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Cadmium-induced multistep transformation of cultured Indian muntjac skin fibroblasts

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Summary. During the past five years we have made a series of cadmium-transformed and resistant fibroblast cell lines by continuous low-level exposure to cadmium. In the present paper we describe the use of four of these lines with varying degrees of transformation to investigate the multistep nature of cadmium carcinogenesis. These include: (a) M cell, an immortal but non-transformed muntjac skin fibroblast line; (b) CCR5, a morphologically transformed and cadmium-resistant line derived from M cells after 20-months continuous exposure to small step-wise increases in cadmium; (c) SCR5, a tumorigenic line derived by selection (in the absence of cadmium) of rapidly growing CCR5 agar colonies; (d) T1, a line derived from an SCR5 tumour growing in a nude mouse. We have compared the morphological characteristics of the four cell lines using light and electron microscopy and evaluated their ability to grow in liquid culture, soft agar and nude mice. We have also examined the changes which have occurred in their cytoskeletons and extracellular matrices using fluorescent antibodies to actin, tubulin and fibronectin and related these to the strength of their cell-cell and cell-substrate attachments and to their levels of transformation and tumorigenesis. We have shown that, while some changes occur in a single step (e.g. intracellular cytoskeletal changes), others are gradual (e.g. changes in extracellular matrix, focus formation and ability to grow in soft agar). We conclude that continuous exposure to low levels of cadmium can initiate growth and structural changes which subsequently lead to cell transformation and tumorigenesis on the removal of cadmium. Though change with cadmium was slow, many of the transformed characteristics are similar to those reported for viral and chemically transformed cells.

Key words: Cadmium - In vitro - Muntjac - Cytoskeleton - Transformation - Extracellular matrix

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Introduction

Reports to date indicate that cadmium can perturb both the structural and functional organization of the mammalian genome (Deavan and Campbell 1980; Hsie et al. 1978), cause chromosomal damage (Deavan and Campbell 1980) and increase mutation frequency at an X-chromosome-linked genetic locus (Hsie et al. 1978). There are also a small but growing number of studies in which cadmium has caused transformation of cultured cells (Dipaolo and Casto 1979) and induced tumours in rats and mice (Poirier et al. 1983; Takenaka et al. 1983) and some indication that cadmium, possibly in combination with other metals and/or chemicals, may cause tumours in man (Lauwerys 1979; Samarawickrama 1979). Yet in spite of the vast number of studies on cadmium, conclusive evidence of its direct role in the multistep process of carcinogenesis remains obscure. Since cadmium is one of our most common environmental pollutants, a clearer understanding of its carcinogenic potential is necessary, particularly under conditions of continuous exposure or in the presence of other metals. We are attempting to redress this problem by examining step-by-step changes which occur in cultured cells during low but continuous cadmium exposure.

Most in vitro transformation studies, including those examining the effects of metals, have been carried out on embryonic cells, generally rodent cells which are known to transform rapidly (Costa and Heck 1982; Meyer 1983; Landolph 1989), but to follow step-by-step changes we required a slow movement towards the transformed state. Furthermore, rodent cells contain large numbers of small chromosomes which are difficult to analyse for specific chromosome aberrations: our intention is to try to link steps in transformation with specific chromosome changes. Lastly, evaluation of the final transformation step is generally made in nude mice: we wanted to be able to separate our donor cells from any contributing host cells in tumour formation. In response to these special requirements, we have deviated from the common protocol used in transformation studies and are using low continuous cadmium

exposures with Indian muntjac cells. Like 3T3, C3H/101/2, SHE and BHK cells, our muntjac skin fibroblasts (M cells) are immortal but non-transformed, exhibit strong post-confluent-growth inhibition and are anchorage-dependent; they have a low spontaneous transformation rate, a stable phenotype and a well defined cytoskeleton. In contrast to the rodent cells, they have a diploid complement of only seven chromosomes, each with a distinctive morphology and a stable G-banding pattern.

We have now developed a number of cell lines with different levels of cadmium resistance and cell transformation using continuous exposure of M cells to low levels of cadmium. In the present study, we have examined four of the lines with different levels of transformation, focusing on changes in the cytoskeleton and relating them to changes in cell growth and behaviour. We have shown that on developing a transformed phenotype, M cells loose their density-dependent growth regulation and acquire anchorage-independence. Coincident with this, we observed a number of cytoskeleton, membrane and extracellular matrix changes which we believe are responsible for the marked changes in cell-to-cell and cell-to-substratum relationships. Many, though not all, of these changes have been reported for cells exposed to other transforming agents.

Materials and methods

Cell lines and culture conditions

The work described in this paper has been carried out using a male Indian muntjac skin fibroblast culture (ATCC number, CCL157, originally obtained from Flow Laboratories), a stable, contact-inhibited anchorage-dependent cell with a diploid genome 2N=7 (our M cell). The morphologically transformed and cadmium-resistant line, CCR5, was derived following continuous exposure of M cells to step-wise increases of cadmium chloride from 0.1 μM to 5 μM over a 20-month period (Boufler and Ord 1988). Each step-up in cadmium concentration was made only when cells were well adjusted to their medium + cadmium environment and was kept small enough to allow the major portion of the population to survive. Cells took on a transformed morphology at the same time as they acquired resistance to 5 μM cadmium. Increases in cadmium concentrations were continued for some cultures from which cell lines resistant to 10, 20 and 50 μM cadmium were obtained (Ord et al. 1990), while others were moved to medium without cadmium. The transformed line, SCR5, was obtained as follows: 50 CCR5 colonies from single cells plated out in soft agar without cadmium were isolated and placed in multiwell dishes; three of the most vigorously growing well cultures were moved to a flask and established as the transformed SCR5 line. The T1 cell line was derived from the first SCR5 tumour of a nude mouse. The relationship of these four lines is shown in Fig. 1. All cells were grown in minimal essential medium supplemented with 10% foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cultures were passaged once a week on plastic at a split ratio of 1:3. Generation times and saturation densities were determined by counting cells in a haemocytometer at regular intervals after plating 10^4 cells/3-cm culture dish.

