Br. J. Exp. Path. (1989) 70, 305-315

Characterization of excessive collagen production during development of pulmonary fibrosis induced by chronic silica inhalation in rats

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Received for publication 27 September 1988
Accepted for publication 1 February 1989

Summary. The activation of collagen synthesis during development of silicotic fibrosis was studied in rats exposed, in dusting chambers, to respirable SiO_2 for periods of 2, 4, 6 or 12 months. Control animals were exposed similarly to clean air or TiO_2. Development of fibrosis was followed by histological examination, measurement of lung weight and determination of lung collagen content (as hydroxyproline). A steady increase in lung weight and collagen content together with changes in cellularity and metabolic activity of the lungs, as ascertained by chemical determination of DNA and RNA, were measured in the lungs of the SiO_2-exposed animals. Hybridization of total lung RNA, extracted at each time point, with cDNA probes specific for type I and type III procollagen mRNA levels showed that the development of fibrosis was associated with increased levels, as compared to age matched controls, of pulmonary procollagen mRNAs. Interestingly, the highest levels of procollagen mRNAs were observed in young (pretreatment control) animals, suggesting that during pulmonary development collagen metabolism in lungs is even greater than during development of fibrosis. In rats exposed to SiO_2 the increase in type III procollagen mRNA occurred earlier than the increase in type I procollagen mRNAs. These observations demonstrate both age-dependent and silicosis-related changes in pulmonary procollagen mRNA levels. The results suggest that development of silicosis is associated with an altered capacity of the lungs to regulate collagen accumulation.

Keywords: silicosis, pulmonary fibrosis, collagen

Fibrotic lung diseases form a heterogeneous and complex group of pulmonary conditions characterized by fibrosis of alveolar structures (Rennard & Crystal 1982). One group of fibrogenic agents which have been extensively studied, both *in vivo* and *in vitro*, is the inorganic dusts, especially silica (Rennard & Crystal 1982; Heppleston *et al.* 1984; Beck & Bignon 1985). Although much effort has been directed towards correlating biochemi-

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cal changes with histological findings, our understanding of the regulation of collagen metabolism in pulmonary fibrosis is far from complete. Attempts to quantify the accumulation of collagen in fibrotic lungs have given variable results. While total collagen is clearly elevated in the lungs of experimental animals with experimental silicosis (Lehtinen *et al.* 1983; Reiser *et al.* 1983), some animal models and samples from patients with idiopathic pulmonary fibrosis have not revealed any increases in the amount of collagen per lung weight (Fulmer *et al.* 1980).

One of the main problems related to studies on pulmonary fibrosis is the extremely complex structure of the lungs which is further complicated by the various changes in cell populations during the fibrotic process. Other difficulties include the small amount of collagen being synthesized in normal lungs and the fact that age-related changes in collagen synthesis complicate the measurements (Rennard & Crystal 1982). Combined with the long half-life of collagen, it is conceivable that even relatively small increases in the synthesis of this accumulating protein, over a period of months, could result in fibrosis (Kulonen *et al.* (1980).

Development of fibrotic lung diseases is invariably associated with an alveolar inflammation in which mononuclear phagocytes (alveolar macrophages) play an important role (Bitterman *et al.* 1986). Although the underlying mechanisms producing fibrosis are incompletely understood, the concept of soluble fibrogenic macrophage factor(s) liberated from macrophages is generally accepted (Aho *et al.* 1983; Kulonen *et al.* 1984; 1985; Lugano *et al.* 1984).

Of the animal models used to study collagen metabolism in lung fibrosis, experimental silicosis produced in the rat using a single intratracheal instillation of silica has perhaps been most widely used. Such studies have reported increases in pulmonary collagen levels, mainly types I and III, following SiO_2 treatment (Chvapil *et al.* 1979; Lugano *et al.* 1982; Lehtinen *et al.* 1983; Reiser *et al.* 1983; Callis *et al.* 1985). More recently the activities of the enzymes involved in procollagen processing have also been determined as indicators of collagen synthesizing activity (Poole 1985; Poole *et al.* 1985). The development of molecular biology has provided new techniques to determine effectively the levels of mRNAs directing synthesis of various procollagens, and of other proteins including growth factors and proto-oncogenes. These new tools are particularly suitable for studies on complex pathogenic processes and synthesis rates of accumulating proteins. We have previously used this type of approach to study pulmonary procollagen mRNA levels in the acute silicosis model in rats (Makela & Vuorio 1986).

