UNIVERSIDADE ESTADUAL DE CAMPINAS

Estela Maria Gonçalves

EFEITO DA CISPLATINA EM CULTURA DE LINHAGENS ESTABELECIDAS E SUA CAPACIDADE DE INDUZIR TRANSFORMAÇÃO CELULAR *IN VITRO*

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

Orientadora: Profª. Drª. Selma Candelária Genari

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DEDICATÓRIA

A meus pais,

Celso e Maria do Carmo

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ÍNDICE

I. RESUMO

A cisplatina é um agente antineoplásico utilizado no tratamento quimioterápico de tumores como os de testículo, de ovário e de bexiga urinária. Contudo, estudos indicam que a cisplatina apresenta potencial mutagênico, genotóxico e tumorigênico tanto *in vitro* como *in vivo*. Após tratamento com 50 μg/ml de cisplatina durante 24 h, células Vero apresentaram alterações comportamentais e morfológicas associadas à transformação celular *in vitro*. Modificações morfológicas foram investigadas com utilização de imunocitoquímica (fibronectina), microscopia eletrônica de varredura e coloração faloidina-fluoresceína (actina). O estudo proliferativo foi realizado a partir de curvas de crescimento e o padrão de adesão celular foi obtido através de testes de adesão. Características citogenéticas foram avaliadas em células Vero e V79 tratadas com cisplatina, através da determinação dos números modais de cromossomos, das freqüências de poliploidia e dos índices mitóticos. Células Vero controles apresentaram crescimento em monocamadas, enquanto que células Vero transformadas cresceram em múltiplas camadas, formando grumos ou agregados celulares. A proliferação celular e as características morfológicas e de adesão das células Vero transformadas foram acentuadamente diferentes das células controles. Células Vero transformadas e células V79 tratadas com cisplatina apresentaram alterações nos números de cromossomos além de aumento nos índices mitóticos e nas freqüências de poliploidia. Os resultados obtidos indicam que as alterações morfológicas, de crescimento e de adesão observadas em células Vero e as alterações citogenéticas, observadas em células Vero e em células V79, provavelmente relacionam-se com a transformação celular *in vitro* induzida pelo tratamento com cisplatina. Estas células Vero transformadas apresentam características associadas ao crescimento neoplásico, podendo ser utilizadas como modelo de estudo de células tumorais *in vitro.*

II. ABSTRACT

Cisplatin is an antineoplastic agent used to treat solid malignancies, such as testicular, ovarian and bladder tumors. However, both *in vitro* and *in vivo,* cisplatin has been shown to be mutagenic, genotoxic and tumorigenic. Maintained in culture, Vero cells presented behavioral and morphological alterations associated with cellular transformation *in vitro*, after treatment with 50 µg/ml of cisplatin during 24 h. The morphological alterations were investigated using immunocytochemistry (fibronectin), scanning electron microscopy and the actin cytoskeleton was labeling with fluorescein isothiocyanate-phalloidin. The study of proliferation was obtained from the growth curve and the adhesion pattern was obtained from the adhesion assay. In Vero and V79 cells treated with cisplatin, cytogenetical characteristics were obtained by modal chromosome numbers, polyploidy frequencies and mitotic index determinations. Control Vero cells presented growth in a monolayer, while the transformed cells grew in multilayers forming cellular aggregates. The cellular proliferation, adhesion pattern and morphological characteristics of the transformed Vero cells were very different from the control ones. Transformed Vero cells and cisplatin-treated V79 cells presented altered chromosome numbers. Polyploidy frequencies and mitotic indexes were also enhanced in these cells. The results indicate that morphological, growth and adhesion changes observed in Vero cells and cytogenetical alterations, observed in Vero and V79 cells, probably resulted from cellular transformation *in vitro* induced by cisplatin treatment. These transformed Vero cells presented characteristics associated with neoplastic growth, and can be used as a model for tumor cells studies *in vitro*.

III. CAPÍTULO 1

A. INTRODUÇÃO GERAL

1. Câncer – Perspectiva Histórica e Generalidades

As primeiras investigações científicas a respeito das causas do câncer datam de 1775, quando o médico inglês Percival Pott realizou um estudo epidemiológico com limpadores de chaminés da cidade de Londres, na Inglaterra. Percival Pott observou que homens jovens que haviam sido limpadores de chaminés quando meninos apresentavam alta taxa de mortalidade devido ao câncer escrotal. Com isso, sugeriu que a fuligem das chaminés (conhecida hoje como alcatrão) era o agente que causava os tumores e recomendava freqüentes banhos e mudanças de roupas para reduzir a exposição dos limpadores ao "agente carcinogênico". Assim, foi definido o termo "carcinógeno", como também demonstrado pela primeira vez que o câncer poderia se desenvolver muitos anos após a exposição ao "agente carcinogênico" (Hill & Tannock, 1992; Young, 2005).

O potencial carcinogênico de fatores ambientais foi estabelecido posteriormente em experimentos com animais, que apresentavam incidência maior de câncer quando expostos a tais fatores, como vírus, compostos químicos e radiação. Após estes estudos, foi possível também estabelecer que estes agentes apresentavam uma importante propriedade em comum: ocasionavam danos ou alterações no DNA, sugerindo que o DNA é um alvo importante para os agentes carcinogênicos e que o desenvolvimento do câncer poderia ser resultante de alterações no DNA (Hill & Tannock, 1992).

O câncer é uma doença onde ocorre profunda alteração no sistema de regulação da proliferação e da diferenciação celulares. Enquanto na maioria dos tecidos as células dividem-se de forma controlada, no câncer, esse mecanismo de controle é perdido e ocorre

uma proliferação celular acima das necessidades do tecido. O câncer pode ser considerado uma doença genética cujo desenvolvimento se deve a mutações em determinados genes nucleares. Uma mutação em um gene que modula a proliferação ou a diferenciação da célula pode fazer com que seu produto seja hiperativo ou produzido em excesso, e como resultado, tem-se a transformação do fenótipo celular. Esses genes mutantes são, por isso, classificados como oncogenes, ou seja, genes causadores de câncer. As suas contrapartes normais não alteradas são chamadas protooncogenes. Os protooncogenes são genes normalmente expressos durante o desenvolvimento embrionário e mesmo em células maduras. Muitos deles codificam moléculas que induzem as células a se diferenciarem, receptores para essas moléculas, proteínas relacionadas a transdução de sinais e fatores de transcrição. Quando ocorrem mutações nos protooncogenes, com sua conseqüente hiperativação ou superexpressão, temos o desenvolvimento de uma neoplasia. Os oncogenes podem ser alterados por uma série de mecanismos, tais como: 1) inserção de transposons contendo um gene promotor no início do oncogene; 2) mutações que alteram a seqüência da proteína e aumentam sua atividade ou expressão; 3) amplificação gênica, que ocasiona um aumento do número de cópias do oncogene; ou 4) translocações cromossômicas. Uma vez que o câncer é originário da desregulação dos mecanismos que controlam a proliferação e a diferenciação celulares, a célula tumoral representa um modelo em que, na maioria das vezes, há dediferenciação de uma célula já especializada. Ou seja, uma célula já diferenciada passa a se comportar como uma célula indiferenciada (Santos & Wada, 2001).

Em resumo, evidências demonstraram que o desenvolvimento do câncer envolve um número de diferentes alterações genéticas ocorrendo seqüencialmente na célula, incluindo a deleção de genes específicos (genes supressores de tumores), a mutação de oncogenes (que

podem transformar células normais em tumorais), além de outras aberrações cromossômicas. Estas mudanças seqüenciais estão aparentemente associadas à progressão até maiores graus de malignidade. A identificação de alterações genéticas nas células tumorais, além do efeito que os produtos protéicos dos genes envolvidos apresentam no desenvolvimento tumoral, representam focos importantes no estudo da biologia do câncer (Hill & Tannock, 1992).

2. Carcinogênese Química

Desde a sua descrição por Berwald & Sachs em 1963, testes *in vitro* utilizando compostos químicos em ensaios de transformação celular em cultura de células de mamíferos têm sido realizados para avaliação do potencial carcinogênico de diversas substâncias. Em um estudo conduzido para testar 28 substâncias químicas a respeito de sua capacidade de induzir o surgimento de células morfologicamente transformadas em cultura, os autores relataram a concordância deste teste *in vitro*, na faixa de 80%, com testes realizados *in vivo* com roedores, explicitando sua extrema atualidade e importância para a determinação do potencial carcinogênico de substâncias químicas (Engelhardt *et al*., 2004).

Assim, estudos *in vitro* a respeito da indução e progressão da transformação celular induzida por carcinógenos químicos podem proporcionar importantes esclarecimentos a respeito dos mecanismos envolvidos neste processo e ser de utilidade para o desenvolvimento de estratégias quimiopreventivas ou quimioterapêuticas para uso humano. Além disso, a compreensão dos eventos celulares que acompanham a progressão da transformação até o fenótipo neoplásico resultante, oferece a possibilidade de intervenção e

prevenção seletivas durante os múltiplos estágios da transformação celular (Zhu & Gooderham, 2002).

3. Transformação Celular *In Vitro*

A carcinogênese é um processo com múltiplas etapas, envolvendo alterações genéticas seqüenciais nas células alvo, levando a alterações no controle do crescimento e, conseqüentemente, à formação de tumores malignos. Na década de 60 foi demonstrado que células embrionárias de hamster podiam ser transformadas por hidrocarbonetos policíclicos aromáticos e que tal sistema poderia ser quantificado. Tal modelo de investigação estimulou o campo de estudo da *transformação celular*, também chamada de carcinogênese *in vitro* ou oncogênese em cultura celular (Heidelberger *et al.*, 1983).

Dessa maneira, o estudo do processo de carcinogênese foi grandemente facilitado pela descoberta da transformação morfológica *in vitro*, que apresenta relações com processo carcinogênico *in vivo* segundo reconhecimento em 1985 pelo IARC/NCI/EPA Working Group, que normatizou os testes de transformação celular em linhagens celulares estabelecidas após exposição a carcinógenos químicos (IARC, 1985). Assim, o objetivo do estudo da transformação *in vitro* é entender a tumorigênese em organismos vivos, assim como os eventos celulares e moleculares envolvidos nesse processo (Pónten, 1976; Smets, 1980).

3.1. Células Transformadas – Algumas Características

3.1.1. Perda da Inibição por Contato do Crescimento

As junções celulares do tipo aderente têm um papel fundamental na organização celular e tecidual, por mediarem a adesão e a sinalização celulares. Estas junções consistem em grandes complexos multiprotéicos que unem o citoesqueleto de actina à membrana plasmática para formar contatos adesivos entre as células ou entre células e a matriz extracelular (Tepass, 2002). A manutenção dos contatos celulares é fisiologicamente significativo, dado o fato que o comprometimento do mecanismo de adesão celular, mediado pelas proteínas caderinas e cateninas, consiste numa etapa importante para o desencadeamento do processo tumoral (Collares-Buzatto, 2001).

Células transformadas apresentam freqüentemente baixa adesividade com outras células e com a matriz extracelular combinadas a uma alta motilidade celular. O processo de transformação inicia-se com a perda de adesão intercelular quando, então, as células transformadas tornam-se extremamente móveis. Devido em parte a essa baixa adesividade, cessa-se a inibição por contato do crescimento celular, que ocorre quando uma célula não transformada entra em contato com outra do mesmo tipo; a célula transformada começa, então, a se multiplicar desordenadamente. Num grau mais intenso de transformação, as células podem se tornar metastáticas e sair do seu local de origem, invadindo outros órgãos via circulação sanguínea e/ou linfática (Collares-Buzatto, 2001).

