Asbestos promotes morphological transformation and elevates expression of a gene family invariably induced by tumor promoters in C3H/10T1/2 cells

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Abstract

The murine proliferin gene family, which has been shown to respond consistently to tumor promoters and other cellular pro-oxidant agents in C3H/10T1/2 cells, was used to monitor responses after treatment of these cell cultures with toxic, pro-oxidant asbestos fibres. Proliferin mRNA levels were increased by amosite, crocidolite or chrysotile asbestos fibres, especially in the presence of fresh serum and at low cell densities. Promotion of morphological transformation was confirmed in two-stage focus formation assays using crocidolite at a fibre density that induced proliferin expression. Asbestos-induced gene expression was inhibited by millimolar levels of N-acetylcysteine (NAC), supporting a linkage between: (i) induced oxidant stress that was sufficient to promote morphological transformation; (ii) induction of proliferin expression. Other anti-oxidant compounds (dithiothreitol and pyrrolidine dithiocarbamate) or enzymes (superoxide dismutase and catalase) did not inhibit induced expression. Non-fibrous powders (titanium dioxide, quartz or silica gel) were also effective inducers of proliferin mRNA accumulation. Latex beads and activated charcoal were effective at higher particle densities, implying that ubiquitous particle-induced surface membrane effects can lead to an NAC-reversible step necessary for proliferin induction. The results showed that asbestos resembled all other promoters of morphological transformation in C3H/10T1/2 cells in that an anti-oxidant-sensitive induction of the proliferin gene family occurred following treatment.

Introduction

Permanent alterations in arrangement, structure and expression of growth control or other genes occur in morphologically transformed clones of C3H/10T1/2 cells (1–4), presumably resulting from genotoxic mechanisms arising subsequent to treatment with initiators. The nature of epigenetic processes or further genetic changes at work during the promotional phase of two-stage transformation experiments or how they generate synergistic increases in transformation frequencies subsequent to initiation are little understood. One approach to gaining further insight into these processes is to define molecular markers closely linked to tumor promotion phenomena in specific experimental cell or tissue systems. Markers of potential utility include inducible changes in second messenger molecules, mRNA and protein abundances or modifications to chromatin and DNA which occur consistently after treatment by an assortment of unrelated chemical and physical agents with promotional activity. The murine C3H/10T1/2 cell in vitro model of two-stage carcinogenesis has been open to such analysis because many structurally diverse agents have been tested for promotion of morphological transformation since the methodology was first established (5). Most of the active promoting agents in these cultures have been tested in our laboratory for effects on transcript levels of the gene family encoding proliferin protein (PLF*). A particularly close association between dosages required to induce proliferin gene (plf) expression and dosages required for promotional activity in two-stage assays has been uncovered in C3H/10T1/2 cells while, conversely, non-promoting agents failed to induce any expression of these genes (6, 7). PLF was originally identified as a growth factor-responsive protein secreted into the medium from cultured murine cell lines and has since been shown to be a growth hormone-related angiogenic protein that is active during murine embryogenesis (8, 9) and suppresses in vitro myogenic differentiation when expressed in C3H/10T1/2 cell cultures (10). We have shown that tumor promoter-induced increases in steady-state plf mRNA were likely to have been secondary to increased intracellular pro-oxidant levels in C3H/10T1/2 cells (11).

Several studies have determined that asbestos minerals elicit co-carcinogenic effects in C3H/10T1/2 cultures (12–14). Enhanced in vitro transformation by asbestos in this model aneuploid, immortalized cell line superficially mimics in vivo chrysotile-mediated co-carcinogenesis in rodent lung tissue (15). Asbestos also affects two-stage promotion, as has been demonstrated in rat tracheal explants in vivo (16). Asbestos therefore presents a potential opportunity to further validate the apparent association between plf gene family induction and promotion of C3H/10T1/2 cell transformation, using insoluble, physical carcinogens in place of the soluble chemicals which have consistently given such paired responses.

