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Fibroblast RNA and Macrophage Proteins (Including the Fibrogenic Factor) in Experimental Silicosis

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A hypothesis is presented for the action of silica-treated macrophages on protein synthesis in fibroblasts and also a method for the isolation of silica-attached materials in lung tissue. The increased protein synthesis in the fibroblasts is due, at least partly, to an increase in mRNA. Silica prevents the suppressing "macrophage effect" of macrophage-originated ribonuclease on fibroblasts. However, under certain conditions, collagen synthesis is stimulated by silica-treated macrophage preparations to such an extent that the effect cannot be explained by the inhibition of macrophage ribonuclease alone. We therefore postulate the existence of a fibrogenic factor, which is released by the macrophages. This factor has been demonstrated and can be purified from lung homogenate of SiO_2-treated rats.

Introduction

Macrophage preparations have been shown to suppress protein synthesis in adjacent fibroblasts (*1*, *2*) but this effect can be canceled out by pretreatment with silica (Fig. 1). We have now demonstrated the effect of SiO_2 on protein synthesis *in vitro*, thus imitating the effect of silica *in vivo*, both in disease and under experimental conditions (*3*, *4*). This model has provided the opportunity for studying in detail several problems relevant to silicotic fibrosis (*5*, *6*) and to fibroses in general (*7*, *8*).

The problems can be divided into two groups: changes in protein synthesis in the fibroblasts and changes in macrophage proteins or secretion products caused by the silica treatment, which may have subsequent effects on the fibroblasts.

Materials and Methods

We used SiO_2-treated rat peritoneal macrophages and fibroblasts from experimental granulation tissue *in vitro*. The experimental conditions have already been described (*3*, *4*, *9-11*), but we would like to stress two important details: only intact, non-elicited macrophages can be used, and the fibroblasts should be "active," (e.g., from experimental granulation tissue or synovial tissue). Established cell lines or "committed" fibroblasts cultured from, e.g., skin, are not suitable. Human monocytes and related malignant cell lines can be used instead of peritoneal macrophages (*12*).

We checked our *in vitro* results with experiments using rats treated intratracheally with silica dust (DQ 12) (*6*, *7*). The *in vivo* procedure gave better yields of biologically active materials for purification than did analogous preparations from rat peritoneal macrophages.

In the nucleic acid experiment, (^{3}H)-cytidine was used as a precursor. It was incorporated in both DNA and RNA, which were separated by conventional KOH treatment according to Schmidt and Thannhauser (*13*).

The chemical assays were performed by routine methods described previously (*3*, *4*, *10-14*). Ribonuclease was estimated according to Liu et al. (*15*). The RNA was purified as described by Rowe et al. (*16*) and translated with commercial rabbit reticulocyte lysate (The Radiochemical Centre, Amersham, U.K., No 50495) according to the manufacturer's instructions. The gradient gel electrophoresis was performed as described by O'Farrel (*17*).