3.1.2. Crescimento Independente de Ancoragem

Células fibroblásticas, epiteliais e endoteliais necessitam estar aderidas a componentes apropriados da matriz extracelular para sobreviverem – um fenômeno denominado dependência de ancoragem. Assim, a proliferação celular é controlada não apenas pela presença de mitógenos, mas também por componentes da matriz extracelular,

como a fibronectina, à qual as células se aderem através de receptores transmembrana pertencentes à família das integrinas (Danen & Yamada, 2001). Por outro lado, a capacidade de algumas células de sobreviverem e proliferarem *in vitro* na ausência da adesão mediada por integrinas a componentes da matriz extracelular, como a fibronectina, é um fenômeno denominado independência de ancoragem e está relacionado à tumorigênese *in vivo*, por capacitar células tumorais a sofrerem metástase e crescerem em locais inapropriados do corpo (Freedman & Shin, 1974).

3.1.3. Alterações na Adesão

Células que crescem aderidas a um substrato, quando transformadas, podem apresentar alterações no padrão de adesão à matriz extracelular. Um dos mais marcantes efeitos da transformação de fibroblastos de camundongos pelo vírus do sarcoma de aves, é a perda de componentes da matriz extracelular, que pode ser devido à diminuição de sua síntese ou por decréscimo na capacidade de depositar a matriz sintetizada nas estruturas pericelulares. Tais características podem ocasionar alterações na adesão das células ao substrato, que pode relacionar-se com a perda da inibição por contato do crescimento e do movimento observadas nestas células. Em animais, tais alterações podem levar à tumorigênese (Alitalo *et al*., 1982).

3.1.4. Alterações Genéticas

Tem sido demonstrado que a formação de tumores é um processo com múltiplas etapas no qual as células adquirem múltiplas alterações genéticas, seguidas pela expansão

clonal seletiva levando ao fenótipo neoplásico. Em um estudo *in vivo* utilizando-se como modelo o camundongo, a progressão tumoral ocorreu através da seguinte seqüência: células diplóides ⇨ células tetraplóides ⇨ células aneuplóides. Esta seqüência de alterações genéticas foi associada a uma progressão morfológica, partindo de células morfologicamente normais até a hiperplasia, seguidas pela displasia e finalmente, pela formação de carcinomas (Ornitz *et al.*, 1987).

Nesse sentido, Zhu & Gooderham (2002) demonstraram que no processo de transformação química de fibroblastos humanos em cultura, surgiram alterações na ploidia celular juntamente com alterações morfológicas nucleares e celulares seguidas por mudanças substanciais no padrão de crescimento *in vitro*. Alterações no conteúdo de DNA celular têm sido consideradas como um indicativo do aumento da instabilidade genética, que pode desencadear a transformação celular com o surgimento de variáveis mutantes, sendo que as mais favoráveis são selecionadas pela pressão do ambiente (Remvikos *et al*., 1988; Shackney *et al*., 1989; Blagosklonny, 2002).

No modelo de estudo de Zhu & Gooderham (2002), um evento-chave necessário para o desenvolvimento da transformação neoplásica induzida pelo carcinógeno químico benzo[*a*]pireno, foi a modificação genética quimicamente induzida. O composto químico utilizado ocasiona a formação de adutos no DNA. Como agentes que induzem a formação de adutos no DNA podem também induzir quebras cromossômicas, o resultado pode ser observado na forma de ganhos cromossômicos através de fusões ou através de quebras não reparadas, com subseqüente não disjunção, ou perdas de cromossomos defeituosos durante a mitose, levando a alterações no conteúdo de DNA celular. Assim, células com conteúdo anormal de DNA podem não ser efetivamente excluídas durante o ciclo celular e podem

continuar a se dividir. Estas células poderão estar sujeitas a segregação assimétrica dos cromossomos a cada divisão (Holliday, 1989; Zhu & Gooderham, 2002).

Além disso, muitos exemplos já foram descritos a respeito da amplificação gênica observada em tumores malignos, tanto na forma de cromossomos extranumerários como na forma de expansões *IN TANDEM* de genes específicos. Estes genes podem ser amplificados por um grande número de oncogenes, cuja atuação produz malignidade (Harris, 1986).

3.1.5. Modificações Morfológicas

Células fibroblásticas transformadas em cultura freqüentemente apresentam diversas alterações morfológicas. Fibroblastos não transformados são alongados e achatados, sendo que os fibroblastos transformados são geralmente mais arredondados e apresentam diversas alterações na superfície celular. Quando observadas em microscopia eletrônica de varredura, as células transformadas são geralmente recobertas por microvilos e lamelipódios, às vezes também por bolhas ou *blebs* (Linstead *et al*., 1988). Outra importante característica de fibroblastos transformados é sua diminuição de adesão ao substrato, o que colabora diretamente com as alterações observadas em sua morfologia (Pastan, 1979).

3.1.6. Alterações no Citoesqueleto de Actina

Diversas pesquisas, nos últimos anos, têm proporcionado importantes informações a respeito de como o citoesqueleto de actina contribui para o controle do crescimento, tanto em células transformadas, como em células não transformadas, indicando sua participação

na regulação de diversos processos celulares relacionados à transformação, como a proliferação, o crescimento independente de ancoragem e a inibição por contato do crescimento (Pawlak & Helfman, 2001).

A transformação celular em fibroblastos é caracterizada por diversas alterações morfológicas, sendo que o fenótipo arredondado e a diminuição dos feixes de actina do citoesqueleto e das fibras de estresse são características marcantes (Janmey & Chaponnier, 1995; Ben-Ze'ev, 1997). A desorganização dos microfilamentos de actina está associada à redução da expressão de proteínas ligantes de actina, tais como a tropomiosina, gelsolina, α-actinina e vinculina (Kaneko *et al*., 1995). Assim, a restauração destas proteínas pode reverter o fenótipo transformado (Glück *et al*., 1993; Braverman *et al*., 1996; Kwon *et al*., 1997). Um estudo demonstrou a "reversão da transformação" em células transformadas por vírus, que após serem transfectadas com cDNA (DNA complementar) da proteína α-actinina, apresentaram reversão ao fenótipo não transformado. A α-actinina está presente nas junções de adesão, estabilizando os microfilamentos de actina. Uma possibilidade para explicar este resultado é que o aumento na expressão desta proteína nas células transformadas, que receberam o cDNA da α-actinina, possa ter levado a formação de junções de adesão mais estáveis, assim diminuindo a motilidade celular, aumentando a adesão ao substrato e, com isso, gerando sinais intracelulares que afetaram o crescimento e a tumorigenicidade destas células (Glück *et al*., 1993).

4. Quimioterapia Antineoplásica

A quimioterapia antineoplásica ou antiblástica é o método que utiliza compostos químicos, os quimioterápicos, no tratamento do câncer. O primeiro quimioterápico

antineoplásico foi desenvolvido a partir do gás mostarda, usado nas duas Guerras Mundiais como arma química. Após a exposição de soldados a este agente, observou-se que os mesmos desenvolveram hipoplasia medular e linfóide, o que levou ao seu uso no tratamento de linfomas malignos. A partir da publicação, em 1946, dos estudos clínicos feitos com o gás mostarda e das observações sobre os efeitos do ácido fólico em crianças com leucemias, verificou-se um avanço crescente na quimioterapia antineoplásica (Koller, 1976; Dehner, 1998).

As interações entre os compostos quimioterápicos e o DNA têm um papel muito importante em suas funções biológicas. De fato, o DNA pode ser considerado como o principal receptor macromolecular para estas substâncias. Existem diversas classes de compostos antineoplásicos que agem sobre o DNA. Alguns formam complexos não covalentes com o DNA, tais como a daunorubicina, a doxorubicina e a distamicina. Outros compostos, entre eles a cisplatina e a mitomicina C, formam ligações covalentes com o DNA. Por último, existem os que causam clivagens no DNA, como a bleomicina e a pepleomicina (Yang & Wang, 1999).

5. Cisplatina

Em 1965, um experimento realizado para estudar os efeitos de campos magnéticos sobre o crescimento bacteriano revelou que ao ser aplicada uma baixa voltagem a uma cultura de bactérias as mesmas cresciam, porém não se dividiam mais (Rosenberg *et al*., 1965). Os agentes que produziram estes efeitos foram identificados como aqueles formados pela eletrólise dos eletrodos de platina, sendo denominados c*is*-diaminotetracloroplatina (IV) e c*is*-diaminodicloroplatina (II) ou cisplatina (Rosenberg *et al*., 1967). Como a

cisplatina inibia o crescimento bacteriano, poderia também inibir o crescimento de células tumorais. Assim, Rosenberg e colaboradores iniciaram testes a respeito da atividade antitumoral da cisplatina em animais. Após a demonstração de tal atividade (Rosenberg *et al*., 1969), a cisplatina foi liberada para testes clínicos em 1972, sendo seu uso aprovado pelo FDA em 1979 (FDA, 1979).

A cisplatina é um agente antineoplásico que exibe uma vasta faixa de atividade em diversos tipos de tumores humanos, seja isolada ou em combinação com outras drogas, como a vimblastina e a bleomicina. A introdução da cisplatina em protocolos quimioterapêuticos foi altamente benéfica para o tratamento de diversos tipos de tumores (Pinto & Lippard, 1985). Assim, a cisplatina é altamente efetiva no tratamento de tumores como os carcinomas de testículo, de ovário e de bexiga urinária, além de uma variedade de outras neoplasias, como o câncer de cérvix uterino, de cabeça e pescoço, de esôfago e de pulmão (Giaccone, 2000).

A quimioterapia com cisplatina, que corresponde ao tratamento padrão para mulheres com câncer ovariano avançado, aumenta em cerca de 38% a sobrevida destas pacientes (Hershman *et al*., 2004) e promove a remissão completa dos tumores, em cerca de 75% dos pacientes com câncer testicular metastático (El-Helw & Coleman, 2004). Além disso, a quimioterapia com cisplatina é efetiva em 40 a 60% dos pacientes com câncer metastático de bexiga urinária (Sonpavde & Petrylak, 2005).

A cisplatina é um complexo inorgânico que contém um átomo metálico, a platina, rodeada, de forma planar, por dois átomos de cloro e duas moléculas de amônia na posição *cis* (Bristol-Myers Squibb, 1999) (Figura 1).

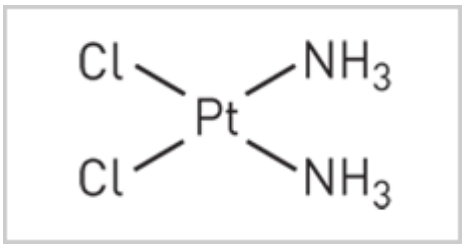

Figura 1. Estrutura plana da cisplatina.

5.1. Mecanismos Bioquímicos de Ação da Cisplatina

Compostos que formam ligações químicas com o DNA representam classes importantes de compostos antitumorais. Um grande número destes compostos têm sido sintetizados e testados para atividade antineoplásica, e muitos deles estão em uso clínico. Dentre eles, podem ser citados: a mostarda nitrogenada, as aziridinas, os alcano-sulfonados, as nitrosuréias e os compostos contendo platina (Lawley & Phillips, 1996; Tannock & Goldenberg, 1998). Todas estas drogas interagem com uma variedade de moléculas celulares, porém o mais importante aspecto responsável por sua citotoxicidade parece ser a inibição da divisão celular ou a estimulação da apoptose ocasionada pela formação dos adutos no DNA (Tannock & Goldenberg, 1998). Exemplos importantes destas drogas incluem a cisplatina, a mostarda nitrogenada, a mitomicina e a carmustina (Dronkert & Kanaar, 2001).

Desde a introdução da cisplatina na prática oncológica, os estudos sobre seu mecanismo molecular de ação têm fornecido consideráveis informações a respeito de como a cisplatina induz seus efeitos antitumorais. Os mecanismos bioquímicos da citotoxicidade da cisplatina envolvem a sua ligação ao DNA e a outros componentes celulares, com a

subseqüente indução da morte celular por apoptose, por necrose ou por ambos os mecanismos (González *et al*., 2001).

