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Reaction of Mineral Dusts with Primary Lung Fibroblast Cultures

by R. J. Richards* and J. Hunt*

The complex metabolism of rabbit lung fibroblast cultures maintained over 24 days *in vitro* and their interaction with the fibrogenic group of asbestos minerals is reviewed. The differential effects of nine dusts given as a single dose to 3-day-old fibroblast cultures which were then analyzed for DNA, protein and cell mat hydroxyproline after 3 weeks are discussed.

A comparison is made of the ability of these minerals to promote reticulin deposition *in vivo* following instillation into rats with the ability of each mineral to alter cell mat hydroxyproline levels in fibroblast cultures. Because this comparison is poor for some of the minerals investigated, it is concluded that with the current methodology the fibroblast cultures do not provide an adequate *in vitro* test system for assessing the potential fibrogenicity of a mineral *in vivo*.

Introduction

Lung fibroblastlike cells are potential target cells following dust deposition in the lung, or they may respond to factors released from other dust-affected cells. Thus at some stage in any mineral-induced fibrogenic process, the fibroblast has an important role in the laying down of collagen. This role has received limited attention from *in vivo* studies but attempts have been made to understand the biochemical potential of lung fibroblasts *in vitro* and their direct interaction with mineral particles.

Rabbit lung fibroblasts maintained *in vitro* over 3 weeks show at least two, and possibly more, phases of development (*1,2*) (Fig. 1). During the growth phase (8-10 days) DNA levels rise in the culture, deposition of cell mat collagen as measured by hydroxyproline levels remains low and the ratio of the two major proteoglycans/glycosaminoglycans [hyaluronic acid (HA) and dermatan sulfate (DS)] released into the culture medium is high. Growing fibroblasts are actively mobile with extensive undulating membrane activity (*3*). In stationary phase (10-19/22 days) growth is arrested, mitotic activity stops, DNA levels remain constant, but increasing amounts of protein are detected and collagen deposition is increased as the HA/DS ratio falls (Fig. 1). During stationary phase, undulating membrane activity is rare; the cells apparently assume different membrane morphology with the development of extensive microvilli (*4*). Less is known of the final stages of culture, designated "aging" and "death," but collagen deposition continues to increase to days 24/26 and DNA levels fall, possibly due to more rapid turnover of this latter component or perhaps cell death.

The development of mineral-induced fibrosis *in vivo* is generally accepted as being due to excessive deposition of reticulin or collagen. Based on this definition, what criteria would be acceptable for a measurement of mineral-induced fibrogenesis *in vitro*?

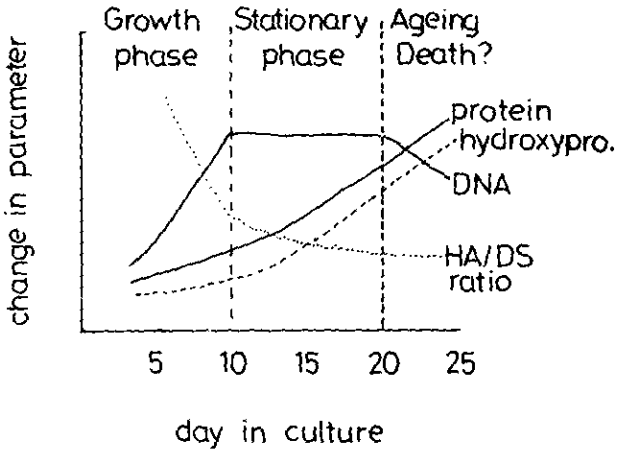

FIGURE 1. Alterations in parameters studied in normal rabbit lung fibroblast cultures over a period of 24 days. HA = hyaluronic acid; DS = dermatan sulfate.

*Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, United Kingdom.

Various biochemical parameters have been considered indicative of fibrogenesis *in vitro*: an elevation of prolyl hydroxylase activity, or an increase in the incorporation of radiolabeled proline into hydroxyproline. Such studies are undoubtedly useful in determining the synthesis of collagen, but further factors may then be important in collagen deposition from the newly synthesized material (*5*).

In the present studies, the estimation of mineral-induced fibrogenesis is based on the amount of fibrous collagen (hydroxyproline) deposited in the cell mat of lung fibroblast cultures and does not include the level of hydroxyproline-containing moieties released into the medium by these cells. As growing fibroblasts may be more abundant following mineral-induced granuloma *in vivo*, single mineral additions of arbitrary dose were added to 3-day-old fibroblast cultures. As collagen deposition appeared maximal after 24 days in culture, this time period was mostly chosen to assess the effects of minerals on hydroxyproline levels in the cell mat.

