Deferasirox Induces Mesenchymal–Epithelial Transition in Crocidolite-Induced Mesothelial Carcinogenesis in Rats

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Abstract
Asbestos was used worldwide in huge quantities in the past century. However, because of the unexpected carcinogenicity to mesothelial cells with an extremely long incubation period, many countries face this long-lasting social problem. Mesothelioma is often diagnosed in an advanced stage, for which no effective therapeutic protocols are yet established. We previously reported on the basis of animal experiments that the major pathology in asbestos-induced mesothelial carcinogenesis is local iron overload. Here, we undertook to find an effective strategy to prevent, delay, or lower the malignant potential of mesothelioma during asbestos-induced carcinogenesis. We used intraperitoneal injections of crocidolite to rats. We carried out a 16-week study to seek the maximal-tolerated intervention for iron reduction via oral deferasirox administration or intensive phlebotomy. Splenic iron deposition was significantly decreased with either method, and we found that Perls’ iron staining in spleen is a good indicator for iron reduction. We injected a total of 10 mg crocidolite at the age of six weeks, and the preventive measures were via repeated oral administration of 25 to 50 mg/kg/d deferasirox or weekly to bimonthly phlebotomy of 4 to 10 mL/kg/d. The animals were observed until 110 weeks. Deferasirox administration significantly increased the fraction of less malignant epithelioid subtype. Although we found a slightly prolonged survival in deferasirox-treated female rats, larger sample size and refinement of the current protocol are necessary to deduce the cancer-preventive effects of deferasirox. Still, our results suggest deferasirox serves as a potential preventive strategy in people already exposed to asbestos via iron reduction. Cancer Prev Res; 6(11): 1222–30. ©2013 AACR.

Introduction
Although malignant mesothelioma is a relatively rare cancer originating from mesothelial cells on the somatic cavity, epidemiologic studies have firmly linked this tumor with asbestos exposure in the 1960s (1–4). Asbestos is a group of natural fibrous minerals that reveal unique properties such as heat-, acid-, and friction-resistance with versatility and low cost for industrial use. Thus, these materials were used all over the world throughout the twentieth century and are not yet banned legally in the developing countries (5, 6). Because of the long latency period (30–40 years) after asbestos exposure, the number of patients with mesothelioma is estimated to increase in most of the countries. In Japan, the number of the patients will peak in 2025 and 100,000 new patients are expected in the coming 40 years (7).

The prognosis of mesothelioma is so poor that more than half of the patients die within a year after diagnosis. Even though multiple chemotherapy regimens have been tested, none of them could dramatically improve the prognosis (8). Therefore, there is a pressing need for early detection and preventive intervention in high-risk people. Considering the long incubation period, strategies to prevent, delay, and lower the malignant potential of asbestos-induced mesothelioma would be of particular interest.

Currently, there are several lines of hypotheses on the molecular mechanisms of asbestos-induced carcinogenesis, which include free radical generation, chromosome tangling, molecular adsorption, and chronic inflammation (9–11). We hypothesized that appropriate intervention may reduce free radical generation even after exposure to asbestos fibers. This is because generation of free radicals largely depends on catalytic iron (12, 13).

Iron, the most abundant transition metal in mammals, plays an essential role in various activities such as oxygen transport and energy production through redox reactions. However, excess iron is a risk for various cancers (12, 14, 15). For example, hepatocytes infected with hepatitis virus B or C accumulate iron through iron dysregulation by low hepcidin, and are frequently transformed to hepatocellular...
carcinoma (16). Indeed, iron reduction can lower the risk for hepatic cancer (17) and probably in other cancers (18).

Concerning mesothelioma, iron overload as a major pathogenic process has been recently studied (3, 19, 20). We reported that intraperitoneal injection of iron saccharate into rats induced mesothelioma (21) with loss of the Cldn2a/2b tumor suppressor gene, the same genetic feature as asbestos-induced mesothelioma, supporting the hypothesis that iron overload is responsible for asbestos-induced mesothelial carcinogenesis (22). More recently, we showed that administration of nitritilotriacetate, an iron chelator to enhance catalytic activity of iron, significantly reduced the period of asbestos-induced mesothelial carcinogenesis in rats not only by crocidolite or amosite but also by chrysotile (20). Crocidolite and amosite contain abundant iron in mineral contents. In contrast, chrysotile can adsorb iron (heme from hemoglobin) on its surface through hemolytic activity (13).

