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Advances in Brief

Cadmium-induced Malignant Transformation of Human Prostate Epithelial Cells[1]

William E. Achanzar, Bhalchandra A. Diwan, Jie Liu, Salmaan T. Quader, Mukta M. Webber, and Michael P. Waalkes[2]

Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [W. E. A., J. L., M. P. W.]; Intramural Research Support Program, Science Applications International Corporation-Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702 [B. A. D.]; and Departments of Medicine and Zoology, Michigan State University, East Lansing, Michigan 48824 [S. T. Q., M. M. W.]

Abstract

Prostate cancer has become epidemic, and environmental factors such as cadmium may be partly responsible. This study reports malignant transformation of the nontumorigenic human prostatic epithelial cell line RWPE-1 by *in vitro* cadmium exposure. The cadmium-transformed cells exhibited a loss of contact inhibition *in vitro* and rapidly formed highly invasive and occasionally metastatic adenocarcinomas upon inoculation into mice. The transformed cells also showed increased secretion of MMP-2 and MMP-9, a phenomenon observed in human prostate tumors and linked to aggressive behavior. Cadmium-induced malignant transformation of human prostate epithelial cells strongly fortifies the evidence for a potential role of cadmium in prostate cancer.

Introduction

Prostate malignancies are a leading cause of cancer-related deaths in men in the United States (1), yet the etiology of prostate cancer remains an enigma. Epidemiological and animal studies provide substantial evidence implicating cadmium, a known human carcinogen, as a prostate carcinogen (2–4), although the mechanisms involved are undefined. The recent development of immortalized but nontumorigenic human prostate epithelial cell lines has opened new avenues for defining mechanisms in prostate carcinogenesis (5–7). *In vitro* transformation of human prostate cells has been used to examine molecular events linked to malignant transformation induced by radiation (5), oncogene expression (6), and organic carcinogens (7). Regarding cadmium, there is evidence it can induce malignant transformation of rat prostate epithelial cells *in vitro* (8). We have extended these studies into a human model using the immortalized, nontumorigenic human prostate epithelial cell line RWPE-1 (6). Here we report malignant transformation of RWPE-1 cells induced by chronic cadmium exposure *in vitro*. The CTPE[3] cells exhibited loss of contact inhibition *in vitro* and rapidly produced poorly differentiated invasive adenocarcinomas when inoculated into Nude mice. CTPE cells showed increased secretion of active MMP-2 and MMP-9, both of which are implicated in prostate cancer invasion (9, 10) and are typical of aggressive tumors. This is the first report of cadmium-induced malignant transformation in a cell line analogous to a potential *in vivo* target cell population in humans and may have important implications in human prostate cancer etiology.

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[2] To whom requests for reprints should be addressed, at National Cancer Institute, National Institute of Environmental Health Sciences, MD F0-09, 111 Alexander Drive, Research Triangle Park, NC 27709. E-mail: waalkes@niehs.ni.

[3] The abbreviations used are: CTPE, cadmium-transformed prostate epithelial; MMP, matrix metalloproteinase; K-SFM, keratinocyte serum-free medium; PSA, prostate-specific antigen.

Materials and Methods

Chemicals and Reagents. $CdCl_2$ (Sigma Chemical Co., St. Louis, MO), K-SFM, epidermal growth factor, bovine pituitary extract, antibiotic/antimycotic solution (Life Technologies, Inc., Grand Island, NY).

Cells and Cell Culture. RWPE-1 cells were grown in K-SFM containing 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and 1× antibiotic/antimycotic solution. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and passed weekly. For cadmium exposure, cells were maintained continuously in medium containing 10 μM $CdCl_2$.

Zymographic Analysis of MMP Activity. Cultures at 70–80% confluence were washed twice with PBS, and the medium was changed to K-SFM without supplements. After 48 h, the conditioned medium was collected and centrifuged for 5 min at 400 × *g*. A 500-μl aliquot was concentrated to <100 μl in a Microcon concentrator at 14,000 × *g* at 4°C. Protein concentration was determined using a commercial assay (Bio-Rad, Hercules, CA), and 1 μg of total protein (6–10 μl) from each sample was electrophoresed on a 10% zymography gel containing 0.1% gelatin (Novex, San Diego, CA). MMP activity was detected by incubating the gel in 1× zymogram renaturing buffer for 30 min at room temperature and then in 1× zymogram developing buffer (Novex) overnight at 37°C, followed by staining with GelCode Blue (Pierce Corp., Rockford, IL). After staining, the bands were quantified using the 1D version 2.0 software (Eastman Kodak, Rochester, NY).

