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Iron Mobilization From Crocidolite as Enhancer of Collagen Content In Rat Lung Fibroblasts

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ABSTRACT. Asbestos exposure causes pulmonary fibrosis by mechanisms that remain uncertain. There is increasing evidence that iron from asbestos is responsible for many of its effects. In this paper, we investigated the effect of iron mobilized from crocidolite asbestos on collagen content in rat lung fibroblast cultures under serum-free conditions. Crocidolite (2, 4, 6 μg/cm^2 well) increased collagen content in a dose-dependent manner (+42 ± 8, +92 ± 10, and +129 ± 13% vs controls). This effect was specific for collagen, since it did not alter total protein content and was inhibited by the iron chelator deferoxamine (DFO). Preincubation of crocidolite with citrate (1 mM) for 48 hr resulted in iron mobilization (51 μM) and increased collagen production (>3-fold) in treated cells. These effects occurred without the intervention of serum factors. The absence of cell damage, proliferation or lipid peroxidation leads to the supposition that iron from crocidolite *per se* may act as a profibrogenic agent. Although the *in vivo* participation of other cells and factors cannot be excluded, we conclude that iron released from crocidolite plays a role in collagen increase occurring during asbestosis. BIOCHEM PHARMACOL 53;11:1659–1665, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. asbestos; pulmonary fibrosis; collagen metabolism; iron chelates

Asbestos exposure is associated with an increased risk of progressive interstitial pulmonary fibrosis (asbestosis) and malignancies such as bronchogenic carcinoma and mesotheliomas [1, 2].

Despite intensive research, the molecular mechanism by which asbestos causes injury is still unknown. The pathogenicity of asbestos is related in part to the fiber type, surface area, and durability [1, 2]. Acute and chronic responses to dust exposure involve a variety of interacting cells, including macrophages, T cells and B cells, epithelial cells and pulmonary fibroblasts. These responses involve direct effects of the dust particles, as well as indirect effects mediated by soluble factors, including cytokines and eicosanoids [see Refs. 2 and 3 for review].

Evidence is accumulating that asbestos-induced damage at the airway surface may involve cellular injury through oxidant-mediated pathways [4]. Reactive oxygen species (ROS), generated either by the fibers or by activation of phagocytic cells, can be important second messengers of asbestos cytotoxicity [1, 2, 4, 5]. This oxidant generation appears to be iron-dependent and the complete chelation of the metal by deferoxamine (DFO) diminishes the toxic effects of the dusts [6, 7]. Asbestos contains iron to levels as high as 36% by weight (e.g. crocidolite) and catalyzes many of the same reactions that iron does, e.g. generation of reactive oxygen species and oxidative damage to DNA and lipids [reviewed in 4, 8]. Asbestos appears to act as an insoluble iron chelator maintaining the reactivity of iron with O_2 lower than low-molecular-weight chelators such as citrate, EDTA or nitriloacetate (NTA) [9, 10]. Intracellular mobilization of iron from asbestos by low-molecular-weight chelators may result in more widespread damage within the cell.

It is known that iron leaches from asbestos both *in vitro* [11, 12] and *in vivo* [13, 14]. Mobilization of iron from asbestos by chelators, such as citrate, EDTA, or NTA enhances some biological effects of asbestos *in vitro* [15]. *In vivo*, this iron mobilization may determine an "iron overload" in cells which have phagocytized the fibers and may be responsible for asbestos-dependent cytotoxicity and other biological effects [16].