The SiO_2-bound material was isolated by density

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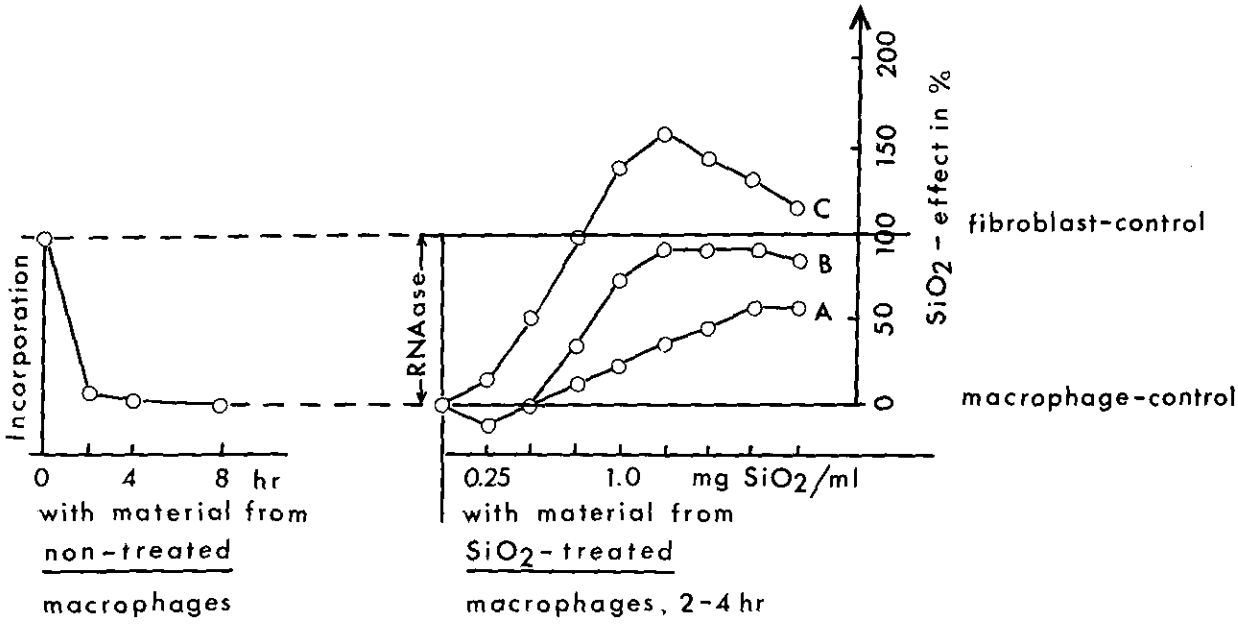

FIGURE 1. Scheme of the action of silica through macrophages on protein synthesis in cultured fibroblasts: (*A*, *B*, *C*) examples of SiO_2 dust with various fibrogenic activities. Pretreatment (silica + macrophages) time is 2-4 hr. Incubation time for the fibroblasts + macrophage preparation + (^{3}H)-proline is 24 hr. The SiO_2 effect is given as a percentage of the effect of the macrophage preparations without SiO_2 (RNAase effect of macrophages treated without SiO_2 to the left).

gradient centrifugation. The separation of the subcellular particles in a stepwise sucrose gradient has been described earlier (*18*). The silica particles with the attached material sediment with the debris and nuclei to the bottom of the tube. The DNA and collagen are degraded with specific enzymes and the remnants washed. The materials adhering to silica were solubilized, the lipids by chloroform:methanol extraction, the proteins with sodium dodecyl sulfate-urea solution, and were then studied by conventional methods.

Results

RNA and Other Tissue Components

The essential feature in the silicosis was the increased protein synthesis which was due, at least partially, to an increase of mRNA. In silicotic rat lung, DNA and nitrogen remained relatively constant with regard to the increasing wet weight (*19*), but whole RNA and hydroxyproline (indicating collagen) increased. A most remarkable 12-fold increase was seen in purified, translatable RNA. Surprisingly, the *in vitro* translation products mainly contained proteins other than collagen (Fig. 2). This was confirmed to a certain extent by experiments with whole cultured fibroblasts. The specific trans-

