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Crocidolite Induces Cell Transformation and *p53* Gene Mutation in BALB/c-3T3 Cells

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Cell transformation is one of the most common assays used to study morphological changes in the multistep process of carcinogenesis. The present study was initiated to investigate the ability of crocidolite to induce cell transformation in BALB/c-3T3 cells and to analyze the relationship between *p53* mutations and crocidolite-induced cell transformation, if any. Cell transformation was carried out according to standard procedures. Exponentially growing cells were exposed to different concentrations (0.2–20 μg/cm^2) of crocidolite fibers for 72 h. Foci obtained from cell transformation were analyzed for their ability to grow in soft agar (anchorage-independence) and *p53* alterations. The results of this study demonstrate that there was an increase in transformation frequency (TF) with an increase in concentration of crocidolite. Also, focal cells were able to grow on soft agar, indicating anchorage-independence. cDNA was prepared from RNA isolated from Type 3 foci and subjected to mutational analysis. Eleven exons of the *p53* gene from eight transformed cell lines were analyzed for alterations using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). Alterations were found in seven of eight cell lines, two of them were in exons 4–6, and five in exons 9–11. The alterations were randomly scattered among the crocidolite dose groups. These results suggest that crocidolite induces mutations predominantly in exons 9–11 of the *p53* gene in a nondose-dependent manner. *Teratogenesis Carcinog. Mutagen. 20:273–281, 2000.*

Key words: crocidolite; BALB/c-3T3 cells; cell transformation; *p53* gene mutation analysis

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INTRODUCTION

Asbestos fibers have been described as a physical carcinogen and their carcinogenic effects appear to be related primarily to fiber dimensions [1]. Long, thin-diameter asbestos fibers are much more carcinogenic than short, thick fibers. Crocidolite fibers, in particular, have been associated with the development of lung cancer, pulmonary interstitial fibrosis, and malignant mesotheliomas of the pleura, pericardium, and peritoneum [2–4]. Although the exact mechanism by which asbestos fibers cause disease remains largely unknown [5], strong evidence shows that asbestos is a physical carcinogen acting both as an initiator and a promoter [1].

Cell transformation is a valid model to study chemical carcinogenesis. It has been reported in the Syrian hamster embryo (SHE) assay that longer, thinner fibers of asbestos are more potent inducers of morphologic transformation [6], an observation consistent with the results of cytotoxicity studies in a variety of cell types. Asbestos also induces transformation in human and rat mesothelial cells. All of these systems used early-passage diploid normal cells as the target cells, in contrast to other transformation models that used established cell lines. A recent report indicated that asbestos induced cell transformation in a human bronchial epithelial cell line [7]. Studies have also shown that both asbestos and crocidolite induce cell transformation in BALB/c-3T3 cells [8,9].

Cytogenetically, asbestos induces both structural and numerical chromosomal abnormalities in a wide variety of mammalian cells [10–12]. In mesotheliomas and bronchogenic carcinomas, the abnormalities include deletions, translocations, and aneuploidy of specific chromosomes [10,11,13,14]. Studies in our laboratory and other laboratories have shown that crocidolite induces micronuclei and multinuclei in BALB/c-3T3 cells [5,15]. These abnormalities are believed to be the mechanism by which cell transformation and tumor progression occur in asbestos-induced cancer, possibly by activating proto-oncogenes and/or altering tumor suppressor genes [12,16,17]. Therefore, we investigated the ability of crocidolite to induce cell transformation and anchorage independence in BALB/c-3T3 cells. In addition, the objective was to analyze the relationship between *p53* mutations and transformation induced by crocidolite.

