Original Article

Distinct affinity of nuclear proteins to the surface of chrysotile and crocidolite

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The inhalation of asbestos is a risk factor for the development of malignant mesothelioma and lung cancer. Based on the broad surface area of asbestos fibers and their ability to enter the cytoplasm and nuclei of cells, it was hypothesized that proteins that adsorb onto the fiber surface play a role in the cytotoxicity and carcinogenesis of asbestos fibers. However, little is known about which proteins adsorb onto asbestos. Previously, we systematically identified asbestos-interacting proteins and classified them into eight sub-categories: chromatin/nucleotide/RNA-binding proteins, ribosomal proteins, cytoprotective proteins, cytoskeleton-associated proteins, histones and hemoglobin. Here, we report an adsorption profile of proteins for the three commercially used asbestos compounds: chrysotile, crocidolite and amosite. We quantified the amounts of adsorbed proteins by analyzing the silver-stained gels of sodium dodecyl sulfate-polyacrylamide gel electrophoresis with ImageJ software, using the bands for amosite as a standard. We found that histones were most adsorptive to crocidolite and that chromatin-binding proteins were most adsorptive to chrysotile. The results suggest that chrysotile and crocidolite directly interact with chromatin structure through different mechanisms. Furthermore, RNA-binding proteins preferably interacted with chrysotile, suggesting that chrysotile may interfere with transcription and translation. Our results provide novel evidence demonstrating that the specific molecular interactions leading to carcinogenesis are different between chrysotile and crocidolite.

***Key Words*:** **asbestos, adsorptive proteins, carcinogenesis, DNA injury, ImageJ**

Asbestos was previously considered a "miraculous mineral" and used for a variety of purposes in manufacturing and construction because of its outstanding physicochemical characteristics. It was tough, durable, lightweight, fire-resistant and very inexpensive.[1,2] As indicated by previous studies, however, the chronic inhalation of asbestos causes multiple respiratory diseases, including asbestosis, lung cancer and mesothelioma.[3–5] In Japan, it is estimated that the number of mesothelioma patients will increase each year, reaching a maximum in 2025.[6] Considering that the use of asbestos is not yet banned in many developing countries,[7,8] it is of social and global importance to elucidate the mechanism of asbestos-induced carcinogenesis.

We previously studied four possible mechanisms in the carcinogenicity of asbestos.[2,9–12] These mechanisms included free radical generation,[13–17] mitotic disturbance,[18–22] molecular adsorption[23–27] and chronic inflammation.[28–32] Several key factors should be considered in evaluating the mechanism of carcinogenicity of asbestos. First, the surface of asbestos can act as a catalyst to produce free radicals via the Fenton reaction. Some amphibole asbestos, such as crocidolite and amosite, include iron as an integral component of their mineral structure, and other types of asbestos contain iron as a surface impurity.[33] Second, asbestos fibers physically interact with chromosomes directly and/or mitotic spindles, thereby inducing chromosomal aberrations. This is indeed a specific event caused by fibrous particles. The early induction of chromosomal aberrations was observed in Syrian hamster cells exposed to asbestos.[34] Third, the surface of asbestos fibers adsorb various endogenous and/or exogenous molecules, including DNA,[12] proteins[26,27] and chemicals,[24] thereby disturbing intracellular signaling pathways. Finally, the needle-like structure[35] of asbestos fibers and their extremely high biopersistence[36] lead to the continuous activation of macrophages and induce chronic inflammation. Cytokines and free radicals that are secreted by activated macrophages may contribute to initiation and promotion during carcinogenesis.[29]

Among the four proposed mechanisms, we hypothesize that molecular adsorption will affect the other three mechanisms. For example, asbestos fibers showed higher activity as a catalyst after hemoglobin adsorption, suggesting that the adsorptive properties resulting from the large surface area of asbestos contributes to free radical generation.[12] Furthermore, the adsorption of cytoskeletal proteins and histones to asbestos may increase the risk of mitotic disturbance. However, information about specific proteins that bind to the surface of asbestos is still unknown. Thus, in the present study, we quantified the amount of adsorptive proteins using densitometry of silver-stained gels to evaluate the difference in adsorptive characteristics between each type of asbestos.

