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Induction of morphological transformation by coal-dust extract in BALB/3T3 A31-1-13 cell line

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Summary

The transforming activity of coal dust extracts was studied using BALB/3T3 clone A31-1-13 cells. Coal-dust extracts, both nitrosated and nonnitrosated, induced cell transformation in a dose–response manner. However, the transformation frequency was higher with the nitrosated than with the nonnitrosated extract. All transformed cell lines derived from coal-dust extract-induced foci showed biological characteristics of neoplastic transformation such as loss of contact inhibition and anchorage-independent growth. These results appear to support a hypothesis of coal mine dust causation of gastric cancer in coal miners.

Several epidemiological studies have shown that there is an elevated mortality of gastric cancer among coal miners (Stocks, 1962; Enterline, 1964; Matolo et al., 1972; Rockette, 1977; Falk and Jurgelski, 1979). The actual etiology of gastric carcinogenesis is not known. However, occupational exposure has been postulated as a possible risk factor for increased stomach cancer in coal miners (Ames, 1982). A close link between coal-dust exposure and gastric cancer has been found by Jacobsen (1976) who reported a positive relationship between gastric cancer mortality and pneumoconiosis progression which is largely a function of coal-mine-dust exposure.

Recently a hypothesis has been proposed by Ong et al. (1983) to explain the elevated incidence of gastric cancer in coal miners. According to their hypothesis, inhaled coal-mine-dust is cleared from the lung and tracheobroncheal tree by mucociliary functions, swallowed, and introduced into the stomach. Organic and/or inorganic materials associated with the dust undergo intra-nitrosation or interaction with exogenous chemicals (from smokeless tobacco or diet) to form carcinogenic compounds which in turn cause precancerous lesions and subsequently develop into gastric cancer.

Whong et al. (1983) have found that an organic solvent extract of coal dust was either non-muta-

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genic or weakly mutagenic with the Ames/Salmonella assay. However, high mutagenic activities were found when the extracts were reacted with nitrite under acidic conditions. The nitrosated coal-dust extracts induced gene mutations in bacteria and mammalian cells, and sister-chromatid exchanges, micronuclei formation, and chromosome aberrations in mammalian cells (Tucker et al., 1984; Tucker and Ong, 1985; Ong et al., 1985; Krishna et al., 1987).

Although shown to be genotoxic in several short-term assays, coal-dust extracts have not yet been shown to be carcinogenic. Since cell transformation assays are generally considered to be more relevant than other short-term assays to in vivo carcinogenesis (Heidelberger et al., 1983; Kuroki and Sasaki, 1985; Sivak and Tu, 1985), it is important to determine whether coal-dust extracts can induce transformation of mammalian cells in vitro.

In the present study the transforming activities of nitrosated and nonnitrosated coal-dust extracts in BALB/3T3 clone A31-1-13 cells were investigated.

Materials and methods

Chemicals and media

Minimum essential medium, fetal bovine serum, penicillin–streptomycin and trypsin were purchased from Gibco, Grand Island, NY. L-Glutamine, gentamicin sulfate, essential vitamin mixture, and Hepes buffer solution were obtained from Whittaker M.A. Bioproducts Inc., Walkersville, MD. Benzo[*a*]pyrene (BaP) and streptomycin were from Sigma Chemical Co., St. Louis, MO. Dimethyl sulfoxide (DMSO) and sodium nitrite were purchased from Mallinckrodt, Paris, KY. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from Aldrich, Milwaukee, WI. BaP was dissolved in DMSO at a concentration of 1 mg/ml and was diluted with growth medium to the desired concentrations immediately before use.

Sample extraction and nitrosation

The procedure of extraction and nitrosation was previously described (Whong et al., 1983). Briefly, 100 g of coal dust (subbituminous from New Mexico) were extracted first with 250 ml of dichloromethane (DCM) for 24 h at room temperature with shaking (250 r.p.m.) in a rotary shaker and then were extracted with 250 ml of 1 : 1 mixture of methanol plus acetone (M + A) under the same conditions. Each extract was concentrated to 0.5 ml with a rotary evaporator. Then, 20 ml of DMSO were added to the extract, which was further concentrated to about 20 ml under a nitrogen stream.