Elevated intracellular oxyradical levels are a principal factor in asbestos toxicity and carcinogenesis (17). Various molecular transformations coupled to cellular oxidant stresses have been measured subsequent to asbestos exposures in many cells and tissues, including one important target tissue for carcinogenesis, the lung (18). DNA, lipid and protein are all subject to damage by oxygen radicals during exposure to asbestos and stress response genes are also induced. Morphological transformation in C3H/10T1/2 cultures is promoted by active oxygen species (superoxide and hydrogen peroxide) (19), which probably also underlie the appearance of increased oxy-adducts in DNA and lipids found in these cells following asbestos exposure (20). Other experiments have established increases in a ‘second messenger’ molecule, diacylglycerol, in asbestos-treated C3H/10T1/2 cells (21, 22), indicating that particle-induced physiological changes are induced that have potential to alter patterns of gene expression. In agreement with changes in second messenger molecules, some primary response genes (c-fos,

*Abbreviations: MCA, 20-methylcholanthrene; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, foetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide; NAC, N-acetylcysteine; PLF, proliferin protein.
c-jun) are induced by asbestos in cultured cells (23) and increases are known for certain secondary response genes (24–27). The present report describes experiments employing selected anti-oxidants as response modifiers and semi-quantitative assays of transcript levels of the plf gene family. The experiments sought to provide evidence for the suggestion that asbestos resembles the many other chemical promoters known for C3H/10T1/2 cells in that the concentrations of asbestos required to promote morphological transformation in two-stage assays also create a cellular condition of oxidative stress that is sufficient to induce elevated expression of the plf gene family.

Materials and methods

**Chemicals and enzymes**

UIUC samples of crocidolite [Na2(Fe3+2Fe2+)2O5(OH)2], amosite (FeMg5Si3O10(OH)2) and chrysotile [Mg6Si4O10(OH)2] asbestos were obtained from V.Timbrell (MRC Epidemiology Unit, Llandough Hospital, Penarth, UK). Physical characteristics of these samples have been published (28). All fibres (particles with a ≥3:1 ratio of length to diameter) are longer than 0.2 µm in length and 0.5 µm in width, with modal diameters of approximately 1.2, 2 and 2 µm respectively. Fused quartz powder (5 µm average particle size; maximum 45 µm) was from Goodfellow Corp. (Berwyn, PA). Titanium(IV) oxide powder (≤5 µm) was from Aldrich Chemical Co. (Milwaukee, WI) and activated charcoal (75% of particles >10 µm, 25% of particles >3.5 µm) was from J.T.Baker Inc. (Toronto, Ontario, Canada). Latex beads (polystyrene, 1.3-30 µm diameter) and 2.12 µm spherical silicas gel (average particle size 5 µm), 20-methylcholanthrene (MCA) (technical grade, 90%), and 12-0-tetradecanoylphorbol-13-acetate (TPA) (99% by TLC) were purchased from Sigma (St Louis, MO). Superoxide dismutase was obtained from Lipoxygenase from Hencshhoff Mannheim (Laval, Quebec, Canada). Cultase was from ICN Biomedicals (Montreal, Quebec, Canada).

**Cells and cell culture**

The mouse embryo-derived C3H/10T1/2 cell line (ATCC CCL 226) was maintained, in humidified incubators with a 5% CO2 atmosphere at 37°C, in Penroast broth supplemented with 10% fetal bovine serum (FBS; Hazelton Biologics Inc., Lexena, KS), 100 µg/ml streptomycin sulphate, 100 U/ml penicillin G and 4.5 g/l glucose. Cultures were discarded upon reaching passage 30 and new cultures were inoculated from stock cultures frozen in liquid N2 at passage 11–12. No contamination by mycoplasma was found by Hoechst dye staining (29). Gene induction experiments were begun by inoculating 0.5–1.0 X 10^6 cells in 150 × 15 mm dishes (Lux; Nunc, Naperville, IL) containing 25 ml DMEM supplemented with 3% FBS. Particulates were added 24–72 h following inoculation, depending upon experimental design.