Materials and Methods

Materials. We analyzed the pooled data from silver-stained gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described[12] using the method developed by MacCorkle *et al.*[27] Briefly, three types of asbestos fibers (chrysotile, Chry; crocidolite, Cro; amosite, Amo; all acquired from Union for International Cancer Control; Geneva, Switzerland) were incubated with lysates from MeT5A mesothelial cells (American Type Culture Collection; Manassas, VA), or organs (lung, kidney, liver, brain and tunica vaginalis) isolated from male Wistar rats. After washing several times and centrifuging the asbestos, proteins that were adsorbed onto asbestos fibers were recovered by the addition of SDS-PAGE sample buffer and boiling. The proteins were then separated by SDS-PAGE, and the gel was subjected to silver staining. We excised the bands from the gels and identified the proteins by matrix-assisted laser desorption ionization-time of flight mass spectro-

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metry (MALDI-TOF/MS). The entire list of proteins was published in a previous report (refer to the supplementary table online[12]). This experiment was approved by the animal experiment committee at Nagoya University Graduate School of Medicine.

Densitometry of silver-stained gels. We used ImageJ software (http://rsb.info.nih.gov/ij/) to quantify the silver-stained density of each protein band separated with SDS-PAGE. We photographed each gel and analyzed them with the software. The picture was originally captured in RGB mode and was converted into 8-bit mode. The color intensity of each band, corresponding to the previously identified proteins, was quantified following subtraction of the background density. To investigate the difference between each type of asbestos, we used the protein band density of amosite as a standard. For the integrative data of each classification of proteins, a simple summation and average of ratios of either chrysotile or crocidolite to amosite was used for the analysis.

Statistics. All statistics were calculated with Prism 5 software (GraphPad Software, Inc.; La Jolla, CA). The data are expressed as the mean ± SEM. All the comparisons were made between chrysotile and crocidolite with Student's *t* test because quantitation with densitometry was already normalized by the amosite data.

Results

For a comparison of the amount of protein adsorbed to each type of asbestos, we used the pooled data from the silver-stained gels that we previously published.[12] In short, we separated proteins that were adsorbed onto the surface of asbestos using SDS-PAGE and visualized the gel using a silver staining method. We quantified the color intensity of each band, as shown in Fig. 1. Due to the lack of appropriate controls, we normalized the color intensity of the protein bands of chrysotile and crocidolite by the corresponding protein band of amosite. We quantified the proteins that were identified by MALDI-TOF/MS wherever possible. The proteins were classified into eight sub-categories according to their biological roles (chromatin/nucleotide/RNA-binding proteins, ribosomal proteins, cytoprotective proteins, cytoskeleton-associated proteins, histones and hemoglobin), and we compared the amount of each adsorptive protein using this classification. The quantitation results are summarized in Table 1.

The high affinity of histones for crocidolite, but not chrysotile, was an important observation (Fig. 2A). Potent adsorption of histones to crocidolite is consistent with the surface charges of crocidolite and histones, which are negative and positive, respectively.[37,38] Conversely, we found that chromatin-binding proteins (e.g., ATP-dependent DNA helicase 2 subunit 1 and 2, and DNA replication licensing factor MCM6 and MCM7) were more adsorptive to chrysotile than crocidolite (Fig. 2B). Accordingly, chrysotile and crocidolite are distinct in accommodating different types of chromatin components on their surface.

In addition to nuclear proteins, we found that RNA-binding proteins demonstrated a high adsorptive capacity for the chrysotile surface (Fig. 3A). Chrysotile also showed an adsorptive tendency for nucleotide-binding proteins (Fig. 3B), though this trend was