Since the 'instillation model' described above may not reflect the biological responses following chronic inhalation, we have investigated silicosis produced in rats by long-term exposure to SiO_2 in a dusting chamber, which more closely parallels the natural course of the human disease. To analyse the changes in collagen production more refined techniques of molecular biology were used, including determination of types I and III collagen mRNA levels, using species-specific cDNA probes, at various time points during development of fibrosis.

Materials and methods

Silica. The silica particles were prepared from a sample of Min-U-Sil, using a procedure similar to that described by Dauber *et al.* (1980). The size distribution of the sample, as determined by electron microscopy, was 1.87 ± 1.26 μm (mean+s.d.), i.e. no particles greater than 5 μm were present. Respirable TiO_2 was obtained from the Institute of Occupational Medicine, Edinburgh, UK.

Animals. The experimental animals were Fischer F334 rats originally obtained from Charles River Breeding Laboratories Inc., Wilmington, USA. They had been bred under barrier-maintained conditions and the colony subjected to regular histological and

microbiological screening. There was no evidence of pulmonary infection in the rats used in this investigation.

Silica exposure. Forty-eight rats (24 males and 24 females), approximately 10 weeks old and weighing between 190 and 260 g were randomly allocated to treatment and control groups. The treated rats were exposed to the respirable silica in an inhalation chamber as described previously (Wagner *et al.* 1974). Exposure was carried out for 6 h per day, 5 days per week at an average dust concentration of 10 mg/m^3 of air. The animals were sacrificed after 2, 4, 6 and 12 months of treatment (one group of six animals, three males and three females, at each time-point). The control animals, grouped similarly in identical chambers but exposed to clean air, were sacrificed at the same time as the animals in the experimental groups. In addition to the 'non-treated' controls, six rats, four males and two females, were simultaneously exposed to titanium dioxide (TiO_2) using the same dosing regime as for the silica-treated animals. These rats were killed after 2 and 4 months of exposure, two males and one female at each time-point. (Animals were treated only for up to 4 months because of lack of standard TiO_2 samples.) Four animals (pretreatment group) were sacrificed and analysed at the beginning of the study to give pretreatment base-line values.

At the end of treatment the rats were weighed, anaesthetized with pentobarbital, the thorax opened and the animals bled out from the right ventricle. The lungs were gently perfused with sterile isotonic saline before being removed and weighed. The lungs were fixed either in 1% neutral formalin for subsequent histological processing (to be reported separately) or frozen at −70°C for biochemical determinations.

Preparation of total RNA. For isolation of total RNA, frozen lung tissue was pulverized by grinding in a mortar under liquid nitrogen, followed by homogenization in 1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 10 mM Tris-HCl, pH 7.5, containing 200 μg/ml proteinase K using an Ultra-Turrax homogenizer. Thereafter total RNA was extracted as described by Rowe *et al.* (1978).

RNA gel transfers and slot blots. Total cellular RNAs were fractionated by electrophoresis on 0.7% agarose gels after denaturation with glyoxal and dimethylsulphoxide (Thomas 1980) Duplicate samples were analysed: one set was used for staining with ethidium bromide, the other set was transferred by blotting onto a Pall Biodyne membrane. Most of the total RNA consisted of the large (28S) and the small (18S) rRNAs, which were visualized by the ethidium bromide staining. Densitometric analyses were always performed to ensure that equal amounts of RNA were applied into each lane. After the transfer the RNAs were fixed to the filters by baking for 2 h at 80°C. The filters were prehybridized and hybridized with nick-translated plasmid DNAs at 45°C for 20 h as described by Thomas (1980). After stringent washes at 55°C, the filters were exposed with X-ray films.

Total RNAs were also analysed without fractionation. Serial dilutions (corresponding to 0.5–5 μg) of each RNA preparation were applied onto nitrocellulose filters, using a vacuum manifold (Schleicher and Schuell). The filters were baked, prehybridized and hybridized as described above.

The above methods were used to determine changes in the relative amount of specific mRNAs in the total RNA samples. As lungs from the same experimental group were also analysed for changes in DNA and RNA levels (see below), it was also possible to determine the changes in the total amount of procollagen mRNAs per lung at the various time-points.