**Cytotoxicity and cell transformation assays**

Toxicity in confluent C3H/10T1/2 cell cultures was determined rapidly by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye reduction assays, as described previously (3), following 18 h exposure to various fibres and particles. Fibre concentrations up to the levels that reduced MTT dye reduction by 50% were used for plf induction experiments (see above). Two-stage morphological transformation assays (5, 30–32) at plf-inducing concentrations of asbestos were performed in 60 mm plastic tissue culture dishes (20 per group) containing 4 ml DMEM and supplements as described for maintenance of cell cultures. The experiments were performed with a serum batch permissible for TPA-induced plf expression (one of four tested serum batches was non-permissive for plf induction) and previously tested in vanadium-induced morphological transformation experiments (5). Each dish was inoculated with 500 cells from a culture at passage 9. After incubation for 48 h, several sets of plates received the initiator carcinogen, MCA (from a 3.73 mM solution freshly prepared in acetone), to give final concentrations of 0.093 µM. Carcinogen-containing medium was removed after 24 h and cells rinsed once with DMEM before addition of fresh DMEM supplemented with serum and antibiotics. Asbestos at a concentration of 10 µg/ml (1.92 µg/cm²) was added as promoter to MCA-treated and untreated control cultures following 2–12 days further incubation after the time of MCA removal. Medium (with 1 µg/ml asbestos or without asbestos) was refreshed on all plates twice weekly until cultures grew to confluence and then weekly until termination of the experiment at 7 weeks post-inoculation. Type III transformed foci were enumerated on Giemsa stained cell monolayers according to the suggestions of Landolph (30).

**RNA extraction, Northern transfer and conversion to 32P-labelled cDNA**

Total cellular RNA from control and asbestos-exposed cultures was prepared by a guanidinium thiocyanate/acid phenol extraction method (33). RNA extraction and UV cross-linking to nylon membranes (Zeta Probe; BioRad) after electrophoresis were performed by standard methods described previously (6,7).

**cDNA probes and hybridization**

Murine β2-microglobulin and proliferin recombinant cDNA plasmid clones used to probe cDNA hybridization probes for Northern blot were those employed previously (6,7). A murine calreticulin recombinant cDNA plasmid, pUC3-49-1, was obtained from a differential hybridization screening for growth-responsive genes (34) (R.Hofbauer and J.Xuan, unpublished results). cDNA inserts were released from vector DNA molecules by appropriate restriction enzyme digestions and purified by electrophoresis from agarose gels prior to use for radiolabelling with [32P]dCTP and a commercial T4 DNA polymerase oligolabelling kit (Pharmacia Biotech Inc.). Prehybridization of Northern transfer blots on Zeta Probe membranes and hybridization and stripping of cDNA probes were performed with minor modifications to the manufacturer's instructions. Radiolabelled, denatured cDNA probes (1.3 × 10^7 c.p.m./ml, 1.6 × 10^6 c.p.m./mg) were incubated with Northerm blot membranes in rotating glass bottles for 18 h at 43°C, in a hybridization solution containing 50% formamide, 7% SDS, 0.25 M NaCl, NaHPO4, pH 7.2, 1 mM EDTA and 1 µg/ml single-stranded salmon sperm DNA.

**Autoradiography and densitometry**

Northern blots were washed for 30 min, in sequence, in 2 × SSC, 0.1% SDS at 20°C, 0.2 × SSC, 0.1% SDS at 20°C and finally 0.2 × SSC, 0.1% SDS at 43°C for Northern blots. Washed blots were sealed in plastic stretch wrap and exposed to Kodak XAR-5 film, with intensifying screens at –80°C for 18–72 h. Developed autoradiographic images of 1 kb plf mRNA bands and control β-microglobulin or calreticulin bands were scanned with a Molecular Dynamics 300B computing densitometer. Autoradiographic images with optical densities determined to within the linear range of the film were obtained by varying the exposure time.

