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BIOCHEMICAL EFFECTS OF ASBESTIFORM MINERALS ON LUNG FIBROBLAST CULTURES

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Summary.—The ability of different types of asbestiform minerals to enhance or suppress the levels of fibrous collagen in cultures of lung fibroblasts was tested. All commercially important asbestos dusts produced both effects, a lower dose favouring a fibrogenic response whereas a higher dose favoured an opposite effect.

EXPOSURE to asbestos, either occupationally or experimentally, often leads to the development of fibrosis, emphysema, bronchial cancer and pleural mesothelioma.

The series of events leading to these diseases are unknown, particularly the effects produced at the molecular level in cells exposed to asbestos. Earlier studies in this laboratory (Richards and Morris, 1973; Richards *et al.*, 1974) exposing lung fibroblasts to UICC Rhodesian chrysotile asbestos have revealed that 50 μg dust/ml of culture medium induces many morphological and biochemical variations, culminating in higher levels of fibrous collagen than those found in normal cultures.

Recent studies have suggested that this effect could be reversed: *i.e.* that chrysotile at the same concentration (50 μg/ml culture medium) could also depress the level of fibrous collagen in test cultures. The results of these studies indicated that the fibrogenic effect (enhancement of fibrous collagen levels) was favoured in cultures given low doses of chrysotile (< 50 μg/ml) whereas depression of fibrous collagen was predominant in cultures receiving higher levels (> 50 μg/ml) of this dust (Richards *et al.*, 1975).

The present study was designed to compare and contrast the effects of chrysotile with other commercially important asbestiform minerals on the activity of lung fibroblast cultures, since these cells play an important role in any fibrogenic reaction. Particular attention was paid to the changes induced in DNA, RNA, fibrous collagen and protein levels in these cells and the proteoglycans secreted into the culture medium after 3 weeks' exposure to differing concentrations of the different dusts.

MATERIALS AND METHODS

Cell cultures.—Rabbit lung fibroblasts were grown as described previously (Richards *et al.*, 1971). Cultures were set up by seeding approximately $0{\cdot}5 \times 10^6$ cells in medical flat bottles (100×40 mm), the number being constant for any one series of bottles. The cells were allowed to form a monolayer for 3 days before the dusts were added. Culture medium (10 ml of 20% {v/v} foetal bovine serum plus Waymouths MB 752/1 medium containing additional ascorbic acid {50 μg/ml} and antibiotics, see Richards *et al.*, 1971), was replaced twice weekly.

Dust samples.—The samples of Rhodesian chrysotile, anthophyllite, amosite and crocidolite employed were standard reference samples prepared under the auspices of the International Union against Cancer (UICC). The 20% and 80% Mg^{2+}-depleted chrysotile samples (Morgan *et al.*, 1973) were kindly supplied by Dr A. Morgan, A.E.R.E., Harwell, U.K., and were stored in ethanol. Prior to use they were washed with distilled water and freeze-dried. Brucite fibres were kindly supplied by Dr F. Pooley, Department of Mineral Exploitation, University College, Cardiff, U.K., and were finely ground using a glass pestle and mortar.

Dust additions.—Dusts were heat sterilized

(140° for 2 h), suspended in Medium 199 by shaking vigorously for 10 s, and added as a single dose (1 ml) to 3-day-old cultures.

Assays.—Prior to analysis cell cultures were treated as described previously (Richards *et al.*, 1971). DNA was estimated by the method of Burton (1956) as modified by Richards (1974) and RNA by the method of Ceriotti (1955). Cell protein was determined by the method of Oyama and Eagle (1956) and cell mat hydroxyproline (a determination of fibrous collagen) by the method of Stegeman (1958) after hydrolysis in 6M-HCl for 24 h at 110°. Proteoglycans in the culture medium were isolated and their uronic acid content determined as described previously (Richards *et al.*, 1971).

Media and chemicals.—Foetal bovine serum and Medium 199 were purchased from Flow Laboratories Ltd, Irvine, Scotland, U.K., and Waymouths MB752/1 powdered medium from Burroughs Wellcome, Beckenham, Kent, U.K. All chemicals were of "Analar" quality and were purchased from B.D.H. Ltd, Poole, Dorset, U.K.

RESULTS AND DISCUSSION

The effect of differing concentrations of different asbestiform minerals on lung fibroblast cultures, in terms of the number of experiments in which enhancement or depression of fibrous collagen was found, is shown in Table I. It is evident that the fibrogenic effect is predominant when the lower doses of anthophyllite, amosite and crocidolite are used, thus confirming the previous findings with chrysotile (Richards *et al.*, 1975).