É geralmente aceito que a ligação da cisplatina ao DNA genômico (gDNA) no núcleo da célula seja altamente responsável por suas propriedades antitumorais (González *et al*., 2001). Os danos induzidos pela ligação da cisplatina ao DNA genômico podem interferir com os mecanismos normais de transcrição e/ou replicação do gDNA. Eventualmente, estes distúrbios no processamento do DNA genômico podem disparar processos citotóxicos que resultam na morte celular. Porém, sabe-se também que a cisplatina forma uma grande quantidade de adutos no DNA mitocondrial (mtDNA). Dessa forma, não deve ser descartada a possibilidade de que o mtDNA também possa ser um importante alvo para a ação farmacológica da cisplatina (Pérez, 1998; Jamieson & Lippard, 1999).

A cisplatina é um composto neutro, sendo seus grupos cloreto estáveis na concentração extracelular de cloro, já que as altas concentrações de cloreto nos fluidos extracelulares (100 mM) suprimem a formação de formas mono- ou dihidratadas de cisplatina, nas quais um ou ambos os grupos cloreto seriam trocados por moléculas de água. Em contraste, no interior da célula, a concentração de cloreto varia entre 2 e 30 mM. Nestas concentrações, ocorre a hidrólise da cisplatina sendo o resultado final a formação do cátion $[Pt(H_2O)_2(NH_3)_2]^{2+}$. Esta forma dihidratada da cisplatina é eletrofílica, bifuncional e altamente reativa com centros nucleofílicos de biomoléculas intracelulares. Assim, previamente à ligação da cisplatina ao DNA genômico, ao DNA mitocondrial ou a outras moléculas celulares, é necessária a perda de seus grupos cloreto (Eastman, 1990; Fuertes *et al*., 2003). Ao entrar na célula, as baixas concentrações de cloreto intracelulares facilitam a troca dos mesmos por água ou por grupos hidroxila (OH). Com isso, a cisplatina pode

interagir com proteínas, RNA e DNA, sendo esta última reação a principal responsável por sua citotoxicidade através da indução da morte celular (Eastman, 1990).

5.2. Ligação da Cisplatina ao DNA

Os sítios nucleofílicos mais acessíveis e reativos para a ligação da cisplatina no DNA, são os átomos de nitrogênio dos anéis imidazóis da guanina e da adenina localizados na curvatura maior da dupla hélice do DNA (Yang & Wang, 1999). Diversos tipos de adutos de platina podem ser formados pela ligação da cisplatina ao DNA: adutos interfitas de DNA, intrafitas de DNA, DNA-proteínas ou ainda intermoleculares. A cisplatina pode ligar-se a todas as bases do DNA, contudo parecem existir ligações preferenciais (Tandon & Sodhi, 1985; Eastman, 1990). Assim, a ligação cisplatina-DNA pode levar a formação de vários tipos de adutos estruturalmente diferentes. Inicialmente, são formados adutos monofuncionais, porém a maior parte deles posteriormente produz ligações cruzadas interfitas ou intrafitas de DNA, que então bloqueiam a replicação do DNA e/ou previnem a sua transcrição (Payet *et al*., 1993). Foi determinado que entre 60 a 65% dos adutos formados pela cisplatina no DNA correspondem a ligações cruzadas intrafitas entre duas guaninas vizinhas e que, entre 20 a 25% dos adutos, correspondem a ligações cruzadas intrafitas entre uma adenina e uma guanina. Os demais adutos, em percentagens menores, ocorrem geralmente entre duas guaninas separadas por uma ou mais bases (ligações cruzadas intrafitas do tipo GXG, sendo G a guanina e X, qualquer uma das bases), além de ligações cruzadas interfitas (Fichtinger-Schepman *et al*., 1985). Por outro lado, a formação de ligações cruzadas entre DNA e proteínas assim como ligações intermoleculares (como

por exemplo, entre DNA, proteína e glutationa) são também induzidas pela cisplatina (Eastman, 1990).

A ligação da cisplatina ao DNA não é suficiente para acarretar a morte celular. A inibição da síntese correta do DNA é o ponto chave para a sua toxicidade, com eventos essenciais ocorrendo na fase G2 do ciclo celular, etapa onde deverá ocorrer o reparo das quebras ocorridas no DNA após a fase S, pois a ligação entre a platina e os nucleotídeos do DNA pode levar ao pareamento incorreto ou a quebras na fita de DNA. Caso estas quebras não sejam reparadas, a célula poderá então entrar em processo de morte celular (Tandon & Sodhi, 1985; Eastman, 1990).

Existe muito debate a respeito do qual tipo de aduto cisplatina-DNA é o mais importante para a mediação de seus efeitos citotóxicos. Aparentemente, o papel principal na indução da morte celular pela cisplatina é desempenhado pelo aduto intrafitas entre duas guaninas, uma vez que o isômero inativo da cisplatina (a transplatina) não é capaz de formar estes adutos. De fato, a transplatina forma principalmente ligações cruzadas intrafitas do tipo GXG e ligações cruzadas interfitas (Eastman & Barry, 1987).

5.3. Vias de Processamento do DNA Após a Formação dos Adutos de Platina

Uma vez formados os adutos de platina no DNA, a célula deve ser capaz de remover ou tolerar a presença dos mesmos, para garantir as funções normais de replicação e transcrição do DNA. Geralmente, o sistema BER (*base excision repair*) remove bases danificadas que causam distorções relativamente pequenas na molécula de DNA (Memisoglu & Samson, 2000). Já o sistema NER (*nucleotide excision repair*) é a via principal pela qual as células removem adutos que distorcem a estrutura helicoidal do DNA

(Friedberg *et al*., 1995). Além disso, o sistema MMR (*mismatch repair*) corrige erros criados durante a replicação do DNA, tais como inserções, deleções e/ou substituições de bases (Harfe & Jinks-Robertson, 2000). Os sistemas de reparo de recombinação homóloga (*RR*) e de síntese translesão (*TLS*) também proporcionam rotas alternativas pelas quais as células podem continuar sua replicação apesar da presença dos adutos. Os sistemas *RR* e *TLS* são freqüentemente considerados como vias de tolerância aos danos no DNA, uma vez que permitem que as células completem sua replicação e mitose às expensas de freqüências maiores de mutação e de recombinação gênica (Doetsch *et al*., 2001).

5.4. Toxicidade da Cisplatina

A cisplatina é altamente tóxica e sua aplicação é limitada por seus severos efeitos colaterais, que incluem a citotoxicidade (Blasiak *et al*., 1999), nefrotoxicidade (Goldstein & Mayor, 1983), ototoxicidade e perda auditiva (Vermorken *et al*., 1983), neurotoxicidade (Schattschneider *et al*., 2001), mielotoxicidade (Badari-Osama *et al*., 2000), mielosupressão e anemia (Canaparo *et al*., 2000), além de graves efeitos sobre o trato gastrintestinal (Hidaka *et al*., 1995).

Estudos *in vitro* têm demonstrado também que a cisplatina apresenta uma forte interação com as cabeças polares dos fosfolipídios da bicamada lipídica das biomembranas. Aparentemente a cisplatina não interage de forma a causar o rompimento da bicamada lipídica, mas ocasiona danos aos canais iônicos da biomembrana, resultando em aumento da condutividade iônica que pode ser parcialmente responsável por seus efeitos adversos (Maheswari *et al*., 2000).

Além de seus efeitos colaterais, a cisplatina apresenta também atividade mutagênica (Turnbull *et al*., 1979), carcinogênica (Leopold *et al*., 1979) e teratogênica (Mazur *et al*., 2000). *In vitro* a cisplatina acarreta mutagenicidade em bactérias (Bristol-Myers Squibb, 1999), como também produz aberrações cromossômicas em células animais em cultura (Van Den Berg & Roberts, 1975; O'Neill *et al*., 1977; Turnbull *et al*., 1979; Bristol-Myers Oncology, 1993). *In vivo* a cisplatina demonstra ser também carcinogênica, como em camundongos e ratos (Leopold *et al*., 1979), além de teratogênica e embriotóxica em ratos (Bristol-Myers Squibb, 1999). Assim, estudos sobre o seu potencial carcinogênico sugerem que este composto químico é um carcinógeno humano em potencial, além da existência de um elevado risco de indução de tumores secundários em pacientes que sobreviveram por longos períodos após o tratamento quimioterápico com cisplatina (Turnbull *et al*., 1979; Wienke *et al*., 1979). Contudo, em seres humanos, uma relação direta entre a utilização de cisplatina e o aumento no risco de desenvolvimento de tumores secundários após a quimioterapia, não foi ainda completamente estabelecida (Johnson *et al*., 1980; Mead *et al*., 1983; Van Imhoff *et al*., 1986; Chambers *et al*., 1989; Osanto *et al*., 1991; Greene, 1992; Zoller *et al*., 1994; Schneider *et al*., 1999).

Tendo em vista os fatos descritos acima e devido às fortes evidências de que a cisplatina pode induzir carcinogênese em células, tecidos e órgãos animais, existe a necessidade do potencial tumorigênico da cisplatina ser investigado em diferentes modelos de estudo para um melhor entendimento de seus efeitos nas propriedades de diferentes tipos celulares.

6. Células Vero como Modelo de Estudo

A linhagem Vero, constituída por células não tumorais, de morfologia semelhante a fibroblastos, isoladas a partir de rim de macaco verde da África (*Cercopithecus aethiops*) foi utilizada neste trabalho, por ser recomendada para estudos envolvendo citotoxicidade (ISO 10993-5, 1992). As células Vero têm sido utilizadas para estudos de transformação celular, induzida por diferentes tratamentos, no Laboratório de Cultura de Células do Departamento de Biologia Celular da Universidade Estadual de Campinas (Genari & Wada, 1995; Genari *et al*., 1996; 1998; Carvalho *et al*., 1999), sendo um ótimo modelo de estudo para a carcinogênese experimental, devido as suas propriedades de crescimento e comportamento em cultura. Como as células Vero apresentam características de crescimento em cultura bastante definidas, pequenas alterações em função de tratamentos por compostos químicos poderão facilmente ser observadas através de modificações nas suas propriedades de crescimento e características morfológicas.

7. Células V79 como Modelo de Estudo

A linhagem V79, desenvolvida a partir de células pulmonares não tumorais provenientes de um macho jovem da espécie *Cricetulus griseus* (Hamster chinês), tem sido utilizada em estudos de transformação celular e em ensaios citogenéticos no Laboratório de Cultura de Células do Departamento de Biologia Celular da Universidade Estadual de Campinas (Carvalho *et al*., 1999; Petinari *et al*., 2004). As células V79 correspondem a um modelo excelente para estudos citogenéticos com a utilização de compostos químicos, já que esta linhagem fibroblástica diplóide apresenta cariótipo e características citogenéticas altamente estáveis (Bradley *et al*., 1981).

B. OBJETIVOS

Objetivo Geral:

O objetivo geral deste trabalho foi investigar os efeitos da cisplatina, agente quimioterápico utilizado no tratamento de tumores sólidos e de outras neoplasias, utilizando-se cultura das linhagens estabelecidas Vero e V79. Para tal foram investigadas diferentes propriedades morfológicas, comportamentais e citogenéticas, utilizando culturas tratadas pela cisplatina e não tratadas como controle.

Objetivos Específicos:

1 – Observar alterações morfológicas em células Vero, através de microscopia invertida de contraste de fase.

2 – Analisar alterações no padrão de crescimento e proliferação celular em células Vero, através da obtenção de curvas de crescimento.

3 – Investigar a viabilidade celular em células Vero, através de testes de citotoxicidade.

4 – Estudar alterações na adesão celular em células Vero, através da obtenção de curvas de adesão.

5 – Avaliar alterações morfológicas ultra-estruturais em células Vero, utilizando Microscopia Eletrônica de Varredura.

6 – Analisar componentes da matriz extracelular e do citoesqueleto de células Vero, através de marcador imunocitoquímico (fibronectina) e de coloração com faloidina (actina).

7 – Efetuar análise citogenética em células Vero e em células V79, através da obtenção dos índices mitóticos, das freqüências de poliploidia e do número modal de cromossomos.

C. ORGANIZAÇÃO DA DISSERTAÇÃO

A presente dissertação de doutorado encontra-se estruturada em 6 Capítulos, incluindo este.