Primary studies (*6*) indicated that glass powder, coal samples and Min-u-sil (99% quartz) had little effect on cell mat hydroxyproline in fibroblast cultures exposed to 500 μg of each mineral for 3 weeks, but UICC chrysotile asbestos, a known fibrogenic agent *in vivo*, directly promoted cell mat hydroxyproline at this time period despite proving toxic to the cells. This ability of chrysotile to directly cause excessive deposition of collagen appeared confirmed with subsequent studies on different fibroblast strains (*1*), and parallel investigations showed the toxic effects of this mineral at the light microscopic level, indicating that reticulin deposition in chrysotile-exposed cultures was abnormal (*7*). Electron microscope studies suggested that chrysotile initiated an early maturation process in fibroblasts, an event which would seem to explain the ability of the mineral to induce excessive collagen deposition in the cultures (*4*).

The studies adding chrysotile to 3-day-old fibroblast cultures and measuring cell mat hydroxyproline after 3 weeks exposure were repeated with approximately 10 different primary cell strains over a period of 3 years. In these studies, chrysotile (500 μg) produced elevation in cell mat hydroxyproline in 60% of the strains, but in 40% a depression in cell mat was detected (*3*). Similar findings were reported with other asbestiform minerals such as amosite, anthophyllite and crocidolite (*8*) when it was suggested that exposure of fibroblasts to lower dose levels of minerals would favor *in vitro* fibrogenesis, whereas higher doses of minerals would tend to suppress collagen formation and deposition. The variability of fibroblast response to the asbestiform minerals, albeit due to differences between fibroblast strains or passage numbers of any given strain, raised doubts about the suitability of the chosen methodology and use of this system for assessing potential fibrogenicity of mineral particles. However, constant long-term exposure of a lung fibroblast strain to chrysotile asbestos indicated that enhancement of cell mat collagen can occur in the mineral-treated strain (*9*). This type of study has not been repeated with other minerals, mainly because it is extremely time-consuming and as such would not provide a simple *in vitro* model for assessing mineral fibrogenicity.

As part of an overall comparative investigation of the reactivity of minerals both *in vivo* and *in vitro*, we have studied the effects of a number of different dusts administered at a variety of doses to lung fibroblast cultures.

Materials and Methods

Primary rabbit lung fibroblasts were established and grown in 20% fetal bovine serum plus Waymouth's medium with additional ascorbic acid as described previously (*6*). In the studies described, cells in passages 4-6 only were used with between 3.5-4.0 × 10^5 cells seeded into medical flat bottles in 10 mL medium at day 0. Dust samples suspended in saline were added at day 3 unless otherwise stated and cultures maintained at 37°C with twice weekly media changes, for a period of up to 3 weeks thereafter. The treatment of cultures and assays for DNA, protein and hydroxyproline have all been described previously (*6*).

Minerals

The use of sonicated and finely prepared samples of UICC chrysotile and sonicated and respirable preparations of the calcium silicate samples has been described previously (*10*). Aerosil A380 silica is a finely divided preparation which tends to aggregate in solution although 90% of the aggregates are less than 1 μm in diameter when viewed by electron microscopy. The carbon sample was obtained by grinding carbon fiber cloth, and electron microscopy revealed that 90% of the "fibers" were less than 10 μm in length. Titanium dioxide (anatase) and activated charcoal were obtained from British Drug Houses, Poole, Dorset, U.K. and DQ 12 quartz, a standard reference sample, was a generous gift from Dr. C. Hardy, Huntingdon Research Center, U.K.

Results and Discussion

The effect of sonicated UICC chrysotile and sonicated calcium silicates A and B on DNA, protein

