Scand J Work Environ Health 1995;21(2):30-34
Issue date: 1995

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This article in PubMed: www.ncbi.nlm.nih.gov/pubmed/8929685

Print ISSN: 0355-3140 Electronic ISSN: 1795-990X

Scand J Work Environ Health 1995;21 suppl 2:30—4

Transforming growth factor β1, *ras* and *p53* in silica-induced fibrogenesis and carcinogenesis

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Williams AO, Saffiotti U. Transforming growth factor β1, *ras* and *p53* in silica-induced fibrogenesis and carcinogenesis. *Scand J Work Environ Health* 1995;21 suppl 2:30—4.

The pathogenesis of mesenchymal and epithelial lung reactions was studied after a single intratracheal instillation of quartz into rats. Relationships between transforming growth factor β1 (TGF-β1) and the *ras* and *p53* genes were investigated in silicosis and associated lung cancer. Immunohistochemical reactivity to mature TGF-β1 was localized intracellularly in fibroblasts and macrophages at the periphery of silicotic granulomas and in stroma adjacent to hyperplastic alveolar type II cells and extracellularly in connective tissue matrix adjacent to hyperplastic alveolar type II cells. TGF-β1 precursor was localized intracellularly in hyperplastic alveolar type II cells adjacent to granulomas and in the cells of adenomas, but not in carcinomas. Hematite-treated controls showed no reactivity to TGF-β1. Immunohistochemical localization of pan-reactive p21 ras protein in quartz-treated rat lungs was increased in hyperplastic alveolar type II cells adjacent to granulomas, but not in adenomas and carcinomas. Foci of nuclear immunoreactivity to *p53* protein were observed in 25% of the carcinomas.

Key terms alveolar type II cells, immunohistochemistry, *p53*, p21 ras, rat, TGF-β1.

Quartz-induced lung cancer in rats was reported in several experiments using exposure by either inhalation or intratracheal instillation (1, 2). Evidence for the carcinogenic activity of crystalline silica also derives from the induction of localized malignant histiocytic lymphomas by intrapleural administration (1), quartz-induced neoplastic transformation of cells in culture (3, 4), and increased lung cancer risk in many, but not all, epidemiologic studies on human subjects with silicosis (1).

A series of experiments in our laboratory (2, 5) investigated the histopathogenesis of lung reactions to crystalline silica in F344 rats of both sexes, as well as in mice and hamsters. Marked species differences were detected. Rats developed silicotic granulomas with fibrosis accompanied by focal hyperplasia of type II cells, adenomatoid proliferation, and eventually high incidences of carcinomas. Mice developed silicotic granulomas with fibrosis, but no epithelial hyperplasia and no tumor induction. Hamsters developed extensive macrophagic silica-storage lesions which did not progress to fibrosis and showed no epithelial hyperplasia. Alveolar type II cell hyperplasia and lung tumors were not found in long-term studies in mice and hamsters exposed to quartz (1, 2). These different host responses offer experimental models to investigate the underlying mechanisms by which silica particles induce the complex mesenchymal and epithelial reactions leading to silicosis or to lung cancer in susceptible hosts.

The rat model resembles the human type of fibrosis-associated lung cancer described as scar cancer, and it lends itself to investigations of the mechanisms of interaction between pulmonary silicosis and hyperplasia of adjacent type II cells leading to the development of carcinoma.

Several cytokines have been shown to play a role in the pathogenesis of experimental silicosis (2). They include interleukin-1, interleukin-6, tumor necrosis factor-α and transforming growth factor β1 (TGF-β1)(2). Their effects on adjacent epithelial cells are still poorly understood. We suggest that cytokines, released by macrophages and other cells during fibrogenic reaction to crystalline silica, stimulate the proliferation of adjacent alveolar epithelial cells, some of which may in turn have undergone direct DNA (deoxyribonucleic acid) damage or chromosome aberrations induced by silica particles. Cytokines are believed to be required for tracheal and bronchial epithelial cell proliferation, differentiation, and growth (6, 7). Alveolar type II cells have been shown in rats to respond to quartz-induced lung injury with early hypertrophy and hyperplasia (8) and with persistent hyperplastic and proliferative changes, eventually giving rise to alveolar cell tumors adjacent to the granulomatous lesions (2, 5).