These observations suggested that iron reduction might cause preventive effects on asbestos-induced mesothelial carcinogenesis. Recently, an oral iron chelator, deferasirox, was for the first time developed and is used for decreasing iron-reduction procedures by administering deferasirox (male, n = 45; female, n = 38) or saline with 1-week interval (male, n = 9; female, n = 9). One month after the initial injection of crocidolite, we initiated the iron-reduction procedures by administering deferasirox (male, n = 9; female, n = 7; and n = 3 for each saline-treated group of both sexes) or by performing phlebotomy to rats (male, n = 8; female, n = 4; and n = 3 for each saline-treated group of both sexes). The dose of deferasirox was changed twice during the experiment to avoid false-negative results and also to reduce side effects. The rats were treated with 25 mg/kg/d deferasirox for the first 60 days, then 50 mg/kg/d for 120 days, and finally 25 mg/kg/d until sacrifice (520-day period for the longest). We also adjusted the dose and frequency of phlebotomy, considering the hematocrit and general condition of rats. Each phlebotomy was done once

Materials and Methods

Materials
Crocidolite was obtained from Unio Internationale Contra Cancrum (UICC). All rats used were Fischer 344/Brown Norway F1 hybrids, which do not generate spontaneous mesothelioma (20, 22). Fischer 344 female rats and Brown Norway male rats were purchased from Charles River Laboratories. The animal experiment committee of Nagoya University Graduate School of Medicine (Nagoya, Japan) approved these animal experiments. Oral iron chelator, deferasirox, was a kind gift from Novartis Pharma.

Experiments to determine maximal tolerated level of iron-reduction procedures
Six-week-old female rats were injected with 1 mL of 1 mg/mL crocidolite solution (crocidolite was suspended in saline with sonication) or saline control (n = 3–4 for each group). One week after the injection, we started to administer deferasirox or to conduct phlebotomy to rats to reduce body iron stores. Before use, we dissolved deferasirox at a concentration of 100 mg/mL in H₂O containing 0.5% hydroxypropyl cellulose (Sigma-Aldrich). We used a pair of mortar and pestle to prepare the deferasirox solution, which was given to each rat via a gavage at a dose of 100 mg/kg/d every weekday (5 days a week) until sacrifice. We conducted phlebotomy under anesthesia using a hep-
per week and scheduled as follows: 4 mL/kg/d for week 10, 14, and 16 after birth, 6 mL/kg/d for weeks 18 to 23, 8 mL/kg/d for week 24, and 10 mL/kg/d for weeks 25 to 30, 40, 50, and 60. Gradual increase in the removal dose of phlebotomy had to be stopped during the experiment due to sudden death of a rat by week 30, presumably because of circulatory disturbance associated with uncompensated anemia. We collected small amount of blood from all the rats at week 6, 10, 14, 18, 20, 30, 40, 50, and 60 to measure hematocrit and serum iron. Rats were sacrificed when ascites or weight loss was prominent. The remaining rats were killed at week 110 after birth.

Macroscopic grading and histopathologic analysis of mesothelioma

Concerning the macroscopic grading, we recorded pictures of abdominal organs of every rat at dissection. We classified the tumor size and dissemination extent into three grades, respectively. The maximum tumor size of $\geq 5\, \text{mm}$, 1 to 5 mm, and $<1\, \text{mm}$ corresponds to tumor-size grade of 3, 2, and 1, respectively. Tumor spread $\geq 50\%$ of the surface area of the intraperitoneal organs, 25% to 50% and $<25\%$ correspond to tumor-dissemination grade 3, 2, and 1, respectively. By adding these two grade numbers, we defined the tumor-progression grade. Each mesothelioma was determined as low grade when the total score was 2 or 3, moderate grade when it was 4, or high grade when it was 5 or 6. Two registered pathologists (Y. Okazaki and S. Toyokuni) diagnosed each mesothelioma as epithelioid, biphasic, or sarcomatoid subtype. We made a diagnosis of biphasic type whether epithelial or sarcomatoid fraction does not occupy 90% or more of the examined area on pathologic specimens stained with H&E.

