

Potential role of ferritin heavy chain in oxidative stress and apoptosis in human mesothelial and mesothelioma cells: implications for asbestos-induced oncogenesis

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Exposure to asbestos is a known etiological factor in malignant mesothelioma (MM). However, *in vitro* cell culture studies have provided paradoxical evidence that asbestos exposure to mesothelial cells causes cytotoxicity or apoptosis rather than malignant transformation. Although it has been shown that the iron associated with asbestos participates in the cell toxicity and probably MM pathogenesis via generation of reactive oxygen species (ROS), the molecular mechanisms largely remain unknown. Here, we demonstrate that ferritin heavy chain (FHC), a core subunit of iron-binding protein ferritin, works as an anti-apoptotic protein against toxic asbestos and oxidative stress in human mesothelial cells and MM cells. We found that FHC was induced in asbestos-exposed MeT-5A human mesothelial cells. The mesothelial cell line stably expressing FHC generated less amount of hydrogen peroxide (H₂O₂), one of the main ROS, after asbestos exposure and was more resistant to apoptosis induced by H₂O₂ compared with the cells transfected with the empty vector. Next, we investigated biological roles of FHC in human MM cell. We found that NCI-H2052, a human MM cell line, had a higher expression of endogenous FHC than MeT-5A and used the cell to address FHC function in MM. NCI-H2052 showed reduced H₂O₂ production and an apoptosis-resistant phenotype compared with MeT-5A. Suppression of the over-expressed FHC by using FHC small interfering RNA rendered the MM cells sensitive to apoptosis, suggesting the contribution of FHC to apoptosis resistance of the MM cells. Our findings highlight the potential role of FHC in the pathogenesis of asbestos-induced mesothelioma.

Introduction

Exposure to asbestos is associated with pulmonary fibrosis and malignant diseases such as lung cancer and malignant mesothelioma (MM). Epidemiological studies have suggested that 2000–3000 cases per year are diagnosed as MM in the USA (1), and that 250 000 and 103 000 people will die from MM in next four decades in Europe or Japan, respectively (2). MM will be, or is indeed, one of the major health problems to be solved not only for occupational high-risk group with a history of severe asbestos exposure but also for general populations. So far, there is no effective therapeutics for this disease. Establishment of early diagnosis before the onset and specific therapies are urgently needed to control MM.

Asbestos is a generic term for a group of naturally occurring hydrated silicate fibers and the family of asbestos minerals can be subdivided into two major groups: amphiboles and serpentine (3). The amphibole fibers include crocidolite, amosite, anthophyllite, tremolite and actinolite. Chrysotile, the most common and industrially important form, is a serpentine representative. Since they differ in their

Abbreviations: DFO, deferoxamine; FHC, ferritin heavy chain; FI, fluorescence intensity; HRP, horseradish peroxidase; H₂O₂, hydrogen peroxide; MM, malignant mesothelioma; ROS, reactive oxygen species; siRNA, small interfering RNA.

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physical and chemical properties such as durability and composition, the biological effects should vary depending on the fiber types. For example, crocidolite is often considered the most oncogenic type of asbestos.

Although causative linkage between asbestos exposure and MM has been well established epidemiologically, the molecular mechanisms by which asbestos induces the deleterious effects are still poorly understood. The long latency period between asbestos exposure and tumor development implies that multiple and likely diverse processes occur in malignant transformation of mesothelial cells. It was found that asbestos caused malignant transformation of human mesothelial cells after long-term exposure in cooperation with simian virus 40 (4,5). Asbestos on its own, however, appears to induce cytotoxicity or apoptosis in human mesothelial cells (4–8). These reports have raised a paradoxical question, ‘How does asbestos that induces cytotoxicity or apoptosis cause malignant transformation?’.

There are numerous reports suggesting that reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), hydroxyl radicals (HO·) and reactive nitrogen species are important mediators of asbestos toxicity (9). One of the principle mechanisms to generate ROS is associated with the contents of asbestos fiber, especially iron (10). In asbestos-exposed cell, molecular oxygen mainly located in mitochondria is converted into O₂⁻ by reduction catalyzed by the iron. This O₂⁻ is a moderately reactive species capable of generating H₂O₂, which in turn can produce highly reactive hydroxyl radicals (HO·) via iron-dependent catalytic reactions, Fenton reaction and/or Haber–Weiss reaction (9,10). Indeed, the chemical properties of asbestos fibers, especially iron content, can contribute to the formation of ROS that is closely related to asbestos-induced pathogenesis (10).

There exist intrinsic defense systems that cancel ROS toxicity in mammalian cells (11). A major iron storage protein ferritin is one of the examples (12). The protein is a 24-subunit protein composed of two subunit types, termed heavy chain and light chain. Ferritin has enzymatic properties, converting Fe²⁺ to Fe³⁺, as iron is internalized and sequestered in the ferritin mineral core. This function is an inherent feature of ferritin heavy chain (FHC) subunit that exhibits ferroxidase activity that is required for iron sequestration (13,14). It has been shown that FHC regulates the intracellular iron which catalyses the formation of toxic ROS (15).