TGF-β1 is a multifunctional regulatory peptide that plays a major role in the physiological and pathological processes affecting cell growth and differentiation, as well as in metabolic activities, and it is present in a variety of normal human and animal tissues, both benign and malignant (7). TGF-β1 has been shown to play a key role in inflammation and tissue repair, and it is capable of stimulating the formation of collagen and connective tissue (7). Studies in vitro have shown its marked stimulatory effects on the

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formation of collagen in rodent and human fibroblasts. Activated lymphocytes can also stimulate proline incorporation into collagen in rodent fibroblasts (9), which can be partially abolished by specific antibodies to TGF-β1 (7).

In view of the multifunctional roles of TGF-β isoforms, we studied three isotypes of TGF-β1 in three rodent models. We investigated immunohistochemically its location, distribution, and possible roles in the pathogenesis of experimental silicosis and the pathogenesis of associated pulmonary carcinogenesis (5). We also studied the cellular immunolocalization of ras and p53 proteins, which are the proteins of the two most common genes known to be altered in human and rodent lung carcinomas. The relationship between changes in these two genes and TGF-β1 may form the basis for a possible mechanism in silica-associated lung carcinogenesis.

Materials and methods

Lung tissues, fixed in 10% buffered formalin and embedded in paraffin, were obtained from 6 male and 10 female F344/NCr rats, which had received a single intratracheal instillation of 12 mg of Min-U-Sil 5 α-quartz at eight weeks of age. These tissues were chosen to include lesions representative of those observed in a total of 37 male and 36 female rats sacrificed at intervals up to 17 months and 14 male and 6 female rats examined at unscheduled death from 17 up to 26 months. For comparison, lung tissues were also observed from eight mice and eight hamsters (equally divided by sex) that were treated with quartz. Control tissues of all three species were from two male and two female animals treated with hematite and as many untreated animals. Experimental details have been described previously, as were the methods used for the preparation of antibodies to TGF-β1, employed for immunohistochemical studies (5). Briefly, the antibodies were raised in rabbits to the NH_2-terminal 1-30 amino acids of mature TGF-β1 (anti-CC and anti-LC) and to amino acids 266—278 of the TGF-β1 precursor/latency-associated peptide (LAP) (anti-Pre). Anti-CC stained extracellular matrix-associated TGF-β1, while anti-LC and anti-Pre stained intracellular TGF-β1.

Localization of antibodies to ras and p53 proteins on paraffin-embedded tissues was studied with immunohistochemical methods. Mouse monoclonal antibody to pan-reactive p21 ras (Cetus Boston, Massachusetts, and Oncogene Science, New York, New York, United States), monoclonal antibodies to p53 [pAb 421 (ABl) and pAb 240 (Ab3)], and polyclonal antisera to p53 (CM-1) were applied as primary antibodies, using the immunoperoxidase technique. For controls, nonreactive rabbit serum immunoglobulin was used instead of the primary antibody.

Results

Pathology. The histopathological findings from the quartz-treated rats showed the development of silicotic granulomas with progressive fibrosis accompanied by marked hyperplasia of alveolar type II cells, sometimes with adenomatoid pattern, adenomas, and a progressive development of lung carcinomas (2). After the instillation of the same quartz sample, mice developed moderate fibrosis, but no alveolar hyperplasia, and hamsters developed only macrophagic storage lesions without significant fibrosis or epithelial reactions (2). A new schematic of the histopathological events observed at early stages (1—45 d) and late stages (≥ 60 d) among the three species of rodents exposed to quartz is given in table 1.