Soft agar assay. Anchorage-independent growth was evaluated by growing cells in soft agar (Bacto-Difco). 10^4 cells in 1.5 ml medium with 0.35% agar were mixed and overlaid on a 4-ml basal layer of 0.6% agar in 6-cm culture dishes. Four replicate cultures were prepared for each cell line. After allowing the agar to set at room temperature, the dishes were incubated at 37° C and fed

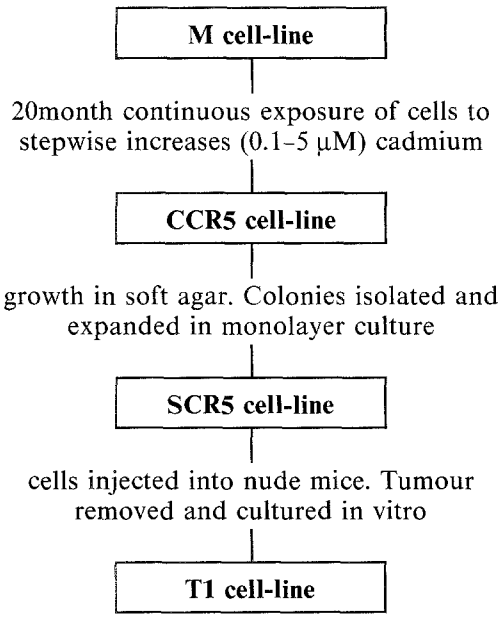

Fig. 1. Relationship between the four cell lines used in the present study

Table 1. Summary of some phenotypic properties of M, CCR5, SCR5 and T1 cells

Property	M cell	CCR5	SCR5	T1
Shape	Flat	Spindle	Spindle	Spindle
Focus morphology	Nil	type I	type II/III	type II
Generation time	54 h	54 h	32 h	32 h
Saturation density	1.4×10^5 cm^{-2}	1.9×10^5 cm^{-2}	2.3×10^5 cm^{-2}	1.2×10^5 cm^{-2}
Growth in soft agar	0	13	33	15
No. of tumours in nude mice/total no. of nude mice injected	0/12	0/6	5/12[a]	2/7[b]

Saturation density is expressed as the average number of cells/cm^2 in triplicate culture. Growth in soft agar was measured as colonies/10^4 cells, averages of 2–4 plates/experiment repeated four times. All tumours were fibrosarcomas

[a] One of the five mice had two tumours

[b] In five mice T1 was repassaged in the form of tumour pieces and in two animals T1 cells were used; the two tumours were one from each group

with small amounts of medium every 2 weeks. Colony formation was determined after 6–8 weeks.

Tumorigenicity in nude mice. Nude mice were inoculated subcutaneously on the shoulder with approximately 2×10^6 cells. The mice were examined regularly for tumor formation. Animals bearing tumours were killed and the tumours removed under sterile conditions. Part of each tumour was removed for histopathological examination; the remainder was finely minced with scapels, trypsinized in (10×) trypsin EDTA (Gibco) and placed in 25-cm^2 culture flasks to establish tumour cell lines.

Immunological localization of actin, tubulin and fibronectin. Cells were cultured on 22-mm glass coverslips at least 48 h before immunolabelling experiments. For staining of actin and tubulin, the cells were fixed for 20 min at room temperature with 3.7% formaldehyde in phosphate-buffered saline ($NaCl/P_i$) at pH 7.6, washed three times in $NaCl/P_i$ and permeabilised by treatment with 0.1% Triton X-100 for 15 min. After three washes in $NaCl/P_i$, coverslips were incubated at 37° C with either chicken anti-actin (Polysciences) diluted 1:10, or rabbit anti-tubulin (Polysciences) diluted 1:50, washed three times in $NaCl/P_i$ and treated with fluorescein-conjugated anti-(rabbit IgG) (Sigma) diluted 1:32. After further washes in $NaCl/P_i$, coverslips were mounted on glass slides with Citifluor and examined with an Olympus fluorescence microscope. For immunofluorescent labelling of fibronectin, cells were fixed as for actin and tubulin but were not permeabilized by detergent treatment. After fixation they were washed in $NaCl/P_i$ and incubated at room temperature with anti-(bovine fibronectin) (UCB Ltd, Belgium) diluted 1:32, washed in $NaCl/P_i$ and treated with fluorescein-labelled anti-(rabbit IgG) (Sigma) diluted 1:32.

Isolation of the pericellular matrix. The method of Hedman et al. (1979) was used to isolate the pericellular matrix from cultures of M, CCR5, SCR5 and T1 cells. Briefly, cells were cultured on glass coverslips to near confluency, washed three times in $NaCl/P_i$ treated for three 10-min periods at 0° C with 0.5% sodium deoxycholate in 10 mM Tris Cl-buffered saline, pH 8.0. The cells were then treated for a further three 5-min periods at 0° C with 2 mM Tris Cl pH 8.0. The pericellular matrices on coverslips were fixed for 30 min with 3.7% formaldehyde, washed in $NaCl/P_i$ and stained with anti-(bovine fibronectin) as described above.

Ultrastructural studies. Cells for electron microscopy were fixed in Karnovsky's fixative for 1 h, double fixed for a second 1 h period in 2% osmic acid, and embedded in Spurr resin following alcohol dehydration. For further details see Ord et al. (1988). Thin sections were cut with a Dupont diamond knife, stained with 2% uranyl acetate followed by lead acetate and examines using a Jeol 1200 EX electron microscope.