Thus, it is possible that asbestos-containing iron modulates FHC expression in the exposed cells and the modulation may be related to asbestos-mediated pathogenesis including MM development. A previous study has shown that exposure to crocidolite induced FHC expression in human lung epithelial cells (16). However, the role of FHC in asbestos-exposed mesothelial cells and in MM cells has not been described yet. In this paper, possible linkages between asbestos exposure, FHC, and apoptosis resistance are explored and discussed.

Materials and methods

Cells

The immortalized human pleural mesothelial cell line (MeT-5A) was purchased from American Type Culture Collection (Manassas, VA). MeT-5A was grown in 199 medium supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 400 nM hydrocortisone, 870 nM insulin and 3.3 nM epidermal growth factor, containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. NCI-H2052, NCI-H226, NCI-H28 and NCI-H2452 were also purchased from American Type Culture Collection. ACC-MESO-1 (17) was purchased from RIKEN BRC cell bank. They were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. MeT-FHC and MeT-Hyg cell lines were established from MeT-5A by transfection with pIRESHyg3 (Clontech, Mountain View, CA) plasmid carrying FHC cDNA and null vector, respectively. MeT-5A was seeded in a six-well dish at a density of 5 × 10⁵ per

well 24 h before transfection with 4 μg of plasmid. Transfection was carried with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the protocol recommended by the manufacturer. Stable cell lines were obtained by selection with 200 $\mu\text{g}/\text{ml}$ hygromycin B (Invitrogen) for >2 weeks.

Asbestos fiber preparation

Crocidolite (JAWE331: Cape, South Africa, 2–30 μm length, >99% purity) and chrysotile (JAWE131: CA, <5 μm length, >95% purity) were obtained from Japanese Association for Working Environment Measurement. Fibers were weighted and suspended in distilled water. The fiber suspension was triturated six to eight times with a 20 gauge syringe needle. A stock solution of the fibers was sterilized by autoclaving. For experiments on the role of iron, iron chelation of crocidolite fibers was done by incubating with 10 mM deferoxamine (DFO) mesylate salt (Sigma–Aldrich, St Louis, MO) for 17 h at room temperature, followed by centrifugation, washing with Hanks' balanced salt solution and re-suspension in distilled water.

Western blot analysis

Cell lysate was prepared by 50 mM Tris-buffered saline with 0.5% Triton X-100. Total protein content was determined by DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Ten to 20 μg of the protein was separated on a 12.5% polyacrylamide gel and transferred to Immobilon-P membrane (MILLIPORE, Billerica, MA). For detection of FHC, the blot was blocked by Tris-buffered saline BLOTTO A (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 h and incubated with primary antibody at room temperature for 2 h. As a primary antibody, anti-human FHC antibody (Santa Cruz Biotechnology) was used at dilution of 1/100. The membranes were washed three times with Tris-buffered saline containing 0.05% Tween 20, incubated with an anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (GE Healthcare Bioscience Corp., Piscataway, NJ). For loading control, an antibody against actin (Santa Cruz Biotechnology) and anti-goat IgG conjugated with HRP (Santa Cruz Biotechnology) were used. The blots were developed with enhanced chemiluminescence plus western blotting detection system (GE Healthcare Bioscience Corp.). Images were obtained by Chemi-Smart 5000 (Vilber Lourmat, France).

H₂O₂ generation assay

We measured the release of H₂O₂, one of the main ROS, from cells exposed to asbestos by HRP-dependent oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) (Invitrogen). First, we prepared the reaction mixture containing 50 μM Amplex Red reagent and 0.1 U/ml HRP in Krebs Ringer phosphate glucose (145 mM NaCl, 5.7 mM Na₂HPO₄, 4.86 mM KCl, 0.54 mM CaCl₂, 1.22 mM MgSO₄, 5.5 mM glucose, pH 7.35). One hundred microliters of the reaction mixture was pipetted into each well of 96-well microplate (Becton Dickinson, Franklin Lake, NJ). Graded doses (6, 12 and 24 $\mu\text{g}/\text{cm}^2$) of asbestos fibers (crocidolite, chrysotile and DFO-treated crocidolite) were added to microplate and incubated at 37°C for 15 min. Then, we added 20 μl of Krebs Ringer phosphate glucose containing 4×10^4 cells into the microplate and incubated them at 37°C for 4 h. By using the fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) equipped for excitation in the range of 530–560 nm and emission at 585 nm, fluorescence intensity (FI) of each well was measured. Percent increase of H₂O₂ generation was calculated as (FI in the presence of asbestos – FI in the absence of asbestos)/FI in the absence of asbestos $\times 100$.