Os capítulos 2, 3 e 4 apresentam os resultados dos estudos sob a forma de artigos, sendo o artigo referente ao Capítulo 2 submetido ao periódico *Cell Biology International*, enquanto os demais serão submetidos à publicação.

O Capítulo 5 apresenta as Conclusões Gerais do trabalho e o Capítulo 6, as Referências Bibliográficas referentes ao Capítulo 1 – Introdução.

Em anexo encontram-se os artigos: Artigo I "Morphological characterization of a human glioma cell line"; Artigo II "TEL, a plant lectin, induces apoptosis in cultured mammalian cell line"; Artigo III "*Talisia esculenta* lectin (TEL) induces cytogenetic and morphological alterations in Vero cells", que referem-se a artigos de co-autoria que foram provenientes de estudos paralelos desenvolvidos ao longo das atividades de doutorado.

IV. CAPÍTULO 2

Morphological and growth alterations in Vero cells transformed by cisplatin.

Artigo submetido para publicação no periódico *Cell Biology International*.

Morphological and growth alterations in Vero cells transformed by cisplatin

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Abstract

Cisplatin is an antineoplastic agent used to treat solid tumours, such as ovarian, testicular and bladder tumours. However, studies *in vitro* and *in vivo* have shown that cisplatin is mutagenic, genotoxic and tumourigenic in other tissues and organs. In this work, we examined the effect of cisplatin on Vero cells, a fibroblast-like cell line. The morphological characteristics were investigated using phase contrast microscopy, scanning electron microscopy and the actin cytoskeleton was labeling with fluorescein isothiocyanate-phalloidin. Cell proliferation was assessed based on the growth curve. Cultured Vero cells treated with cisplatin showed behavioural and morphological alterations associated with cellular transformation. The transformed cells grew in multilayers and formed cellular aggregates. The proliferation and morphological characteristics of the transformed cells were very different from those of control ones. Since transformed Vero cells showed several characteristics related with neoplastic growth, these cells could be a useful model for studying tumour cells *in vitro*.

Keywords: Cellular transformation; Cisplatin; *In vitro* carcinogenesis; Vero cells.

1. Introduction

Cisplatin is an antineoplastic agent that has been used since the 1970s (Rosenberg *et al*., 1969; Rozencweig *et al*., 1977) and was tested in clinical trials in 1971 and 1972. In 1979, cisplatin was approved by the FDA to treat testicular, ovarian and bladder tumours (FDA, 1979) and has been used in the chemotherapy of solid malignancies and other neoplasias (Philip *et al*., 1988; Swenerton *et al*., 1992). However, cisplatin is a highly toxic drug and its usefulness is limited by several adverse reactions (Badari-Osama *et al*., 2000; Blachley and Hill, 1981; Blasiak *et al*., 1999; Canaparo *et al*., 2000; Conklin-Kenneth, 2000; Goldstein and Mayor, 1983; Hidaka *et al*., 1995; Lieberthal *et al*., 1996; Schattschneider *et al*., 2001; Vermorken *et al*., 1983). In addition, several studies have shown that cisplatin is mutagenic (O`Neill *et al*., 1977; Turnbull *et al.*, 1979; Wiencke *et al*., 1979), genotoxic (Srb and Prochazkova, 1983; Tandon and Sodhi, 1985; Tominaga *et al*., 1986; Van den Berg and Roberts, 1975) and tumourigenic (Leopold *et al*., 1979) in human and animal cells in culture. *In vivo*, cisplatin is carcinogenic in mice and rats (Bristol-Myers Squibb, 1999). However, in humans, a direct relationship between the use of cisplatin and an increased risk for secondary tumours has not been completely established (Chambers *et al*., 1989; Greene, 1992; Johnson *et al*., 1980; Mead *et al*., 1983; Osanto *et al*., 1991; Schneider *et al*., 1999; Van Imhoff *et al*., 1986; Zoller *et al*., 1994).

Carcinogenesis is a multiple-step process involving serial genetic modifications in the target cell, that lead to alterations in growth control and, consequently, to the formation of malignancies. The study of carcinogenesis process has been greatly facilitated by the standardization of assays for assessing morphological transformation *in vitro* and neoplastic transformation *in vivo* (IARC, 1985). The process of mammalian cell transformation has

been extensively studied. Many studies have examined how tumours originate and progress in animals (IARC, 1985; Pónten, 1976; Terzaghi-Howe, 1993; Zhu and Gooderham, 2002) and have elucidated the signalling pathways involved in cellular transformation (Glück *et al*., 1993; Otto *et al*., 2002; Sattler and Salgia 2004; Vara *et al*., 2004; Yuhong *et al*., 2002). Since cisplatin can induce carcinogenesis in animal cells and organs, an understading of its effects in differents models is essential.

The Vero cell line, an immortal, non-tumourigenic fibroblastic cell line established from kidney cells of the African green monkey (*Cercopithecus aethiops*), has a characteristic growth pattern in culture (Bianchi and Ayres, 1971) and have been used to study cell growth, differentiation (Genari *et al*., 1996; Haas *et al*., 2001; Lombello *et al*., 2000; Santos and Wada, 1999; Santos *et al*., 2001, 2003), cytotoxicity (Estacia *et al*., 2002; Malmonge *et al*., 1999) and cell transformation induced by different agents or conditions (Carvalho *et al.*, 1999; Genari and Wada, 1995; Genari *et al*., 1996; 1998). These cells are recommended for such investigations in standard protocols (ISO 10993-5, 1992-E) and provide an excellent model for studying carcinogenesis *in vitro* because of their characteristic growth pattern and behaviour in culture. Since Vero cells have very well-defined properties, subtle alterations in their growth and morphology induced by chemical carcinogens are easily observed.

In this study, we used phase contrast microscopy, scanning electron microscopy, fluorescein isothiocyanate-phalloidin (actin cytoskeleton labeling) and growth curves to investigate the morphology and growth characteristics of Vero cells treated with cisplatin compared to control Vero cells.

2. Materials and Methods

2.1. Control Culture Conditions

Vero cells, obtained from the Adolfo Lutz Institute, São Paulo, SP, Brazil, at passage 199, were maintained in Ham F10 medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 5% of fetal calf serum (FCS - Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. The medium was renewed at 48 h intervals, and the cells were always subcultured when the monolayers become confluent.

2.2. Cytotoxicity Assay

The cytotoxicity of cisplatin on Vero cells was assessed using the crystal violet method (CVM) described by Murakami *et al*. (1998) and the MTT (bromet 3-(4,5-dimetiltiazol-2-yl)-2,5-difenil tetrazolium) reduction assay described by Mosmann (1983).

In the first protocol (CVM), Vero cell suspensions containing $1.5x10^5$ cells/ml in 100 μl of Ham F10 medium (Sigma) supplemented with 5% of FCS (Nutricell) were transferred to the wells of a 96-well culture plate (Iwaki SciTech Div., Asahi Techno Glass, Japan) and cultured for 24 and 48 h at 37°C (control cells). Treated cells were incubated with 25, 50, 100 or 200 μg of cisplatin/ml (Sigma) (Rao *et al.*, 1998) and were grown under the same conditions as non-treated (control) cells. The cisplatin solutions were prepared in complete medium at the desired concentrations (Allavena *et al.*, 1990). After incubation, the culture medium was removed and the cells were washed with 0.1 ml of 0.1 M

phosphate-buffered saline (PBS - Nutricell), pH 7.4 at 37°C, then fixed in 10% formalin (Labsynth, Diadema, SP, Brazil), washed in PBS and stained with 0.05% crystal violet (Riedel-de Haën, Hannover, Germany) in 20% methanol (Merck KgaA, Darmstadt, Germany). The cells were then washed twice in PBS and incubated in 0.1 M sodium citrate, pH 4.2, in 50% ethanol (Chemco, Campinas, SP, Brazil) for 30 min. The plate with the remaining viable cells was read at 540 nm in a microplate reader (Bio-Rad, Philadelphia, PA, USA).

In the second protocol used (MTT reduction), the cells were treated and incubated as described above for the CVM. After incubation, the culture medium containing cisplatin was removed and the cells were washed with 0.1 ml of 0.1 M PBS, pH 7.4 at 37°C, followed by the addition of 100 μl of MTT (Sigma) (5 μg/ml in Ham F-10 medium) and incubation for 2 h at 37°C. The cells were then treated with isopropanol (Isofar, Jacaré, RJ, Brazil). The plate with the remaining viable cells was read at 540 nm as described above.

A total of 12 samples for each concentration of cisplatin and control, were used in three separate experiments for each protocol. The results were expressed as the mean ± standard deviation.

2.3. Morphological and Growth Characteristics (Phase Contrast Microscopy)

Vero cells at passage 199 were seeded at a density of $1.5x10^5$ cells in culture flasks (Iwaki) and then incubated with cisplatin (50 μg/ml). This concentration of cisplatin was chosen based on the results of the cytotoxicity assays and was prepared as indicated above (Allavena *et al.*, 1990). After 24 h, the culture medium with cisplatin was removed and the

treated cells were then maintained in control culture conditions. The morphological and growth characteristics were assessed by daily observation of the cultures using an inverted phase contrast microscope (Olympus IX50 with a PMC35Dx photo micrograph system) during 10 successive subcultures. After this period, the Vero cells showed behavioural and morphological alterations characteristic of transformed cells. This assay was done in triplicate using non-treated Vero cells as a control.

2.4. Actin cytoskeleton labeling

After 10 successive passages during which treated Vero cells maintained their transformed phenotype, control and transformed Vero (VT) cells were seeded at $5x10^4$ cells/ml in Ham F-10 medium supplemented with 5% of FCS. After a 72 h incubation, the culture medium was removed and the cells were washed with PBS at 37°C. The material was fixed in 1% glutaraldehyde (Sigma) for 60 min and permeabilized in 0.2% Triton X-100 (Sigma) for 30 min. After three washes in PBS, coverslips were incubated with a 5 µg/ml fluorescent phalloidin conjugate (Phalloidin-FITC, Sigma) solution in PBS for 1 h to specifically stain filamentous actin. The cells were observed with a Nikon Eclipse E800 microscope (Japan) equipped with a filter set for fluorescein. This experiment was done in triplicate.

2.5. Scanning Electron Microscopy (SEM)

Transformed and control Vero cells were seeded in a 6-well culture plate (Iwaki)

provided with coverslips (Glasstécnica, São Paulo, SP, Brazil), at a density of $75x10^5$ cells/ml and cultured under normal conditions for 24 and 72 h. The cells were subsequently fixed in 2.5% glutaraldehyde (Sigma) and post-fixed in 1% osmium tetroxide (Sigma), dehydrated in a graded ethanol series (Labsynth), critical point dried (Balzers CDT 030) and coated with gold in a sputter coater (Balzers CDT 050). The cells were examined with a JEOL JSM 5800 LV scanning electron microscope. These experiments were done in triplicate.

2.6. Growth Curve

Control and transformed Vero cells were seeded at a density of $25x10^3$ cells/ml in culture tubes containing 2 ml of Ham F10 medium supplemented with 5% FCS, and grown under normal conditions for 1 to 21 days. At selected times, the number of viable cells was counted in a hemocytometer chamber and the inviable cells were excluded based on the uptake of trypan blue. The growth curves obtained were the average of triplicate samples for each time interval. The results were expressed as the mean ± standard deviation.

2.7. Statistical Analysis

The results of the cytotoxicity assays and the growth curve were analyzed using one-way ANOVA (Johnson and Wichern, 1988), with a significance level of 5% ($p<0.05$).

3. Results

3.1. Cytotoxicity Assay

Figs. 1 and 2 show that the two methods used to study the cytotoxicity of cisplatin on Vero cells gave similar results. At the concentrations used, cisplatin was not cytotoxic to Vero cells over the time periods analyzed. However, significant differences were observed between control and cisplatin-treated cells after 24 h of incubation (Figs. 1A and 2A). These differences problably reflect a transient proliferative effect of cisplatin.