MATERIALS AND METHODS

Cell Culture and Reagents

BALB/c-3T3 clone A31-I-13 cells were a generous gift from Dr. T. Ong (NIOSH, Morgantown, WV). The cells were maintained in Dulbecco's modified essential medium (Gibco, St. Louis, MO) supplemented with 10% fetal calf serum and recommended antibiotics. All cell cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2. Crocidolite was obtained from fields in Yaoan County, Yunnan Province, China. The distribution of the crocidolite fibers were as follows: 22% of the fibers were >10 μm in length, 51% were 5–10 μm, and 27% were <5 μm in length. All fibers had a diameter of <1 μm. Crocidolite was suspended in sterile saline. Positive control benzo[a]pyrene (B[a]P; Fluka, Ronkonkoma, NY) was dissolved in dimethyl sulfoxide (Merck, Whitehouse Station, NJ), to a final concentration of 0.2 μg/ml in cell culture medium.

Cytotoxicity Assay

The cytotoxic effect of crocidolite fibers on colony forming efficiency (CFE) was assayed according to the recommended protocol [18]. The cells were seeded in

60 mm dishes at a density of 200 cells/dish. These cells were treated with different concentrations of crocidolite (0.2–20 μg/cm^2) for 72 h. Saline was used as a solvent control and B[a]P was used as positive control at a concentration of 0.2 μg/ml. All experiments were carried out in triplicate. The concentrations of crocidolite at which the relative colony forming efficiencies (RCFEs) ranged from 5–90% were selected to detect morphological transformation.

Cell Transformation Assay

The cell transformation assay was performed according to standard procedures [18]. Briefly, cells were seeded at a density of 1×10^4 cells in each 60-mm dish. Exponentially growing cells (24 h after seeding) were treated with crocidolite (0.2 and 20 μg/cm^2) for 72 h. The cells were allowed to grow for 5 weeks with two media changes per week. At the end of 5 weeks, clones from six transformed foci (Type 3) in the crocidolite groups were individually picked and cultured. The transformation frequency (TF) in each group was statistically evaluated by exact probabilities.

Anchorage-Independent Assay

Individual transformed colonies induced by crocidolite were separately passaged before proceeding with the soft agar assay [19]. Six percent noble agar (Difco, Detroit, MI) was autoclaved for 20 min and allowed to cool to 48–50°C in a sterile water bath. A portion of the 6% agar was diluted with complete culture medium and 15 ml of this mixture was poured into 100-mm culture dishes and allowed to solidify (bottom agar); 100 μl of the cultures containing 1,000 cells and 0.3% agar was poured on top of the solidified bottom agar and incubated at 37°C at 5% CO_2 for 4 weeks. Three replicates of each sample were used. The anchorage-independence of transformed focal cells were measured by the ability of the cells to form colonies containing greater than 50 cells.

Isolation of RNA and RT-PCR

Total RNA was isolated from transformed and nontransformed cells according to Sambrook et al. [20] and stored at –20°C. A small quantity of RNA was electrophoresed on a 1.5% agarose gel to check for integrity. RNA was reverse-transcribed using M-MLV reverse transcriptase (Gibco, Gaithersburg, MD). The cDNA obtained was further used for PCR-SSCP analysis.

PCR-SSCP

The mouse *p53* gene sequence (11 exons) was amplified in four fragments (exons 1–3 (353 bp), exons 4–6 (347 bp), exons 7–9 (359 bp), exons 9–11 (266 bp)). The PCR primers were: exons 1–3 P1(5′): ATGGAGGAGTCACAGTCGGA, P2 (3′) CGTGCACATAACAGACTTGG; exons 4–6 P3 (5′): TTCCACCTGGGCTTCCTGGA, P4 (3′) CTCGGGTGGCTCATAAAGGTA; exons 7–9 P5 (5′): TTTCGCCACAGCGTGGTGGT, P6 (3′) CTTGAGGGTGAAATACTCTC; exons 9–11 P7 (5′) GCAAAGAGAGCGCTGCCCAC, P8 (3′) TGAGTCAGGCCCCACTTTCT. The PCR amplifications were performed in a GeneAmp PCR system (Perkin-Elmer, Foster City, CA). The following conditions were used for 40 amplification cycles: denaturing, 1 min at 95°C; annealing, 1 min at 60°C; extension, 1 min at 72°C. The products were electrophoresed in a 1.5% agarose gel.