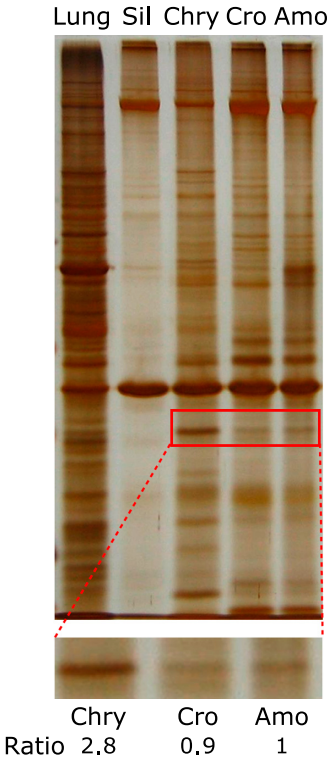

Fig. 1. Quantitation of protein bands in silver-stained gels with ImageJ. The square portion is magnified below. The color intensity of the protein bands in the square was measured. After subtraction of the background, we normalized the values for chrysotile- and crocidolite-bound proteins by that of amosite. Proteins in each lane are adsorbed proteins to each type of asbestos or silica, as indicated at the top of the figure. Sil, silica; Chry, chrysotile; Cro, crocidolite; Amo, amosite.

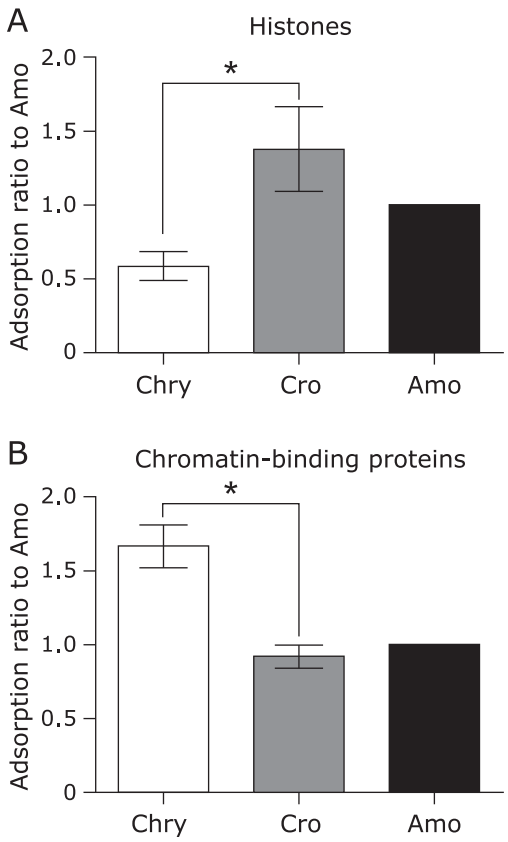

Fig. 2. Selective adsorption of histones and chromatin-binding proteins to crocidolite and chrysotile. (A) Histones include histone H2A type 3, H2B type 1, H3.3 and H4. (B) Chromatin-binding proteins include ATP-dependent DNA helicase 2 subunit 1 and 2, DNA replication licensing factor MCM6 and MCM7, Flap endonuclease 1 and interleukin enhancer-binding factor 2. Refer to Table 1 for each quantitation. Chry, chrysotile; Cro, crocidolite; Amo, amosite (N = 4 for A and N = 6 for B; mean ± SEM; *$p<0.05$).

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Table 1. A list of asbestos binding proteins