3.2. Morphological and Growth Characteristics (Phase Constrast Microscopy)

The control cells grew in a monolayer until confluence and showed the typical elongated shape of fibroblast-like cells, with little cytoplasmic granulation (Figs. 3A and 3B). After reaching confluence, the cells stopped dividing and died. In contrast, after confluence, transformed Vero cells (VT) grew in multilayers and formed cellular aggregates, indicating a loss of contact inhibition and anchorage-independent growth (Figs. 3H to 3K).

After exposure to cisplatin, the treated cells began to show changes in their morphological characteristics and structural alterations was observed, including some aberrations (Figs. 3C to 3G). During this incubation, a number of cells also detached from the culture flasks and died. The remaining cells grew slowly and, when confluency was achived, the behavioural and morphological alterations were similar to those associated with cellular transformation (Figs. 3J and 3K). From this point onwards (3rd subculture)

until the 10th subculture, these characteristics were maintained, indicating that the cells had been transformed.

3.3. Actin Cytoskeleton Labeling

Fluorescein isothiocyanate-phalloidin revealed thin actin filaments filling the cytoplasm in control cells. Stress fibers and organized actin filaments were observed at the cell periphery (Figs. 4A, 4C and 4E). VT cells showed more irregular contours along their borders and actin was observed close to the cell periphery in the form of stress fibers. In these cells, the morphology was irregular and showed organized actin filaments in the cytoplasm (Fig. 4B, 4D and 4F).

3.4. Scanning Electron Microscopy (SEM)

After 24 h in culture, control cells formed a semi-confluent monolayer of elongated, regular-shaped, flattened cells with some vesicles and microvilli on their surface (Figs. 5A, 5C, 5E and 5G); these cells showed contact inhibition and anchorange-dependent growth. At semiconfluency, VT cells were very irregular and the borders of the cells had numerous cytoplasmic prolongations (Figs. 5B, 5D, 5F and 5H).

Control cells were confluent at 72 h and formed a monolayer with further growth being prevented by contact inhibition (Figs. 6A, 6C, 6E and 6G). In contrast, VT cells initially grew as a monolayer but, after 72 h (at confluence), some regions of the monolayer grew as multilayers and formed cellular aggregates (Fig. 6B, 6D, 6F and 6H), that grew

independently in an uncontrolled manner. In addition, cells in aggregate showed an increase in the number of microvilli and in the formation of little vesicles on their surface (Fig. 6H).

3.5. Growth Curve

The growth curves of control and transformed Vero cells are shown in Fig. 7. The growth of control and VT cells was very similar at the beginning of the proliferative phase. However, from day 7 until day 13, VT cells grew faster than control cells. At the end of the proliferative phase, the control Vero cells reached senescence, stopped dividing and died. In contrast, after 18 days, VT cells were still viable and showed stable growth.

4. Discussion

The precise mechanism of action of cisplatin has not been determined, but the drug has biochemical properties similar to those of alkylating agents (Huitema *et al.*, 2000; Roberts and Pascoe, 1972). Platinum-containing antineoplastic agents appear to exert their effects by binding to DNA, thereby inhibiting DNA synthesis (Drewinko and Gottlieb, 1975; Howle and Gale, 1970; Micetich *et al*., 1985). The cytotoxicity of cisplatin and its analogues has been shown to be a consequence of the DNA damage caused by the formation of platinum-DNA adducts (Reedjik, 1987). Cisplatin is not cycle-phase specific (Roberts and Pascoe, 1972) and the adducts may inhibit DNA replication, transcription and, ultimately, cell division (Weiss and Christian, 1993).

At the concentrations used, cisplatin was not cytotoxic to Vero cells during the time periods analyzed and a transient proliferative effect was observed at 24 h. This observation can be explained by the fact that after the DNA damage caused by the formation of cisplatin-DNA adducts, genotoxic stress induces numerous signaling pathways that can influence cell growth and cytotoxicity, including members of the MAP kinase pathways (Eastman, 1990). The MAP kinase pathways are parallel cascades of structurally related serine/threonine kinases that serve to integrate numerous extracellular signals, involved in the regulation of cell proliferation, differentiation and cell survival (Seger and Krebs, 1995; Treisman, 1996). The induction of MAP kinase activity after treatment with cisplatin, suggests that MAP kinase activation can partially protect cells from cisplatin cytotoxicity (Persons *et al*., 1999). However, as seen here, the apparent initial proliferative effect was short-lived and did not last longer than 24 h after the initial exposure to cisplatin.

After three successive subcultures, the treated Vero cells began to show changes in their morphological characteristics and structural alterations, including morphological aberrations, cell dethachment and death. These changes indicate a deleterious effect of cisplatin on Vero cells after an initial lack of cytotoxicity. The surviving cells grew very slowly and, when confluency was achived, cisplatin-treated cells showed behavioural and morphological alterations associated with cellular transformation, including growth in multilayers and the formation of cellular aggregates.

This abnormal cellular behaviour, known as *morphologic transformation* (Smets, 1980), occurs because of the loss of contact inhibition (Heidelberger and Iype, 1966), and is frequently observed in cells extracted from tumours and grown in culture (Bridges *et al*., 1991; Ebert *et al*., 1990; Persky *et al*., 1989; Sridhar *et al*., 1989) or in cells transformed by viruses, carcinogenic chemicals and other agents (Alitalo *et al*., 1982; Brown *et al*., 1986; Duesberg and Vogt, 1970; Zhu and Gooderham, 2002). Cell transformation *in vitro* can also be defined as the acquisition of permanent disturbances in the control of growth and/or locomotion (Pónten, 1976; Smets, 1980).

In mammalian cells, the process of transformation is characterized by the loss or down-regulation of tumour-suppressor genes and/or the mutation or overexpression of proto-oncogenes, the products of which promote uncontrolled proliferation of cells and extend their life span. Deregulation of intracellular transduction pathways generates mitogenic signals that promote abnormal cell growth and the acquisition of an altered phenotype (Danesi *et al*., 2003). An interesting example of these intracellular transduction pathways is the activation of two similar GTPases, H-Ras and R-Ras, that have differing effects on cellular phenotypes: activated H-Ras strongly transforms many fibroblast cell lines, causing dramatic changes in cell shape and cytoskeletal organization. In contrast, R-

Ras transforms fewer cell lines and the transformed cells display only some of the morphological changes associated with H-Ras transformation (Hansen *et al*., 2002).

The morphological characteristics of the control cells were typical of Vero cells, and included growth in monolayers, an elongated shape similar to fibroblast-like cells, and little cytoplasmic granulation (Genari and Wada, 1995; Janik and Greco, 1976; Leary and Blair, 1980). In contrast, the VT cells grew in multilayers and forming cellular aggregates. The cells in these aggregates were covered by a large number of microvilli and small vesicles. These structures are frequently observed in transformed cells (Genari *et al*., 1996; Petinari *et al*., 2004). The changes in surface and cellular shape are associated with increased intracellular protein production or the elimination of this material (Linstead *et al*., 1988). The vesicles seen in VT cells suggested the elimination of material to the extracellular environment whereas the microvilli appeared to be involved in cell-to-cell and cell-to-matrix binding (Takezawa *et al*., 1993). This *in vitro* growth appears to simulating the formation of miniature tumour colonies (Bridges *et al*., 1991; Genari *et al*., 1996; Persky *et al*., 1989; Sridhar *et al*., 1989).

Actin filaments are the main components of a large group of cytoskeletal structures. The analysis for actin indicated thin actin filaments filling the cytoplasm and stress fibers in control Vero cells, with the formation of actin filaments at the cell periphery. The organization of the actin cytoskeleton is frequently altered by cellular transformation. The ability to spread on the substrate is reduced in transformed cultures (Bershadsky and Vasiliev, 1988). Actin filaments were generally disrupted in the malignant transformed cells. The actin network is a complex and important structural and functional system that provides the basic infrastructure for maintaining cell morphology and functions such as adhesion, motility, exocytosis, endocytosis, and cell division. Actin polymerization and

actin remodeling play a pivotal role in regulating the morphological and phenotypical events of a malignant cell. Actin remodeling reflects the activation of oncogenic actin signaling pathways (e.g., Ras and Src), or the inactivation of several important actin-binding proteins that have tumour suppressor functions (e.g., gelsolin) (Rao and Li, 2004). Transformed Vero cells showed more irregular contours along their borders and actin was observed close to the cell periphery in the form of stress fibers. In these cells, the morphology was very irregular, indicating that polymerization of the the actin cytoskeleton and/or the activity of actin-binding proteins are compromised in cisplatin-transformed Vero cells.

The transformed and cancer cells, when compared with their genitive ancestral cells or their tissue of origin, may differ significantly in their growth curves. These cells have prolonged survival in low nutritional conditions and an accelerated growth index (Abercrombie, 1979; Golombick *et al*., 1990). As seen in the growth curves, control Vero cells stopped dividing upon reaching senescence and died. In contrast, VT cells had a higher growth rate in the proliferative phase and continued to proliferate, with the cells growing in multiple layers. After 18 days, VT cells were still viable, with remaining cells showing stable growth, whereas control cells died during this period. Thus, control Vero cells maintained the characteristics of contact inhibition whereas the transformed cells lost this characteristic (Heidelberger and Iype, 1966). A loss of contact inhibition has been observed in various transformed cell types after exposure to viruses, carcinogenic chemicals, radiation, and other factors (Abercrombie, 1979; Borek, 1972; Borek and Sachs, 1966; Genari and Wada, 1995; Petinari *et al*., 2004), and also in malignant cells obtained from tumours and maintained in culture (Bridges *et al*., 1991; Ebert *et al*., 1990; Persky *et al*., 1989; Sridhar *et al*., 1989).

In conclusion, the results described here indicate that the treatment of Vero cells with cisplatin resulted in changes in cell morphology and growth. The multiple-step nature of this process is characteristic of the development of neoplastic disease. Since transformed Vero cells showed several characteristics associated with neoplastic growth, these cells could be a useful model for studying tumour cells *in vitro*.

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Figure Legends

Fig. 1. Cytotoxicity of cisplatin in Vero cells after incubation for 24 h (A) and 48 h (B). Cytotoxicity was assessed by the crystal violet method. The experiments were done in triplicate as described in the Methods section. The columns represent the mean ± standard deviation. ***** $P<0.05$ compared to control Vero cells.

Fig. 2. Cytotoxicity of cisplatin in Vero cells after incubation for 24 h (A) and 48 h (B). Cytotoxicity was assessed by the MTT reduction method. The experiments were done in triplicate as described in the Methods section. The columns represent the mean ± standard deviation. ***** $P<0.05$ compared to control Vero cells.

Fig. 3. Phase contrast photomicrographs of control and cisplatin-treated Vero cells. (3A) and (3B): Control Vero cells; (3C) to (3K): Cisplatin-treated Vero cells. (3C) and (3D): Cell with multiple nucleous (arrow); (3E) to (3G): Cells with morphological aberrations (arrow) and (3H) to (3K): Cells with multilayered growth (*). (3A): 250X; (3B), (3D), (3E) and (3F): 700X; (3C), (3I) and (3K): 350X; (3G): 900X; (3H) and (3J): 140X.

