SELMA CANDELÁRIA GENARI

"EFEITO DO ESTRESSE NUTRICIONAL EM CULTURAS DE CÉLULAS PRIMÁRIAS E EM LINHAGENS ESTABELECIDAS"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato(a) Selma Candelária Genari e aprovada pela Comissão Julgadora.
10/12/98

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para a obtenção do título de Doutor em Ciências, área de concentração em Biologia Celular.

Orientadora: Profa. Dra. Maria Lúcia Furlan Wada

Campinas, SP
1998.

UNICAMP
BIBLIOTECA CENTRAL

UNICAMP BC
N.º CHAMADA:
V. Ex.
TOMBO BC/ 36601
PROC. 229/99
C [ ] D [x]
PREÇO R$ 11,00
DATA 20/02/99
N.º CPD

CM-00120836-3

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

Genari, Selma Candelária

G285e Efeito do estresse nutricional em culturas de células primárias e em linhagens estabelecidas/Selma Candelária. Genari. -- Campinas, SP: [s.n.], 1998.

108f.:ilus.

Orientadora: Maria Lúcia Furlan Wada

Tese (doutorado) - Universidade Estadual de Campinas. Instituto de Biologia.

1.Cultura de células. 2.Stress. 3.Morfologia. 4.Citogenética. 5. Citoesqueleto. I. Wada, Maria Lúcia Furlan. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Titulo.

LOCAL E DATA: Campinas, 10 de Dezembro de 1998.

BANCA EXAMINADORA:

TITULARES:

Orientadora:
Profa. Dra. MARIA LUCIA FURLAN WADA

Assinatura

Profa. Dra. ELOIZA HELENA TAJARA DA SILVA

Assinatura

Profa. Dra. ANA ELIZABETE SILVA

Assinatura

Profa. Dra. MARIA LUIZA SILVEIRA MELLO

Assinatura

Prof. Dr. HERNANDES FAUSTINO DE CARVALHO

Assinatura

SUPLENTES:

Profa. Dra. CHRISTINE HACKEL

Assinatura

Profa. Dra. MARY ANNE HEIDE DOLDER

Assinatura

O presente trabalho foi realizado no Laboratório de Cultura de Células Animais do Departamento de Biologia Celular, Instituto de Biologia da Universidade Estadual de Campinas, sob a orientação da Profa. Maria Lúcia Furlan Wada e com o suporte financeiro das seguintes entidades:

- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).
- Fundo de Apoio ao Ensino e à Pesquisa (FAEP/UNICAMP).
- Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)

Durante a realização deste trabalho foram publicados ou submetidos para publicação os seguintes artigos originais, os quais encontram-se em anexos:

1) GENARI, S. C.; DOLDER, M. A. H. and WADA, M. L. F. Scanning and transmission electron microscopy of transformed Vero cells with altered *in vitro* growth characteristics. *J. Submicrosc. Cytol. Pathol.* **28**(4), 565-572, 1996.

2) GENARI, S. C.; GOMES, L. and WADA, M. L. F. Alterations in the growth and adhesion pattern of Vero cells induced by nutritional stress conditions. *Cell Biol. Int.* **20**(2), 1998.

3) GENARI, S. C. and WADA, M. L. F. Influence of the nutritional stress conditions on differentiation and tubulogenesis of epithelial cells *in vitro*. *J. Cell. Physiol.* (submetido para publicação).

4) GENARI, S. C. and WADA, M. L. F. Effects of nutritional stress conditions on the ploidy and growth of human amniotic cells. *In Vitro Cell. Dev. Biol.* (submetido para publicação).

5) GENARI, S. C. and WADA, M. L. F Cytogenetics and cytotoskeleton alterations in Vero cells transformed for nutritional stress conditions. *J. Histochem. Cytochem.* (a ser submetido para publicação).

Ao German Alejandro
por seu amor, carinho
e compreensão...

À nossa filha
que irá chegar e
já compartilha das
emoções deste momento...

Dedico.

AGRADECIMENTOS

Durante a elaboração deste trabalho foram muitas as pessoas e as Instituições que direta ou indiretamente contribuiram em sua execução. Gostaria de expressar meus profundos agradecimentos à todas elas... Em especial agradeço a:

Profa. Dra. Maria Lúcia Furlan Wada pelo carinho, compreensão e dedicação na orientação deste trabalho, sempre apoiando e estimulando a minha vida profissional e sobretudo pela amizade surgida desta convivência, a qual irá nos acompanhar nas etapas futuras de nossas vidas.

À Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), pela bolsa a mim concedida durante o curso de Doutoramento, sem a qual eu não teria condições de ter realizado o presente trabalho.

Às professoras Dras. Maria Luiza S. Mello, Eloiza H. Taraja, Ana Elizabeth Silva e ao professor Dr. Hernandes F. de Carvalho pela leitura cuidadosa e sugestões que muito contribuiram para a versão final deste trabalho.

Às professoras Dras. Maria Luiza S. Mello, Eloiza H. Taraja, Ana Elizabeth Silva, Christine Hackel, Mary A. Heide Dolder e ao professor Dr. Hernandes F. de Carvalho, por aceitarem participar como membros da banca examinadora desta tese.

À professora Dra. M. A. Heide Dolder pelo seu valioso auxilio e colaboração na execução dos estudos morfológicos em microscopia eletrônica.

Ao professor Dr. Hernandes F. de Carvalho pela doação de reagentes, por sua inestimável assistência durante os experimentos de imunocitoquímica e pela revisão crítica dos manuscritos.

A todos os professores do Departamento de Biologia Celular que muito contribuiram para a minha formação profissional através de seus ensinamentos, de seu exemplo e dedicação.

Ao Instituto de Química pela utilização do Microscópio Eletrônico de Varredura.

Aos funcionários do Departamento de Biologia Celular e aos colegas de Pós-Graduação que na amizade e incentivo diários, contribuiram para tornar esse trabalho ainda mais gratificante.

Aos meus pais que, por seu imenso amor, não mediram esforços para que eu pudesse ter uma formação profissional e mais oportunidades de realizações do que eles próprios tiveram em sua vida...

ÍNDICE:

I - INTRODUÇÃO

A transformação celular envolve mudanças num grande conjunto de propriedades, podendo causar alterações nas interações célula-célula (LOEWENSTEIN, 1979; KLAUNIG & RUCH, 1990; REN et alii, 1990; YAMASAKI, 1990), célula-substrato (MURRAY et alii, 1980; KESKI-OJA et alii, 1985; NERMUT, 1991)) e célula-meio (HOLZER et alii, 1986; CROSS & DEXTER, 1991), induzindo a mudanças no comportamento e padrão de crescimento em cultura. Como a transformação celular *in vitro* apresenta relações com o processo de carcinogênese *in vivo* e pode ser induzida por diferentes agentes (IARC/NCI/EPA Working Group, 1985), ela tem sido grandemente estudada como um análogo ao desenvolvimento neoplásico, embora a correspondência exata entre esses dois processos não tenha sido totalmente estabelecida (PONTÉN, 1976). Atualmente o objetivo do estudo da transformação *in vitro* é entender a tumorigênese em organismos vivos, assim como os eventos moleculares envolvidos nesse processo (SMETS, 1980; BISHOP, 1991).

Entre as alterações comportamentais mais frequentemente observadas depois da transformação, está o crescimento celular em múltiplas camadas, indicando a perda de inibição por contato (ABERCROMBIE, 1979). A transformação celular *in vitro* geralmente está associada a alterações no complemento cromossômico, podendo apresentar mudanças no grau de ploidia celular ou aberrações cromossômicas individuais (BIANCHI & AYRES, 1971; BIEDLER, 1976; GILVARRY et alii, 1990).

Algumas modificações nas propriedades celulares decorrentes da transformação envolvem alterações dos componentes e/ou funções da superfície celular. Várias proteínas e

glicoproteínas de superfície estão presentes em concentrações reduzidas, ou mesmo ausentes nas células transformadas (HYNES, 1976; VAHERI & MOSHER, 1978; SMETS & VAN BEEK, 1984). Entre essas proteínas encontra-se a fibronectina, uma proteína da matriz extracelular que desempenha várias funções, como na migração, adesão e espalhamento celular à superfície, e frequentemente está presente em quantidades reduzidas após a transformação induzida por agentes virais (HAYMAN et alii, 1981; ALITALO et alii, 1982, NERMUT et alii, 1991) e em culturas celulares provenientes de certos tipos tumorais (HYNES, 1976; MURRAY, 1980). Alguns estudos indicam ainda que o decréscimo dessa proteína pode ser consequência da diminuição de sua síntese ou à inabilidade desta célula transformada em reter a fibronectina na sua superfície. Essas modificações acabam interferindo no processo de adesão, levando alguns tipos celulares transformados a crescer em suspensão, sem se fixar a um substrato, ou apresentando menores índices de adesão (HAYMAN et al. 1981; COOK & CHEN, 1988; PERSKY et al., 1989).

As alterações morfológicas e de adesão podem estar relacionadas, ainda, ao citoesqueleto, uma vez que este encontra-se associado à manutenção da forma célular assim como aos elementos de matriz extracelular (Ben-Ze'Ev, 1985).

Diferentes organismos como leveduras, até células eucarióticas de todos os níveis filogenéticos, parecem responder de maneira similar quando expostas a vários tipos de estresse metabólico que podem causar transformação (MORIMOTO, 1991; WELCH, 1993). Entre esses vários tipos de estresse estão mudanças de temperatura (MORIMOTO, 1991; WELCH, 1993), alterações iônicas e no pH (MATSUOKA, et alii, 1979; ZURA & GRANT, 1981), irradiação (WITTE et al, 1989; BOENSEN, 1992; TERZAGHI-HOWE,

1993), estresse hiperosmótico (WOLLNIK, et al., 1993), estresse nutricional (GALEGO, et alii, 1984; BOREK, 1972) e outros.

Condições não fisiológicas de tratamento por estresse nutricional têm sido associadas a indução de efeitos genotóxicos e transformação em cultura de células de mamíferos. A demonstração do fenômeno de transformação celular por processos de estresse nutricional pode ter uma possível aplicação para algumas classes de neoplasias desenvolvidas (BOREK, 1972). As células epiteliais do fígado, quando submetidas ao estresse nutricional, passam a apresentar características associadas à malignidade, como o crescimento em múltiplas camadas (BOREK, 1972). Um outro exemplo é o protozoário *Tetrahymena pyriformis* que, quando submetido ao estresse nutricional, passa a sintetizar quatro proteínas com pesos moleculares de 100kDa, 70kDa, 50kDa e 30kDa (GALEGO, et alii, 1984). Esses estudos também podem auxiliar o entendimento da necrose que ocorre em determinadas regiões tumorais, onde as células sobreviventes sofrem privação de oxigênio e nutrientes, por estarem em locais pobremente vascularizados (SHERIDAN et alii, 1981).

A linhagem celular Vero, estabelecida a partir de células renais de macaco verde da África (*Cercopithecus aethiops*), apresenta comportamento característico em cultura, com crescimento em monocamadas. Em estudos anteriores, foi demonstrado que, por processo de transformação, essas células passam a apresentar alterações morfológicas, comportamentais e de crescimento em relação ao padrão da linhagem inicial. A população Vero alterada apresentou crescimento em múltiplas camadas formando grumos celulares, indicando perda de inibição por contato. Foram descritas, também, alterações citogenéticas referentes ao grau de ploidia celular, número modal de cromossomos e ainda alterações no cariótipo da população transformada em relação à população inicial (GENARI & WADA, 1995).

Baseados nos dados encontrados na literatura e descritos acima, a transformação das células Vero pode ter ocorrido em função de condições não fisiológicas de tratamento, já que essas células passam a crescer em múltiplas camadas e a apresentar várias alterações descritas em células submetidas ao estresse nutricional.

II - OBJETIVOS DO TRABALHO

O objetivo geral do presente trabalho é estudar o efeito do estresse nutricional em diferentes tipos celulares de linhagem estabelecida (Vero e MDCK) e primária (células amnióticas humanas), avaliando aspectos celulares morfológicos, citogenéticos e de crescimento em cultura.

Os principais objetivos específicos são:

Observar as alterações de padrão comportamental ocorridas nos tipos celulares de linhagens e cultura primária submetidos ao estresse nutricional, comparando-os com seus respectivos controles mantidos em condições normais de tratamento.

Estudar as diferenças morfológicas e de crescimento celular em cultura, através da utilização da microscopia eletrônica de varredura e da obtenção das curvas de crescimento das populações controle e sob estresse.

Estudar a distribuição da fibronectina, através de métodos imunocitoquímicos, nos diferentes tipos celulares em condições normais de cultura e sob o efeito do estresse nutricional.

Analisar, através de métodos imunocitoquímicos, a distribuição de actina e vimentina nas populações controle e sob estresse, para detecção de possíveis alterações ocorridas no citoesqueleto das células.

Efetuar estudo citogenético nas células sob estresse e seus controles, através da determinação do índice mitótico, grau de ploidia celular, número modal de cromossomos e estudo do cariótipo.

III - ARTIGOS ANEXOS

J. SUBMICROSC. CYTOL. PATHOL., **28** (4), 565-572, 1996

Scanning and transmission electron microscopy of transformed Vero cells, with altered *in vitro* growth characteristics

S.C. GENARI, M.A.H. DOLDER and M.L.F. WADA

Department of Cellular Biology, State University of Campinas, UNICAMP, São Paulo, Brazil

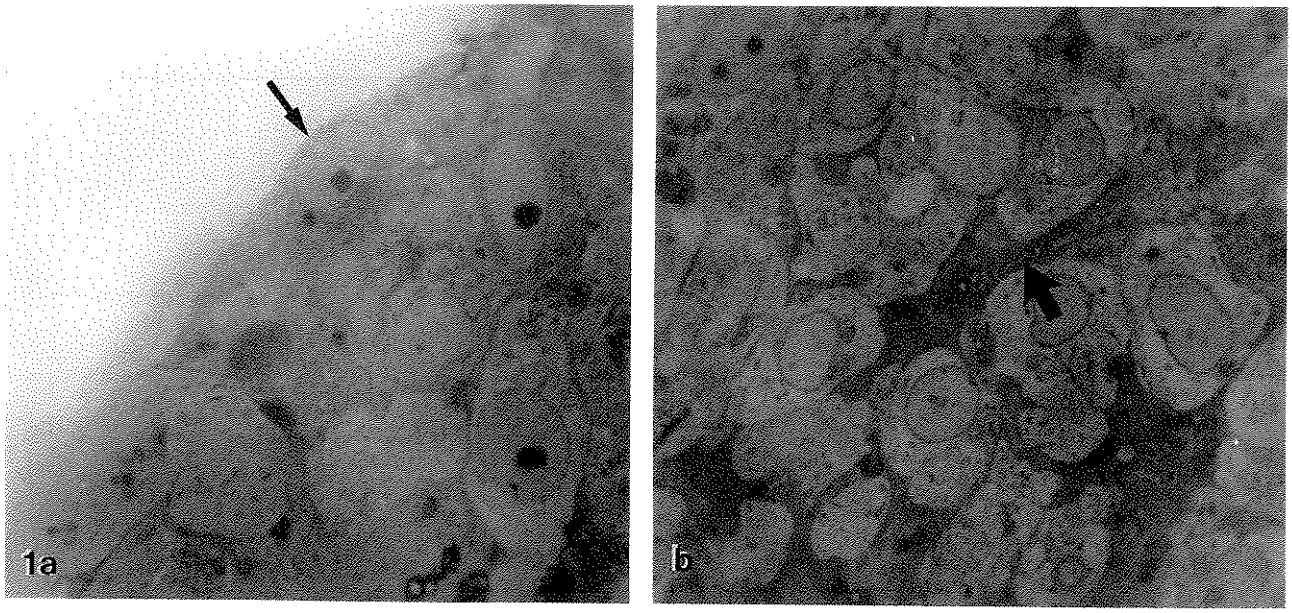
SUMMARY - The Vero lineage, established from kidney cells of the green African monkey, presented fibroblasts-like cells and growth in monolayers. Maintained in culture, the Vero cells presented behavioural and morphologic alterations, associated with cellular transformation. The morphological alterations were investigated using scanning and transmission electron microscopy. The study of proliferation and determination of the cellular doubling time was obtained from the growth curve. The initial population presented growth in a monolayer, while the altered cells grew in multilayers forming cellular aggregates, with flattened cells on the surface and globular cells in the inner region of the aggregate, together with extracellular matrix material. The cell surface of the altered population presented innumerable structures similar to little vesicles, microvilli and cytoplasmic prolongations. The cellular proliferation of both populations was very similar. Our results indicate that morphological and growth changes probably resulted from cellular transformation of the initial Vero cells. These transformed cells presented several characteristics associated with neoplastic growth, and can be used as a model for tumor cells studies *in vitro*.

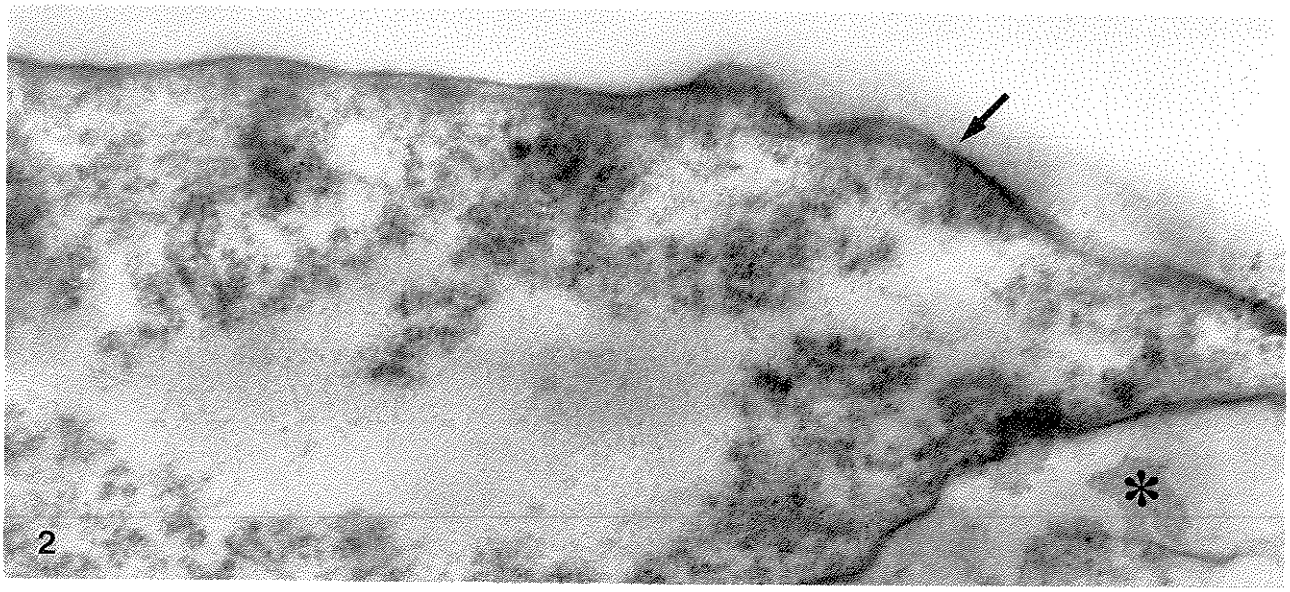
KEY WORDS *cellular transformation - Vero cells - multiple layers - cellular aggregates - morphologic alteration*

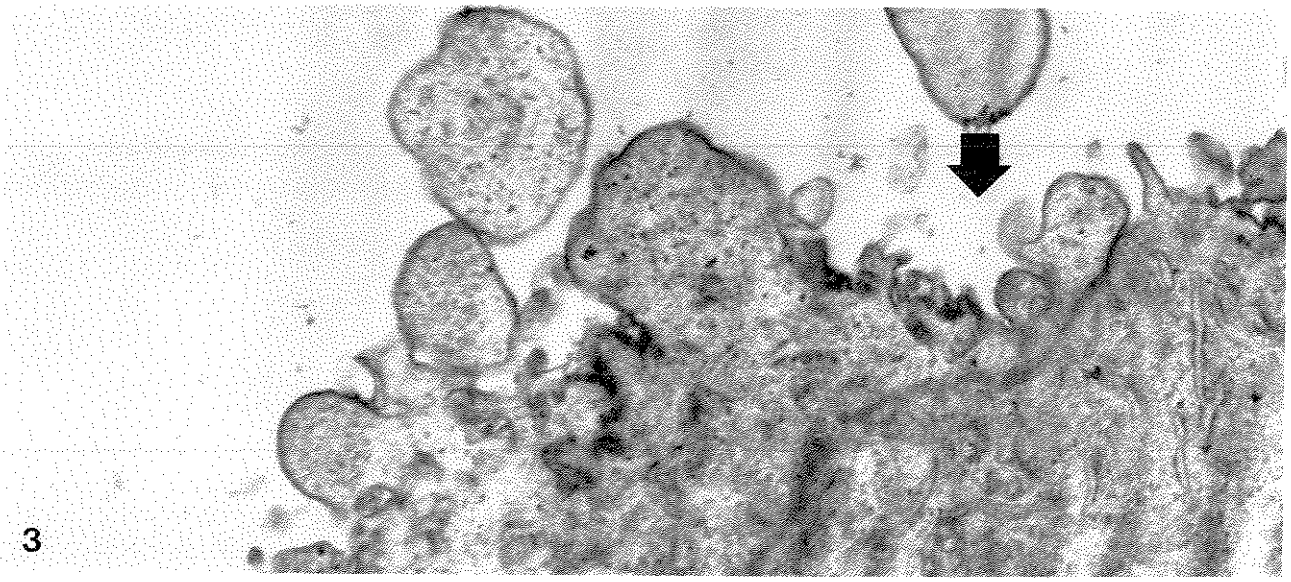
INTRODUCTION

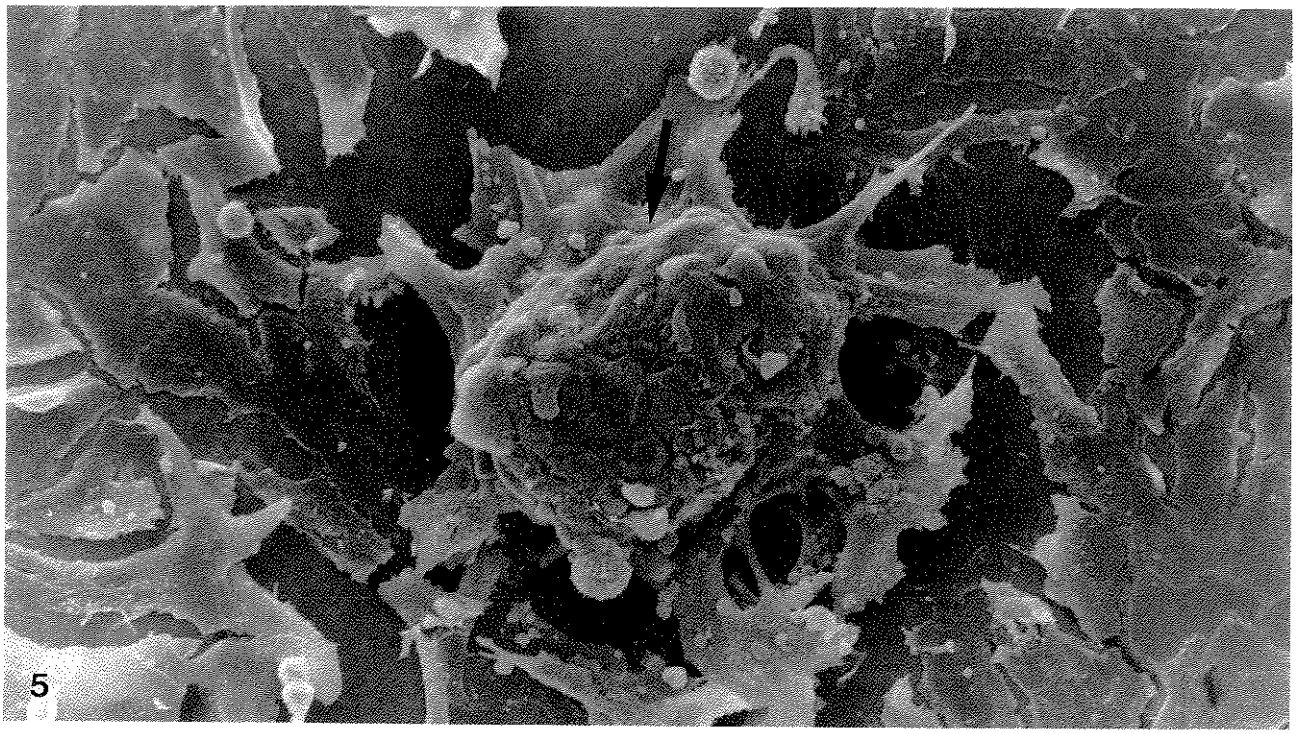
The Vero cell lineage, established from kidney cells of the green African monkey (*Cercopithecus aethiops*), has a characteristic growth pattern in culture. This lineage presented fibroblastic-like cells, growing in monolayers (Janik and Greko, 1976; Fleming and Kistler, 1977; Lee and Engelhardt, 1977; Araki and Nakamura, 1978; McClane and MacDonel, 1979; Leary and Blair, 1980). Maintained in culture, the Vero cells began to alter their initial behavioral and growth characteristics. This altered Vero population grew in multiple layers, forming cellular aggregates (Genari and Wada, 1995). This abnormal social behavior is called morphologic transformation (Smets, 1980), and occurs due to the loss of the contact inhibition (Heidelberger and Iype, 1966), frequently observed in cells extracted from tumors and grown in cell culture (Persky *et al.*, 1989; Sridhar *et al.*, 1989; Ebert *et al.*, 1990; Bridges *et al.*, 1991) or cells transformed by virus and carcinogenic chemicals (Duesberg and Vogt, 1970; Alitalo *et al.*, 1982; Brown *et al.*, 1986; Perocco *et al.*, 1993). The transformed and the cancer cells, when compared with their genitive ancestral cells or their tissue of origin, may present significant differences in their growth curve. These cells consistently have prolonged survival in low nutritional conditions and an accelerated growth index (Abercrombie, 1979; Golombick *et al.*, 1990). The process of mammalian cell transformation has been the subject of many investigations. The objective of this study *in vitro* is to help understand how tumors originate and progress in animals (Pontén, 1976; IARC/NCI/EPA Working Group, 1985; Terzaghi-Howe, 1993). The study described in this paper investigated the morphologic and growth characteristics of the initial Vero cells and the altered population, by transmission and scanning electron microscopy, and their respective growth curves.

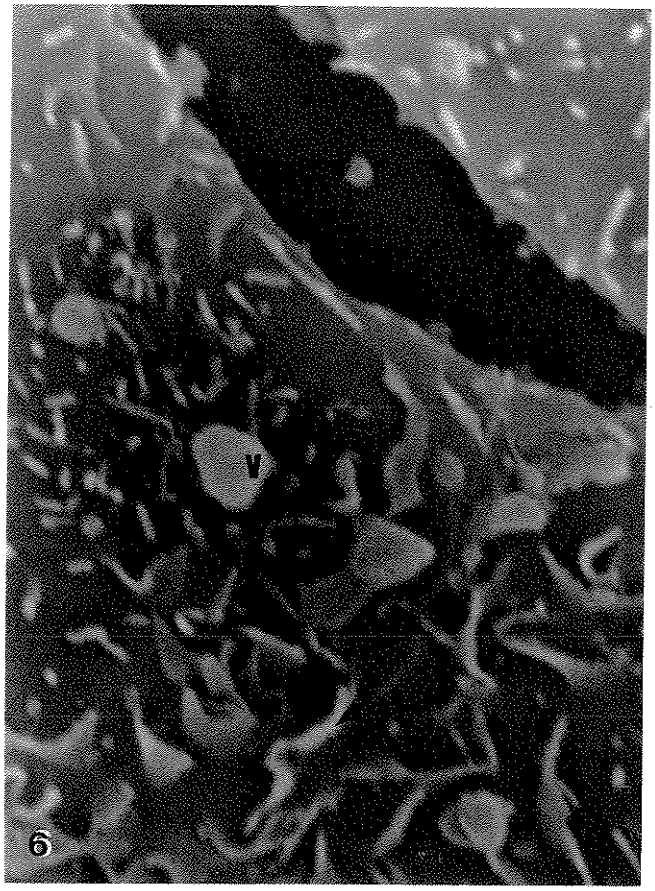
Mailing address: Dr. Selma Candelária Genari, Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas, UNICAMP, Caixa Postal 6109, CEP 13 083-970, Campinas, São Paulo, Brazil.