Results

Growth changes induced by cadmium treatment

M cells are well spread and flattened on their substratum; they exhibit a high degree of contact inhibition and proliferate slowly with a generation time of 54 h. The cadmium-transformed cells, CCR5, SCR5 and T1, are phenotypically distinct from the parent M cell. They are smaller, spindle-shaped and have a higher nu-

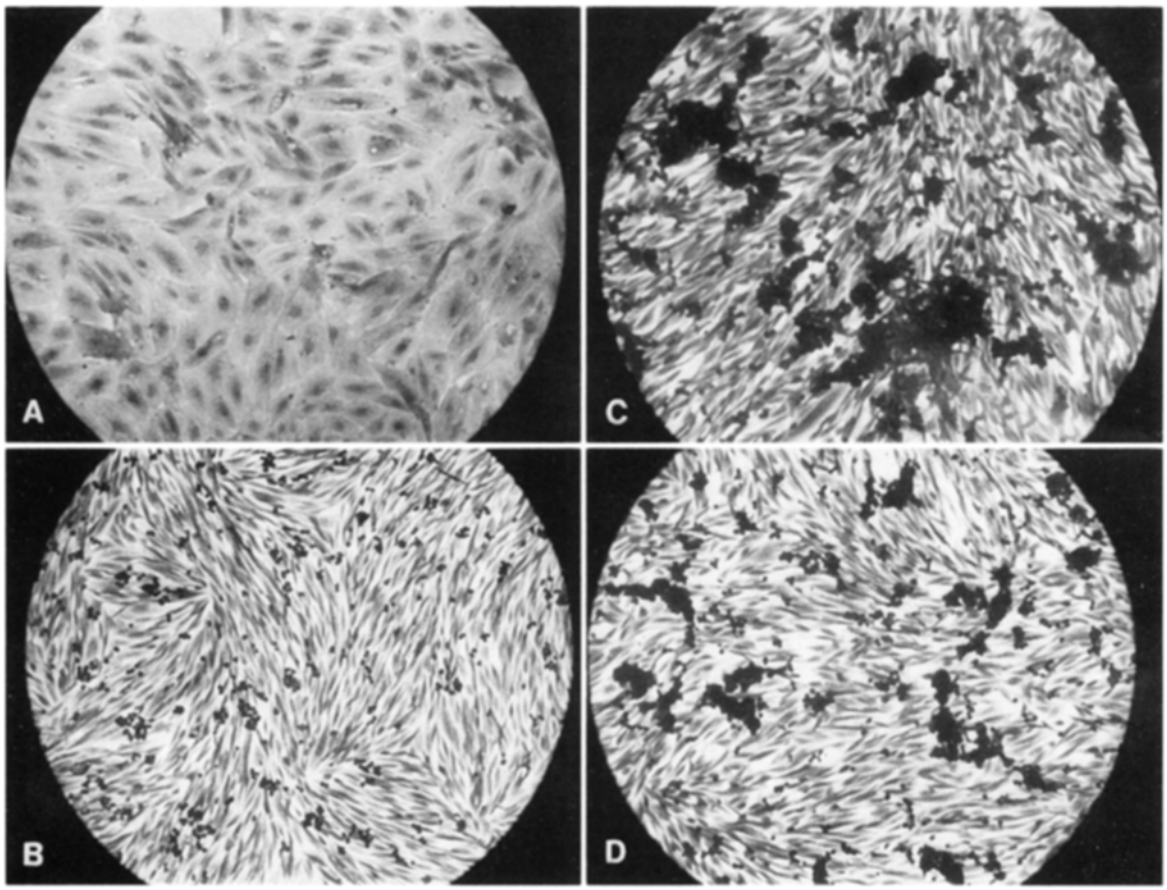

Fig. 2. Morphology of confluent cultures of the four muntjac cell lines: (**A**) M cells, (**B**) CCR5 cells, (**C**) SCR5 cells, (**D**) T1 cells. Cells were grown to near confluency, fixed with formaldehyde and stained with 0.2% Coomassie blue. Dark-stained areas are overlaying foci. (Magnification ×60)

