

Silicosis and lung cancer: a fifty-year perspective

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Abstract. The development of our studies on silica carcinogenesis and its mechanisms is reviewed. Starting from an analysis of the cellular reactions to silica in the pathogenesis of silicosis in the rat, followed by an analysis of the carcinogenic response to silica in the lungs of rats (but not in mice and hamsters), we went on to develop cellular models for culture and neoplastic transformation of rat alveolar epithelial cells. We studied the binding of silica to DNA, the generation of reactive oxygen species and the DNA damage mediated by hydroxyl radicals, showing marked differences among silica samples of varying purity. Then we investigated the role of peptides induced by silica in various cells, including cytokines and growth factors. Tumor necrosis factor (TNF)- α , which can cause activation of DNA transcription and is required for silica-induced fibrosis, was found to inhibit neoplastic transformation by quartz in cell cultures. Transforming growth factor (TGF)- β was found to be produced in hyperplastic alveolar type II cells and to reach fibroblasts, macrophages and the connective tissue matrix adjacent to silicotic granulomas. Neuroendocrine cells and their peptides were found to be increased in alveolar and bronchiolar epithelia of silica lesions in rats, in contrast with mice and hamsters. Expression of adhesion molecules was found to be altered in silica-induced carcinogenesis and epithelial-mesenchymal transition was revealed by mesenchymal markers in the induced carcinomas. Promoter hypermethylation of adhesion genes in the induced carcinomas indicated a role for epigenetic mechanisms.

Keywords: Silica-induced carcinogenesis, neoplastic transformation of alveolar type II cells in culture, DNA binding and DNA damage, tumor necrosis factor- α , transforming growth factor- β , neuroendocrine cells, adhesion molecules

Introduction

In October, 1955, after training in pathologic anatomy in Milan, and three years of research in experimental carcinogenesis at the Division of Oncology of the Chicago Medical School, I returned to the University of Milan at the invitation of Professor E. C. Vigliani as an assistant in Occupational Medicine responsible for the laboratory of pathology of the Clinica del Lavoro. There, we studied the histopathogenesis of experimental silicosis in rats treated with a single intratracheal instillation of one of several defined dusts (α -quartz, tridymite, cristobalite, HF-etched quartz and ferric oxide) and also dusts from

furnace-bricks, and we analyzed the development of the complex cellular reactions to silica in the lungs and lymphnodes, including macrophages (and their necrosis), mast cells (and their degranulation), plasma cells, leucocytes, lymphocytes and fibroblasts, leading to fibrosis and hyalinosi (1-6).

With my background in carcinogenesis studies, I was impressed with the finding that rats treated with fibrogenic silica dusts also showed epithelial changes characterized by atypical hyperplasias of the bronchiolar and alveolar epithelia, often with enlarged nuclei and hyperchromatic cytoplasm. Some of these lesions were seen as early as 10 days after treatment and extended further in the following months. The epithelial

lesions occurred in proximity to areas of developing silicotic granulomas and fibrosis. No tumors were observed in these rats, which were sacrificed mostly within 6 months. These early observations were reported later in detail with my working hypothesis that "one, or probably several of the cell mediators released by macrophages and other reticuloendothelial cells during the complex process of fibrogenesis are acting upon the adjacent epithelial cells of the distant airways to induce further cell damage, to stimulate cell proliferation, or both." (7). I also observed an increased incidence of lung cancers, adjacent to silicotic granulomas and fibrosis, in the autopsies of silicotic patients of the Clinica del Lavoro (6 cases in 52 autopsies, or 11.5%, at a time when lung cancer incidence was still low in the general population). For comparison, in Great Britain lung cancer was reported in 13.2% of cases of deaths with asbestosis in 1949 (8), and the causal relationship of asbestos and lung cancer was fully documented by 1955 (9). However, the prevailing belief until the early 1980s was that no relationship existed between silicosis and lung cancer.