Dye-staining apoptosis assay

Occurrence of apoptosis induced by oxidant (H₂O₂) in *in vitro* cultured cells was evaluated by APOPercentage Apoptosis Assay (Biocolor Ltd., Northern Ireland). Cells (1×10^5) were plated into each well of BIOCOAT Collagen 1 cellware (96-well plate) (Becton Dickinson). Twenty-four hours after incubation at 37°C, different concentrations of H₂O₂ (0.5 and 1 mM) were added into the wells and incubated for next 4 h. Thirty minutes before the indicated time is reached, we replaced the medium with 100 μl of fresh culture medium containing 5 μl APOPercentage dye and H₂O₂. Then, we incubated the cells for the remaining 30 min of the assay. After that, we removed the culture–dye mixture and gently washed the cells with 200 μl phosphate-buffered saline per well and transferred immediately to an inverted microscope and obtained the images of random places. Apoptosis committed cell undergoes phosphatidylserine transmembrane movement that permits dye entry to cell and its accumulation in cell was visualized as purple–red color.

Flow cytometry and quantification of apoptotic cells

Detection and quantification of apoptotic cells was obtained by flow cytometric analysis by using Guava PCA and Guava Nexin Assay (Guava Technologies, Hayward, CA). The protocol was performed according to the manufacturer's instructions. Four hours after H₂O₂ (0.5 and 1 mM) treatment, cell suspensions

were incubated with two staining reagents to distinguish the apoptotic and non-apoptotic cell population: assay utilizes Annexin V–phycoerythrin to detect early apoptotic cells and 7-AAD to detect late apoptotic and dead cells. For comparison between cell lines, ratio of percent apoptotic cell for each cell line was calculated as (percent apoptotic cell in H₂O₂-treated sample/percent apoptotic cell in H₂O₂ non-treated sample).

RNAi

NCI-H2052 was seeded in 12-well dishes at a density of 5×10^4 per well 24 h before transfection with 100 nM of ON-TARGETplus SMART pool human FTH1 (L-019634-00, Dharmacon, Lafayette, CO). Transfection was carried with DharmaFECT transfection reagent (Dharmacon) following the protocol recommended by the manufacturer. We evaluated the transfection efficiency by using siGLO Red transfection indicator (Dharmacon) and estimated that 70–80% of the cells were positive for transfection.

Results

Asbestos induces FHC expression in MeT-5A human mesothelial cell line

MeT-5A, an immortalized human pleural mesothelial cell, is a near-diploid non-tumorigenic cell line exhibiting typical mesothelial characteristics (18). Also, cytotoxicity of amosite asbestos in MeT-5A was similar to that in normal human pleural mesothelial cells (19). Therefore, we used it as *in vitro* model of mesothelial cell and evaluated asbestos-induced FHC expression. Two different types of asbestos, crocidolite and chrysotile, with graded doses in order (1, 2, 5 and 10 $\mu\text{g}/\text{cm}^2$) were used to expose MeT-5A for 24 h. By western blotting, apparent dose-dependent FHC expression was observed with crocidolite exposure (Figure 1A). Chrysotile also appeared to induce dose-dependent FHC expression, but it was less than that induced by crocidolite (Figure 1A). Crocidolite (5 $\mu\text{g}/\text{cm}^2$) induced FHC in time-dependent manner. As shown in Figure 1B, FHC was up-regulated within 1 h after exposure and gradually increased up to sustained level during the 48 h that we observed.

FHC induction is hampered by iron chelation of the crocidolite

Crocidolite contains ~27% iron in the crystalline structure (9). To examine the specific role of iron of the fiber in FHC induction, crocidolite was pre-treated with DFO, an iron-chelating agent, and exposed to MeT-5A for 9 h. DFO-treated crocidolite (5 and 10 $\mu\text{g}/\text{cm}^2$) induced less FHC compared with non-treated fiber of same doses (Figure 2).

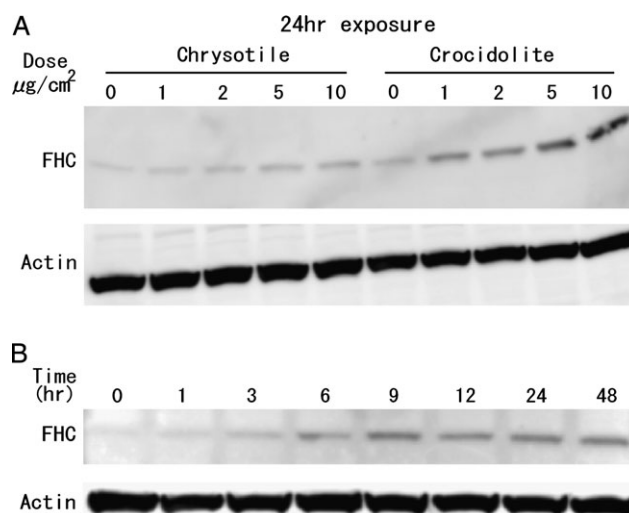


Fig. 1. Asbestos exposure induces FHC expression in MeT-5A human mesothelial cells. (A) Dose-dependent induction of FHC by chrysotile and crocidolite exposure. The concentrations of asbestos exposed and exposure time are indicated. Actin as a loading control. (B) Time-dependent induction of FHC by crocidolite exposure (5 $\mu\text{g}/\text{cm}^2$). The exposure time is indicated. Actin as a loading control.