Hybridization probes. The levels of procollagen mRNAs in the various hybridization filters were measured using the following cDNA probes: pα1R2, for rat proα1(I) collagen mRNA and pα2R2, for rat proα2(I) collagen mRNA (Genovese *et al.* 1985), and

pRGR5 for rat proαI (III) collagen mRNA. The plasmids were labelled by nick-translation with ^{32}P-dCTP. As an additional control of total RNA levels, the cDNA probe for rat glyceraldehyde 3-phosphate dehydrogenase, pRGAPDH, was used in slot blot hybridizations (Fort *et al.* 1985).

Chemical determinations. From each group, two lung samples were homogenized in 10 volumes of distilled water with an Ultra-Turrax homogenizer, followed by lyophilization of the homogenate. Nucleic acids were extracted from the lungs as described by Schmidt & Tannhauser (1945). DNA was measured with the method of Burton (1956), and RNA as described by Ceriotti (1955).

Aliquots of the homogenates were hydrolysed in 6 M HCl at 140°C for 3 h for determination of hydroxyproline with the method of Juva and Prockop (1966).

Results

Microscopic examination of the histological sections clearly revealed progressive development of pulmonary inflammation and fibrosis in the SiO_2 group. Detailed analysis of the histological changes will be published separately.

Measurement of changes in lung weight Fig. 1(a) showed a time-dependent increase in the SiO_2 group to a level of approximately 2 to 2.5-fold higher than control animals or TiO_2-exposed animals. At the same time, total animal weights remained practically constant (Table 1). To determine biochemically the extent of fibrosis the collagen content of lungs was estimated at each time-point by measurement of hydroxyproline in homogenized lungs. The increase observed in the lung collagen content in the SiO_2 group paralleled the increase in lung weight (Fig. 1 (b)).

Pulmonary RNA contents were measured to determine changes in the overall metabolic synthetic activity of the lungs. As shown in Fig. 2 (a) the RNA content of the

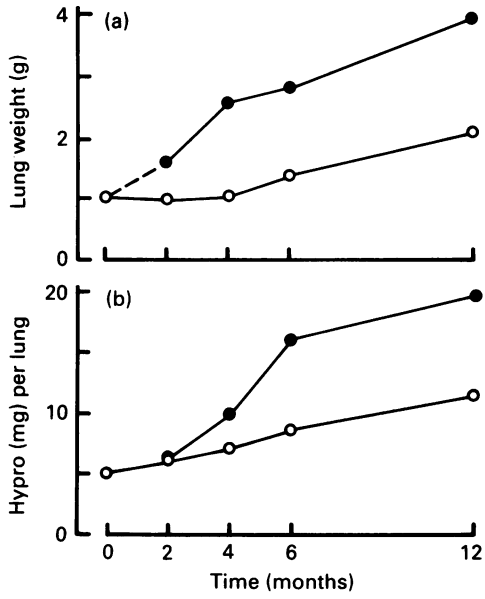

Fig. 1. Changes in lung weight and collagen (hydroxyproline) content as a measure of the development of silicotic fibrosis in the chronic inhalation model. The SiO_2 inhalation was started at day 0. The experimental animals and their controls were sacrificed after 2, 4, 6 and 12 months. The lungs were removed, perfused, weighed, and analysed for hydroxyproline content. Panel a, determination of lung weight (the average of four animals), panel b, hydroxyproline content (average of two animals). Open symbols, control animals; closed symbols, SiO_2-exposed animals.

Table 1. Changes in the weights of the experimental animals.

Exposure time	Control	SiO_2	TiO_2
Pre-dose control	227		
2 months	251	260	267
4 months	282	289	264
6 months	288	285	
12 months	242	242	

Each number is the average weight (in grams) of the animals in the group (four animals in control and SiO_2 groups; two in the TiO_2 group).