In 1960, I returned with a faculty appointment to Chicago, with the opportunity to greatly extend my experimental work. Based on my experience with studies of mineral particles in the lung, I devised a method for the induction of bronchogenic carcinoma by intratracheal instillation of non-fibrogenic mineral particles (such as hematite) acting as carriers on which organic carcinogens (such as benzo[*a*]pyrene) had been adsorbed (10). The animal of choice was the Syrian golden hamster, for its resistance to pulmonary inflammation. This became a standard method for lung carcinogenesis, and led to many new findings, including the synergistic effects of systemically administered carcinogens (such as diethylnitrosamine) (11) and the inhibition of carcinogenesis by vitamin A (12), which in turn opened up the field of chemoprevention.

In 1968, I joined the National Cancer Institute (NCI) as Associate Scientific Director for Carcinogenesis, with the task of developing an extensive program on chemical and physical carcinogenesis, which we organized in three main areas: (a) identification of carcinogenic activity by long term bioassays in animals (rats, hamsters and mice) of selected chemicals, especially those involving environmental and occupational

exposures; (b) development of biological models for carcinogenesis, in animals and in cell cultures; and (c) identification of carcinogenesis mechanisms (13). Particular emphasis was given to a special lung cancer program. In my Laboratory of Experimental Pathology at NCI, my colleagues and I continued to investigate respiratory tract carcinogenesis using *in vivo* and *in vitro* models in the 1970s and '80s.

In vivo carcinogenesis

In 1972 M.M.F and J.C. Wagner reported the induction of malignant histiocytic lymphomas in rats injected intrapleurally with crystalline silica (14). In 1983, Holland et al. reported the induction of lung tumors in rats after intratracheal instillation of quartz (15). Then, at the First International Symposium on Silica, Silicosis and Cancer, held in 1984 (16), new extensive evidence of pulmonary carcinogenicity by crystalline silica was reported from three separate laboratories (17-19), following inhalation or intratracheal instillation in rats. Epidemiologic evidence of the carcinogenicity of silica in humans was also reported at the 1984 Symposium (16), and later extensively confirmed, as shown in the Monographs of the International Agency for Research on Cancer (20, 21).

This new evidence led me to report my previous observations on the pathology of early epithelial proliferative lesions induced by silica in rats (7, 22), and to resume studies on silica carcinogenesis with new approaches. The reports at the 1984 Symposium showed major species differences in the response to intrapulmonary silica: in contrast with the effect in rats, silica induced neither fibrosis nor tumors in the lungs of hamsters, only macrophage storage lesions (19, 23). We confirmed this finding, and also showed that mice of several strains developed silicosis, but no epithelial proliferations and no carcinogenic response (24). Thus, a choice of animal models is available to study mechanisms and pathways associated with both silicosis and cancer (rats), or silicosis only (mice), or neither (hamsters) (24, 25). In our experimental studies on silica rat carcinogenesis, we observed that the early hyperplastic and proliferative lesions of the alveolar and bronchiolar epithelia progressed to extensive

hyperplasias, adenomatoid hyperplasias and eventually to adenomas and carcinomas (7, 24). In our study on F344 rats treated with a single intratracheal instillation of 12 mg of Min-U-Sil 5 quartz (24), adenocarcinomas were the most frequent tumor type in both sexes (74%), with lower incidences of epidermoid carcinomas (6%), undifferentiated carcinomas (4%), mixed type carcinomas (7%) and adenomas (8%). Similar hyperplastic and proliferative lesions were observed also in human silicotic lungs.

With new methods now available for molecular analysis of selected cells in fixed tissues, such as laser-capture microdissection (26), it is possible to compare the molecular changes of genes and gene products that characterize the early stages of silica-induced carcinogenesis, as discussed below.

Neoplastic transformation of cells in culture

The direct interaction between a carcinogenic agent and the target cells can be effectively investigated using *in vitro* cultures of appropriate cell types. Initial evidence of neoplastic transformation induced by quartz was obtained by Hesterberg and Barrett in cultures of Syrian hamster embryo cells (27). In the mouse embryo cell line BALB/3T3/A31-1-1, which can be transformed by a broad spectrum of chemical carcinogens, we demonstrated neoplastic transformation as well as chromosomal damage by several different samples of quartz: all samples induced dose-dependent transformation up to a plateau level; interestingly, the F600 sample showed minimal cytotoxicity, but transforming activity comparable to the other more toxic samples (28). Hamster embryo cells and the mouse embryo cell line are both of mesenchymal origin and are transformed by silica, even if silica did not induce lung tumors in these two species.