SSCP was performed as described by Orita et al. [21] on a normal vertical elec-

trophoresis system (15 × 10 × 1.5 mm). PCR products were diluted 1:1 in denaturing buffer (95% methyl mercury hydroxide, 0.25% bromophenol blue, and 0.25% xylene cyanol), denatured at 100°C for 10 min, then held at 0°C. Samples were loaded onto the gel and ran at 160V for 10 h. The gels were dried and autoradiographed according to Ainsworth's method [22].

RESULTS

Cytotoxicity Induced by Crocidolite in BALB/C-3T3 Cells

As shown in Table I, the RCFE of cells ranged from 84.5% to 5.0% with concentrations of crocidolite ranging from 0.2–20 μg/cm^2. The percent CFE ranged from 37.2 in the lowest dose to 2.2 in the highest treatment group. Based on the results of the cytotoxicity assay, crocidolite concentrations were chosen for cell transformation and anchorage-independent assay.

Cell Transformation Induced by Crocidolite in BALB/c-3T3 Cells

Morphologically transformed foci classified as Type 3 were identified and analyzed. The criteria for transformed foci of BALB/c-3T3 cell were as follows: fibroblastic, multilayered, crisscrossing, densely stained cells with random orientation at edges of foci, and basophilic [18]. The morphology of nontransformed and transformed foci are shown in Figure 1.

As shown in Table I, the average number of transformed foci per culture dish with the solvent control was very close to the standard for spontaneous transformation. Transformed foci in B[a]P-treated group (positive control) was significantly higher than in the solvent control ($P \leq 0.01$). Crocidolite increased the number of transformed foci at concentrations greater than 0.2 μg/cm^2 ($P \leq 0.05$), in a dose-dependent manner, except at 20 μg/cm^2, where the number of transformed foci decreased. However, the transformation frequency was higher since the cells were normalized for the surviving cells.

TABLE I. Cytotoxicity and Morphological Transformation Induced by Crocidolite in BALB/c-3T3 Cells

Crocidolite (μg/cm^2)	% CFE[a] (mean ± SE)[c]	% RCFE[b]	No. of transformed foci	TF[d] ‰
Saline	44.0 ± 3.0	100	4	0.045
B(a)P	43.3 ± 4.0	98.4	26	0.300**
0.2	37.2 ± 2.5	84.5	5	0.067
1	29.3 ± 4.7	66.6	9	0.154*
5	14.8 ± 3.3	33.6	16	0.541**
10	5.5 ± 1.7	12.5	13	1.182**
20	2.2 ± 1.0	5.0	8	1.818**

[a]CFE = colony-forming efficiency.
[b]RCFE = relative colony-forming efficiency.
[c]Mean ± SE: data from three replicate dishes.
[d]TF: transformation frequency = (number of transformed foci/number of inoculated cells X CFE)X1000‰
*$P \leq 0.05$.
**$P \leq 0.01$.

