 400×.) (*F*) Cytokeratin staining. (Original magnification: ×400.) Pan-cytokeratin stain highlighted the surface mesothelial cells lining the peritoneum. (*G*) Rat IgG isotype control staining. (Original magnification: 400×.). (*H*) Rabbit IgG isotype control staining. (Original magnification: 400×.)

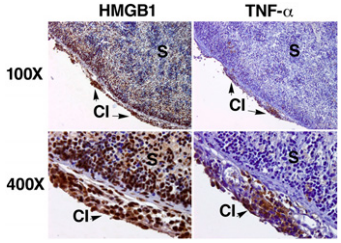


Fig. S5. Immunohistochemical analyses in hamster specimens. (Original magnification: top row, 100×; lower row, 400×.) (*Left*) HMGB1 staining. (*Right*), TNF-α staining. CI, chronic inflammation; S, spleen.

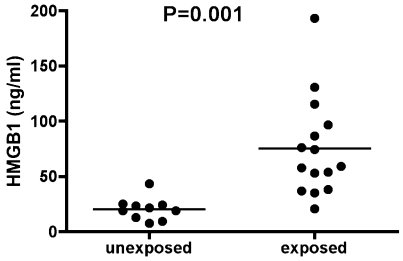


Fig. S6. HMGB1 levels in serum from individuals with or without asbestos exposure. Bars show mean of HMGB1 levels. Mean serum HMGB1 level in asbestos-exposed individuals was significantly higher than in nonexposed controls (P = 0.001).

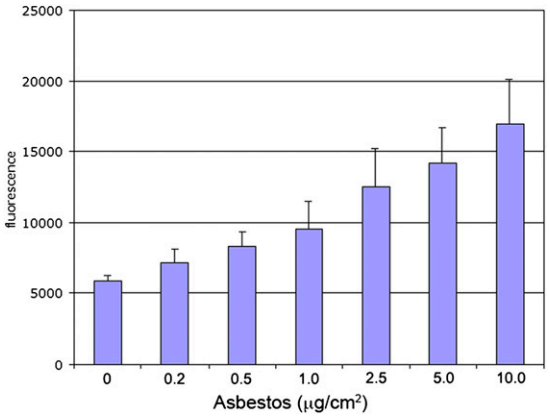


Fig. S7. Asbestos induces H_2O_2 release from HM in a dose-dependent manner (*Discussion*). H_2O_2 release from HM was determined using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes). Approximately 1.5×10^4 HM were incubated with asbestos at 0, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0 μg/cm^2 for 5 h. Asbestos stimulated H_2O_2 release from HM in a dose-dependent manner (representative results showing average of three separate experiments).