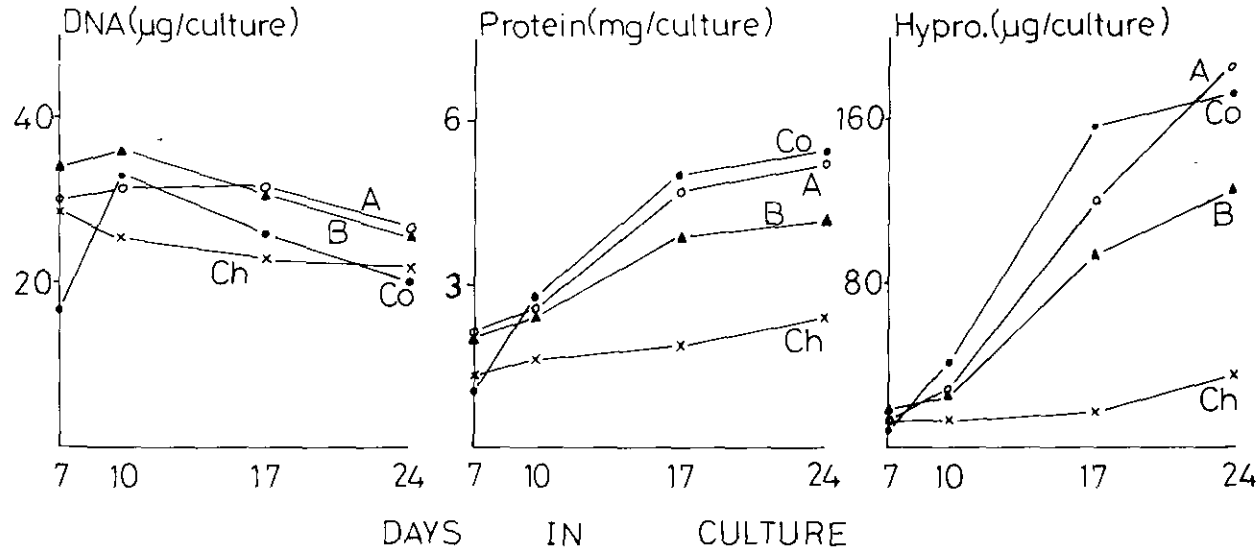

FIGURE 2. Effects of sonicated samples of UICC chrysotile asbestos A and two calcium silicates (A and B) on DNA, protein and cell mat hydroxyproline levels in fibroblast cultures maintained for 24 days. Dust additions (500 µg) given on day 3 and analysis performed on day 24.

