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Transforming and carcinogenic potential of cadmium chloride in BALB/c-3T3 cells

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Abstract

A large number of workers are potentially exposed to cadmium during mining and processing. Therefore, there is a concern regarding the potential carcinogenic hazards of cadmium to exposed workers. Studies have been performed to determine if cadmium chloride ($CdCl_2$) can induce morphological cell transformation, DNA from $CdCl_2$-induced transformed cells can transform other mammalian cells, and the transformed cells induced by $CdCl_2$ can form tumors in nude mice. BALB/c-3T3 cells were treated with different concentrations of $CdCl_2$ for 72 h. The frequency of transformed foci from each treatment was determined after cells were cultured for 4 to 5 weeks. DNAs from five $CdCl_2$-induced transformed cell lines were isolated and gene transfection assay was performed using NIH-3T3 cells. Non-transformed BALB/c-3T3 cells and cells from 10 transformed cell lines induced by $CdCl_2$ were injected into both axillary regions of nude mice. Mice were screened once a week for the appearance and size of tumors. $CdCl_2$ caused a statistically significant, concentration-related increase in the transformation frequency. DNA from all five $CdCl_2$-induced transformed cell lines tested was found to induce varying degrees of transfection-mediated transformation in NIH-3T3 cells. All 10 $CdCl_2$-induced transformed cell lines formed fibrosarcomas in nude mice within 39 days of inoculation. Within this time period, no tumors were found in nude mice injected with non-transformed BALB/c-3T3 cells. These results indicate that $CdCl_2$ is capable of inducing morphological cell transformation and that the transformed cells induced by $CdCl_2$ are potentially tumorigenic.

Keywords: Cell transformation; Gene transfection; Tumorigenicity; Cadmium chloride; BALB/c-3T3 cells

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1. Introduction

Approximately 500,000 workers in the United States are exposed to cadmium [1]. Workers may be exposed to cadmium and cadmium compounds in a variety of occupational settings such as smelting, refining of zinc, electroplating, manufacturing of cadmium alloys, nickel–cadmium batteries, and welding. Mainstream cigarette smoking also contains cadmium [2]. Laboratory studies have demonstrated that cadmium is mutagenic and carcinogenic in experimental animals [3]. DNA strand breaks [4], gene mutations [5], chromosomal aberrations [6], micronucleus [7] and cell transformation [8] have been observed in vitro as a result of cadmium chloride ($CdCl_2$) exposure. Cadmium compounds inhibit the repair of DNA damaged by other agents, thereby enhancing their genotoxicity [9–11]. Chromosomal aberrations (CA) were seen in mouse bone marrow cells exposed to $CdCl_2$ in vivo [12]. Frequency of CA increased in peripheral blood lymphocytes of workers exposed to cadmium in the metal industry [3]. Results of epidemiological studies seem to indicate that there is a correlation between occupational exposure to this metal and lung cancer in workers [3]. However, the possible mechanism of cadmium-induced carcinogenesis has not been adequately studied.

Cell transformation coupled with the nude mouse/carcinogenesis assay is a very useful approach to study the carcinogenic potential of environmentally and occupationally related agents. It is relatively sensitive, inexpensive, and easy to perform. Moreover, it enables the study of mechanistic and/or sequential processes during carcinogenesis. It is generally believed that the mechanisms of in vitro cell transformation and in vivo carcinogenesis are similar [13]. Therefore, the tumorigenic potential of any transformed cells can be assessed by the development of tumors. It has been reported by Saffiotti and Bertolero [8] that $CdCl_2$-induced morphological transformation in BALB/c-3T3 cells and that cells from a transformed cell line induced by $CdCl_2$ developed tumor in nude mice. In our laboratory, studies have also been performed to determine the carcinogenic potential and possible carcinogenic mechanism of $CdCl_2$. In this report, we describe the transforming activity of DNA and the tumorigenic potential of cells from five and 10 transformed cell lines induced by $CdCl_2$, respectively.

2. Materials and methods

2.1. Cell culture and chemicals

BALB/c-3T3 clone A31-I-13 cells, kindly provided by Dr. M.A. Cifone (Covance Laboratories, Vienna VA) was used in this study. The cultures were grown in 75 cm^2 tissue culture flasks (Corning, NY) containing 15 ml of Minimum Essential Media (MEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma). Cells at passage 7 were used for the cell transformation studies. $CdCl_2$ (Sigma) was diluted fresh each time to final concentrations of 6, 9, and 12 μM.