Studies on hepatic fibrosis occurring in iron overload diseases suggest that excess of tissue iron may be involved in the stimulation of collagen synthesis [17, 18]. In iron-storage diseases (e.g. idiopathic hemochromatosis (IH), thalassemic syndromes, transfusional iron overload etc.) the liver is the main target organ of injury. Hepatic iron overload results in increased collagen biosynthesis by hepatic cells as well as stimulation of type I procollagen mRNA expression [19]. In general, hepatic fibrosis is viewed as a secondary effect following cell injury and death. However, in metabolic liver diseases such as IH or Wilson's disease, early periportal fibrosis may occur with heavy

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Abbreviations: DFO, deferoxamine; ROS, reactive oxygen species; NTA, nitriloacetate; IH, idiopathic hemochromatosis; RLF, rat lung fibroblasts; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; NBD-Cl, 7-chloro-4-nitrobenzen-2-oxa-1,3-diazole; PCA, perchloric acid; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

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parenchymal iron deposition—but in the absence of obvious cell necrosis and inflammation—suggesting that excess tissue iron provides a direct stimulus to collagen synthesis [19].

As reported above, cells that are exposed to asbestos internalize the fibers. This mechanism of iron entry into cells circumvents the controlled transport and storage of iron *in vivo* by proteins such as transferrin and ferritin, creating within the cell a potential "iron overload" condition [16] which may interfere with collagen metabolism.

Taken together, these observations led us to hypothesize that iron may play an important role in the lung collagen increase during asbestosis.

The aim of this work was to investigate the role of iron in asbestosis by means of crocidolite asbestos, an amphibole with a high percentage of iron. In particular, we tested the ability of iron associated with or mobilized from crocidolite to enhance collagen content in cultures of lung fibroblasts, a well-known target cell of asbestosis.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade and purchased from Sigma (St. Louis, Missouri, USA) unless otherwise indicated. DFO was kindly supplied by Ciba Geigy (Basel, Switzerland), trypsin was from Gibco (S. Giuliano Milanese, Milan, Italy).

Mineral Dust

Samples of crocidolite asbestos (containing 27% iron by weight) were kindly supplied by the Istituto di Medicina del Lavoro, Siena University, Siena, Italy.

Preparation of Crocidolite for Treatment of Cells

Crocidolite was suspended in 50 mM NaCl, pH 7.5 immediately before use, vortexed for 1 min, and diluted to the appropriate concentration with complete growth medium without serum. To assess the role of iron in crocidolite-induced collagen production, 100 μM DFO, an iron chelator, was added to asbestos 24 hr before cell treatment.

Effect of Iron Mobilization by Citrate on Crocidolite-induced Collagen Production

Crocidolite (1 mg/mL) was preincubated in 50 mM NaCl, pH 7.5, with or without citrate (1 mM) for 48 hr on a wrist-action shaker. An aliquot of the incubation mixture was centrifuged at 1200 g for 10 min to remove crocidolite fibers. The amount of iron in the supernatant, i.e. mobilized iron, was determined using an iron assay with ferrozine as described by Lund and Aust [15]. Complete incubation mixture, supernatant fraction (containing mobilized iron) and pellet (containing fibers after iron mobilization) were diluted to appropriate concentrations with complete growth medium without serum and used for cell treatment. In addition, cultured cells were treated with freshly prepared solutions of citrate and ferric chloride ($FeCl_3$), at a concentration that was similar to the amount of iron mobilized by citrate during the 48 hr preincubation. A sample in which crocidolite was preincubated for 48 hr with DFO (100 μM) was added in the experimental protocol.

Fibroblast Cultures

Rat lung fibroblasts (RLF) were isolated from specific pathogen-free Sprague–Dawley rats (2–3 weeks of age) (Nossan, Correzzana, Milan, Italy) using the explantation method. Briefly, the rats were anesthetized, the inferior vena cava severed, and the trachea cannulated. The lungs were lavaged with five 10 mL aliquots of PBS solution with antibiotics. The lungs were then removed from the chest, minced and incubated 1:20 (w/v) with a trypsin solution (0.25% trypsin in sterile PBS) for 30 min at 37°C. Clumps of minced tissue were removed by filtering over 100–200 μm mesh. The cells in the filtrate were washed (600 g × 10 min at 4°C), resuspended in 199 medium plus 10% fetal bovine serum (FBS) with antibiotics, and plated at a density of 2×10^5 cells/cm^2 in 25 cm^2 flasks (Costar, Cambridge, MA, USA). The cells were incubated in 95% air–5% CO_2 at 37°C for 7–10 days. On reaching visual confluence, cells were passaged and plated at 2×10^5 cells/cm^2 in 24-well plates in 199 medium plus 10% FBS for 4–5 days.

