Parallel development of cadmium resistance and in vitro transformation in cultured Indian muntjac cells

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Summary

The development of cadmium resistance in an Indian muntjac cell line has been investigated. The parent cell line is highly sensitive to cadmium ions. Resistance was obtained by continuous growth of cells in low levels of cadmium with stepwise increments. Four cell lines were developed with resistances of between 50- and 200-fold greater than that of the parental line. Early in the development of resistance an unstable cell line displaying extensive chromosomal rearrangement and an elevated sister chromatid exchange frequency was identified. The more stable resistant lines produced from this original cell line have normal karyotypes. Having passed through the initial period of genome rearrangement the resultant cells acquired several characteristics of morphologically transformed cells. It is concluded that long-term exposure to low levels of cadmium can transform cells in vitro concurrently with their acquiring cadmium resistance.

Key words: muntjac, cadmium, transformation.

Introduction

Cadmium, a common environmental contaminant, is of considerable toxicological interest. Among the many properties of the metal, its accumulation in the body (e.g. see Bernard & Lauwreys, 1984) and its possible carcinogenicity (e.g. see Hallenbeck, 1984) are of particular concern. Although the epidemiological evidence for cadmium being a carcinogen is fragmentary and contradictory (Hallenbeck, 1984; Sorahan & Waterhouse, 1983; Armstrong & Kazantzis, 1983; Thun et al. 1985), an in vitro assay for carcinogenicity, the transformation of cells in culture, has produced consistent positive results (Heck & Costa, 1982). Thus cadmium salts have been shown to cause morphological transformation alone and to potentiate the transforming activity of simian adenovirus (Casto et al. 1976) and benzo(a)pyrene (Rivedal & Sanner, 1981) when used in combination.

Possibly the most intensively studied of the cellular effects of cadmium is the development of resistance. Cadmium-resistant lines have been produced from cells of a variety of species (Rugstad & Norseth, 1975, 1978; Hildebrand et al. 1979; Beach & Palmiter, 1981; Gick & McCarty, 1982; Shworak et al. 1983). Most research utilizing such cell lines has concentrated on elucidating the mechanisms of tolerance to cadmium and other metals (e.g. see Beach et al. 1981; Hildebrand et al. 1982; Enger et al. 1986); few authors have looked at the changes occurring during the development of resistance.

We know of no cellular studies that followed the development of metal resistance and the process of in vitro transformation simultaneously. In this report we describe the development of cadmium resistance in four Indian muntjac cell lines. Cadmium resistance was gained with the simultaneous acquisition of phenotypic traits indicative of in vitro transformation. The magnitude of the positive results in transformation assays increased with the increasing levels of metal resistance, i.e. the greater the level of cadmium resistance attained by a cell, the more transformed it appeared.

Materials and methods

The Indian muntjac skin fibroblast line, ATCC number CCL157, was originally obtained from Flow Laboratories. Cells were maintained at 37°C in Hepes-buffered Eagle's Minimal Essential Medium (Flow) supplemented with 2 mM-glutamine, 50 units ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin and 10% foetal calf serum. Latterly, some cells have been maintained in bicarbonate-buffered medium in an atmosphere of 5% CO₂.

Cadmium was added to cells as cadmium acetate, concentrated stock solutions were prepared weekly and stored at 4°C in polyethylene bottles.

Cell growth was determined in a simple assay involving the plating of 10⁴ cells per 30 mm diameter dish, adding cadmium as appropriate, followed by trypsinizing and counting after 4
days of culture. Results are expressed as percentage of control (untreated) cell number and plotted as for a standard cell killing curve.

Chromosome preparations were made by standard hypotonic KCl/methanol–acetic acid fixation following a 2-hour treatment with colcemid. For sister chromatid exchange (SCE) assays cells were grown for 48 h (approximately two cell cycles) in the presence of 10 µM bromodeoxyuridine (Sigma). SCEs were stained by the method of Alves & Jonasson (1978), i.e. with 2% Giemsa in 0.3 M Na₂HPO₄, pH 10.2–10.6 for 10 min, rinsing in distilled water and drying. A minimum of 25 metaphases per treatment were scored for SCE analysis; chromosome aberration data were obtained from scoring 100 cells per treatment. Duplicate experiments were performed in all cases.