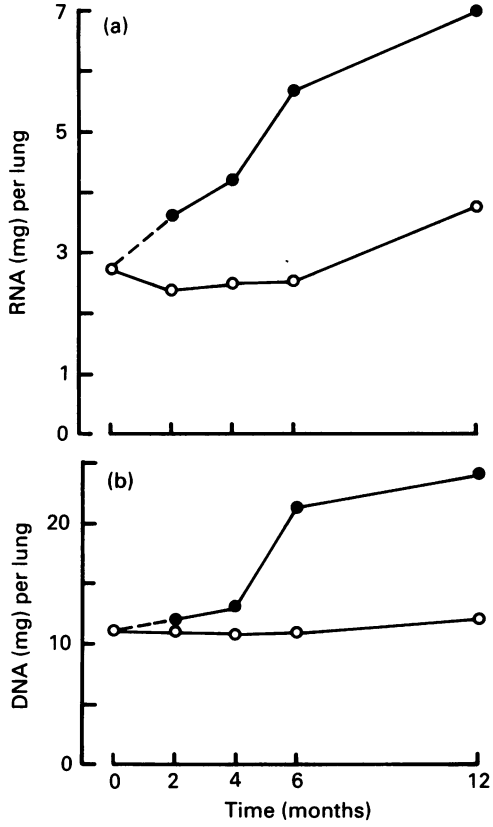

Fig. 2. Changes in pulmonary RNA and DNA levels during ageing and development of silicotic fibrosis. Aliquots of lung homogenates from two animals at each time point (described in Fig. 1) were analysed for RNA and DNA content. The results are shown as the amount of RNA (panel a) and DNA (panel b) per the whole lung. Open symbols, control group; closed symbols, SiO_2-group.

lungs from the control animals remained fairly constant throughout the study although the animals killed at 12 months did show slightly increased levels as compared to pre-dose values. In contrast, the SiO_2-exposed animals showed time-dependent increases in pulmonary RNA content with levels being significantly greater ($P<0.05$) than controls at each time-point. Determination of pulmonary DNA levels revealed that most of the increase in RNA levels could be explained by a concomitant increase in the number of cells in the silicotic lungs (Fig. 2(b)). However, during the early part of the study (2 and 4 months) the RNA levels increased faster than DNA levels suggesting that the protein-synthesizing activity per cell was increased at this stage.

Determination of type I and type III procollagen mRNAs was performed by hybridization of Northern blots and slot blots containing serial dilutions of total RNAs. The specificity of the hybridizations was good as exemplified in Fig. 3, illustrating a Northern blot hybridized with the cDNA probes. At each time-point, 2–3 lungs were used for RNA isolation, each analysed separately and at least twice in Northern hybridization and once in slot blots with all the three procollagen cDNA probes. Rather surprisingly the results showed a marked age-dependent decrease in the pulmonary procollagen mRNA levels (Figs 3, 4(a) and 5(a)). This decrease, particularly evident in the lungs of the control animals, was also seen in the animals exposed to silica. The proportion of type I and type III procollagen mRNAs in silicotic lungs increased above the level seen in controls only after 6 months' exposure. If one takes into account the changes observed in total lung RNA (Fig. 2(a)), the difference between control and silicotic groups in the amount of procollagen mRNAs per lung becomes markedly greater (Fig 4(b) and 5(b)). The hybridization data could also be used to estimate the ratio of type I and type III procollagen mRNAs at each time-point. As shown in Figs 4 and 5, during the development of silicosis the amount of type III procollagen mRNA per lung exhibited an earlier increase than that of type I procollagen mRNAs. This suggests that the lungs respond to silica by increasing the level of proα1(III) collagen mRNA more than the level of proα1(I) collagen mRNA.

Discussion

Despite numerous studies the development of fibrotic lung disease still presents a number of puzzling problems. These are related not only