**Results**

**Asbestos-mediated toxicity in C3H/10T1/2 cells**

Gene induction and two-stage morphological transformation experiments require that cells continue to be viable and metabolically active for extended periods while exposed to the test agent. Accordingly, non-toxic exposure conditions for crocidolite and amosite asbestos fibres were determined by metabolic dye reduction assays conducted with confluent C3H/10T1/2 cell monolayers to which various concentrations (µg/cm²) of UIUC amosite or crocidolite had been added for 18 h. These conditions closely represent cells at densities that encountered asbestos during the promotional phase of two-stage assays or received asbestos during treatment prior to a gene induction assay. The MTT assay avoids clonal densities employed during estimations of toxicity by colony formation assays, as originally prescribed by Resnikov et al. (32) for C3H/10T1/2 cells, which, like BALB/c 3T3 cells, may display density-dependent cytotoxicity (35). Under the particular conditions of culture employed asbestos fibres did not remain in suspension in the medium, but settled upon the surface of the cell monolayer. Cellular metabolic capacity (MTT dye reduction) was reduced to ~50% of control values at 2 and 8 µg/cm² crocidolite and amosite asbestos respectively within 18 h (Figure 1). Viability determined in this way was in agreement with the ~50% reduction in colony forming ability previously determined at the same fibre density (14); however, side-by-side comparisons of the two toxicity assays were not attempted. No distinct threshold for MTT dye reduction was apparent between 0.01 and 10 µg/cm² amosite or crocidolite, nor were losses of monolayer cell numbers noted within this range (data not shown). A range of asbestos fibre concentrations between those producing no measurable toxicity and those causing metabolic capacity to drop by >50% were therefore...
plf mRNA levels compared with untreated controls, but in detectable minimum) above control cultures receiving 5% FBS were observed at fibre densities 5*0.43 (ig/cm²) amosite. BADO causes no change in serum (5% v/v) or 2.8 (J.g/cm²) an ASBESTOS) (ng/cm²) amosite. BAL causes no change in serum (5% v/v) or 2.8 (J.g/cm²) a plf mRNA in response to added asbestos minerals, in consideration of known effects of cell density, serum concentration and redox status of the medium. A proliferin and other murine cell lines (11,36). An effective protocol that on /j/fmRNA accumulation in the cytoplasm of C3H/10T1/2 and the Northern transfer process (Figure 3A).

Chloramphenicol or calreticulin transcripts were used to account for plf mRNA levels if either p-micro- 

Asbestos-induced elevation of transcript levels persisted for at least 72 h after asbestos fibres were added to the culture media (Figure 3A, inset). Hybridization ratios were also calculated for calreticulin and p-microglobulin transcripts in this experiment as an independent check on relative increases in plf mRNA. Calreticulin transcripts fell moderately (3-fold) relative to control cultures following exposure to TPA (Figure 3B and data not shown) and were also reduced by similar magnitude, in most cultures, following exposure to higher densities of asbestos fibres (Figure 3B). Comparable results were obtained for estimates of relative plf mRNA levels if either p-microglobulin or calreticulin transcripts were used to account for lane-to-lane loading variations created during electrophoresis and the Northern transfer process (Figure 3A).

Two additional forms of asbestos minerals, crocidolite and chrysotile, were tested for inductive effects on plf mRNA accumulation relative to non-responsive p-microglobulin transcripts. Combined treatment of cells with 5% FBS and either fibre type prompted synergistic increases in plf mRNA to levels above those resulting from individual treatments with either 5% FBS or asbestos (Figure 4A and B). Maximal
mRNA levels (data not shown).

...from the dishes did not produce any discernible increase in... 

...replating for 24 h in the conditioned medium first removed... 

...solution of 0.25% trypsin and 0.1% EDTA, then washing in... 

...monolayers obtained by incubating cell monolayers in a... 

...levels of expression observed in cultures achieving 80% of... 

...mRNA in Swiss 3T3 cells (36). Increasing cell densities... 

...plf mRNA levels, as had previously been noted for the lower... 

...and asbestos addition to cultures that had grown to 12 and... 

...and l.5l X 10^5 cells/dish (150 mm), which... 

...counted at the time of harvest, giving values of 3.03 X 10^6... 

...were anticipated to have suppressive potential for plf expression... 

...to combined serum and asbestos treatments. To test this possibility, several cultures were inoculated with... 

...in C3H/10T1/2 cultures as growth continued to confluence... 

...in C3FI/10T1/2 cell transformation had shown that anti-oxidants... 

...in the presence of 2.8 μg/cm^2 added prior to the extra 5% FBS. 