Culture Treatment

One day before the experiment, the cells were preincubated with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, antibiotics, 0.1 mM proline and 50 μg/mL ascorbic acid. After 24 hr, cells were washed with DMEM without serum and the media were replaced with supplemented DMEM (without serum) containing 50 μg/mL ascorbic acid and 50 μg/mL β-aminoproprionitrile. Cell cultures were then exposed to crocidolite (2, 4, 6 μg/cm^2) with or without DFO for 48 hr. Parallel cultures were exposed for 48 hr to crocidolite preincubated with citrate (complete mixture, top or pellet) or freshly prepared solutions of $FeCl_3$–citrate.

Cytotoxicity Assay

Cells were grown to confluence and crocidolite cytotoxicity was assessed by measuring the release of cytoplasmic lactate dehydrogenase (LDH) following 48 hr exposure to various treatments. Medium was aspirated and centrifuged (5 min at 11600 g) and assayed spectrophotometrically as described by Bergmeyer and Bernt [20]. Results were expressed as percent release of total LDH, obtained by sonication of cells. Viability of cells was assessed by staining with trypan blue.

Collagen Content Assay

Collagen secreted in the medium during the culture period was assayed using a high-pressure liquid chromatography

method essentially as described by Campa *et al.* [21]. Briefly, the media (0.5 mL) were treated 1:1 with hydrochloric acid (12 M) and incubated at 110°C for 16 hr. Hydrolysates were mixed with 35 mg charcoal, filtered (Millipore, type DA, pore size 0.65 μm), and evaporated to dryness under vacuum at 45°C. The residue was then dissolved in 0.5 mL of water. A 100 μL aliquot was buffered with 0.1 mL potassium tetraborate (0.4 M, pH 9.5) and reacted with 0.1 mL of 12 mM 7-chloro-4-nitrobenzen-2-oxa-1,3-diazole (NBD-Cl) in methanol. Samples were protected from light with aluminium foil and incubated at 37°C for 20 min. The reaction was stopped by addition of hydrochloric acid (50 μL, 1.5 M), and finally 150 μL sodium acetate (167 mM) in acetonitrile (26% v/v) was added. Samples were filtered (Millipore, type GV, pore size 0.45 μm) and a 100 μL aliquot was loaded onto the column and eluted with acetonitrile gradient as previously described [21]. The hydroxyproline content in each sample was determined by comparing peak areas of samples from the chromatogram to those generated from standard solutions, derivatized, and separated under identical conditions. Hydroxyproline was taken as an index of collagen content and expressed as picomoles hydroxyproline per microgram DNA. Collagen content was calculated by multiplying the amount of hydroxyproline by a factor of 7.69. Control experiments were performed to verify that asbestos and iron did not affect the assay itself. No interference on collagen assay was found for either. Total protein content was assessed in cell culture media by the method of Lowry *et al.* [22].

DNA Estimation

Fibroblast cultures similar to those used for collagen studies were set up in order to measure the DNA content of each well as an estimate of cell number, according to Taylor *et al.* [23]. At the end of incubation, media were discarded and cells suspended in 0.5 N perchloric acid (PCA) (300 μL/well) and allowed to stand at 90°C for 30 min. After cooling, samples were incubated at 30°C overnight with 0.6 mL diphenylamine reagent for the colorimetric assay. Following centrifugation (600 g for 10 min), the absorbance of supernatants was measured at 595 nm spectrophotometrically. Measurements of DNA in each sample were obtained from the absorbance of a standard curve with known amounts of DNA.