Statistical analysis

Data are shown as means ± SEM. The cumulative probability of death was calculated for each group according to the Kaplan–Meier method and compared using the log-rank test. We used an unpaired t test, which was modified for unequal variances when necessary. The $\chi^2$ test and one-way ANOVA with a post hoc Tukey test were used for the other analyses as described in each figure legend (Fig. 1–6). All the statistics were calculated with Prism 5 (GraphPad Software Inc.).

Results

Both deferasirox and phlebotomy reduce iron storage but not serum iron

To optimize iron-reducing treatments for rats, we carried out a 16-week experiment in advance. We intended to find the maximal tolerated treatments to avoid false-negative results. We gave deferasirox to rats at 100 mg/kg/d 5 days a week and the phlebotomy was 4 mL/kg/d every week. We weighed the rats every week and confirmed that there was no significant acute effect on weight by either treatment up to the sixth week of treatment (Fig. 1A). However, deferasirox induced the reduction of rat weight at the seventh week presumably because of the renal damage as described below. We evaluated the body iron stores by several different means including serum iron concentration, hematocrit, histologic imaging analysis of spleen, and direct measurement of splenic iron concentration. We found that serum iron was rather increased after deferasirox administration in the saline vehicle group (Fig. 1B), which was not observed in the crocidolite-injected group. Hematocrit was measured every week. Both treatments slightly but significantly reduced hematocrit in the crocidolite-injected group (Fig. 1C). Thus, we confirmed the iron reduction effects of deferasirox and phlebotomy as tolerable with minimally decreased hematocrit and normal serum iron concentration. Still, relatively high dosage of deferasirox induced vascular degeneration in renal cortex (Fig. 1D).

We then evaluated splenic iron with two distinct methods. Here, we developed a method to histologically quantify body iron by combining Perls’ iron staining and imaging analysis. We found with Perls’ iron staining that there was a large difference in the splenic iron among the experimental groups (Fig. 2A; nontreatment, deferasirox, and phlebotomy). We took the picture of whole spleen section with a virtual slide system and subjected it to imaging analysis to calculate an area occupied by hemosiderin iron (see Materials and Methods for details). Integrated iron area divided by splenic area, as shown in Fig. 2B, was in good agreement with the splenic iron concentration which was determined by ICP-MS (Fig. 2B and C). Thereafter, we used this imaging analysis to semiquantitatively measure the iron content of spleen in the long-term carcinogenesis experiments.

By studying adverse effects of the high-dosage deferasirox, we conducted histopathologic analysis and found that there was vascular degeneration, a well-known drug-induced reversible renal injury (26, 27), in deferasirox-treated rats (Fig. 1D), which we believe is at least partially responsible for weight loss (Fig. 1A).

Appropriate iron reduction reduces body iron without apparent side effects

One month after the initial asbestos administration, we started administrating deferasirox to rats at 25 mg/kg/d or performing phlebotomy. The doses of deferasirox and phlebotomy were modified during the experiment in order both to eliminate side effects of each treatment and to avoid false-negative results (see Materials and Methods). We weighed rats every week, but in this experiment we did not find significant weight loss in any group (Fig. 3A and B, top). Consistent with the short-term experiment, the concentration of serum iron did not change throughout the experiment (Fig. 3A and B, middle). There was a slight decrease in hematocrit in the iron-reduction treatment groups (Fig. 3A and B, bottom). Gradual hematocrit decrease in rats subjected to intensive phlebotomy at week 24 to 30 (weeks 14–20 after the initiation of phlebotomy) was due to the repeated procedures. The dose of phlebotomy was modified thereafter because a rat did not tolerate the protocol (see Materials and Methods for details). Consequently, we confirmed that the iron-reducing treatments were effective by the imaging analysis of splenic iron at sacrifice (Fig. 3C).
Concerning the adverse effect of deferasirox on the kidney, we analyzed the histopathologic specimens and found that there was slight degeneration of tubular cells and lipofuscin deposition in both control- and deferasirox-treated groups, suggesting that these changes were aging-associated (Fig. 4A). Furthermore, scoring of renal degeneration confirmed that there was no significant renal injury after long-term deferasirox treatment both in male and female groups, whereas aging-associated alterations were more prominent in the male groups (Fig. 4B). The lack of renal injury in this long-term experiment might be due to the lower dosage of deferasirox in this chronic experiment than the acute phase experiment as shown in Fig. 1D.