1a
1b


2


3





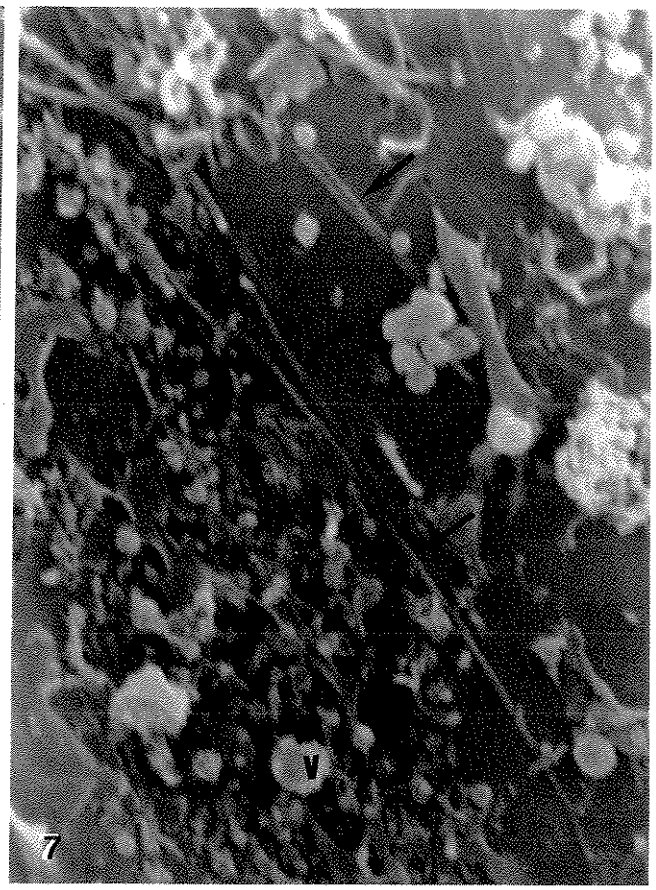


FIGURE 5 The altered Vero cells after confluency, with 15 days culture, continue to develop indefinitely, growing in multiple layers and forming cellular aggregates (arrow). × 800.

FIGURE 6 Cellular surface of the initial Vero cells presented little vesicles (V), microvilli (arrowheads) and cytoplasmatic prolongations of various lengths (arrow). × 16,000.

FIGURE 7 Cellular surface of the altered Vero cells presented innumerable vesicles (V) and fibrillar structures (arrows), which may represent extracellular matrix elements. × 10,000.

(Persky *et al.*, 1989; Sridhar *et al.*, 1989; Bridges *et al.*, 1991).

The formation of miniature tumor colonies requires as critical events, the production of matrix elements as well as cell-to-extracellular matrix interaction (Persky *et al.*, 1989). Another important aspect may be the possible relation between fibrillar structures of extracellular matrix elements and the physical stability of the cellular aggregates, since these structures are frequently observed in dividing cells (Takezawa *et al.*, 1993).

The surface of altered cells was covered with innumerable structures which can be described as microvilli, vesicles or ruffles. These structures are frequently observed in transformed cells.

These changes in surface and cellular shape were associated with the increased intracellular protein production or the elimination of this material (Linstead *et al.*, 1988). The vesicles observed in altered cells appear to indicate the elimination of material to the extracellular environment. In the transformed Vero cells, the microvilli seem to play roles for the binding of cell-to-cell and cell-to-collagen or other matrix elements (Takezawa *et al.*, 1993). Fibroblasts first bind directly or indirectly to collagen fibrils through binding sites located on the cell membrane of microvilli (Grinnell and Bennett, 1981; Asaga *et al.*, 1991; Lu *et al.*, 1992).

In relation to the growth curves, in the proliferative phase no differences are observed between the initial Vero and the altered population, but after the end of the proliferative phase the initial Vero cells stopped cellular division, reached senescence and died. The altered population continued the proliferative phase for an indefinite time, since the cells begin growing in multiple layers. This fact demonstrated that the initial Vero cells maintained the characteristics of contact inhibition, while the altered population lost this characteristic (Heidelberger and Iype, 1966). The loss of contact inhibition was observed in various transformed cellular types after viral action, exposure at the carcinogenic chemicals, radiation and other factors (Borek and Sachs, 1966; Borek, 1972; Abercrombie, 1979) and also in malignant cells obtained from tecidual tumors and maintained in culture (Persky *et al.*, 1989; Sridhar *et al.*, 1989; Ebert *et al.*, 1990; Bridges *et al.*, 1991).

In this manner, the morphological and growth changes described in this paper were probably the result of cellular transformation mechanisms in culture. These altered cells can be used as a model for tumor cell studies *in vitro*.

ACKNOWLEDGEMENTS

This work was carried out with support from the Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

ABERCROMBIE M., 1979. Contact inhibition and malignancy. *Nature*, **281**, 259-262.

ALITALO K., KESKI-OJA J., HEDMAN K. and VAHERI A., 1982. Loss of different pericellular matrix components of rat cells transformed with a T-class *ts* mutant of Rous sarcoma virus. *Virology*, **119**, 347-357.

ARAKI H. and NAKAMURA K., 1978. Effect of sera from cystic fibrosis homozygotes and heterozygotes on glucose metabolism in Vero cells. *The Lancet*, **15**, 793-795.

ASAGA H., KIKUCHI S. and YOSHIZATO K., 1991. Collagen gel contraction by fibroblasts requires cellular fibronectin but not plasma fibronectin. *Exp. Cell Res.*, **193**, 167-174.

BOREK C., 1972. Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. *Proc. Natl. Acad. Sci. USA*, **69**, 956-959.

BOREK C. and SACHS L., 1966. *In vitro* cell transformation by X-irradiation. *Nature*, **210**, 276-278.

BRIDGES M.A., WALKER D.C., HARRIS R.A., WILSON B.R. and DAVIDSON A.G.F., 1991. Cultured human nasal epithelial multicellular spheroids: polar cyst-like model tissues. *Biochem. Cell Biol.*, **69**, 102-108.

BROWN K., QUINTANILLA M., RAMSDEN M., KERR I.B., YOUNG S. and BALMAIN A., 1986. *V-ras* genes from Harvey and BALB murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. *Cell*, **46**, 447-456.

DUESBERG B.H. and VOGT P.K., 1970. Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. *Proc. Natl. Acad. Sci. USA*, **67** (4), 1673-1680.

EBERT T., BANDER N.H., FINSTAD C.L., RAMSAWAK R.D. and OLD L.J., 1990. Establishment and characterization of human renal cancer and normal kidney cell lines. *Cancer Res.*, **50**, 5531-5536.

FLEMING N. and KISTLER G., 1977. Morphology, histochemistry and isozymes of kidney cells during long-term exposure to cigarette smoke. *Acta Histochem. Bd.*, **60**, 132-145.

GENARI S.C. and WADA M.L.F., 1995. Behavioural differences and cytogenetic analysis of transformed cellular population derived from a Vero cell line. *Cytobios*, **81**, 17-25.

GOLOMBICK T., DANSEY R., BEZWODA W.R. and ROSENDORFF J., 1990. Establishment and characterization of two new human ovarian cancer cell lines UWOV1 and UWOV2 and a subline UWO2(Sf) growing in serum-free conditions: growth characteristics, biochemical and cytogenetic studies. *In Vitro Cell. Dev. Biol.*, **26**, 447-454.

GRINNELL F. and BENNETT M.H., 1981. Fibroblast adhesion on collagen substrata in the presence and absence of plasma fibronectin. *J. Cell Sci.*, **49**, 19-34.

HEIDELBERGER C. and IYPE P.T., 1966. Malignant transformation *in vitro* by carcinogenic hidrocarbons. *Science*, **155**, 214-217.

IARC/NCI/EPA WORKING GROUP, 1985. Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols. *Cancer Res.*, **45**, 2395-2399.

JANIK B. and GREKO M.A., 1976. Secondary association of exogenous polynucleotides with cells and nuclei in uptake experiments. *Exp. Cell. Res.*, **103**, 420-423.

JENSEN M.D., WALLACH D.F.H. and LIN P.S., 1974. Comparative growth characteristics of Vero cells on gas-permeable and conventional supports. *Exp. Cell Res.*, **84**, 271-281.

LEARY K. and BLAIR C.D., 1980. Sequentional events in the morphogenesis of japanese encephalitis virus. *J. Ultrastruct. Res.*, **72**, 123-129.

BNAME: No Job Name PAGE: 1 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
ings2/journals/academic/167/80250

Cell Biology International 1998, Vol. 22, No. 0, 000–000
Article No. cb980250

ALTERATIONS IN THE GROWTH AND ADHESION PATTERN OF VERO CELLS INDUCED BY NUTRITIONAL STRESS CONDITIONS

SELMA CANDELÁRIA GENARI*, LAURECIR GOMES and MARIA LÚCIA FURLAN WADA

Department of Cell Biology, Biology Institute, State University of Campinas, UNICAMP—CP6109—CEP13083-970, Campinas, São Paulo, Brazil

Accepted 31 March 1998

The pattern of growth, adhesion and protein synthesis in Vero cells submitted to nutritional stress conditions was investigated. The control cells presented a characteristic pattern, with monolayer growth, while the stressed cells presented multilayered growth, with aggregate or spheroid formation which detached on the flask surface and continued their growth in another region. In the soft agar assay, with reduced amount of nutrients, only the stressed cells presented growth, indicating physical and nutritional independence. A 44-kDa protein was observed in stressed cells and was absent in non-stressed cells. The adhesion index and fibronectin synthesis and distribution were altered in stressed cells. After confluence, control cells presented fibronectin accumulation in lateral cell–cell contact regions, while this fibronectin accumulation pattern was not observed in stressed cells. These alterations may be responsible for the multilayered growth and decreased adhesion index observed in stressed cells which were transformed by nutritional stress conditions.
© 1998 Academic Press

KEYWORDS: cellular transformation; adhesion alterations; soft agar; fibronectin; 44 kDa protein; nutritional stress.

INTRODUCTION

The Vero cell lineage obtained from kidney cells of the African green monkey (*Cercopithecus aethiops*) has a characteristic growth pattern in culture (Bianchi and Ayres, 1971). The cells of this lineage grow in monolayers, are elongated in shape and are similar to fibroblast cells, with little cytoplasmic granulation. When a Vero population reaches cellular confluence, subculture should be carried out, otherwise the cells degenerate and lift off the culture flask surface (Genari and Wada, 1995). Cultured fibroblasts and epithelial cells in culture can undergo neoplastic evolution for the cellular transformation process. The transformation involves a modification or serial modifications in the cellular genome, which lead to the transformed phenotype (Smets, 1980). Morphological manifestations of the transformation are observed in the growth, adhesion and spreading characteristics, which are frequently altered in transformed and tumour cells (Keski-Oja *et al.*, 1985; Vasiliev, 1985; Nermut *et al.*, 1991). Since cellular transformation is inducible *in vitro* by several agents such as viruses, chemicals, radiation and metabolic injury incurred by some treatments, these transformed cells have been used to study a process analogous to neoplastic transformation *in vivo*, to help understand how tumours originate and progress in animals (Pónten, 1976; IARC/NCI/EPA Working Group, 1985; Terzaghi-Howe, 1993). Several treatments which lead to physiological stress can induce transformation in cultured cells. Thus, in the present study, we investigated the growth, adhesion index, protein synthesis patterns and fibronectin distribution of Vero cells submitted to nutritional stress conditions and compared these parameters to those of a control non-stressed population.

*To whom the correspondence should be addressed: Selma Candelária Genari, Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas, UNICAMP, CP 6109—CEP 13083-970—Campinas, São Paulo, Brasil; email: nutricel@correionet.com.br

1065–6995/98/080000+00 $25.00/0

© 1998 Academic Press

)BNAME: No Job Name PAGE: 2 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
ings2/journals/academic/167/80250

MATERIALS AND METHODS

Control culture conditions

The Vero cells obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were maintained in Ham F10 medium (Sigma Chemical Co., St Louis, MO), supplemented with a 5% fetal bovine serum (Nutricell, Campinas, SP, Brazil) in 37°C with 5% of CO_2. The medium was renovated at 48-h intervals, and subculture was always performed at confluence of the monolayers.

Stress culture conditions

Stressed cells at confluence were maintained without change of culture medium for 15 days and then returned to control culture conditions. The period of 15 days was chosen because no changes in growth characteristics were observed over shorter periods of stress, and most cell cultures presented senescence and death over longer periods of stress even after they were returned to normal culture conditions.

Pattern growth analysis

The control and stressed cells at a density of 3×10^5 cells/ml were seeded on to 35-mm culture plates and their growth characteristics observed. The stressed cells presented multilayered growth with the formation of spheroids that spontaneously detached from the plate surface after prolonged culture times. The detached spheroids were collected and added to other culture flasks and their growth pattern was observed and photographed under an inverted phase-contrast microscope.

Adhesion assay

Suspensions of control or stressed cells at a density of 2×10^4, were seeded on to 35 mm culture plates with Ham F10 medium supplemented with 5% fetal bovine serum (FBS). The cells were maintained at 37°C with 5% CO_2 for different periods of time i.e. 10, 20, 40, 60, 80 and 100 min. At the end of each period, the plates were washed in saline solution, fixed in methanol/acetic acid (3:1 v/v) and stained with 5% Giemsa (Murray *et al.*, 1980). The number of adhered cells was estimated by counting ten random fields per plate. The assay for each time period was carried out in triplicate. The mean rate of adhered cells was obtained for each time period and the data were plotted graphically.

Soft agar assay

The cells were cultured in 35-mm plates with a 1.5-ml underlay containing 0.6% agar (cell culture tested, Sigma) in Ham F12 medium (Sigma) at the concentration indicated by the manufacturer and 10% FBS. The plating layer was the same medium in 0.3% agar, but with half the concentration of the above nutrients. Cells were plated at a concentration of 6×10^5 cells per dish and incubated for 8 days at 37°C, with 5% CO_2. The Sp2 cell line obtained from mouse myeloma, which grows in a suspension system, was used as control. The assay was performed with ten culture plates for each cell population.

Cell extract and total protein content

After detachment from the flask walls, cells were maintained for 2 h in Ham F10 medium supplemented with 5% FBS for the reestablishment of the cell surface proteins. After this time they were washed three times in phosphate-buffered saline (PBS) and a density of 6×10^7 cells was obtained. The cells were lysed in 1% Triton X100 in Tris–HCl buffer, pH 7.5 (1:1 v/v). Total protein content of the cell extracts was determined by the method of Bradford (1976).

Electrophoresis

Samples of the control and stressed cell extracts, with 50 μg of protein, were submitted to electrophoresis in 10% polyacrylamide gel (SDS–PAGE) (2% sodium dodecyl sulfate, 10% glycerol, 10 mM ethylene diamine tetraacetic acid (EDTA) and 0.01% bromophenol blue) under reducing (2-mercaptoethanol 0.2%) and non-reducing conditions (Zingales, 1984).

Immunocytochemistry

Immunodetection was performed on subconfluent and confluent monolayer cultures of control and stressed Vero cells grown on glass coverslips. The cells were seeded at a density of 5×10^4 cells/ml on glass coverslips in Ham F10 medium with 5% FBS and further cultured for 24 and 72 h before immunostaining. After washing in 0.1 M PBS, pH 7.4, the cells were fixed in 4% paraformaldehyde in PBS for 15 min. The material was preincubated for 10 min in 1% bovine serum albumin (BSA) in PBS to block nonspecific staining. The preparations were then incubated overnight in a moist chamber at 4°C with a monoclonal anti-cellular fibronectin antibody

BNAME: No Job Name PAGE: 3 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
ngs2/journals/academic/167/80250

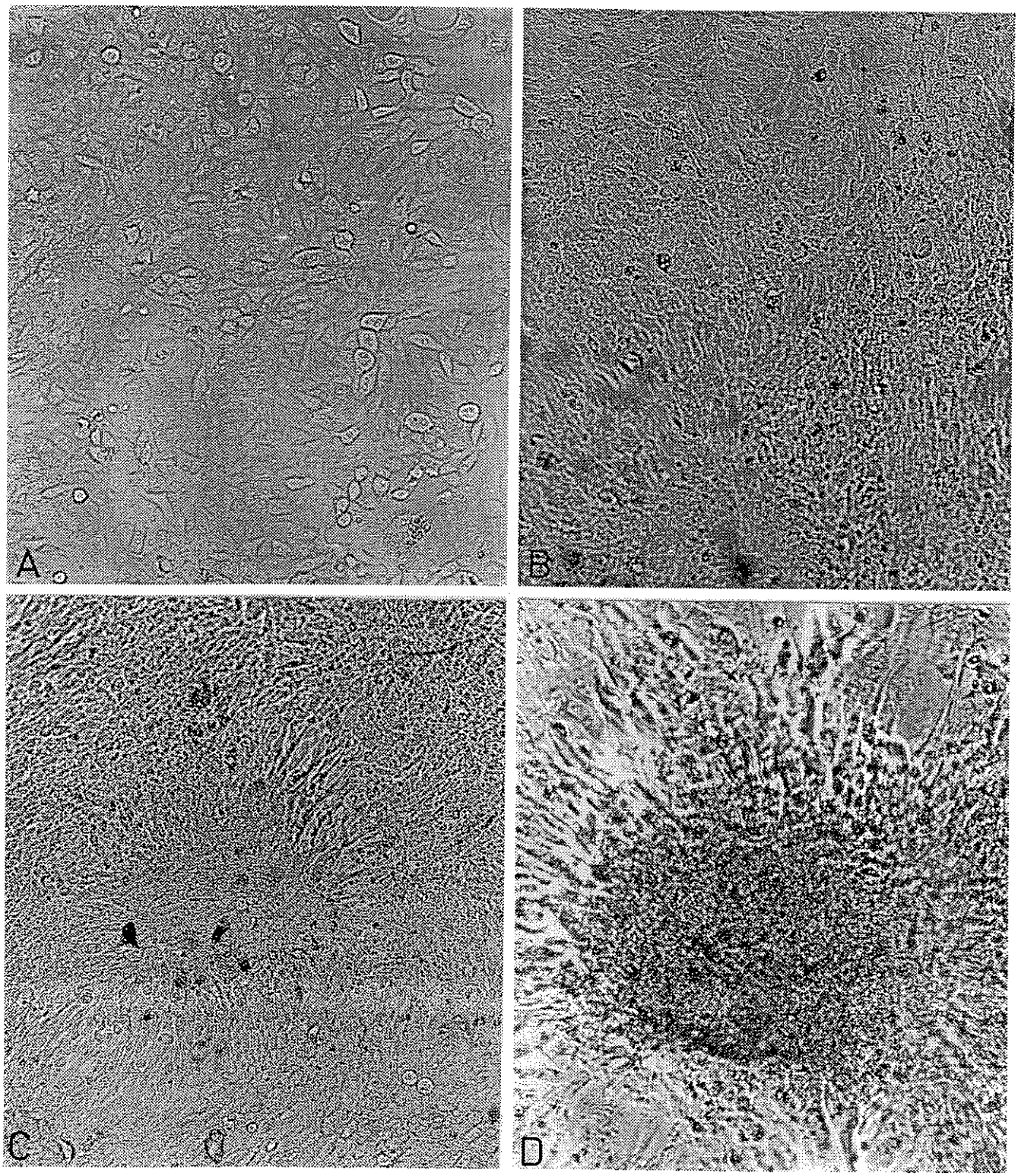


Fig. 1. Phase contrast photomicrographs of the same field of nutritionally stressed Vero cells, in the process of spheroid formation. (A) Confluent monolayer after 24 h of culture. (B) After confluence, the stressed cells grown for 48 h presented multilayered growth. (C) After 96 h, the stressed cells presented cellular aggregations and initial spheroid formation. (D) Cell aggregates or spheroid formation after 120 h of culture. × 160.

NAME: No Job Name PAGE: 4 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
gs2/journals/academic/167/80250

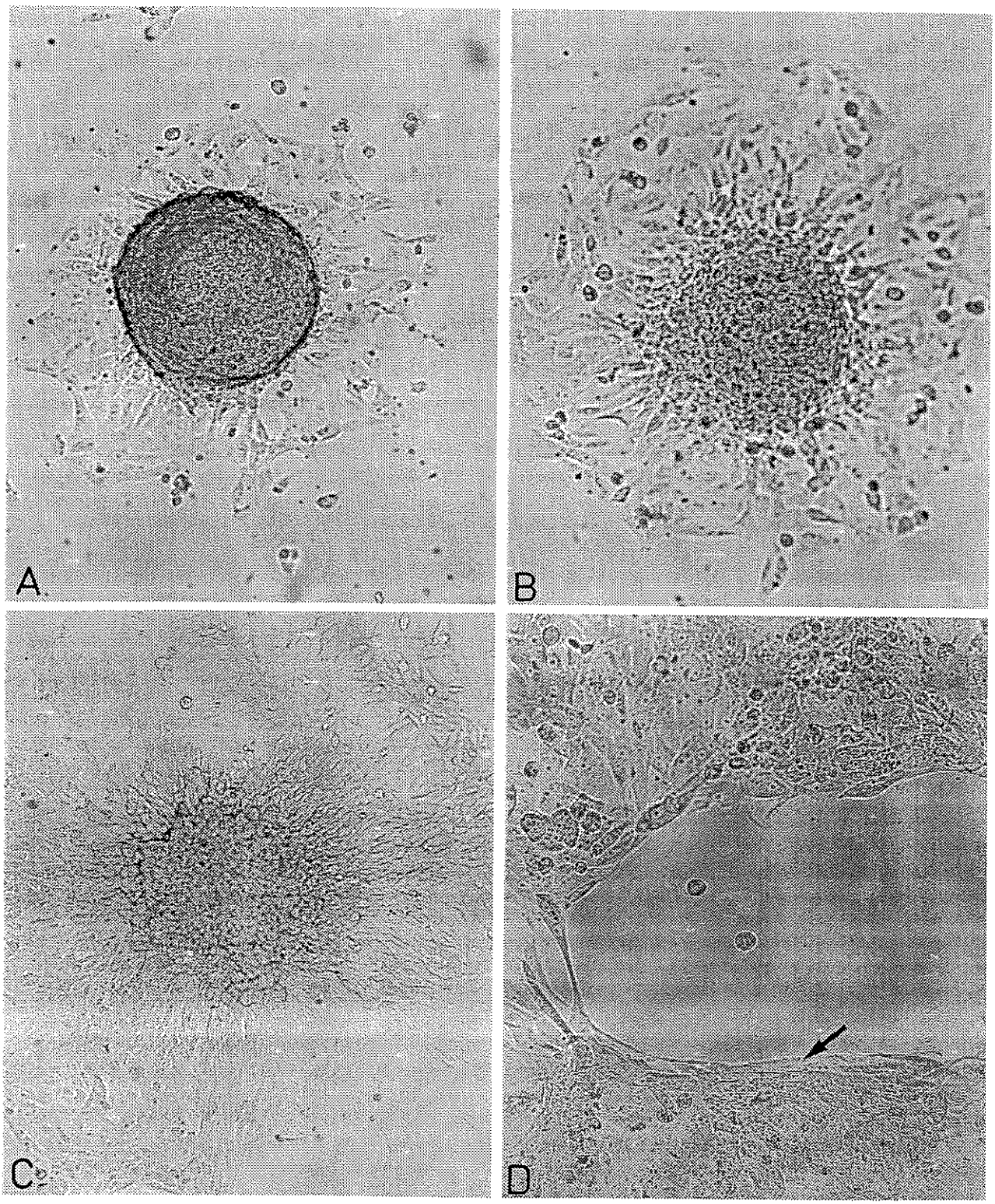


Fig. 2. Phase-contrast photomicrographs of spontaneously detached cellular spheroids returned to culture conditions. The spheroid presented cellular adhesion and migration on the culture flask surface. (A), 24 h; (B), 48 h; (C), 120 h. × 90. (D), The area initially occupied by the detached spheroid remained unoccupied by the surrounding cells, even though multilayered growth was observed at the periphery (arrow). × 160.

)BNAME: No Job Name PAGE: 5 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
lings2/journals/academic/167/80250

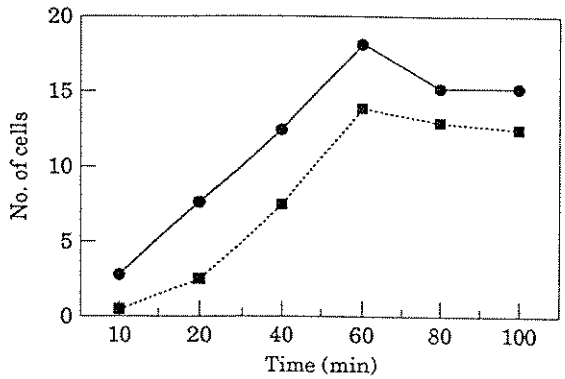


Fig. 3. Analysis of cell adhesion for control (Vero-C, ●) and stressed (Vero-S, ■) cells in Ham F10 medium, supplemented with 5% FBS over different time periods. The stressed cells showed a smaller adhesion rate than normal cells for all time periods analysed.

(Sigma) obtained from mouse ascite fluid (Clone FN-3E2). After washing in PBS, the antigenic sites were observed using FITC conjugated anti-mouse IgG (F0257—whole molecule) (Sigma) developed in a goat. The coverslips were mounted in Vectashield mounting medium (Vector, Burlingame, CA, U.S.A.) and analysed with an Axioskop Zeiss fluorescence microscope. Control experiments were performed including omission of primary antibody and under serum-free conditions.

RESULTS

Pattern growth analysis

The control Vero cells presented monolayered growth and, after confluence, they stopped dividing and entered senescence followed by death. The stressed cells, after confluence, presented multilayered growth with the formation of cell aggregates or spheroids which grew for 15 to 20 days and then spontaneously detached from the plate's surface (Fig. 1). The spheroids were collected and placed in other culture flasks, where they presented adhesion to the substrate and cell migration. A monolayer was initially followed by multiple layer growth (Fig. 2A, B and C). The place initially occupied by the detached spheroids remained unoccupied by the surrounding cells, and multilayered growth was observed at the periphery of these areas (Fig. 2D).

Adhesion assay

The mean rate of adhered control and stressed cells obtained for each time period is graphically presented in Figure 3, showing a lower adhesion rate for stressed cells than normal cells for all time periods analysed.

Soft agar assay

In the soft agar assay, a lower concentration of medium and FBS was used than suggested by the manufacturer. The Sp2 myeloma cells and control Vero cells did not present colony formation and were observed as individual cells on all soft agar culture plates (Fig. 4A and B). Eight day stressed cells in soft agar culture presented initial colony formation on all culture plates which slowly developed for two days (Fig. 4C).

Total protein analysis and electrophoresis

The total protein concentration in control Vero cells was 8.7 μg/μl, while the stressed cells presented 21.0 μg/μl. The electrophoresis gel showed some differences between the proteins of control and stressed cells. The stressed cells presented a 78-kDa band which was less visible in control cells, and a 44-kDa protein which was highly visible in the stressed cells and absent in the control cells (Fig. 5). No noticeable differences were observed between the reducing and non-reducing conditions.

Immunocytochemistry

Before confluence, the control cells presented dispersed fibronectin distribution on their surface and a more concentrated distribution in some regions of contact fibres between neighbouring cells (Fig. 6A and B). The stressed cells did not present this concentration in specific areas, showing a dispersed fibronectin distribution (Fig. 6C and D). At monolayer confluence, the control cells were actively synthesizing fibronectins which accumulated between adjacent cell–cell contact regions (Fig. 7A and B), while the stressed cells presented diffuse fibronectin localization (Fig. 7C and D).