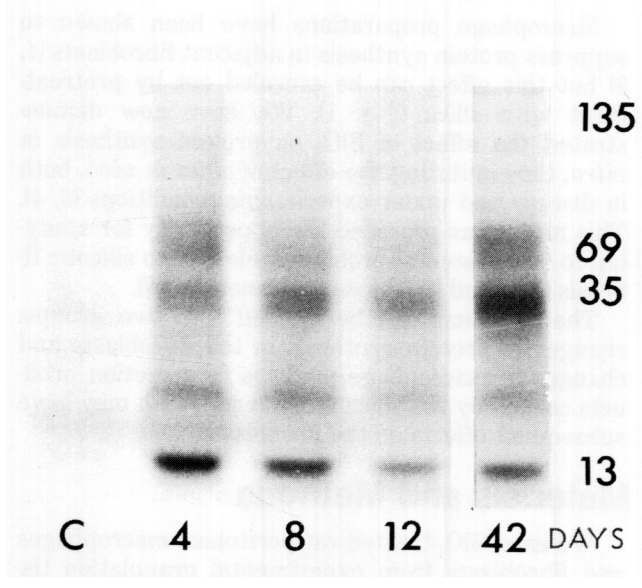

FIGURE 2. Translation products of the RNA purified from silicotic rat lung as seen in gel electrophoretic pattern. The reticulocyte lysate was used with (^{35}S)-methionine as isotope marker; 4-42 days indicates the time interval after intratracheal injection of silica, C control lung. The numbers in the right-hand margin give the molecular weights in 10^3. The procollagen band (135 × 10^3) was very weak.

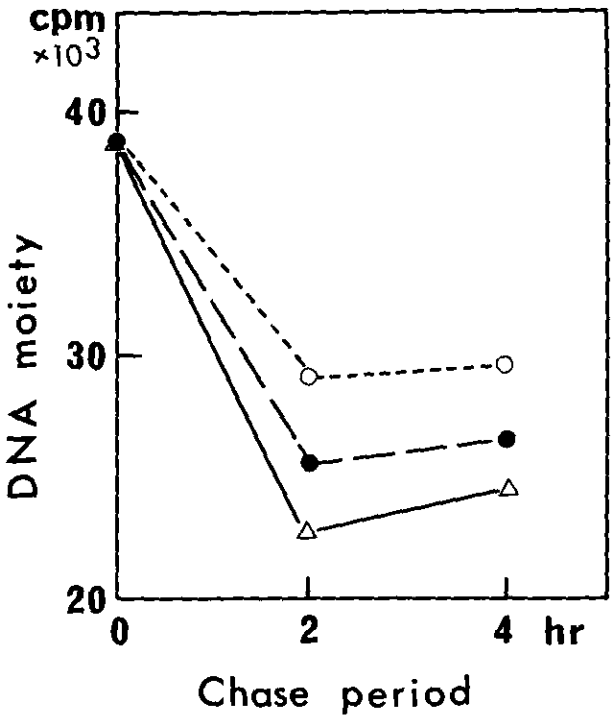

FIGURE 3. Stabilization of fibroblast DNA by SiO_2- and control macrophage preparations as shown by a "chase" experiment after initial incorporation of (^{3}H)-cytidine: (○) SiO_2-treated macrophages; (●) control macrophages; (Δ) no macrophages.

lational capacity of the purified lung RNA preparations remained relatively constant during the development of the silicosis, which was contrary to our findings with RNA from cultured granulation-tissue fibroblasts incubated with extracts from SiO_2-treated peritoneal macrophages (unpublished results with E. Vuorio).

We reported earlier that the RNase effect of macrophages was suppressed following SiO_2-treatment (*2*). We tried to elaborate on this by incorporating (^{3}H)-cytidine in incubated fibroblasts and occasionally following it with a "chase." In nonconfluent granulation-tissue fibroblasts, the incorporated activity of DNA and RNA was highest in the presence of extracts from SiO_2-treated macrophages in comparison with the respective controls from untreated macrophages. In the degradation of DNA from confluent fibroblasts, we observed that the SiO_2-macrophage preparation had a retarding effect during the "chase" (Fig. 3). The effect on DNA (*20*) may be primary and during longer time intervals may be extended to hnRNA and then to mRNA (*19*).

Products of Macrophages

Ribonuclease. Although macrophage ribonuclease has been isolated (*21*), it has proved difficult to study both as a protein and as an enzyme. In lung