Prior to nitrosation, equal amounts of DCM extract and M + C extract of the coal dust were mixed. An equal volume of sodium nitrite (30 mg/ml in 50% DMSO) was added to the mixed extract. The mixture of coal-dust extract and nitrite was adjusted to pH 3.0 with 12 N HCl. The coal-dust extract and the sodium nitrite alone served as controls and were also adjusted to pH 3.0 under the same conditions. All the pH-adjusted solutions were incubated at 37 °C for 3 h with shaking on a rotary shaker. At the completion of incubation, the solutions were kept at −20 °C until used.

Cell culture

The stock of BALB/3T3 cells was maintained in liquid nitrogen. Cell cultures were initiated from a stock by rapid thawing in a 40 °C water bath. Cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% v/v essential vitamin mixture, 100 U of penicillin/ml, 100 μg of streptomycin/ml and 5 μg/ml gentamicin at 37 °C in a humidified incubator containing 5% CO_2 in air. Cultures initiated from cryopreserved cells were serially subcultured and used over a range between 10 and 12 passages (Sheu et al., 1987). Cells were subcultured twice a week. The medium for the transformation assay was identical to the culture medium except that it had a lower concentration of FBS (7.5%) (Sheu et al., 1987; Bertram, 1977) and was without penicillin (Bertram, 1979).

Cytotoxicity assay

The toxic effect of extracts was determined using the relative cloning efficiency (RCE) assay. 200 cells were seeded in a 60-mm Corning dish containing 3 ml medium. 5 dishes were used for each treatment concentration and for the solvent

control. 24 h after seeding, the cultures were exposed to pre-selected concentrations of coal-dust extracts or solvent for 72 h. Then the medium containing the extract was removed and the cultures were washed with PBS and refed with fresh medium. After 6 days incubation, cultures were fixed and stained with 10% Giemsa solution. The colonies were counted and the RCE was determined.

Transformation assay

The transformation assay and the scoring of transformed foci were carried out as described in the literature (IARC/NCI/EPA Working Group, 1985; Kakunaga and Yamasaki, 1985; Matthews, 1986). Exponentially growing 3T3 cells were seeded for each treatment at 2×10^4 cells/25-cm^2 flask in 20 replicates each containing 5 ml medium. Cultures were incubated at the same conditions as for the normal cell culture for 48 h. Cells were then exposed to test extracts, BaP (positive control) or DMSO (solvent control) for 72 h. At the end of treatment, the medium was removed and the cells were rinsed once with PBS and refed with fresh medium. Cells were cultivated for 4 weeks with a medium change twice a week. Type III foci were scored after fixation and staining.

Soft-agar cloning assay

Soft-agar suspension medium was prepared as described by Freshney (1983). 6 ml of F12 medium containing 0.33% Difco Noble agar, 20.8% FBS and 111–1000 cells were added to each 60-mm dish. The cultures were incubated for 3 weeks at 37°C. Colony counting was done under a dissecting microscope.

Results

The results of the survival assay of BALB/3T3 cells treated with various concentrations of coal-dust extracts are shown in Fig. 1. Cloning efficiency is about 50% at the concentration of 2.08 mg coaldust/ml. A similar dose-dependent reduction in cloning efficiency by both nitrosated and nonnitrosated extracts was found at the concentrations tested (from 0.26 to 8.3 mg/ml).