We need to learn more about the specific mechanisms of neoplastic transformation in target cells *in vitro* and *in vivo*. These concerns apply to the selection of cellular models used for mechanism studies. For example, studies that used a tumor cell line (such as the human lung A549 cell line) may not be amenable to reveal mechanisms active in the induction of transformation. If the target cells are not suscepti-

ble to silica-induced carcinogenesis, the molecular pathways elicited by silica in them may not be the ones that are critical for the mechanisms of carcinogenesis.

The target cells of silica carcinogenesis *in vivo* are those of the alveolo-bronchiolar epithelium. It was therefore our goal to study neoplastic transformation in cultures of such cell types. We first used the FRLE cell line, derived from fetal rat lung epithelial cells retaining markers of alveolar type II pneumocytes, and showed that α -quartz (Min-U-Sil 5) induced morphologically transformed colonies, that grew as carcinomas when inoculated in nude mice (29). This transforming activity was obtained initially by treatment of the FRLE cells with a high dose of quartz (100 $\mu\text{g}/\text{cm}^2$), and later confirmed for doses as low as 12.5 $\mu\text{g}/\text{cm}^2$ (30). By electron-microscopy of the treated cells (both FRLE and BALB/3T3/A31-1-1), we observed that, in addition to the usual cytoplasmic localization of quartz particles, some fine particles (<0.5 μm) were localized inside the nuclei and in the mitotic spindles of dividing cells, suggesting direct contact with chromatin (31). The FRLE cell line was not an ideal model, because the cells were isolated at passage 35 in a medium with high serum content (16%) and were subtetraploid with several marker chromosomes. Therefore we undertook to develop new cell lines from primary cultures of alveolar type II pneumocytes from young adult rats, using our LEP/RTE-1 medium (32), previously selected for serum-free growth of rat tracheal epithelial cells; the alveolar cells required addition of a low concentration (1.5-2%) of fetal bovine serum. The cells, which remained diploid or near diploid and grew well in such medium, were immortalized by transfection with plasmids containing either the SV40 large T antigen (line AE5) or the adenovirus-2 E1A gene (line AE6). The transfected cells maintained good epithelial morphology for over 40 passages (33). When AE6 cells were treated at passage 8 with 25 $\mu\text{g}/\text{cm}^2$ quartz (Min-U-Sil 5), they developed distinct morphological changes indicative of transformation, confirmed by anchorage-independent growth in soft agar and by inoculation in nude mice where they grew as undifferentiated carcinomas (33). The molecular mechanisms involved in the direct transformation of alveolar cells by silica remain to be further investigated.

Silica-DNA interactions

The formation of oxygen free radicals at the quartz surface is a key mechanism for silica toxicity and carcinogenicity (34). We showed that crystalline silica can induce oxygen-dependent DNA strand breakage (35), and that silanol groups on the silica surface bind strongly to the phosphate-sugar backbone of DNA, at physiologic pH (36). Hydroxyl radicals, responsible for DNA damage, have an extremely short half-life and are active only over distances of approximately 15 angstroms, or one-half the diameter of a DNA helix. We suggested that the binding of DNA to the silica surface provides an anchoring mechanism whereby the hydroxyl radicals are generated close enough to the target DNA to generate strand breaks (31, 36, 37). DNA strand breakage, thymine glycol production, oxygen consumption and hydroxyl radical generation were found to vary considerably for different samples of crystalline silica *in vitro*, and were not directly correlated with the surface area (38). Studies with a sample of cristobalite, heated up to 1300° C, showed that thermal treatments deeply affected surface properties; tests on the AE6 cell line showed that surface radicals and cytotoxicity were greatly lowered by treatment of the dust at 800° and completely inactivated at 1300° C, indicating that hydrophobicity is at least one of the surface properties determining the cytotoxic potential of the dust (39).