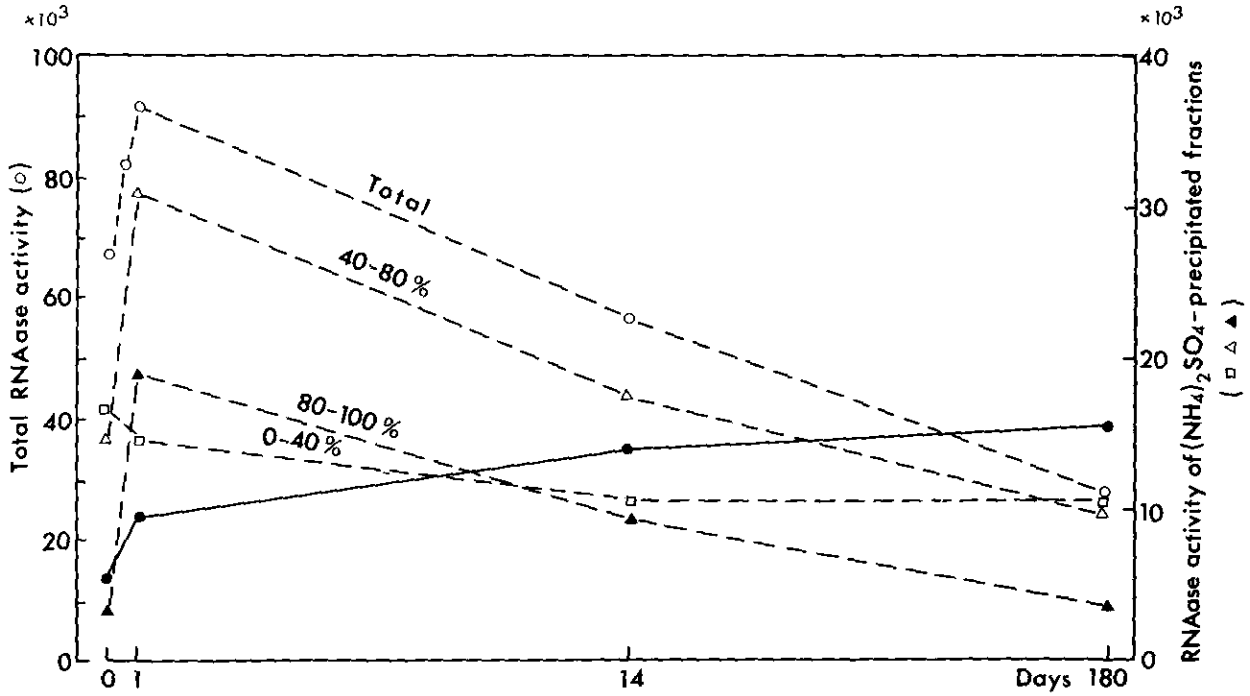

FIGURE 4. Changes in the activities of various lung RNase fractions (— —) calculated in units per wet weight; (● — ●) proportions of the aggregate forms of phosphocellulose- and poly(G)-bound fraction (arbitrary units). The abscissa gives the time interval after an intratracheal injection of 50 mg DQ 12 silica to adult rats.

homogenate we found changes in the activities of several RNase fractions following intratracheal silica administration (Fig. 4). The aggregation state of one RNase moiety increases during the silicotic process. The migration of inflammatory cells from the blood stream may contribute to the initial increase in RNase activity. The RNase activity that was precipitated at 40% saturation of ammonium sulfate and which by immunochemical evidence was supposed to originate from macrophages decreased.

Fibrogenic Factor and Other Macrophage Proteins. The isolation of a fibrogenic factor, although only in small yield, from the peritoneal macrophage medium, has been described previously (*6, 10*), as has the preparation of antiserum in rabbits (*22*). We found that the whole supernatant from silicotic rat lung homogenate contained an activity which stimulated the incorporation of proline to collagen in cultured granulation-tissue fibroblasts (in preparation). The fractionation of this supernatant with ammonium sulfate precipitation combined with gel-filtration chromatography revealed many differences in the protein pattern between the silicotic and control rats (*7*).