Immunoreactivity to TGF-β1. The results of the immunolocalization of TGF-β1 in the lungs of quartz-treated rats (5), mice, and hamsters are summarized in table 2, together with the results observed among hematite-treated and untreated controls of all three species. The results, observed for all the rats examined and previously illustrated (5), showed that intracellular mature TGF-β1 was localized in fibroblasts and macrophages at the periphery of silicotic granulomas and in the stroma adjacent to hyperplastic alveolar type II cells. Extracellular mature TGF-β1 was localized in the connective tissue matrix adjacent to hyperplastic alveolar type II cells. TGF-β1 precursor/LAP was localized intracellularly in hyperplastic type II cells adjacent to granulomas and in the cells of adenomas, but not in the carcinomas. Hematite-treated controls showed no reactivity to TGF-β1. Only a few focal groups of macrophages in the silicotic granulomas were immunoreactive to anti-Pre, but their staining pattern was not punctate as observed in type II cells.

Immunoreactivity to p21 ras and p53 proteins. In the silicotic rat lungs, immunoreactivity to pan p21 ras protein was constantly detected in the hyperplastic alveolar type II cells, including those forming adenomatoid patterns (figures 1 and 2). There was no reactivity in the adenomas and carcinomas (figure 3). Nuclear immunoreactivity to p53 was seen in two out of eight (25%) lung carcinomas (figure 4). These findings are summarized in table 3.

Discussion

Chronic lung lesions induced by crystalline silica in rats represent a model for studying the interactions of fibrogenesis, epithelial hyperplasia, and carcinogenesis. Our observations suggest that TGF-β1 is an important factor in the complex cell-cell interactions in the pathogenesis of mesenchymal-epithelial responses to silica (5). Elevated levels of TGF-β1 have been reported in pulmonary fibrosis in rats (10), in murine hepatic fibrosis due to carbon

Table 1. Histopathological findings in quartz-treated lungs of three rodent species. (+ = present, ++ = moderately increased, +++ = markedly increased in numbers, – = not present, early = 1—45 days, late = ≥ 60 days)

Lesions	Rats		Mice		Hamsters		Controls[a]	
	Early	Late	Early	Late	Early	Late	Early	Late
Alveolar macrophages	+++	+++	++	++	++	+++	–[b]	–[b]
Interstitial macrophages	+	+++	+	++	++	+++	–[b]	–[b]
Alveolar type II cells	+	+	+	+	+	+	+	+
Hyperplastic alveolar type II cells	±	+++	+	++	–	–	–	–
Fibrosis	+	++++	+	++	++	–	–	–
Carcinoma	–	++	–	–	–	–	–	–

[a] Controls (untreated and hematite-treated) for all three species.
[b] Increased in hematite controls.

Table 2. Immunohistochemical localization of transforming growth factor β1 (TGF-β1) in quartz-treated rodents. (± = weakly reactive, + = reactive, – = nonreactive, NA = not available, early = 1—45 days, late = >45 days)

	Rats		Mice		Hamsters		Controls[a]
	Early	Late	Early	Late	Early	Late	
TGF-β1 (Anti-LC)							
Intracellular							
Alveolar macrophages	+	+	–	+	–	+	–
Interstitial macrophages	+	+	–	+	–	–	–
Fibroblasts	±	+	–	±	–	–	
Alveolar type II cells	–	–	–	–	–	–	–
Hyperplastic alveolar type II cells	–	–	NA	NA	NA	NA	NA
Carcinoma	NA	–	NA	NA	NA	NA	–
TGF-β1 (Anti-CC)							
Extracellular							
Alveolar macrophages	–	–	–	–	–	–	–
Interstitial macrophages	–	–	–	–	–	–	–
Alveolar type II cells	–	–	–	–	–	–	–
Hyperplastic alveolar type II cells	–	–	NA	NA	NA	NA	NA
Carcinoma	NA	–	NA	NA	NA	NA	NA
Matrix	+	+	±	+	–	–	–
TGF-β1 (Anti-Pre)							
Precursor/LAP							
Alveolar macrophages	–	–	–	–	–	–	–
Interstitial macrophages	+	+	–	±	–	–	–
Fibroblasts	–	–	–	–	–	–	–
Alveolar type II cells	+	+	–	±	–	–	–
Hyperplastic alveolar type II cells	+	+++	NA	NA	NA	NA	NA
Carcinoma	NA	–	NA	NA	NA	NA	NA
Matrix	–	–	–	–	–	–	–

[a] Controls (untreated and hematite-treated) for all three species.