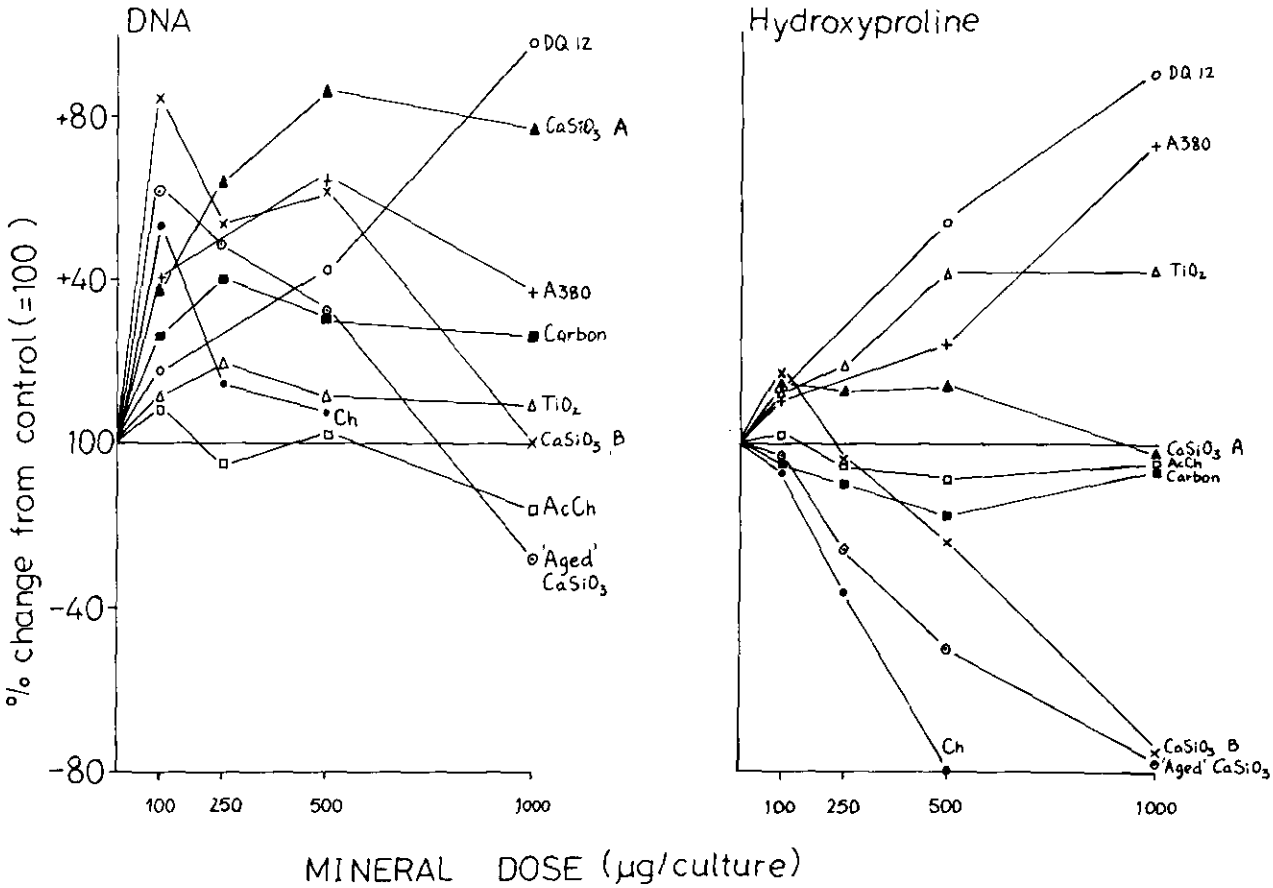

FIGURE 3. Effects of different doses of minerals on lung fibroblast cultures following a single exposure (day 3) with analysis for DNA and cell mat hydroxyproline on day 24. All the calcium silicate samples are respirable and the chrysotile is a finely prepared sample; AcCh = activated charcoal.

and cell mat hydroxyproline levels in lung fibroblasts over a period of 3 weeks are shown in Figure 2. The effects of each mineral are dose-dependent (*10*) but the effects of the dose shown (500 μg mineral/culture) reflect the differential toxicity of each dust. All the dust-treated cultures have higher levels of DNA at day 7 (4 days after mineral administration), an effect usually maintained by both calcium silicate samples throughout the culture period, but not by chrysotile. After day 10, calcium silicate A has similar levels of protein and cell mat hydroxyproline to that found in control cultures. The effects of calcium silicate B on protein and hydroxyproline are seen after day 10 and are intermediate between those found for calcium silicate A and chrysotile. Each mineral thus affects the cultures in a different manner; the alterations produced may occur at different time intervals after dust addition and the most extensive effects on hydroxyproline levels are detected at 3 weeks after exposure.

These studies were repeated with a number of different mineral samples of assumed or unknown fibrogenic potential *in vivo*, and a summary of the effects of different doses (10-1000 μg/culture), administered to cultures on day 3, on the levels of DNA and cell mat hydroxyproline assayed 3 weeks later is shown in Figure 3. Two dusts, titanium dioxide and activated charcoal, have little if any effect on DNA levels at any dose employed. Both the carbon sample and A380 silica-treated cultures have higher levels of DNA than that found in normal cultures but increasing the dose of each dust is not correlated with an increase in DNA. In contrast, DQ 12 quartz-treated cultures have elevated levels of DNA directly related to dose, an effect also seen with calcium silicate A up to 500 μg. Calcium silicate B, the "aged" calcium silicate and chrysotile all reduce cell mat hydroxyproline on a dose-response basis. Cultures treated with A380 silica and titanium dioxide have elevated levels of hydroxyproline at doses above 500 μg.

How do these findings relate to the reactivities of the same minerals in other *in vitro* systems (hemolysis and macrophage toxicity) and to their effects *in vivo*? These comparisons are made in Table 1, and it can be seen that titanium dioxide proves a good "control" mineral, in the sense that it is relatively inactive *in vivo* or *in vitro* and yet at doses of 500 μg or above it elevates hydroxyproline levels in fibroblast cultures. The carbon sample also proves relatively inactive except in the macrophage toxicity test which is similar to the findings with activated charcoal, although this latter compound produces alveolar wall thickening and patchy excessive reticulin deposition upon instillation into rats. DQ 12 quartz and A380 silica are highly reactive in most studies; they both produce alveolar wall thickening and excessive reticulin deposition *in vivo* (DQ 12 > A380) which correlates with additional total lung hydroxyproline measurements and increases in DNA and hydroxyproline in fibroblast cultures. Both silicas are highly hemolytic but exhibit differential macrophage toxicity.

The worst correlations between *in vitro* and *in vivo* effects are found with the silicate samples. Considerable differences are seen between the hemolytic properties of calcium silicates A and B, although both are equally toxic to macrophages and calcium silicate B is toxic to fibroblasts. Neither silicate promotes reticulin deposition *in vivo* but cal-

Table 1. Summary of the effects of mineral reactivity *in vitro* and *in vivo*.