Protein name	Gi No.	Adsorption ratio to Amosite			Classification
		Chrysotile	Crocidolite	Amosite	
Histone H4	gi51317315	0.87	0.75	1	Histone
Histone H2B type 1	gi399856	0.50	1.27	1	Histone
Histone H3.3	gi55977042	0.50	1.27	1	Histone
Histone H2A type 3	gi90101452	0.49	1.34	1	Histone
DNA replication licensing factor MCM6	gi2497824	2.11	1.22	1	Chromatin-binding
ATP-dependent DNA helicase 2 subunit 2	gi125731	1.80	0.97	1	Chromatin-binding
DNA replication licensing factor MCM7	gi20981696	1.80	0.97	1	Chromatin-binding
Interleukin enhancer-binding factor 2	gi62510764	1.55	1.14	1	Chromatin-binding
ATP-dependent DNA helicase 2 subunit 1	gi125729	1.17	1.06	1	Chromatin-binding
Flap endonuclease 1	gi729475	0.68	0.62	1	Chromatin-binding
Cleavage and polyadenylation specificity factor subunit 5	gi74735411	2.64	1.08	1	RNA-binding
Splicing factor, proline- and glutamine-rich	gi1709851	2.11	1.22	1	RNA-binding
Putative pre-mRNA-splicing factor-ATP-dependent RNA helicase DHX15	gi13124667	2.00	0.85	1	RNA-binding
RNA-binding protein EWS	gi544261	2.00	0.85	1	RNA-binding
Heterogeneous nuclear ribonucleoprotein U (Human)	gi126302554	1.59	1.00	1	RNA-binding
Heterogeneous nuclear ribonucleoprotein A0	gi8134660	1.54	1.28	1	RNA-binding
Heterogeneous nuclear ribonucleoprotein A1	gi133254	1.54	1.28	1	RNA-binding
FUS glycine rich protein	gi4210363	1.01	1.03	1	RNA-binding
Probable ATP-dependent RNA helicase DDX5	gi129383	0.77	0.98	1	RNA-binding
KH domain-containing-RNA-binding signal transduction-associated protein 1	gi62511098	0.77	0.98	1	RNA-binding
Heterogeneous nuclear ribonucleoprotein U (Rat)	gi16923996	0.59	1.14	1	RNA-binding
ATP synthase subunit O	gi543880	2.63	1.39	1	Nucleotide-binding
Succinyl-CoA ligase	gi135025	1.63	0.78	1	Nucleotide-binding
Elongation factor Tu	gi1706611	1.12	0.88	1	Nucleotide-binding
Carbamoyl-phosphate synthase	gi117492	0.76	0.99	1	Nucleotide-binding
ATP synthase subunit alpha	gi83300587	0.72	0.90	1	Nucleotide-binding
Elongation factor 1-alpha 1 (Rat)	gi50402095	0.62	0.73	1	Nucleotide-binding
Elongation factor 1-alpha 2	gi50402096	0.58	0.43	1	Nucleotide-binding
Glutamate dehydrogenase 1	gi92090591	0.48	0.95	1	Nucleotide-binding
39S ribosomal protein L40	gi21263795	2.64	1.08	1	Ribosomal protein
39S ribosomal protein L48	gi118573683	2.64	1.08	1	Ribosomal protein
60S ribosomal protein L23a	gi51338637	2.64	1.08	1	Ribosomal protein
40S ribosomal protein S3a (Rat)	gi1350987	1.15	1.22	1	Ribosomal protein
60S ribosomal protein L7a (Rat)	gi54039228	1.15	1.22	1	Ribosomal protein
40S ribosomal protein S9 (Rat)	gi52788199	0.61	1.56	1	Ribosomal protein
60S ribosomal protein L8	gi51702823	0.56	1.34	1	Ribosomal protein
40S ribosomal protein S16	gi54039370	0.50	1.27	1	Ribosomal protein
60S ribosomal protein L22	gi1172995	0.50	1.27	1	Ribosomal protein
60S ribosomal protein L31	gi51702803	0.50	1.27	1	Ribosomal protein
39S ribosomal protein L28	gi85695426	0.50	1.18	1	Ribosomal protein
Alpha actinin 1	gi13591902	2.54	0.87	1	Cytoskeleton-associated
Alpha actinin 4	gi77539778	2.54	0.87	1	Cytoskeleton-associated
Actin (Human)	gi4501885	1.55	1.14	1	Cytoskeleton-associated
Keratin type I cytoskeletal 18 (Rat)	gi73621121	1.07	1.15	1	Cytoskeleton-associated
Actin (Rat)	gi55577	0.99	0.98	1	Cytoskeleton-associated
Myosin 10	gi13431672	0.85	1.38	1	Cytoskeleton-associated
Myosin 11	gi81175185	0.85	1.38	1	Cytoskeleton-associated
Predicted: similar to tubulin polymerization-promoting protein	gi62638424	0.78	1.42	1	Cytoskeleton-associated
Tubulin beta-5 chain	gi56754676	0.72	0.90	1	Cytoskeleton-associated
Myosin 9	gi13431671	0.70	1.24	1	Cytoskeleton-associated
Keratin type I cytoskeletal 18 (Human)	gi125083	0.68	0.62	1	Cytoskeleton-associated
Ezrin	gi68067388	0.66	1.08	1	Cytoskeleton-associated
Moesin	gi13540689	0.59	1.14	1	Cytoskeleton-associated
Spectrin alpha chain	gi17380501	0.55	1.10	1	Cytoskeleton-associated
Septin-7 (Rat)	gi9789715	0.54	0.85	1	Cytoskeleton-associated
Cytoskeleton-associated protein 4	gi109481770	0.50	1.09	1	Cytoskeleton-associated
Radixin	gi56799432	0.50	1.09	1	Cytoskeleton-associated
Myosin light polypeptide 6	gi2842665	0.50	1.27	1	Cytoskeleton-associated
Keratin type II cytoskeletal 8	gi1708592	0.48	0.95	1	Cytoskeleton-associated
Predicted: similar to septin-11	gi109499524	0.40	1.09	1	Cytoskeleton-associated
Myosin binding protein C	gi149056049	0.06	0.90	1	Cytoskeleton-associated
Predicted: similar to Myosin 11	gi109487680	0.06	0.90	1	Cytoskeleton-associated
Filamin-A	gi116241365	0.02	0.03	1	Cytoskeleton-associated
Septin-7 (Human)	gi67472677	0.00	0.89	1	Cytoskeleton-associated
Hemoglobin subunit alpha 1/2	gi122477	0.87	0.75	1	Hemoglobin
Hemoglobin subunit beta 1	gi122514	0.60	0.81	1	Hemoglobin
Hemoglobin subunit beta 2	gi122529	0.60	0.81	1	Hemoglobin
Superoxide dismutase [Mn]	gi134678	1.25	1.06	1	Cytoprotective
Glutathione peroxidase 1	gi121668	1.25	1.06	1	Cytoprotective
Heat shock 70 kDa protein 1/2	gi147744565	1.17	1.06	1	Cytoprotective
Heat shock cognate 71 kDa protein	gi123648	1.17	1.06	1	Cytoprotective
78 kDa glucose-regulated protein	gi121574	0.92	0.80	1	Cytoprotective
Peroxiredoxin 1 (Rat)	gi2499470	0.61	1.56	1	Cytoprotective