Cytoprotective role of FHC in human mesothelial cells

Since it has been shown that FHC has an anti-oxidative function in cells (15,20,21), we investigated whether FHC acted as a cytoprotective protein against toxic asbestos and oxidative stress in mesothelial cells. We established MeT-5A stably expressing FHC (MeT-FHC) and assayed for its ability to generate H₂O₂ under asbestos exposure and sensitivity to apoptosis induced by H₂O₂. We used MeT-5A stably expressing not FHC but hygromycin-resistant gene (MeT-Hyg) as a control. Western blot analysis confirmed higher FHC expression in MeT-FHC when compared with that in MeT-Hyg (Figure 3A). The release of H₂O₂ from asbestos-treated MeT-FHC or MeT-Hyg cells was measured based on HRP-catalyzed oxidation of fluorescent

Amplex Red reagent. There was crocidolite dose-dependent induction of H₂O₂ generation (Figure 3B). The percent increase of H₂O₂ generation in MeT-FHC was smaller compared with that generated by control MeT-Hyg. We also observed the same effects with chrysotile, although it was less compared with that by crocidolite (Figure 3C). Crocidolite pre-treated with DFO showed further decrease of H₂O₂ generation (Figure 3D). To assess the anti-apoptotic role of FHC on oxidant-induced apoptosis, we stained apoptotic cells by purple-red dye after apoptosis induction by H₂O₂. The results revealed that 4 h after exposure to the oxidant stimulus (H₂O₂, 0.5 or 1 mM), MeT-FHC exhibited less apoptotic cells compared with the control cells (Figure 3E). Using flow cytometry, we quantified apoptotic cells in MeT-Hyg and MeT-FHC after H₂O₂ challenge. The sum of the early apoptotic cells and late apoptotic cells (including dead cells) in MeT-FHC was smaller than that in MeT-Hyg at the H₂O₂ concentration of both 0.5 and 1 mM. Figure 3F shows representative results of repeated independent experiments (23.1 versus 43.6% and 34.4 versus 48.0%, respectively). The ratio of percent apoptotic cell in H₂O₂-treated sample to that in non-treated sample in MeT-FHC was also smaller than that in MeT-Hyg (Figure 3G).

Human MM cells that over-express FHC show an apoptosis-resistant phenotype

Previous studies have shown that MM is refractory to chemotherapy (22) and radiotherapy (23), suggesting that MM cells are highly resistant to apoptosis. Thus, we hypothesized that FHC may contribute to apoptosis resistance not only in mesothelial cells but also in MM

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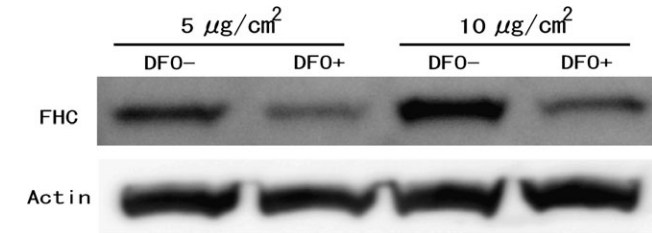


Fig. 2. Iron chelation of crocidolite by DFO hampers FHC induction. Concentrations of non-treated (DFO-) and treated (DFO+) crocidolite are indicated. Exposure time was 9 h. Actin as a loading control.