	Effects of fibroblast cultures (24 days)[a]		Effect in other *in vitro* systems		Effects *in vivo*[d]		
Mineral	DNA	Hydroxyproline	Hemolysis[b]	Macrophage toxicity[c]	Total lung hydroxyproline[e]	AWT[f]	Excess reticulin
TiO_2	O	↓(500)	O	O	94	O	O
Carbon	O	O	>20.0	High	90	O	O
Activated charcoal	O	O	> 5.0	Very high	96	X	X
$CaSiO_3$ A	↑(250)	O	2.8	Moderate	146	O	O
$CaSiO_3$ B	↑(100)	↓(500)	25.0	Moderate	118	O	O
Aged $CaSiO_3$	↑(100)	↓(500)	3.0	?	?	W	W
Chrysotile A	↑(100)	↓(250)	0.4	Very high	?	?	?
A380 silica	↑(100)	↓(500)	0.9	High	141	X	X
DQ 12 quartz	↑(500)	↓(500)	0.4	Moderate	153	X	X

[a]Dose that is reactive is given in parentheses (μg). O = no effect; ↑ = increase; ↓ = decrease.

[b]Studies with rabbit erythrocytes (*10*); amount of mineral given (mg) to produce 20% hemolysis; O = no effect; ? = effect unknown.

[c]Studies with rabbit alveolar macrophages incubated in absence of serum for up to 1 hr; results based on viable cells surviving.

[d]Instillation studies with 5 mg quantities of minerals given to SPF Charles River rats, examined after 15 weeks exposure: O = no effect; X = effect noted; ? = result unknown; W = probable weak reaction.

[e]Results expressed as percentage relative to control levels (= 100%).

[f]AWT = alveolar wall thickening.

Table 2. Effects of different doses of finely prepared UICC chrysotile A on lung fibroblast cultures after 3 weeks exposure to the mineral.[a]

Dose chrysotile, μg	DNA, μg/culture	Protein, mg/culture	Hydroxyproline, μg/culture
0 (control)	19.5 (± 0)	5.25 (± 0.03)	41.6 (± 0.40)
10	16.5 (± 0)	5.92 (± 0.22)	52.5 (± 0.75)
50	24.0 (± 3.0)	5.51 (± 0.64)	51.4 (± 4.10)
100	18.7 (± 0.7)	5.17 (± 0.23)	45.7 (± 0.70)

[a]Dust added day 3; analyses day 24; range from means given in parentheses.

cium silicate A raises total lung hydroxyproline. Chrysotile is highly hemolytic, toxic to macrophages, and is the only mineral examined which is highly toxic to fibroblasts. It might also be expected to promote alveolar wall thickening and cause excessive deposition of reticulin in rat lungs following intratracheal instillation (5 mg for 15 weeks). In view of the high toxicity of chrysotile, other experiments were carried out with fibroblast cultures, exposing them to low doses (10-100 μg) of a finely prepared sample of the mineral. The results suggest that hydroxyproline levels may be slightly elevated following exposure to low doses of the mineral after 24 days *in vitro* but this study requires confirmation (Table 2).

In conclusion, many minerals, particularly at low doses, appear to promote DNA levels in growing lung fibroblast cultures; whether or not this is related to initial elevated growth in the cultures has not been established in the present studies, but one mineral (α quartz) has been shown to enhance proliferation of human lung fibroblasts *in vitro* (*11*). A direct relationship between DNA and hydroxyproline levels in fibroblast cultures exposed to minerals for up to 3 weeks *in vitro* is obtained with some dusts (DQ 12 quartz, A380 silica and activated charcoal). With other minerals (calcium silicate A, carbon) elevated levels of DNA are found with little change in hydroxyproline levels. The most toxic compounds chrysotile, calcium silicates ("aged" and B) may well promote DNA levels at low doses but this effect is lost with increasing mineral concentration whereupon protein metabolism in the cultures appears primarily affected in that reduced amounts of collagen are deposited.

The differential effects of mineral produced in fibroblast cultures and the fact that the same dust exhibits different toxicities with macrophages and red blood cells present an interesting phenomenon for further study. However, the fibroblast culture, using the methodology described, does not yet provide an adequate *in vitro* test system for assessing potential fibrogenicity of a mineral *in vivo*.

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