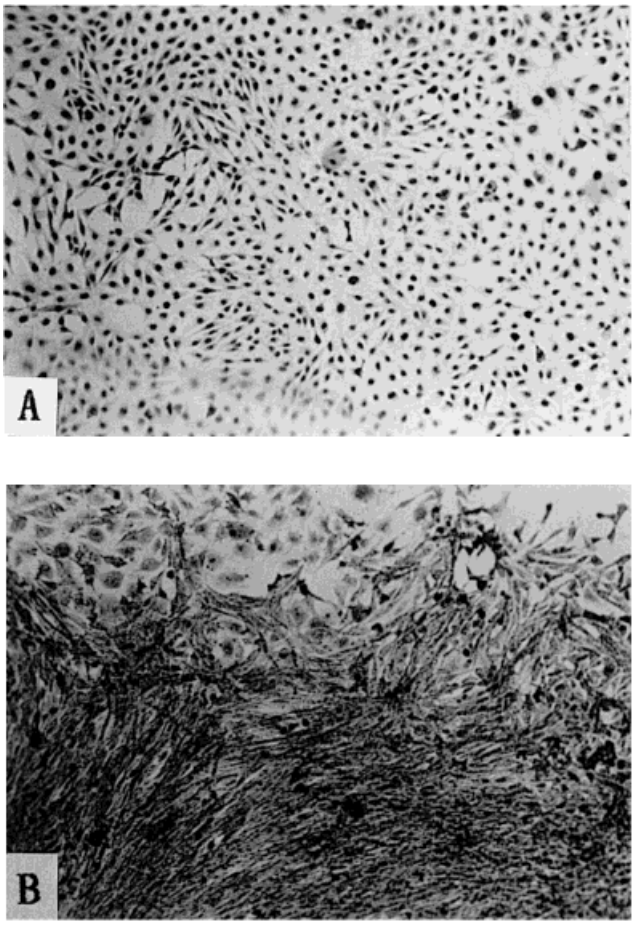

Fig. 1. Morphology of BALB-c/3T3 cells. **A**: Monolayer of nontransformed cells. **B**: Transformed cells exhibiting multilayered, crisscrossing nature of cells with dense staining and random orientation at the edges of foci.

Anchorage-Independent Assay

Cells from six transformed foci obtained from different crocidolite groups were assayed for their anchorage-independence (two duplicate culture dishes from cells of each focus). The results showed that cells from all six transformed foci formed colonies in soft agar (Table II). A dose-dependent increase in the number of colonies was seen with increasing concentration. The maximum number of soft agar colonies (84.5) was seen in the highest dose group.

Analysis of *p53* Gene Mutations by PCR-SSCP

PCR-amplified double stranded DNA fragments were denatured and analyzed as single strands on a nondenaturing polyacrylamide gel. Band shifts were detected as a result of differences in the strand mobility due to changes in the folded structure of the single-stranded DNA. Two transformed cell lines showed band shifts in exons

TABLE II. Effect of Crocidolite on Transformation and Growth in Soft Agar

Crocidolite ($\mu g/cm^2$)	No. of transformed foci	Soft agar (average no. of colonies)
0.2	5	—
1	9	2.5
5	16	23.3
10	13	40.3
20	8	84.5
B[a]P	26	97.0

4–6 (an additional band was observed compared to the control). Figure 2 shows five transformed cell lines with abnormal bands in exons 9–11 (three with an additional band compared to the control, two with different banding patterns). No band shifts were found in exons 1–3 and exons 7–9 for any of the transformed cell lines. Therefore, in total seven of eight transformed cell lines carried *p53* mutations; two mutations in exons 4–6 and five in exons 9–11. No mutations were observed in nontransformed BALB/c-3T3 cells.

DISCUSSION

In vitro carcinogenesis is generally thought to be a multistep process involving the stages of initiation, promotion, and progression [23–25]. Important phenotypic alterations of cell transformation include a number of aberrant growth characteristics, such as loss of contact inhibition, the ability to grow to higher cell density, the ability to grow on confluent monolayers of nontransformed cells, and the ability to grow in soft agar [26]. Transformed cells also result in malignancy, which is not per se an in vitro event, but implies that the cells will grow into a malignant tumor if they are implanted into an isogenic organism or into an animal lacking an immune system [26]. Morphological transformation in vitro may, therefore, be analogous to in vivo carcinogenesis (although it is not clear that the processes are identical) and has been used extensively as a model system for the investigation of chemical carcinogenesis [27].

The results of cell transformation in our study indicate that crocidolite induced