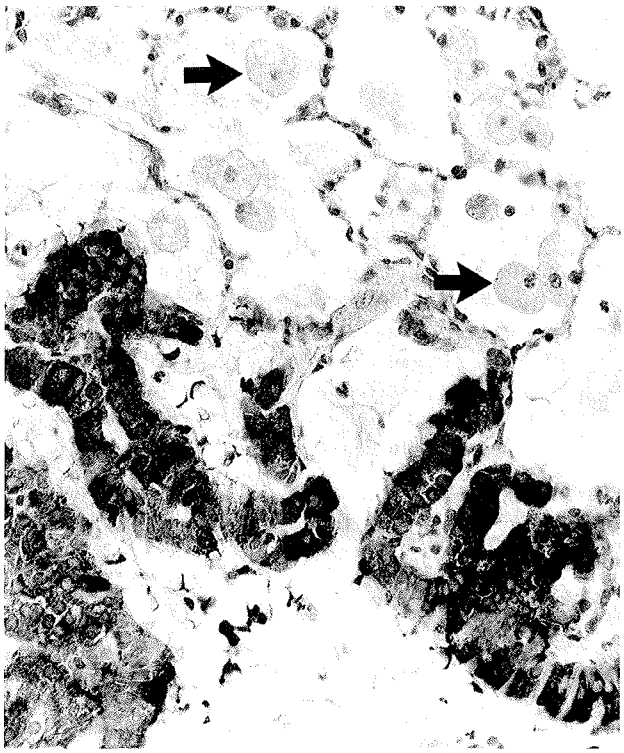

Figure 1. Pan-reactive p21 ras protein immunoreactivity localized in hyperplastic alveolar type II cells in quartz-treated rat lung (dark staining cells). The upper part of the figure shows normal alveolar walls; arrows point to alveolar macrophages.

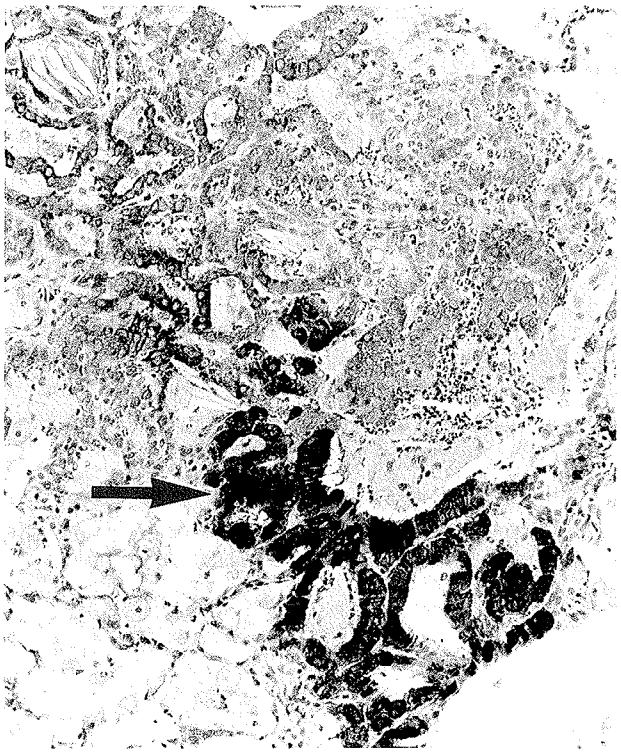

Figure 2. Pan-reactive p21 ras protein immunoreactivity localized in hyperplastic alveolar type II cells in quartz-treated rat lung (dark staining cells). The figure shows nonreactive epithelial cell proliferation in the upper right corner and hyperplastic alveolar type II cells positive for p21 ras protein (arrow).