**Long-term administration of therapeutic-dose deferasirox causes mesenchymal–epithelial transition with possible suppression of crocidolite-induced mesothelioma**

We analyzed the survival of crocidolite-injected rats, macroscopic progression and histopathology of the induced mesothelioma. Although we could not find significant suppression of mesothelial carcinogenesis following deferasirox or phlebotomy treatments (Fig. 5A and B), the survival rate at 110 weeks of deferasirox-treated female rats (3 alive and 4 dead) was slightly higher \( (P = 0.0496; \chi^2\text{ test}) \) than that of nontreated female rats (3 alive and 24 dead). Mesotheliomas varied in their size and dissemination (Fig. 6A and B) as well as histopathology (Fig. 6C and D). Tumor progression was evaluated on the basis of the summation of the two different phenotypes, nodular size of tumors, and dissemination area of the mesotheliomas (see Materials and Methods for details). The results indicated that deferasirox slightly reduced the progression of tumors though statistically not significant (Fig. 6B). Surprisingly, deferasirox modulated histopathology of the mesothelioma. The fraction of less malignant epithelioid subtype (20) was significantly higher in both sexes of the deferasirox-treated group than in the control group (Fig. 6D and E). Taken together, deferasirox had a modulatory effect on histopathology of crocidolite-induced mesotheliomas with a possible tumor-suppressive activity. Intensive phlebotomy, however, was not as effective as deferasirox in suppressing carcinogenesis in the light of rat survival (Fig. 5A and B), and rather had controversial effects on tumor progression and histopathology (Fig. 6C and D).
Discussion

Asbestos-induced mesothelioma is still an annoying social problem in many countries (4, 7). Considering the long incubation period of 30 to 40 years, there is a possibility to conduct certain preventive interventions especially for the high-risk population after asbestos exposure. Recently, we found that local iron overload is a major pathology in asbestos-induced mesothelial carcinogenesis for all the commercially used asbestos, namely, crocidolite, amosite, and chrysotile (20). In the present experiments, we undertook to use iron reduction as a means to prevent crocidolite-induced mesothelial carcinogenesis in rats as a premier preclinical study. And we showed that repeated administration of deferasirox at a therapeutic dose currently used for humans modulated the histopathology to increase the fraction of less aggressive epithelioid subtype over sarcomatoid subtype. This tumor-modulatory effect of deferasirox was via the mesenchymal–epithelial transition, a reverse of epithelial–mesenchymal transition (28). It is possible that ample iron is necessary for the transformed mesodermal cells to proliferate efficiently. Therefore, reduction of body iron stores such as by deferasirox can be a novel preventive strategy against crocidolite-induced mesothelioma.

Here, we used two distinct means to reduce body iron stores, oral administration of deferasirox and phlebotomy, considering the actual human clinical intervention. Although administration of deferasirox modulated histopathology of mesothelioma, phlebotomy did not. The
reason why phlebotomy did not affect crocidolite-induced mesothelioma type in this study should be carefully considered. At first, phlebotomy was conducted too intensively to avoid false-negative results even though we conducted an assessment of iron status in advance. It is possible that intensive phlebotomy induced mild inflammation, thus cytokinemia, leading to adverse effects in carcinogenesis, which is a situation specific to animal experiments. It is also possible that intensive phlebotomy induced anemia and thus hypoxia-inducible factor-1 (HIF-1) activation, which is commonly expressed in human mesothelioma, though HIF-1 does not seem to be a prognostic factor (29, 30). Sudden drop of hematocrit at 30 weeks after birth occurred only in the phlebotomy group. This is probably the additive effects of iron depletion in the bone marrow for erythroid hematopoiesis and loss of red blood cells by phlebotomy. In this sense, deferasirox presents milder and gradual iron depletion de novo, which would be more compliant with humans.