Table 1 shows the results of the transformation experiments. The concentrations of coal-dust ex-

TABLE 1

TRANSFORMATION RESPONSE OF BALB/3T3 CELLS TREATED WITH NITROSATED AND NONNITROSATED COAL-DUST EXTRACTS

Treatment	Concentration (mg [a]/ml)	Relative cloning efficiency (%)	Transformation frequency [d]	Foci/flask (mean ± SD) [e]
Coal-dust extract, nonnitrosated	0.00	100.0	5/ 5	0.19 ± 0.13
	2.08	65.0	39/15	1.58 ± 0.28 [f]
	4.16	9.2	53/20	2.28 ± 0.21 [f]
	8.32	0.0	63/18	2.65 ± 0.26 [f]
Coal-dust extract, nitrosated	0.00	100.0	5/ 5	0.19 ± 0.13
	1.04	76.9	23/ 9	0.72 ± 0.29 [g]
	2.08	51.5	59/17	2.28 ± 0.29 [f]
	4.16	13.4	85/19	3.54 ± 0.26 [f]
	8.32	0.0	128/20	6.03 ± 0.15 [f]
Dimethyl sulfoxide	5.00 [b,c]	ND	7/ 7	0.28 ± 0.15
Sodium nitrite	0.05 [c]	92.8	3/ 3	0.11 ± 0.11
BaP	0.20 [b]	ND	180/20	8.33 ± 0.19

[a] Original amount of the coal dust.
[b] μl(μg)/ml.
[c] The concentrations of $NaNO_2$ and DMSO were equal to those used in the highest concentration of coal-dust extract.
[d] Type III foci/flasks with type III foci. 20 flasks were used in each treatment.
[e] Calculated according to the method of Matthew (1986).
[f] $P < 0.001$.
[g] $P < 0.05$.

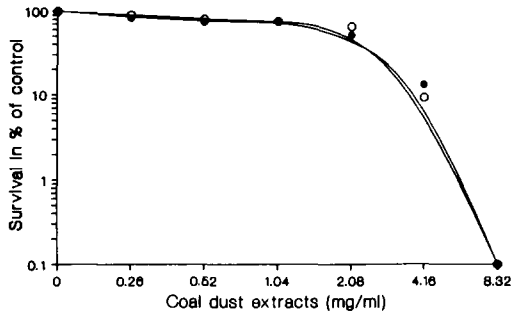

Fig. 1. Effect of coal-dust extracts on relative cloning efficiency of BALB/3T3 after 72 h treatment. Each point represents an average of three 60-mm plates. ●, coal-dust extract nitrosated, ○, coal-dust extract nonnitrosated.

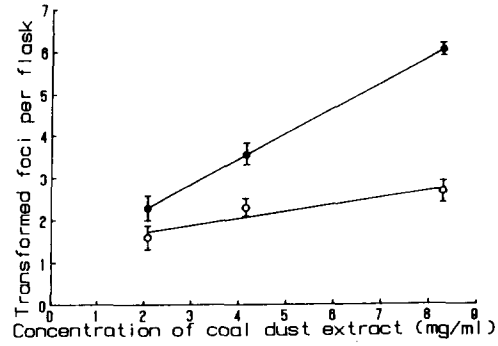

Fig. 2. Induction of cell transformation by different concentration of coal-dust extract. Cells were treated with nitrosated (●) or nonnitrosated (○) coal-dust extract for 72 h. Each point represents an average of 20 flasks.

tracts and sodium nitrite used for the experiments were based on the relative cloning efficiencies. BaP was used as a positive control and 0.5% v/v DMSO was used as a solvent control. Morphological transformation was induced both by nitrosated and nonnitrosated coal-dust extracts. The number of transformed foci in the coal-dust extract-treated cultures were higher than background levels ($P < 0.05$). A dose-related response was observed (Fig. 2). The response was about 1.5–2.3-fold higher with nitrosated than with nonnitrosated coal-dust extract.

For determining characteristics of transformation, cells from transformed foci were isolated and suspended in 5 ml of growth medium and seeded in T25 flasks. The cultures from the isolated foci were subcultured twice a week. The anchorage-independent growth and other biological properties of these cells were characterized at the 9th, 19th and 27th passages. The growth rate of the coal-dust extract-transformed cells was higher than that of normal BALB/3T3 cells. The maximum growth density of transformed cells (13.94×10^4 cells/cm^2) was 2.7 times higher than that of nontransformed cells (5.17×10^4 cells/cm^2).