2.2. Cytotoxicity and cell transformation

Exponentially growing cells were seeded for cytotoxicity assay. Cells were exposed to different concentrations of $CdCl_2$ (6, 9, 12 μM) for 72 h. The cytotoxicity was measured using trypan blue exclusion method by counting the number of live cells to the number of dead cells. This assay was done simultaneously with the cell transformation assay.

For cell transformation assay, approximately, 2×10^5 BALB/c-3T3 cells/flask ($n = 5$) were treated with $CdCl_2$ at concentrations from 6–12 μM for 72 h and then rinsed with 5 ml phosphate-buffered saline solution (PBS, pH 7.2) three times. When confluent, each flask was split into five flasks for a total of 25 flasks. Thereafter, the cultures were maintained in 5 ml MEM containing 7.5% FBS for a period of 5–6 weeks in humidified chamber with 5% CO_2 at 37°C. At the end of incubation, Type III foci were scored as transformed cells, which were isolated and established as transformed cell lines.

2.3. DNA extraction

DNA was extracted from non-transformed BALB/c-3T3 cells and five different transformed foci induced by $CdCl_2$ using the standard

phenol/chloroform extraction and ethanol precipitation method [14]. The concentration of DNA was determined using a spectrophotometer.

2.4. Gene transfection

For gene transfection, the calcium–phosphate precipitation method was followed [15] with minor modifications. In this study, a series of 75 cm^2 culture flasks were seeded with 5×10^5 NIH-3T3 cells/flask in 10 ml Dulbecco's modified essential medium (DMEM; Sigma). After 24 h of incubation, 30 μg of DNA extracted from each of five different transformed cell lines were diluted in 450 μl of distilled water. Fifty μl of solution 1 (2.5 M $CaCl_2$) (Stratagene Mammalian Transfection Kit, Stratagene, LaJolla, CA) and 500 μl of solution 2 (2× *N*, *N*-bis[2-hydroxyethyl]-2-amino-ethane sulfonic acid and buffered saline) were added to DNA, gently mixed and allowed to incubate for 10–20 min at room temperature for the precipitation of DNA. After incubation, the DNA precipitate was mixed carefully and then added drop by drop to the pre-seeded NIH-3T3 cell culture. The plate was gently swirled to evenly distribute the precipitate. Cell cultures were incubated for 12–24 h at 37°C in a 3% CO_2. At the end of the incubation, medium was removed and cultures were rinsed twice with PBS. Fresh culture medium was added and cells were further incubated for 24 h. Cells in each culture were then split at a 1:20 ratio and incubated for four more weeks with the medium changed biweekly. At the end of incubation, transformed type III foci in the culture were scored as DNA transfectants.

2.5. Nude mouse tumorigenicity assay

Athymic BALB/c (4 weeks old) female nude mice (nu/nu) were obtained from Harlan–Sprague–Dawley (Indianapolis, IN). Mice were housed in a centralized animal facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The animals were housed in ventilated microisolators and provided with sterile feed and water ad libitum.

Ten $CdCl_2$-transformed cell lines were selected for the study of tumorigenic potential in athymic nude mice. Three animals/group were injected subcutaneously in both axillary regions with approximately 2 million cells/site. Mice were observed for tumor formation by palpating at weekly intervals. When the tumors were 1.5–2.0 cm in size, the tumors were removed aseptically and used for histological examination and establishing tumor-derived cell lines.

2.6. Statistical analysis

Statistical analysis was carried out according to standard procedures. Student's *t*-test was used to calculate the cytotoxicity and transformation frequencies. Statistical significance value of $P \leq 0.05$ was considered significant. Mean and standard deviations were calculated for all the data presented.

3. Results

The results of this study indicate that $CdCl_2$ induced cell toxicity in a dose dependent manner. The percent live cells decreased as the concentration of $CdCl_2$ increased. Approximately 14% cells survived in the highest concentration tested (Table 1). Cell transformation studies also gave similar result. Average number of transformed foci per flask increased in a concentration-related manner (Table 1). However, only two of the higher concentrations (9 and 12 μM) resulted in a significant increase in $CdCl_2$-induced cell transformation.

Morphologic features of BALB/c-3T3 cells changed in a consistent manner after transformation. The non-transformed BALB/c-3T3 cells grew in a monolayer (Fig. 1, panel A). $CdCl_2$ transformed

Table 1
Cytotoxicity and transforming potential of $CdCl_2$

Concentration (μM)	Cytotoxicity (% live cells)	Transformed foci/flask
0	100.00	0.6
6	56.07	1.0
9	31.03	1.5*
12	13.92	3.1*

* Significantly different when compared to control ($P \leq 0.05$).