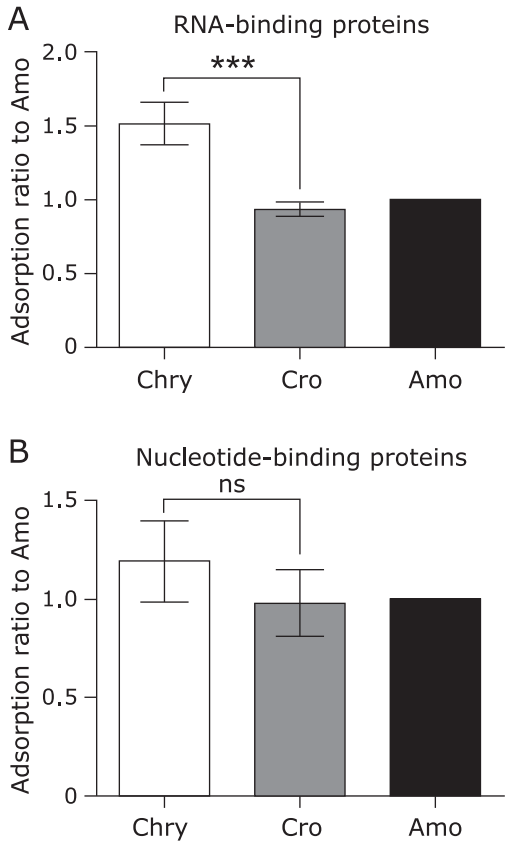

Fig. 3. Selective adsorption of RNA-binding proteins to chrysotile. Refer to Table 1 for a list of all proteins and their respective quantitation. Chry, chrysotile; Cro, crocidolite; Amo, amosite (N = 11 for A and N = 8 for B; mean ± SEM; *p<0.05; ns, not significant).

not statistically significant. These data indicate that chrysotile is more likely to interfere with transcription and translation processes than other asbestos.