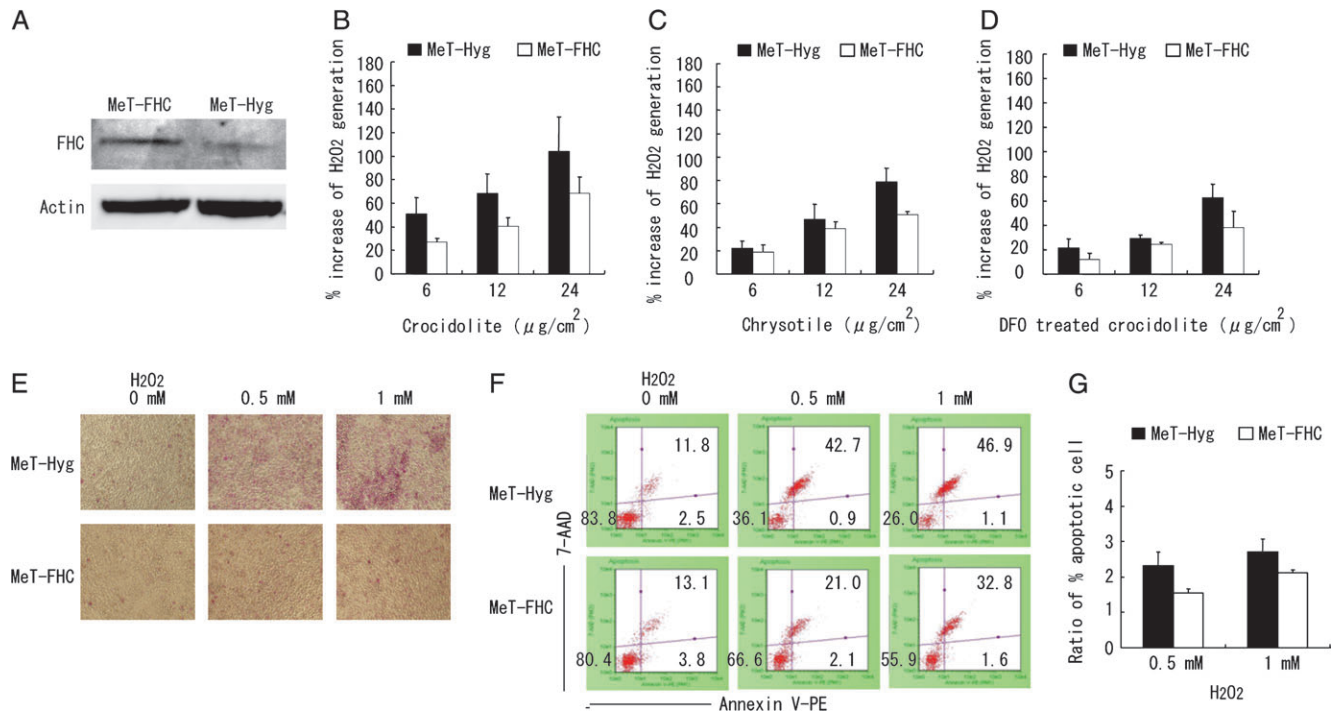


Fig. 3. FHC as an anti-apoptotic protein in MeT-5A human mesothelial cells. (A) Western blot of FHC expression in MeT-FHC and MeT-Hyg. (B) Evaluation of H₂O₂ generation by crocidolite exposure in MeT-Hyg and MeT-FHC with fluorescent microassay by using Amplex Red reagent. Asbestos dose-dependent H₂O₂ generation was seen in both cell lines. However, the percent increase of H₂O₂ generation was less in MeT-FHC. The data represent the mean + SE from three experiments. (C) H₂O₂ generation by chrysotile exposure in MeT-Hyg and MeT-FHC. Similar to crocidolite, chrysotile dose-dependent H₂O₂ generation was observed in both cell lines and the amount from MeT-FHC was less than that from MeT-Hyg. The data represent the mean + SE from two experiments. (D) H₂O₂ generation by DFO pre-treated crocidolite exposure. DFO pre-treated crocidolite induced less H₂O₂ generation than non-treated crocidolite. Compare with (B). The data represent the mean + SE from two experiments. (E) With APOPercentage assay, apoptotic cells were visible as purple-red color *in vitro* culture at 4 h after incubation with H₂O₂. MeT-FHC contained less apoptotic cells. The images are representative from one of three experiments. (F) Flow cytometric analysis of apoptotic cells by two stains after 4 h treatment of cells with H₂O₂. Numbers in the corner of lower left, lower right and upper right of each graph indicate the percent of live cells (Annexin V-phycoerythrin negative/7-AAD negative), early apoptotic cells (Annexin V-phycoerythrin positive/7-AAD negative) and late apoptotic and dead cells (Annexin V-phycoerythrin positive/7-AAD positive), respectively. MeT-FHC was more resistant to apoptosis. The representative results of three independent experiments are shown. (G) Ratio of percent apoptotic cell in H₂O₂ treated to that in non-treated samples for each cell lines were calculated and compared in bar graph. The data represent the mean + SE from three experiments.

cells. We examined endogenous FHC expression in human MM cell lines by western blotting. We found that NCI-H2052 (H2052) showed the highest expression of endogenous FHC among the MM cells we tested and the level was higher than that of non-malignant MeT-5A (Figure 4A). Thus, we used H2052 for further investigation to address the biological role of FHC in human MM cells. We found that the percent increase of H₂O₂ generation in H2052 was smaller compared with that in MeT-5A when exposed to crocidolite and chrysotile (Figure 4B and C). Not surprisingly, crocidolite induced more H₂O₂ generation than chrysotile. To address whether H2052 expresses an apoptosis-resistant phenotype, we stained apoptotic cells in H2052 after adding 0.5 or 1 mM of H₂O₂ in the culture medium. These experiments revealed that H2052 exhibited less apoptotic cells compared with MeT-5A (Figure 4D). Consistent with the results obtained by dye-staining experiments, apoptotic cell fraction in H2052 was clearly smaller than that in MeT-5A at two different concentrations of H₂O₂. Figure 4E shows representative results of repeated independent experiments (17.9 versus 53.1% and 20.3 versus 61.4%, respectively). And, the MM cell showed small ratio of percent apoptotic cell in H₂O₂-treated sample to that in non-treated sample (Figure 4F).