The ability of cells to grow in semi-solid medium was assessed by plating cells in 0.3% agar over a base layer of 0.6% agar in 60 mm diameter Petri dishes. After incubation at 37°C for approximately 4 weeks, cultures were stained and colonies visible by eye were enumerated.

The concanavalin A agglutination assay of Borland & Hard (1974) was employed to determine lectin agglutinability. This involves incubation of 4 x 10⁵ cells in 5 or 50 µg/ml concanavalin A (Sigma) for 5 min at room temperature. Agglutination was assessed by observation of cells under a phase-contrast microscope and estimating the number of cells per clump.

For time-lapse video analysis cells were grown in 60 mm dishes, and during recording were placed on the stage of an inverted phase-contrast microscope contained within a constant temperature (37°C) tent. The microscope was linked to a video recorder by means of a camera. Recordings were made over 24 h, using the 80-h speed setting on the recorder. For analysis tapes were played back at normal speed and stopped at intervals to allow the tracing of cell outlines on acetate sheets.

Results

Cell lines generated during cadmium exposure and development of cadmium resistance

Cadmium-resistant variants of the parental Indian muntjac cell line (M) were produced by the following steps: (1) four weeks continuous exposure to 0.1 µM Cd²⁺; (2) four weeks continuous exposure to 0.5 µM Cd²⁺; (3) four weeks continuous exposure to 1 µM Cd²⁺; (4) exposure to 1 µM increments in Cd²⁺ concentration up to 5 µM for varying lengths of time.

This comparatively lengthy procedure (totalling seven months) was required as the M cells are highly sensitive to cadmium toxicity (Fig. 1). Each of the increments in Cd²⁺ concentration referred to in step (4) led to considerable cell death, the variable time between increments being required to allow the cells to return to a relatively normal growth rate.

The initial cell population adapted to growth in 5 µM Cd²⁺ are known as CR5. Their growth was poor; electron-microscopic studies suggest that this was due to the massive damage sustained on each change of medium and cadmium, i.e. they 'tolerated' the cadmium but were not protected from its damaging action (Ord et al. 1988). Following a prolonged period of growth in this poor state (approximately 100 generations) the CR5 cells produced a line of cells with an increased proliferation rate and displaying generally healthier growth. The cell line with improved growth rate is distinguished from the original CR5 by being named CCR5.

Although the onset of the change from tolerance to resistance (i.e. from CR5 to CCR5) took more than a year, once started it progressed rapidly. A doubling of the cadmium concentration from 5 to 10 µM in CCR5 cultures, though again leading to large-scale cell death, produced a healthily growing cell line with resistance to 10 µM-cadmium, the CCR10 line. CCR20 was produced in a similar fashion from CCR10 by the addition of 20 µM-Cd²⁺ to the cells and growing the survivors. The experiments described below have been carried out on the five cell lines, M, CR5, CCR5, CCR10 and CCR20. CR5 and CCR5 were routinely maintained in standard medium with 5 µM-Cd²⁺, CCR10 and CCR20 were routinely maintained in medium containing 10 µM-Cd²⁺ and 20 µM-Cd²⁺, respectively.

Sensitivity of the new lines to cadmium

Cadmium toxicity to all five cell lines was determined in the simple proliferation assay described in Materials and methods. All resistant cell lines were grown in the absence of cadmium for one week before the experiment, to clear any accumulated cadmium and damaged cell components. Results (Fig. 1) clearly demonstrate the increased resistance of the C5, CCR5, CCR10 and CCR20 lines to cadmium. Each transition (M to CR5, CR5 to CCR5, CCR5 to CCR10 and CCR10 to CCR20) leads to a greater tolerance of the growth-inhibitory action of cadmium ions. In terms of D₃₇ values (the dose required to reduce cell proliferation to 37% of controls) the resistant cells are approximately eightfold (CR5), 18-fold (CCR5), 30-fold (CCR10) and 58-fold (CCR20) more resistant to short-term exposures to cadmium. The levels of resistance are even more striking when expressed in terms of the concentration of cadmium tolerated in long-term growth. The parent M cell can continuously proliferate in up to 0.1 µM-cadmium; the increased resist-