Anchorage-independent growth of the cells derived from transformed foci induced by nitrosated coal-dust extract was measured by their colony-forming efficiency (CFE) in soft agar medium.

TABLE 2

THE GROWTH OF TRANSFORMED CELLS IN SOFT AGAR MEDIUM

Cell	Seeding density					
	1000		333		111	
	Number of colony	CFE	Number of colony	CFE	Number of colony	CFE
BALB/3T3-P_{5a}	4.25 ± 1.26	0.435	2.00 ± 0.82	0.60	0.25 ± 0.5	0.23
-P_{5b}	ND	ND	2.00 ± 0.00	0.60	0.00	0.00
CDN/3T3-P_9	87.00 ± 12.08	8.70	29.25 ± 11.35	8.78	9.75 ± 2.62	8.78
-P_{19}	ND	ND	37.5 ± 6.36	11.26	12.5 ± 2.12	11.26
BaP/3T3-P_9	96.75 ± 11.47	9.68	48.50 ± 6.76	14.56	21.00 ± 3.92	18.92
-P_{19}	ND	ND	56.50 ± 7.52	16.97	22.00 ± 4.24	19.82

CFE, colony-forming efficiency in agar = number of colony/number of cells plated × 100%.
P, passage, the number represents passage number.
BALB/3T3-P_{5a}, BALB/3T3 cells, passage 5, Expt. a.
CDN/3T3-P_9, coal-dust extract-transformed BALB/3T3 cells, passage 9.
BaP/3T3-P_9, BaP-transformed BALB/3T3 cells, passage 9.
ND, not determined.

When cells from transformed foci were plated in soft agar medium, colonies developed. There was no effect of initial seeding density on CFE in soft agar medium. The average CFE of the transformed cells in soft agar medium ranged from 8.7% to 11.2%, which was significantly higher than the 0.23 to 0.6% of nontransformed cells (Table 2).

Discussion

The present study showed that coal-dust extracts with or without nitrosation are capable of inducing morphological transformation in BALB/3T3 cells with a dose–response manner. The transformation frequency was much higher (1.5–2.3 times) with the nitrosated than the nonnitrosated coal-dust extracts. The differences are statistically significant ($p < 0.05$). These results are in agreement with the findings of Whong et al. (1983), indicating that the nitrosated extract is more active than the nonnitrosated extract. All transformed cell lines derived from coal-dust extract-induced foci showed characteristics of neoplastic transformation such as random orientation, piling up of cells, a high saturation density and the ability to grow in soft agar medium.

The transforming activity of coal-dust extracts did not require metabolic activation. Similar findings were reported for their mutagenic and cytogenetic effects. In the absence of metabolic activation, nitrosated coal-dust extracts induced gene mutation (Whong et al., 1983; Tucker et al., 1984), sister-chromatid exchanges, and chromosomal aberrations (Tucker et al., 1984; Tucker and Ong, 1985). It has been suggested that nitrosation products from nitrite-treated coal-dust extracts are direct-acting nitroso compounds (Whong et al., 1983). The formation of direct-acting mutagens from refined coal distillates treated with nitrite has also been reported (Pelroy and Stewart, 1981). Therefore, it is likely that the chemicals responsible for the transforming activity products in coal-dust extracts may also be direct-acting. However, since the BALB/3T3 cells are capable of activating precarcinogens to ultimate carcinogens (Cortesi and Dolara, 1983; Cortesi et al., 1983), indirect-acting substances which are responsible for the transformation activity of coal-dust extracts cannot be ruled out.