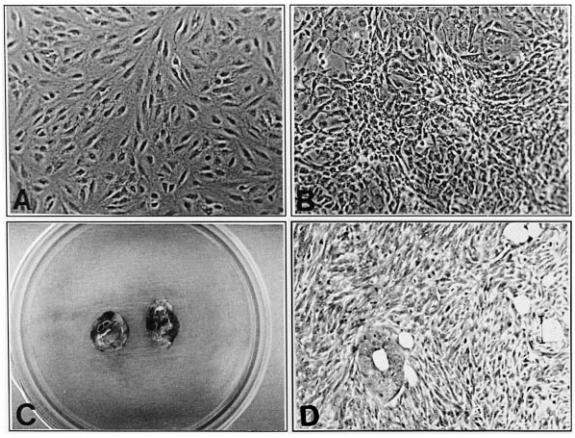

Fig. 1. Morphological and histological changes in non-transformed and transformed cells; (A) Non-transformed BALB/c-3T3 cells growing as a monolayer of cells, (B) Type 3 transformed foci with multilayering, criss-cross and basophilic characteristics, (C) Tumors aseptically dissected from the mouse, (D) Histopathological changes of the tumor indicating fibrosarcoma.

cells demonstrated morphological changes including loss of contact inhibition, invasiveness, disoriented (criss-cross) organization, multilayered growth, piling up and forming foci (Fig. 1, panel B).

Five transformed cell lines induced by 12 μM $CdCl_2$ were selected to study the ability of these cells to transform NIH-3T3 cells. The results of gene transfection study demonstrate that the DNA from $CdCl_2$-transformed cells induced transfection-mediated transformation. As shown in Fig. 2, the transfection-mediated transformation in host NIH-3T3 cells by $CdCl_2$-transformed cells was 8–35 times higher than that of non-transformed BALB/c-3T3 cells. The results of this study demonstrate that the $CdCl_2$-transformed cells may carry activated proto-oncogenes and/or inactivated tumor suppressor genes.

Carcinogenic potential of transformed cells induced by $CdCl_2$ has been determined by the nude mice/tumorigenicity study. In this study, nude mice were injected with normal and transformed cells in the axillary region and observed for tumor formation. Transformed cells were capable of forming tumors when injected into athymic nude mice. As shown in Fig. 3, tumors developed in 20% of the nude mice as early as 3 weeks after injection of $CdCl_2$-transformed cells. All 10 $CdCl_2$-induced transformed cell lines produced tumors at both injection sites 39 days after cell inoculation. No tumors were found in nude mice even 49 days after injection of non-transformed cells. The size and type of tumors 49 days after cell inoculation are listed in Table 2, Fig. 1, panel C. All tumors derived from $CdCl_2$-transformed cells were histopathologically characterized as fibrosarcomas (Fig. 1, panel D). No metastases were found in the lung of tumor-bearing nude mice. These results sug-

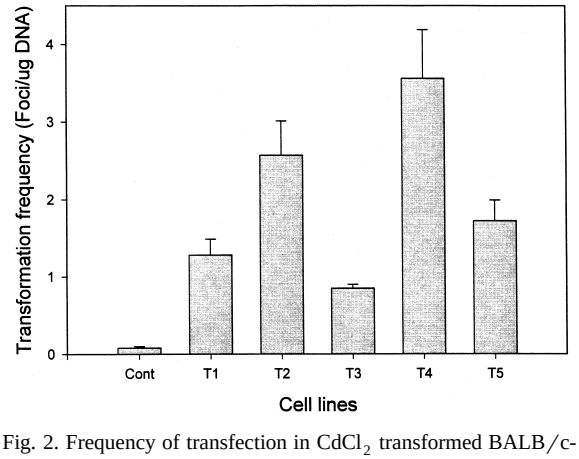

Fig. 2. Frequency of transfection in $CdCl_2$ transformed BALB/c-3T3 cells; Cont = non-transformed BALB/c-3T3 cells, T1–T5 = five different transformed cell lines induced by $CdCl_2$.

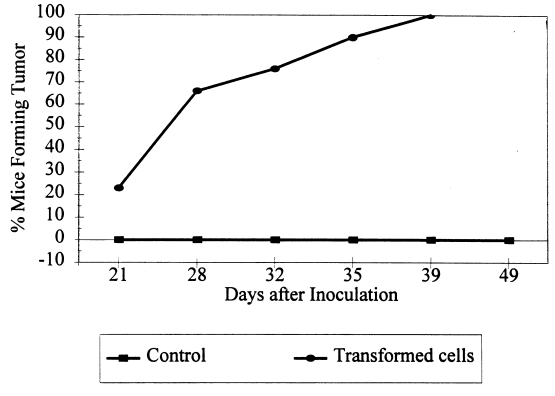

Fig. 3. Tumorigenicity of $CdCl_2$ induced transformed cells; 100% mice injected with transformed cell lines formed tumors within 39 days of inoculation and none of the mice injected with non-transformed BALB/c-3T3 cells shows tumors even after 49 days of inoculation.