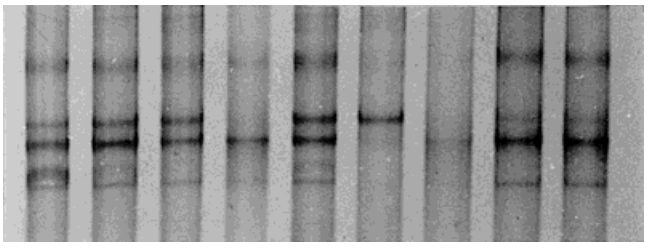

Fig. 2. PCR-SSCP results of exons 9–11 of the *p53* gene. Lane 1, clone 3 in B[a]P-treated group; Lane 2, clone N in 1 $\mu g/cm^2$ dose group; Lane 3, clone F3 in 5 $\mu g/cm^2$ dose group; Lane 4, clone F1 in B[a]P-treated group; Lane 5, clone E in 10 $\mu g/cm^2$ dose group; Lane 6, clone Q in 10 $\mu g/cm^2$ dose group; Lane 7, clone L in 5 $\mu g/cm^2$ dose group; Lane 8, clone A in 20 $\mu g/cm^2$ dose group; Lane 9, nontransformed BALB/c-3T3 cell line.

cell transformation in a dose-dependent manner. The ability of these transformed cells to grow on semisolid media has been well documented in the literature. Transformed cells show anchorage-independent growth before becoming tumorigenic to form progressively growing tumors in nude mice. Our study supports the data from the literature that all the transformed foci tested for their anchorage-independence formed colonies in soft agar. However, it was interesting to note that the cells derived from Type 3 foci had a dose-dependent response increase to form semisolid agar colonies. This indicates that not only do the number of Type 3 foci increase with the crocidolite dose, but also the extent of transformation increases with dose, as indicated by the increasing semisolid agar growth potential. It is possible that the degree of genetic damage in Type 3 act as a function of dose. This study in turn demonstrates that not all Type 3 foci are equal, and that additional events are necessary for the anchorage-independent growth in addition to those involved in morphological cell transformation. Other studies have shown cell transformation induced by asbestos fibers in human epithelial cells [8], C3H/10T1/2 fibroblastic cells [28], and SHE cells [29]. The mechanism by which asbestos induces cell transformation is not yet clear. However, it has been reported that many types of cells (SHE, Chinese hamster embryo cells) are able to endocytose asbestos fibers [30,31], and that the cell transformation in SHE cells induced by asbestos was related to the cytogenetic damage [5]. Although electron microscopy has not been used to study the endocytosis of asbestos fibers in BALB/c-3T3 cell lines, Ault et al. [32] have shown in epithelial cells that thin, long fibers get caught in the keratin cage, protrude into the spindle, and show the ability to snag or block moving chromosomes. We have found that crocidolite induces micronuclei and multinuclei in BALB/c-3T3 cell lines [15], and the dose–response relationship was consistent with the present study. This indicates that the mechanism by which crocidolite induces cell transformation in BALB/c-3T3 cell lines may be related to its cytogenotoxic activities. The effects of asbestos on cellular genetic substances may also involve disturbance in the movement of chromosomes during mitosis by asbestos fibers in the cell [32,33] and an increase in active oxygen species induced by asbestos, which may exert a genotoxic effect on DNA and/or chromosomes [34].

In order to study the possible mechanism of transformation induced by crocidolite at the molecular level, we tested all 11 exons of *p53* gene in this study by PCR-SSCP. The results showed there were mutations in exons 4–6 (2/7) and exons 9–11 (5/7). The hot spots of *p53* gene mutation in human cancer are located in exons 5–8 and about 80% of mutations are observed in this area. Our data, on the contrary, showed most mutations (5/7) in exons 9–11. However, we only tested eight transformed cell lines, and whether *p53* gene mutations in transformed mouse cell line are different from those that are found in humans needs to be investigated further.

The PCR-SSCP method allows us to establish that a mutation exists, but gives no information as to the type of mutation or the exact location of the mutation. Several studies have reported that *p53* gene mutations were G-T transversions in 80% of small-cell lung carcinomas and in 60% of nonsmall-cell lung carcinomas [35]. Mutations of *p53* were G-A transitions in mesothelioma induced by asbestos [36]. Differences in *p53* gene mutation in lung cancer induced by different carcinogens have also been reported. G-T transversions were commonly obtained in lung cancer induced by smoking [37]; there were no G-T transversions of the *p53*

gene in seven uranium miner lung cancer patients [38]. Further studies are needed to determine whether transformed cells induced by asbestos carry specific mutations in the *p53* gene.

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