DISCUSSION

The aim of this study was to investigate the influence of nutritional stress conditions on Vero cell growth and adhesion characteristics. The multilayered growth observed in the stressed cells, with spheroid formation, is a manifestation of morphological transformation and was irreversible, even when the stressed cells were returned to normal culture conditions. Cell transformation *in vitro* can be defined as the acquisition of permanent distur-

BNAME: No Job Name PAGE: 6 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
lings2/journals/academic/167/80250

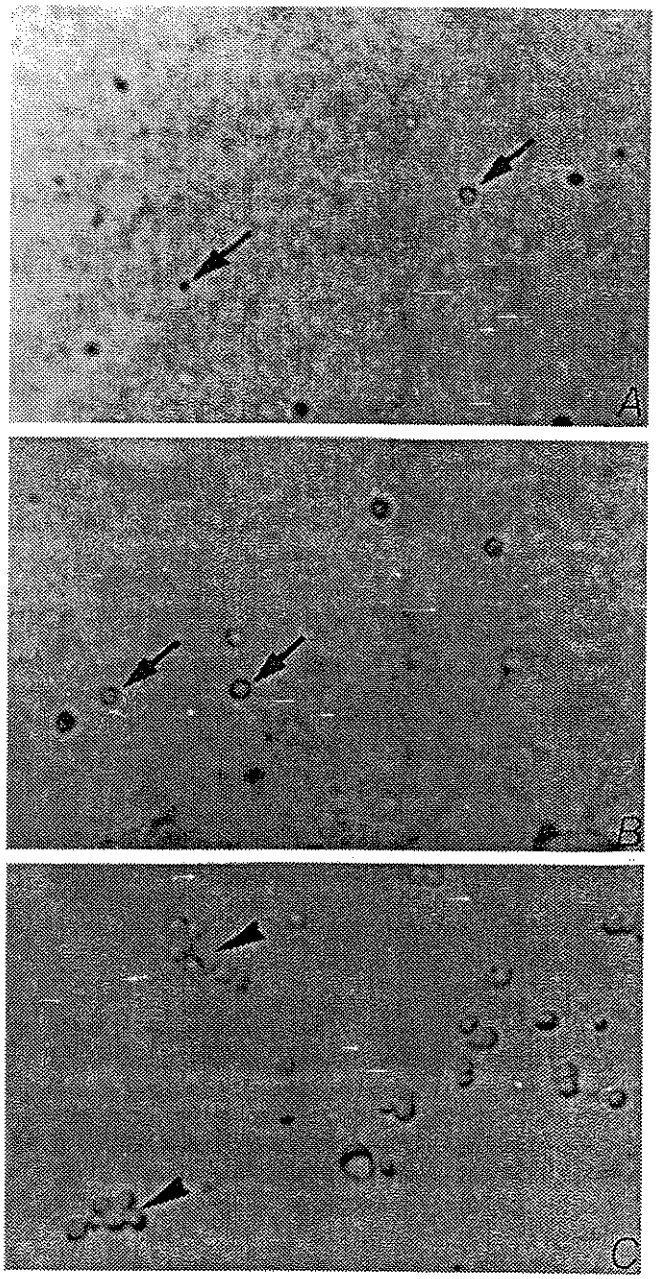


Fig. 4. Phase contrast photomicrographs of a cellular culture in soft agar medium with reduced nutrient concentration. (A), Sp2 myeloma cells (arrow); (B), control Vero cells (arrow); (C), initial colony formation of stressed Vero cells (arrowhead). × 150.

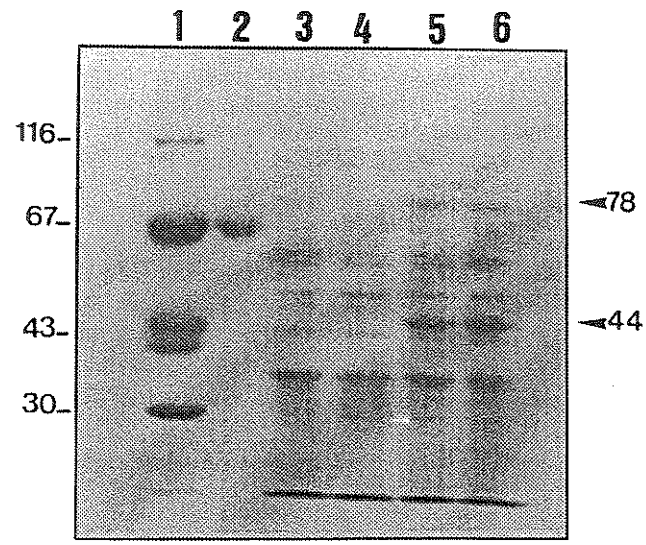


Fig. 5. Comparison of the protein pattern of control and stressed Vero cells by 10% polyacrylamide gel SDS–PAGE gel. Lanes 1 and 2: molecular weight protein standards (Pharmacia, Uppsala, Sweden); Lanes 3 and 4: Cell extract of the control Vero cell population under reduced and non-reduced conditions, respectively; Lanes 5 and 6: Cellular extract of the stressed Vero cell population under reduced and non-reduced conditions, respectively. Proteins on the gel were stained by Coomassie brilliant blue.

bances in the growth and/or locomotion control (Pontén, 1976; Smets, 1980) and involves alterations in several cell–cell (Klaunig and Ruch, 1990; Ren *et al.*, 1990; Yamasaki, 1990), cell–substrate (Keski-Oja *et al.*, 1985; Nermut, 1991) and cell–medium (Holzer *et al.*, 1986; Cross and Dexter, 1991) interactions. When the Vero monolayers were submitted to nutritional stress the cells were not only exposed to the effects of a depleted medium, but also to the consequences of this condition, such as the products of their own metabolism as well as a lowered pH. Nutritional stress (Borek, 1972) and low pH (Morita *et al.*, 1989; Zura and Grant, 1981) have been associated with the induction of cell transformation in mammalian cell culture and of some kinds of stomach and bladder tumour development in mice (Ashby and Ashidate, 1986; Brusick, 1987). Thus, the altered morphological and growth characteristics observed in the stressed cells were probably caused by the nutritional stress conditions and their consequences.

The spheroid is a spherical mass composed of cells and extracellular matrices (Landry, 1985; Takezawa *et al.*, 1993; Genari and Wada, 1996) and their formation in multilayer growth indicates the loss of contact inhibition (Abercrombie, 1979), which has been associated with malignancy. The fact that cells do not occupy the space left by the detached spheroid may indicate the production of some substance which prevents the attachment and spreading of surrounding cells.

Normal fibroblasts or epithelial cells require adhesion and spreading on the substrate for growth *in vitro*, while some kinds of tumour cells present the capacity to grow in suspension or in a soft agar medium, a property known as anchorage independence (Beremblum and Armuth, 1981; Vasiliev,

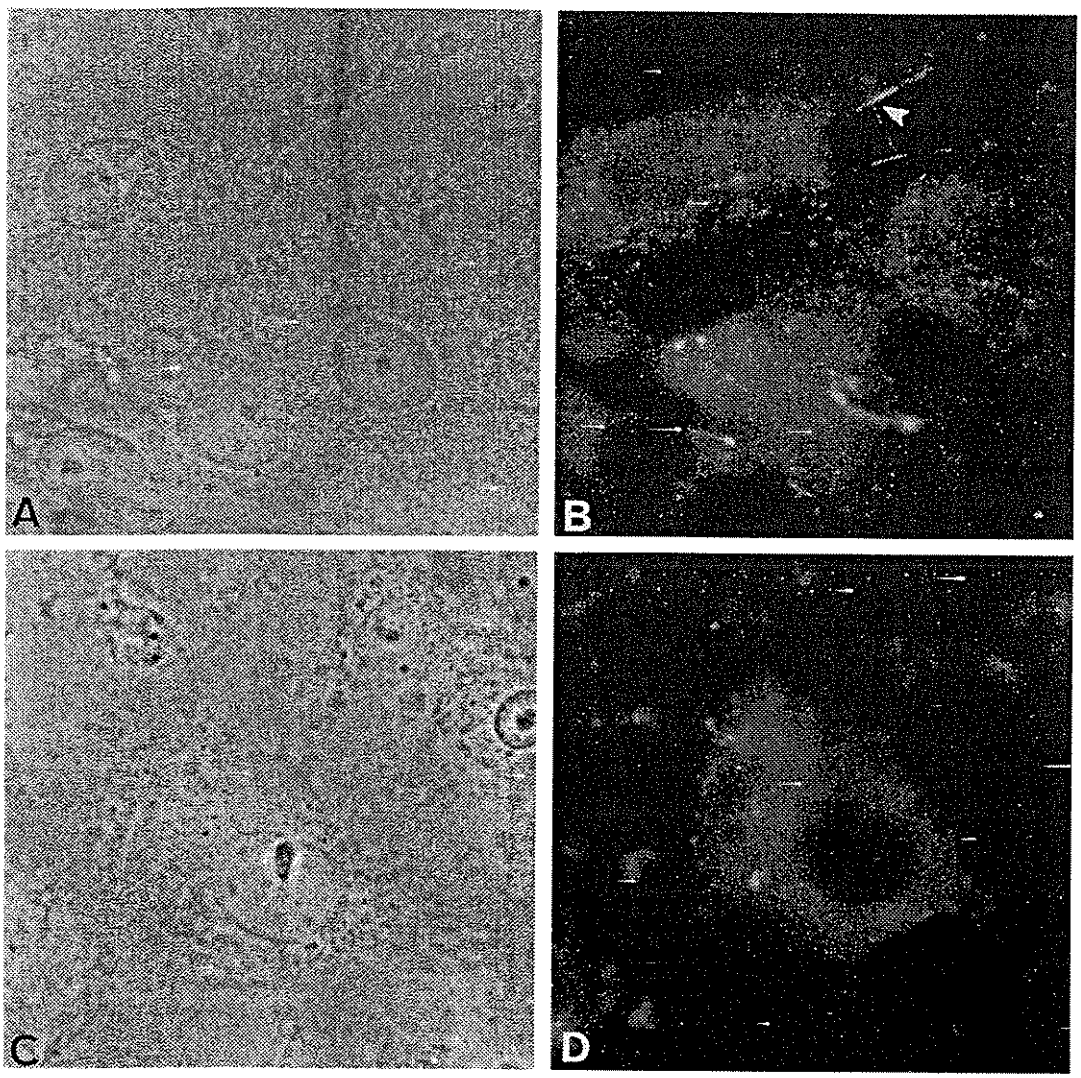


Fig. 6. Immunostaining of fibronectin distribution after 24 h of culture in sparse cells of the control Vero population (A and B), and in the stressed population (C and D). (A) Phase contrast of the control sparse cells; (B) same field showing control cells presenting fibronectin accumulation in fibres between neighbouring cells (arrowheads) × 3500. (C) Phase contrast of stressed sparse cells and (D) same field with stressed Vero cells presenting diffuse fibronectin distribution. × 3500.

1985). For the soft agar assay, only the stressed cells presented initial growth and colony formation, indicating not only a physical, but also a nutritive independence, since these cells were growing in reduced amounts of nutrients and serum, while the control population did not present development or colony formation (Holzer *et al.*, 1986; Persky *et al.*, 1989).

Several studies demonstrated altered proteins or new protein synthesis during the carcinogenic process *in vivo*. Some of these proteins are able to induce cellular transformation *in vitro* (Beremblum and Armuth, 1981). The best studied abnormal protein in cells transformed by viruses or chemical agents or spontaneously, as well as in cancer cells, is a 53-kDa protein, called pp53. This phosphoprotein is absent in normal cells and has been associated with maintaining the transformed phenotype (Lane and Crawford, 1979; Senger *et al.*, 1979; Gurney *et al.*, 1980; McCormick and Harlow, 1980; Crawford *et al.*, 1981). More recent studies have demonstrated that the p53 gene is frequently affected in several kinds of human tumours, being involved in the control of cellular growth and playing an important role in the suppression of the abnormal cell proliferation and tumour development (Milner, 1991).

During cellular transformation obtained by several treatments such as high temperatures (Morimoto, 1991; Welch, 1993), irradiation (Witte *et al.*, 1989; Boensen, 1992; Terzaghi-Howe, 1993), nutritional stress (Borek, 1972) and hyperosmotic

)BNAME: No Job Name PAGE: 8 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
lings2/journals/academic/167/80250

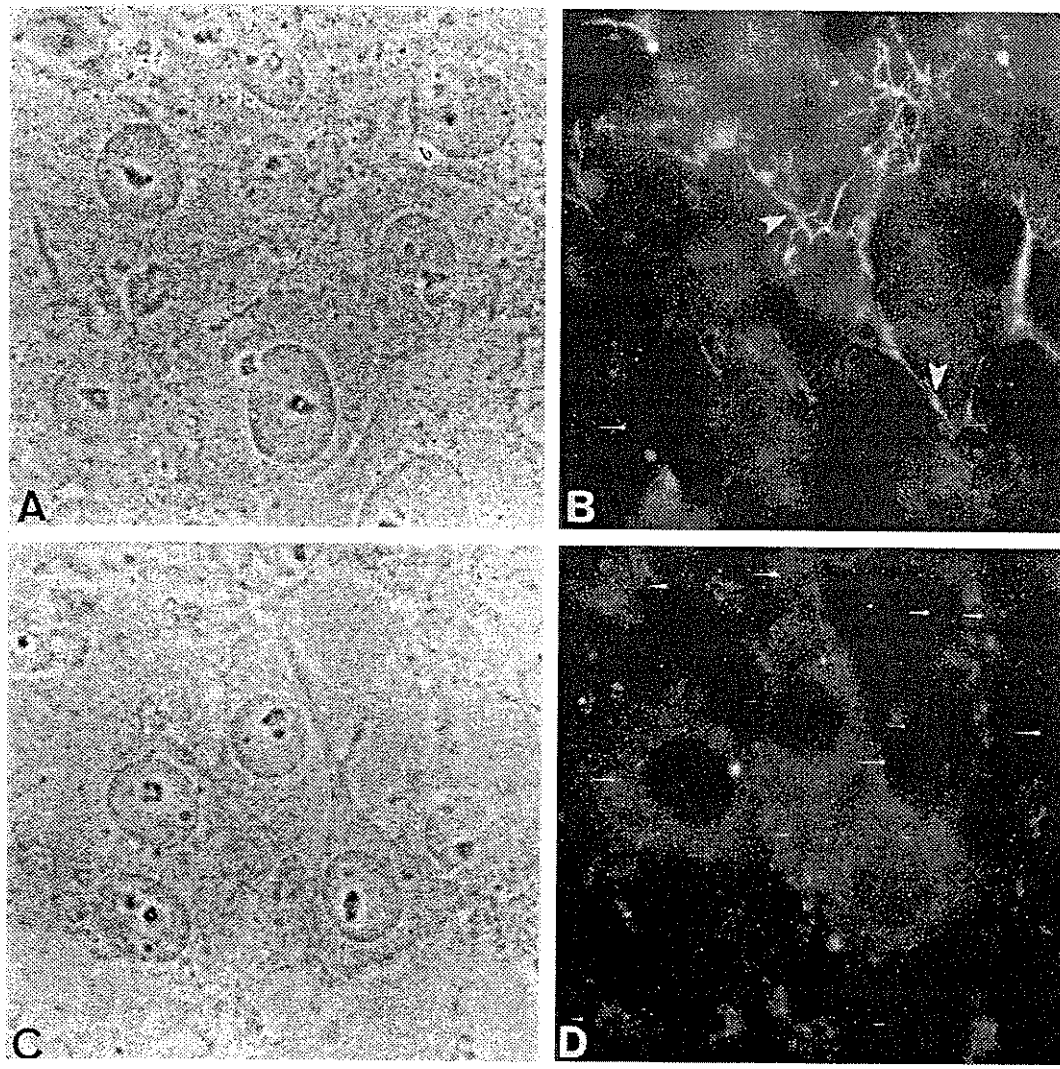


Fig. 7. Immunostaining of fibronectin distribution after 72 h of culture in confluent monolayers of control Vero population (A and B), and in the stressed population (C and D). (A) Phase contrast of a monolayer control cell population; (B) same field showing fibronectin accumulation between adjacent cell–cell contact regions (arrowheads) in control cells, × 3500. (C) Phase-contrast of a monolayer stressed population and (D) same field showing the stressed cells presenting diffuse fibronectin distribution. × 3500.

(Wollnik *et al.*, 1993) or other environmental stress, the synthesis of a group of cellular proteins has been observed. These proteins are collectively called HSP (heat shock proteins) or stress proteins because they were initially observed in cells exposed to heat shock. HSP production is observed in all organisms, from the simplest prokaryotes, yeast, plants on up to higher eukaryotes and their production indicates an apparent defence mechanism (Welch and Suhan, 1986). Most HSP are in fact expressed in all cells maintained under normal growth conditions, but the expression of these proteins can increase as the result of several metabolic insults, which reduce the level of normal ATP (Welch, 1993). Some stress proteins have been shown to function as mediators of protein maturation, leading to the more general concept of molecular chaperones, i.e. proteins which assist the correct non-covalent assembly of other polypeptide structures, but which are not components of these assembled structures, when they perform their normal biological functions (Ellis, 1993). Some stress proteins were synthesized by *Tetrahymena pyriformis* submitted to stress conditions by starvation. These proteins are included in the 100-kDa, 70-kDa, 50-kDa and 30-kDa family (Galego, 1984). The stressed Vero cells presented a 44-kDa protein which was not observed in the unstressed population, and a 78-kDa protein which was more visible than in control cells, where it was found in very

BNAME: No Job Name PAGE: 9 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
ings2/journals/academic/167/80250

small quantities. The synthesis of large amounts of an approximately 35-kDa protein was observed in studies using various mouse fibroblast cells after their viral, chemical or spontaneous transformation. These studies showed that this protein is associated with growth control and may possibly be a marker for cellular transformation (Gottesman, 1978). A 37-kDa protein was also described on the cellular surface of mouse fibrosarcoma, being associated *in vitro* with invasiveness, since the use of specific antibodies inhibited cell invasion and the contact inhibition was restored (Parish *et al.*, 1987). The data presented above may indicate a possible function of the 44-kDa protein and other HSP in the altered characteristics of stressed cells and in the maintenance of their transformed phenotype.

Other proteins frequently altered in transformed cells are the fibronectins, which are glycoproteins found in plasma and secreted by a variety of cell types including fibroblasts, endothelial cells and hepatocytes (Grinnell and Bennett, 1981). The principal function of fibronectins is the adhesion of cells to extracellular materials or to a solid substrate. This process includes initial attachment, spreading, the organization of cytoskeletal fibrils and focal attachments to the substrate (Hynes, 1990). Fibronectin was lost from the surface of virally, chemically or spontaneously transformed cells and from tumour-derived cells (Murray *et al.*, 1980; Bannikov *et al.*, 1982). The loss of or diminished fibronectin production in transformed cells may be caused by alterations in cell adhesion, cytoskeletal organization and cell migration. The distribution of fibronectin in stressed Vero cells was considerably altered in relation to the control cells. In the sparse cultures the control cells presented fibronectin accumulation in contact fibres between neighbouring cells, while this pattern of fibronectin accumulation was not observed in stressed cells. At confluence, control cells presented fibronectin accumulation between adjacent cells, while the stressed cells presented a diffuse distribution. This localization suggests that fibronectin may have an important function in the attachment, adhesion and migration of these cells to the substratum because, in fact, the stressed cells presented a smaller adhesion index than control cells. Decreased spreading and attachment, accompanied by reduced adhesion, are frequently observed in transformed or tumorigenic cells, and are associated with the deficient interaction and production of extracellular matrix elements (Grinnel and Bennett, 1981; Kleinman *et al.*, 1981; Alitalo *et al.*, 1982; Vasiliev, 1985). The absence of fibronectin accumulation in lateral cell–cell contact regions may signify a reduced fibronectin synthesis or deficient fibronectin accumulation in stressed cells. The deficient formation of continuous cell–cell contact regions may lead to disorganization in the growth pattern, with multilayer growth and aggregate or spheroid formation (Bannikov, 1982; Hynes, 1990). The fibronectin accumulation in cell–cell contact regions may contribute to the maintenance of the monolayered organization, as observed in the growth characteristics of control Vero cells. The decreased synthesis or accumulation of fibronectin can lead to the disorganization of cell growth pattern, with multilayered growth *in vitro*, contributing to the metastasis process *in vivo*.

The data presented here indicate that nutritional stress conditions may promote the transformation of Vero cells, leading to alterations in their growth characteristics, adhesion index and protein synthesis.

ACKNOWLEDGEMENTS

This work was carried out with the support of CAPES and FAEP.

REFERENCES

ABERCROMBIE M, 1979. Contact inhibition and malignancy. *Nature* **281:** 259–262.

ALITALO K, KESKI-OJA J, HEDMAN K, VAHERI A, 1982. Loss of different pericellular matrix components of rat cells transformed with a T-class ts mutant of Rous Sarcoma Virus. *Virology* **119:** 347–357.

ASHBY J, ISHIDATE M, 1986. Clastogenicity in vitro of the Na, K, Ca and Mg salts of saccharin; and of magnesium chloride; consideration of significance. *Mutation Res* **163:** 63–73.

BANNIKOV GA, GUELSTEIN VI, MONTESANO R, 1982. Cell shape and organization of cytoskeleton and surface fibronectin in non-tumorigenic and tumorigenic rat liver cultures. *J Cell Sci* **54:** 47–67.

BEREMBLUM I, ARMUTH V, 1981. Two independent aspects of tumor promotion. *Biochem et Biophys Acta* **651:** 51–63.

BIANCHI NO, AYRES J, 1971. Heterochromatin location on chromosomes of normal and transformed cells from African green monkey (*Cercopithecus aethiops*). *Exp Cell Res* **68:** 253–258.

BOENSEN JJB, DIETEREN N, BAL E, 1992. A possible factor in genetic instability of cancer cells: stress-induced secreted proteins lead to decrease in replication fidelity. *Carcinogenesis* **13:** 2407–2413.

BOREK C, 1972. Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated Hepatoma-like cells under conditions of nutritional stress. *Proc Natl Acad Sci USA* **69:** 956–959.

BRADFORD MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72:** 248–254.

BNAME: No Job Name PAGE: 10 SESS: 4 OUTPUT: Thu Jul 9 09:05:55 1998
ngs2/journals/academic/167/80250

BRUSICK DJ, 1987. Implications of treatment-condition-induced genotoxicity for chemical screening and data interpretation. *Mutation Res* **189:** 1–6.

CRAWFORD LV, PIM DC, GURNEY EG, 1981. Detection of common feature in several human tumor cell lines—a 53,000-dalton protein. *Proc Natl Acad Sci* **78:** 41–46.

CROSS M, DEXTER TM, 1991. Growth factors in development, transformation and tumorigenesis. *Cell* **64:** 271–280.

ELLIA RJ, 1993. The general concept of molecular chaperones. *Phil Trans R Soc Lond* **339:** 257–261.

GALEGO L, BARAHORA I, RODRIGUES-POUSADA C, 1984. Response of Tetrahymena pyriformis to stress induced by starvation. *Eur J Biochem* **139:** 163–171.

GENARI SC, WADA MLF, 1995. Behavioural differences and cytogenetic analysis of a transformed cellular population derived from a Vero cell line. *Cytobios* **81:** 17–25.

GENARI SC, WADA MLF, 1996. Scanning and transmission electron microscopy of transformed Vero cells, with altered in vitro growth characteristics. *J Submicrosc Cytol Pathol* **28:** 565–572.

GOTTESMAN MM, 1978. Transformation-dependent secretion of a low molecular weight protein by murine fibroblasts. *Proc Natl Acad Sci* **75:** 2767–2771.

GRINMELL F, BENNETT H, 1981. Fibroblast adhesion on collagen substrata in the presence and absence of plasma fibronectin. *J Cell Sci* **49:** 19–34.

19GURNEY EG, HARRISON RO, FENNO J, 1980. Monoclonal antibodies against Simian Virus 40 T antigens: Evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. *J Virol* **34:** 752–763.

HOLZER C, MAIER P, ZBINDEN G, 1986. Comparison of exogenous growth stimuli for chemically transformed cells: growth factors, serum and cocultures. *Exp Cell Biol* **54:** 237–244.

HYNES RO, 1990. *Fibronectins.* Springer-Verlag, New York.

IARC/NCI/EPA WORKING GROUP, 1985. Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols. *Cancer Res* **45:** 2395–2399.

KESKI-OJA J, ALITALO K, BARLATI S, VAHERI A, 1985. Pericellular matrix changes in fibroblastic and epithelial cells induced by oncogenic transformation. In: Santi L, Zardi L, eds. *Theories and Models in Cellular Transformation.* Instituto Scientifico Tumori, Genoa, Italy, Academic Press, 55–70.

KLAUNIG J, RUCH RJ, 1990. Biology of disease—role of inhibition of intercellular communication in carcinogenesis. *Lab Invest* **62:** 135–146.

KLEINMAN HK, KLEBE R, MARTIN GR, 1981. Role of collagenous matrices in the adhesion and growth of cells. *J Cell Biol* **88:** 473–485.

LANE DP, CRAWFORD LV, 1979. T antigen is bound to a host protein in SV 40-transformed cell. *Nature* **278:** 261–263.

LANDRY J, BERNIER D, OUELLET C, MORCEAU N, 1985. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* **101:** 914–923.

MCCORNICH F, HARLOW E, 1980. Association of a murine 53,000-Dalton phosphoprotein with Simian Virus 40-T antigen in transformed cells. *J Virol* **34:** 1213–1224.

MILNER J, 1991. A conformation hypothesis for the suppressor and promoter functions of p53 in cell growth control and in cancer. *Proc R Soc Lond B* **245:** 139–145.

MORIMOTO RI, 1991. Heat shock: The role of transient inducible responses in cell damage, transformation and differentiation. *Cancer Cells* **3:** 295–301.

MORITA T, TAKEDA K, OKOMURA K, 1990. Evaluation of clastogenicity of formic acid, acetic acid and lactic acid on cultured mammalian cells. *Mutation Res* **240:** 195–202.

MURRAY JC, LIOTTA L, RENNARD SI, MARTIN GR, 1980. Adhesion characteristics of murine metastic and nonmetastic tumor cells in vitro. *Cancer Res* **40:** 347–351.

NERMUT MV, EASON P, HIRST EMA, KELLIE S, 1991. Cell/substratum adhesions in RSV-transformed rat fibroblasts. *Exp Cell Res* **193:** 382–397.

PARISH RW, SCHUMIDHAUSER C, SCHMIDT T, DULDER R, 1987. Mechanisms of tumor cell metastasis. *J Cell Sci* (Suppl.) **8:** 181–197.

PÓNTEN J, 1976. The relationship between in vitro transformation and tumor formation in vivo. *Biochem et Biophys Acta* **458:** 397–422.

PERSKY B, MEYSKENS FL, HENDRIZ MJC, 1989. Immunohistochemical and ultrastructural study of human melanoma colonies grown in soft agar. *Am J Anat* **184:** 212–224.

REN J, HAMADA J, TAKEICHI N, FUJIKAWA S, KOBAYASHI H, 1990. Ultrastructural differences in junctional intercellular communication between highly and weakly metastatic clones derived from rat mammary carcinoma. *Cancer Res* **50:** 358–362.

SENGER LA, 1979. Transformed mammalian cells secrete specific proteins and phosphoproteins. *Cell* **16:** 885–893.

SMETS LA, 1980. Cell transformation as a model for tumor induction and neoplastic growth. *Biochim Biophys Acta* **605:** 93–111.

TAKEZAWA T, MORI Y, YONAHA T, YOSHIZATO K, 1993. Characterization of morphology and cellular metabolism during the spheroid formation by fibroblasts. *Exp Cell Res* **208:** 430–441.

TERZAGHI-HOWE M, 1993. Factors regulating the emergence of spontaneous and X-ray-induced variants in primary rat tracheal epithelial cell culture. *In Vitro Cell Dev Biol* **29**(A): 120–126.