Lipid Peroxidation Assay

To evaluate the extent of lipid peroxidation in fibroblast cultures treated with crocidolite asbestos for 48 hr, cells from 4 culture wells were cumulated for each sample and analyzed for thiobarbituric acid reacting substance (TBARS) formation as previously described [24].

Statistical Analysis

All data are presented as means ± SD from six replicate cultures, unless otherwise indicated. Statistical evaluation was performed using the Student's *t*-test for single group comparisons and one-way analysis of variance (F test) for multiple group comparisons. A P value < 0.05 was considered significant.

TABLE 1. Toxicity of crocidolite (CRO) and crocidolite preincubated with citrate (CRO + CT) in rat lung fibroblast cultures after 48 hr treatment

	Treatment	Viability (% controls)	LDH (% release)
Control		98.7 ± 0.2	7.2 ± 2.2
CRO	2 μg/cm^2	95.0 ± 0.5	9.7 ± 2.6
	4 μg/cm^2	94.9 ± 0.5	10.2 ± 2.9
	6 μg/cm^2	94.6 ± 0.5	14.7 ± 3.5*
Control		98.6 ± 0.3	7.3 ± 2.2
CRO-CT	(pellet + top)	94.7 ± 0.5	11.0 ± 3.1
	(top)	94.9 ± 0.4	10.4 ± 2.7
	(pellet)	94.8 ± 0.5	10.7 ± 2.8

CRO: crocidolite; CRO-CT: Crocidolite (4 μg/cm^2) after preincubation with 1 mM citrate as described under METHODS. Values are means ± SD from 6 experiments. Percentage of total lactate dehydrogenase (LDH) released was determined as described in METHODS after incubation for 48 hr. *$P < 0.05$ vs control values.

RESULTS

Cytotoxicity Associated with Crocidolite Asbestos

Asbestos caused a dose-related decrease in cell viability in rat lung fibroblasts as assessed by trypan blue exclusion (Table 1). Concentrations of 6 μg crocidolite/cm^2 well were minimally toxic (defined as <95% viability with respect to controls).

Table 1 also shows the effects of increasing concentrations of crocidolite on LDH release by RLF cultured under serum-free conditions. Control cells show a basal release of ~7% of total LDH activity. At doses of 2 and 4 μg crocidolite per cm^2 of culture well, there was a slight but not significant increase in LDH release compared with controls. A significant increase in enzyme release was observed in RLF cultures treated with 6 μg crocidolite per cm^2 culture well ($P < 0.05$). No significant change in LDH release and cell viability was observed when cells were treated with crocidolite (4 μg/cm^2) preincubated for 48 hr with citrate (Table 1).

Crocidolite is a primarily iron-containing asbestos. To determine whether the iron content of crocidolite influenced cytotoxicity in fibroblast cultures, DFO, an iron chelator, was added to asbestos 24 hr prior to their addition to cell cultures. The pretreatment with 100 μM DFO resulted in a complete inhibition of asbestos-associated LDH release, with values similar to those obtained for controls (~7%) (data not shown).

Effects of Crocidolite Asbestos on Collagen Content in RLF Cultures

Preliminary experiments were performed to evaluate the direct effects of crocidolite fibers on collagen content by RLF cultures under serum-free conditions. Fig. 1 shows that the addition of various amounts of crocidolite to fibroblast