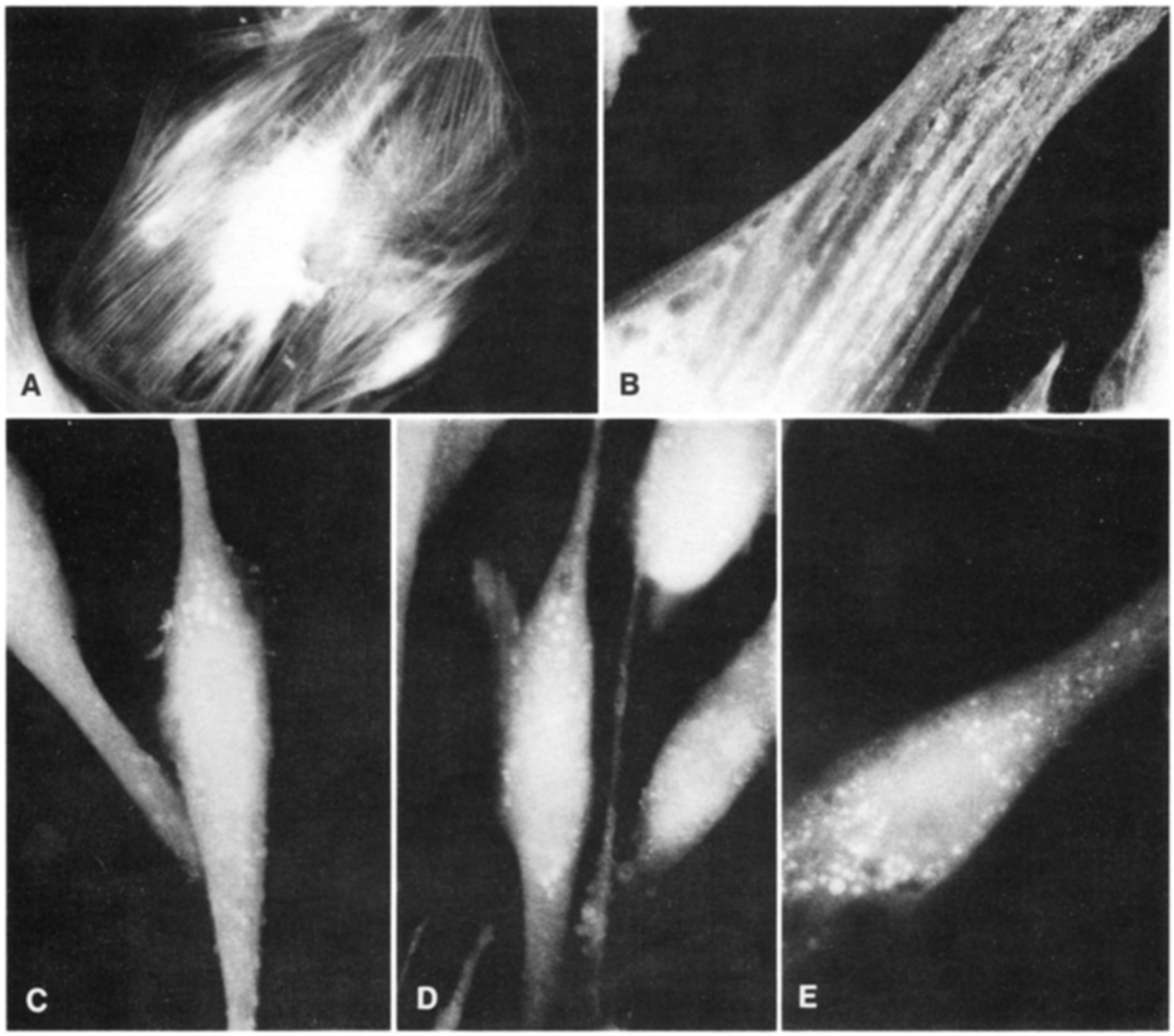

Fig. 3. Immunofluorescent staining of actin in (**A**) M cell (×380), (**B**) M cell (×950) and (**C**) CCR5 cell, (**D**) SCR5 cell, and (**E**) T1 cell (all ×950). The long well-organized actin-containing filaments typical of M cells are absent in the transformed cells which instead show a diffuse background staining with bright fluorescence coming from an array of actin patches

clear cytoplasmic ratio. While the mean generation times for SCR5 and T1 cells became significantly shorter, i.e. approximately 32 h (see Table 1), that of CCR5 cells remained similar to the parent non-transformed M cell. The growth patterns for M and the transformed lines differ markedly. In confluent monolayer cultures, M cells show no sign of cell–cell contacts or of foci formation (Fig. 2A). The cadmium-transformed cell lines, on the other hand, all show intercellular associations with different degrees of focus formation (Fig. 2B–D). Using the classification of Reznikoff et al. (1973), we define these foci as type I for CCR5, i.e. foci of tightly packed cells with diffuse boundaries, and as type II/III for SCR5, i.e. multilayered foci with distinct boundaries which are formed by localized piling-up of rounded cells. In addition, dense cultures of SCR5 cells display the criss-cross pattern of cell arangement typical of most transformed cells in culture while the CCR5 cells do not. T1 cells form mainly type II foci even at low cell densities. Monolayer cultures of T1 cells are unstable, continually shedding viable cells to the growth medium and seldom forming confluent cultures.

To help define a level of transformation for the four cell lines, we examined their saturation densities. The saturation densities for CCR5 and SCR5 cells were higher than M cells (Table 1). Thus 7 days after plating 10^4 cells/3-cm dish, CCR5 and SCR5 cells reached saturation densities of 1.9×10^5 and 2.3×10^5 cells/cm^2 respectively, compared with 1.4×10^5 cells/cm^2 for M cells. T1 cells, with their habit of cell shedding, grow to a saturation density of only 1.2×10^5 cells/cm^2.

Since growth of cells in soft agar is the event most closely correlated with tumorigenicity in rat and mouse transformants (Shin et al. 1975), we assayed anchorage-independent growth for the muntjac cell lines. Approximately 10^4 cells were plated in soft agar and examined macroscopically after 6–8 weeks in culture. While M cells never formed colonies in soft agar, the transformed cell lines, CCR5, SCR5 and T1 cells, formed colonies at frequencies of 13, 33 and 15 per 10^4 cells,