We also studied the selectivity of adsorptive proteins that were categorized as ribosomal proteins, cytoprotective proteins, cytoskeleton-associated proteins and hemoglobin, but we did not find any significant differences (Fig. 4A–D).

Discussion

In 1987, the International Agency for Research on Cancer (IARC) designated asbestos fibers as Group 1 carcinogens to humans (http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C-11.pdf). The number of new patients with malignant mesothelioma is still increasing worldwide. Therefore, elucidating the carcinogenic mechanisms of asbestos is important. Currently, crocidolite is considered to be the most carcinogenic asbestos compound (500 times more than chrysotile), but this is still controversial.[39–41] We recently proposed that the surface of asbestos acts as a niche for oxidative modifications that can lead to the formation of oxidized DNA and proteins, such as 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal. Additionally, we identified more than 100 asbestos-interacting proteins.[12]

In the present study, we re-analyzed and quantified the proteins that are specifically adsorptive to each type of asbestos. There was no significant difference in the total amount of protein adsorbed to each type of fiber (data not shown). Therefore, the predominant adsorption of proteins either to chrysotile or crocidolite was not due to the difference in the total amount of proteins adsorbed. Although the adsorbed amounts on asbestos were different for each protein, we used a sum of the ratios to amosite for simplicity.

We found that there was a selective adsorption of proteins, namely, histones and chromatin-binding proteins, to crocidolite and chrysotile, respectively (Fig. 2). Although, unlike histones, chromatin-binding proteins are not constitutive components of chromatin, it is plausible that chrysotile would interfere with DNA maintenance by interacting with helicases and replication factors in DNA damage, repair and replication processes. In theory, negatively charged DNA is more adsorptive to positively charged chrysotile than to crocidolite, as previously discussed.[12] Indeed, chrysotile and crocidolite directly interact with chromatin components via differential adsorption to biomolecules, including DNA and proteins. Accordingly, we hypothesize that each type of asbestos is involved in direct DNA injury or mitotic disturbance, though different mechanisms may be involved (Fig. 5), based on the ability of asbestos to enter the cytoplasm and nucleus in various cells, including mesothelial cells.[9,42]

We also found that RNA-binding proteins are more adsorptive to chrysotile than to crocidolite. We hypothesize from this evidence that chrysotile may interfere with transcription and translation, though this should be confirmed in future studies. Interestingly, it was reported that cells exposed to crocidolite exhibit a general trend of up-regulated gene expression following acute exposure (6 h), but subchronic exposures (24 and 48 h) result in an overall decrease in gene expression.[43] We believe that the accommodation of RNA-binding proteins may play a role in this down-regulation of gene expression.