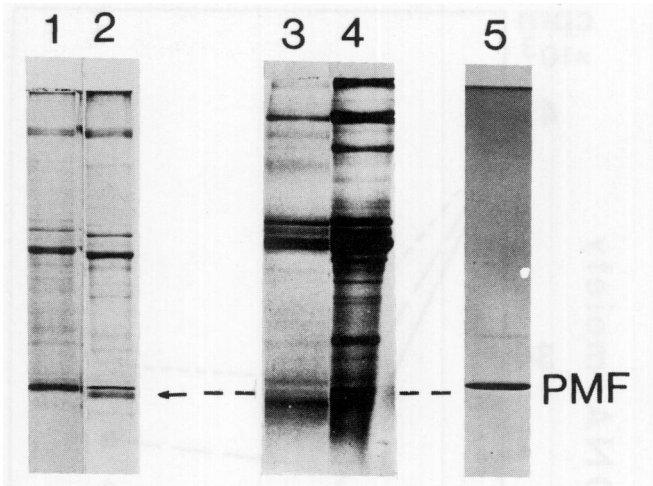

FIGURE 5. Gel electrophoresis of the SiO_2-induced protein fraction in lung homogenates from silica-treated and normal rats: (1) whole homogenate, control rat; (2) whole homogenate 12 weeks after intratracheal injection of 50 mg DQ 12 silica; (3) homogenate of alveolar macrophages, control rat; (4) homogenate of alveolar macrophages, SiO_2-treated rat; (5) PMF, pooled isolated protein fraction preparation with fibrogenic activity.

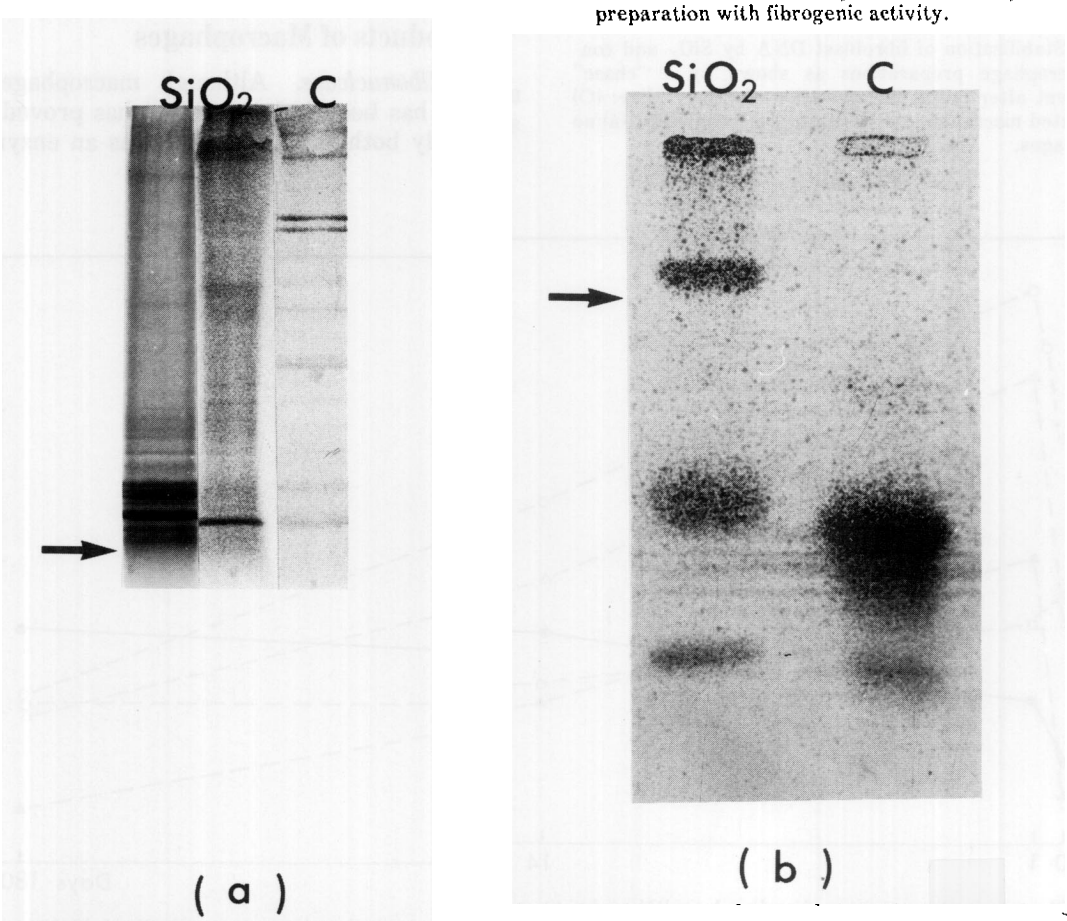

FIGURE 6. The patterns of (*a*) silica-attached proteins in electrophoretic strips and (*b*) lipids developed with chloroform:methanol: formic acid:water (291:291:6:13) in thin-layer chromatogram. The findings related to silica are marked by arrows.