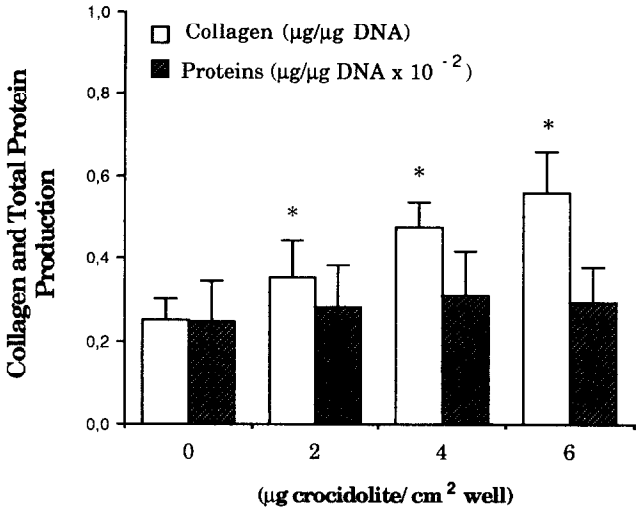

FIG. 1. Effect of crocidolite asbestos on collagen and total protein content. RLF were treated with crocidolite for 48 hr as described in Materials and Methods. Collagen values are expressed as μg/μg DNA; protein values are reported as μg/μg DNA × 10^{-2}. Each value represents mean ± SD from 6 replicate cultures. *P < 0.05 vs control values.

cultures resulted in a significant increase of collagen secreted in the media but did not affect total protein content. Collagen content was enhanced in a dose-dependent manner (+42, +92, and 129% in the presence of 2, 4, and 6 μg crocidolite per cm^2 well, respectively) (Fig. 1). These results, obtained in fibroblast cultures deprived of serum, demonstrate that crocidolite can exert its effect without the intervention of serum factors.

To determine whether the iron content of crocidolite influenced its effects on collagen content in RLF cultures, the iron chelator DFO was added to fibers 24 hr prior to their addition to cell cultures. As shown in Fig. 2, DFO

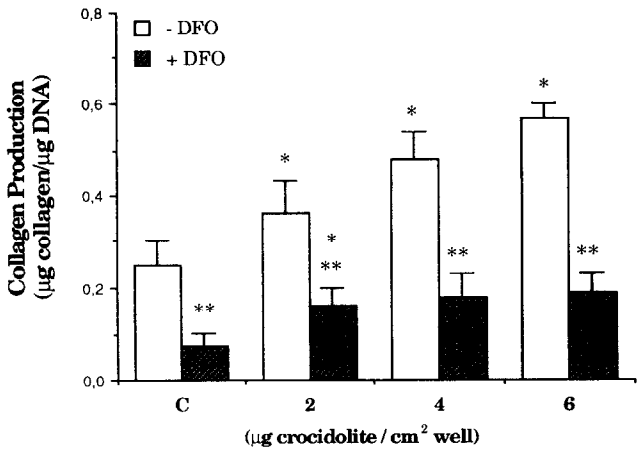

FIG. 2. Effect of DFO on crocidolite-induced collagen increase. DFO (100 μM) was incubated with crocidolite 24 hr before cell treatment as described in Materials and Methods. Values are expressed as μg of collagen per μg of DNA. Data are means ± SD from 6 experiments. *P < 0.05 vs control incubated in the absence of DFO; **P < 0.05 vs the corresponding sample incubated in the absence of DFO.