The effects of different concentrations of the dusts on a variety of parameters studied in these experiments are shown in Tables II and III. These show that all the asbestos samples tested in this system (including Mg^{2+}-depleted chrysotile) are capable, below a dose of 100 μg/ml, of producing a fibrogenic response (Table II). Higher levels of crocidolite and amosite never produce such a response. Brucite, a $Mg(OH)_2$ polymer, which is the major component of the outer layer of chrysotile, did not significantly affect the level of cell mat hydroxyproline in the cultures. This would suggest that the brucite coating of chrysotile has little biological reactivity in producing the *in vitro* fibrogenic response. However, it should be remembered that brucite is highly soluble and therefore as a fibre it would tend to lose Mg^{2+} and dissolve in the culture medium. Excess Mg^{2+} in solution (administered as $MgCl_2$) has little effect on the hydroxyproline levels in cultured fibroblasts. It is therefore difficult to assess whether or not pure fibrous brucite behaves in a similar manner to the brucite coat on chrysotile, although studies by Morgan and Holmes (1971) have revealed that the outer coating of chrysotile is readily removed *in vivo*.

At first sight the removal of surface Mg^{2+} (and other ions?) does alter the fibrogenic potency of chrysotile (Table II). Both 20% and 80% Mg^{2+}-depleted chrysotiles (at a dose of 50 μg/ml culture medium) produce a fibrogenic response lower than that found with an equivalent weight of chrysotile. However, magnesium accounts for over 30% by weight

TABLE I.—*The Enhancement or Depression of Cell Mat Fibrous Collagen in Lung Fibroblast Cultures Exposed to Asbestiform Minerals*

Dust	Concentration of dust (μg/ml culture medium)	Total number of experiments	Enhancement of fibrous collagen (no. of experiments)	Depression of fibrous collagen (no. of experiments)
Chrysotile	50	52	37	15
20% Mg^{2+}-depleted chrysotile	50	14	6	8
80% Mg^{2+}-depleted chrysotile	50	14	6	8
Anthophyllite	50	23	15	8
Amosite	50	23	15	8
	100	14	10	4
	200	8	0	8
Crocidolite	50	26	15	11
	100	11	3	8
	200	8	0	8
Brucite	50	16	0	0

TABLE II.—*The Relative Content of Protein, Hydroxyproline, DNA and RNA in the Cell Mat of Normal and Dust-exposed Fibroblast Cultures Showing Enhanced Fibrous Collagen (Hydroxyproline Levels) after 24 days, and the Total Release of Proteoglycans into the Medium During this Time. (Normal Cultures are taken as 100%. ND = Not determined)*

Dust	Concentration (μg/ml culture) medium	Protein	Hydroxyproline	DNA	RNA	Proteoglycans: Unsulphated (94% hyaluronic acid)	Proteoglycans: Sulphated (80% dermatan sulphate)
Normal	0	100	100	100	100	100	100
Chrysotile	50	122 (89–177)	271 (102–866)	100 (83–140)	118 (96–142)	71 (21–98)	102 (61–156)
20% Mg^{2+}-depleted chrysotile	50	124	224	86	124	145	211
80% Mg^{2+}-depleted chrysotile	50	107	141	82	112	110	160
Anthophyllite	50	114 (102–133)	270 (165–409)	136 (100–171)	94	130 (117–155)	108 (93–121)
Amosite	50	107 (93–116)	199 (100–315)	115 (111–120)	106	130 (108–145)	109 (95–127)
	100	95 (83–102)	112 (109–115)	124	102	116 (78–154)	68 (28–107)
	200	—	—	—	—	—	—
Crocidolite	50	110 (102–115)	202 (119–330)	103 (85–115)	109	87 (70–105)	102 (93–111)
	100	107	119	ND	ND	138	146
	200						
Brucite	50	93 (85–97)	108 (95–122)	98 (85–110)	97 (81–109)	55	84 (79–89)

TABLE III.—*The Relative Content of Protein, Hydroxyproline, DNA and RNA in the Cell Mat of Normal and Dust-exposed Fibroblast Cultures Showing a Depression in Fibrous Collagen (Hydroxyproline Levels) after 24 Days, and the Total Release of Proteoglycans into the Medium during this Time. (Normal Cultures are taken as 100%. ND = Not determined)*

Dust	Concentration (μg/ml culture) medium	Protein	Hydroxyproline	DNA	RNA	Proteoglycans: Unsulphated (94% hyaluronic acid)	Proteoglycans: Sulphated (80% dermatan sulphate)
Normal	0	100	100	100	100	100	100
Chrystotile	50	81 (67–99)	55 (25–80)	103 (69–151)	70 (57–83)	89 (93–94)	89 (82–95)
20% Mg^{2+}-depleted chrysotile	50	65 (51–78)	31 (17–46)	59 (58–60)	43	ND	20
80% Mg^{2+}-depleted chrysotile	50	73 (69–77)	52 (45–58)	58 (47–69)	80	ND	35
Anthophyllite	50	90 (86–94)	82	100 (88–112)	96 (90–101)	86 (84–88)	68 (61–75)
Amosite	50	80 (79–80)	87 (82–91)	109 (105–113)	108 (94–122)	76	72 (65–78)
	100	67	41	78	73	35	69
	200	80 (69–81)	68 (41–95)	78 (69–87)	73 (65–80)	58 (45–70)	36 (35–36)
Crocidolite	50	86 (75–94)	75 (58–90)	100 (100–119)	95 (90–100)	116	89 (70–121)
	100	80 (76–84)	83 (74–91)	109	80 (71–98)	45	59 (54–64)
	200	82 (69–94)	79 (63–95)	89 (86–92)	84 (75–93)	58	59 (36–81)

of chrysotile and therefore its removal means that more fibres are present per given weight of depleted sample than per given weight of untreated chrysotile. As already shown (Table I; Richards *et al.*, 1975) an increase in the number of fibres given to fibroblast cultures favours depression of the fibrous collagen. Thus it is probable that this effect is seen with the Mg^{2+}-depleted chrysotiles and future studies should include the effects of equivalent numbers of fibres of normal or Mg^{2+}-depleted chrysotiles rather than equivalent weight.