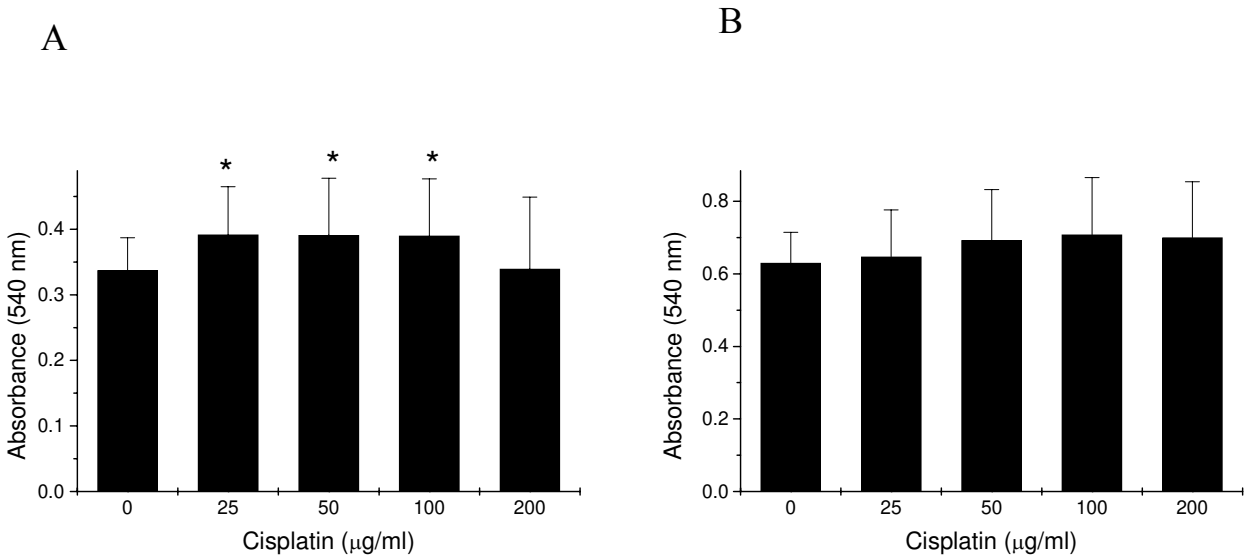
Fig. 4. Actin cytoskeleton labeling in control and cisplatin-transformed Vero cells after a 72 h incubation. (4A), (4C) and (4E): Control Vero cells showing actin filaments filling the cytoplasm. Stress fibers and organized actin filaments were observed at the cell periphery. (4B), (4D) and (4F): Cisplatin-transformed Vero cells showing irregular contours along their borders. Actin was observed as stress fibers close to the cell periphery. In these cells,

the morphology was irregular and showed organized actin filaments in the cytoplasm. (4A), (4C), (4D), (4E) and (4F): 1000X; (4B): 600X.

Fig. 5. Representative scanning electron micrographs of control and cisplatin-transformed Vero cells, at semiconfluency (24 h in culture). (5A), (5C), (5E) and (5G): Control Vero cells with some vesicles (ve) and microvilli (vi) on the cell surface; (5B), (5D), (5F) and (5H): Cisplatin-transformed Vero cells with a large number of cytoplasmic prolongations (cp), vesicles (ve) and microvilli (vi) on the cell surface. (A) and (B): 1000X; (C) to (G): 2500X; (H): 3000X.

Fig. 6. Representative scanning electron micrographs of control and cisplatin-transformed Vero cells, at confluency (72 h in culture). (6A), (6C), (6E) and (6G): Control Vero cells showing monolayer growth; (6B), (6D), (6F) and (6H): Cisplatin-transformed Vero cells, with cell aggregates (ca) and a large number of vesicles (ve) and microvilli (vi). (6A): 1500X, (6B): 1200X, (6C) and (6D): 1000X, (6E): 2500X, (6F): 2000X, (6G): 4000X, (6H): 4300X.

Fig. 7. Growth curves for control (VC) and cisplatin-transformed (VT) Vero cells in Ham F10 medium supplemented with 5% FCS. The transformed cells showed a higher growth rate than control cells from days 7 to 13. The points are the mean ± standard deviation of triplicate experiments done as described in the Methods section. * $P < 0.05$ compared to control Vero cells.


A
B
Absorbance (540 nm)
Cisplatin (μg/ml)
0
25
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200
*

Fig. 1

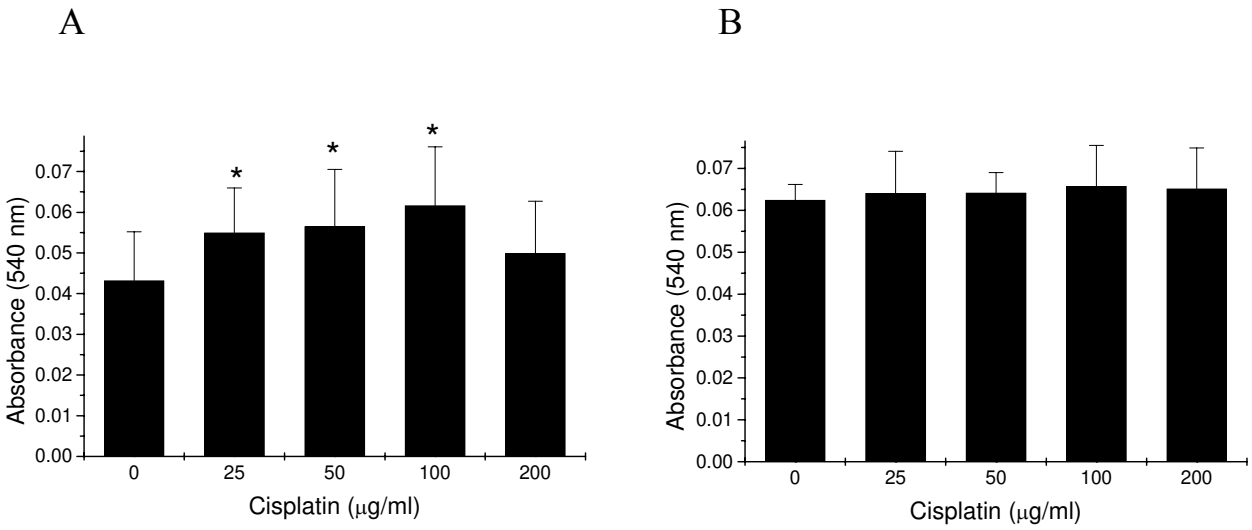
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Fig. 2