VASILIEV JM, 1985. Spreading of non-transformed and transformed cells. *Biochem Biophys Acta* **780:** 289–296.

WELCH WJ, SUHAN JP, 1980. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J Cell Biol* **103:** 2035–2052.

WELCH WJ, 1993. Heat shock proteins functioning as molecular chaperones: their roles in normal and stressed cells. *Phil Trans R Soc Lond B* **339:** 327–333.

WITTE L, FUKZ Z, HAIMOVITZ-FRIEDMAN A, 1989. Effects of irradiation on the release of growth factors from cultured bovine, porcine and human endothelial cells. *Cancer Res* **49:** 5066–5072.

WOLLNIK B, KUBISCH C, MAASS A, 1993. Hyperosmotic stress induces immediate-early gene expression in ventricular adult cardiomyocytes. *Biochem Biophys Res Commun* **194:** 642–646.

YAMASAKI H, 1990. Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis* **11:** 1051–1058.

ZINGALES B, 1984. Analysis of protein by sodium dodecyl sulfate polyacrylamine gel electrophoresis. In: Morel CM, ed. *Genes and Antigens of Parasites.* UNDP World Bank/WHO/Fio Cruz, 357–363, Rio de Janeiro.

ZURA KD, GRANT WF, 1991. The role of hydronium ion in the induction of chromosomal aberrations by weak acid solutions. *Mutation Res* **84:** 349–364.

CYTOGENETICAL AND CYTOSKELETAL ALTERATIONS IN VERO CELLS TRANSFORMED BY NUTRITIONAL STRESS

SELMA CANDELÁRIA GENARI* AND **MARIA LÚCIA FURLAN WADA**

Department of Cell Biology, Biology Institute – State University of Campinas ,

UNICAMP - CP 6109 - CEP 13083-970

Campinas - São Paulo, Brazil.

Condensed title for running head: **CYTOGENETICAL AND CYTOSKELETAL ALTERATIONS**

* To whom the correspondence should be addressed:

Selma Candelária Genari

Departamento de Biologia Celular - Instituto de Biologia

Universidade Estadual de Campinas - UNICAMP

CP 6109 - CEP 13083-970 - Campinas, SP - Brasil.

Phone: 55 19 788 7786

FAX: 55 19 239 3124

E-mail: sgenari@nutricell.com.br

SUMMARY

We have investigated the effect of nutritional stress conditions on different properties of Vero cells in culture. Nutritional stress and lowered pH may promote the transformation of Vero cells, leading to alterations in their growth and adhesion characteristics. In this work we investigated the induction of morphologic transformation of Vero cells submitted to nutritional stress and their correlation with cytogenetical alterations, the cytoskeleton and extracellular matrix components. Control and stressed populations presented different modal chromosome number and karyotypes. Polyploidy was enhanced in stressed cells. Codistribution of fibronectin and actin was observed in control cells, while the expression of fibronectin was reduced in stressed cells and no codistribution was observed. Stressed Vero cells also presented reduced staining for vimentin. We can conclude that the nutritional stress conditions associated with a lowered pH are responsible for the cytogenetical and cytoskeletal alterations in stressed Vero cells.

Keywords: *Transformation; Fibronectin; cytoskeleton; polyploidy; Vero cells.*

INTRODUCTION

Some studies have demonstrated that non-physiological conditions in culture such as ionic alterations, pH lowering (Zura and Grant, 1981 and Morita et al., 1989/1990) and nutritional stress (Borek, 1972, Genari and Wada, 1998) may be associated with genotoxical effect, induction of cellular transformation and cytogenetical alterations (Genari and Wada, 1995). Other studies have also associated these conditions to those observed in some stomach and urinary bladder tumors in the mouse (Brushich, 1987; Ashby and Ishidate, 1986). We have investigated the effect of nutritional stress conditions on different properties of Vero cells in culture. These studies indicate that nutritional stress may promote the transformation of Vero cells, leading to alterations in their growth and adhesion characteristics, and in the proteins synthesized (Genari et al, 1996; Genari et al, 1998).

During long term cultivation, cell lines may change some of their original characteristics, including the karyotype. These changes may be due not only to genetic characteristics of culture cells but also may be induced by alterations in the culture conditions (Mamaeva, 1998). Genetic alteration is frequently associated with changes in cellular growth and morphology, and these characteristics are defined by interactions between extracellular matrix and cytoskeletal components, or between neighbouring cells (Geiger, et al., 1985).

This research investigated the induction of morphologic transformation in Vero cells submitted to nutritional stress conditions and their correlation with cytogenetical alterations as well as cytoskeletal and extracellular matrix components.

MATERIALS AND METHODS:

Control Culture Conditions

Vero cells at passage number 195 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 a 5% fetal bovine serum (Nutricell, Campinas,SP - Brazil) at 37°C with a 5% CO_2 atmosphere. The medium was changed at 48 hours intervals, and the subculture was performed when cells reached confluence.

Stress Conditions

Confluent cells were maintained without culture medium replacement for fifteen days. After this time, the stressed cells were returned to the control culture conditions. This period was standardized because no morphological alterations were observed if cells were subjected to shorter periods. On the other hand, senescence and cellular death were verified in most of the cultures stressed for periods over fifteen days, even when the cells were returned to the normal culture conditions. At the end of the stressing period, the medium reached pH 6.8.

Morphological and Growth Characteristics

The growth and morphological characteristics of control and stressed MDCK cells were assessed by daily observation of the cultures with an inverted microscope.

Cytogenetics and karyotypic analysis

Cells were arrested in metaphase by the addition of colchicine (16 μg/ml) on logarithmic phase cultures (control cells at passage 198 and stressed cells at the passage 201), followed by a 4 h incubation period. Cells were harvested and chromosomes were prepared according to routine techniques. Trypsin-Giemsa (GTG) banding was performed according to a modification of the method of Seabright (1971). The modal chromosome number was determined by counting the chromosomes in 100 metaphases for each cell population. The mitotic and polyploidy index were obtained according to Deitch and Sawicki (1979) and Gylvarry et al. (1990), respectively. The arrangement of the chromosomes was adjusted according to Bianchi & Ayres (1971) and Genari & Wada (1995).

Immunocytochemistry

Immunocytochemistry for fibronectin, actin and vimentin was performed on subconfluent and confluent monolayer cultures of control and stressed cells grown on glass coverslips. The cells were seeded at the density of 5 X 10^4 cells/ml in Ham F10 medium with 5% FBS and further cultured for 24 or 72 h before immunostaining. After washing in PBS, pH 7.4, the cells were fixed and permeabilized in 0.25% glutaraldehyde, 4% formaldehyde in 80mM Pipes pH6.8, containing 1mM $MgCl_2$, 5mM EGTA and 0.2% Triton X-100 for 30 min. After blocking with 100mM sodium borohydride buffer for 20 min and with 3% BSA for 1h they were incubated with the primary antibodies. The cells were incubated with phalloidin-rhodamin (SIGMA) for F-actin during 1 h and further stained with either a monoclonal antibody anti-vimentin (SIGMA - from mouse ascites

fluid, clone V9) or an anti-cellular fibronectin (SIGMA - from mouse ascites fluid, clone FN-3E2). Primary antibodies were visualized by incubation with a goat FITC-conjugated antibody anti-mouse IgG (SIGMA). The material was observed with a Zeiss Axioskop equipped with filter sets for rhodamin and fluorescein.

RESULTS

Morphological and Growth Characteristics

Control Vero cells grew exponentially in a monolayer until confluence and presented an elongated shape, typical of normal fibroblastic-like cells (Figure 1 A). After reaching confluence at day 3 to 4, normal cells stopped dividing and died. The stressed cells, after confluence, presented growth in multilayers with formation of cellular aggregates (Figure 1 B).

Cytogenetics and karyotyping

Control and stressed Vero cells presented polyploidy indices of 5.0% and 12.3% respectively. Mitotic index was the same (7.2%) for both conditions. Control and stressed populations presented a modal chromosome number of 55 and 56, respectively (range: 53 to 58; n = 100 for each condition). The diploid chromosome number distribution in control and stressed cells is presented in Figure 2. Figures 3 and 4 exhibit the karyotype of control and stressed Vero cells, respectively. The karyotype of stressed cells was characterized by trisomy of chromosome 5. Monosomy of some chromosomes was also observed in both conditions.

Immunocytochemistry

In control and stressed cells bundles of actin in the form of stress fibers were observed. They transverse the cytoplasm, and codistributed with fibronectin in control cells, while the expression of fibronectins was reduced in stressed cells and no codistribution was observed (Figure 5). When compared to the control cells, stressed cells presented reduced

staining for vimentin, with a diffuse localization. Control cells presented vimentin fibers located mainly around the nucleus and extending to the cytoplasm (Figure 5). After confluence, control cells presented fibronectin and actin deposition between adjacent cells (Figure 6); the distribution of fibronectin was not altered in stressed cells and continued as before confluence (not shown).

DISCUSSION

The Vero cell lineage obtained from kidney cells of the African green monkey (*Cercopithecus aethiops*) has a characteristic growth pattern in culture. We previously have shown that nutritional stress conditions may induce alterations of different properties of Vero cells, including morphologic transformation (Genari et al, 1996; Genari et al, 1998). We now describe the association of cytogenetical alterations, cytoskeletal and extracellular matrix components, associated with this morphologic transformation in Vero cells.

The normal diploid chromosome number in the African green monkey is 60 (Bianchi and Ayres, 1971) and the karyotype of Vero cells is described as pseudodiploid (ATCC). In this study the karyotype of control and stressed Vero cells, these display a different modal chromosome number, 55 and 56, respectively. The karyotype of stressed cells was characterized by a trisomy of the chromosome 5. Polyploidy was enhanced in stressed cells (12.3%) as compared to control cells (5.0%), and this characteristic has been associated with transformation.

When the Vero monolayers were submitted to nutritional stress the cells were not only exposed to the effects of a depleted medium, but also to other consequences of this condition, such as accumulation of metabolites and lowered pH. Nutritional stress and low pH (Morita, *et al.* 1989; Zura and Grant, 1981; Genari and Wada, 1998) have been associated with the induction of cell transformation in mammalian cell culture and of some kinds of stomach and bladder tumor developed in mice (Ashby and Ashidate, 1986; Brusick, 1987).

Fibronectin expression is important for cellular migration and adhesion, morphogenesis and transformation process. In virally transformed cells (Murray 1980; Hayman et al, 1981; Chen et al, 1984; and Nermut et al., 1991) the expression of fibronectin is often reduced and the cells are typically less adherent to the substrate and no longer show lost of contact inhibition (Chen and Chen, 1987; Hynes, 1990a). Nevertheless, some studies indicated that cellular fibronectin and other matrix components may be effective in promoting assembly and stabilization of actin stress fibers in some cells (Hynes, 1990b). The distribution of fibronectin in stressed cells was altered in relation to control cells. The control cells presented fibronectin accumulation between neighboring cells at confluence, while stressed cells presented a diffuse distribution. The absence of fibronectin accumulation between the cells of the monolayer may signify a reduced fibronectin synthesis or deficient fibronectin accumulation in stressed cells, which may lead at the disorganization of the growth pattern with multilayered growth and formation of cellular aggregates (Banikov, 1982; Hynes, 1990).

Most of cell types attach to fibronectin matrices by an integrin receptor, which that results in cell spreading, accompanied by cytoskeletal reorganization and clustering of integrin receptors into specialized regions, termed focal adhesions, on the basal surface of the cells. These structures are regions of close contact between the cell membrane and the substrate and serve as sites to anchor actin stress fibers (Singer et al., 1988; Christopher et al., 1997). The cytoskeleton plays a major role in maintaining cell shape, alterations in the cytoskeleton of normal cells result in changes in shape and adhesiveness, and may induce expression of matrix metalloproteinases (Hansell et al., 1995). Some studies have described reduction of focal adhesions, actin stress fibers and

UNICAMP
BIBLIOTECA CENTRAL

fibronectin associated with morphological alterations in transformed cells (Boschek et al., 1981). In our results we did not observe decrease of stress fibers and focal adhesion associated with diminished fibronectin in transformed cells. The actin distribution presented the same pattern in control and stressed cells, but another cytoskeleton compound, the vimentin, was reduced in stressed cells. Typically found in cells of mesenchymal origin, vimentin, a cytoskeletal intermediate filament, is also observed in most cultured cell types (Jaeger et al., 1995). Some studies have indicated that an excessive vimentin content can similarly act as a mutagenic protein, leading to a number of morphological, structural and biological abnormalities in epithelial cells (Andreoli and Trevor, 1995). We believe that reduced amounts of vimentin also may be associated with alterations of morphology and growth characteristics, as observed in stressed cells.

We can conclude that the nutritional stress conditions accompanied by lowered pH are responsible for the cytogenetical, cytoskeletal and extracellular matrix alterations in stressed Vero cells.

ACKNOWLEDGEMENTS

We are very grateful to Dr. Hernandes F. de Carvalho for the phalloidin-rhodamin donation and his assistance in immuhistochemistry assays. This work was carried out with funding from Coordenadoria de Aperfeicoamento de Pessoal de Nivel Superior (CAPES) to S. C. Genari, from Fundo de Apoio ao Ensino e à Pesquisa (FAEP/UNICAMP) to M. L. F. Wada and from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) to H. F. Carvalho.

LITERATURE CITED

Andreoli, J. M. and Trevor, K. T. (1995). Structural and biological consequences of increased vimentin expression in simple epithelial cell types. *Cell Motil. Cytoskeleton* **32**, 10-25.

Ashby, J. and Ishidate, M. (1986). Clastogenicity in vitro of the Na, K, Ca and Mg salts of saccharin; and magnesium chloride; consideration of significance. *Mutation Res.* **163**, 63-73.

Bannikov, G. A, Guelstein, V. I. Montesano, R., Tint, I. S., Tomatis, L. Troyanovsky, A. and Vasiliev, J. M. (1982). Cell shape and organization of cytoskeleton and surface fibronectin in non-tumorigenic and tumorigenic rat liver cultures. *J. Cell Sci.* **54**, 47-67.

Bianchi, N. O. and Ayres, J. (1971). Heterochromatin location on chromosomes of normal and transformed cells from African green monkey (*Cercopithecus aethiops*). *Exp. Cell Res.* **68**, 253-258.

Borek, C. (1972). Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated Hepatoma-like cells under conditions of nutritional stress. *Proc. Nat. Acad. Sci. USA* **69**, 956-959.

Boeschek, C. B., Jockusch, B. M., Friss, R. R., Back, R., Grundmann, E. and Bauer, H. (1981). Early changes in the distribution and organization of microfilament proteins during cell transformation. *Cell* **24**, 175-184.

Brushich, D. J. (1987). Implications of treatment-condition-induced genotoxicity for chemical screening and data interpretation. *Mutation Res.* **189**, 1-6.

Chen, J-M., Olden, K., Beranrd, B. A. and Fong-Fong, C. (1984). Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. *J. Cell Biol.* **98**, 1546-1555.

Chen, J-M and Chen, W-T (1987). Fibronectin-degrading proteases from the membranes of transformed cells. *Cell* **48**,193-203.

Christopher, R. A., Kowalczyk, A. P. and McKeown-Longo, P. J. (1997). Localization of fibronectin matrix assembly sites on fibroblasts and endothelial cells. *J. Cell Sci.* **110**, 569-581.

Deitch, A. D. and Sawicki, S. G. (1979). Effects of cordycepin on microtubules of cultures mammalian cells. *Exp. Cell Res.* **118**, 1-13.

Geiger, B., Volk, T. and Volberg, T. (1985). Molecular heterogeneity of adherens junctions. *J. Cell Biol.* **101**, 1523-1531.

Genari, S. C. and Wada, M. L. F. (1995). Behavioural differences and cytogenetical analysis of a transformed cellular population derived from a Vero cell line. *Cytobios* **81**, 17-25.

Genari, S. C. Dolder, M. A. D. and Wada, M. L. F. (1996). Scanning and transmission electron microscopy of transformed Vero cells, with altered *in vitro* growth characteristics. *J. Submicrosc. Cytol. Pathol.* **28**(4), 565-572.

Genari, S. C., Gomes, L. and Wada, M. L. F. Alterations in the growth and adhesion pattern of Vero cells induced by nutritional stress conditions. *Cell Biol. Int.***20** (in press).

Gylvarry, U., Farrel, D., Lynch, V., Moriarty, M., Dooley, M., Law, E. and Clynes, M. (1990). Cytological differences between normal and malignant human cell populations in culture. *Cancer Res.* **50**, 3390-3393.

Hansell, E. J., Frisch, S. M., Tremble, P., Murnane, J. P. and Werb, Z. (1995). Simian virus 40 transformation alters the actin cytoskeleton, expression of matrix metalloproteinases, and inhibitors of metalloproteinas, and invasive behavior of normal and ataxia telangiectasica human skin fibroblasts. *Biochem. Cell Biol.* **73**, 373-389.

Hayman, E., Engvall, E. and Ruoslahti, E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells.*J. Cell Biol.* **88**, 352-357.

Hynes, R. O. (1990)a. Fibronectin and the cytoskeleton. In *Fibronectins.* (ed. Springer-Verlag), pp231-248. New York.

Hynes, R. O. (1990)b. Oncogenic Transformation. In *Fibronectins.* (ed. Springer-Verlag), pp231-248. New York.

Jaeger, R. G., Jaeger, M. M. M., Araújo, V. C. and Bachar, B. (1995). Analysis of the interdependent localization of vimentin and microtubules in neoplastic myoepithelial cell. *Cell Motil. Cytoskeleton* **32,** 289-298.

Mamaeva, S. E. (1998). Karyotype evolution of cells in culture: a new concept. *Int. Rev. Cytol.* **178**:1-40.

Morita, T., Watanabe, Y., Takeda, K. and Okomura, K. (1989). Effects of pH in the in vitro chromosomal aberration test. *Mutation Res.* **225**, 55-60.

Murray, J. C., Liotta, L., Rennard, S. I. and Martin, G. R. (1980). Adhesion characteristics of murine metastic and nonmetastic tumor cells in vitro. *Cancer Res.* **40**, 347-351.

Nermut, M. V., Eason, P., Hirst, E. M. A. and Kellie, S. (1991). Cell/substratum adhesion in RSV-transformed rat fibroblasts. *Exp. Cell Res.* **193**, 382-397.

Reimann, N., Bartnizke, S., Bullerdiek, J., Schmitz, U., Rogalla, P., Nolte, I. and

Seabright, M. (1971). A rapid banding technique for human chromosomes. *Lancet* **1**,971-972.

Singer, I. I., Scott, S., Kawka, D. W., Kazazia, D., Gailit, J. and Ruoslahti, E. (1988). Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. *J. Cell Biol.* **106**,2171-2182.

Zura, K. D. and Grant, W. F. (1981). The role of hydronium ion in the induction of chromosomal aberrations by weak acid solutions. *Mutation Res.* **84**, 349-364.

LEGENDS

Figure 1 - Control and stressed Vero cells growing in culture. Control cells with 48 h of culture form a monolayer and grow as fibroblastic-like cells (A), 100X. Stressed Vero cells after 26 days in culture grow in multilayer forming cellular aggregates (B) (*arrow*), 100X.

Figure 2 - Chromosome number of control (upper panel) and stressed Vero cells (lower panel). Control population presented a modal chromosome number of 55, while the stressed cell population presented a modal chromosome number of 56 (n=100; range 53 to 58, for both conditions).

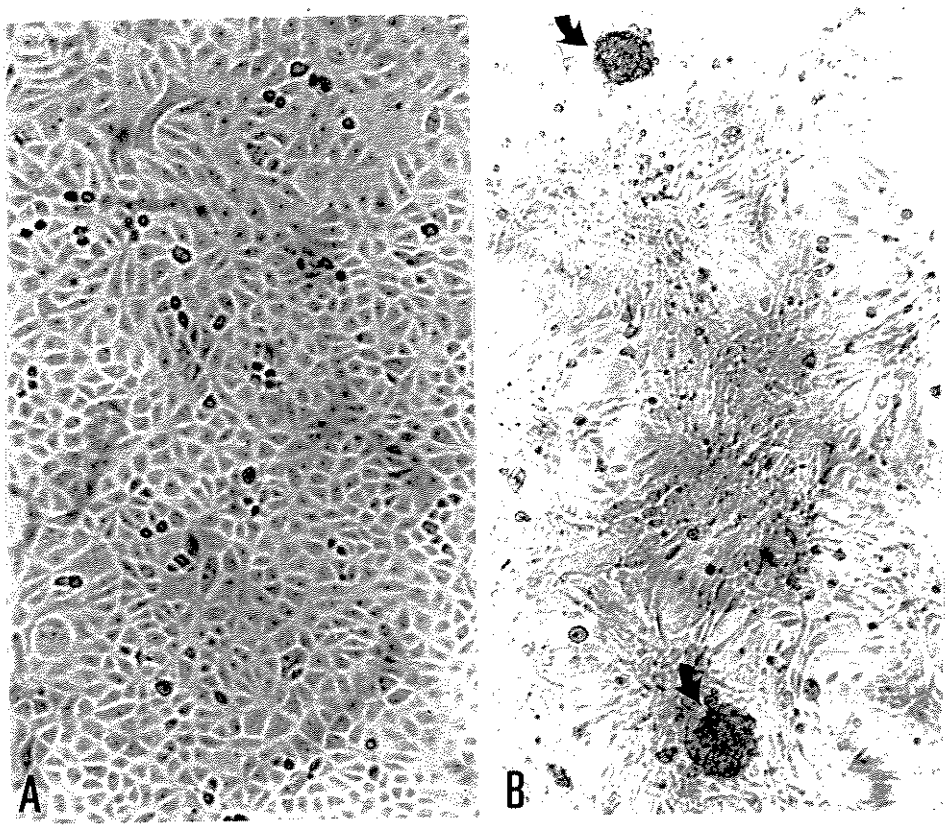
Figure 3 – Representative 55, XX GTG-banding karyotype of control Vero cells. 1500X.

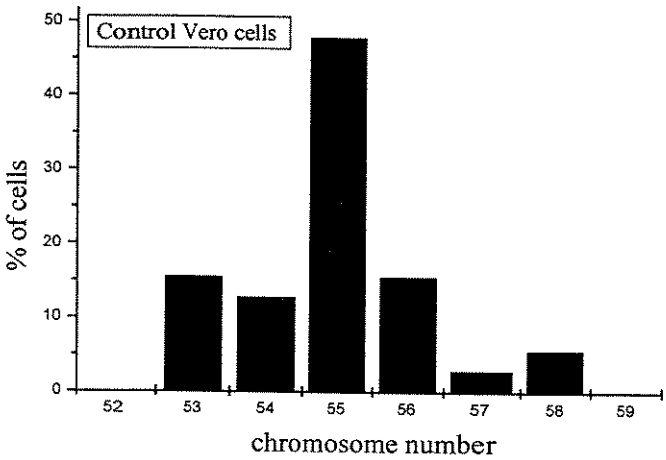
Figure 4 – Representative 56, XX GTG-banding karyotype of stressed Vero cells. 1500x.

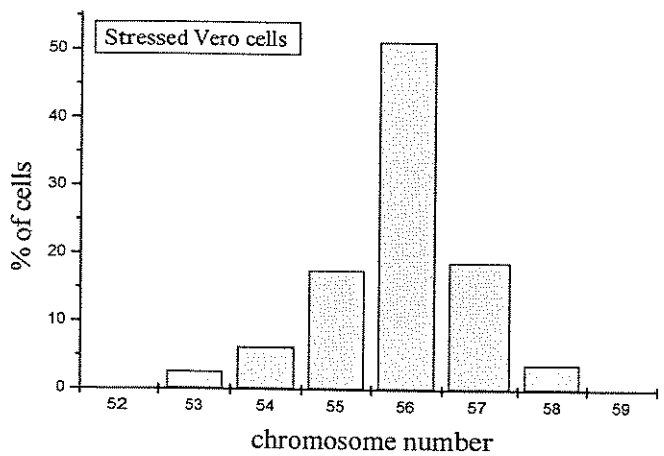
Figure 5 - Indirect immunofluorescence staining of fibronectin, actin and vimentin in control and stressed Vero cells. Cells were seeded on coverslips and cultured for 24 h. Cells were then fixed, permeabilized and stained by indirect immunofluorescence with antibodies against fibronectin or vimentin and phalloidin. Left panel shows the control cells, and right panel shows the stressed Vero cells; A and C correspond to the colocalization of fibronectin and actin and B and D vimentin and actin. In control cells the codistribution of actin and fibronectin, is observed mainly at the stress fibers and at the cell periphery (A). In the stressed cells, fibronectin staining was reduced and no actin

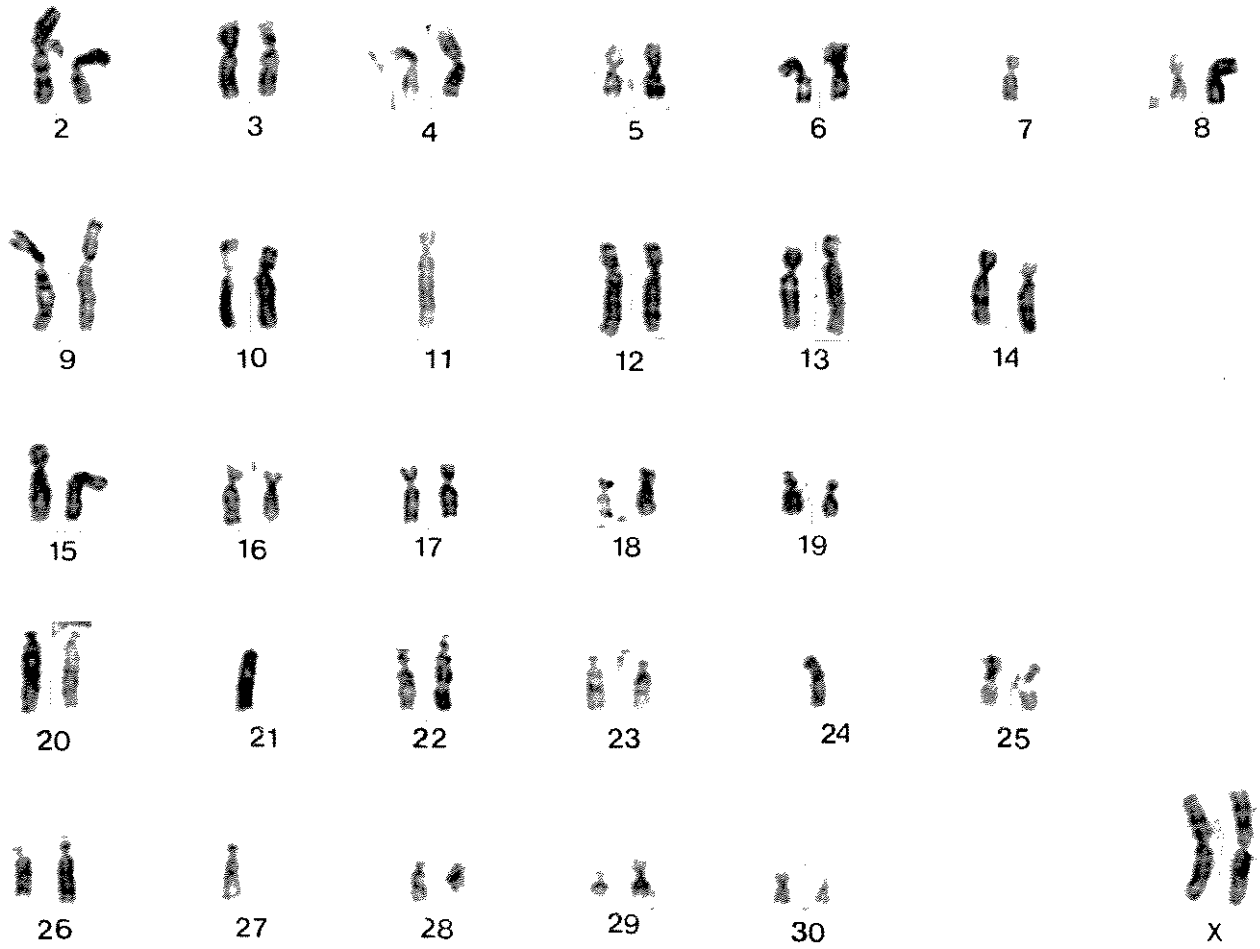
colocalization was observed (C). When compared to control cells (B), stressed cells presented reduced staining for vimentin (D). 350X.