The fibrogenic reaction induced by chrysotile or crocidolite is not accompanied by any significant change in the DNA levels in the cultures suggesting that the fibroblasts produce more fibrous collagen. On the other hand anthophyllite (50 μg/ml culture medium) and amosite (50 and 100 μg/ml culture medium) both increase the level of cell mat DNA, although at the lower dose (50 μg/ml) the increase is not sufficient to account for the excess fibrous collagen (Table II). The deposition of fibrous collagen is not yet fully understood but is considered to be dependent on the correct balance of proteoglycans in the extracellular matrix (Obrink, 1973). Recent studies by Motomiya *et al.* (1975) have shown that during the development of pulmonary fibrosis there is an increase in the amount of dermatan sulphate (formerly called chondroitin sulphate B) present in the lung tissue. It is therefore interesting to note that particularly in the chrysotile, Mg^{2+}-depleted chrysotile and crocidolite-treated cultures the relative amounts of sulphated proteoglycans (80% of which is dermatan sulphate—see Richards and Wusteman, 1973) secreted into the medium are considerably higher than the relative amounts of the unsulphated proteoglycans secreted. Thus there is a direct link between pulmonary fibrosis produced *in vivo* and the fibrogenic effect produced by these asbestos dusts *in vitro*.

The levels of mucopolysaccharides secreted by anthophyllite and amosite-treated cultures are not entirely consistent with the former hypothesis and are more difficult to explain. Nevertheless, both of these dusts increase the level of cellular DNA and during the growth of fibroblast cultures large amounts of hyaluronic acid are produced (Hamerman *et al.*, 1965; Richards and Wusteman, 1973). This suggests that the high levels of hyaluronic acid secreted by cultures treated with anthophyllite and amosite may be due to these dusts producing additional changes, particularly in the growth patterns of the fibroblasts.

Experiments where a depression of fibrous collagen occurs upon treatment with a variety of asbestiform minerals are shown in Table II. No complete explanation can be offered to explain why the same concentrations of any one dust can induce opposite effects, *i.e.* increased or depressed levels of fibrous collagen. It is thought, however, that several factors are important, including the state of growth of the cells at the time of dust addition, but perhaps more important is the number of fibres entering the cells and the retention of masses of fibres in the culture (Richards *et al.*, 1975).

For most of the dusts studied, with the exception of the Mg^{2+}-depleted chrysotiles, there is little significant change in the levels of DNA when fibrous collagen levels are suppressed (Table III). However, with one single exception (cultures treated with 50 μg/ml amosite), depressed RNA levels occur in all the cultures which have lower levels of fibrous collagen. This effect is accompanied by a lower level of protein in all the dust-treated cultures suggesting that RNA metabolism and hence protein synthesis are directly affected by these asbestiform minerals. In addition, the secretion of proteoglycans into the medium of cultures with depressed levels of fibrous collagen (Table III) is quite different from those where enhancement of fibrous collagen levels has occurred after dust addition (Table II). In the former cultures the ratio of unsulphated/sulphated proteoglycans secreted is approximately 1 : 1 or greater,

suggesting that less sulphated material is synthesized and secreted. As already pointed out by Kurtz and Stidworthy (1975) the synthesis of proteoglycans is dependent upon RNA synthesis and in the present study reduced RNA levels are present in all the dust-treated cultures where fibrous collagen levels are depressed.

Thus in summary it can be stated that all the main groups of asbestos dusts studied can enhance or depress the level of fibrous collagen produced in lung fibroblast cultures and that these effects are dose-dependent. In terms of " biological reactivity " in the cultures employed, chrysotile is the most reactive followed by anthophyllite, then amosite/crocidolite, which is similar to findings by Harrington *et al.* (1971) and Desai *et al.* (1975) using haemolysis experiments which indicated that the lower the Mg/Si ratio of the dust the lower its reactivity. Nevertheless, it would be premature to relate the fibrogenic potential of these minerals to their Mg/Si ratio because of the apparent unreactive nature of brucite and the highly reactive nature of Mg^{2+}-depleted chrysotiles in fibroblast cultures.

The results of the present study suggest that asbestos dusts interfere with the balance of unsulphated/sulphated proteoglycans secreted into the culture medium by fibroblast cells which may ultimately determine the deposition of fibrous collagen. It is, however, highly probable that alterations in RNA metabolism precede the synthesis and imbalance in proteoglycan secretion and changes in RNA metabolism as a primary factor in asbestos-induced molecular changes in lung fibroblasts now form the basis of continuing investigations.

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