...Low culture density greatly elevated serum inducibility of plf mRNA in Swiss 3T3 cells (36). Increasing cell densities in C3H/10T1/2 cultures as growth continued to confluence were anticipated to have suppressive potential for plf expression in response to combined serum and asbestos treatments. To test this possibility, several cultures were inoculated with decreasing numbers of C3H/10T1/2 cells, incubated for 48 h, then exposed to asbestos (for 48 h prior to harvest) and 5% FBS (for the 24 h prior to harvest), or both treatments were conducted separately. Cells in parallel untreated cultures were counted at the time of harvest, giving values of 3.03 X 10^6, 4.25 X 10^5 and 1.51 X 10^6 cells/dish (150 mm), which represented 12, 17 and 60% of cells found in confluent dishes (2.5 X 10^6 cells/dish). Upon treatment with crocidolite or combined 5% serum and crocidolite, plf mRNA attained greater levels as culture density decreased (Figure 5). Combined serum and asbestos addition to cultures that had grown to 12 and 17% confluence did not provide a synergistic increase in plf mRNA levels, as had previously been noted for the lower levels of expression observed in cultures achieving 80% of confluence or more (see above). Temporary disruption of cell monolayers obtained by incubating cell monolayers in a solution of 0.25% trypsin and 0.1% EDTA, then washing in phosphate-buffered saline as single cell suspensions, followed by replating for 24 h in the conditioned medium first removed from the dishes did not produce any discernible increase in plf mRNA levels (data not shown).

Effects of anti-oxidants upon asbestos-induced plf mRNA expression

Prior experiments with chemical agents that are promoters of C3H/10T1/2 cell transformation had shown that anti-oxidants in the medium can antagonize or in some circumstances enhance the inductive effect that particular tumor promoters have on plf mRNA levels (7,11). In those experiments, the sulphhydryl compound N-acetylcycteine (NAC) was shown to be effective in reducing to control levels plf mRNA induced in response to organotin compounds or to vanadate compounds. Table I shows that NAC had the same effect on crocidolite and chrysotile asbestos-induced expression. Complete repression of crocidolite-induced expression was achieved at 4 mM NAC, while 8 mM NAC reduced chrysotile-induced expression by nearly 80%. Two other sulphhydryl compounds, diithiothreitol (0.5–50 mM) and pyrrolidine dithiocarbamate (50–300 μM) had no discernible effects on induced plf mRNA levels (not shown). In contrast to these agents, repeated additions (every 12 h for 48 h) of superoxide dismutase and catalase (ranging from 0.1 to 40 U/ml) resulted in up to 5-fold greater plf mRNA accumulation than asbestos alone could induce (Table I).

Promotion of morphological transformation by crocidolite

Work by others has shown that asbestos is co-carcinogenic when administered simultaneously with polycyclic compounds or γ radiation in C3H/10T1/2 cell cultures (12,14). Considering that promotion of transformation in C3H/10T1/2 two-stage transformation assays and induction of plf mRNA levels have been consistently associated as outcomes which follow exposure to diverse chemical agents, it was predicted that the observed capacity of asbestos to induce plf mRNA would be a quality coinciding with a promotional activity in transformation assays. Experiments were therefore conducted to determine if morphological transformation rates could be increased by crocidolite asbestos at a plf-inducing concentration when applied to cultures in a two-stage transformation protocol, with MCA as the initiator. Incubation periods between removal...
Table I. Effects of NAC or superoxide dismutase (SOD) and catalase (CAT) on asbestos-induced plf mRNA accumulation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pI-focusing agents</th>
<th>NAC (mM)</th>
<th>SOD and CAT (U/ml)</th>
<th>pI mRNA (% of TPA-induced level)</th>
<th>SE</th>
<th>n</th>
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<tr>
<td>Experiment 1</td>
<td>None</td>
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<td>2.9</td>
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<td></td>
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<td>37.7</td>
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<tr>
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<td>0.0</td>
<td>3</td>
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<tr>
<td></td>
<td>TPA</td>
<td>0</td>
<td>100</td>
<td>11.0</td>
<td>6</td>
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<tr>
<td>Experiment 2</td>
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<td>2.9</td>
<td>0.6</td>
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<td></td>
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<td>13.9</td>
<td>3</td>
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<tr>
<td></td>
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<td></td>
<td>Chrysotile</td>
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<tr>
<td>Experiment 3</td>
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<td>75.2</td>
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</table>

*Asbestos fibres were added to replicate cultures (n) to 2.12 μg/cm² final concentration; TPA was added to 0.5 μM (0.32 μg/ml).