Fig. 1. Short-term cadmium toxicity in: normal, M, cells (○); CR5 (●); CCR5 (●); CCR10 (▲) and CCR20 (♦). See Materials and methods for experimental details.
Fig. 2. Representative chromosome preparations from M cells (A) and CR5 (B,C,D). All preparations were stained for sister chromatid exchange except D. Note presence of dicentrics (▲), gaps and breaks (▼) and chromosomes of altered morphology (▲) in CR5 metaphases. Bars represent 10 μm.

ances then are: 50-fold (CCR5), 100-fold (CCR10) and 200-fold (CCR20).

The cadmium sensitivity of SVM, an SV40-transformed Indian muntjac cell line (Pillidge et al. 1986a), is similar to that of M cells: D37 = 2 μM (M), D37 = 1.5 μM (SVM).

Chromosome instability

The karyotype of the parent M cell line is typical of the Indian muntjac (Fig. 2A; and Wurster & Benirschke, 1970). The karyotype is fairly stable, the range of chromosome numbers per cell being six to nine. Cells with nine chromosomes are however rare, representing only approximately 1% of the population; if such cells are excluded the range becomes six to seven. In contrast, chromosome preparations of CR5, made soon after the emergence of this cell line, showed many cells to be polyploid with both variable chromosome numbers and morphology (Fig. 2). No consistent trend in type of rearranged chromosome or chromosomal aberration was observed in CR5 metaphases. The extent of rearrangement was so great that few if any chromosomes retained their normal morphology; this is due largely to translocations. In addition, the chromosomes of CR5 tend to be broken, as seen by the frequencies of breaks and gaps recorded in Table 1. Their baseline SCE frequency is also elevated (Table 1), although we have previously demonstrated that short-term cadmium treatment has no effect on the incidence of SCE in M cells (Bouffler & Bell, 1984).

The change from CR5 to CCR5 (from cadmium tolerance to cadmium resistance) was associated with a return to normal karyotype and SCE frequency (Table 1). Similarly, CCR10 and CCR20 have normal M-cell-like chromosomes and baseline SCE frequencies (Table 1). No evidence of homogeneously staining regions or double minute chromosomes was seen in the karyotypes of any of the resistant cell lines.

It is important to note that the culture time in bromodeoxyuridine required to visualize SCEs was the same for M and CR5 cells. Cells must incorporate bromodeoxyuridine into their DNA for two consecutive cell cycles and arrive at mitosis to permit the differential staining of chromosomes and so the determination of SCE frequencies. Consequently, the cell cycle times in the two cell lines must be similar. Since CR5 cultures double more slowly than M or the resistant cell lines CCR5, CCR10 and CCR20, the CR5 cells must be in a
state of normal cycle time but reduced proliferation due to a constant loss of cells from the population. This implies that not all the progeny of a CR5 cell are tolerant to similar levels of Cd\textsuperscript{2+}; many become non-viable. Confirmation of this idea comes from time-lapse video recording and analysis of normal and CR5 cells.

**Living cell behaviour**

Analysis of video recordings of normal M cells in culture revealed the following points: (1) little movement of cells over the culture dish surface. (2) All divisions attempted were successful and normal (i.e. cell rounds up, often with chromosomes becoming visible at the spindle equator, divides into two equally sized daughter cells, daughters move away from each other and flatten, see Fig. 3). No material was seen to be lost from the main cell body/bodies during division. On average, division took 3 h to complete (from first signs of cells rounding until the beginning of daughter cell flattening). (3) A total of 21% of the cells analysed divided during the recording period.