In conclusion, we used silver-stained gels and ImageJ software to generate a comparative profile of proteins that adsorb to each type of asbestos. We found that chrysotile and crocidolite predominantly adsorbed chromatin-binding proteins and histones, respectively, when compared among the three types of asbestos. Regarding the previously reported DNA adsorption to chrysotile, we propose that chrysotile and crocidolite interact with chromatin components by accommodating different sets of adsorptive proteins. Furthermore, we hypothesize that chrysotile would have an inhibitory effect on transcription and/or translation by binding to RNA-binding proteins. Our results provide novel insight into the distinct pathogenic mechanism of each asbestos compound, and may be useful for developing sensitive method to detect each asbestos in tissues.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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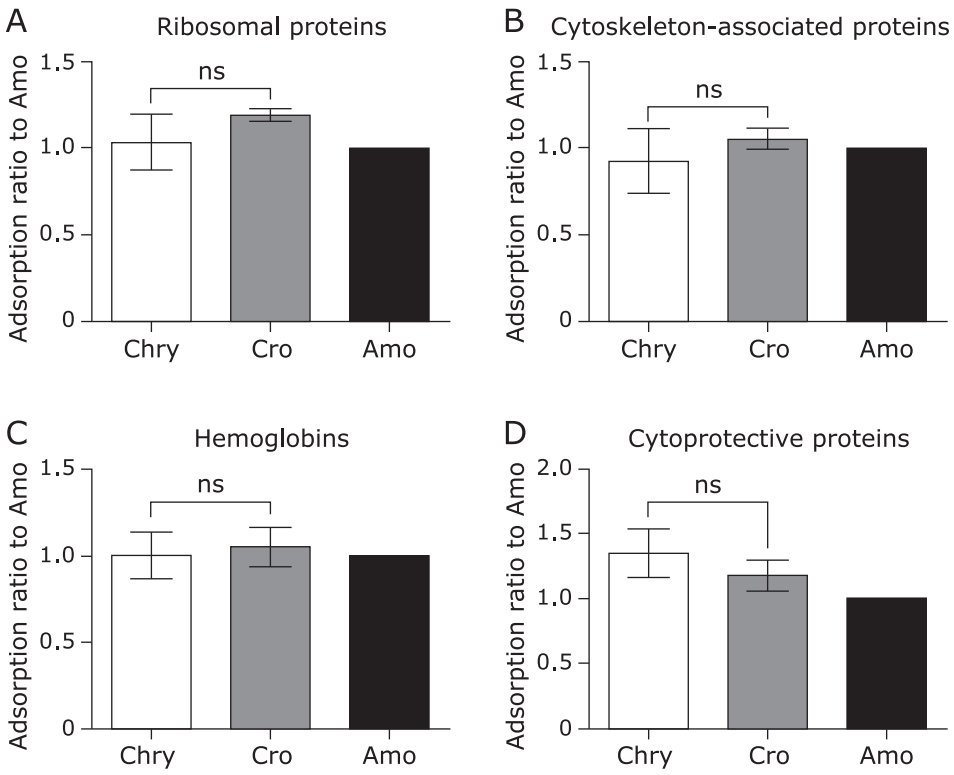

Fig. 4. Lack of selective adsorption of the proteins classified as ribosomal proteins, cytoprotective proteins, cytoskeleton-associated proteins and hemoglobin. Refer to Table 1 for a list of all proteins and their respective quantitation. Chry, chrysotile; Cro, crocidolite; Amo, amosite (N = 11 for A, N = 24 for B, N = 3 for C and N = 6 for D; mean ± SEM; ns, not significant).

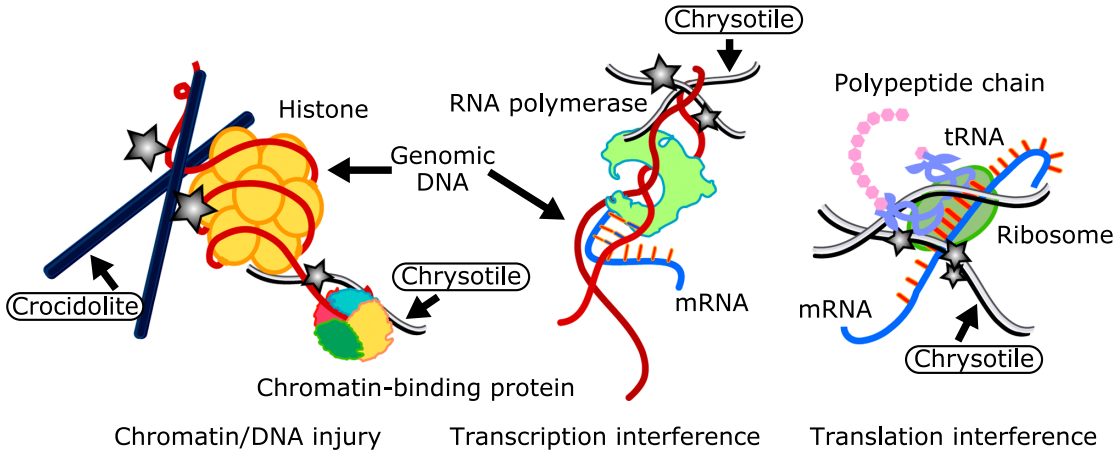

Fig. 5. Schematics that hypothesize different mechanisms of pathogenicity for chrysotile and crocidolite. We propose that chrysotile and crocidolite interact and injure genomic DNA by accommodating different sets of proteins. Furthermore, chrysotile may interfere with transcription and translation in cells by interacting with proteins involved in these signaling pathways.

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