ISSN- 0102-9010

# THE INFLUENCE OF NUTRITIONAL STRESS ON THE DIFFERENTIATION OF EPITHELIAL CELLS *in vitro*

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## ABSTRACT

Madin-Darby canine kidney (MDCK) cells have a distinctive epithelioid morphology and display several functional and anatomical properties of normal kidney tubule cells. This cell line has been considered ideal for studying cell growth and regulation and for understanding the factors involved in the assembly of epithelial cells into organized multicellular units and tubulogenesis. In this work, we examined the effect of nutritional stress on the morphological and growth characteristics of MDCK cells. Control MDCK cells grew in monolayers but ceased to proliferate after confluence and eventually die. In contrast, nutritionally stressed cells showed continuous multilayered growth, with differentiation and the formation of tubular structures during prolonged culture, as well as enhanced polyploidy. Enhanced fibronectin, actin and vimentin deposition were observed in regions of cell-cell contact in stressed cells. These results indicate that nutritional stress may alter the interactions between extracellular matrix components, including fibronectin, and the cytoskeleton. Such alterations may be important in folding of the epithelial monolayer to form tubular structures.

**Key words*:*** Cellular transformation, MDCK, multilayer growth, nutritional stress polyploidy

## INTRODUCTION

Madin-Darby canine kidney (MDCK) cells, derived from the kidney of a normal Cocker Spaniel dog show monolayer growth in culture and have the morphological properties of distal tubular epithelial cells. This cell line retains the differentiated function of polarized cells, with the capacity for vectorial salt and fluid transport and the ability to regenerate tubule-like structures when injected into athymic nude mice [15]. Because of these properties, MDCK cells have been used as a model system for studying fundamental mechanisms of transepithelial transport, tubulogenesis and renal morphogenesis and differentiation *in vitro* [10,14].

Transepithelial transport occurs across monolayers of polarized epithelial cells in an apical to basolateral direction and produces droplets of fluid between the cell layer and the underlying impermeable surface. The presence of these structures, known as domes, in MDCK cells is indicative of transepi-thelial transport that depends on cell differentiation [9]. Various compounds have been identified as potent inducers of differentiation and of dome formation in confluent MDCK cells.

Changes in ionic composition and a decrease in pH may be associated with genotoxicity cell transformation [3-5], and certain types of stomach and urinary bladder tumors in mice [13,16]. In this work, we examined the effect of nutritional stress on dome formation in MDCK cells, and assessed the correlation between these changes and alterations in cytoskeletal and extracellular matrix components.

## MATERIAL AND METHODS

*Culture*

MDCK cells at passage 37 were obtained from the Adolfo Lutz Institute (São Paulo, Brazil), and maintained in Ham F10 medium (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 5% fetal calf serum (FCS; Nutricell, Campinas, SP, Brazil) at 37°C in a 5% $CO_2$ atmosphere. At the start of the incubation, the pH of the medium was adjusted to 7.2, which is ideal for MDCK cells (American Type Culture Collection recommendations). Confluent cells were cultured without replacing the culture medium for fifteen days. The pH of the medium after this incubation was 6.8. The stressed cells were then incubated with regular changes of the medium every 48 h.

*Growth Curve*

To examine the growth curve, control and stressed cells were seeded onto 35 mm tissue culture dishes at a density of $6 \times 10^3$ cells/dish (Corning, New York, NJ, USA) and maintained under

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normal culture conditions in a 5% $CO_2$ atmosphere at 37°C for 1 to 10 days. The number of viable cells at each time interval was assessed, using the trypan blue exclusion test. Triplicate samples were counted at each interval.

*Scanning electron microscopy*

Stressed MDCK cells were seeded onto glass coverslips at a density of $2 \times 10^4$ cells/ml in 35 mm tissue culture dishes and then grown under normal culture conditions until they reached the confluence and for up to 21 days after confluency. The cells were subsequently fixed in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated, critical point dried and coated with gold before being examined with a JEOL-300 scanning electron microscope.