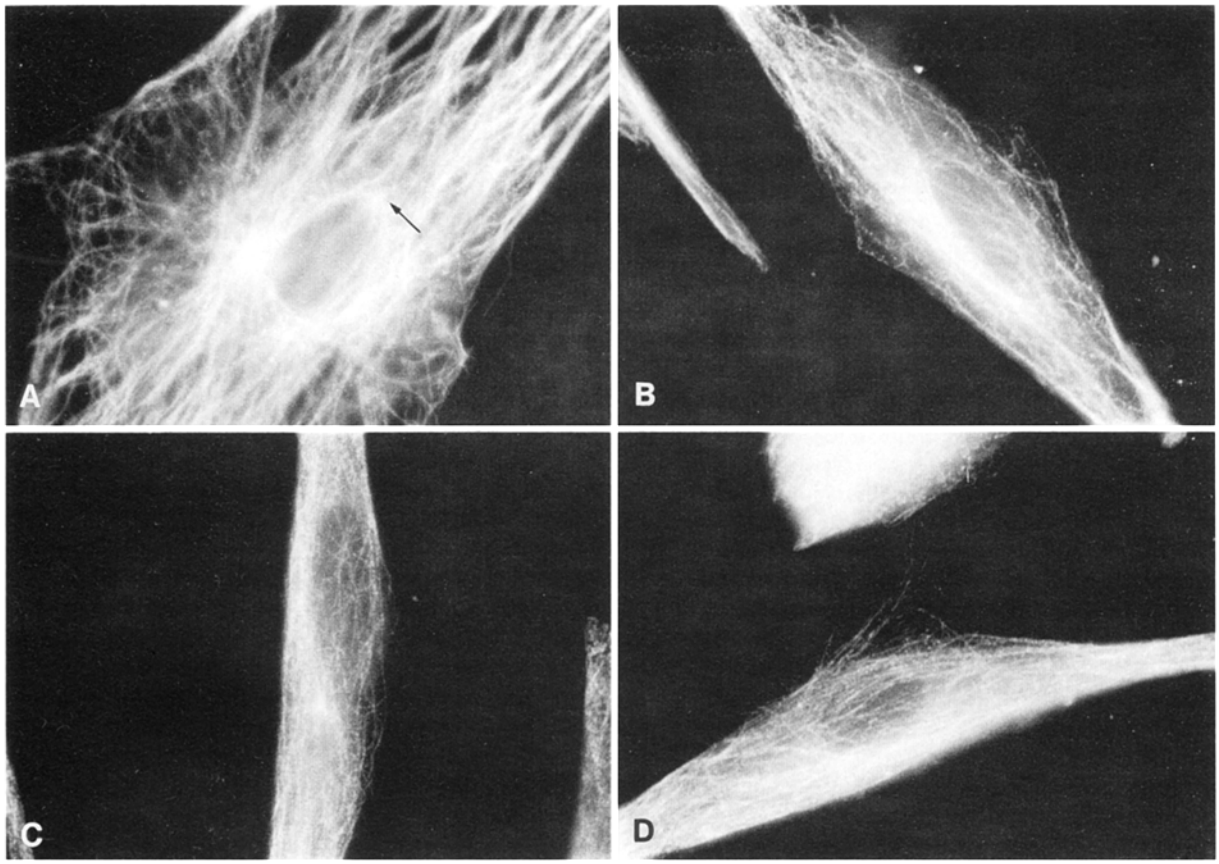

Fig. 4. Immunofluorescent localization of microtubules in the various muntjac cell types: (**A**) M cell, showing numerous cytoplasmic microtubules that radiate from the nucleus and the microtubule organizing centre lying adjacent to the nucleus (arrow); (**B**) CCR5; (**C**) SCR5 and (**D**) T1 cells, showing that extensive networks of microtubules are still present in transformed cells. (Magnification ×950)

respectively; i.e. they all contained some cells competent for anchorage-independent growth though, unexpectedly, T1 cells grew less well in soft agar than SCR5.

To evaluate the tumorigenic potential of the cell lines, approximately 2×10^6 cells were injected into nude mice (a minimum of six mice/cell line) and examined periodically for evidence of tumour formation. The results are tabulated in Table 1. None of the mice receiving M or CCR5 cells developed tumours. In contrast, 4 of 12 mice injected with SCR5 cells, and 2 of 7 mice injected with T1 cells, developed muntjac cell tumours.

Cytoskeletal changes induced by cadmium treatment

Figure 3 shows the actin patterns of the parental M and the cadmium-transformed cell lines obtained using fluorescently labelled anti-actin. In the M cell a multitude of long, well organized actin-containing filaments run parallel to the cell's long axis, while bundles of stress fibres are also oriented in the direction of tension exerted on the substratum (Fig. 3A, B). The transformed variants lack these filaments (Fig. 3 C–E). Instead, they show diffuse staining with bright fluorescence coming from an array of actin patches concentrated particularly in the perinuclear region. No attempt has been made to count these patches or correlate numbers with tumorigenicity since variation among cells of any one line is possibly as great as between the transformed cell lines themselves.

The immunofluorescent staining patterns for tubulin in the four cell lines are shown in Fig. 4. The thinly spread cytoplasm of M cells is laced with numerous fluorescent tracts, many of which appear to run from the perinuclear area to the plasma membrane (Fig. 4 A). Significant numbers of microtubules can also be seen running through the cytoplasm of transformed cells (Fig. 4 B–D) but with little difference between the patterns of CCR5, SCR5 and T1 cells. The very different patterns for M and transformed cells was coincidental with the change in form from a thin flat morphology to a much thicker fusiform cell. This thicker cell morphology makes visualization and photographic documentation of microtubules much more difficult to evaluate and quantification was impossible.

Changes at the cell surface and in the extracellular fibronectin matrix produced by cadmium treatment

Electron microscope examination shows that the prominent glycocalyx covering both dorsal and ventral cell

surfaces of M cells is greatly reduced on cadmium-mediated transformation (Fig. 5). One of the most important proteins of the glycocalyx is fibronectin. Results of immunofluorescent staining for extracellular fibronectin are shown in Figs. 6 and 7. In subconfluent M cells the fluorescence is localized to fibrils and aggregates between and under cells (Fig. 6 A–C). In CCR5 cells, fibrils and aggregates are greatly reduced, appearing in only small quantities in areas of cell-to-cell and cell-to-substratum contacts (Fig. 7 A). In SCR5 and T1 cells fluorescent patches and fibrils are missing altogether (Fig. 7 B, C).

Figure 8 compares the distribution of fibronectin in the pericellular matrix isolated from confluent cultures of M, CCR5, SCR5 and T1 cells. The matrix derived from confluent cultures of M cells has a fibrillar structure in which fibronectin is extensively distributed (Fig. 8 A). In contrast, the pericellular matrices of the transformed cells show a progressive reduction in quantity with increasing levels of transformation and tumorigenicity (Fig. 8 B–D).

Discussion

On treatment with cadmium, M cells underwent alterations in cell shape and in cell–cell and cell-substrate interactions coincident with changes in their organization, growth pattern and cell density. Light, fluorescent and electron microscopy studies suggest, that these changes were related to cadmium-induced changes in the organization of the stress fibres, the cell membrane and the extracellular matrix. The first step in transformation (i.e. from the well-spread, contact-inhibited M cell to the fusiform, morphologically transformed CCR5) did not prove tumorigenic, indicating that the acquisition of a transformed phenotype was not in itself proof of chemically induced neoplastic transformation. The presence of a small agar colony-forming fraction within the CCR5 line, however, suggested that this line was heterogeneous and contained a small subpopulation of transformed cells. Selection of these cells gave the transformed and tumorigenic SCR5 cell line. A second selection step following growth in a nude mouse, T1 cells, did not increase the independent growth potential of SCR5 cells. The relationships of the different transformation form and growth characteristics of M, CCR5, SCR5 and T1 cells have been set out in Table 2.