Cells derived from transformed foci induced by coal-dust extracts showed certain characteristics of a transformed phenotype such as an increase in both saturation density and the ability to grow in soft agar medium. The saturation density of BALB/3T3 cells is closely related to their tumor-producing potential. It has been shown that transformed clones with a high density and a high cloning efficiency in soft agar medium produced tumors in nude mice (Aaronson and Todaro, 1968). Anchorage-independent growth of cells in soft agar medium is often used as a marker of cell transformation (MacPherson and Montagnier, 1964; Styles, 1977; Barrett and Ts'O, 1978). The ability of anchorage-independent growth also appears to correlate with tumorigenicity in vivo (Freedman and Shin, 1974; Shin et al., 1975; Barrett et al., 1979; Rundell et al., 1983). Therefore, our results seem to support the coal-mine-dust hypothesis proposed by Ong et al. (1983) for the causation of gastric cancer in coal miners.

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References

Aaronson, S.A., and G.J. Todaro (1968) Basis for the acquisition of malignant potential by mouse cells cultivated in vitro, Science, 162, 1024–1026.

Ames, R.G. (1982) Gastric cancer among coal miners: some hypothesis for investigation, J. Soc. Occup. Med., 32, 73–81.

Barrett, J.C., and P.O.P. Ts'O (1978) Evidence for the progressive nature of neoplastic transformation, Proc. Natl. Acad. Sci. (U.S.A.), 15, 3761–3765.

Barrett, J.C., B.D. Crawford, L.O. Mixter, L.M. Schechtman, P.O.P. Ts'O and R. Pollack (1979) Correlation of in vitro growth properties and tumorigenicity of Syrian hamster cell lines, Cancer Res., 39, 1504–1510.

Bertram, J.S. (1977) Effect of serum concentration on the expression of carcinogen-induced transformation in the C3H10T 1/2 CL8 cell line, Cancer Res., 37, 514–523.

Bertram, J.S. (1979) Reduction in the formation of carcinogen-induced transformed foci by penicillin G sodium in the C3H10T1/2 CL8 line, Cancer Lett., 7, 289–298.

Cortesi, E., and P.J. Dolara (1983) Neoplastic transformation of BALB/3T3 mouse embryo fibroblasts by the beef extract mutagen 2-amino-3-methylimidazo (4,5-d) quinoline, Cancer Lett., 20, 43–47.

Cortesi, E., V. Saffioti, P.J. Donovan, J.M. Rice and T. Kakunaga (1983) Dose-response studies on neoplastic

transformation of BALB/3T3 clone A31-1-1 cells by aflatoxin B_1, benzidine, benzo[*a*]pyrene, 3-methylcholanthrene, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, Teratogen. Carcinogen. Mutagen., 3, 101–110.

Enterline, P.E. (1964) Mortality rates among coal miners, Am. J. Public Health, 54, 758–768.

Falk, H.L., and W. Jurgelski (1979) Health effects of coal mining and combustion: carcinogen and cofactors, Environ. Health Perspect., 33, 203–226.

Freedman, V.H., and S. Shin (1974) Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium, Cell, 3, 355–359.

Freshney. R.I. (1983) Cultures of Animal Cells, a Manual of Basic Technique, Liss, New York, pp. 133–136.

Heidelberger, C., A.E. Freeman, R.J. Pienta, A. Sivak, J.S. Bertram, B.C. Casto, V.C. Dunkel, M.W. Francis, T.J. Kakunaga, B. Little and L.M. Schechtman (1983) Cell transformation by chemical agents — a review and analysis of the literature, A report of the United States Environmental Protection Agency, Mutation Res., 114, 283–385.

IARC/NCI/EPA Working Group (1985) Cellular and molecular mechanism of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols, Cancer Res., 45, 2395–2399.

Jacobsen, M. (1976) Dust exposure, lung disease, and coal miner's mortality, Ph.D. Dissertation, Univ. of Edinburgh (Scotland).

Kakunaga, T., and H. Yamasaki (1985) Recommended protocol for the BALB/c 3T3 cell transformation assay, in: T. Kakunaga and H. Yamasaki (Eds.), Transformation Assay of Established Cell Lines: Mechanisms and Application, IARC Sci. Publ. No. 67, Lyon, pp. 208–212.