We did not reach the conclusion that administration of deferasirox prevented mesothelial carcinogenesis. However, given that the survival rate at week 110 of deferasirox-treated female rats (3 alive and 4 dead) was higher ($P = 0.0496; \chi^2$ test) than that of nontreated female rats (3 alive and 24 dead), our results indicate that deferasirox may have preventive effects in female rats, which should be corroborated in future studies. The reason why female rats were good responders to the iron reduction treatment may be due to the differences in the iron metabolism between male and female. Females showed higher serum iron in all the corresponding groups. As shown in Fig. 3B, an average of serum iron concentration in female rats with no preventive intervention was approximately 400 mg/dL, whereas it was almost half in the corresponding male rats. The serum richness of iron in female rats may explain the female as a good responder. The levels of serum iron concentration in rats are different from those of humans. The human normal
Serum iron is in the range of 50 to 200 mg/dL and thus female rats in the present study contain excess iron in their serum.

Despite the discrepancy in the amounts of serum iron of rats and humans, the dose of deferasirox used was within the therapeutic ranges for humans. We observed mild histopathologic changes in the renal tubular cells, although they were within aging-associated changes (Fig. 4A). Given that previous reports showed renal impairment in deferasirox-treated children (27), the lack of significant renal injury in the present study might be due to the difference in iron metabolism between rats and humans. Therefore, the adverse effects of deferasirox should be carefully monitored despite the possible beneficial effects in suppressing carcinogenesis.

In addition to the iron reduction effects, deferasirox reportedly may work as an anticancer drug in an iron-independent manner as an NF-κB inhibitor (31). Our present results do not directly support this idea. However, considering that intensive phlebotomy did not work as effectively as deferasirox, we may think of a possibility that the tumor-suppressive effects of deferasirox may have played a partial role in the present study.

There are four limitations of the present study. Although humans are exposed to asbestos fibers through the respiratory system, we used an intraperitoneal injection model to induce mesothelioma faster and efficiently. With this protocol, it takes approximately 2 years to develop mesothelioma in almost all the rats, which is different from human situations concerning the carcinogenic processes during the incubation period. The second is the high dose of serum iron is in the range of 50 to 200 mg/dL and thus female rats in the present study contain excess iron in their serum.

Despite the discrepancy in the amounts of serum iron of rats and humans, the dose of deferasirox used was within

Figure 4. Lack of significant renal damage after long-term deferasirox treatment. A, histologic analysis of kidney after long-term after deferasirox treatment. The presence of slight tubular degeneration (arrowheads) and lipofuscin deposition (arrows) in both control and deferasirox-treated rats. Scale bars, 100 μm. B, the area of degenerative lesions of renal tubules is indicated as a nephrotic index. Statistics was calculated between low degeneration group ("not detected" and "<10%" groups) and high degeneration group (">33%" and ">50%" groups) to let the χ² test valid. Scale bars, 100 μm. P values (ns, not significant) were determined by the χ² test.

Figure 5. Suppression of mesothelial carcinogenesis in crocidolite-injected female rats with long-term treatment of deferasirox. A and B, the survival curves of male (A) and female (B) rats injected with crocidolite. P values were determined with the log-rank test. See Results for details.
crocidolite used. Injection of lower amounts might have generated better results as a cancer prevention study. The third is that we did not use other asbestos in this experiment, whereas crocidolite has the most potent carcinogenicity among them (4). The last limitation is the relatively small number of animals enrolled for the study. Thus, further refinement of protocol should be discussed before the application of iron reduction treatments as a preventive strategy against crocidolite-induced mesothelioma in humans.

In conclusion, we for the first time showed that an oral iron chelator, deferasirox, possesses modulatory effects on crocidolite-induced mesothelial carcinogenesis. This report would be the first step to consider practical human protocols for high-risk populations to asbestos-induced mesothelioma. Simultaneously, the mechanisms by which deferasirox counteracted mesothelial carcinogenesis should be clarified. Given that epithelial–mesenchymal transition is induced by multiple factors such as hypoxia, inflammatory cytokines, and NF-κB (32), deferasirox may have counteracted epithelial–mesenchymal transition, thereby inducing mesenchymal–epithelial transition in the long course of carcinogenesis.

Disclosure of Potential Conflicts of Interest
S. Toyokuni has commercial research support (provided deferasirox) from Novartis Pharma. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: H. Nagai, S. Toyokuni
Development of methodology: H. Nagai, S. Toyokuni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Nagai, Y. Okazaki, S.H. Chew, N. Misawa, H. Yasui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Nagai, Y. Okazaki, N. Misawa, H. Yasui
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References


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