Dramatic changes in stress fibres, with a reduction and/or disorganization of actin-containing structures (Verdarame et al. 1980; Pollack et al. 1976; Carley et al. 1981), microtubules (Brinkley et al. 1975) and membrane proteins (Hynes and Yamada 1982; Alitalo and Vaheri 1982; Vaheri et al. 1978) have been reported to accompany viral and chemical transformation in a

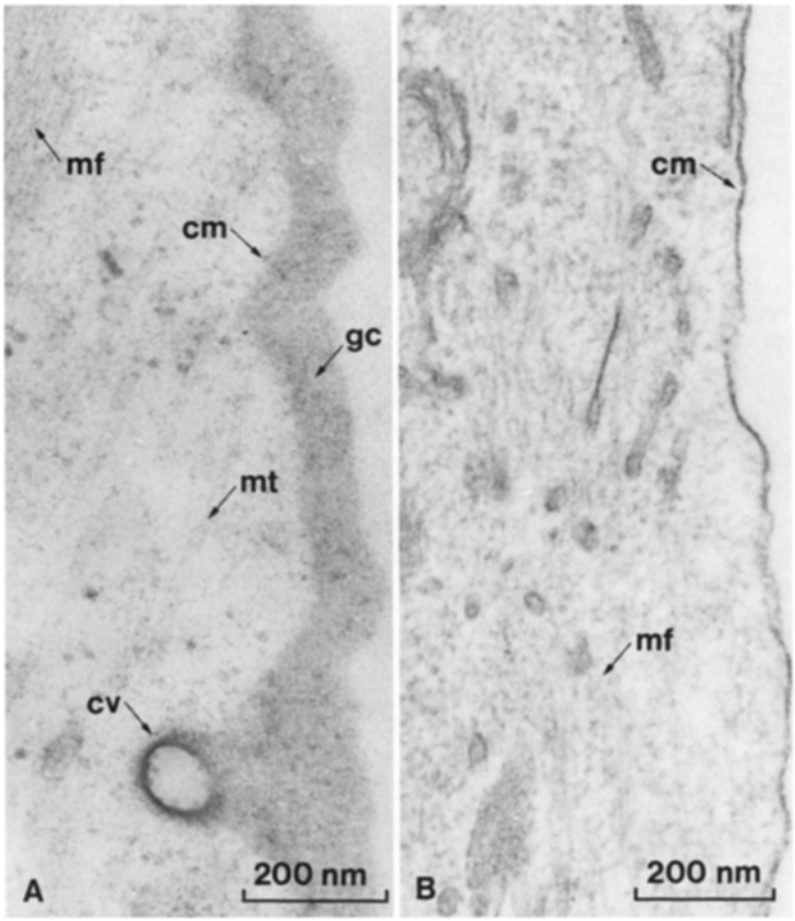

Fig. 5. Section through an M cell (**A**) and a CCR5 cell (**B**) to show glycocalyx change. The M cell has a thick glycocalyx (gc); note the bundled microfilaments (mf) and accompanying microtubules (mt) which run to the cell membrane (cm); also the coated vesicle (cv) forming in the membrane. (B) Section through a CCR5 cell shows no glycocalyx on the outer surface of the cell membrane (cm); the very thin glycocalyx of these cells is only visible when sections cut obliquely across the membrane; the single strand microfilament network (mf) is just visible in the cytoplasm

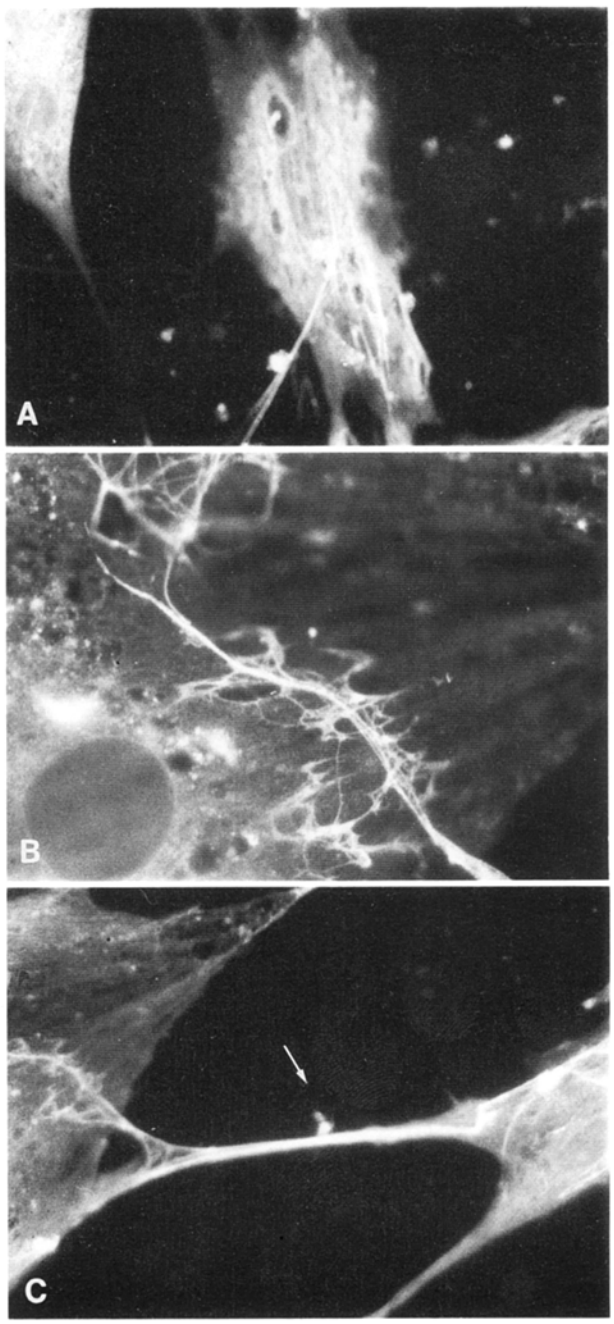

Fig. 6. Immunofluorescent localization of extracellular fibronectin in M cells. Cells were grown on coverslips for 48 h, fixed with formaldehyde, incubated with antibovine fibronectin, then with fluorescein labelled anti-(rabbit IgG): (**A**) focused on the cell (×380); (**B**) focused on the plane of the substratum (×950); (**C**) focused high on the cells to show fibronectin fibrils bridging between two cells (*arrow*) (×950)