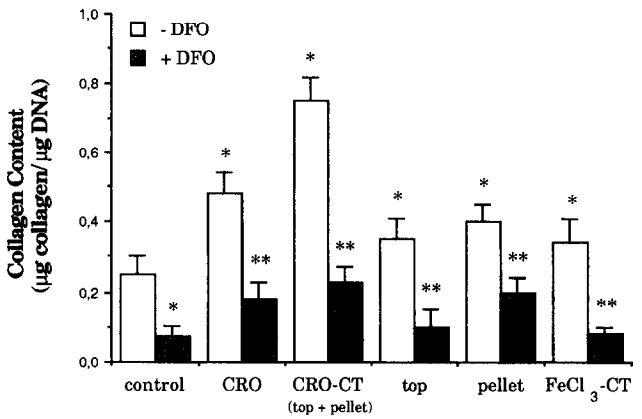

FIG. 3. Effect of iron mobilization on crocidolite-induced collagen increase. Iron was mobilized from crocidolite in the presence of 1 mM citrate, as described in Materials and Methods. Some samples were preincubated with 100 μM DFO for 48 hr before cell treatment. DFO: deferoxamine 100 μM; CRO: crocidolite 4 μg/cm^2 well; CRO-CT: crocidolite preincubated with citrate, complete samples containing mobilized iron (51 μM) plus fibers; top: sample without fibers containing 51 μM mobilized iron; pellet: fibers after iron mobilization; $FeCl_3$-CT: freshly prepared control of 50 μM $FeCl_3$-citrate. Each value represents mean ± SD from 6 experiments. *P < 0.05 vs controls incubated in the absence of DFO; **P < 0.05 vs the corresponding sample incubated in the absence of DFO.

strongly decreased crocidolite-induced collagen increase at all doses tested.

Iron Mobilization in vitro *from Crocidolite*

Incubation of crocidolite in 50 mM NaCl containing 1 mM citrate resulted in iron mobilization which increased with the time. At 48 hr, about 50 nmol of iron per mg of crocidolite were mobilized. No iron was mobilized from 24–36 hr in the mixture without citrate (lower limit of detection was 2 nmol iron/mg asbestos) and only minimal amounts were detected at 48 hr of incubation.

Effect of Iron Mobilization on Crocidolite-induced Collagen Increase

To determine if iron mobilized from crocidolite contributes to collagen increase more than iron associated with crocidolite, the effect of citrate on asbestos-induced collagen increase was investigated. For this study the dose of 4 μg crocidolite/cm^2 well was used. The results (Fig. 3) show that the addition of citrate enhanced collagen content about 3-fold compared with controls and about 1.5-fold compared with crocidolite alone, suggesting that iron mobilization may be an important factor in asbestos-induced collagen increase.

To investigate whether collagen increase was dependent upon iron associated with or mobilized from crocidolite, the collagen increase stimulated by crocidolite plus citrate was compared with that stimulated by the supernatant (from which crocidolite fibers had been removed) or by the pellet

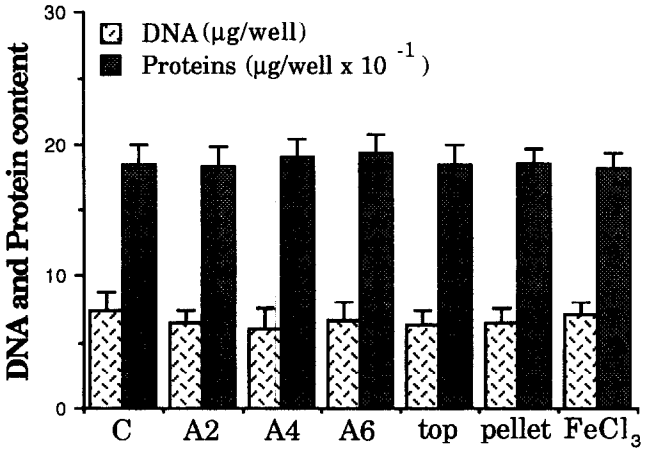

FIG. 4. Effect of crocidolite and iron on cell proliferation in RLF. C: control; A2, A4 and A6: crocidolite 2, 4 and 6 μg/cm² well, respectively; top: supernatant containing iron (51 μM) mobilized from crocidolite; pellet: fibers after iron mobilization; $FeCl_3$: freshly prepared control of 50 μM $FeCl_3$-citrate. DNA values are expressed as μg/culture well, protein values are reported as μg/culture well × 10^{-1}. Data are means ± SD from 6 replicate cultures. No significant changes were detected for DNA and protein content in fibroblast cultures after 48 hr exposure to crocidolite or iron.