*Cytogenetic analysis*

After ten successive passages during which stressed cells maintained their transformed phenotype, control and stressed cells were arrested in metaphase by adding colchicine (16 µg/ml) to logarithmic phase cultures (passage 38 for control cells and passage 42 for stressed cells), followed by a 4 h incubation. Then the cells were harvested and chromosomes were prepared using standard techniques. The modal chromosome number was determined by counting the chromosomes in 100 metaphases for each cell population. The mitotic and polyploidy indices were calculated as described by Deitch and Sawicki [1] and Gilvarry *et al.* [7], respectively, by the analyses of the percentage of cells in division and presenting multiple 2C chromosomes number, based on an analysis of 1000 cells for each procedure.

*Immunocytochemistry*

Immunocytochemistry was done on subconfluent and confluent monolayers of control and stressed cells after ten successive passages. The cells were seeded onto glass coverslips at a density of $5 \times 10^4$ cells in 1 ml of Ham F10 medium containing 5% FCS and further cultured for 24 or 72 h before immunostaining. After washing in phosphate-buffered saline (PBS, pH 7.4), the cells were fixed and permeabilized for 30 min in 80 mM Pipes buffer, pH 6.8, containing 0.25% glutaraldehyde, 4% formaldehyde, 1 mM $MgCl_2$, 5 mM EGTA and 0.2% Triton X-100. After blocking with 100 mM sodium borohydride for 20 min and with 3% BSA for 1 h, the cells were incubated with phalloidin-rhodamine (Sigma) for 1 h to detect F-actin and then stained with a monoclonal antibody for vimentin (Sigma - from mouse ascitic fluid, clone V9) or for cellular fibronectin (Sigma - from mouse ascitic fluid, clone FN-3E2). The primary antibodies were visualized by incubation with a goat FITC-conjugated anti-mouse IgG antibody (Sigma). The cells were observed with a Zeiss Axioskop equipped with filter sets for rhodamine and fluorescein.

## RESULTS

*Morphological and growth characteristics*

The control MDCK cells grew exponentially in a monolayer until confluence and showed the polygonal flattened shape typical of normal epithelial-like cells (Fig.1A). After reaching confluency, these cells stopped dividing and died. In contrast, stressed cells showed dome formation at confluence (Fig.1B). After confluency, these cells grew indefinitely in multilayers and formed cellular aggregates (Fig. 1C). During long-term culture, the stressed cells formed tubule-like structures (Fig. 1D).

*Growth curve*

Control and stressed MDCK cells showed very similar growth rates during the proliferative phase. However, after 150 h in culture, the control cells reached senescence, stopped proliferating and died, whereas the stressed cells continued to grow (Fig. 2).

*Scanning electron microscopy*

Stressed MDCK cells initially grew as a monolayer, with the cells firmly attached to each other. These cells were flattened and showed several microvilli and vesicular structures (Fig. 3A,B). After confluence, some regions of the monolayer formed dome-like structures. The cells in dome showed numerous surface microvilli (Fig. 3C,D). During prolonged culture after confluence, the stressed cells grew as multilayers and formed cellular aggregates (Fig. 3E,F).

*Cytogenetics characteristics*

Control and stressed MDCK cells showed polyploidy indices of 1.8% and 4.4%, respectively. The mitotic index was the same (9.3%) for both groups. Control and stressed population had a modal chromosome number of 80 (range: 78 to 82; n = 100 for each condition).

*Immunocytochemistry*

In control cells, stress fibers formed from bundles of actin were observed. These fibers crossed the cytoplasm and codistributed with fibronectin at the cell periphery (Fig. 4A). In stressed cells, the codistribution of fibronectin and actin was observed in regions of cell-cell contact in both non-confluent (Fig. 4C) and confluent monolayers (not shown). Compared to the control cells, stressed cells presented a slightly enhanced staining for vimentin (Fig. 4B,D).

## DISCUSSION

Cell differentiation is a complex sequence of events that involves coordinated changes in multiple biochemical and morphological parameters [9]. In this work, we examined the influence of nutritional stress on the differentiation of MDCK cells. Cells under