Down-regulation of FHC renders MM cells sensitive to apoptosis

In order to confirm that FHC contributes to apoptosis resistance in H2052 cells, we suppressed FHC expression in the cells by small interfering RNA (siRNA) and assayed their apoptosis sensitivity. Apoptotic cell population in H2052 transfected with FHC siRNA was larger than that transfected with control siRNA at concentration of 0.5 and 1 mM H₂O₂ exposure (52.8 versus 48.8%, 58.1 versus 49.3%, respectively) (Figure 5).

Discussion

In the present study, at first, we showed that asbestos induced FHC protein in human mesothelial cells. FHC induction by crocidolite was more apparent than that by chrysotile. This induction was mainly due to iron in the fiber because removal of iron from the fiber by iron chelator hampered the induction. Mesothelial cells stably expressing FHC blunted ROS production in asbestos exposure, compared with control cells, and were relatively resistant to apoptosis induced by H₂O₂. We also demonstrated that endogenous FHC over-expression was found in a human MM cell line and that the cells generated lower ROS when exposed to asbestos and were more resistant to oxidant-induced apoptosis compared with non-malignant mesothelial cells with lower FHC expression. Knockdown experiment using siRNA-targeting FHC revealed that FHC down-regulation rendered some population of the MM cells sensitive to apoptosis. These data suggest that FHC up-regulation is an acute response of human mesothelial cells against asbestos exposure and that FHC exerts an anti-apoptotic activity against oxidative stress not only in mesothelial cells but also in MM cells.

Although it is still controversial, crocidolite has been generally regarded as a more carcinogenic form than chrysotile (3,24). Crocidolite has a high iron content (~27%), whereas chrysotile has a lower but significant iron content (~1 to 6%) (9). With this regard, iron content in the fiber matrix can be a critical factor, because iron-derived ROS can cause many pathologic conditions in cells. In our asbestos exposure experiments, we found that both fibers induced FHC. The induction by crocidolite was more apparent than that by chrysotile. Amount of H₂O₂ production by crocidolite exposure was more than that by chrysotile, although chrysotile also generated ROS in mesothelial cells and MM cells. Furthermore, we showed that