**NAC or SOD and CAT were added to cultures immediately prior to asbestos and remained in the medium until cells were harvested; anti-oxidant enzymes were renewed twice daily.

Cells were harvested and total RNA was prepared 44 h after asbestos additions or 24 h after TPA additions.

Table II. Two-stage morphological transformation in C3H/10T1/2 cultures exposed to 0.093 μM MCA (initiator) and 1.92 μg/cm² crocidolite (promoter)

<table>
<thead>
<tr>
<th>Stage</th>
<th>MCA or acetone (48 h)</th>
<th>Days between initiation and promotion</th>
<th>Stage II asbestos (6 weeks)</th>
<th>No. of Type II or III foci/no. dishes (mean no. foci/dish ± SEM)</th>
<th>No. of Type III foci/no. dishes (mean no. foci/dish ± SEM)</th>
<th>Fraction of dishes with Type II or III foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9.3 X 10^-8 M</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>II</td>
<td>Acetone</td>
<td>NA</td>
<td>-</td>
<td>2/23 (0.087 ± 0.06)</td>
<td>0/23</td>
<td>2/23</td>
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<tr>
<td>II</td>
<td>MCA</td>
<td>7</td>
<td>+</td>
<td>9/24 (0.38 ± 0.61)</td>
<td>4/24 (0.17 ± 0.078)</td>
<td>9/24</td>
</tr>
<tr>
<td>II</td>
<td>Acetone</td>
<td>7</td>
<td>+</td>
<td>3/24 (0.13 ± 0.069)</td>
<td>0/24</td>
<td>3/24</td>
</tr>
<tr>
<td>II</td>
<td>MCA</td>
<td>2</td>
<td>+</td>
<td>36/24 (1.5 ± 0.22)</td>
<td>18/24 (0.75 ± 0.17)</td>
<td>19/24*</td>
</tr>
<tr>
<td>II</td>
<td>MCA</td>
<td>7</td>
<td>+</td>
<td>17/24 (0.71 ± 0.18)</td>
<td>3/24 (0.13 ± 0.069)</td>
<td>12/24</td>
</tr>
<tr>
<td>II</td>
<td>MCA</td>
<td>12</td>
<td>+</td>
<td>9/24 (0.38 ± 0.12)</td>
<td>2/24 (0.083 ± 0.058)</td>
<td>8/24</td>
</tr>
</tbody>
</table>

**P < 0.01, **P < 0.001, t-test, single tail; spontaneous foci subtracted from the data; comparison between additive numbers of foci resulting from individual treatments and actual obtained foci after combined treatments.

Influence of diverse particulate materials on plf mRNA levels

The similar concentration ranges of plf gene induction by the three asbestos minerals pointed to the possibility that the response followed as a consequence of physical perturbation at the cell surface membrane and not as a result of surface chemical properties of the particles contacting the membrane. Various organic and inorganic powders were therefore examined for their effects on plf mRNA levels in low density cultures. All powders tested, including activated charcoal, polystyrene beads, titanium dioxide, amorphous silica gel and fused quartz, were found to increase plf mRNA levels (Figure 6). Table III compares (on the base of weight/culture area and estimates of particles/cell) then lowest concentrations at which these powders induced 3-fold greater levels than found in respective control cultures. Latex beads were the least potent particles in that a density of 11 μg/cm² was required to induce plf mRNA levels 3-fold above background, which was a particle density >150 times that of chrysotile and >16-50 times the other inorganic dusts. Carbon particles appeared to have a potency intermediate between latex beads and > 16-50 times the other inorganic dusts. Carbon particles...
iodoacetic acid, butylated hydroxytoluene, Tween 60, tri-n-
from cultures previously initiated with mutagenic agents. This
frequency of morphologically transformed foci which arise
concentrations that, after prolonged exposure, increase the
between the concentrations of chemical that induce plf/mRNA
levels to rise above those in untreated cultures and the
Discussion
required 21 particles/cell.
followed by silica gel at 1.3 particles/cell, while latex beads
mRNA increases at a density calculated to be 0.7 fibres/cell,
ile was again the most potent material, with detectable
densities inducing 3-fold
mRNA accumulation was less
and the inorganic dusts. The spread of values for particle
densities inducing 3-fold plf mRNA accumulation was less
when compared on a particles/cell basis. On this basis, chrysotile
was again the most potent material, with detectable plf
mRNA increases at a density calculated to be 0.7 fibres/cell,
followed by silica gel at 1.3 particles/cell, while latex beads
required 21 particles/cell.