Table 2
Size of tumors in nude mice within 49 days after cell inoculation

No. of cell lines[a]	Average size of tumor[b] (cm)	
	Right (mean ± SE)	Left (mean ± SE)
C	–	–
T1	1.83 ± 0.17	1.83 ± 0.11
T2	1.43 ± 0.13	1.33 ± 0.13
T3	2.33 ± 0.18	1.93 ± 0.13
T4	1.66 ± 0.22	1.43 ± 0.17
T5	1.90 ± 0.21	1.96 ± 0.21
T6	1.60 ± 0.21	1.63 ± 0.08
T7	1.50 ± 0.08	1.46 ± 0.17
T8	1.60 ± 0.15	1.43 ± 0.22
T9	1.80 ± 0.17	1.36 ± 0.20
T10	2.00 ± 0.27	1.96 ± 0.16

[a]C, non-transformed (BALB/c-3T3) cells; T1–T10, transformed cell lines.
[b]All tumors were fibrosarcomas.

gest that the transformed cells induced by $CdCl_2$ do possess tumorigenic potential.

4. Discussion

Carcinogenesis is a multistep process involving initiation, promotion, and progression. Morphological transformation of cells in vitro is a phenomenon that has been noted for many years as a complex series of events with patterns of growth control changes similar to carcinogenesis [16,17]. Morphological transformation involves three major areas of phenotypic alterations. The first change is immortalization, which may be caused by mutations in certain proto-oncogenes or tumor suppressor genes resulting in unlimited growth [13]. The second change in transformation is the acquisition of a number of aberrant growth characteristics, including the loss of contact inhibition, the ability to grow on a confluent monolayer of non-transformed cells [13]. The third alteration is 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. Morphological cell transformation in vitro may, therefore, be analogous to in vivo carcinogenesis [13]. It has been used extensively as a useful model system for investigation of chemical carcinogens. Therefore, cell transformation coupled with the tumorigenesis assay is a very useful approach to study the carcinogenic potential of environmentally and occupationally related chemicals. It enables the study of mechanistic and/or sequential processes during carcinogenesis.

Several studies have shown BALB/c-3T3 cell system is a sensitive system for cell transformation. Exposure of cells to physical or chemical agents leads to a change in single or many cells and this change is transmitted to the progeny of the surviving cells. A consequence of this change is an enhanced probability of cell transformation when these cells are maintained under conditions of confluence. Some metals are known to induce cell transformation and malignant tumors. Studies conducted by Saffiotti and Bertolero [8] showed morphological neoplastic transformation of BALB/c-3T3 using sodium arsenite, sodium arsenate, cadmium chloride and potassium chromate. Results of our studies are in agreement with that reported by Saffiotti and Bertolero [8], although a different cell clone and different concentrations of $CdCl_2$ were used.

The gene transfection assay provides a means whereby DNA samples can be screened for the presence of activated transforming proto-oncogenes or inactivated tumor suppressor genes. If these genes are present, the cells taking up the DNA will be transformed and foci will result. Our gene transfection assay demonstrated increased transformants — a finding consistent with the presence of activated transforming genes. Thus, morphological transformation of BALB/c-3T3 cells may be associated with activation of proto-oncogenes or inactivation of tumor suppressor genes.

Malignancy of cultured cells has been demonstrated by the production of fibrosarcomas following injection into nude mice. Nude mice offer an excellent animal model to test the tumorigenesis of morphologically transformed cells. In our studies, we found that $CdCl_2$ induced dose-responsive cytotoxicity and cell transformation of BALB/c-3T3 cells. Most importantly, these transformants rapidly produced tumors in vivo with 100% of mice inoculated with 10 different transformed cell lines developing tumors less than 6 weeks after injection. Previous studies have shown that in vitro exposure of $CdCl_2$

leads to malignant progression in vivo in nude mice [8], and rat L6 myoblasts [18]. Our studies also show that $CdCl_2$ not only induce morphological cell transformation but induce a series of genetic changes which rapidly lead to fibrosarcomas in nude mice. The ability to transfect these neoplastic changes and the ability to rapidly produce fibrosarcomas in vivo, could provide a useful system for investigating mechanisms of cadmium-induced carcinogenesis. Analyses of mutation, expression and amplification of selected proto-oncogenes and tumor suppressor genes in transformed cells and in tumor cells derived from transformed cells induced by $CdCl_2$ are in progress.

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