Tumorigenicity in Nude Mice. To test for malignant transformation, 1×10^6 RWPE-1 or CTPE cells were inoculated s.c. in the dorsal thoracic midline of 20 nude (NCr-*nu*) mice (National Cancer Institute-Frederick Cancer Research and Design Center Animal Production Area, Frederick, MD). Tumor formation and growth were assessed weekly. All mice were sacrificed by 10 weeks after injection or when clinical conditions dictated euthanasia. Tumor samples were paraffin-embedded, sectioned, stained with H&E, and analyzed by light microscopy. Immunostaining was performed according to standard techniques using a monoclonal antibody specific for human PSA (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom).

Statistical Analyses. Zymography data represent the mean ± SE of three determinations and were analyzed by Student's *t* test. Incidence data (tumorigenicity studies) were analyzed by Fisher's exact test. A two-sided value of $P < 0.05$ was considered significant in all cases.

Results

Cadmium-induced Malignant Transformation of RWPE-1 Cells. To achieve transformation, RWPE-1 cells were continuously exposed to 10 μM cadmium, a concentration very near the estimated range in the prostates of people with no known occupational exposure to cadmium (11–28 μM, assuming 1 g wet tissue equals 1 ml; Ref. 11). Subtle morphological differences were observed between the cadmium-treated and passage-matched control cells after 8 weeks. The treated cells, designated CTPE, were subsequently cultured in cadmium-free medium and soon began to form cell mounds, even when subconfluent (Fig. 1*A*), whereas mounding was not observed in passage-matched control cells (Fig. 1*B*). This mounding indicates loss of contact inhibition and was the first indication of transformation in the CTPE cells.

Definitive evidence of cadmium-induced malignant transformation

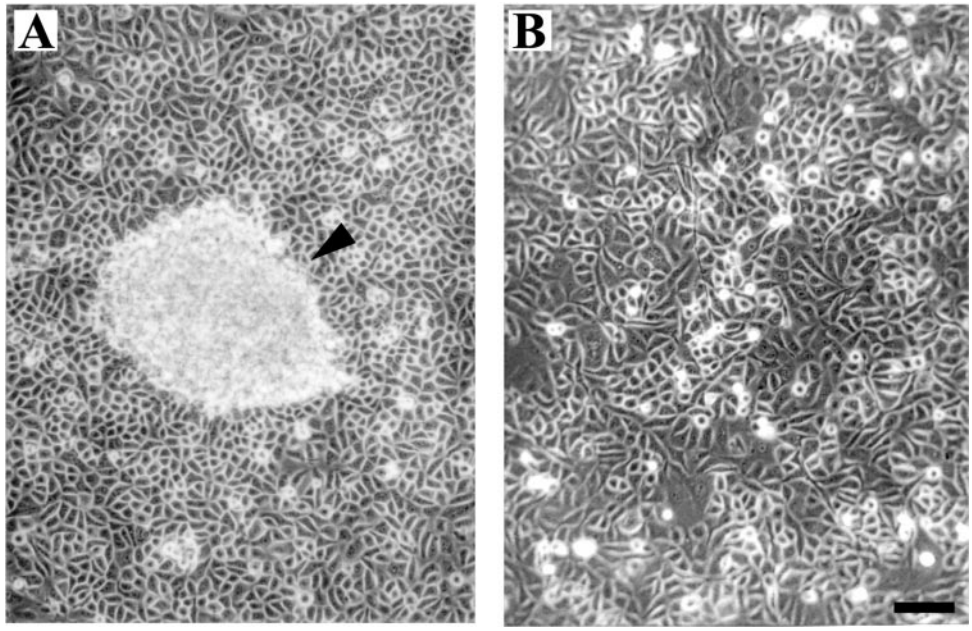

Fig. 1. Apparent loss of contact inhibition in RWPE-1 cells chronically treated with cadmium. *A*, representative focus (*arrowhead*) formed by CTPE cells. *B*, passage-matched control monolayer lacking cell foci. *Bar*, 100 μm.