Table III. Summary of plf mRNA induction by diverse powdered materials

<table>
<thead>
<tr>
<th>Powder type</th>
<th>Powder fineness (particles/μg)</th>
<th>Particle density inductive for plf mRNA&lt;sup&gt;a&lt;/sup&gt; (μg/cm²)</th>
<th>Particle density inductive for plf mRNA&lt;sup&gt;b&lt;/sup&gt; (particles/cell)</th>
</tr>
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<tbody>
<tr>
<td>Charcoal</td>
<td>3.0 x 10⁴</td>
<td>2.00</td>
<td>3.5</td>
</tr>
<tr>
<td>Latex beads</td>
<td>3.3 x 10⁴</td>
<td>11.00</td>
<td>21.0</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>6.3 x 10⁴</td>
<td>0.65</td>
<td>2.4</td>
</tr>
<tr>
<td>Silica gel</td>
<td>3.3 x 10⁴</td>
<td>0.70</td>
<td>1.3</td>
</tr>
<tr>
<td>Quartz</td>
<td>7.2 x 10⁴</td>
<td>0.20</td>
<td>8.3</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>6.3 x 10⁴</td>
<td>0.65</td>
<td>8.0&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asbestos</td>
<td>4.9 x 10⁴</td>
<td>0.45</td>
<td>6.5&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chrysotile</td>
<td>3.5 x 10⁴</td>
<td>0.07</td>
<td>0.7&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fold above control levels, determined by manual interpolation from graphical data in Figures 3, 4 and 6.
<sup>b</sup>Counted in buffered saline suspension by phase contrast microscopy, in a Neubauer-type haemocytometer.
<sup>c</sup>Based upon a confluent density of 17 000 C3H/10T1/2 cells/cm².
<sup>d</sup>Fibre numbers adjusted to include the proportion of fibres <2 μm in length that are not resolved by light microscopy (28).

and the inorganic dusts. The spread of values for particle
densities inducing 3-fold plf mRNA accumulation was less
when compared on a particles/cell basis. On this basis, chrysotile
was again the most potent material, with detectable plf
mRNA increases at a density calculated to be 0.7 fibres/cell,
followed by silica gel at 1.3 particles/cell, while latex beads
required 21 particles/cell.

Discussion
The work presented here adds weight to a consistent correlation
between the concentrations of chemical that induce plf mRNA
levels to rise above those in untreated cultures and the
concentrations that, after prolonged exposure, increase the
frequency of morphologically transformed foci which arise
from cultures previously initiated with mutagenic agents. This
correlation has been demonstrated for TPA, mezerein, anthatrin,
idoacetic acid, butylated hydroxytoluene, Tween 60, tri-n-
butyltin, ammonium metavanadate and activated oxygen
species produced by xanthine oxidase (6,7), and also for
saccharin, formaldehyde and diethylstilbestrol (C.Parfett and
R.Pilon, in preparation). Earlier studies by others have found
asbestos minerals to be co-carcinogenic in C3H/10T1/2 cells
(12-14), but our work revealed that true promotional activity
also occurs, since asbestos addition to initiated cultures could
be delayed until several days after initiator-containing medium
and continued to produce synergistic increases in foci numbers.
Enhanced transformation frequency by facilitated entry of
MCA into cells by adsorption onto asbestos fibres (12,37) is
an unlikely mechanism under these circumstances. Crocidolite
at 0.017 or 0.18 μg/cm² was ineffective in promoting C3H/
10T1/2 cells with γ irradiation as initiator (13), but our results
(see Figure 3A) indicated that these asbestos concentrations
would not have induced plf mRNA levels, in contrast to
1.4 μg/cm², which we showed to both induce plf expression
and promote transformation. When crocidolite addition was
delayed until cultures had achieved confluence, 12 days after
removal of the initiating agent (MCA), no increases in trans-
fomed foci were observed, which was consistent with the
large inhibitory effect on asbestos-induced plf mRNA levels
exhibited by increased cell density measured under conditions
of serum co-treatment, which are more likely to be reflective
of conditions encountered during the promotional phase of a
two-stage transformation assay. Thus, for each experimental
variable examined (i.e. asbestos concentration, culture density),
prior monitoring of plf mRNA for accumulation above levels
found in untreated cultures was predictive of treatments that
would facilitate promotional effects in the two-stage trans-
formation assay. The experiments were not intended to assess
whether the magnitudes of the effects on plf mRNA accumula-
tion and on promotion of foci were closely correlated.