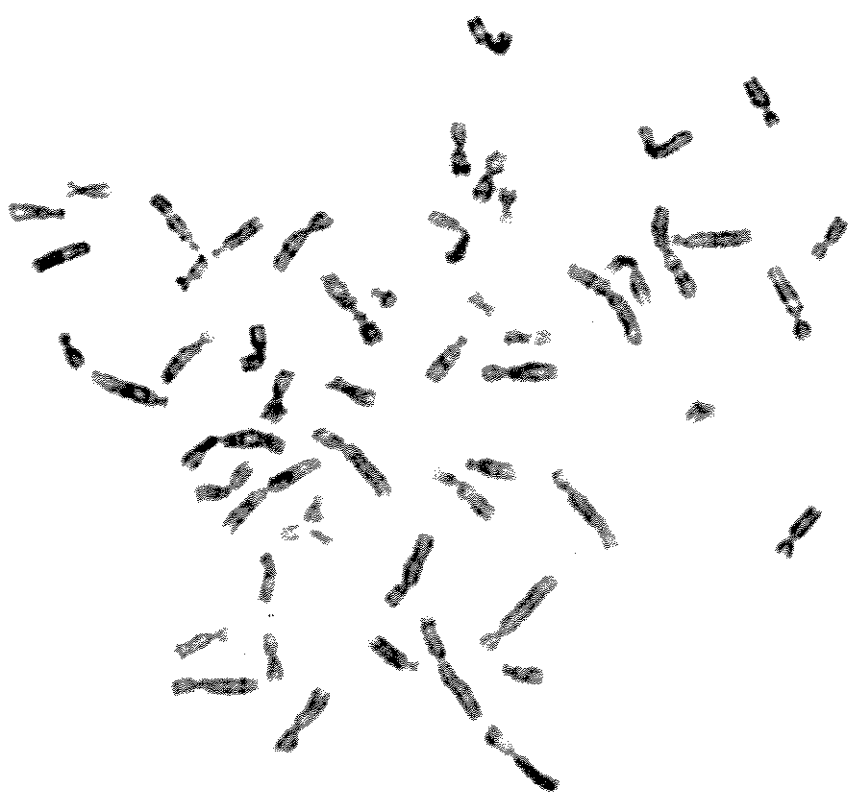
Figure 6 - Indirect immunofluorescence staining of fibronectin (B) and actin (C) in control Vero cells cultured for 72 h. After confluence control cells presented codistribution of fibronectin and actin between adjacent cells (D), while the distribution of fibronectin was not altered in stressed cells that continued as before confluence (not shown). 350X.

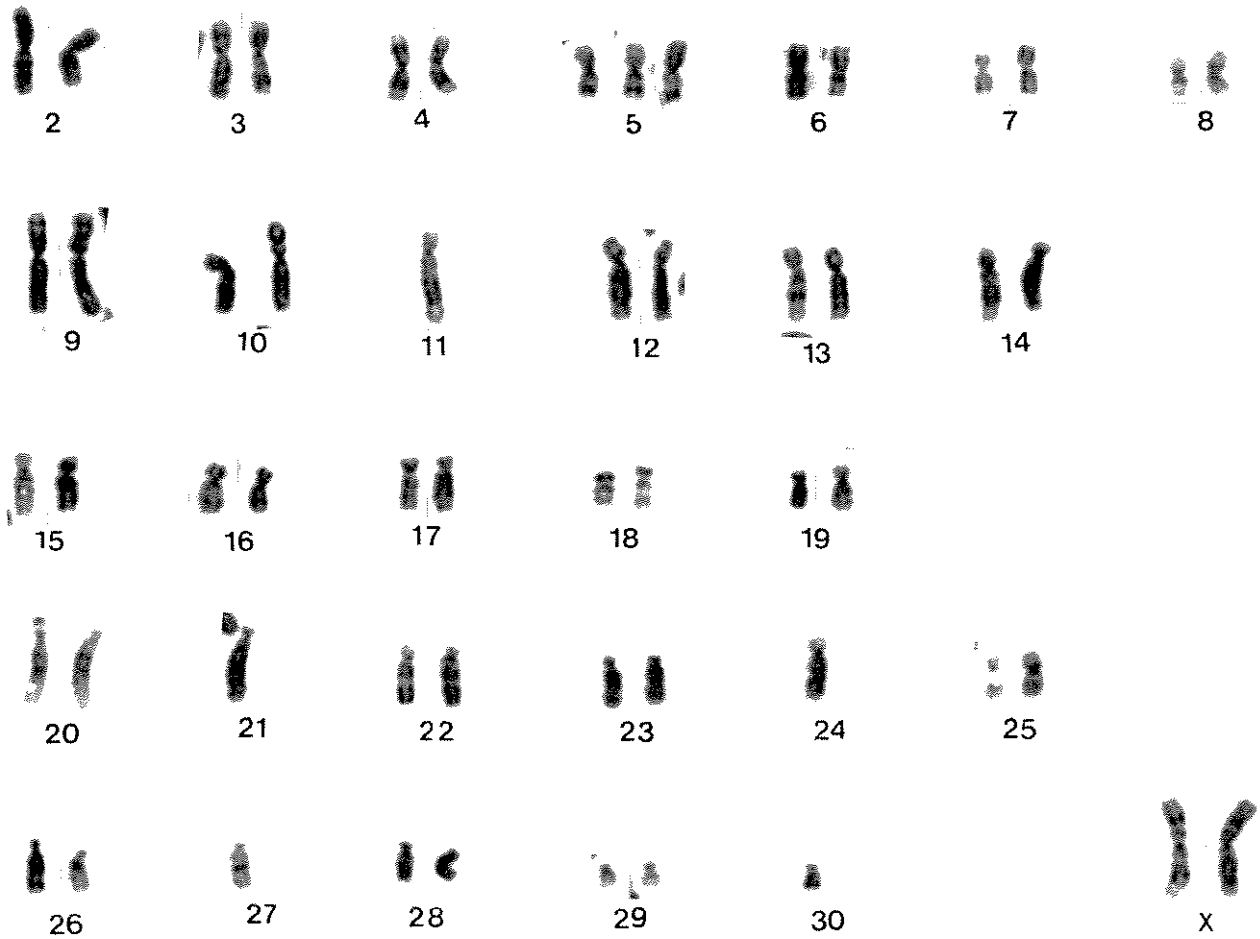

A
B

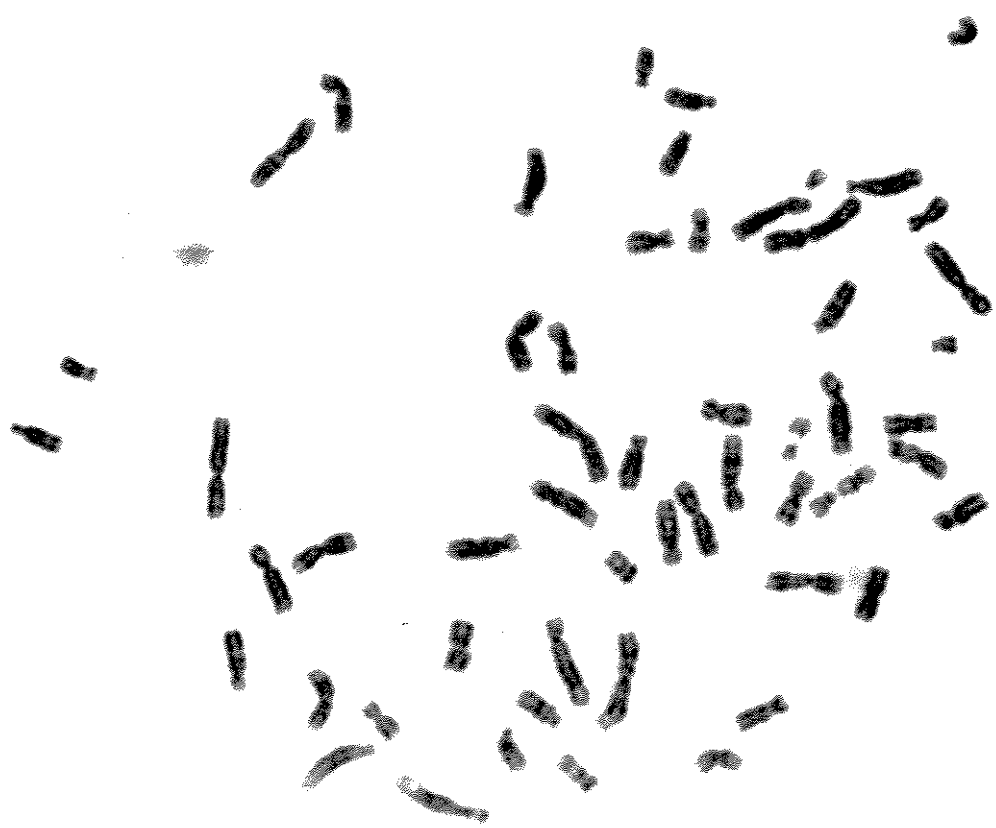

Control Vero cells
% of cells
0
10
20
30
40
50
52
53
54
55
56
57
58
59
chromosome number

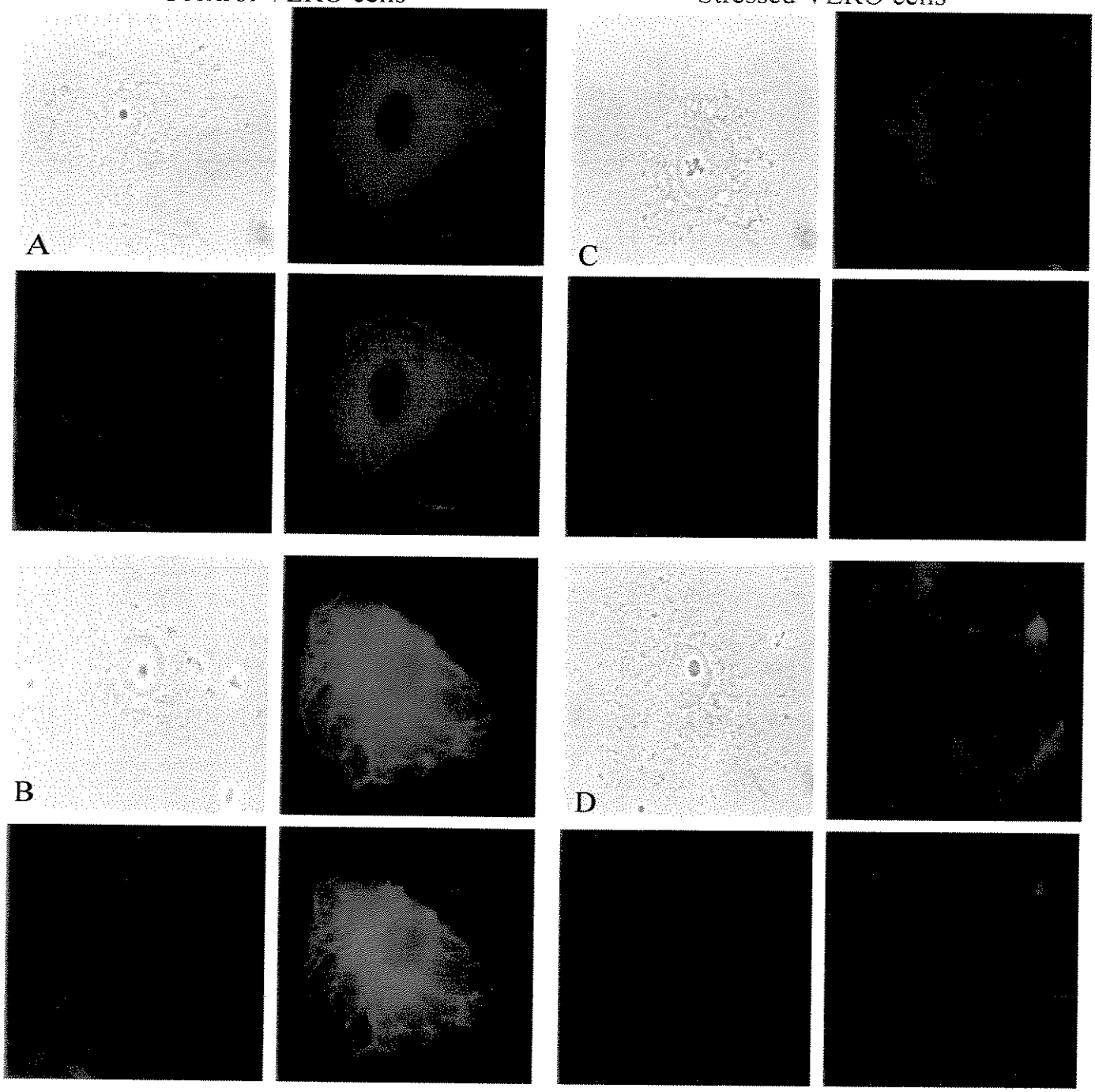

Stressed Vero cells
% of cells
0
10
20
30
40
50
52
53
54
55
56
57
58
59
chromosome number

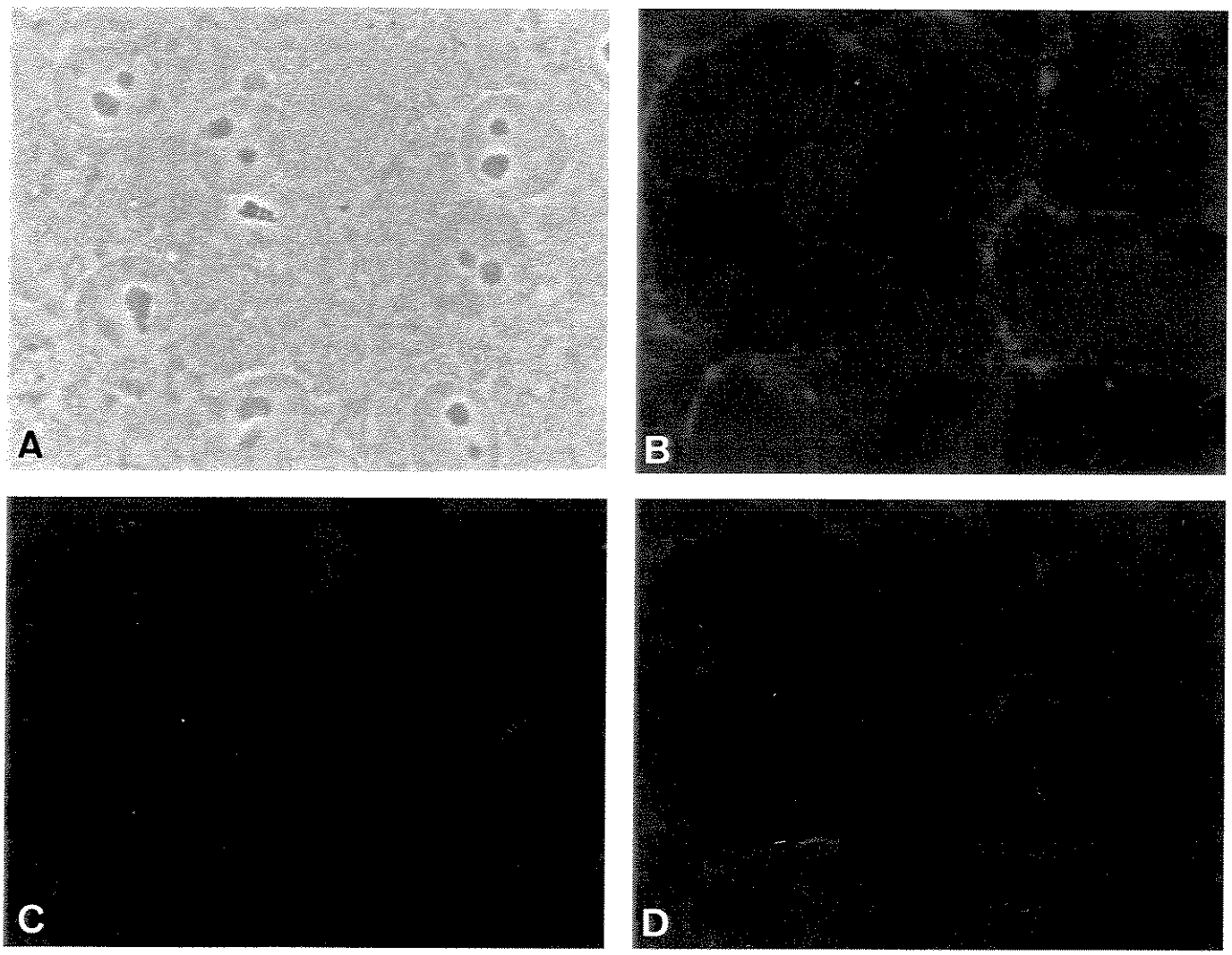

2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
X




2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
X




Control VERO cells
Stressed VERO cells
A
C
B
D


A
B
C
D

Influence of the Nutritional Stress Conditions on Differentiation and Tubulogenesis of Epithelial Cells *In Vitro*

SELMA CANDELÁRIA GENARI* AND **MARIA LÚCIA FURLAN WADA**

Department of Cell Biology, Biology Institute – State University of Campinas, UNICAMP - CP 6109 - CEP 13083-970 Campinas - São Paulo, Brazil.

Condensed title for running head: **NUTRITIONAL STRESS-INDUCED TUBULOGENESIS *IN VITRO***

* To whom the correspondence should be addressed:

Selma Candelária Genari

Departamento de Biologia Celular - Instituto de Biologia

Universidade Estadual de Campinas - UNICAMP

CP 6109 - CEP 13083-970 - Campinas, SP - Brasil.

Phone: 55 19 788 7786

FAX: 55 19 239 3124

E-mail: sgenari@nutricell.com.br

Total number of text figures and tables = 7 figures.

SUMMARY

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 studies concerning cell growth regulation and to investigate the factors involved with the assembly of epithelial cells into organized multicellular units and tubulogenesis. We have focused our investigations on the effect of physiological conditions, more specifically on nutritional stress accompanied by lowered pH, on the morphological and growth characteristics of MDCK cells. Control MDCK cells grow in monolayer and after the confluence, decreased cellular proliferation and die. The nutritionally stressed cells presented continuous multilayered growth, with differentiated characteristics and formation of tubular structures in long culture time. Cytogenetical studies showed differences between control and stressed cells and enhanced polyploidy index in the latter. Fibronectin, actin and enhanced vimentin deposition was observed at the cell-cell contact regions in stressed cells. These results suggest that nutritional stresses may induce alterations in the interactions between extracellular matrix components including fibronectin, with the cytoskeleton, which may be important factors in the folding of an epithelial monolayer to form tubular structures.

INTRODUCTION

The Madin-Darby Canine Kidney (MDCK), originally derived from the kidney of a normal cocker spaniel dog, is a well characterized cell line which presents monolayer growth in culture and has the morphological properties of distal tubular epithelial cells (Rindler, et al., 1979). This cell line was shown to retain the differentiated function of polarized cells, which have the capacity for vectorial salt and fluid transport, occluding junctions formation, sensitivity to growth regulation and the ability to regenerate tubule-like structures when injected into athymic nude mice (Rindler, et al., 1979; Meza, et al., 1980; Cereijido, et al., 1981; Saier, 1981). With these properties, the MDCK cell line has been used as a model system for studying fundamental mechanisms of transepithelial transport (Cobb, et al., 1982; Wollner and Nelson, 1992), tubulogenesis (Montesano, et al. 1991) and renal morphogenesis and differentiation processes *in vitro* (Rodrigues-Boulan, 1983; Patrone, et al., 1992; Devuyst, et al., 1994; Orellana, *et al.*, 1996). Transepithelial transport occurs across the epithelial polarized cells monolayer, in an apical to basolateral direction, making up bubbles of fluid between the cell layer and the underlying impermeable surface. These structures are called "domes" and their presence in MDCK cells are evidence for the occurrence of thansepithelial transport that is dependent on differentiation processes (Lever, 1990). A number of compounds has been identified as potent inductors of differentiation and dome formation by confluent MDCK cells (Lever, 1979). These inductors include nonphysiological polar compounds such as dimethylsulfoxide, dimethylformamide, hexamethylene bis-acetamide, and compound of

physiological occurrence such as butyrate, hypoxantine, adenosine (Lever, 1990), steroid hormones (Oberleithner, et al., 1990) and mineralocorticoids (Lever, 1979).

Some studies have demonstrated that non physiological conditions such as ionic alterations, pH lowering (Zura and Grant, 1981 and Morita et al., 1989/1990) and nutritional stress (Borek, 1972, Genari and Wada, 1998) may be associated with genotoxical effect, induction of cellular transformation and cytogenetical alterations (Genari and Wada, 1995). Nevertheless, other studies associated these conditions to some types of stomach and urinary bladder tumors in the mouse (Brushich, 1987; Ashby and Ishidate, 1986).

This work investigated the induction of *dome* and tubular structures formation and the alterations in morphological, growth and adhesion characteristics of MDCK cells submitted to nutritional stress conditions and their correlation with the cytoskeleton and extracellular matrix components.

MATERIALS AND METHODS:

Control Culture Conditions

MDCK cells at passage number 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 a 5% fetal bovine serum (Nutricell, Campinas,SP - Brazil) at 37°C with a 5% CO_2 atmosphere. The medium was changed after intervals of 48 hours, and the subculture was performed when cells reached confluence.

Stress Conditions

Confluent cells were maintained without culture medium replacement for fifteen days. After this time, the stressed cells were returned to the control culture conditions. This period of time was standardized because no morphological alterations were observed if cells were subjected to shorter periods. On the other hand senescence and cellular death were verified in most of the cultures stressed for periods over fifteen days, even when the cells were returned to the normal culture conditions. At the end of stressing time, the medium pH was 6.8. Long term cultures of 60 days were performed in stressed cells.

Morphological and Growth Characteristics

The growth and morphological characteristics of control and stressed MDCK cells were assessed by daily observation of the cultures with an inverted microscope.

Growth Curve

For the growth curve control and stressed cells were seeded at the density of 6 X 10^3 cells in 35 mm tissue culture dishes (Corning, New York, NJ - USA) and maintained under normal culture conditions at 37°C with a 5% CO_2 atmosphere by the periods of 1 to 10 days. The number of viable cells in each period was assessed and the data are presented as the mean of triplicate samples.

Scanning Electron Microscopy

The stressed MDCK cells were homogeneously seeded at the density of 2 X 10^4 cells/ml on glass coverslips in 35mm tissue culture dishes and maintained under normal culture conditions until they reached the confluence and after confluence for up 21 days. These samples were fixed in 2.5% glutaraldehyde, post fixed with 1% osmium tetroxide, dehydrated, critical point dried and coated with gold. Observation of the specimens was performed with a JEOL-300 microscope.

Cytogenetics and karyotypic analysis

Cells were arrested in metaphases by the addition of colchicine (16 μg/ml) on logarithmic phase cultures (control cells at passage 38 and stressed cells at the passage 42), followed by a 4 h incubation period. Cells were harvested and chromosomes were prepared according to routine techniques. Trypsin-Giemsa (GTG) banding was performed according to a modification of the method of Seabright (1971). The modal chromosome number was determined by counting the chromosomes in 100 metaphases to each cell

population. The mitotic and polyploidy index were obtained according Deitch and Sawicki (1979) and Gilvarry et. al (1990), respectively. The arrangement of the chromosomes was adjusted to the nomenclature of the canine karyotype, according to Reimann et. al (1996).

Immunocytochemistry

Immunocytochemistry was performed on subconfluent and confluent monolayer cultures of control and stressed cells grown on glass coverslips. The cells were seeded at the density of 5 X 10^4 cells/ml in Ham F10 medium with 5% FBS and further cultured for 24 or 72 h before immunostaining. After washing in PBS, pH 7.4, the cells were fixed and permeabilized in 0.25% glutaraldehyde, 4% formaldehyde in 80mM Pipes pH6.8, containing 1mM $MgCl_2$, 5mM EGTA and 0.2% Triton X-100 for 30 min. After blocking with 100mM sodium borohydride buffer for 20 min and with 3% BSA for 1h they were incubated with the primary antibodies. The cells were incubated with phalloidin-rhodamin (SIGMA) for F-actin for 1 h and further stained with either a monoclonal antibody anti-vimentin (SIGMA - from mouse ascites fluid, clone V9) or an anti-cellular fibronectin (SIGMA - from mouse ascites fluid, clone FN-3E2). Primary antibodies were visualized by incubation with a goat FITC-conjugated antibody anti-mouse IgG (SIGMA). The material was observed with a Zeiss Axioskop equiped with filter sets for rhodamin and fluorescein.

RESULTS

Morphological and Growth Characteristics

The control MDCK cells grew exponentially in monolayer until the confluence and presented a polygonal flattened shape, typical of normal epithelial-like cells (Figure 1 A). After reaching confluence at day 3 to 4 normal cells stops dividing and died. The stressed cells presented dome formation at the confluence (Figure 1 B). After confluence they started to grow indefinitely in multilayers with formation of cellular aggregates (Figure 1 C). In long term cultures the stressed cells formed tubule-like structures (Figure 1 D).

Growth Curve

Control and stressed MDCK cells presented very similar growth rates in the proliferative phase. However, after 150 h in culture the stressed population, presented a continuous proliferative phase, while the control population reached senescence, stopped proliferating and died. The growth curve of control and stressed MDCK cells are presented in Figure 2.

Scanning Electron Microscopy

The stressed MDCK grew initialy as a monolayered cell sheet and cells are firmly attached to each other. These cells were flattened and presented several microvilli and vesicular structures (Figure 3 A and B). After confluence, some regions of the monolayer formed dome structures. The dome cells showed numerous microvilli at the cellular

surface (Figure 3 C and D). On prolonged culture times after confluence the stressed cells started to grow in multilayers and formed of cellular aggregates (Figure 3 E and F).

Cytogenetics and karyotyping

Control and stressed MDCK cells presented polyploidy indices of 1.8% and 4.4% respectively. The mitotic index was the same (9.3%) for both conditions. Control and stressed population presented a modal chromosome number of 80 (range: 78 to 82; n = 100 for each condition). The diploid chromosome number distribution in control and stressed cells is presented in Figure 7. Figures 8 and 9 exhibit the karyotype of the control and stressed MDCK cells, respectively. The karyotype of control cells was characterized by one sexual X chromosome and three telomeric extra autosomes, while the stressed cells presented two sexual X chromosome and two telomeric extra autosomes.

Immunocytochemistry

In control cells bundles of actin in the form of stress fibers were observed. They transverse the cytoplasm, and codistributed with fibronectin, also at the cell periphery (Figure 7A). In stressed cells, the codistribution of fibronectin and actin was observed at the cell-cell contact regions in non confluent (Figure 7C) and in confluent monolayers (not shown). When conparated to the control cells, stressed cells presented enhanced staining for vimentin, which colocalized with actin at the cell-cell contact regions of stressed cells (Figure 7B and D).

DISCUSSION

Cell differentiation represent a complex sequence of events leading to coordinated changes in multiple biochemical and morphological parameters (Lever, 1990). Little is known about the factors and conditions responsible for the regulation of growth and expression of the differentiated state in epithelial cells. The MDCK cell line has a distinctive epithelioid morphology and displays several functional properties of normal kidney tubule cells. So, these cells were considered to be ideal for studies concerned with transmembrane transport and growth regulation (Saier, *et al.* 1981; Valentich, 1981; Oberleithner, et al., 1990) and to investigate factors that govern the ordered assembly of epithelial cells into spatially organized multicellular units (Montesano, et al, 1997). Thus, in this work we have focused our investigations in the physiological conditions affecting the several morphological and growth characteristics of MDCK cells.