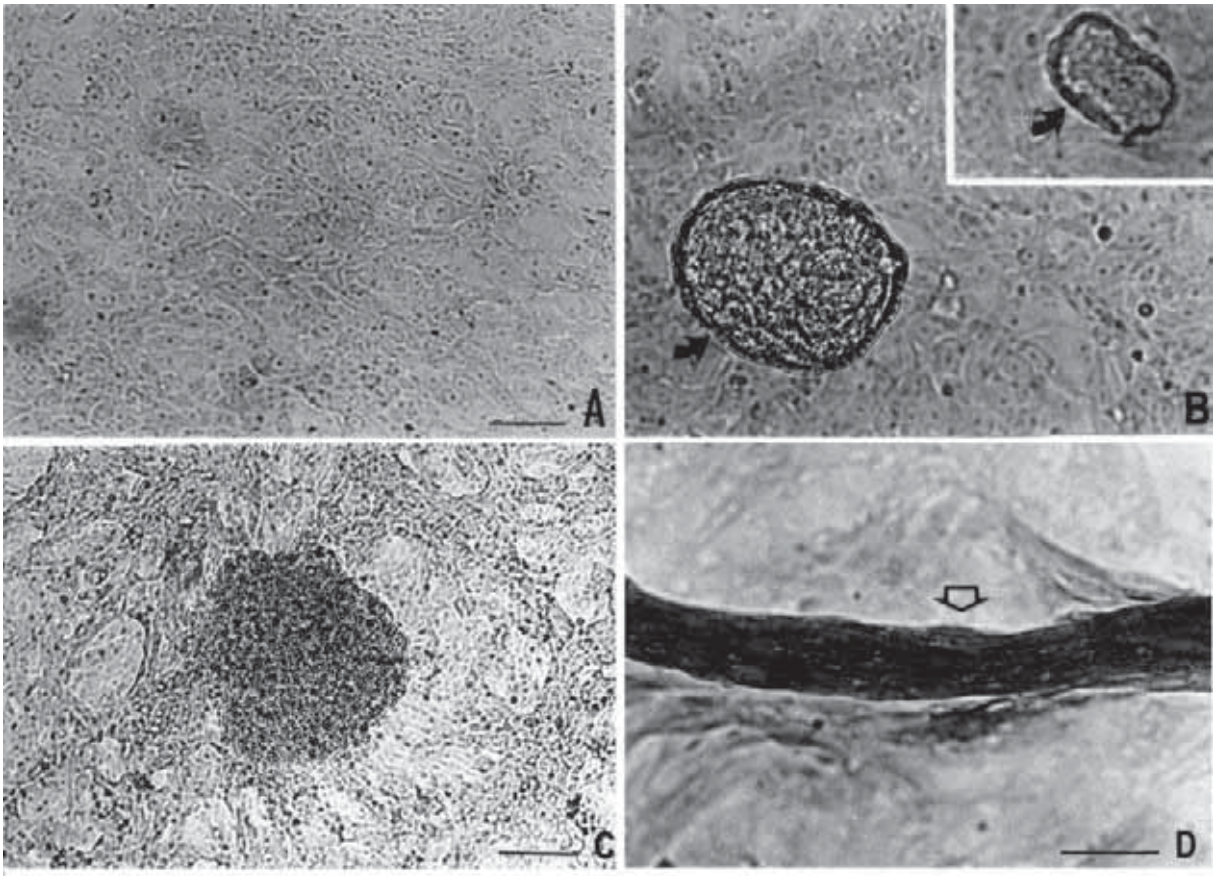


**Figure 1.** Phase-contrast micrographs of control (**A**) and nutritionally stressed (**B**-**D**) MDCK cells. After 48 h in culture control MDCK cells were growing exponentially in a monolayer and showed the typical flattened shape of normal epithelial-like cells (A). Stressed MDCK cells cultured for 48 h showed dome-like formation (**arrow**, B; inset). After confluence, stressed cells cultured for 21 days grew in multilayers and formed cellular aggregates (C). During long-term culture (60 days), stressed cells formed tubular structures (**arrow**, D). Bars = A,B = 50 μm, C = 100 μm, D = 10 μm.

nutritional stress, are also exposed to the effects of accumulated metabolites and a decreased pH. Nutritional stress and a low pH [4,12] have been associated with the transformation of cultured mammalian cells and with the development of certain types of stomach and bladder tumor in mice [13,16]. The appearance of characteristics such as dome formation, microvilli, loss of contact inhibition leading to multilayer growth, and the formation of tubular structures may be induced by nutritional stress and a low pH. Since these altered properties and growth characteristics were not reverted by returning the stressed cells to normal culture conditions, nutritional stress could be responsible for cell transformation. Additionally, the intracellular pH plays a critical role in controlling the cell cycle and is often associated with an abnormal proliferation [6]. Thus nutritional stress accompanied by a decreased in pH could also be responsible for the cytogenetic alterations in MDCK cells,