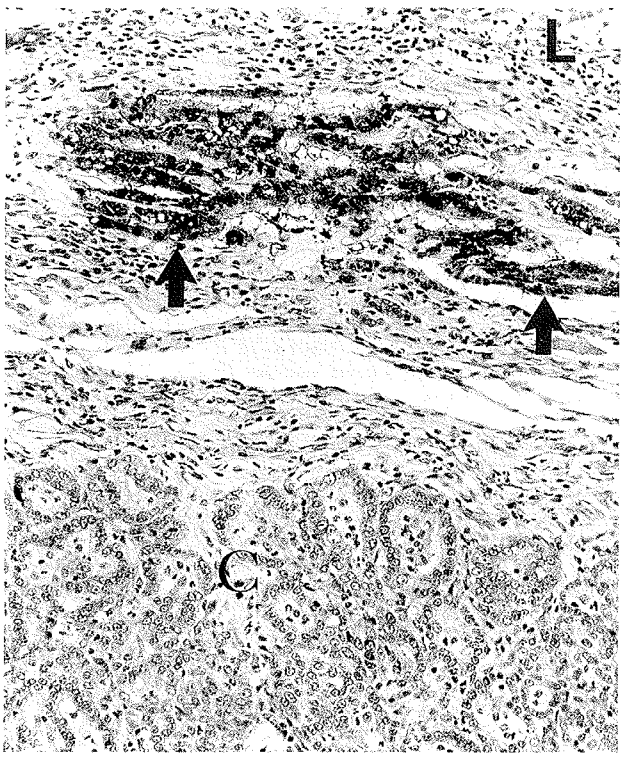

Figure 3. Well-differentiated alveolar adenocarcinoma (C) in the lung of a quartz-treated rat, showing no reactivity to p21 ras protein. Adjacent hyperplastic alveolar type II cells forming adenomatoid patterns (arrows) show reactivity to p21 ras protein (dark staining cells). Uninvolved lung tissue (L) is shown at the top of the figure.

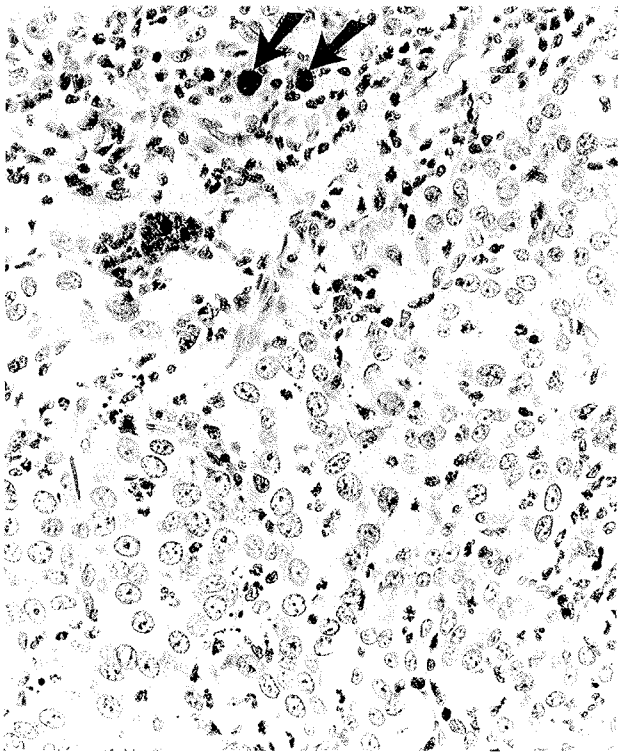

Figure 4. Nuclear immunoreactivity for p53 (arrows) in cells of an undifferentiated carcinoma in a quartz-treated rat lung.

tetrachloride or schistosomiasis, and in humans with the ocular fibrotic disease called proliferative vitreoretinopathy (11). Increased levels of TGF-β1 mRNA (messenger ribonucleic acid) have also been reported in patients with hepatic cirrhosis with regenerative nodules and increased fibrogenic activity (12).