came after inoculation of cells into Nude mice. Tumors arose in 18 of 20 mice within 6 weeks, and several arose as early as 3 weeks after inoculation of CTPE cells. In sharp contrast, tumors were not observed in mice inoculated with control cells. Tumors developing from CTPE cells were exclusively poorly differentiated adenocarcinomas (Fig. 2*A*), consistent with prior reports of tumors formed by human prostate epithelial cells transformed *in vitro* by nonmetallic agents (5–7). In addition, ~80% of these tumors invaded into the subdermal muscle, fat, or connective tissue, a strong indication of aggressive behavior (Fig. 2*B*). One lung metastasis occurred. The strong staining with an antibody specific for human PSA established these carcinomas as arising from human prostatic epithelial cells (Fig. 3).

CTPE Cells Exhibit Increased MMP-2 and MMP-9 Secretion. MMPs are secreted enzymes that selectively degrade the extracellular matrix and have been implicated in tumor cell invasion (12). Zymographic analysis revealed marked increases in secretion of active MMP-2 and MMP-9 from CTPE cells (Fig. 4*A*). The activity of these enzymes in CTPE-conditioned medium was 2.4-fold (MMP-2) and 3.6-fold (MMP-9) higher than in RWPE-1 medium (Fig. 4*B*). The ratio of secreted MMP-9:MMP-2 was also increased in CTPE (0.72 $\pm$ 0.03) *versus* RWPE-1 (0.48 $\pm$ 0.02), a result strikingly similar to that reported in primary cultures of human prostate tumors when compared with cells from benign lesions (10). The hypersecretion of these MMPs is consistent with the highly aggressive nature of the tumors derived from these cells.

Discussion

Cadmium is a suspected human prostatic carcinogen and can induce prostate tumors in rats (3, 4), making the prostatic epithelium a suspected *in vivo* target of cadmium. However, the underlying mechanisms involved in cadmium carcinogenesis remain unclear. To help elucidate these mechanisms, in the present study we succeeded in malignantly transforming a normal human prostate epithelial cell line by chronic exposure to cadmium *in vitro*. Malignant transformation was established by the rapid formation of highly invasive adenocarcinomas after inoculation of CTPE cells into Nude mice. There are two major implications of this work: (*a*) the malignant transformation of normal human prostate epithelial cells is compelling evidence that cadmium indeed has the potential to be a human prostatic carcinogen;

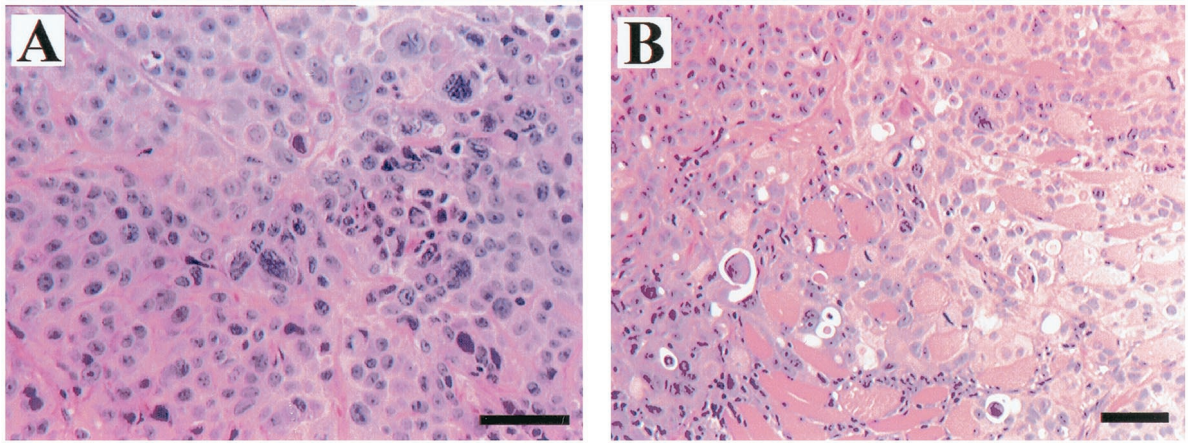

Fig. 2. *A*, tumor produced by CTPE cells after inoculation s.c. into a nude mouse. The tumor is a poorly differentiated adenocarcinoma showing pronounced cellular pleiomorphism and frequent mitotic bodies. *Bar*, 50 μm. *B*, area of tumor invasion into the subdermal muscle layers. A large number of muscle fibers can be seen in the lower portion of the photomicrograph. Invasion into the muscle was a frequent occurrence, as was invasion into the fat and connective tissue. *Bar*, 50 μm.