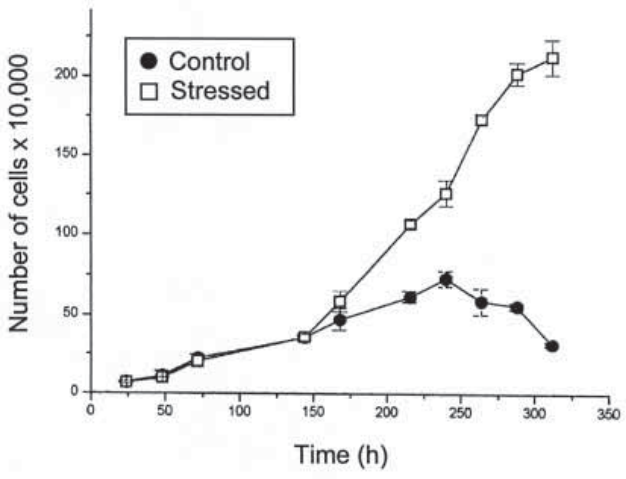


**Figure 2.** Growth curves for control and stressed MDCK cells. Both groups of cells showed the same growth rate during the first 150 h of culture, after which the control cells stopped dividing (stationary phase) and then died. Stressed cells showed continuous growth. The points are the means ± S.E.M. of three experiments.