MDCK control cells formed an uninterrupted monolayer with the characteristic morphology of epithelial cells (Meza, et al., 1980). After the confluence, control cells stopped proliferation and entered senescence, followed by death. The stressed cells initially presented the same proliferation velocity as control cells, and at the confluence, they presented differentiated characteristics as *dome* formation and microvilli. Nevertheless, after confluence, these stressed MDCK cells grew indefinitely in multilayers forming aggregates what indicates the loss of the contact inhibition (Abercrombie, 1979; Genari, et al., 1996). After long term cultures these stressed cells formed tubule-like structures.

When the MDCK monolayers were submitted to nutritional stress the cells were not only exposed to the effects of a depleted medium, but also to the consequences of this condition, such as accumulation of metabolites and the lowered pH. Nutritional stress and low pH (Morita, *et al.* 1989; Zura and Grant, 1981; Genari and Wada, 1998) have been associated with the induction of cell transformation in mammalian cell culture and some kinds of stomach and bladder tumor development in mice (Ashby and Ashidate, 1986; Brusick, 1987). Other studies described different clones or subtypes of MDCK cells and showed the effect of culture conditions in their phenotype (Nakasato, et al., 1989; Devuyst, et al., 1994). Among these conditions, the alkaline stress was described as a potent transformation stimulus for MDCK cells (Oberleithner, et al., 1991; Wünsch, et al., 1995). Thus, the appearance of differentiated properties such as *dome* formation, microvilli, loss of contact inhibition and multilayer growth, and formation of tubular structures may be induced by nutritional stress conditions. Since these altered properties and growth characteristics were irreversible by returning stressed cells to normal culture conditions, we suspected that cellular transformation was also induced by nutritional stresses. Moreover, these results indicate that MDCK cells may differentiate or/and undergo transformation not only by alkaline pH, but also by acid pH as observed in the present nutritional stress conditions.

The normal diploid chromosome number in the domestic dog (*Canis familiaris*) is 78 (Reiman, et al., 1996). The karyotype of MDCK cells is described as pseudodiploid (Saier, 1981). In the present study, the karyotype of control and stressed MDCK cells displays the same chromosome number, but stressed population presented two extra telocentric autosomes and a biarmed sexual X chromosome, while control population

displays three extra telocentric autosomes. Wünsch et al. (1995) described the presence of an extra biarmed chromosome, apparently resulting from nondisjunction during mitosis, in MDCK cells transformed by alteration in the intracellular pH. The intracellular pH is known to play a critical role in controlling cell cycle and is often associated with abnormal proliferation (Gillies, et al., 1992). We can conclude that the nutritional stress conditions accompanied by pH alterations are responsible for the karyotype differences and phenotype alterations in MDCK stressed cells. Polyploidy was enhanced in stressed cells as compared to the control cells, and this characteristic has also been associated with transformation.

Some of the factors that may induce epithelial cell differentiation in culture, are the nature of the cell substratum, the extracellular matrix components and their influence on the nutrient accessibility to the basal cell surface (Emerman, 1979; Rabito, et al, 1980; Reid, et al., 1981; Greenberg and Hay, 1982; Lever, 1990). It been described that MDCK cells cultured in collagen gels form branching tubules and spherical cysts (Montesano, et al, 1997). This may be a factor that contributes to the differentiation of stressed MDCK cells, since the after a long culture time, cells can modify their substrate by increasing or reducing production and deposition of extracellular matrix components. Indeed, stressed MDCK cells presented fibronectin accumulation between adjacent cells. Fibronectin expression is important for the cellular migration and adhesion, morphogenesis and transformation process. In virally transformed cells (Chen et al, 1984; Hayman et al, 1981; Nermut et al., 1991 and Murray 1980) the expression of fibronectins is often reduced and these cells are typically less adherent to the substrate and no longer show lost of contact inhibition (Chen and Chen, 1987; Hynes, 1990a). Nevertheless, some

studies that indicated that cellular fibronectin and some other matrix components may be effective in promoting assembly and stabilization of actin stress fibers in epithelial cells. This result suggest that extracellular matrix elements including fibronectin, interacts with intracellular microfilaments in epithelial tissues and this interaction may be important in inducing and/or mantaining the morphology and polarity of epithelial MDCK cells (Hynes, 1990b).

Changes in cell shape and contacts have long been recognized as processes with central role in the regulation of tissue morphogenesis, cellular growth (Folkman and Moskona, 1978) and differentiation (Ben-Ze'ev, 1987) and it is defined by interactions between extracellular matrix and cytoskeletal components, or between neighbouring cells (Geiger, et al., 1985). In this work we also investigated the distribution pattern of actin and vimentin, which are proteins that participate of the main anchoring junctions in epithelial cells: the adherens junction and desmossomes, respectively (Birchmeier, et al., 1993).The anchoring junctions are clearly important for cell adhesion. Genetic studies have also indicated that adherens junctions are also sites of developmentally significant signalling between epithelial cells, controlling the spatial pattern of differentiation (Woods and Bryant, 1993). The confluent and non confluent stressed cells presented enhanced vimentin and actin deposition at the cell-cell contact regions, as compared to the control cells. It is thought that the oriented contraction of the actin filaments running along adherens junctions causes the epithelial cells to narrow their apex and it plays an important function in the folding of an epithelial sheet to form tubes (Geiger et al., 1985; Geiger and Ginsberg, 1991). Since the stressed cells formed tubule-like structures in long term cultures, we can conclude that actin accumulation at the cell-cell contact regions

contributes in this process. Other studies have described alterations of the intermediate filament proteins in response to changes in culture conditions (Ben-Ze'ev, 1987); and increase amounts of vimentin in cells treated with tumor promoters(Lazlo and Bissel, 1983) and in some epithelial tumors (Caselitz, et al., 1981; Miettinen, et al., 1984: Araújo and Araújo, 1990; Jaeger, et al., 1995). Moreover, these studies associate the increased vimentin expression with a more invasive behaviour (Schaafsma, et al., 1994; Andreoli and Trevor, 1995).

Besides the fact that cytoskeleton plays a major role in maintaining cell shape, there is increasing evidence that the cytoskeleton contributes to these specialized interactions between epithelial cells, having critical roles in epithelial biogenesis (Yap et al., 1995). The surface contacts and cell shape changes are transduced via the cytoskeleton to bring about biochemical responses from the nucleus (Ben-Ze'ev, 1985). Many proteins of the cytoskeleton are modified by second messenger and can collectively behave as a complex biological network that integrates information from signal transduction pathways and results in changes on cell morphology (Fowler and Vale, 1996). Our results show that expression of these cytoskeletal elements is related to changes in cell shape and cell contacts, and that such changes may be central to the acquisition of the differentiated and/or transformed phenotype.

In conclusion, our data indicate that nutritional stress conditions accompanied by pH alteration are important stimuli that induce alterations in growth, morphology and interactions of renal epithelial cells, leading to tubular morphogenesis *in vitro*.

ACKNOWLEDGEMENTS

We are very grateful to Dr. Hernandes F. de Carvalho for the phalloidin-rhodamin donation and his assistance in immuhistochemistry assays, and for his critical comments on the manuscript. This work was carried out with funding from Coordenadoria de Aperfeicoamento de Pessoal de Nivel Superior (CAPES) to S. C. Genari, from Fundo de Apoio ao Ensino e à Pesquisa (FAEP/UNICAMP) to M. L. F. Wada and from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) to H. F. Carvalho.

LITERATURE CITED

Abercrombie, M. (1979). Contact inhibition and malignancy. *Nature*, **281**: 259-262.

Andreoli, J. M. and Trevor, K. T. (1995). Structural and biological consequences of increased vimentin expression in simple epithelial cell types. *Cell Motil. Cytoskeleton* **32**, 10-25.

Ashby, J. and Ishidate, M. (1986). Clastogenicity in vitro of the Na, K, Ca and Mg salts of saccharin; and magnesium chloride; consideration of significance. *Mutation Res.* **163**, 63-73.

Behrens, J., Mareel, M. M., Van Roy, F. M. and Birchmeier, W. (1989). Dissecting tumor cell invasion: epithelial cells acquire invasive properties after loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* **108**, 2435-2447.

Ben-Ze'Ev, A. (1987). The role of changes in cell shape and contacts in the regulation of cytoskeleton expression during differentiation. *J. Cell Sci. Suppl.* **8**, 293-312.

Ben-Ze'Ev, A. (1985). The cytoskeleton in cancer cells. *Biochem. Biophys. Acta* **780**, 197-212.

Birchmeier, W., Weidner, K. M. and Behrens, J. (1993). Molecular mechanisms leading to loss of differentiation and gain of invasiveness in epithelial cells. *J. Cell Sci. Suppl.* **17**, 159-164.

Borek, C. (1972). Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated Hepatoma-like cells under conditions of nutritional stress. *Proc. Nat. Acad. Sci. USA* **69**, 956-959.

Brushich, D. J. (1987). Implications of treatment-condition-induced genotoxicity for chemical screening and data interpretation. *Mutation Res.* **189**, 1-6.

Button, E., Shapland, C. and Lawson, D. (1995). Actin, its associated proteins and metastasis. *Cell Motil. Cytoskeleton* **30**, 247-251.

Caselitz, J.: Osborn, M. and Weber, K. (1981). Intermediate-sized filament protein (prekeratin, vimentin, desmin) in the normal parotid and parotid gland tumors. *Cancer* **54**,2959-2967.

Cereijido, M., Ehrenfeld, J., Fernandez-Castelo, S. and Meza, I. (1981). Fluxes, junctions and blisters in cultured monolayers of epithelioid cells (MDCK). *Ann. N. Y. Acad. Sci.* **372**, 422-441.

Cereijido, M., González-Mariscal, L., Contreras, R. G., Gallardo, J. M., Garcia-Villegas, R. and Valdés, J. (1993). The making of a tight junction. *J. Cell. Sci. Suppl.* **17**, 127-132.

Chen, J-M., Olden, K., Beranrd, B. A. and Fong-Fong, C. (1984). Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. *J. Cell Biol.* **98**, 1546-1555.

Chen, J-M and Chen, W-T (1987). Fibronectin-degrading proteases from the membranes of transformed cells. *Cell* **48**,193-203.

Cobb, M. H., Yang, C-P. H., Jefferson, D. M., Pasnikowski, E. and Scott, W. N. (1982). Mineralocorticoid-induced membrane proteins in MDCK cells. *Mol. Cell. Endocrinol.* **27**, 129-137.

Deitch, A. D. and Sawicki, S. G. (1979). Effects of cordycepin on microtubules of cultures mammalian cells. *Exp. Cell Res.* **118**, 1-13.

Devuyst, O., Beawens, R., Denef, J-F., Crabbé, J. and Abramow, M. (1994). Subtypes of Madin-Darby canine kidney (MDCK) cells defined by immunocytochemistry: Further evidence for properties of renal collecting duct cells. *Cell Tissue Res.* **277**, 231-237.

Emerman, J. T., Burwen, S. J. and Pitelka, D. R. (1979). Substrate properties influencing ultrastrutural differentiation of mammary epithelial cells in culture. *Tissue Cell.* **11**,19-119.

Folkman, J. and Moscona, A. (1978). Role of cell shape in growth control. *Nature* **273**, 345-349.

Fowler, V. M. and Vale, R. (1996). Cytoskeleton. *Curr. Opin. Cell Biol.* **8**, 1-3.

Geiger, B. and Ginsberg, D. (1991). The cytoplasmic domain of adherens-type junction. *Cell Motil. Cytoskeleton* **20**, 1-6.

Geiger, B., Volk, T. and Volberg, T. (1985). Molecular heterogeneity of adherens junctions. *J. Cell Biol.* **101**, 1523-1531.

Genari, S. C. and Wada, M. L. F. (1995). Behavioural differences and cytogenetical analysis of a transformed cellular population derived from a Vero cell line. *Cytobios* **81**, 17-25.

Genari, S. C. Dolder, M. A. D. and Wada, M. L. F. (1996). Scanning and transmition electron microscopy of transformed Vero cells, with altered *in vitro* growth characteristics. *J. Submicrosc. Cytol. Pathol.* **28**(4), 565-572.

Genari, S. C., Gomes, L. and Wada, M. L. F. Alterations in the growth and adhesion pattern of Vero cells induced by nutritional stress conditions. *Cell Biol. Int.***20** (in press).

Gillies, R. J., Martinez-Zeguilan, R., Peterson, E. P., and Perona, R. (1992) Role of intracellular pH in mammalian cell proliferation. *Cell Physiol. Biochem.* **2**, 159-179.

Gylvarry, U., Farrel, D., Lynch, V., Moriarty, M., Dooley, M., Law, E. and Clynes, M. (1990). Cytological differences between normal and malignant human cell populations in culture. *Cancer Res.* **50**, 3390-3393.

Greenberg, G. and Hay, E. D. (1982). Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J. Cell. Biol.* **95,** 333-339.

Hayman, E., Engvall, E. and Ruoslahti, E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells.*J. Cell Biol.* **88**, 352-357.

Huotari, V., Vääräniemi, J., Lehto, V-P and Eskelinen, S. Regulation of the disassembly/assembly of the membrane skeleton in Madin-Darby canine kidney cells. *J. Cell. Physiol.* **167**, 121-130.

Hynes, R. O. (1990)a. Fibronectin and the cytoskeleton. In *Fibronectins.* (ed. Springer-Verlag), pp231-248. New York.

Hynes, R. O. (1990)b. Oncogenic Transformation. In *Fibronectins.* (ed. Springer-Verlag), pp231-248. New York.

Jaeger, R. G., Jaeger, M. M. M., Araújo, V. C. and Bachar, B. (1995). Analysis of the interdependent localization of vimentin and microtubules in neoplastic myoepithelial cell. *Cell Motil. Cytoskeleton* **32,** 289-298.

Lazlo, A and Bissel, M.(1983). TPA induces simultaneous alterations in the synthesis and organization of vimentin. *Exp. Cell. Res.* **148**,221-234.

Lever, J. E. (1981). Regulation of dome formation in kidney epithelial cell culture. *Ann. N. Y. Acad. Sci.* **372**, 371-381.

Lever, J. E. (1990). Inducers of dome formation in epithelial cell cultures including agents that cause differentiation. In *Tissue Culture of Epithelial Cells* (ed. Mary Taub), pp.1-22. New York.

Mauchamp, J., Chambard, M., Verrier, B., Gabrion, J., Chabaud, O., Gerard, C., Penel, C., Pialat, B. and Anfosso, F. (1987). Epithelial cell polarization in culture: orientation of cell polarity and expression of specific functions, study with cultured thyroid cells. *J. Cell Sci. Suppl.* **8**, 345-358.

Meza, I., Ibarra, G., Sabanero, M., Martinez-Palomo, A. and Cereijido, M. (1980). Occluding Junctions and Cytoskeletal Components in a Cultured Transporting Epithelium. *J. Cell Biol.* **87**,746-754.

Miettinen, M.; Franssila, K.; Lehto, V. B.; Paasivuo, R. and Virtanen, I. (1984). Expression of intermediate filament protein in thyroid gland and thyroid tumors. *Lab. Invest* **50**, 262-270.

Montesano, R.; Matsumoto, K.; Nakamura, T. and Orci, L. (1991). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901-908.

Montesano, R., Soriano, J. V., Pepper, M. S. and Orci, L. (1997). Induction of epithelial branching tubulogenesis *in vitro*. *J. Cell Physiol.* **173**,152-161.

Morita, T., Watanabe, Y., Takeda, K. and Okomura, K. (1989). Effects of pH in the in vitro chromosomal aberration test. *Mutation Res.* **225**, 55-60.

Murray, J. C., Liotta, L., Rennard, S. I. and Martin, G. R. (1980). Adhesion characteristics of murine metastic and nonmetastic tumor cells in vitro. *Cancer Res.* **40**, 347-351.

Nakazato, Y., Suzuki, H. and Saruta, T. (1989). Characterization of subclones of Madin-Darby canine kidney renal epithelial cell line. *Biochem. Biophys. Acta* **1014**, 57-65.

Nermut, M. V., Eason, P., Hirst, E. M. A. and Kellie, S. (1991). Cell/substratum adhesion in RSV-transformed rat fibroblasts. *Exp. Cell Res.* **193**, 382-397.

Oberleithner, H., Vogel, U. and Kersting, U. (1990). Madin-Darby canine kidney cells. I. Aldosterone-induced domes and their evaluation as a model system.*Pflügers Arch.* **416**, 526-532.

Oberleithner, H., Westphale, H. J., Gaβner, B. (1991). Alkaline stress transforms Madin-Darby canine kidney cells. *Pflügers Arch.* **419**, 418-420.

Orellana, S. A., Neff, C. D., Sweeney, W. E. and Avner, E. D. (1996). Novel Madin Darby canine kidney cell clones exhibit unique phenotypes in response to morphogens. *In vitro Cell. Dev. Biol.* **32**, 329-339.

Patrone, L. M., Cook, J. R., Crute, B. E. and Van Buskirk, R. G. (1992). Differentiation of epithelial cells on microporous membranes. *J. Tiss. Cult. Meth.* **14**, 225-234.

Rabito, C. A ., Tchao, R., Valentich, J. and Leighton, J. (1980). Effect of cell-substratum interaction on hemicyst formation by MDCK cells. *In vitro* **16**,461- .

Reid, L.; Morrow, B.; Jubinsk, P.; Schwartz, E. and Gatmaitan, Z. (1981). Regulation of growth and differentiation of epithelial cells by hormones, growth factors and substrates of extracellular matrix. *Ann. N. Y. Acad. Sci.* **372**,354-370.

Reimann, N., Bartnizke, S., Bullerdiek, J., Schmitz, U., Rogalla, P., Nolte, I. and RØnne, M. (1996). An extended nomenclature of the canine karyotype. *Cytogenet. Cell Genet.* **73**, 140-144.

Rindler, M. J., Chuman, L. M., Shaffer, L. and Saier Jr, M. H. (1979). Retention of differentiated properties in na established dog kidney epithelial cell line (MDCK). *J. Cell Biol.* **81**, 635-648.

Rodrigues-Boulan, E., Paskiet, K. T. and Sabatine, D. D. (1983). Assembly of enveloped viruses in Madin-Darby canine kidney cells: Polarized budding from single attached cells and from clusters of cells in suspension. *J. Cell Biol.* **96**,866-874.

Saier Jr, M. H. (1981). Growth and differentiated properties of a kidney epithelial cells line (MDCK). *Am. J. Physiol.* **240**, C106-C109.

Schaafsma, H. E.; Van Der Velden, L. A.; Manni, J. J.; Peters, H.; Link, M.; Rutter, D. J. and Ramaekers, F. O. (1993). Increased expression of cytokeratin 8, 18 and vimentin in the invasion front of mucosal squamous cell carcinoma. *J. Pathol.* **170**, 77-86.

Seabright, M. (1971). A rapid banding technique for human chromosomes. *Lancet* **1**,971-972.

Valentich, J. D., Tchao, R. and Leighton, J. (1979). Hemicyst Formation Stimulated By Cyclic AMP in Dog Kidney Cell Line MDCK. *J. Cell. Physiol.* **100**, 291-304.

Valentich, J. D. (1981). Morphological similarities betweenthe dog kidney cell line MDCK and the mammalian cortical collecting tubule. *Ann. N. Y. Acad. Sci.* **372**, 384-405.

Vasiliev, J. M. and Gelfand, I. M. (19). Mechanisms of Morphogenesis in cell culture. *Int. Ver. Cytol* **50**, 159-273.

Webb, C. P., Lane, K., Dawson, A. P., Vande Woude, G. F. and Warn, R. M. (1996). C-Met signalling in na HGF/SF-insensitive variant MDCK cell line with constitutive motile/invasive behaviour. *J. Cell Sci.* **109**, 2371-2381.

Wojnowski, L., Hoyland, J., Mason, W. T., Schwab, A., Westphale, H. J. and Oberleithner, H. (1994). Cell transformation induces a cytoplasmatic Ca-2+ oscillator in Madin-Darby canine kidney cells. *Pflüegers Arch.* **426**, 89-94.

Woods, D. F. and Bryant, P. J. (1993). Apical junctions and cell signalling in epithelia. *J. Cell Sci. Suppl.* **17**, 171-181.

Wollner, D. A. and Nelson, W. J. (1992). Establishment and maintaining epithelial cell polarity: Roles of protein sorting, delivery and retention. *J. Cell Sci.* **102**, 185-190.

Wünsch, S., Gekle, M., Kersting, U., Schuricht, B. and Oberleithner, H. (1995). Phenotypically and karyotypically distinct Madin-Darby canine kidney cell clones respond differently to alkaline stress.*J. Cell. Physiol.* **164**, 164-171.

Zura, K. D. and Grant, W. F. (1981). The role of hydronium ion in the induction of chromosomal aberrations by weak acid solutions. *Mutation Res.* **84**, 349-364.

Yap, A. S.; Stevenson, B. R. Waters, M. J.; Keast, J. R. and Manley, S. W. (1995). Vinculin localization and actin stress fibers differ in thyroid cells organized as monolayers or follicles. *Cell Motil, Cytoskeleton* **32**, 318-331.

LEGENDS

Fig. 1 Phase-contrast micrographs of control (A) and nutritionally stressed MDCK cells (B, C, D). Control MDCK cells after 48 h of culture, growing exponentially in monolayer show the flattened shape typical of epithelial-like normal cells (A), 200X. Stressed MDCK cells after 48 h of culture show *dome* formation (*arrow*) (B), 200X. After the confluence stressed cells with 21 days of culture grow in multilayers and form cellular aggregates, (C), 100X. In long term culture (60 days) stressed cells formed tubular structures (D), 1400X.

Fig. 2 Growth curve of control and stressed MDCK cells. Control and stressed cells presented the same growth rate until 150 h of culture. After this point control cells stopped cellular division, reached the stationary phase, and died. Stressed cells presented continuous extended phase.

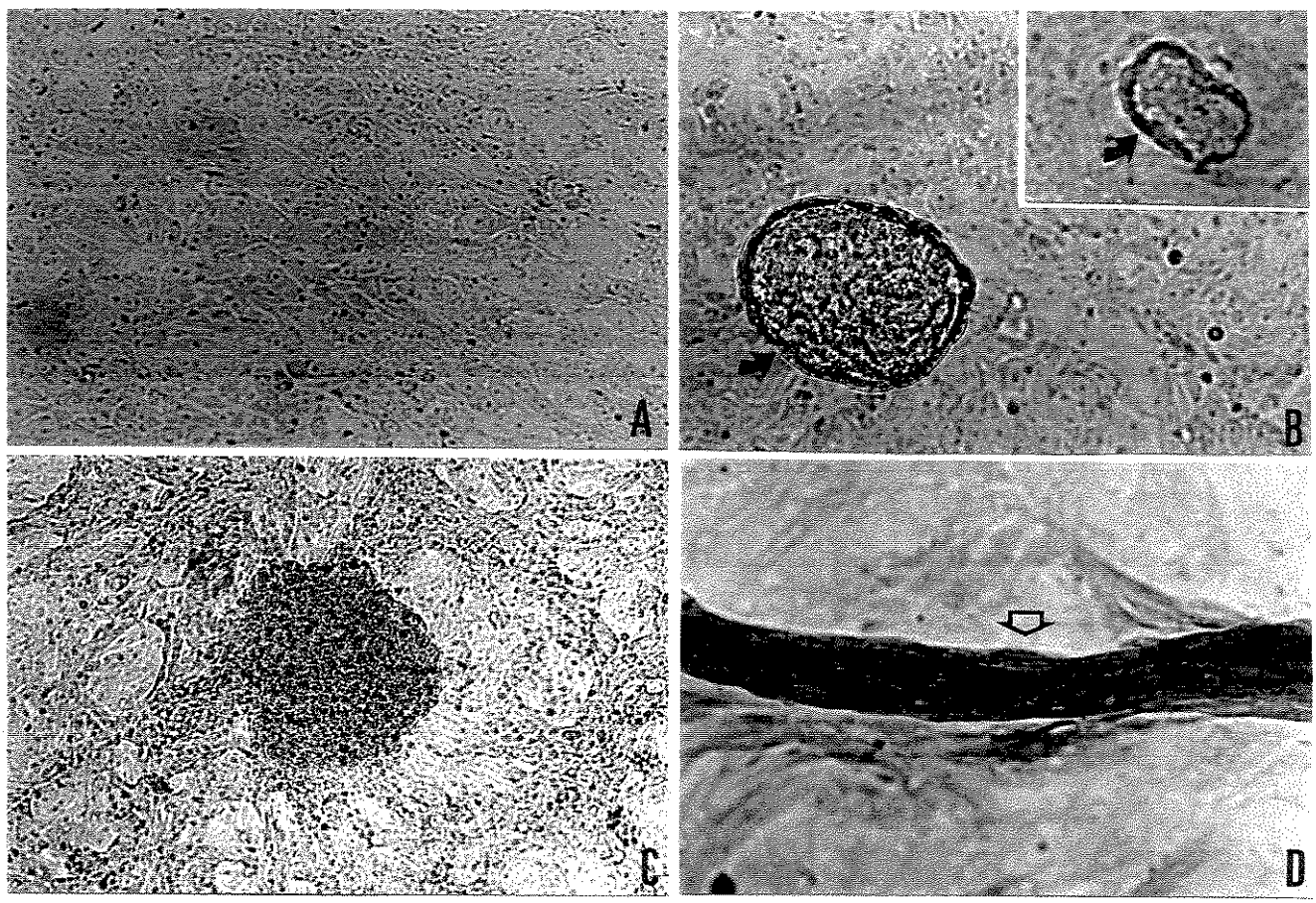
Fig. 3 Scanning electron microscopy of MDCK epithelial cells. The stressed cells grow as a monolayered in which cells are firmly attached to each other. These cells with flattened shape presented stub-like microvilly (asterisk and star) and vesicular structures (arrowheads) (A), 6000X. Detail of the stub-like microvilli (B), 10000X. After the confluence, the stressed MDCK cells formed *dome* structures with numerous microvilli (C), 2000X. Detail of the *dome* surface showing microvilli (D), 5000X. On extended periods after confluence, the stressed cells started to grow in multilayers (E), 1500X; and formed cellular aggregates (F), 1350X.

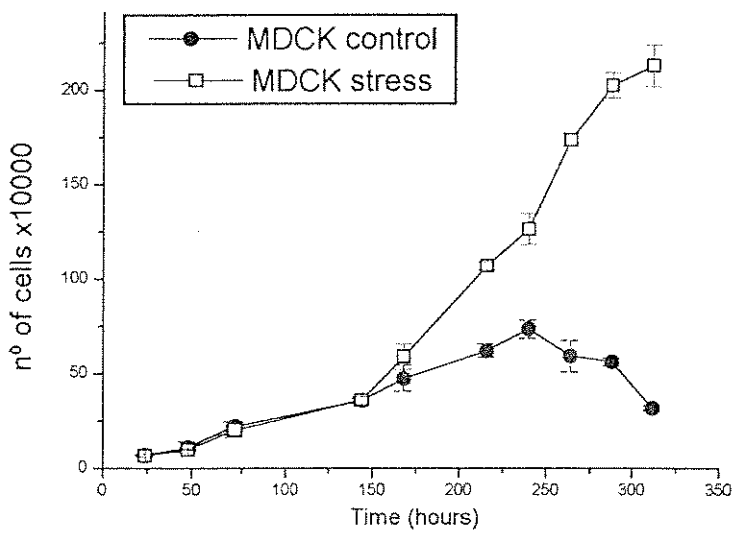
Fig. 4 Diploid chromosome number of control (upper panel) and stressed MDCK cells (lower panel). Both cellular populations presented a modal chromosome number of 80 (range 78 to 82 chromosomes; n=100).