Molecular mediators

Several cytokines and growth factors have been shown to be involved in the process of silica fibrogenesis and carcinogenesis, and their mechanisms and pathways require further study. In rats, silica increased the release of tumor necrosis factor(TNF)- α from alveolar macrophages (40); in mice, TNF- α is required for the development of silica-induced pulmonary fibrosis and silica induces a marked increase of lung TNF- α mRNA (41). Following a report that TNF- α enhanced neoplastic transformation of mouse BALB/3T3/A31-1-1 cells, we tested it again in this cell line, and confirmed that it enhanced trans-

formation by 3-methylcholanthrene, but surprisingly found that it inhibited transformation by quartz (33, 42). This effect may occur through a block of silica toxicity due to oxygen radicals, as was suggested by a similar inhibitory effect of the free radical scavenger dimethylsulphide. TNF- α is part of the mechanism of the nuclear factor(NF)- κ B, which is a transcription factor for several genes and an important mediator in the pathogenesis of silica-induced pulmonary pathology (43). The binding of TNF- α to its receptor triggers phosphorylation and destruction of I κ B (the inhibitor of NF- κ B in the cytosol), which allows NF- κ B to enter the nucleus. NF- κ B is activated by silica in alveolar macrophages and other lung cells by mechanisms involving the hydroxyl radical, and in turn regulates the production of TNF- α and other inflammatory mediators. These mechanisms are important in the pathogenesis of silica-induced lung diseases (43-45)

Transforming growth factor(TGF)- β is a multifunctional regulatory peptide with key roles in inflammation and tissue repair, capable of stimulating the formation of collagen. We studied its sites of production and destination by immunohistochemical methods in the rat silicosis model (46). Antibodies were raised in rabbits to the NH₂-terminal 1-30 amino acids of mature TGF- β 1 (for intracellular and extracellular location), and to amino acids 266-278 of the TGF- β 1 precursor. Immunoreactivity to the precursor (indicative of the site of production) was localized intracellularly in hyperplastic alveolar type II pneumocytes adjacent to granulomas, and in their proliferative lesions and adenomas, but not in adenocarcinomas. Mature TGF- β 1 was localized intracellularly in fibroblasts and macrophages at the periphery of silicotic granulomas and adjacent to hyperplastic type II cells, and extracellularly in the connective tissue matrix of the granulomas and in the stroma of both hyperplastic type II cells and well-differentiated adenocarcinomas. These findings point to hyperplastic type II cells as sites of production and secretion of TGF- β 1 (46). In FRLE cells, quartz increased secretion of TGF- β 1 into the medium about 2.5-fold, as shown by enzyme-linked-immunosorbent assays (47). Human silicotic lungs also showed positive immunoreactivity for TGF- β 1 precursor in foci of alveolar epithelial hy-

perplasia and adenomatoid formations adjacent to areas of fibrosis (47). TGF- β receptors are single-pass transmembrane proteins which, when bound to their extracellular ligands, activate intracellular kinases that attach phosphate groups to serine or threonine residues of target proteins and regulate gene transcription (43). Mitogen-activated protein kinases (MAPKs) have also been recently shown to be induced in silica treated mouse cell lines (43).

Strong immunohistochemical localization of a panreactive p21 ras protein was shown in silica-treated rats in hyperplastic alveolar type II cells adjacent to granulomas and in adenomatoid lesions, but not in adenomas and carcinomas; foci of nuclear immunostaining to p53 protein were found in neoplastic cells of 2 out of 8 examined silica-induced undifferentiated lung carcinomas (47, 48). Studies *in vivo* and *in vitro* by Castranova and coll. (49) have recently shown that silica induces apoptosis, mostly in alveolar macrophages, in the lungs of p53+/+ mice, but not in p53-/- mice, suggesting that p53 plays a crucial role in the signal transduction pathways of silica-induced apoptosis.