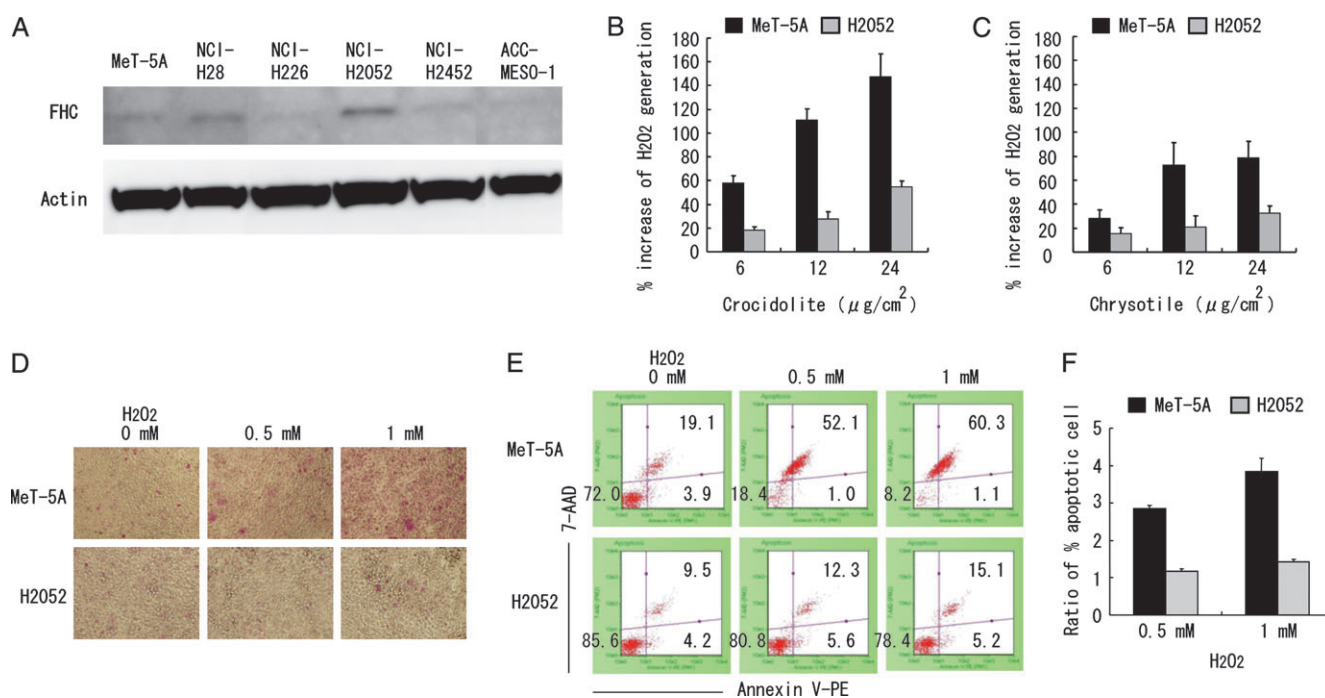


Fig. 4. Reduced oxidative stress and apoptosis-resistant phenotype in a human MM cell line NCI-H2052 (H2052) that over-expresses FHC. (A) FHC expression in human MM cells. FHC was over-expressed in H2052 compared with MeT-5A. Actin as a loading control on the blot. (B) Crocidolite dose-dependent percent increase of H₂O₂ generation was evidently smaller in H2052. The data represent the mean + SE from four experiments. (C) H₂O₂ generation by chrysotile. Note that chrysotile induced less H₂O₂ generation than crocidolite. Compare with (B). The data represent the mean + SE from three experiments. (D) Dye-staining of apoptotic cells under H₂O₂ attack. H2052 cells contained less apoptotic cells at 4 h after incubation with H₂O₂. The images are representative images from one of three experiments. (E) Flow cytometric analysis after 4 h treatment with H₂O₂. Refer to figure legend of Figure 3E about numbers in the corner of lower left, lower right and upper right of each graph. H2052 cells were more resistant to apoptosis. The representative results of three independent experiments are shown. (F) Ratio of percent apoptotic cell in H₂O₂ treated to that in non-treated samples for H2052 cell line was calculated and compared with that of MeT-5A. The data represented in bar graph is the mean + SE from three experiments.

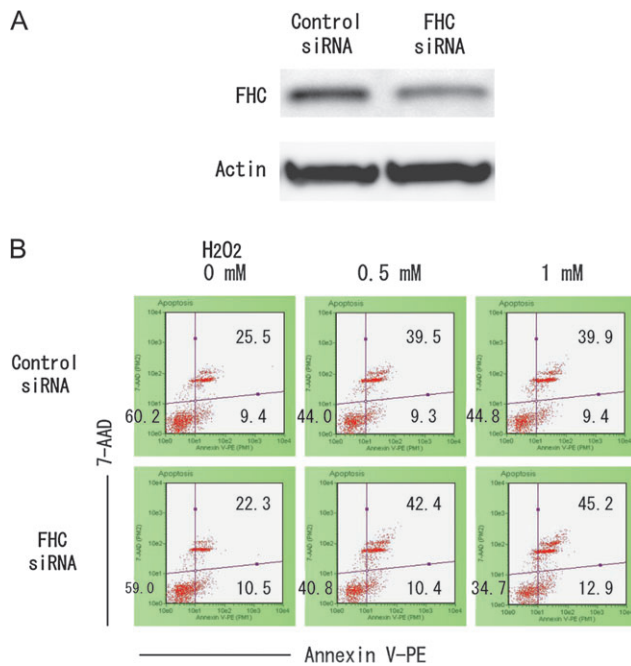


Fig. 5. Knockdown of FHC by siRNA. (A) Western blot of FHC knockdown by siRNA. Actin as a loading control. (B) Flow cytometry of apoptotic cells by oxidant in H2052 transfected with control siRNA or FHC siRNA. The representative results of four independent experiments are shown.

removal of iron from crocidolite by DFO treatment reduced the FHC induction in mesothelial cells and H₂O₂ generation. These results strongly support the idea that iron in the asbestos is a critical factor to induce FHC and to generate oxidative stress in asbestos exposure. In addition, it was reported that asbestos can cause rapid hemolysis of red blood cells *in vitro* and hemorrhage in lung was observed in asbestos exposure *in vivo*, suggesting effects of iron released from hemoglobin in MM (25).

It has been shown that cells have various mechanisms to regulate ROS by maintaining the well-tuned balance of oxidant and antioxidant enzymes. For examples, enzymes such as superoxide dismutases, catalase and glutathione peroxidases convert ROS into less-active species (26). Another antioxidant system that cells assume to control ROS is to restrict the cellular availability of redox active iron (Fe²⁺) whose concentration seems to be critical in ROS toxicity. This control of redox active iron levels is partly obtained through metal sequestration mediated by synthesis of ferritin, highly conserved iron-binding protein (13,14). Therefore, it is reasonable that ferritin, especially core subunit FHC, is up-regulated under exposure to iron-containing asbestos.