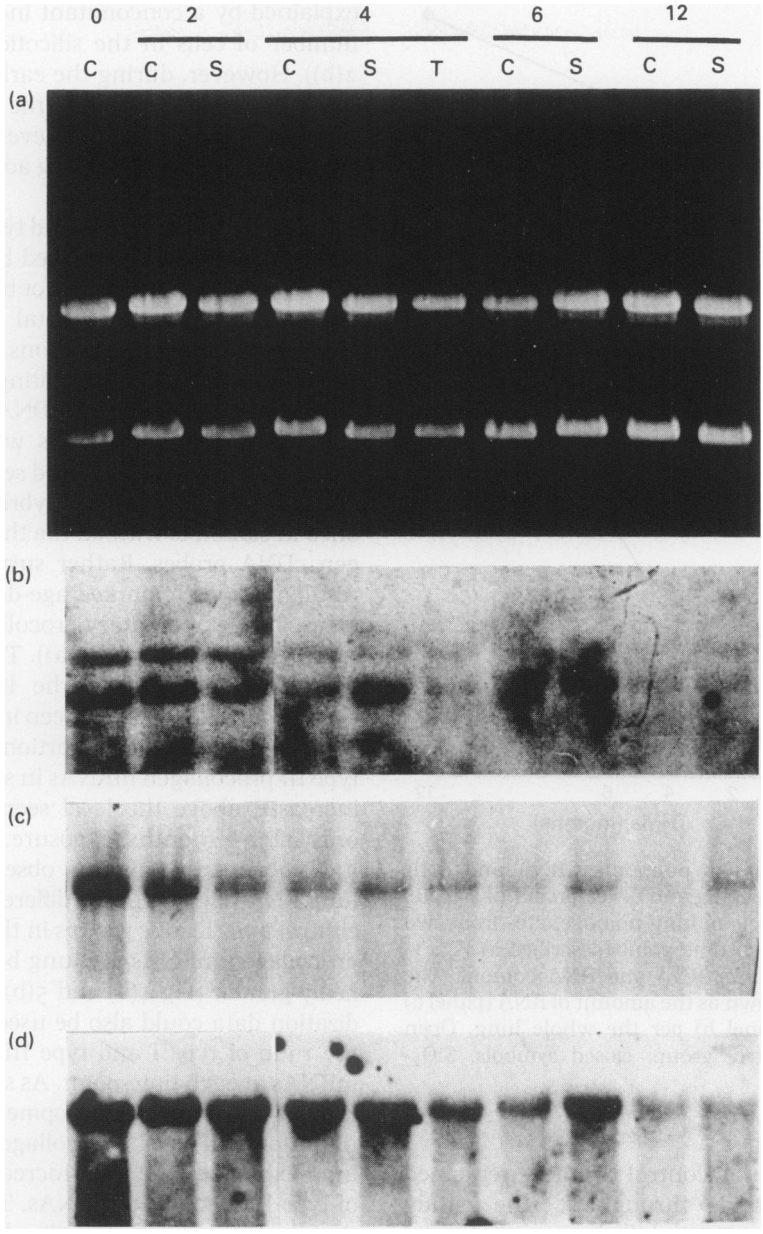

Fig. 3. Northern hybridization of total pulmonary RNAs for the levels of procollagen mRNAs. Total RNA was isolated at various time-points from perfused lungs, fractionated under denatured conditions by electrophoresis and stained with ethidium bromide (panel a), or transferred by blotting to Pall Biodyne membranes, and hybridized with cDNA probes for procollagens: panel b, the probe specific for rat proα1(I) collagen mRNA; panel c, for rat proα2(I) collagen mRNA; and panel d, for rat proα1 (III) collagen mRNA. The letters above each lane indicate that RNA was isolated from control animals (C), from SiO_2 exposed animals (S), or from TiO_2 exposed animals (T). The numbers indicate the time (in months) after beginning of the exposure.

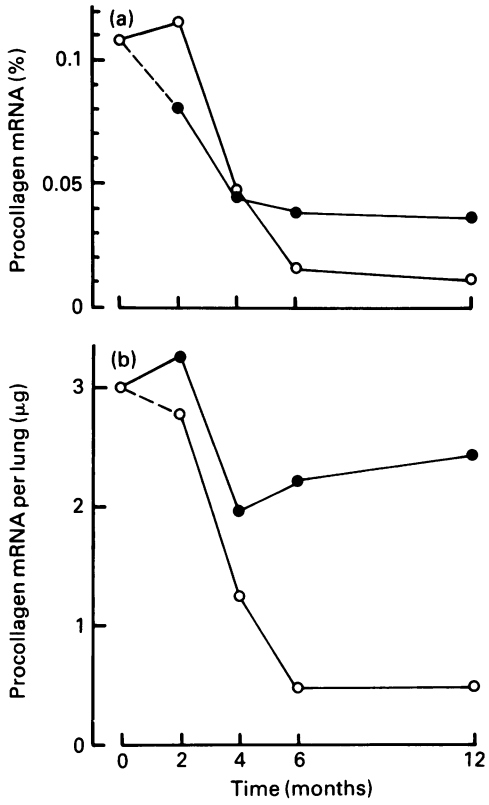

Fig. 4. Changes in type I procollagen mRNA levels during ageing and development of silicotic fibrosis. For each time-point total RNA was isolated from lungs of 2 or 3 animals described in Figs 1 and 2, and analysed and quantified by hybridization of Northern blots (Fig. 3) and slot blots for the levels of proα1(I) and proα2(I) collagen mRNAs. Panel a, the proportion of type I procollagen mRNAs in total lung RNA; panel b, total amount of type I procollagen mRNAs per lung. Open symbols, control group; closed symbols, SiO_2 group.