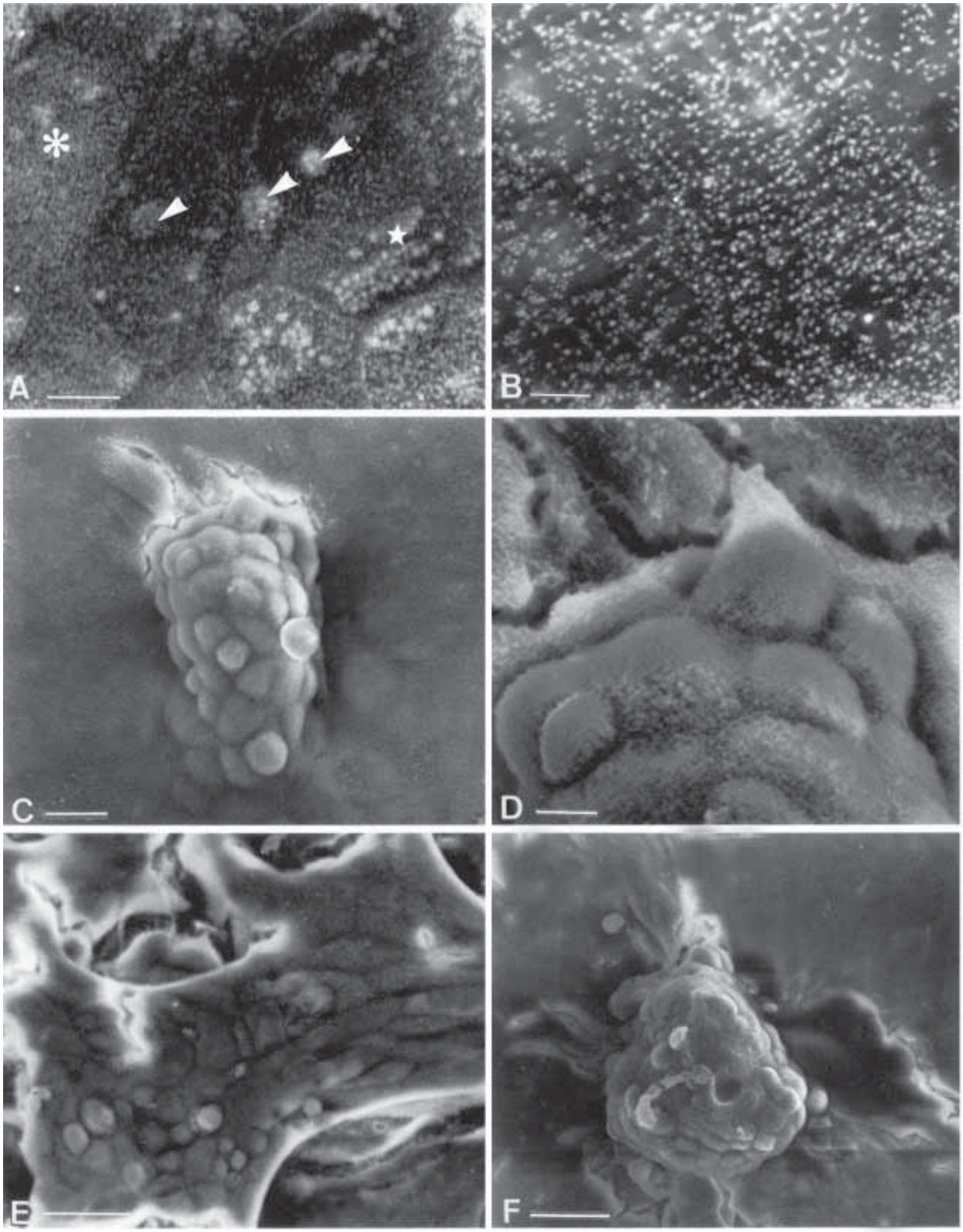


**Figure 3.** Scanning electron microscopy of MDCK cells. The stressed cells grew as a monolayer, with the cells firmly attached to each other. These cells had a flattened shape with stub-like microvilli (**asterisk** and **star**) and vesicular structures (**arrowheads**) (**A**; detail in **B**). After confluence, the stressed MDCK cells formed *dome* structures with numerous microvilli (**C**; detail in **D**). After prolonged culture, the stressed cells started to grow in multilayers (**E**) and formed cellular aggregates (**F**). Bars = A = 2 µm, B = 1 µm, C = 5 µm, D = 2 µm, E and F = 10 µm.