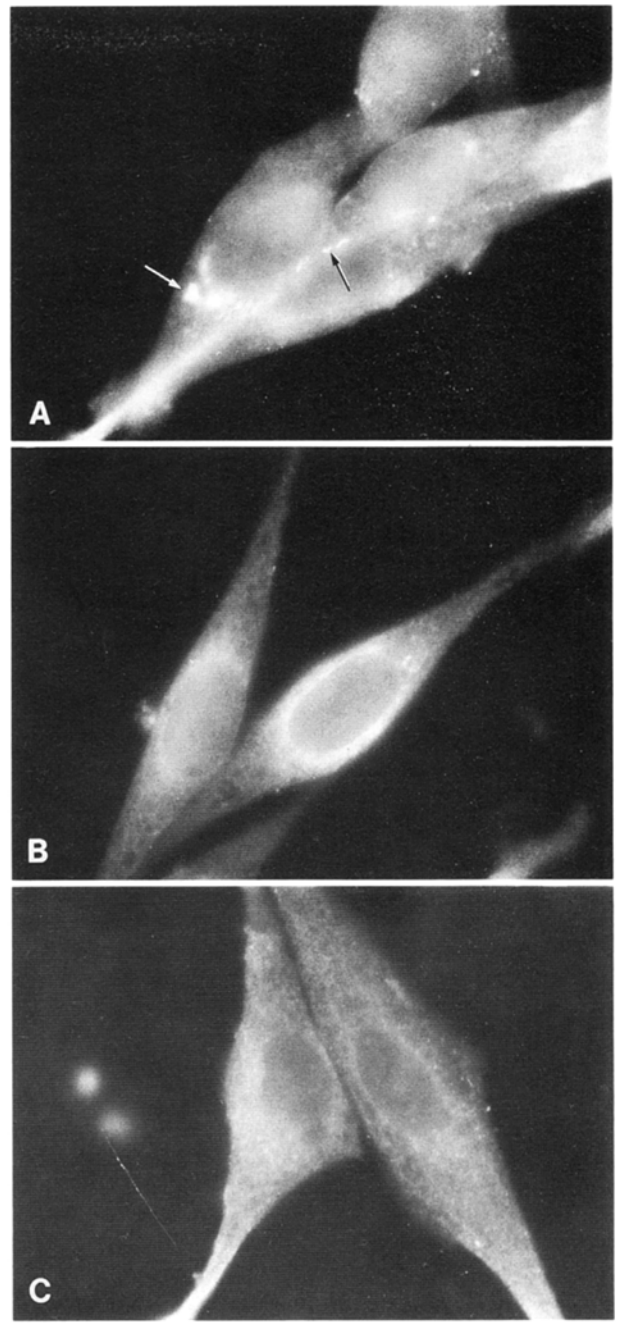

Fig. 7. Immunofluorescent localization of extracellular fibronectin in the muntjac transformed cell types. (**A**) CCR5 cells with small patches of fibronectin present between cells and forming cell-to-substratum contacts (*arrows*); (**B**) SCR5 cells; (**C**) T1 cells. (Magnification ×950)

number of cell types. Most of these changes were observed in M cells as they assumed a fusiform morphology on transformation with cadmium. Cadmium treatment of muntjac cells showed that the breakdown of M cell actin occurred as a single step between the M and the CCR5 cell lines and resembled that reported by Carley et al. (1981) for virally and chemically transformed rat kidney cells, i.e. the breakdown of actin cables and filaments into numerous actin aggregates. No further changes could be detected as cells became fully transformed and tumorigenic. The change in microtubules has been far less marked than that of actin, even in the tumorigenic SCR5 and T1 cells. In this respect the cadmium-transformed cytoskeleton may deviate from that reported for many other transformed cell lines (Brinkley et al. 1975).