Of special interest was a protein with a molecular weight of about 16,000. It was related to the action of silica (*6*) and originated from macrophages. It could be isolated by gel filtration in almost pure state (Fig. 5). It stimulated the incorporation of proline to collagen by 35-40% at a concentration of 1.5 $\times 10^{-6}M$, in the same way as a fibrogenic factor (in preparation). In its molecular weight and acidity, it resembled the light chain of macrophage myosin, calmodulin or troponin C, but differed in the characteristic details in its amino acid composition. The fibrogenic activity seemed to be released, not synthesized, during the disintegration of the macrophages by silica.

SiO_2-Binding Components of Macrophages. The SiO_2 particles were attached to vesicular subcellular structures of the macrophages (*18*). The isolation of the attaching subcellular material has been summarized in the experimental section. The resulting simple patterns (Fig. 6) provide a tool for the preparation and analysis of the "attachment sites" between SiO_2 particles and cell structures.

Discussion

Both direct analyses and translation experiments show that messenger RNA is increased in experimental silicosis. Although general gene expression is enhanced, the minor role of collagen is surprising. This may be due to increased DNA, increased transcription to hnRNA, increased conversion of hnRNA to cytoplasmic mRNA or a decreased degradation of mRNA for which there is some evidence. DNA is also a target, even in isolated nuclei, for SiO_2-macrophage preparations (*20*) which stimulated the incorporation of cytidine (*5*).

Tolstoshev et al. (*23*) discuss at length how collagen synthesis in the lung is regulated by the level and utilization of pro-collagen mRNA, and by procollagen degradation. The amount and quality of mRNA are key factors (*24*) for understanding the molecular biology of silicosis. The role of the fibrogenic factors is as yet unclear, as is the nature of the other proteins whose mRNA's show simultaneous increase.

There are many pharmacological means known for supressing collagen synthesis in a developing fibrosis but they are all nonspecific and produce harmful side effects. When the mechanism of silicotic fibrosis can be understood in terms of molecular biology and the role of macrophages defined, silicosis and other fibroses can then be treated by methods designed to have local and specific effects.