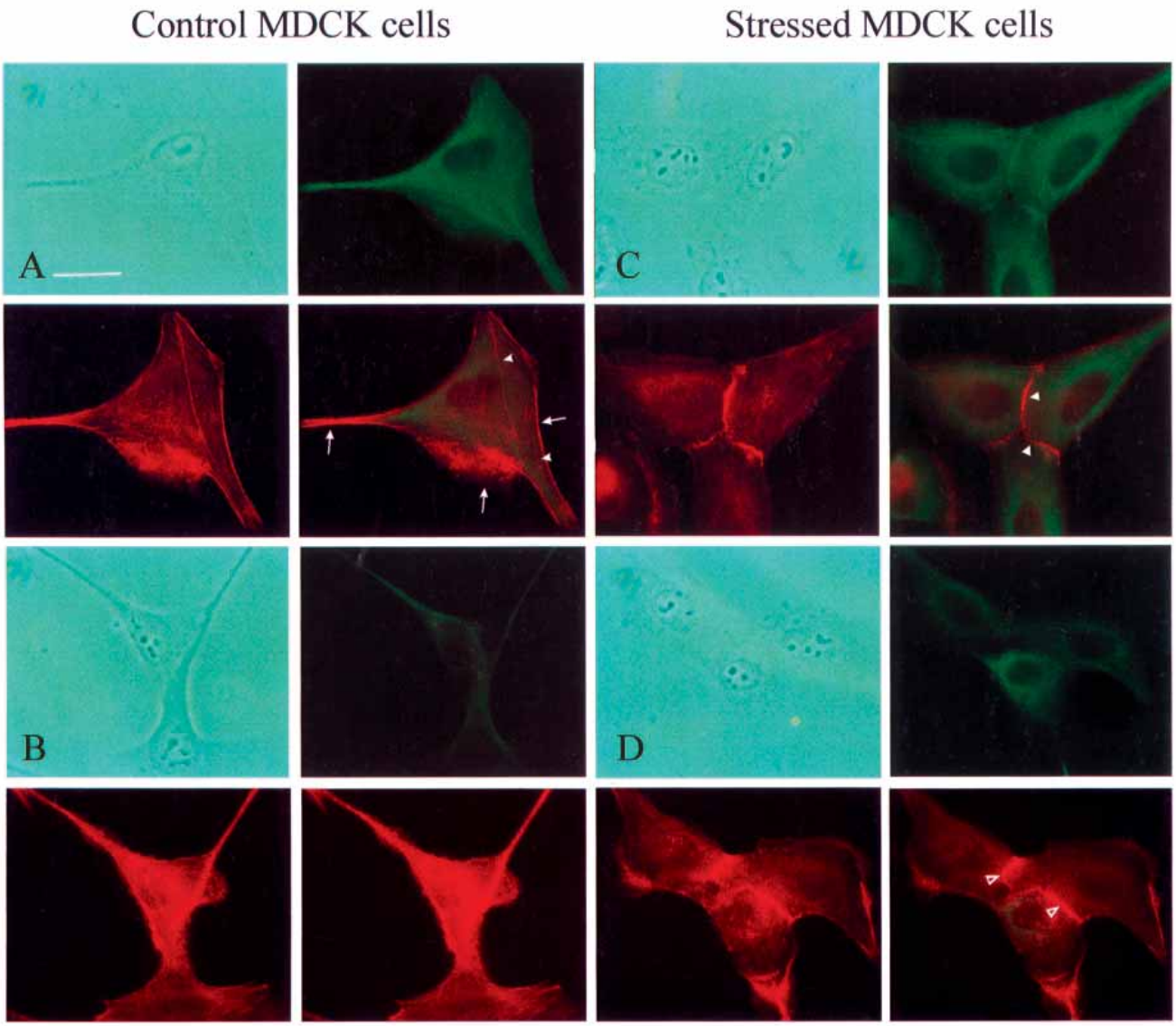


**Figure 4.** Indirect immunofluorescence staining of fibronectin, actin and vimentin in control and stressed MDCK cells. The cells were seeded on coverslips and cultured for 24 h, after which they were fixed, permeabilized and stained with antibodies against fibronectin or vimentin and phalloidin. The first two panels on the left show control cells, and the two on the right show stressed MDCK cells. **A** and **C** correspond to the colocalization of fibronectin and actin, and **B** and **D** show vimentin and actin. In control cells, the codistribution of actin and fibronectin was observed mainly in stress fibers (**arrowheads**) and at the cell periphery (**arrows**) (A). In stressed cells, the codistribution of fibronectin and actin was observed in regions of cell-cell contact regions (**arrowheads**) (C). Compared to control cells (B), stressed cells showed slightly enhanced staining for vimentin (D), which colocalized with actin in regions of cell-cell contact (**arrow**). Bar = 30 μm in all panels.

leading to errors in mitosis that could increase the polyploidy index and affect the modal number of chromosomes.

Changes in cell shape and contact have a central role in regulating of cellular growth and differentiation and tissue morphogenesis. The alterations involve interactions between the extracellular matrix and cytoskeletal components, or between neighboring cells [2]. Fibronectin has an important function in cell migration and adhesion, and its expression is often reduced in transformed and tumor cells, which are typically less adherent to the substrate and no longer show a loss of contact inhibition. Fibronectin and other matrix components may be effective in promoting the assembly and stabilization of actin stress fibers in epithelial cells [8]. These findings suggest that extracellular matrix elements, including fibronectin, interact with intracellular microfilaments in

epithelial tissues, and that this interaction may be important in inducing and/or maintaining the morphology and polarity of MDCK cells [8].

MDCK cells cultured on collagen gels form branching tubules and spherical cysts [11]. These structures may contribute to the differentiation of stressed MDCK cells since during prolonged culture the cells can modify their substrate by increasing or reducing the deposition of extracellular matrix components. Indeed, stressed MDCK cells showed an accumulation of fibronectin between adjacent cells. Confluent and non-confluent stressed cells had enhanced vimentin and actin deposition in regions of cell-cell contact when compared with control cells. The accumulation of actin in these regions could contribute to the formation of tubule-like structures during prolonged culture.

Our results show that expression of certain cytoskeletal elements is related to changes in cell shape and cell contacts, and that such changes may be central to the acquisition of a differentiated and/or transformed phenotype. Nutritional stress and a change in pH are important stimuli that lead to alterations in the growth, morphology and interactions of cultured renal epithelial cells, and may result in tubular morphogenesis *in vitro*.

**ACKNOWLEDGMENTS**

The authors thank Dr. Mary Anne Heidi Dolder for her help with electron microscopy and Dr. Hernandes F. de Carvalho for providing the phalloidin-rhodamin–conjugate. This work was supported by Coordenadoria de Aperfei-çoamento de Pessoal de Nível Superior (CAPES) and Fundo de Apoio ao Ensino e à Pesquisa (FAEP/UNICAMP).

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Received: September 18, 2002
Accepted: June 17, 2003