The following mechanisms are suggested for the role of TGF-β in the quartz-induced lesions of rat lung: (i) progressive repair and healing of the silicotic granuloma require an adequate supply of TGF-β1, leading to an increase in the production of TGF-βl precursor in the proliferating alveolar type II cells; (ii) the subsequent activation of the TGF-β1 precursor to mature TGF-β1 stimulates collagen production; (iii) the deposition of collagen and extracellular matrix continues to provide a substrate for repeated cycles of epithelial proliferation (13); (iv) the development of malignancy, which results from malignant transformation of type II cells, in a high proportion of quartz-treated rats may be due to clonal outgrowth during active epithelial proliferation or the escape of cells from the negative regulatory effects of TGF-β1 (7).

The intracellular localization of TGF-β1 precursor/LAP is indicative of its production, first detected in macrophages during the early stages of reaction to silica and then, more conspicuously, in the hyperplastic alveolar type II cells (5). Although the precursor was localized in some hyperplastic epithelial cells at the periphery of carcinomas, it was not detected in malignant cells of the carcinomas. This observation suggests that reactivity to this antibody is lost with progressive loss of cell differentiation. The localization of TGF-β1 precursor can be used to identify the transition between hyperplastic type II cells, which are immunoreactive, and those which have undergone malignant change and have lost their immunoreactivity.

Table 3. Immunoreactivities to transforming growth factor β1 (TGF-β1), ras and p53 in the rat silicosis model. (LAP = latency-associated peptide)

Antibody	Type II cell	Hyperplastic type II cell	Adenoma	Carcinoma
TGF-β1 precursor/LAP	+	+	+	–
p21 ras	+	+	–	–
p53	–	–	–	+ (25%)[a]

[a] Positivity in 25% of the carcinoma cases associated with silicosis.

TGF-β1 is a unique peptide with regard to the regulation of normal and pathological physiology. It is an endogenous cell component that contributes to the healing process (7) and is capable of inducing an inflammatory reaction with the production of granulation tissue. TGF-β1 is likely to be a significant stimulus for the epithelial hyperplasia and proliferation observed in experimental silicosis, as previously observed in other epithelia. The control of cell growth and differentiation by TGF-β1 appears to be coordinated within epithelial cells of various types, in which TGF-β inhibits proliferation and induces terminal differentiation (6, 7). It is noteworthy that, in silicotic rat lungs, TGF-β1 precursor is demonstrable in hyperplastic alveolar type II cells, and in some interstitial macrophages, but not in alveolar macrophages, even though these cell types appear to be capable of internalizing silica particles (Williams et al, unpublished data).

The localization and distribution of TGF-β1 suggests that it plays a role in the pathogenesis of silicotic lesions, and possibly in the development of associated pulmonary carcinomas. It is conceivable that, in the rat silicotic lesions, the synthesized TGF-β1 inhibits further cell proliferation and promotes epithelial differentiation, as suggested for idiopathic pulmonary fibrosis (14). The development of carcinoma from the hyperplastic alveolar type II cells in the rat model may be due to the reduction or failure of TGF-β1 synthesis, leading to uncontrolled proliferation and loss of differentiation.

The immunohistochemical localization of p21 ras protein in hyperplastic type II cells and in their adenomatoid proliferations, but not in the adenomas and carcinomas, suggests its association with phenotypic change. Lack of immunoreactivity to p21 ras antibodies in malignant phenotypes suggests that the *ras* gene may have been activated, but this possibility requires confirmation. The absence of detectable p21 ras protein in the adenomas, which are positive for TGF-β1 precursor/LAP, suggests that the negative regulatory control by TGF-β1 is still maintained after the ras protein is down-regulated. It has been shown in human bronchial epithelial cells, murine skin keratinocytes, and thyroid follicular cells that the down-regulation of TGF-β1 gives rise to an increase of mutant p53, through *myc* transcription (15—17). This mechanism may be pertinent to our observations in the silica rat model, according to which 25% of the observed carcinomas showed nuclear accumulation of p53 protein.

Acknowledgments

We thank Dr Kathleen C Flanders for her help with the immunohistochemical studies of TGF-β1 in rat tissues and to Mr Ricardo Dreyfuss for the photomicrographs.

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