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REFERENCES

1. Jimenez, S. A., McArthur, W., and Rosenbloom, J. Inhibition of collagen-synthesis by mono-nuclear cell supernates. J. Exptl. Med. 150: 1421-1431 (1979).
2. Aho, S., and Kulonen, E. Involvement of ribonuclease in the interactions of macrophages and fibroblasts in experimental silicosis. Experientia 36: 29-30 (1980).
3. Aalto, M., Potila, M., and Kulonen, E. Effects of silica-treated macrophages on synthesis of collagen and other proteins *in vitro*. Exptl. Cell Res. 97: 193-202 (1976).
4. Aho, S., and Kulonen, E. Effect of silica-liberated macrophage factors on protein synthesis. Exptl. Cell Res. 104: 31-38 (1977).
5. Kulonen, E., Aalto, M., Aho, S., Lehtinen, P., and Potila, M. The SiO_2-liberated fibrogenic macrophage factors with reference to DNA. In: The *In Vitro* Effects of Mineral Dusts (R. C. Brown, I. P. Gormley, M. Chamberlain and R. Davies, Eds.), Academic Press, London, 1980, pp. 281-287.
6. Kulonen, E., Aalto, M., Aho, S., Lehtinen, P., and Potila, M. Increase of RNA and appearance of new protein in silicotic lung tissue. In: Inhaled Particles, V (W. H. Walton, Ed.), Ann. Occup. Hyg., 26: 463-471 (1982).
7. Kulonen, E., Aalto, M., Aho, S., Lehtinen, P., and Potila, M. Recent investigations on macrophage proteins and on fibroblast RNA in silicotic lung fibrosis. In: Contributions in Microbiology and Immunology, Vol. 7 (P. Gigase, Ed.), Karger, Basel, 1982, pp. 204-211.
8. Heppleston, A. G. Pulmonary repair and fibrosis. In: Handbook of Inflammation (L. E. Glynn, Ed.), Vol. 3, Elsevier/North Holland, Amsterdam, New York and Oxford, 1981, pp. 393-456.
9. Heppleston, A. G., and Styles, J. A. Activity of a macrophage factor. Nature 214: 521-522 (1967).
10. Aalto, M., and Kulonen, E. Fractionation of connective-tissue-activating factors from the culture-medium of silica-treated macrophages. Acta Pathol. Microbiol. Scand. C 87: 241-250 (1979).
11. Dehm, P. J., and Prockop, D. J. Synthesis and extrusion of collagen by freshly isolated cells from chick-embryo tendon. Biochim. Biophys. Acta 240: 358-369 (1971).
12. Aalto, M., Kulonen, E., Rönnemaa, T., Sundström, C., and Vilpo, J. Liberation of a fibrogenic factor from human-blood monocytes, ascites-cells, cultured histiocytes and transformed mouse macrophages by treatment with SiO_2. Scand. J. Clin. Lab. Invest. 40: 311-318 (1980).
13. Schmidt, G., and Thannhauser, S. J. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161: 83-89 (1945).
14. Lehtinen, P., and Kulonen, E. Preparation of subcellular fractions from granulation-tissue by density gradient centrifugation. Acta Chem. Scand. B33: 327-336 (1979).
15. Liu, D. K., Williams, G. H., and Fritz, P. J. Alkaline ribonuclease and ribonuclease inhibitor in mammary-gland during lactation cycle and in R3230AC mammary tumor. Biochem. J. 148: 67-76 (1974).
16. Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. H., Bornstein, P., and Palmiter, R. D. Correlation of procollagen messenger-RNA levels in normal and transformed chick-embryo fibroblasts with different rates of procollagen synthesis. Biochemistry 17: 1581-1590 (1978).
17. O'Farrel, P. H. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021 (1975).
18. Aalto, M., Turakainen, H., and Kulonen, E. Effect of SiO_2-liberated macrophage factor on protein-synthesis in connective tissue *in vitro*. Scand. J. Clin. Lab. Invest. 39: 205-213 (1979).

19. Lehtinen, P., Aho, S., and Kulonen, E. Effect of silica on the rat lung with special reference to RNA. Ann. Occup. Hyg., in press.
20. Lehtinen, P., and Kulonen, E. Subcellular targets of the soluble SiO_2-liberated macrophage factors in experimental granulation-tissue. Biochim. Biophys. Acta 564: 132-140 (1979).
21. Aho, S. Purification of two ribonucleases from macrophage culture-medium and studies on their effect on granulation-tissue fibroblasts. Acta Chem. Scand. B34: 707-714 (1980).
22. Aalto, M., Viljanen, M., and Kulonen, E. Neutralization of the fibrogenic silica-released macrophage factor by antisera. Exptl. Pathol., 22: 181-184 (1982).
23. Tolstoshev, P., Berg, R. A., Rennard, S. I., Bradley, K. H., Trapnell, B. C., and Crystal, R. G. Procollagen production and procollagen messenger-RNA levels and activity in human-lung fibroblasts during periods of rapid and stationary growth. J. Biol. Chem. 256: 3135-3140 (1981).
24. Tolstoshev, P., Haver, R., Trapnell, B. C., and Crystal, R. G. Procollagen messenger-RNA levels and activity of collagen-synthesis during the fetal development of sheep lung, tendon and skin. J. Biol. Chem. 256: 9672-9679 (1981).