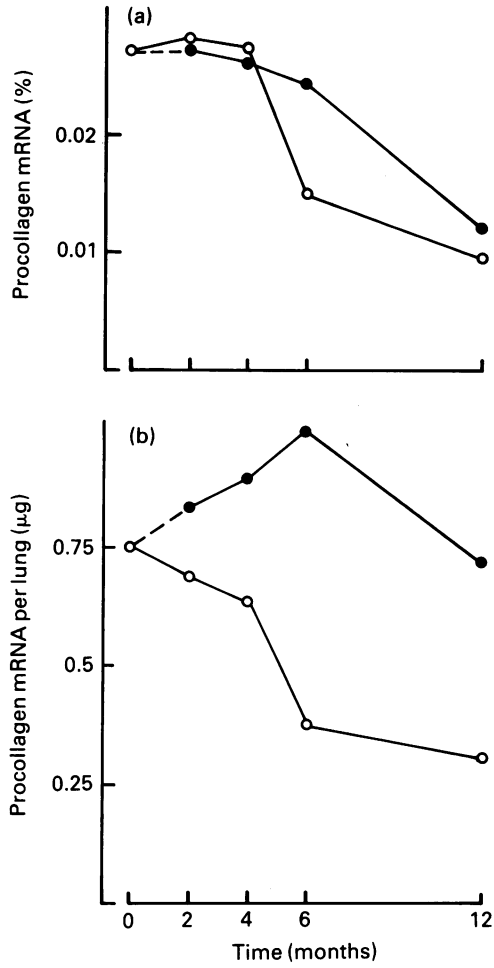

Fig. 5. Changes in type III procollagen mRNA levels during ageing and development of silicotic fibrosis. For each time-point total RNA isolated from lungs of 2 or 3 animals described in Figs 1 and 2, and analysed and quantified by hybridization of Northern blots (Fig. 3) and slot blots for the level of proα1(III) collagen mRNA. Panel a, the proportion of type III procollagen mRNAs in total lung RNA; panel b, total amount of type III procollagen mRNAs per lung. Open symbols, control group; closed symbols, SiO_2 group.

to the pathogenesis of the disease but also to discrepancies between results obtained *in vivo* and *in vitro*, and between models and human disease. One of the interesting questions has been: why does the collagen/lung weight ratio remain unaltered in human lung fibrosis (Fulmer *et al.* 1980)? The present study adds to this confusion by showing that during normal growth and development the lungs exhibit a higher collagen-synthesizing potential (higher procollagen mRNA levels) than is seen during the actual development of silicotic fibrosis (Fig. 4). The results presented here show that lung collagen production was high in the

animals killed at 2 months from the start of the study; that is, when the rats were approximately 19 weeks old. Using recombinant cDNA fragments as standards it was estimated that at this time-point type I collagen mRNAs accounted for approximately 0.1% of total lung RNA. Assuming that mRNA comprises 5% of total RNA, the type I collagen mRNAs would account for approximately 2% of total lung mRNA (protein-synthesizing capacity). This compares well with earlier estimates which have suggested lung parenchymal cells to devote 2–5% of their total protein-synthesizing capacity to collagen production (Bradley al. 1974, 1975).

Although comparative studies on procollagen mRNA levels are not available, a lot of the protein chemical evidence supports our findings. A detailed study on development of rabbit lungs revealed marked increases in pulmonary collagen content during foetal development and maturation (Bradley *et al.* 1974). Similarly, a tenfold increase in collagen content in human lung parenchyma has been reported during development from a 17-week foetus to adult (Bradley *et al.* 1975).

Several studies have shown a marked reduction in the rate of collagen production after maturity is reached (Rennard & Crystal, 1982). The correlation between the drop in collagen synthesis and onset of maturity was emphasized in this investigation where pulmonary procollagen in RNA levels in the rats killed at 4, 6 and 12 months were considerably lower than those killed at 2 months, that is, the time-point correlating with cessation of weight gain. Similarly, our estimates for the ratio of type I and type III collagen mRNAs in the normal lungs agree with earlier protein chemical studies (Hance *et al.* 1976).