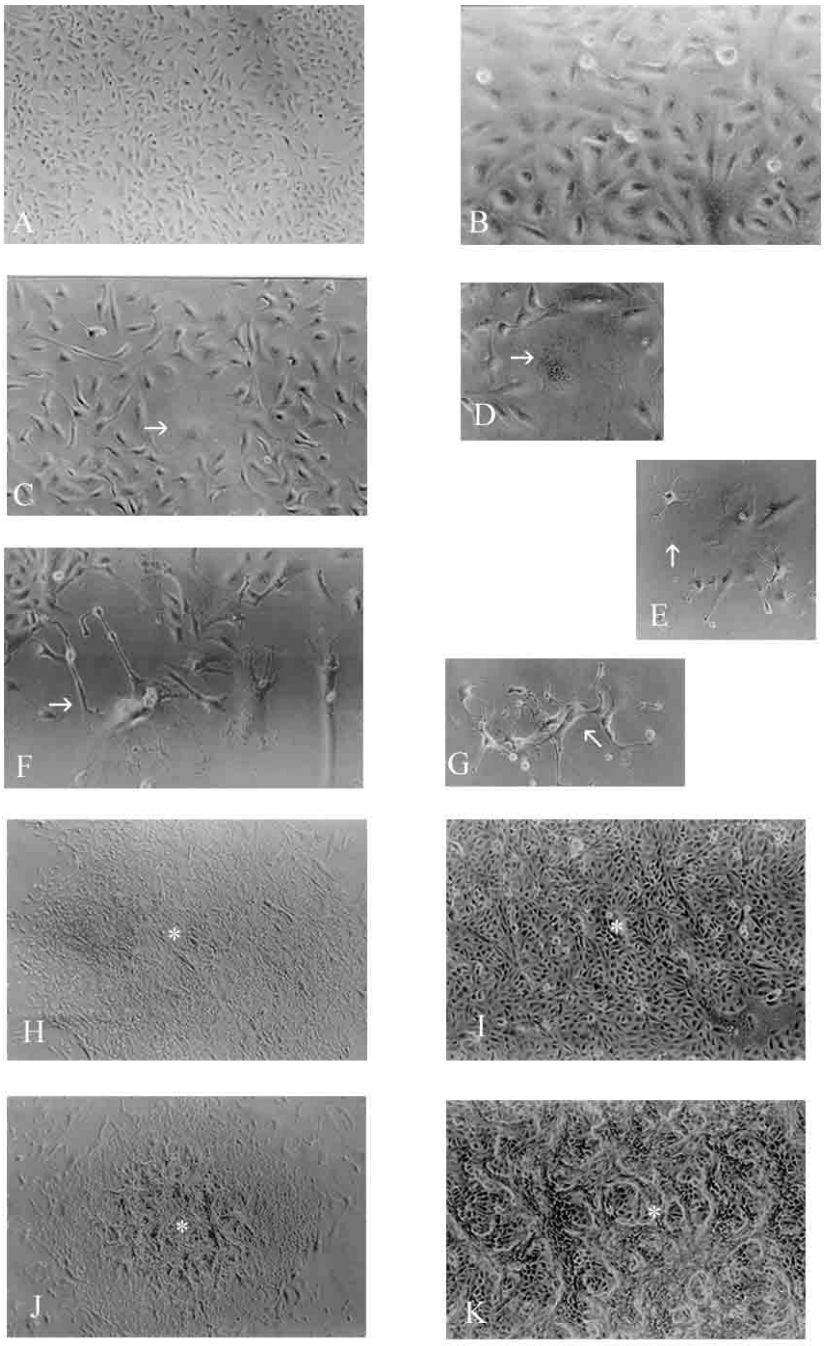

Fig. 3

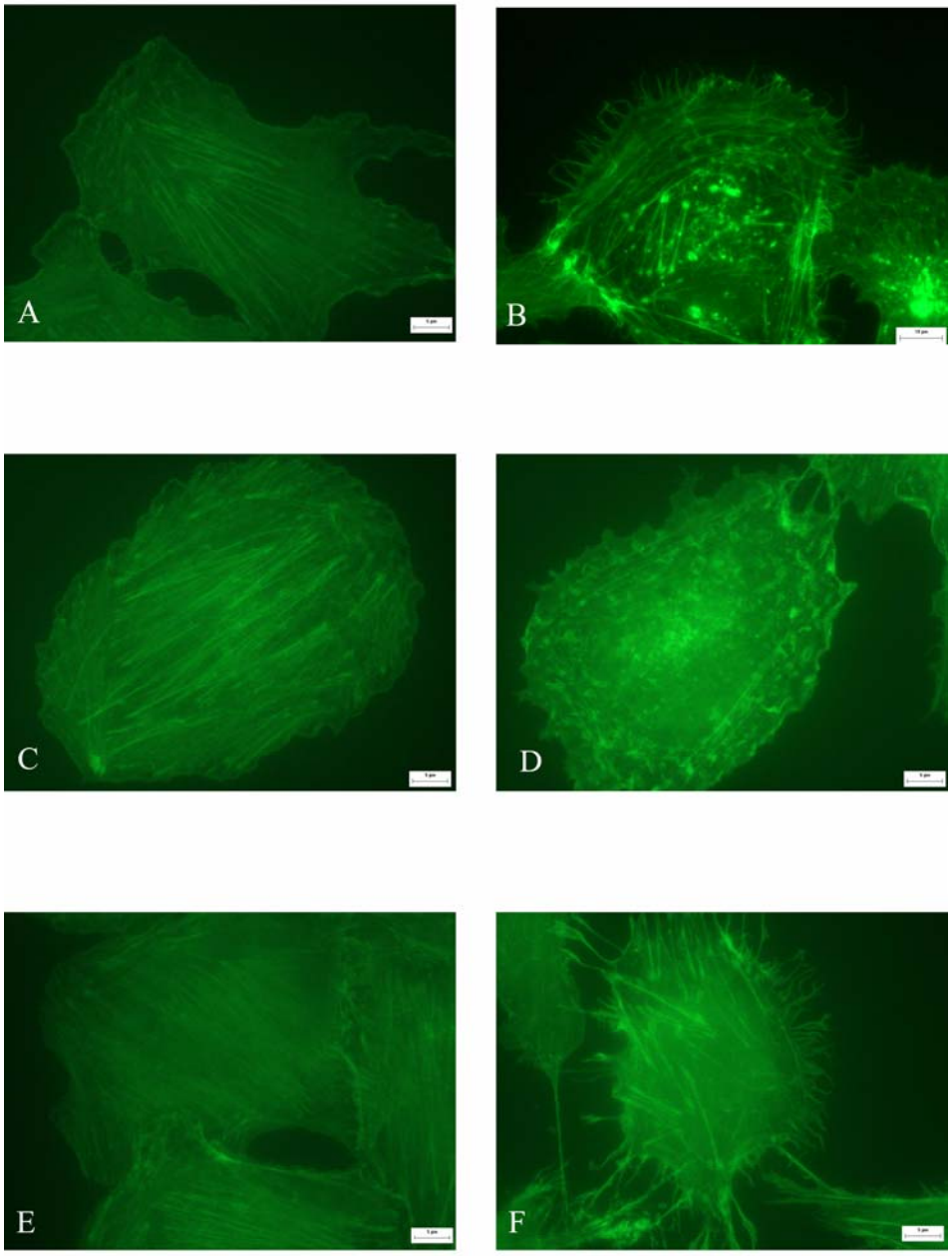

Fig. 4

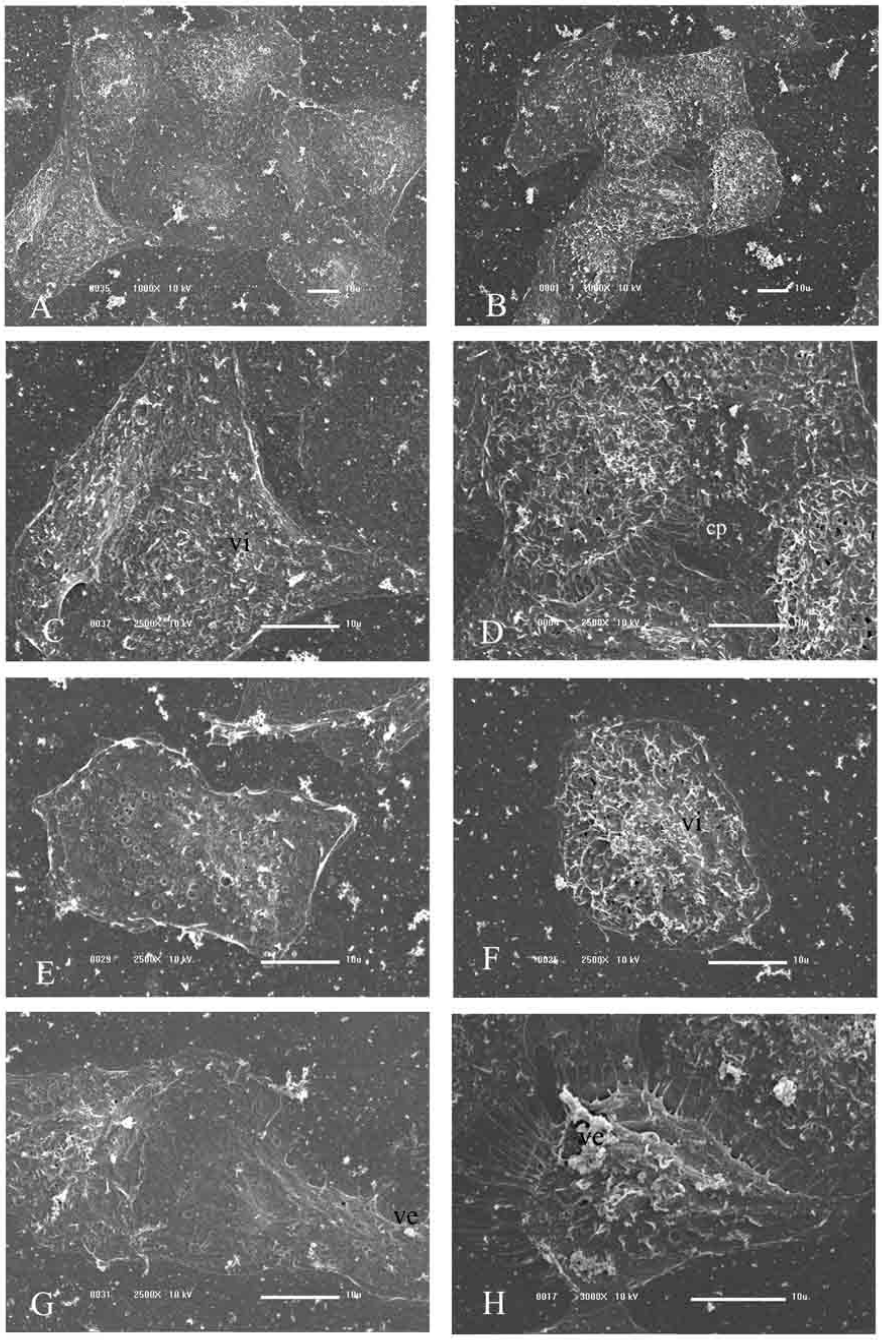

Fig. 5

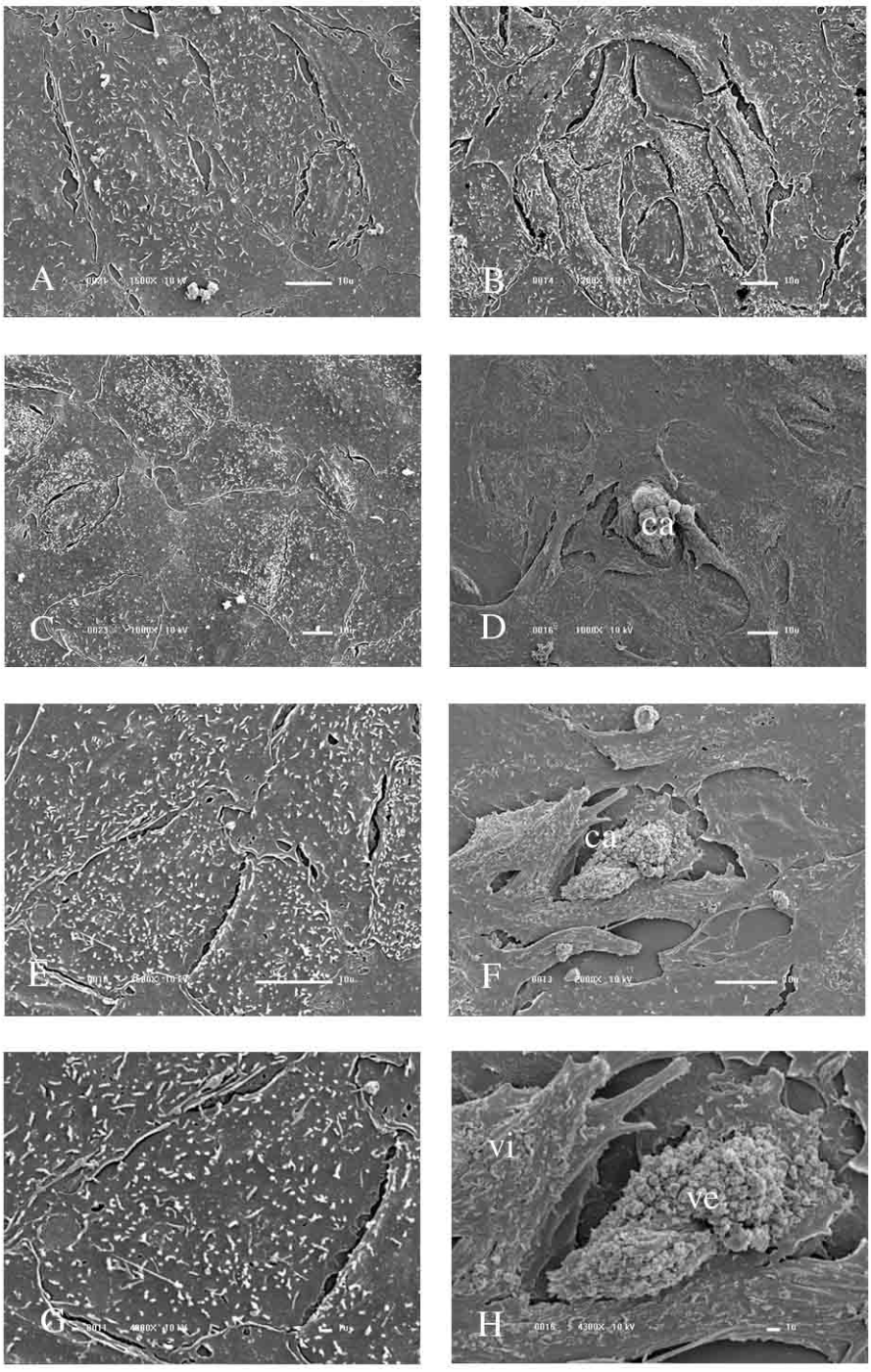

Fig. 6

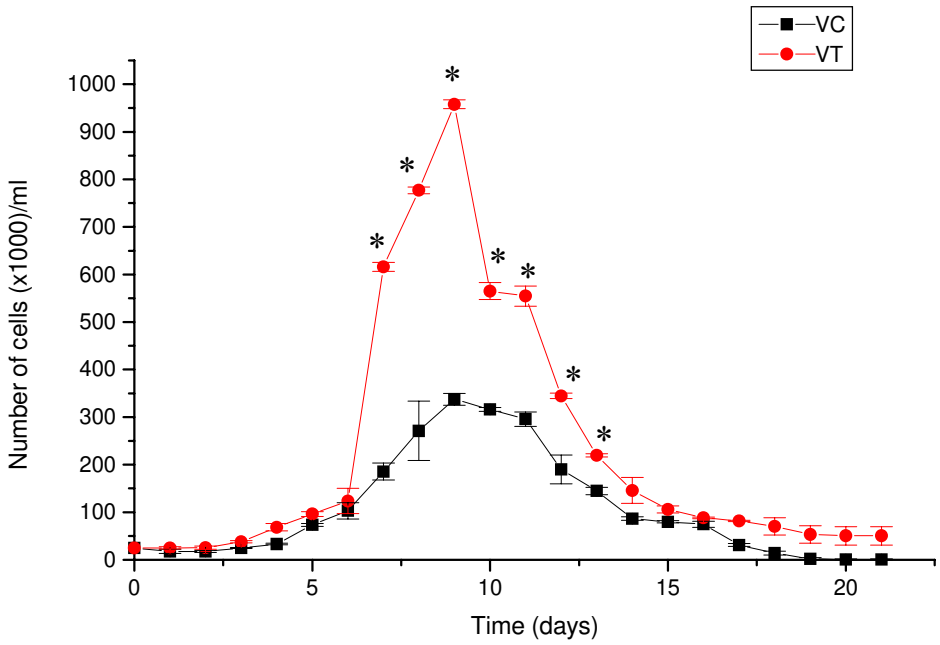
VC
VT
Number of cells (x1000)/ml
Time (days)
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Fig. 7

V. CAPÍTULO 3

Alterations in the pattern of adhesion and fibronectin deposition of Vero cells transformed by cisplatin

Artigo a ser submetido para publicação.

Alterations in the pattern of adhesion and fibronectin deposition of Vero cells transformed by cisplatin

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Running title: Adhesion and fibronectin deposition in cisplatin-transformed Vero cells

Keywords: Adhesion; Cellular transformation; Cisplatin; Fibronectin; Vero cells

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Original Submission

Abstract

Cisplatin is an antineoplastic agent used to treat solid malignancies, such as ovarian, testicular and bladder tumors. However, cisplatin can induce genotoxic effects and chromosomal alterations in non-tumoral cells that can result in mutagenesis and carcinogenesis. In this study, we investigated the effects of cisplatin on the cytogenetic and morphological characteristics and adhesion pattern of cultured Vero cells based on an analysis of the modal chromosome number, mitotic index, polyploidy frequency, fibronectin deposition and adhesion curves. The treatment of Vero cells with cisplatin resulted in characteristics typical of cellular transformation that included multilayered growth and the formation of cellular aggregates. The distribution of fibronectin and the pattern of adhesion were altered in transformed Vero cells. Control cells showed an accumulation of fibronectin in the regions of lateral cell-cell contact, whereas this pattern of accumulation was not seen in transformed cells. This alteration could contribute to the multilayered growth and the decresed adhesion in transformed Vero cells; these cells also showed an altered modal chromosome number and a higher mitotic index and polyploidy frequency. These changes in the cytogenetic characteristics of cisplatin-transformed Vero cells, including the altered morphology and adhesion, are characteristic of a transformed phenotype. These results indicate that cisplatin can transform Vero cells *in vitro* and that it is therefore a potential human carcinogen.