There was no evidence to implicate asbestos iron content
as a factor in induction of plf mRNA accumulation. Minimal
induction concentrations were estimated to range from
0.43 μg/cm² for chrysotile to 1.4 μg/cm² for amosite, but iron
content varies from 3% in chrysotile to 37% in crocidolite and
amosite. It remains possible, however, that 0.1 μg/ml Fe(NO₃)₃
in DMEM medium could adsorb to and mask relative differ-
ences in iron composition of the fibre surfaces. DNA strand
breakage in C3H/10T1/2 cells occurring at high asbestos
densities (25-200 μg/cm²) is dependent upon available iron
which could be blocked by chelation (20).

C3H/10T1/2 cells were found to be sensitive to an assortment
of particle morphologies and compositions. In contrast, rat and
human primary pleural mesothelial cell cultures are more
dependent on particle geometry for induction of responding
genes (23,25). Although some particulates are known to
transform C3H/10T1/2 cells directly (38,39), correlations
between gene induction and promotion of transformation in
C3H/10T1/2 cells were not tested using the various particle
types, other than with a single concentration of crocidolite.
Responses to inert particles revealed in this investigation
should provide further avenues to extend this correlation.

Inhibition of asbestos-induced plf mRNA accumulation was
achieved in the range 4–8 mM NAC in the culture medium,
which closely matched NAC levels required to inhibit plf
induction by two other soluble compounds shown previously
to promote C3H/10T1/2 cell morphological transformation:
tri-n-butyltin and ammonium metavanadate (7,11). It is interest-
ing, in the light of the fact that members of the proliferin
gene family contain functional AP-1 transcription factor bind-
lating sites (40–42), that the same NAC concentrations were also effective in reducing c-fos and c-jun proto-oncogene induction to control levels in cultures of rat pleural mesothelial cells, which are the target cell population for asbestos-induced mesothelioma in vivo (43). Although added NAC was likely to have increased glutathione levels in C3H/10T1/2 cells, two additional observations indicated that there probably exists a complex interplay among: (i) the balance of oxidized and reduced sulphydryl species; (ii) the balance of active oxygen species; (iii) the accumulation of specific mRNA molecules. Lack of any suppressive effects on plf induction by high levels of two other sulphydryl compounds (dithiothreitol and pyrrolidine dithiocarbamate) might indicate that NAC plays some specific role with regard to sulphydryl groups within the cell, while enhanced plf mRNA accumulation generated by a combination of exogenous anti-oxidant enzymes, superoxide dismutase and catalase, suggests that active oxygen species in accessible cellular compartments are an inhibitory factor. Possible mechanisms for opposing effects of anti-oxidants on promoter-induced gene expression were discussed previously (11).

Gene induction assays can provide useful adjunct information on variables associated with cell culture and carcinogen exposure conditions, prior to confirming suspected promotional ability in two-stage morphological transformation experiments. In work reported elsewhere, we have shown that plf expression is one of several genes with expression consistently elevated following exposure of C3H/10T1/2 cultures to pro-oxidant tumor promoters such as asbestos. Relative temporal changes in gene expression can be detected by various methodologies and, due to close parallels with or possible functional roles in gene expression can be detected by various methodologies and, due to close parallels with or possible functional roles in gene expression, can be detected by various methodologies and, due to close parallels with or possible functional roles in gene expression can be detected by various methodologies

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References


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