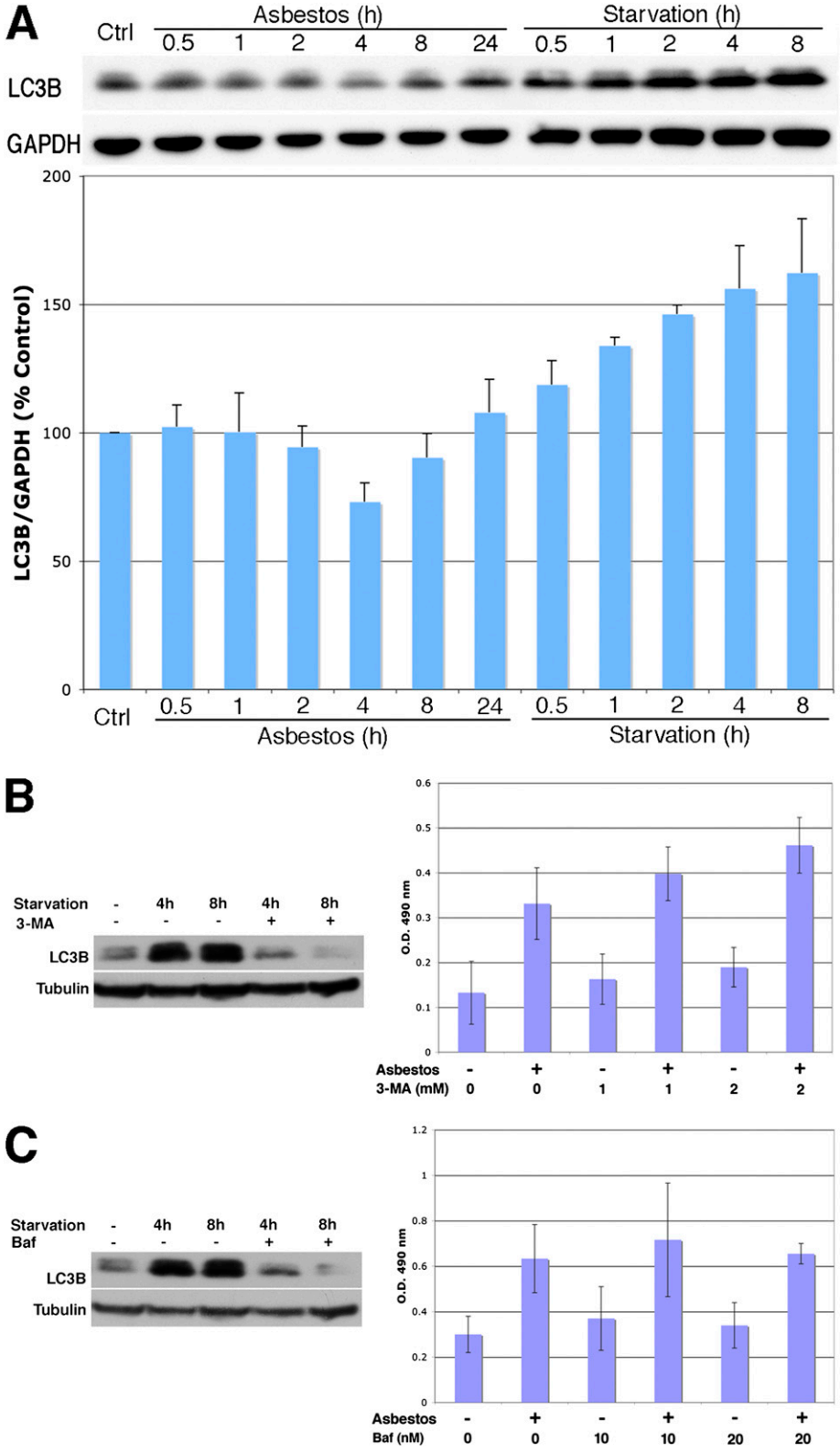


Fig. S8. Autophagy is not involved in asbestos-induced cell death. (*A*) Western blot of LC3B shows that asbestos does not induce autophagy. LC3 is widely used to monitor autophagy. The amount of LC3-II is closely related to the number of autophagosomes, serving as a good indicator of autophagic activity. Expression levels of LC3B were checked in HM exposed to 5 $\mu g/cm^2$ of crocidolite asbestos for 0.5, 1, 2, 4, 8, and 24 h. Starvation was used as positive control for autophagy. Note that the levels of LC3B are induced by starvation but not by asbestos exposure. GAPDH was used as loading control. Densitometry was performed to quantify the changes of LC3B levels. (*B*) 3-Methyladenine (3-MA) efficiently inhibits starvation-induced autophagy and does not influence asbestos-induced cell death. (*Left*) Western blot of LC3B. LC3B, an indicator of autophagic activity, is induced by starvation (positive control for autophagy). (*Right*) Autophagy inhibitor 3-MA does not inhibit asbestos induced cell death. HM were incubated with or without autophagy inhibitor 3-MA (1 or 2 mM) for 1 h, followed by addition of asbestos for 24 h. Cytotoxicity was measured by LDH assay. Results show that 3-MA does not inhibit asbestos-induced cell death. (*C*) Identical results were observed using Bafilomycin A1 (Baf), another inhibitor of autophagy. (*Left*) Western blot of LC3B. (*Right*) LDH assay measuring cytotoxicity. Results show that autophagy inhibitor Bafilomycin A1 also does not inhibit asbestos-induced cell death.

Table S1. Characteristics of smoker cohort tested for HMGB1 serum levels

Subject ID no.	Sex	Age, y	Packs/y
1	Male	73	45
2	Male	57	90
3	Male	73	45
4	Male	53	35
5	Male	69	100
6	Male	50	30
7	Male	68	50
8	Male	74	na
9	Male	64	35
10	Male	62	68
11	Male	69	100
12	Male	60	39
13	Male	60	60
14	Male	60	39
15	Male	59	50
16	Male	54	36
17	Male	53	32
18	Male	53	32
19	Female	64	40
20	Male	53	35

Additional details are provided in Fig. 6. ID, identification.