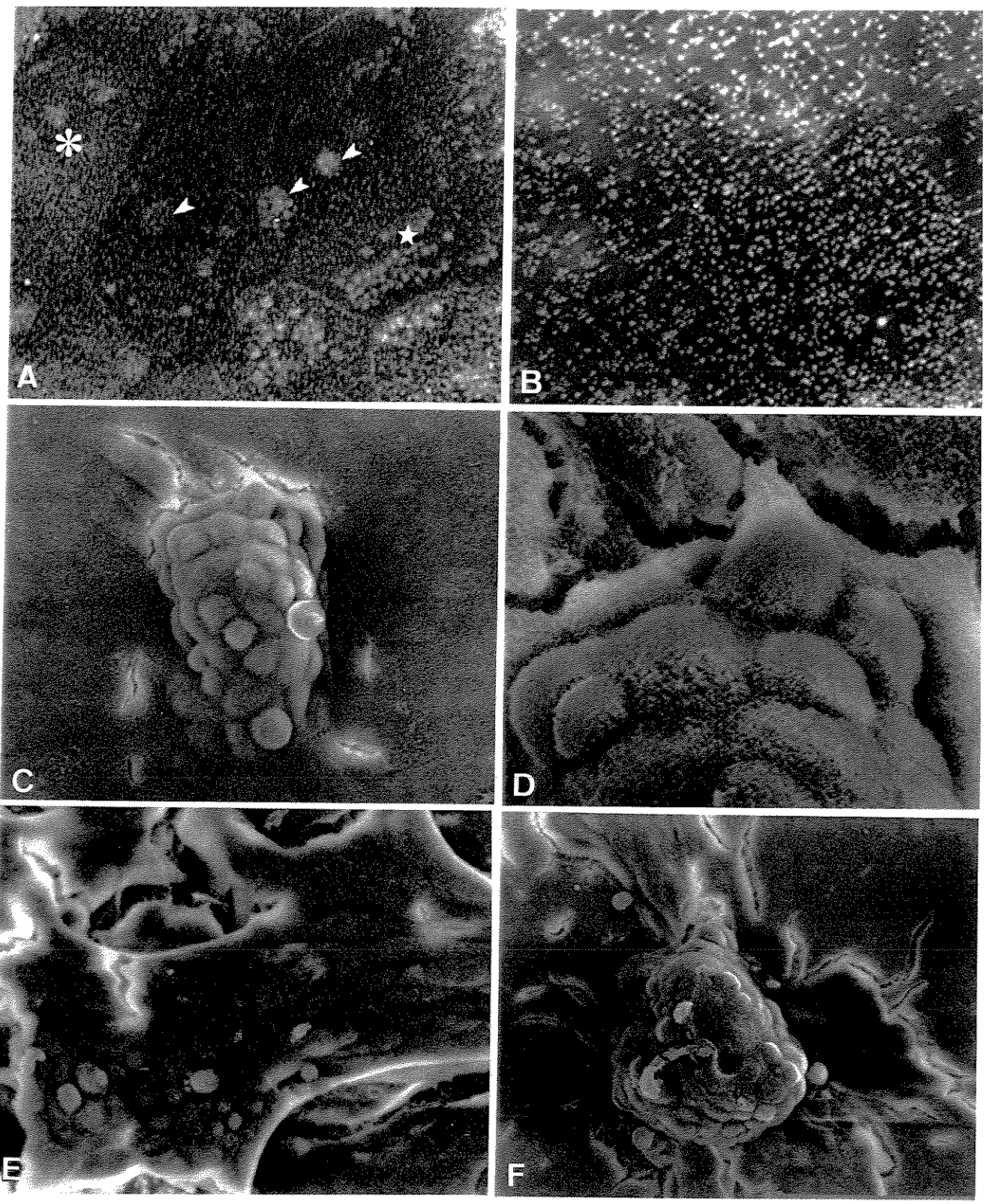
Fig. 5 Representative GTG-banding karyotype of control MDCK cells with one sexual X chromosome and three telomeric extra autosomes. 1500X.

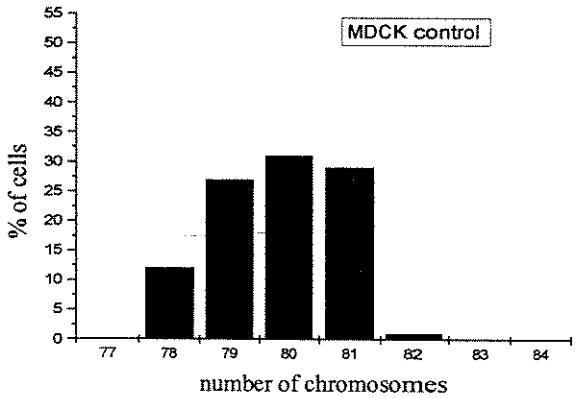
Fig. 6 Representative GTG-banding karyotype of stressed MDCK cells with presented two sexual X chromosome and two telomeric extra autosomes. 1500X.

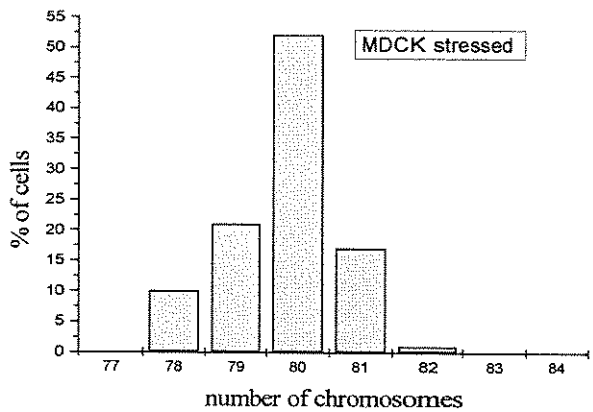
Fig. 7 Indirect immunofluorescence staining of fibronectin, actin and vimentin in control and stressed MDCK epithelial cells. Cells were seeded on coverslips and cultured for 24 h. Cells were then fixed, permeabilized and stained by indirect immunofluorescence with antibodies against fibronectin or vimentin and phalloidin. Left panel shows the control cells, and right panel shows the stressed MDCK cells; A and C corresponds to the colocalization of fibronectin and actin and B and D vimentin and actin. In control cells the codistribution of actin and fibronectin, is observed mainly at the stress fibers (*arrowheads*) and at the cell periphery (*arrow*) (A). In the stressed cells the codistribution of fibronectin and actin was observed at the cell-cell contact regions (*arrow*) (C). When compared to control cells (B), stressed cells presented enhanced staining for vimentin (D), which colocalizes with actin at the cell-cell contact regions (*arrow*). 350X.

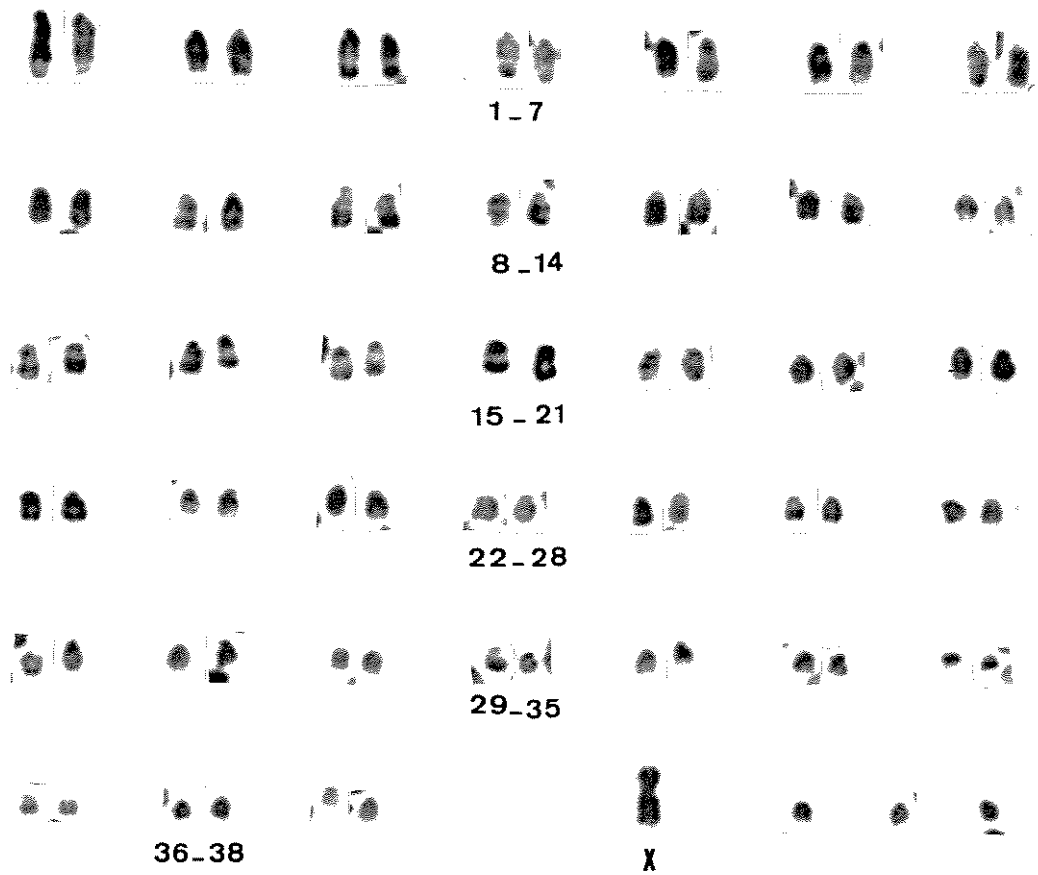

A
B
C
D

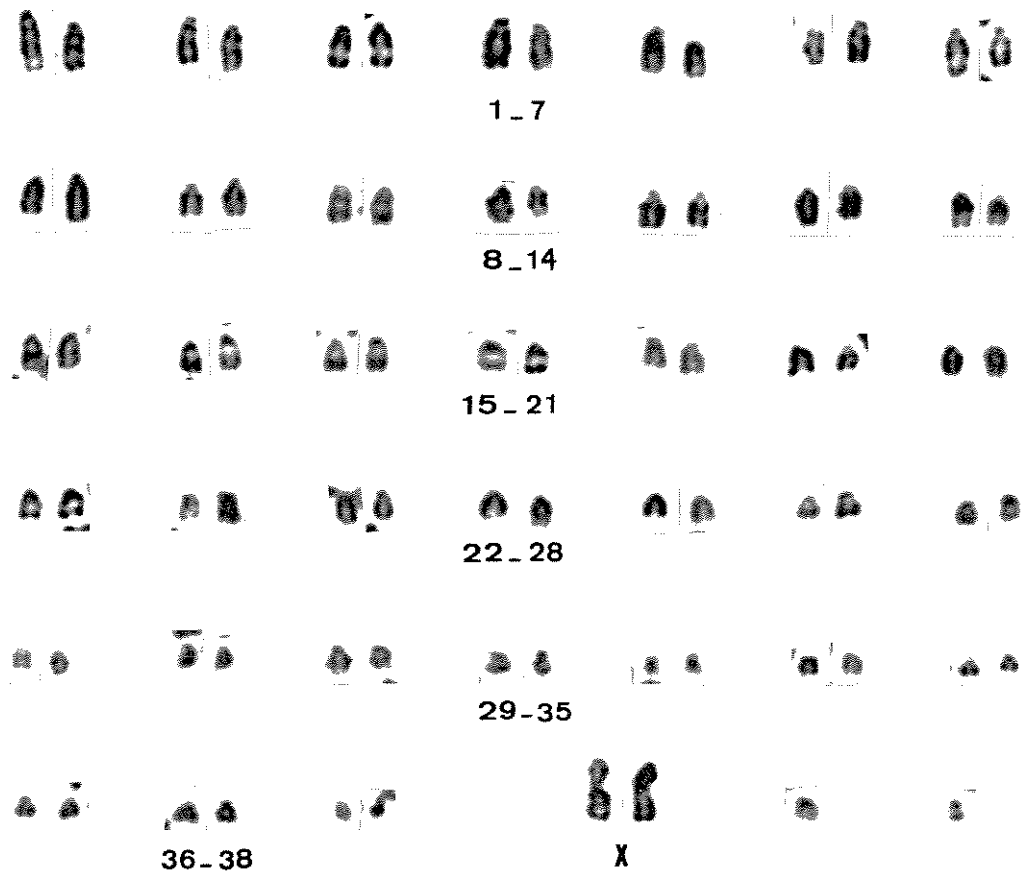

MDCK control
MDCK stress
n° of cells x10000
Time (hours)
0
50
100
150
200
250
300
350

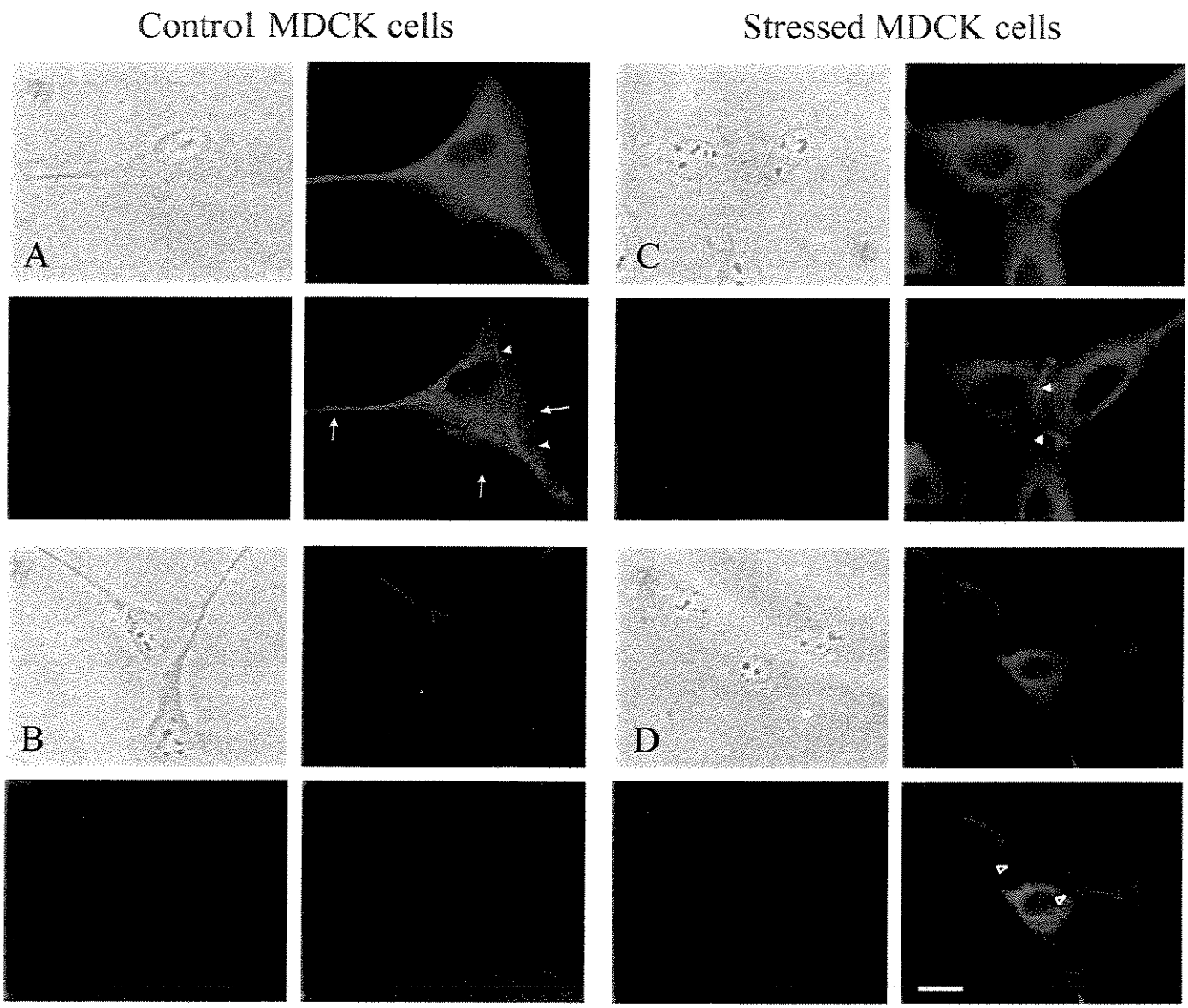

A
B
C
D
E
F


MDCK control
% of cells
55
50
45
40
35
30
25
20
15
10
5
0
77
78
79
80
81
82
83
84
number of chromosomes


MDCK stressed
% of cells
55
50
45
40
35
30
25
20
15
10
5
0
77
78
79
80
81
82
83
84
number of chromosomes


1 - 7
8 - 14
15 - 21
22 - 28
29 - 35
36 - 38
X


1_7
8_14
15_21
22_28
29_35
36_38
X


Control MDCK cells
Stressed MDCK cells
A
C
B
D

Letter to the Editor

EFFECTS OF NUTRITIONAL STRESS CONDITIONS ON THE PLOIDY AND BEHAVIOUR OF HUMAN AMNIOTIC CELLS.

SELMA CANDELÁRIA GENARI* AND MARIA LÚCIA FURLAN WADA

Department of Cell Biology, Biology Institute – State University of Campinas ,

UNICAMP - CP 6109 - CEP 13083-970

Campinas - São Paulo, Brasil.

Condensed title for running head: **POLIPLOYDIZATION ON AMNIOTIC CELLS**

* To whom the correspondence should be addressed:

Selma Candelária Genari

Departamento de Biologia Celular - Instituto de Biologia

Universidade Estadual de Campinas - UNICAMP

CP 6109 - CEP 13083-970 - Campinas, SP - Brasil.

Phone: 55 19 788 7786

FAX: 55 19 239 3124

E-mail: sgenari@nutricell.com.br

Total number of text figures and tables = 3 figures.

SUMMARY

The extensive use of permanent cell lines and primary cultures in various fields has stimulated cytogenetic studies of cells in culture. The analyses of variability of karyotype have been of fundamental importance when associated with phenotypical cell characteristics in identifying peculiarities of karyotype evolution during the establishment of cell lines. Some studies have demonstrated that non physiological conditions may be associated with induction of cellular transformation and cytogenetical alterations. We investigated the effect of nutritional stress conditions on the phenotypic and cytogenetical characteristics of primary human amniotic cells. Control amniotic cells grew in monolayer as fibroblastic-like cells and after confluence showed decreased proliferation followed by cell death. Stressed amniotic cells presented multilayered growth and formed aggregates. Control cells presented a modal number of chromosomes equal to 46 and a normal 46, XY karyotype, and polyploidy was founded in 6.6% of the cells. Stressed cells presented a modal number of chromosomes equal to 82, with a high variable karyotype and 100% of cells were polyploid. We can conclude that the nutritional stress conditions accompanied by pH alterations may be responsible by the polyploidization and the phenotype alterations in amniotic stressed cells or for the selection of polyploid clones which have more potential to adapt to the culture conditions as compared to the diploid cells. The variability in the karyotype characteristics is then intimately related with the establishment of a cell line.

Key words: *transformation, human amniotic cells, polyploidy, nutritional stress.*

Dear Editor

For the past few years, extensive use of permanent cell lines and primary cultures in various fields of biology, medicine and biotechnology has stimulated cytogenetic studies of cells in culture. The analyses of regularity or variability of the karyotype, which are both common features of cells growing *in vitro* and *in vivo*, have been of fundamental importance when associated with the phenotypical cell characteristics in identifying peculiarities of karyotype evolution during the establishment of cell lines and the effect of various experimental factors (Mamaeva, 1998). During cell cultivation, changes in some of original characteristics, including the karyotypic ones, may happen. These changes can be due not only to genetic peculiarities of cultured cells but also to conditions of their cultivation, particularly in function of the culture medium, growth factors, serum factors, and high doses of antibiotics, for example. Some studies have been demonstrated that non physiological conditions such as ionic alterations, pH diminution (Zura and Grant, 1981 and Morita et al., 1989) and nutritional stress (Borek, 1972, Genari and Wada, 1998) may be associated with genotoxical effect, induction of cellular transformation and cytogenetical alterations (Genari and Wada, 1995). In this study we then investigate the effect of nutritional stress conditions on the phenotypic and cytogenetical characteristics of primary human amniotic cells, during the processes of establishment of the lineage.

Amniotic cells were obtained by transabdominal punction on 16th week of pregnancy and cultured in Ham F10 medium supplemented with 30% fetal calf serum

(NUTRICELL), at 37°C, with a 5% CO_2 atmosphere. Cytogenetical preparation and analysis were performed according to Priest (1991). After the cytogenetical analysis the amniotic cells were submitted to nutritional stress conditions by a period of 21 days, and their growth characteristics were accompanied during this time. At the end of stress period, cytogenetical preparations of stressed cells were also performed by the same methodology mentioned above. The modal chromosome number was determined by counting the chromosomes in 100 metaphases from each cellular population. The mitotic indices were determined according to Gilvarry et al., (1990).

Control non stressed amniotic cells grew in monolayer with flattened shape, typical of fibroblastic-like cells and formed aligned arrangements (Fig 1 A). After confluence the control cells showed decreased proliferation and presented aspects of senescence, followed by cell death. Stressed amniotic cells, after 21 days of culture, presented multilayered growth and formed aggregates (Fig 1 B), indicating loss of contact inhibition, what is frequently associated with malignancy (Abercrombie, 1979; Genari, et al., 1996). Stressed cells also demonstrated to be sensitive to subcultivation by treatment with tripsin-EDTA. So the subcultivation was performed by dissociating cells mechanically. When these cell aggregates were mechanically removed and subcultived, they adhered to the flask culture surface and grew and migrated (Fig 1 C). Initially these cells presented rounded shape and grew as monolayer (Fig 1 D). Then they started growing as multilayer (Fig 1E). Control cells presented a modal number of chromosomes equal to 46, and a normal 46, XY karyotype (Fig 2). Polyploidy was founded in 6.6% of the cells. Stressed cells presented a modal number of chromosomes equal to 82, with a high variable karyotype (not shown) and 100% of cells were polyploid (Fig 3).

Cell lines are characterized, as a rule, by karyotypic heterogeneity of the initial cell population and selection of cell clones that are the most adapted to the *in vitro* conditions. Many factors including nutritional stress, clonal evolution and adaptations to *in vitro* conditions may contribute to the cytogenetic diversity. At this stage that significant numeric and structural rearrangments of chromosomes occur. Duration of the establishment stage of a cell line is determined by both the amount of karyotypically different cell clones in the initial material and the duration of karyotypic evolution of dominating cell clones that results in a balanced karyotype stabilization (Mamaeva, 1998).

Polyploidy has been associated with the establishment of cells lines from human tumors. At the earlier passages, these cells gradually become hyperdiploid with replacement of near-haploid cell clones by near-diploid or near-diploid near-tetraploid cells (Kohno et al., 1980; More et al., 1985; Drouim et al., 1993). Based on the currently available data from these works, it is evident that near-diploid cell clones tend to be replaced by polyploid cells. Unfortunately, the karyotypes evolution in these lines at the establishment stage was not studied in detail, and the presence of at least two modal classes, near-diploid and near-tetraploid, in most of cell lines indicate that these characteristc may be associated with the process of the line establishment (Waters et al., 1988).

Malignancy is a dynamic process in which the transformed cells grow without restriction or control. These altered cells frequently exhibit mitotic irregularities and genomic instability (Brison, O., 1995; Cheng & Loeb, 1993; Otto et al., 1989). An increase in chromosome number is a common feature in many malignancies and appear

to be a crucial step in the progression of many solid tumors. Furthermore polyploid karyotypes have been described in several *in vivo* and *in vitro* tumor models. Whether a malignant tumor arises from a single cell or from more than one cell is still contradictory. However, a large number of evidence suggests that cancers are monoclonal at the initial phase and the clonal evolution during tumor progression plays a fundamental role in the generation of heterogeneity (Nowell, 1976; Woodruff, 1989; Marx, 1982). Biological heterogeneity has been observed in primary tumor as well as within and among metastatic lesions (Runowics et al., 1990; Talmadge, et al., 1984) and appears to be more common in metastatic models than in nonmetastatic tumors (Nguyen, et al., 1993).

When the amniotic cells were submitted to nutritional stress they were not only exposed to the effects of a depleted medium, but also to the consequences of this condition, such as the accumulation of metabolites and to a low pH. Nutritional stress and low pH (Morita, *et al.* 1989; Zura and Grant, 1981; Genari and Wada, 1998) have been associated with the induction of cell transformation in mammalian cells in culture and with the development of some kinds of stomach and bladder tumour in mice (Ashby and Ashidate, 1986; Brusick, 1987).

Since intracellular pH is known to play a critical role in controlling cell cycle and is frequently associated with abnormal proliferation (Gillies, et al., 1992), we can conclude that the nutritional stress conditions accompanied by pH alterations may be responsible by the polyploidization and the phenotype alterations in amniotic stressed cells. These non physiological conditions may also be responsible for the selection of polyploid clones which have more potential to adapt to the culture conditions as

compared to the diploid cells. The variability in the karyotype characteristics is then intimately related with the establishment of a cell line.

ACKNOWLEDGEMENTS

This work was carried out with the support of Coordenadoria de Aperfeicoamento de Pessoal de Nivel Superior (CAPES) and Fundação de Apoio ao Ensino e Pesquisa (FAEP).

REFERENCES

Abrercrombie, M. Contact inhibition and malignancy. Nature 281:259-262; 1979.

Ashby, J. and Ishidate, M. Clastonenicity in vitro of the Na, K, Ca and Mg salts of saccharin; and magnesium chloride; consideration of significance. Mutation Res.163:63-73; 1986.

Borek, C. Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated Hepatoma-like cells under conditions of nutritional stress. Proc. Nat. Acad. Sci. USA 69:956-959; 1972.

Brison, O. Gene amplification and tumor progression. Biochem. Biophys. Acta. 1155: 25-41; 1993.

Brushich, D. J. Implications of treatment-condition-induced genotoxicity for chemical screening and data interpretation. Mutation Res. 189:1-6; 1987.

Cheng, K. C.; Loeb, L. A. Genomic instability and tumor progression: mechanistic considerations. Adv. Cancer Res. 60: 21-156; 1993.

Drouin, V.; Viguie, F. and Debesse, B. Near-haploid karyotype in a squamous cell lung carcinoma. Genes, Chrom Cancer 7:209-212; 1993.

Genari, S. C. and Wada, M. L. F. Behavioural differences and cytogenetical analysis of a transformed cellular population derived from a Vero cell line. Cytobios 81:17-25; 1995.

Genari, S. C. Dolder, M. A. D. and Wada, M. L. F. Scanning and transmition electron microscopy of transformed Vero cells, with altered in vitro growth characteristics. J. Submicrosc. Cytol. Pathol. 28:565-572; 1996.

Genari, S. C., Gomes, L. and Wada, M. L. F. Alterations in the growth and adhesion pattern of Vero cells induced by nutritional stress conditions. Cell Biol. Int. (in press), 1998.

Gillies, R. J., Martinez-Zeguilan, R., Peterson, E. P., and Perona, R. Role of intracellular pH in mammalian cell proliferation. Cell Physiol. Biochem. 2:159-179; 1992.

Gylvarry, U., Farrel, D., Lynch, V., Moriarty, M., Dooley, M., Law, E. and Clynes, M. Cytological differences between normal and malignant human cell populations in culture. Cancer Res. 50:3390-3393; 1990.

Kohno, S. I.; Minowada, J. and Sandberg, A. A. Chromosome evolution of nearhaploid clones in a established human acute lymphoblastic leukemia cell line (Nalm-16). JNCI, J. Natl. Cancer Inst. 64:485-493; 1996.

Mamaeva, S. E. Karyotype evolution of cells in culture: a new concept. Int. Rev. Cytol., 178:1-40; 1998.

Marx, J. L. Tumors: a mixed bag of cells. Science 215:275-277;1992.

More, D. E.; Weise, K.; Zawydiwski, K. and Thompson, E. B. The karyotype of the glucocorticoid-sensitive, lymphoblastic human T-cell line CCRF-CEM shows a unique deleted and inverted chromosome 9. Cancer Genet. Cytogenet. 14:89-94; 1985.

Morita, T., Watanabe, Y., Takeda, K. and Okomura, K. Effects of pH in the in vitro chromosomal aberration test. Mutation Res. 225:55-60; 1989.

Nguyen, H. N.; Sevin, B.-U.; Averette, H. E. Evidence of tumor heterogenity in cervical cancer and lymph node metastases as determined by flow cytometry. Cancer 71:2543-2550, 1993.

Nowell, P. C. The clonal evolution of tumor cell populations. Science 194:23-28, 1996.