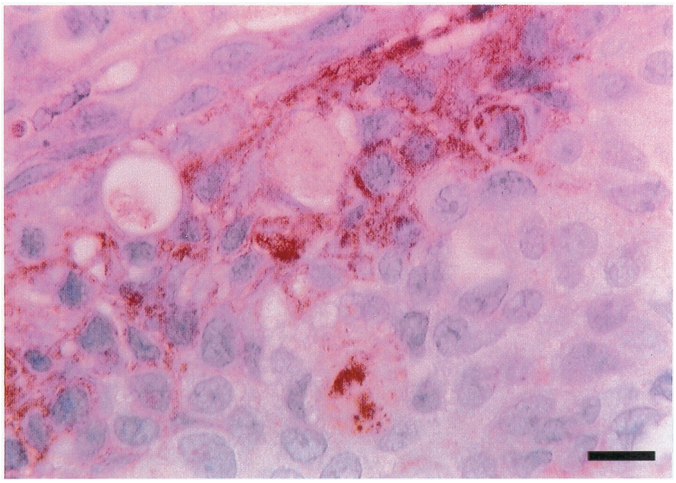

Fig. 3. Positive staining for PSA, indicated by the *pinkish* to *reddish-brown* color, confirmed that the tumors were derived from human prostatic epithelial cells. *Bar*, 25 μM.

and (*b*) we now have a model with human relevance in which we can elucidate the genetic events involved in cadmium-induced malignant transformation by comparing the CTPE and RWPE-1 cells at the cellular and molecular levels and potentially develop a genetic "signature" for cadmium-induced prostatic tumors.

Worldwide, prostate cancer incidence and mortality rates have been increasing steadily for the past several decades (13). Although the basis for this increase remains unclear, epidemiological evidence suggests that environmental factors, such as pollutants, may play a role given that prostate cancer mortality rates vary greatly between geographic regions (13). Additionally, prostate cancer incidences in the United States immigrant populations from countries with historically low incidence eventually tend toward the prevailing local rate (14). One environmental pollutant repeatedly implicated in increased prostate cancer risk is cadmium (3, 4). Because of its wide industrial usage (2) and very limited recycling, cadmium is accumulating in the biosphere, consequently increasing the likelihood of human exposure. Numerous reports have revealed a significant correlation between cadmium exposure and prostate cancer (2–4). These studies suggest that occupational or environmental cadmium exposure is a risk factor for the development of prostate malignancies, although there is some controversy because several studies have not found such an association (2–4). The present study fortifies the positive human epidemiological data by providing clear and compelling evidence that human prostatic epithelial cells are susceptible to cadmium-induced malignant transformation. This, in combination with accumulating evidence that shows that the rat prostate is a target for cadmium carcinogenesis (3, 4), strongly supports its potential role in human prostate cancer.

The increased secretion of MMP-2 and MMP-9, together with the highly invasive and occasionally metastatic nature of CTPE cells, are consistent with a potential role of cadmium in both initiation and enhancement of tumor progression. A role for cadmium in enhanced tumor progression has been proposed recently based on both *in vitro* and *in vivo* studies. For instance, tumorigenic myoblasts exposed to cadmium *in vitro* prior to inoculation into Nude mice showed increased malignant progression that more frequently caused host death (15). Cadmium also increases invasiveness human fibrosarcoma cells in *in vitro* model systems (16). *In vivo*, repeated cadmium exposures in rats clearly enhanced malignant progression of ensuing injection site tumors, as assessed by rate of regional invasiveness and distant metastases (17). The elevated MMP-2 and MMP-9 levels observed in CTPE cells are also consistent with data from primary cultures derived from human prostate tumors, which show elevated secretion of MMP-2 (9) or MMP-9 (10) compared with normal prostate cells. Additionally, an increased ratio of MMP-9:MMP-2 activity has been observed in cells from prostate carcinoma when compared with cells from benign lesions (10), paralleling the progression to a malignant state. A similar increase in the ratio of MMP-9:MMP-2 was observed in CTPE cells, indicating that these cells possess characteristics in common with prostate carcinoma cells arising *in vivo*. Overall, the rapid development and pronounced invasiveness of tumors derived from CTPE cells provide persuasive evidence that cadmium can enhance tumor progression.