Human mesothelial cells stably over-expressing FHC generated less H₂O₂ when challenged by asbestos and were resistant to apoptosis induced by oxidant stimuli compared with control cells. These data suggest FHC reduces intracellular oxidative stress triggered by asbestos exposure in mesothelial cells and contributes to apoptosis resistance by diminishing ROS generation. Our results are consistent with the results obtained by others, using murine erythroleukemia cells and HeLa cells (15,20,21). Recently, Pham *et al.* (27) reported the key role of FHC in regulating apoptosis during inflammation. They showed that FHC is required to prevent sustained c-Jun N-terminal kinase cascade activation, thus inhibiting apoptosis induced by tumor necrosis factor- α . FHC-driven inhibition of c-Jun N-terminal kinase signaling depends on suppressing ROS generation and is achieved through its ability to sequester iron.

Our findings that asbestos exposure induces FHC expression in the exposed cells and that the FHC expression confers apoptosis resistance on mesothelial cells allow us to propose a model for early stage of MM development: When mesothelial cells are exposed to asbestos,

especially more potently carcinogenic crocidolite asbestos, they acutely respond to the stimuli with FHC induction. Nevertheless, majority of the exposed cells undergo cell death or apoptosis. In mesothelial cells that escape the cell death, which have sustained over-expression of FHC as one of the adopted protective mechanisms, the cells continuously receive toxic stimulation from penetrated asbestos fibers and generate ROS during the long latency period. This model agrees with an earlier study that asbestos exposure generated a mesothelial cell population with an extended life span after massive apoptosis (7). Thus, we speculate that asbestos exposure would be a ‘driving force’ to select stochastic cell populations that are able to become origin of MM. In addition, we showed that in comparison with weak carcinogenic chrysotile, the most carcinogenic asbestos crocidolite induced more FHC expression, which would be advantageous for avoiding apoptosis. Since asbestos is a genotoxic agent (28,29) and causes chromosomal changes (30), accumulation of genetic alternations would be accelerated in the cell clones under the persistence of the fibers. In this situation, cytokines such as a tumor necrosis factor- α released from itself or neighboring cells may promote the cell survival via nuclear factor- κ B activation (31).

We found that NCI-H2052, a human MM cell, expressed relatively high amounts of endogenous FHC protein. Given that FHC expression opposes to asbestos-induced apoptosis, constitutive activation of endogenous FHC in H2052 might occur as one of the acquired capabilities of cancer cells to escape apoptosis (32). Our study, however, has also shown that not all human MM cells over-expressed FHC and the expression level was various among the cell lines. We speculate that during the process of evolution from the initiated asbestos-resistant cells and partially transformed cells to fully transformed cells, some subclones stay FHC dependent but others that acquire apoptosis resistance by alternative pathways could become FHC independent and lose the over-expression of FHC. This may account for the various expression level of FHC in human MM cells.

Several studies have reported that ferritin is differentially expressed in tumor cells and normal cells. For example, increased expression of FHC was observed in colon cancer cell line that showed highly tumorigenic ability (33). Increased ferritin concentration in tumor versus normal tissue has also been confirmed in several malignancies, such as colon cancer (34), breast cancer (35), seminoma (36) and renal cell carcinoma (37). However, the relationship between FHC and malignancy is rather complex, as there is a report that down-regulation of FHC was required for cell transformation by c-MYC (38), and the possibility that it is cell type, tissue or stage-specific nature could not be excluded. Further study will be needed to determine a more detailed role of FHC in cancer formation.

We demonstrated that H2052 that over-expressed endogenous FHC exhibited an apoptosis-resistant phenotype compared with MeT-5A and that down-regulation of FHC by siRNA rendered the MM cells sensitive to apoptosis, suggesting that FHC contributes to apoptosis resistance in H2052 cells. Clinically, MM is known to be refractory to chemotherapy and radiotherapy, but the mechanism is largely unknown. Since we have not profiled the status of apoptosis related genes inclusively in H2052, we cannot conclude that the apoptosis resistance in H2052 is solely dependent on FHC. Partial shift of H2052 cells from apoptosis insensitive to sensitive by FHC siRNA may indicate that molecules other than FHC are also involved in the insensitivity of H2052 to apoptosis. For example, Narasimhan *et al.* (39) has reported that the resistant nature to apoptosis in MM cell lines was explained by mechanisms that counteract the pro-apoptotic effect of Bax. It has been reported that an apoptosis inducer tumor necrosis factor-related apoptosis-inducing ligand alone had little effect on mesothelioma cells (40) and significant degree of apoptotic resistance in human mesothelioma cells in tumor fragment spheroids remained, despite maximum apoptosis induction (41), suggesting complex mechanisms of apoptosis resistance in MM. It will be very important to identify the molecules associated with apoptosis resistance in MM and analyze the pathway to treat the disease effectively.

In conclusion, our study provides evidence that FHC plays an anti-apoptotic role not only in asbestos-exposed mesothelial cells but also

in MM cells. Evading apoptosis is a hallmark of cancer and the targeting the pathway holds a promising strategy for antitumor treatment (32). Therefore, FHC and the molecules identified through further investigation to be associated with apoptosis resistance in MM may provide potential targets for efficient treatment and diagnosis of MM.

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Conflict of Interest Statement: None declared.

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