Some striking differences, particularly in cell division behaviour, were noted when CR5 cells were observed. A slightly lower proportion of cells attempted division (14%). Of these 38% showed some form of abnormality. Four types of abnormal division were observed in video recordings of CR5 cells. (1) Cell rounds up and reaches a metaphase-like stage; when attempting to divide, the cell 'writhes', putting out and retracting blebs of various shapes and sizes; cell shape is abnormal and rapidly changing during this phase. After a variable length of time (always longer than 2.5 h) the cell ceases to move, remains rounded and in some instances detaches from the substratum. These cells do not resettle during the course of recording. (2) Cell rounds up and goes through an extended and abnormal metaphase similar to that described above. Eventually the cell ressetles as a single multinucleate cell. (3) Cell rounds and divides as normal but one daughter cell fails to settle after mitosis. (4) Division is essentially normal but some bundles of cytoplasm are lost during mitosis. Fig. 3 shows tracings of cells typifying normal division and some of the abnormal classes of division. Normal divisions in CR5 took, on average, 2 h 45 min. Abnormal divisions were protracted and variable in duration. An indication of time is given in the panels of Fig. 3. A summary of the video analysis can be seen in Table 2. These abnormalities in division in CR5 can account for the slow growth of the cell line while it maintained an approximately normal cell cycle duration. In addition, the division abnormalities suggest a mechanism for the generation and maintenance of the high level of polyploidy in this cell line.

**Cell morphology and transformation**

During the development of resistance several changes in cell morphology were noted. M and CR5 grow as monolayers of well-flattened cells firmly attached to their substratum. Little evidence of cell overlapping or piling up was noted. CCR5 and CCR10 have a more elongate, fusiform morphology. As a consequence of this change in morphology, the cells lie closer together and so grow to higher densities. Additionally, the cells appear to be less firmly attached to their culture dish surface as the length of trypsin treatment required to detach them is significantly less than that needed for M and CR5. There is a slight tendency for these cells to form diffuse foci but the piling of cells is not a striking feature. The tendency to focus formation is very prominent in CCR20; distinct foci composed of many layers of cells are common. Even at low cell densities foci are formed leaving areas of culture dish free of cells. Clearly there is no contact inhibition of growth in CCR20.

In themselves, the changes in cell morphology described above are indicative of transformation. To confirm whether or not true in vitro morphological transformation had occurred, two assays: (1) growth in soft agar and (2) concanavalin A agglutinability, were employed to compare the relative extent of transformation in the five cell lines. Results of these assays are seen in Table 3. Assay (1) shows that M cells do not have the anchorage-independent growth phenotype, while CR5, CCR5, CCR10 and CCR20 are positive for this trait. Furthermore, each increment in cadmium resistance is accompanied by an increased ability to grow in soft agar. The differences in frequency of anchorage-independent colonies among the four cadmium-grown cell lines are similar to those found with anchorage-independent lines of human cells (Newbold et al. 1982). Assay (2) data for lectin agglutination reveal the same trends as the soft agar growth results, i.e. the four resistant lines show a progressive increase in their agglutinability. Again the implication is that there is a progressive increase in in vitro transformation status associated with increased cadmium resistance.

**Table 1. Chromosomal aberration and SCE in cadmium-resistant muntjac cells**

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>Aberrations*</th>
<th>SCE per chromosome</th>
</tr>
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<tbody>
<tr>
<td>Cell line</td>
<td>CDG</td>
<td>CDB</td>
</tr>
<tr>
<td>M</td>
<td>7</td>
<td>6-9</td>
</tr>
<tr>
<td>CR5</td>
<td>9</td>
<td>3-27</td>
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<tr>
<td>CCR5</td>
<td>7</td>
<td>5-9</td>
</tr>
<tr>
<td>CCR10</td>
<td>7</td>
<td>5-9</td>
</tr>
<tr>
<td>CCR20</td>
<td>7</td>
<td>5-9</td>
</tr>
</tbody>
</table>

*CDG, chromatid gap; CDB, chromatid break; CSG, chromosome gap; CSB, chromosome break.

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Fig. 3. Tracings of cell outlines from time-lapse video recordings of normal and abnormal types of cell division. See the text for details of the types of abnormal division. Numbers on the top right-hand side of each panel refer to the sequence of panels, those at the bottom on the left indicate real time in h and min.

Discussion

The Indian muntjac cell line employed in this study is highly sensitive to cadmium, 0-1 μM is the maximum concentration tolerated on continuous exposure. It has been possible over a period of five years to isolate and maintain variant cell populations resistant to continuous growth in cadmium concentrations of up to 200 times the parent cell tolerance. The biochemical and cellular basis of this cadmium resistance is the subject of a further paper (Ord et al. 1988).