Otto,e. McCord, S.; Tisty, T.D. Increased incidence of CAD gene amplification in tumorigenic rat lines as indicator of genomic instability of neoplastic cells. J. Biol. Chem. 264:3390-3396, 1990.

Priest, J. H. Prenatal Chromosomal Diagnosis and Cell Culture. In The ACT Cytogenetics Laboratory Manual. Margaret J. Barch, M. S., CLSp (CG) Editor. 2o edition. Raven Press, New York, pp149-326; 1991.

Runowicz, C. D.; Nuchtern, L. M. Braunstein, J. D. et al. Heteroneity in hormone receptor status in primary and metastatic endometrial cancer. Gynecol. Oncol. 38:437-441; 1990.

Talmadge, J.E.; Wolman, S. R.; Fidler,I. J. Evidence for the clonal origin of spontaneous metastases. Cancer Res. 44:3801-3805, 1984.

Waters, J. J.; Ibson, J. M.; Twentyman, P. R.; Bleehen, N. M. and Rabbitts, P. H. Cytogenetic abnormalities in human small cells lung carcinoma: cell lines characterized for myc gene amplification. Cancer Genet. Cytogenet. 30:213-223; 1988.

Woodruff, M. F. A. Tumor clonality and its biological significance. Adv. Cancer Res. 50:197-229, 1989.

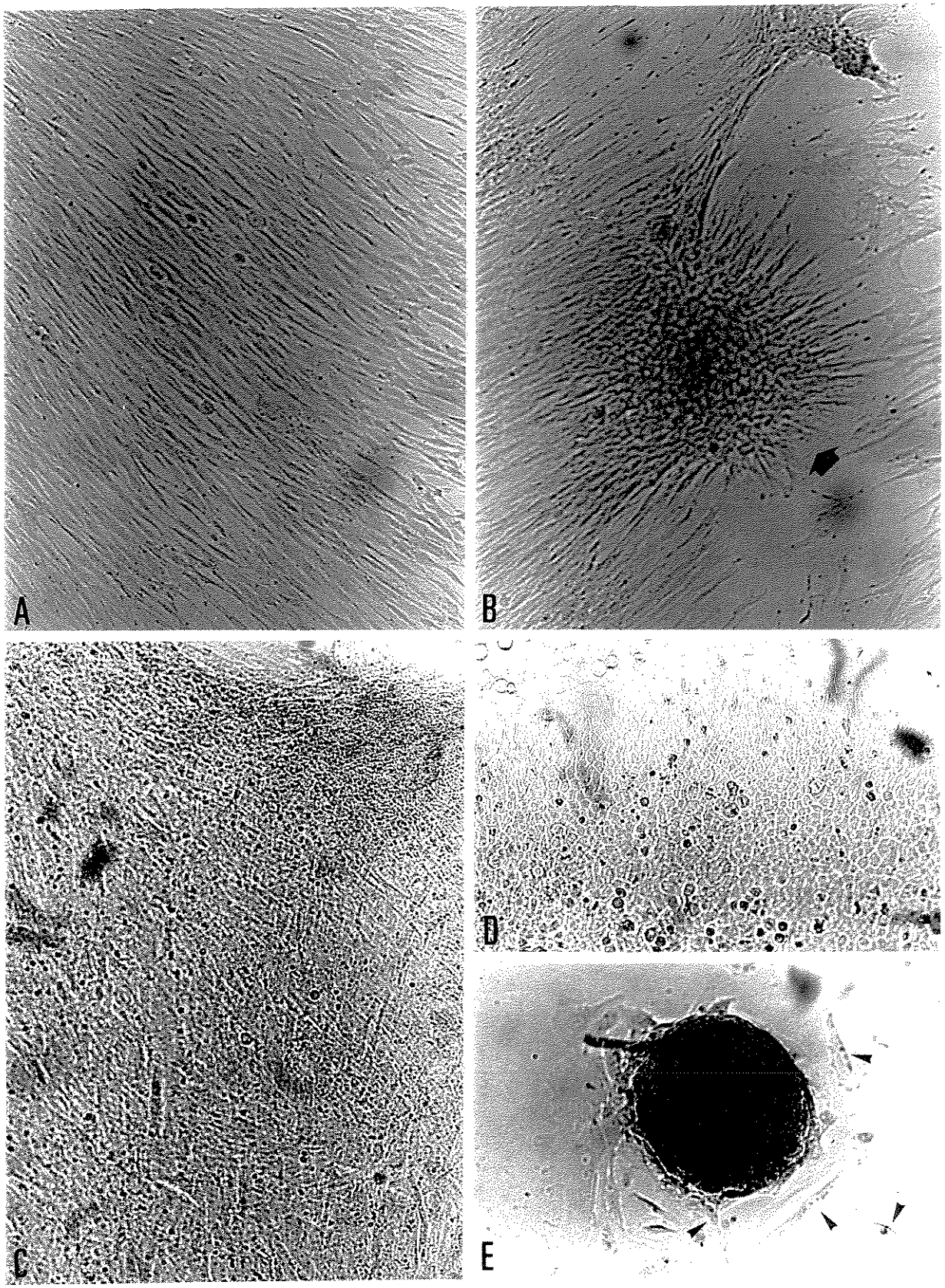
Zura, K. D. and Grant, W. F. The role of hydronium ion in the induction of chromosomal aberrations by weak acid solutions. Mutation Res. 84:349-364; 1981.

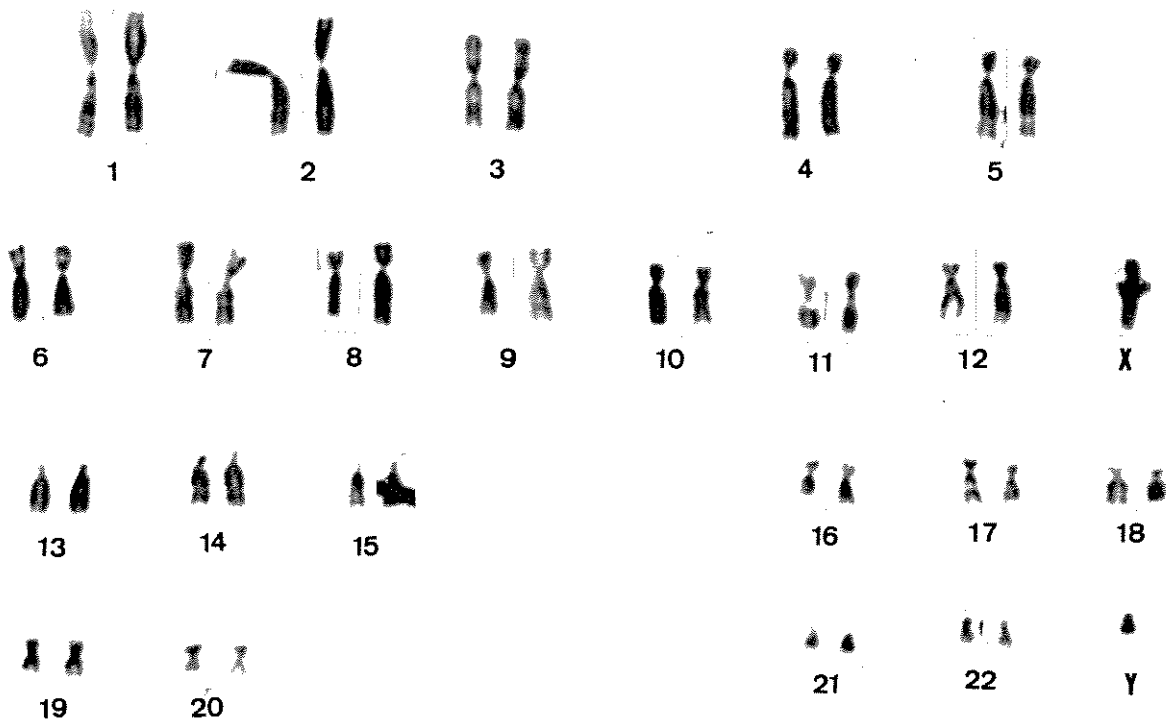
LEGENDS

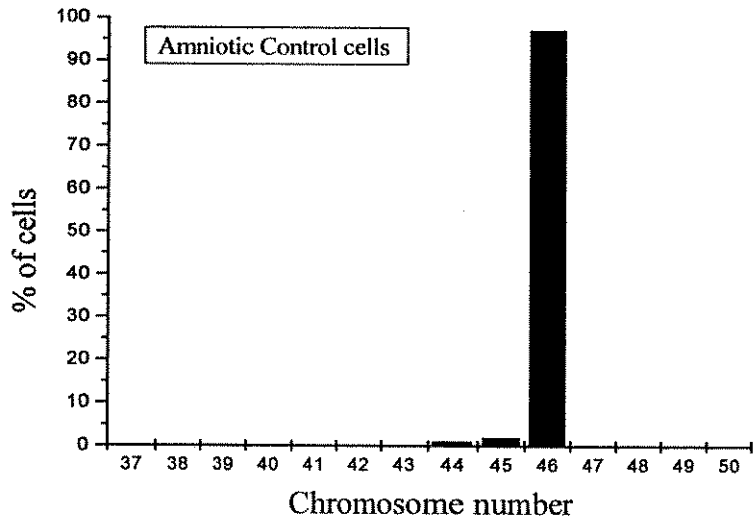
Figure 1 - Control and stressed human amniotic cells growing in culture. Control cells with 10 days of culture form a monolayer and grow as aligned fibroblastic-like cells (A), 100X. Stressed amniotic cells after 21 days in culture showing growth as multilayer and forming cell aggregates (arrow) (B), 100X. Mechanically removed cells adhered on the flask surface and migrated before forming aggregates (arrow) (E), 400X. Cells initially presented a rounded and grew in monolayer (D) up to confluence, then they grow in multilayers in a disorganized fashion (C), 100X.

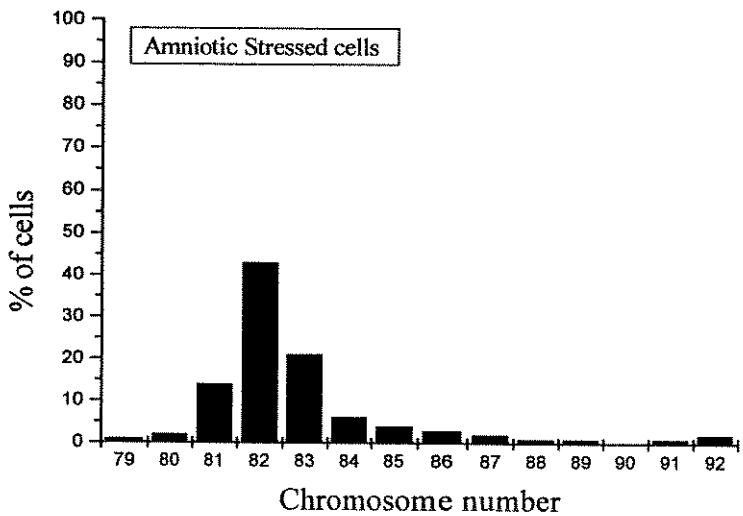
Figure 2 - Representative GTG-banding of a normal 46, XY karyotype control amniotic cell. 1200X.

Figure 3 - Chromosome number of control (upper panel) and stressed human amniotic cells (lower panel). Control population presented a modal chromosome number of 46 (n=100; range 45 to 46 chromosomes), while the stressed cell population presented a modal chromosome number of 82 (n=100; range 79 to 92).


A
B
C
D
E


1
2
3
4
5
6
7
8
9
10
11
12
X
13
14
15
16
17
18
19
20
21
22
Y


Amniotic Control cells
% of cells
100
90
80
70
60
50
40
30
20
10
0
37 38 39 40 41 42 43 44 45 46 47 48 49 50
Chromosome number


Amniotic Stressed cells
% of cells
100
90
80
70
60
50
40
30
20
10
0
79 80 81 82 83 84 85 86 87 88 89 90 91 92
Chromosome number

IV – CONCLUSÕES FINAIS

As linhagens estabelecidas Vero e MDCK assim como as células de cultura primária de líquido amniótico, que possuem inibição por contato e crescimento em monocamadas, apresentaram crescimento em múltiplas camadas com formação de agregados celulares após serem submetidas ao estresse nutricional, perdendo portanto a inibição por contato.

A linhagem Vero submetida ao estresse sofreu alterações morfológicas na superfície celular, caracterizado pelo aumento da presença de estruturas vesiculares e microvilosidades, pouco observadas na população controle.

As células presentes nos agregados ou grumos da população Vero estressada apresentaram ainda um certo grau de diferenciação, com células achatadas na superfície do grumo e células arredondadas no interior do mesmo e presença de material extracelular, simulando a formação de pequenas colônias tumorais.

Nas monocamadas celulares da população MDCK estressada houve a formação de estruturas denominadas *domes*, evidenciando a ocorrência de transporte transepitelial. Após a confluência e crescimento em múltiplas camadas, as células MDCK estressadas organizaram-se formando estruturas tubulares.

As células Vero controle apresentaram codistribuição de fibronectina e actina, as quais concentraram-se principalmente nas fibras de estresse e acumuladas nas regiões de

contato entre as células da monocamada. Nas células Vero estressadas houve redução da evidenciação de fibronectina sem codistribuição com a actina. Nas células MDCK controle e estressadas observou-se codistribuição de fibronectina e actina especialmente evidenciada nas regiões de contato entre as células estressadas.

A diminuição da fibronectina nas células Vero sob estresse provavelmente encontra-se associada a desorganização no padrão de crescimento, levando à formação de múltiplas camadas e diminuição nos índices de adesão. Na população Vero controle a presença da fibronectina entre as células da monocamada manteria a adesão ao substrato e o crescimento em monocamadas.

A resposta imunocitoquímica para vimentina em função do estresse nutricional foi reduzida nas células Vero e aumentada nas células MDCK.

A codistribuição de fibronectina e actina entre as regiões de contato entre células adjacentes da população MDCK estressada, assim como o aumento da evidenciação de vimentina encontra-se associada a formação de estruturas tubulares por estas células.

A alteração no número modal de cromossomos de células Vero, MDCK e de líquido amniótico submetidas ao estresse, assim como elevados índices de poliploidia, indicam que o estresse nutricional associado ao abaixamento do pH é responsável não somente por alterações morfológicas, mas também genéticas nos tipos celulares estudados.

Pelo presente trabalho pudemos concluir que condições não fisiológicas de tratamento por estresse nutricional associada a redução do pH do meio, foram responsáveis pela indução da transformação em todos os tipos celulares estudados, levando a alterações em vários aspectos celulares analisados.

V – RESUMO

Condições não fisiológicas de tratamento por estresse nutricional e diminuição do pH, têm sido associadas a indução de efeitos genotóxicos e transformação em cultura de células de mamíferos. As linhagens celulares Vero, estabelecida a partir de células fibroblásticas renais de macaco verde da África (*Cercopithecus aethiops*) e MDCK (Madin-Darby canine kidney) a partir de células epiteliais renais, assim como células de cultura primária de líquido amniótico humano foram submetidas ao estresse nutricional, com o objetivo de se investigar o efeito desse sobre vários caracteres celulares. As células estressadas assim como seus respectivos controles foram analisadas com relação a aspectos morfológicos e de crescimento em cultura, características citogenéticas e alterações no citoesqueleto e elementos de matriz extracelular. As linhagens estabelecidas Vero e MDCK assim como as células de cultura primária de líquido amniótico, apresentam inibição por contato e crescimento em monocamadas. Após serem submetidas ao estresse nutricional essas populações passaram a crescer em múltiplas camadas com formação de agregados celulares, perdendo portanto a inibição por contato. A linhagem Vero estressada mostrou alterações morfológicas na superfície celular, com aumento em estruturas vesiculares e microvilosidades, pouco observadas na população controle, que exibia superfície regular. As células presentes nos agregados ou grumos da população Vero estressada apresentaram ainda um certo grau de diferenciação, com células achatadas na superfície do grumo e células arredondadas no interior do mesmo e presença de material extracelular, simulando a formação de pequenas colônias tumorais. Nas monocamadas celulares da população MDCK estressada houve a formação de estruturas denominadas *domes*, evidenciando a ocorrência de transporte transepitelial. Após a confluência e crescimento em múltiplas

camadas, as células MDCK estressadas organizaram-se formando estruturas tubulares. As células Vero controle apresentaram codistribuição de fibronectina e actina, as quais concentraram-se principalmente nas fibras de estresse e se acumularam nas regiões de contato entre as células da monocamada. Nas células Vero estressadas houve redução da resposta imunocitoquímica para fibronectina sem codistribuição com a actina, bem como a redução da coloração para vimentina. A diminuição de fibronectina nas células Vero estressadas provavelmente encontra-se associada à desorganização no padrão de crescimento, levando à formação de múltiplas camadas e diminuição nos índices de adesão, enquanto a presença da fibronectina entre as células da monocamada manteria a adesão ao substrato e o crescimento em monocamadas. As células MDCK controle e estressadas exibiram codistribuição de fibronectina e actina especialmente nas regiões de contato entre as células estressadas, enquanto a vimentina apresentou-se mais intensamente corada nas células MDCK estressadas. A codistribuição de fibronectina e actina nas regiões de contato entre células adjacentes da população MDCK estressada, assim como o aumento da evidenciação de vimentina encontram-se associados à formação de estruturas tubulares por estas células. Células Vero, MDCK e de líquido amniótico apresentaram número modal de cromossomos diferentes de suas populações celulares controle, assim como elevados índices de poliploidia, indicando que o estresse nutricional associado ao abaixamento do pH é responsável não somente por alterações morfológicas, mas também genéticas nos tipos celulares estudados. Pelo presente trabalho pudemos concluir que condições não fisiológicas de tratamento por estresse nutricional associado à redução do pH do meio, foram responsáveis pela indução da transformação em todos os tipos celulares estudados, levando a alterações em vários dos aspectos celulares analisados.

VI – ABSTRACT

Non-physiological treatment conditions by nutritional stress and lowered pH have been associated with genotoxic effect and cellular transformation in mammalian culture cells. Vero cell line, obtained from fibloblastic kidney cells of African green monkey (*Cercopithecus aethiops*), and MDCK (Madin-Darby canine kidney) epithelial cells as well as a primary culture of amniotic human cells were submitted to nutritional stress conditions, to investigate the effect of this treatment, in several cellular characteristics in these cell types. The cellular morphological, growth, cytogenetical, cytoskeleton and extracellular characteristics of cells submitted to stress conditions and their respective controls were analyzed. Vero and MDCK cell lines as well as the primary culture of amniotic cells presented contact inhibition and monolayer growth. After submitted to nutritional stress conditions all these cell types presented multilayered growth with formation of cellular aggregates, losing the contact inhibition property. Vero stressed cells presented morphological alterations at the cellular surface, with enhanced of vesicular and microvilli structures, not often observed in control cells. At the periphery of Vero cell aggregates, flattened cells were observed, while in the aggregate, the inner cells presented a rounded shape with extracellular material, indicating an ability to stimulate miniature tumoral colony formation. On the monolayer of stressed MDCK cells specific structures called *domes* were observed, which indicate that transepithelial transport has been performed. After confluence an multilayered growth of stressed MDCK cells were organized in tubular structures. Control Vero cells presented fibronectin and actin codistribution at stress fibers and between adjacent cells in the monolayer. Stressed Vero cells showed reduced immunocytochemical response to fibronectin and vimentin without actin codistribution.

The reduction of fibronectin response in Vero stressed cells probably is associated to the growth pattern disorganization, with multilayer growth and a reduced adhesion index, while fibronectin between adjacent cells is responsible forthe substrate adhesion and monolayer growth. Control and stressed MDCK cells presented fibronectin and actin codistribution at the cell-cell contact regions, while the vimentin immunocytochemical response was enhanced in stressed cells. These characteristics in stressed MDCK cells probably are associated with the formation of the tubular structures observed. Vero and MDCK lines as well as amniotic human cells presented altered modal chromosome number and enhanced polyploidy index when compared at their controls, showing that nutritional stress conditions and lowered pH are associated, not only to morphological, but also to genetical alteration in the analyzed cell types. In the present research we can conclude that non-physiological conditions by nutritional stress associated with lowered medium pH were responsible by transformation induction in all the cellular types studied.

VII – REFERÊNCIAS BIBLIOGRÁFICAS

ABERCROMBIE, M. (1979). Contact inhibition and malignancy. *Nature*, **281**: 259-62.

ALITALO, K., KESKI-OJA, J., HEDMAN, K. and VAHERI, A. (1982). Loss of different pericellular matrix components of rat cells transformed with a T-class ts mutant of Rous Sarcoma Virus. *Virology*, **119**: 347-357.

BEN-ZE'EV, A. (1985). The cytoskeleton in cancer cells. *Biochem. Biophys. Acta.*, **780**: 197-212.

BIANCHI, N. O. and AYRES, J. (1971). Heterochromatin location on chromosomes of normal and transformed cells from African green monkey (*Cercopithecus aethiops*). *Exp. Cell Res.*, **68**: 253-258.

BIEDLER, J. L. (1976). Chromosome abnormalities in human tumor cells in culture. In Fogh, J. (ed): "Human tumor cells *in vitro*". New York, Academic Press.

BISHOP, J. M. (1991). Molecular themes in oncogenesis. *Cell*, **64**:235-48.

BOESEN, J. J. B., DIETEREN, N., BAL, E., LOHMAN, P. H. M. and SIMONS, J. W. I. M. (1992). A possible factor in genetic instability of cancer cells: stress-induced secreted proteins lead to decrease in replication fidelity. *Carcinogenesis*, **12**: 2407-2413.

BOREK, C. (1972). Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated Hepatoma-like cells under conditions of nutritional stress. *Proc. Nat. Acad. Sci. USA*, **69**: 956-959.

COOK, J. R. and CHEN, J. (1988). Enhancement of transformed cell growth in agar by serins proteases inhibitors. *J. Cell Physiol.*, **136**: 188-193.

CROSS, M. and DEXTER, T. M. (1991). Growth factors in development, transformation and tumorigenesis. *Cell*, **64**: 271-80.

GALEGO. L., BARAHONA, I. and RODRIGUES-POUSADA, C. (1984). Response of *Tetrahymena pyriformis* to stress induced by starvation. *Eur. J. Biochem.*, **139**: 163-171.

GENARI, S. C. and WADA, M. L. F. (1995). Behavioural differences and cytogenetic analysis of a transformed cellular population derived from a Vero cell line. *Cytobios*, **81**: 17-25.

GILVARRY, U., FARRELL, D., LYNCH, V., MORIARTY, M., DOOLEY, M., LAW, E. and CLYNES, M. (1990). Cytological differences between normal and malignant human cell populations in culture. *Cancer Res.*, **50**, 3390-3393.

HAYMAN, E. ENGVALL, E. and RUOSLAHTI, E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells. *J. Cell Biol.*, **88**: 352-357.

HOLZER, C., MAIER, P. and ZBINDEN, G. (1986). Comparison of exogenous growth stimuli for chemically transformed cells: growth factors, serum and cocultures. *Exp. Cell Biol.*, **54**: 237-44.

HYNES, R. O. (1976). Cell surface proteins and malignant transformation. *Biochem. et Biophs. Acta*, **458**: 73-107.

IARC/NCI/EPA Working Group (1985). Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chemicals: overview and recommended protocols. *Cancer Res.*, **45**: 2395-2399.

KESKI-OJA, J., ALITALO, K., BARLATI, S. and VAHERI, A. (1985). Pericellular matrix changes in fibroblastic and epithelial cells induced by oncogenic transformation. In: Theories and models in cellular transformation. Edited by L. Santi and Luciano Zardi, Instituto Scientifico Tumori, Genoa, Italy - Academic Press. 55-70.

KLAUNIG, J. and RUCH, R. J. (1990). Biology of disease: role of inhibition of intercellular communication in carcinogenesis. *Lab. Invest.*, **62**: 135-46.

LOEWENSTEIN, W. R. (1979). Junctional intercellular communications and the control of growth. *Biochem. et Biophs. Acta.*, **560**: 1-65.

MATSUOKA, A.; HAYASHI, M. and ISHIDATE JR., M. (1979). Chromosomal aberration tests on 29 chemicals combined with S9 mix *in vitro*. *Mutation Res.* **66**: 277-90.

MORIMOTO, R. I. (1991). Heat shock: The role of transient inducible responses in cell damage, transformation and differentiation. *Cancer Cells*, **3**: 295-301.

MURRAY, J. C., LIOTTA, L., RENNARD, S. I. and MARTIN, G. R. (1980). Adhesion characteristics of murine metastic and nonmetastic tumor cells *in vitro*. *Cancer Res.*, **40**: 347-351.

NAKAYASU, H. and UEDA, K. (1986). Preferential association of acidic actin with nuclei and nuclear matrix from mouse leukemia L5178Y cells. *Exp. Cell Res.*, 163, 327-336.

NERMUT, M. V., EASON, P., HIRST, E. M. A. and KELLIE, S. (1991). Cell/substratum adhesions in RSV-transformed rat fibroblasts. *Exp. Cell Res.*, **193**: 382-97.

PERSKY, B., MEYSKENS, F. L. and HENDRIZ, M. J. C. (1989). Inmunohistochemical and ultrastructural study of human melanoma colonies grow in soft agar. *Am. J. Anat.*, **184**: 212-224.

PONTÉN, J. (1976). The relationship between *in vitro* transformation and tumor formation *in vivo*. *Biochem. Biophys. Acta.*, **738**: 237-249.

REN, J., HAMADA, J., TAKEICHI, N., FUJIKAWA, S. and KOBAYASHI, H. (1990). Utrastructural differences in junctional intercellular communication between highly and weakly metastatic clones derived from rat mammary carcinoma.*Cancer Res.*, **50**: 358-62.

SHERIDAN, J. W. and SIMMONS, R. J. (1981)a. Studies on a human melanoma cell line:

effect of cell crowding and nutrient depletion on the biophysical and kinetic characteristics of the cells. *J. Cell. Physiol.*, **107**: 85-100.

SHERIDAN, J. W.; BISHOP, C. J. and SIMMONS, R. J. (1981)b. Biophysical and morphological correlates of kinetic change and death in a starved human melanoma cell line. *J. Cell. Sci.*, **49**: 119-137.

SMETS, L. A. (1980). Cell transformation as a model for tumor induction and neoplastic growth. *Biochem. et Biophs. Acta*, **605**: 93-111.

SMETS, L. A. and VAN BEEK, W. P. (1984). Carbohydrates of the tumor cell surface. *Biochem. et Biops. Acta*, **738**: 237-249.

TERZAGHI-HOWE, M. (1993). Factors regulating the emergence of spontaneous and X-ray-induced variants in primary rat tracheal epithelial cell culture. *In Vitro Cell Dev. Biol.*,**29**: 120-126.

VAHERI, A. and MOSHER, D. F. (1978). High molecular weight cell surface-associated glycoproteins (fibronectin) lost in malignant transformation. *Biochem. et Biophs. Acta*, **516**: 1-25.

WELCH, W. J. (1993). Heat shock proteins functioning as molecular chaperones: their roles in normal and stressed cells. *Phil. Trans. R. Soc. Lond. B.*, **339**: 327-33.

WITTE, L., FUKS, Z. HAIMOVITZ-FRIEDMAN, A., VLODAVSKY, I., GOODMAN, D. S. and ELDOR , A. (1989). Effects of irradiation on the release of growth factors from cultured bovine, porcine and human endothelial cells. *Cancer Res.*, **49**: 5066-5072.

WOLLNIK, B., KUBISCH, C., MAASS, A., VETTER, H. and NEYSES, L. (1993). Hyperosmotic stress induces immediate-early gene expression in ventricular adult cardiomyocytes. *Biochem. Biophys. Res. Comm.*, **194**: 642-646.

YAMASAKI, H. (1990). Gap junctional intercellular communication and carcinogenesis.

Carcinogenesis, **11**: 1051-8.

ZURA,K. D. and GRANT, W. F. (1981). The role of hydronium ion in the induction of chromosomal aberrations by weak acid solutions. *Mutation Res.*,**84**: 349-64.