In the present study, malignant transformation was achieved by continuous cadmium exposure for an extended period of time. Continuous exposure of prostatic epithelial cells to cadmium *in vivo* is a likely scenario because the human prostate progressively accumulates cadmium with increasing age (11). In humans, cadmium has a biological half-life measured in decades (4) and, thus, is considered a cumulative toxicant. Therefore, even if an individual receives only small but repeated exposures, chronic exposure of prostatic epithelium would occur as a result of the biokinetics of cadmium, dictating both prostatic accumulation and an extremely long residence time. This is supported by several studies showing that cadmium can be an effective prostatic carcinogen in rats, even after a single systemic exposure (4). Prostatic accumulation of cadmium is likely attributable to the fact that cadmium mimics zinc, an essential element that the prostate accumulates to higher levels than any other tissue (18). This mimicry is probably important in dictating the adverse effects of cadmium. For example, recent evidence indicates that cadmium replaces zinc in p53 and impairs its DNA binding activity and subsequent induction of cell cycle arrest after DNA damage (19). Therefore, the replacement of zinc by cadmium in key regulatory factors in prostatic epithelial cells may potentially result in aberrant gene expression that, in turn, leads to cellular transformation. This is an attractive hypothesis, particularly given the observation that cadmium is generally only poorly muta-

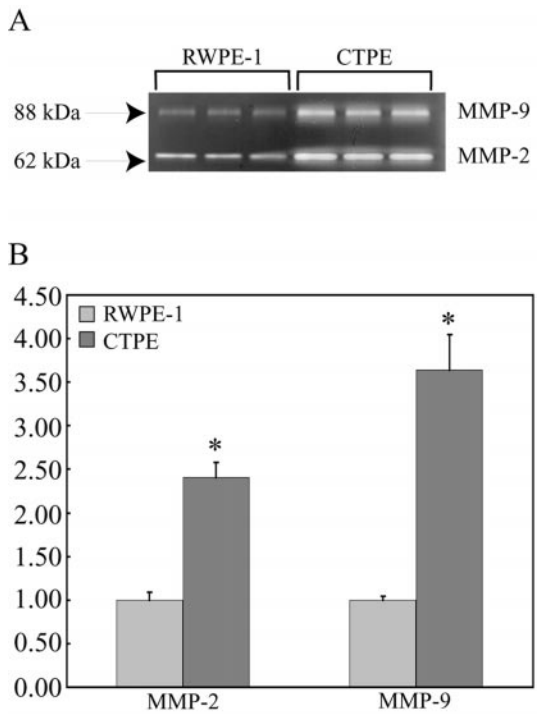

Fig. 4. Analysis of MMP-2 and MMP-9 activity in RWPE-1 and CTPE in conditioned medium. *A*, zymogram gel showing increased MMP-2 and MMP-9 activity in CTPE conditioned medium. *B*, quantitative analysis of zymography results. Data expressed as fold-RWPE-1 activity and are represented as means (n = 3); *bars*, SE. *, significant differences from RWPE-1 activity ($P < 0.05$).

genic at doses allowing reasonable survival (20). However, further investigation is needed to determine the precise carcinogenic mode of action for cadmium.

In summary, chronic cadmium exposure can induce malignant transformation of human prostatic epithelial cells *in vitro*, producing highly aggressive tumors upon inoculation into Nude mice. This is the first report of cadmium-induced malignant transformation of human cells and is particularly significant because transformation occurred using a cell line analogous to a potential *in vivo* target site of cadmium carcinogenesis. In addition, this study provides compelling evidence that cadmium has the potential to be a human prostatic carcinogen. Further comparison between the transformed and control cells should lead to a better understanding of the mechanism involved in cadmium carcinogenesis and, perhaps, a molecular "fingerprint" for identification of cadmium-induced prostatic malignancies.

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