Krishna, G., J. Nath, L. Soler and T. Ong (1987) In vivo induction of sister chromatid exchanges in mice by nitrosated coal-dust extract, Environ. Res., 42, 106–113.

Kuroki, T., and K. Sasaki (1985) Relationship between in vitro cell transformation and in vivo carcinogenesis based on available data on the effect of chemicals, in: T. Kakunaga and H. Yamasaki (Eds.), Transformation assay of established cell lines: mechanisms and application, IARC Sci. Publ., Lyon, No. 67, pp. 93–120.

MacPherson, I., and L. Montagnier (1964) Agar suspension culture for the selective assay of cells transformed by polyoma virus, Virology, 23, 291–294.

Matolo, N.M., M.R. Klauber, W.M. Gorishek and J.A. Dixon (1972) High incidence of gastric carcinoma in a coal mining region, Cancer, 29, 733–737.

Matthews, E.J. (1986) Assessment of chemical carcinogen-induced transforming activity using BALB/c 3T3 cells, J. Tissue Cult. Method, 3, 157–164.

Ong, T., W.-Z. Whong and R.G. Ames (1983) Gastric cancer in coal miners: an hypothesis of coal-mine-dust causation, Med. Hypotheses, 12, 159–165.

Ong, T., W.-Z. Whong, J. Xu, B. Burchell, F.H.Y. Green and T. Lewis (1985) Genotoxicity studies of rodents exposed to coal-dust and diesel emission particulates, Environ. Res., 37, 399–409.

Pelroy, R.A., and D.L. Stewart (1981) The effect of nitrous acid on the mutagenicity of two coal liquids and their genetically active chemical fractions, Mutation Res., 90, 297–308.

Rockette, H.E. (1977) Cause specific mortality of coal miners, J. Occup. Med., 19, 795–801.

Rundell, J.O., M. Gunatakata and E.J. Matthews (1983) Criterion development for the application of BALB/c 3T3 cells to routine testing for chemical carcinogenic potential, in: M.D. Waters, S. Sandhu, J. Lewtas, L. Claxton, N. Shernoff and S. Nesnow (Eds.), Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III, Plenum, New York, pp. 309–324.

Sheu, C.W., F.M. Moreland and V.C. Dunkel (1987) The effect of cell passage in the susceptibility of BALB/3T3 clone A31-1-1-1 cells to 3-methylcholanthrene-induced morphological transformation, Environ. Mutagen., 9, 59–67.

Shin, S., V.H. Freedman, R. Risser and R.P. Pollack (1975) Tumorigenicity of the virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth in vitro, Proc. Natl. Acad. Sci. (U.S.A.), 72, 4435–4439.

Sivak, A., and A.S. Tu (1985) Neoplastic transformation system — their use in studying carcinogenesis, in: J. Celis and A. Grasessmann (Eds.), Cell Transformation, Plenum, New York, pp. 1–19.

Stocks, P. (1962) On the death rates from cancer of the stomach and respiratory diseases in 1940–53 among coal miners and other male residents in counties of England and Wales, Br. J. Cancer, 16, 592–598.

Styles, J.A. (1977) A method for detecting carcinogenic chemicals using mammalian cells in culture, Br. J. Cancer, 36, 558–563.

Tucker, J.D., and T. Ong (1985) Induction of sister chromatid exchanges by coal dust and tobacco snuff extracts in human peripheral lymphocytes, Environ. Res., 7, 313–324.

Tucker, J.D., W.-Z. Whong, J. Xu and T. Ong (1984) Genotoxic activity of nitrosated coal-dust extract in mammalian systems, Environ. Res., 35, 171–179.

Whong, W.-Z., R. Long, R.G. Ames and T. Ong (1983) Role of nitrosation in the mutagenic activity of coal-dust: A postulation for a gastric carcinogenesis in coal miners, Environ. Res., 32, 298–304.