(which contained the fibers). Cultures treated with the supernatant showed an approximate 40% increase in collagen content compared to controls, an effect that was strongly inhibited by DFO (Fig. 3). This result demonstrates that iron mobilized from crocidolite contributes to collagen stimulation in RLF cultures. On the other hand, when cells were treated with the pellet of preincubation mixture, a 60% increase in collagen content in respect to controls was found (Fig. 3). This increase might be accounted for by the fibers. In any case, the addition of 100 μM DFO resulted in a 50% decrease in collagen content, suggesting that iron was still present on the fibers after citrate preincubation.

A control of freshly prepared 50 μM Fe (III) chelated with citrate was examined to compare collagen increase with that observed when 51 μM iron was mobilized from crocidolite by citrate. This control stimulated an increase in collagen content very close (+36%) to that obtained for the supernatant sample that had a similar amount of iron mobilized from crocidolite (Fig. 3).

DNA and Protein Content in RLF Cultures

Under our experimental conditions, crocidolite did not influence cell proliferation. In fact, exposure of RLF for 48 hr to crocidolite (2, 4, 6 μg/cm²), supernatant containing mobilized iron or pellet containing iron-depleted fibers, did not result in a significant increase in DNA or protein content (Fig. 4). In addition, no detectable changes in TBARS, as assessed by using the thiobarbituric acid assay, were found in RLF cultures treated for 48 hr with crocidolite or crocidolite plus citrate (data not shown). These results suggest that, at least under our experimental conditions, crocidolite-induced collagen increase is not associated with cell proliferation or with lipid peroxidation.

DISCUSSION

There is accumulating evidence that iron on- or from-asbestos fibers is responsible for cytotoxicity and other biological effects [8, 11, 16, 25]. The present study was carried out to investigate the role of asbestos-associated iron in collagen production during asbestosis.

The results presented here strongly suggest that iron is involved in the collagen increase occurring in fibroblast cultures challanged with crocidolite. Crocidolite asbestos increased collagen content in a dose-related manner in the range of concentrations observed by us and this effect was completely inhibited by DFO, a potent iron chelator. In addition, mobilization of iron from crocidolite by citrate greatly enhanced collagen content in the media of RLF cultures. This suggests that iron mobilized from asbestos contributes to collagen content increase more than iron associated with fibers. This is in agreement with previous studies on crocidolite-dependent DNA damage, where it was demonstrated that mobilization of iron from crocidolite by chelators greatly increased its *in vitro* biochemical reactivity and DNA single-strand break formation [16, 25]. Supernatant, containing iron mobilized from crocidolite, seems to be responsible for 46% of the collagen increase observed in cells treated with complete preincubation mixture. This increase was completely prevented by DFO pretreatment. In addition, the pellet exhibited a great effect on collagen increase in RLF cultures. This is not surprising since only minimal amounts of the total iron present on asbestos can be mobilized by chelators [9, 10, 15, 16]. Thus, iron is still present on the fibers after citrate preincubation and is likely slowly released in the media during cell treatment. In fact, DFO significantly reduced the effects of pellet on collagen increase. Although we cannot exclude the importance of fibers in asbestos-induced collagen increase, our data strongly suggest that the iron mobilized from crocidolite plays a role in enhancing this effect. Finally, when a freshly prepared solution of $FeCl_3$ was added to RLF cultures at concentrations comparable with the amount of iron mobilized from crocidolite by citrate, a similar response in collagen increase was observed. The latter results further support the idea that iron plays a role in asbestos-dependent collagen increase.

Iron is essential for normal collagen synthesis since it is required as a cofactor for prolyl hydroxylase, a key enzyme in collagen biosynthesis [26]. A shortage of iron has been found to be a limiting factor for enzyme activity [26, 27]. On the other hand, increased prolyl hydroxylase activity and collagen synthesis has been reported in various models of iron overload [18, 28, 29]. In these models, iron overload resulted in increased net and relative collagen biosynthesis by hepatocytes as well as in increased mRNA expression for type I procollagen. The exact mechanism of increased

prolyl-hydroxylase activity and collagen synthesis in response to iron overload is still unknown. Iron could in some way increase the synthesis of the subunits of prolyl-hydroxylase and/or enhance conversion to the active tetramer. It is also possible that the increase in prolyl-hydroxylase activity may be a secondary phenomenon. The iron might increase gene transcription or enhance the expression of mRNA for the formation of the procollagen chains [30]. This remains to be elucidated.