In recent years, I have collaborated with L.M. Montuenga and his coworkers at the University of Navarra in Pamplona, Spain. Using our model of lung carcinogenesis induced by intratracheal instillation of crystalline silica, the expression of neuroendocrine activity was studied immunohistochemically in the lungs from rats, mice and hamsters (50). Neuroendocrine (NE) cells are either clustered as alveolar or bronchiolar neuroepithelial bodies (NEBs) or isolated in bronchioles as pulmonary neuroendocrine cells (PNECs). In rats, silica induced marked increases in the number of alveolar NEBs and in the number of cells per NEB: these cells expressed calcitonin-gene related peptide (CGRP), a general marker for NE cells, and also proadrenomedullin N-terminal 20 peptide (PAMP) and the amidating enzyme peptidyl-glycine α -amidating monooxygenase (PAM). Adrenomedullin (AM) was expressed in isolated PNECs in the bronchiolar epithelium, but NE cells of hyperplastic NEBs were consistently negative. Mice and hamsters showed no significant increases in the number of NEBs or cells per NEB; they showed immunoreactivity for PAM, but not for PAMP; some hamster bronchiolar NEBs

were positive for AM in contrast to rats and mice. Proliferative stimulation of epithelial cells near NE cells may contribute to epithelial hyperplasia in the silica rat model (50).

Adhesion molecules have been recently recognized as having a critical role in cell proliferation and carcinogenesis. Immunohistochemical studies in our rat model showed that expression of E-cadherin and of α - and β -catenins is altered during silica-induced carcinogenesis and patched expression of the mesenchymal markers, vimentin and N-cadherin, was found in silica-induced adenocarcinomas and squamous cell carcinomas, indicating the occurrence of epithelial-mesenchymal transition in silica carcinogenesis (51). Analysis by bisulfite sequencing of the promoter region of E-cadherin in several silica-induced tumors showed that E-cadherin promoter hypermethylation was associated with marked loss of E-cadherin protein expression (by immunohistochemistry). Global methylation, measured by the 5-methylcytosine DNA content, was found to be 19% lower in silica-induced carcinomas, compared with normal lung tissues (52). The role of promoter hypermethylation in the transcriptional repression of adhesion related genes was studied: strong methylation was found in 78% of the tested tumors for the H-cadherin promoter and in 56% for the APC promoter, indicating a role of epigenetic mechanisms in adhesion related genes (52). Hypermethylation patterns of the ras effectors Rassf 1A and Nore 1A were compared with the mutational status of K-ras, N-ras and c-H-ras observed with laser microdissection and genomic sequencing of exons 1 and 2: no genetic or epigenetic alterations were found in the ras genes in the silica-induced tumors. These results point to the importance of adhesion-related genes in silica-induced lung carcinogenesis (52).

The close association of epithelial proliferations with adjacent silicotic granulomas indicate the importance of cell-cell interactions between mesenchymal cells (macrophages, monocytes, fibroblasts, mast cells) and the adjacent epithelial cells. The role of cytokines and growth factors shows their critical importance as mediators of key properties of target epithelial cells, such as progressive proliferation and altered adhesiveness.

Conclusions

The induction of lung cancer by crystalline silica, firmly established by human epidemiological evidence and by experimental evidence from long-term animal studies and from neoplastic transformation of the target cells in culture, has provided a model for investigating pathogenetic mechanisms that were revealed to be progressively more complex as more advanced methods for molecular studies became available. The reactions to silica particles in the lungs stimulate the activation of a number of molecular mediators, including those that are primarily involved with the inflammatory and fibrogenic responses and their immunological aspects, such as interleukins as well as TNF- α . The molecular mechanisms involved in silica-induced carcinogenesis have also been found to include complex molecular mediators and their interactions. The investigation of the molecular mechanisms underlying silica-induced lung carcinogenesis promises to continue to provide new insights into this complex biological response, with its close interaction of mesenchymal and epithelial cells and their cascades of mediators.

The lung pathology induced by silica in susceptible hosts has become a fruitful model for fibrosis-associated lung carcinogenesis. Many aspects of the carcinogenic mechanisms of crystalline silica, which we learned by investigating animal, cellular and molecular models, are of potential interest for future studies aimed at defining the mechanisms of the human diseases, silicosis and lung cancer, with the aim of identifying molecular characteristics of susceptibility to cancer induction and possibly identifying persons at high risk. Knowledge of the molecular pathogenesis of silica-induced lung cancer may eventually open up useful approaches to biological or pharmacological intervention for cancer prevention.

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