The finding that massive chromosomal rearrangement occurred in the original cadmium-tolerant cell line but diminished as cells became resistant is unlikely to be unique to this system. However, there are few other reports available for comparison that examine the nature of cellular changes occurring during the development of cadmium resistance. Published studies, in particular those on metallothionein induction and synthesis, generally concern the characteristics of the final resistant cell lines rather than the intermediate lines produced during the development of resistance. None involves the development of resistance in cervine cells. Extensive chromosomal rearrangement has been implicated in the development of resistance to other chemicals, e.g. methotrexate (Morgan et al. 1986), and could be more common in the development of resistance to cadmium than suggested in the literature. Instability of the genome is common in virally transformed cells, including an SV40-transformed Indian muntjac line (Pillidge et al. 1986).

Of equal interest here is the stabilizing of the genome once cadmium resistance is established. It suggests that in CR5, where each change of medium and cadmium brings fresh waves of nuclear and cytoplasmic damage (Ord et al. 1988), chromosome damage is maintained at a high but, at least for some of the population, sub-lethal level. Once resistance is achieved the genome is able to return to a relatively normal state. The recurrence of increased chromosome number in the cells recently shifted to 50 μM-cadmium suggests that this cadmium concentration is beyond the level that can be handled by the mechanism in use in CCR5, CCR10 and CCR20.

The finding of complex genome rearrangements during the early stages in the development of resistance led

<table>
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<th>Table 2. Summary of time-lapse video analysis data</th>
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<tbody>
<tr>
<td><strong>Cell type</strong></td>
</tr>
<tr>
<td>CR5</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td><strong>Abnormal divisions</strong></td>
</tr>
<tr>
<td></td>
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<tr>
<td>0</td>
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<td>2</td>
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us to examine whether there were more general cellular consequences of long-term exposure to cadmium. Cadmium has been implicated in the development of some human and animal cancers, for example prostate (see Kipling & Waterhouse, 1969), testicular (Gunn et al. 1963), lung (Takenaka et al. 1985) and muscle (Heath et al. 1962), though it is not considered a general carcinogen under most conditions. Transformation of cells in vitro is a process bearing many similarities to tumour formation in whole animals. In vitro transformation is known to be a multistep process. Newbold et al. (1982), using Syrian hamster embryo cells, have shown the initial step in the transformation of rodent cells is the release from senescence (i.e. immortalization) followed by the acquisition of anchorage-independent growth. The parental Indian muntjac skin fibroblast line is immortal, so has completed this step in transformation, but the soft agar growth data presented here demonstrate that their growth is anchorage-dependent. All three cadmium-resistant cell lines CCR5, CCR10 and CCR20 have acquired the anchorage-independent growth phenotype, results showing an increasing number of colonies with increasing cadmium resistance.

Other characteristics of these cells suggest that in vitro transformation has occurred during the acquisition of cadmium resistance. They are more readily agglutinated by concanavalin A in comparison to M (Table 1). The original flat well-spread cell has become fusiform, attachment to the substratum is much weaker and CCR20 cells form foci. All these are characteristics of cells transformed in vitro and so indicate a progression along the pathway of transformation during cadmium exposure. As the cadmium sensitivity of SVM, the SV40-transformed muntjac cell line, is similar to that of M cells, cadmium resistance does not appear to be a common feature of transformed muntjac cells. However, the specificity of the association of transformation with cadmium resistance is less clear. It is possible that long-term selection of cells in any toxic agent might also select for the transformed phenotype. We are not aware of any studies that address this possibility either in muntjac or cells of any other species.

It is well documented that short-term exposure to cadmium can cause morphological transformation of hamster cells, a property common to many metals (Heck & Costa, 1982). This report shows unequivocally that long-term exposure to low levels of cadmium can lead to cell transformation in addition to cadmium resistance. This could be of importance, considering the long-term, low-level cadmium exposure in certain working environments and the population as a whole. The nature of both the resistance and transformation by cadmium is now being explored at the molecular level using the five lines of muntjac fibroblasts described in this study.

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### References


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