Procollagen mRNA levels, however, should not be considered as the only factor regulating collagen production *in vivo*. The complicated process of collagen synthesis (Prockop & Kivirikko 1984; Cheah 1985) offers numerous other steps where collagen production can be regulated. Considerable experimental evidence from fibrotic conditions such as scleroderma and liver cirrhosis (Vuorio *et al.* 1987; Ala-Kokko *et al.* 1987) has been reported which demonstrates that elevated procollagen production is associated with correspondingly elevated procollagen mRNA.

Our findings about the increased procollagen mRNA levels indicates that fibrotic lungs have acquired a potential to produce higher amounts of collagen than normal lungs. Combined with a steady increase in total lung hydroxyproline content (Fig. 1) this observation strongly suggests that increased accumulation of collagen in the model is mediated through elevated levels of procollagen mRNAs. The fact that the rats exposed to chronic inhalation of SiO_2 developed lung fibrosis with the pulmonary (type I and type III) procollagen mRNAs similar to or mostly lower than those observed during normal growth suggests that lungs can tolerate high rates of collagen production without fibrosis. We suggest that a major derangement of the regulatory mechanisms of collagen synthetic pathway must therefore be involved in the fibrotic process. Since degradation of newly synthesized collagen has been shown to be an important mechanism regulating collagen accumulation in lungs (McAnulty & Laurent 1987) it is possible that fibrosis may be associated jointly with increased collagen synthesis and decreased collagen degradation. Our findings that an increase was first observed in the amount of type III collagen mRNA and only later in type I procollagen mRNAs agrees with the observation that inflammation is associated with an early increase in type III collagen (Gay *et al.* 1978). Conversely, in bleomycin-induced lung fibrosis a transient decrease in the ratio of type I and type III collagen has been observed (Brody *et al.* 1982).

It is interesting to note that while lung hydroxyproline content in the chronic SiO_2 inhalation model exhibited a steady time-dependent increase and the weight of the lungs increased proportionally the proportion of collagen per unit dry weight remained

unchanged (Fig. 1). This is different from the findings obtained with the single intratracheal instillation model (Chvapil *et al.* 1979; Lehtinen *et al.* 1983; Reiser *et al.* 1983). This may indicate that the chronic inhalation model more closely parallels the human lung fibrosis, where no proportional increase in lung collagen content has been observed (Fulmer *et al.* 1980).

The single instillation mode of silicosis has been characterized in detail in respect to collagen production. The acute phase appears to involve an early stimulation of collagen production, which is seen as increases in (i) pulmonary collagen contents (Chvapil *et al.* 1979; Lugano *et al.* 1982; Lehtinen *et al.* 1983; Callis *et al.* 1985), (ii) activities of specific processing enzymes (Poole 1985; Poole *et al.* 1985), and (iii) the levels of procollagen mRNAs (Makela & Vuorio 1986). Since macrophages are capable of liberating factors which can increase fibroblast proliferation and collagen gene expression, a number of mechanisms could account for the develpment of fibrosis. These include an increase in the number of collagen-producing cells, an increase in expression of procollagen genes in an unchanged number of fibroblastic cells, or a combination of the two. A number of researchers have divided the development of experimental silicosis in the single instillation model into an acute and a chronic phase (Chvapil *et al.* 1979; Lugano *et al.* 1982, 1984; Kulonen *et al.* 1985; Callis *et al.* 1985). Conceivably these phases could reflect different mechanisms of fibroblast activation.

One of the advantages of our current approach of using isolated RNAs is that the same RNA filters can now be hybridized with any cDNA clone available. With the new probes, changes in the expression of a number of growth factors can now be measured and correlated to changes occurring in the connective-tissue-specific mRNAs. The powerful technique of in-situ hybridization (Sandberg & Vuorio 1987) could then be used to localize this growth factor expression and to relate it to the ingested silica particles and the activated collagen gene expression. These studies are currently under way using the same RNA filters and lung specimens described here.

Acknowledgements

The writers thank Dr David Rowe for cDNA clones pαIR2, Dr Ph. Fort for the cDNA clone pRGAPDH, and Merja Haapanen, Marita Potila and Tuula Divanen for expert technical assistance. Financial support from the Medical Research Council of the Academy of Finland, the Turku University Foundation, and the Finnish Medical Foundation is gratefully acknowledged.

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