Iron mobilized from crocidolite may represent an "iron overload" for the cells challenged with asbestos. As previously reported, mobilization of iron from asbestos by chelators was responsible for ·OH formation [9] and DNA strand breaks in cultured cells [25, 31]. Data reported in this paper demonstrate that iron mobilized from asbestos can also be involved in the increase in collagen production.

At physiological pH, chelators were required for iron mobilization *in vitro* [15] suggesting that *in vivo* as well this process could be mediated by low-molecular-weight chelators such as citrate or others [10]. It has been reported that iron mobilization *in vitro* increases as the pH decreases [16]. The pH within phagosomes, which often contain intracellular asbestos, can be significantly lower than the overall pH of the cell, suggesting an increased iron mobilization in these cellular districts [16]. Iron mobilization from crocidolite by low-molecular-weight cellular chelators may determine an *in vivo* "iron overload" in cells which have phagocytized the fibers and may be responsible for collagen stimulation and other crocidolite-dependent biological effects in target cells of the lung.

Cell injury and repair are important in the pathogenesis of the fibrotic process [32]. Asbestos may cause lung damage by generating ROS, which may then alter vital cellular constituents leading to either cell death or malignant transformation [1, 2, 4]. Under our experimental conditions, crocidolite-induced collagen increase appears to occur in the absence of significant cell damage and necrosis. These data, taken together, suggest that the "iron overload" occurring in crocidolite-treated RLF cultures provides a direct stimulus to collagen synthesis without prior iron-mediated cellular injury.

Iron also induces lipid peroxidation, and increased prolyl hydroxylase activity and collagen synthesis have been reported in fibroblasts exposed to superoxide [33]. Chojkier *et al.* [34] provided information describing a link between lipid peroxidation and fibrogenesis. In these studies, human fibroblast cultures were subjected to iron-induced peroxidation or added malondialdehyde (MDA), and an increase in collagen gene transcription and collagen production was observed. However, under our experimental conditions, the effect of iron on collagen increase does not appear to be correlated with membrane lipid peroxidation of RLF, since iron mobilized from crocidolite does not produce detectable changes in TBARS, as assessed by the thiobarbituric acid assay. The discrepancy between our results and those reported by others may be related to the higher doses of asbestos used by these authors as well as to the difference in cell types. These results support the hypothesis that iron *per se* may act as a profibrogenic agent.

Our study, carried out under serum-free conditions, provides evidence that iron mobilized from crocidolite can enhance collagen production *in vitro* without the intervention of cytokines or other serum factors. *In vivo* responses to asbestos are obviously more complex, involving as they do a variety of interacting cells and soluble factors. Alveolar macrophage is thought to be a major mediator of the pulmonary response to inhaled dust. These cells accumulate at the sites of asbestos deposition in the lung and phagocytize the fibers. The iron mobilization from asbestos within macrophage phagosomes may facilitate iron availability for pulmonary fibroblasts, thereby enhancing collagen production.

In conclusion, in this paper we demonstrate that iron mobilized from crocidolite can enhance collagen production in rat lung fibroblasts without affecting total protein and DNA content. This effect occurs without the intervention of serum factors. The absence of cell damage, proliferation or detectable lipid peroxidation suggests that iron mobilized from crocidolite directly stimulates collagen production by rat lung fibroblasts. The basis for the stimulatory effect of mobilized iron on collagen production in fibroblast cultures remains to be determined. Although we cannot exclude the *in vivo* participation of other cells and factors, we conclude that iron mobilization is responsible *in vitro* for a significant amount of crocidolite-induced collagen production.

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