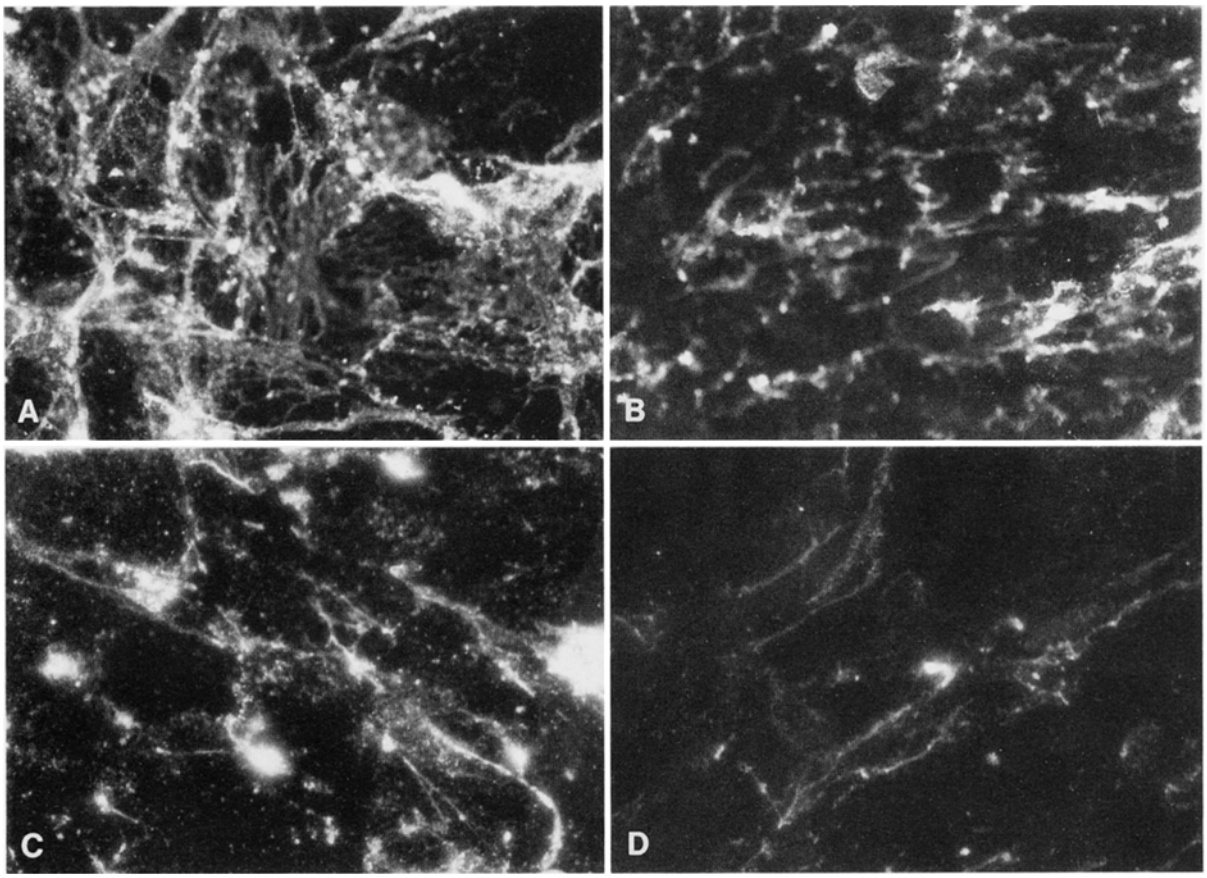

Fig. 8. Fibronectin immunofluorescence of pericellular matrix prepared from confluent cultures of (**A**) M cells, (**B**) CCR5 cells, (**C**) SCR5 cells, (**D**) T1 cells, using the method of Hedman et al. (1979). (Magnification ×190)

In addition to the cytoskeletal changes which were responsible for the changed phenotype of CCR 5 cells and all subsequent muntjac transformed lines, changes also occurred in the extracellular matrix proteins. Fluorescent tagging of fibronectin showed that cadmium transformation was associated with a gradual decrease in cell-surface fibronectin similar to that reported for cells transformed by other agents (Alitalo and Vaheri 1982; Yogeeswaran 1983). This change was coincident with the appearance of direct attachments between cells. In addition, a progressive reduction occurred in the amount of fibronectin deposited in the pericellular matrix. This was coincident with a weakening of cell-to-substratum attachment. The matrix of M cells consists of a stable cloth-like structure in which fibronectin is extensively distributed; this matrix decreases with increasing transformation, reaching its lowest level in the T1 cells. A lack of pericellular matrix probably accounts for two characteristics peculiar to T1 cells: the presence of large numbers of round (but viable) cells and continual shedding of T1 cells from the growth substratum. The relationship of cytoskeletal and extracellular protein characteristics of M, CCR5, SCR5 and T1 cells is shown in Table 2.

These experiments provide evidence for the carcinogenic potential of cadmium in vitro and suggest that although transformation by cadmium was gradual, most of the changes induced were similar to those reported for cells transformed in other ways. The main difference between our results and those reported for rodent cells exposed to carcinogens and/or toxic metals such as nickel or chrysotile (Borland and Hard 1974; Leonard et al. 1981; Costa and Heck 1982; Jaurand et al. 1984) is in the much longer time period required for changes to occur. We do not know yet whether the slow step-by-step movement towards transformation of muntjac cells was due to the use of skin fibroblasts rather than embryonic cells, to our cadmium treatment regime, i.e. low continuous exposure which caused mainly reversible cell damage (Ord et al. 1988), or to a fundamental difference between muntjac and rodent cells. Considerable evidence is accumulating linking neoplastic transformation with both mutation in oncogenes (Weinberg 1985; Bishop 1987) and loss of tumour suppressor genes (Sager 1989). The presence of cadmium in the growth medium caused significant perturbation of muntjac chromosomes, some repetitive chromosome abnormalities, and both loss and addition of whole chromosomes (Bouffler and Ord, 1988). We are now using comparisons of chromosome numbers and banding patterns to determine whether specific changes can be identified in association with transformation and tumorigenesis. We are also using M cells and 3T3 cells with other transforming agents to evalu-

Table 2. Relationships between some cell morphology and growth properties (A) and cytoskeletal and matrix properties (B) of the four cell lines, M cell, CCR5, SCR5 and T1

	Property	Difference
A.	Cell size	M > CCR5 > SCR5 > T1
	Cell cycle length	M = CCR5 > SCR5 = T1
	Glycocalyx	M > CCR5 = SCR5 = T1
	Saturation density	M < CCR5 < SCR5 > T1
	Focus formation	M < CCR5 < SCR5 > T1
	Growth in soft agar	M < CCR5 < SCR5 > T1
	Cell - substratum attachment	M > CCR5 > SCR5 > T1
	Cell - cell attachment	M < CCR5 < SCR5 = T1
B.	Actin cables	M > CCR5 = SCR5 = T1
	Microtubules	M > CCR5 = SCR5 = T1[a]
	ECM[b] fibronectin	M > CCR5 > SCR5 = T1
	Fibronectin of the pericellular matrix	M > CCR5 > SCR5 > T1

[a] Fluorescence studies (confirmed by electron microscopy) showed microtubules present in all four cell lines but the thickness of tumour cells and the greater size of the thinly spread M cells made quantitative evaluations of microtubules/cell impossible; examination at different focal levels suggest a difference in microtubule distribution as well as a decrease in numbers between M cells and the three transformed lines

[b] Extracellular matrix protein

ate the comparative responsiveness of Indian muntjac cells.

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