Introduction

Cisplatin is an antineoplastic agent with a broad range of activity against several human malignancies. Platinum-based chemotherapy, the standard treatment for women with advanced ovarian cancer, improves survival by 38% (Hershman *et al*., 2004) and, in ~75% of patients, complete responses are seen to initial treatment with cisplatin used either alone or in combination with other drugs (Kelland, 2005). Most patients (~75%) with metastatic testicular cancer are cured with cisplatin-based chemotherapy regimens (El-Helw and Coleman, 2004), and the combination of cisplatin plus fluorouracil achieves response rates of about 80% for advanced head and neck cancer (Argiris *et al*., 2005). In addition, cisplatin-based chemotherapy cures 40-60% of patients with metastatic bladder cancer (Sonpavde and Petrylak, 2005).

However, chemotherapeutic drugs can cause secondary malignant neoplasms in long-term survivors of chemotherapy. Alkylating agents, procarbazine and anthracyclins are chemotherapeutic drugs that have a high secondary malignancy risk for patients (Eser *et al*., 2004). Cisplatin is considered to be a DNA-damaging drug because of its ability to form different types of bifunctional adducts in its reaction with cellular DNA, thereby producing intrastrand and interstrand DNA cross-links in a manner similar to that of bifunctional alkylating agents (Reedjik, 1987; Sedletska *et al*., 2005). Cisplatin-associated secondary leukemia after the treatment of osteosarcoma has been reported in children (Escudero *et al*., 2004) and persistent chromosomal aberrations have been reported in peripheral blood lymphocytes of testicular cancer patients treated with this drug (Gundy *et al*., 1990). The treatment of testicular and ovarian cancer is associated with an increased risk of leukemia, with evidence for dose-response relationships in cisplatin-based chemotherapy (Travis *et al*., 1999; 2000). Cisplatin-based chemotherapy to treat ovarian

carcinoma has been also related to secondary non-Hodgkin's lymphoma (Eser *et al*., 2004). In addition, cisplatin has been related to mutagenesis and carcinogenesis *in vitro* and in laboratory animals (Chen *et al*., 2003; Khynriam and Prasad, 2003; Mimoto *et al*., 2000; Moggs *et al*., 1997).

Cultured fibroblasts can undergo cellular transformation involving a series of genetically stable modifications in their morphology and growth control, with such changes arising either spontaneously or following induction by chemical carcinogens or viruses (Pardinas *et al*., 1997; Rubin, 2001; Zhu and Gooderham, 2002). Cultured cells can therefore provide a useful model for studying the multiple processes involved in carcinogenesis.

In this work, we investigated the effects of cisplatin on the cytogenetic and morphological characteristics and adhesion pattern of Vero cells, a non-tumorigenic fibroblastic cell line, based on the modal chromosome number, mitotic index and polyploidy frequency, fibronectin deposition and adhesion pattern compared to control Vero cells.

Materials and Methods

Cell Culture Conditions

Vero cells, an immortal non-tumorigenic fibroblastic cell line established from African green monkey (*Cercopithecus aethiops*) kidney, obtained at passage number 199 from the Aldofo Lutz Institute (São Paulo, SP, Brazil), were cultured in Ham F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% fetal calf serum (Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C.

Treatment with Cisplatin

Vero cells at passage 199 were seeded at a density of 1.5 x 10^5 cells in culture flasks (Iwaki SciTech Div., Asahi Techno Glass, Japan) and incubated with 50 μg of cisplatin (Sigma)/ml, prepared in complete medium at the desired concentration (Allavena *et al.*, 1990). After 24 h, the culture medium containing cisplatin was removed and the treated cells were then maintained in control culture conditions (without cisplatin). After ten successive subcultures, the Vero cells showed growth and morphological alterations characteristic of transformed cells. This assay was done in triplicate using non-treated Vero cells as a control.

Cytogenetic Analysis

Control and transformed Vero cells were arrested in metaphase by adding 16 μg of colchicine (Sigma)/ml followed by incubation for 4 h at 37°C. The cells were then harvested using trypsin-EDTA (Nutricell) and isolated by centrifugation at 1000 rpm for 10

min. Chromosomal preparations were obtained by swelling the cells in 0.075 M KCl (Merck KgaA, Darmstadt, Germany) followed by fixation in methanol:acetic acid (3:1, v/v) (Merck). Slides were prepared according to standard techniques and stained with 5% Giemsa (Sigma) solution.

The modal chromosome number was determined by counting the chromosomes in 100 metaphases of intact control and transformed Vero cells (Genari and Wada, 2003), in the range of 50-60 chromosomes. The mitotic index (MI) and polyploidy frequency (PF) were determined according to Deitch and Sawicki (1979) and Gilvarry *et al*. (1990), respectively. The MI was calculated by counting the metaphases in 1000 cells and then dividing the number of metaphases by the total number of cells analyzed (MI [%] = number of metaphases/1000 x 100). The PF was determined by counting the polyploid metaphases in 1000 metaphases and then dividing the number of polyploid metaphases by the total number of metaphases analyzed (PF [%] = number of polyploid metaphases/1000 x 100).

Immunocytochemistry

Fibronectin was detected immunocytochemically in transformed and control Vero cells. The cells were seeded at a density of 5 x 10^4 cells/ml in Ham F-10 medium (Sigma) supplemented with 5% fetal calf serum (Nutricell). After 72 h, the culture medium was removed and the cells were washed with PBS (Nutricell), pH 7.4, at 37°C, and fixed in 1% glutaraldehyde (Sigma) for 60 min. After blocking nonspecific sites with 3% BSA (bovine serum albumin, Nutricell) for 1 h, the cells were incubated with the primary antibody (anti-cellular fibronectin, clone FN-3E2, from mouse ascites fluid, Sigma) which was subsequently visualized by incubation with a goat FITC-conjugated anti-mouse IgG

(Sigma). The material was observed with a Nikon Eclipse E800 microscope (Japan) equipped with a filter set for fluorescein. This experiment was done in triplicate.

Adhesion Assay

The modified method described by Murakami *et al*. (1998) was used to analyze the adhesion pattern. Transformed and control Vero cells (1.5×10^5 cells/ml, in 100 μl of Ham F10 medium supplemented with 5% fetal calf serum) were transferred 96-well culture plates (Iwaki) and incubated for 10, 20, 30, 40 and 60 min. After each period, the culture medium was removed and the cells were washed with 0.1 ml of 0.1 M PBS (Nutricell), pH 7.4 at 37°C, then fixed in 10% formalin (Labsynth, Diadema, SP, Brazil), washed in PBS (Nutricell) and stained with 0.05% crystal violet (Riedel-de Haën, Hannover, Germany) in 20% methanol (Merck). The cells were then washed twice in PBS (Nutricell) and incubated in 0.1 M sodium citrate in 50% ethanol (Chemco, Campinas, SP, Brazil), pH 4.2, for 30 min. The plate with the remaining adherent cells was read at 550 nm in a Fusion microplate reader (Version 3.02, Packard Bioscience Company) and the results (absorbance) were expressed as the mean ± standard deviation. This experiment was done in triplicate.

Statistical Analysis

The results of the adhesion assay were analyzed using one-way ANOVA with a value of $p < 0.05$ indicating significance.

Results

Cytogenetic Analysis

Control Vero cells had a modal chromosome number of 54 (range 50-60, n=100) whereas transformed Vero cells had a modal chromosome number of 56 (range 50-60, n=100) (Figure 1). Metaphases of control and transformed Vero cells are shown in Figures 2 and 3, respectively. The mitotic index (MI) and polyploidy frequency (PF) were increased in transformed Vero cells; the MI was 3.1% for control cells and 4.5% for transformed cells, while the PF for control and transformed Vero cells was 11% and 19.8%, respectively.

Immunocytochemistry

Control Vero cells showed a dispersed distribution of fibronectin on their surface, with a more concentrated distribution in some regions of contact between neighboring cells (Figure 4A and 4B). Transformed Vero cells did not have fibronectin between neighboring cells, although there was accumulation in specific areas, indicating an altered fibronectin distribution (Figure 4E and 4F). Control cells showed fibronectin accumulation between adjacent cell-cell contact regions (Figure 4C and 4D), whereas transformed cells had a diffuse localization of fibronectin (Figure 4G and 4H).

Adhesion Assay

The adhesion of control and transformed Vero cells is shown in Figure 5. Transformed cells had a significantly lower adhesion than control Vero cells after 60 min.

Discussion

There is considerable evidence that DNA is the principal intracellular target of cisplatin *in vivo*. The inhibition of cell division implies interference with DNA replication, but the concomitant cellular growth indicates that RNA and protein synthesis procede normally (Pinto and Lippard, 1985). In living cells, genomic DNA is constantly damaged by endogenous and exogenous factors, and DNA repair plays a very important role in correcting DNA damage and maintaining genetic fidelity and stability. One of the critical steps in DNA repair is the recognition of DNA damage. The recognition and signal of DNA damage will arrest the cell cycle, allowing DNA repair enzymes to be recruited and the damage to be repaired. If the damage is too severe to be repaired, the signal for DNA damage will activate a programmed cell death pathway that will eventually lead to apoptotic cell death. However, if the DNA damage is not recognized because of defects in the damage recognition mechanism, then cells will escape from the cell cycle checkpoint and enter the cell cycle without repairing the damaged DNA, an event that may enhance the accumulation of mutations. Mutations can lead to defects that give rise to many diseases and a high risk for disease progression, especially for cancer and age-related diseases (Chen *et al*., 2003).

In cellular transformation induced by carcinogens, morphological alterations are observed early after treatment with the chemical carcinogen and may be accompained by numerical and/or structural chromosomal alterations (Barrett, 1985). The modal chromosome number of 54 for control Vero cells may be regarded as the standard for these cells (Genari and Wada, 1995). Transformed Vero cells had extranumerary chromosomes, with a modal chromosome number of 56. The extra chromosomes may represent an amplification of the oncogenes associated with altered concentrations of proteins, and may

be partly responsible for the transformed phenotype (Brown *et al*., 1986). Heinen and Bassleer (1976) observed that chick embryo fibroblasts cultivated *in vitro* and treated with cisplatin were blocked just before entering mitosis (G2 block) and became polyploid. The polyploidy frequency increased in transformed Vero cells, with control and transformed Vero cells having frequencies of 11% and 19.8%, respectively. The mitotic index was 3.1% for control and 4.5% for transformed Vero cells. The higher polyploid frequency and mitotic index in cisplatin-transformed cells are also related to the altered proliferation and growth characteristics of these cells, including the formation of multiple layers and aggregates (Genari and Wada, 1995; Genari *et al*., 1996).

The phenotypic characteristics of transformed cells include stable phenotypic changes, morphological transformation and membrane alterations, such as the loss of extracellular glycoproteins like fibronectin (Smets, 1980). Morphological transformations include phenomena such as the loss of contact inhibition during movement and growth, as well as alterations in the adhesive properties (Smets, 1980). Normal fibroblasts require adhesion and spreading on the substrate for growth *in vitro*. Several studies have demonstrated the presence of altered proteins or new protein synthesis during carcinogenesis *in vivo* and during transformation *in vitro*. Proteins that are frequently altered in transformed cells include fibronectin, a plasma glycoprotein secreted by a variety of cell types, including fibroblasts, endothelial cells and hepatocytes (Grinmel and Bennett, 1981). The principal function of fibronectin is to mediate the adhesion of cells to extracellular material 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 is frequently 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 distribution of fibronectin in transformed Vero cells was altered compared to control Vero cells. Decreased spreading and attachment, accompanied by reduced adhesion, are frequently observed in transformed or tumorigenic cells, and are associated with a defective interaction and/or production of extracellular matrix elements (Grinmel & Bennett, 1981; Kleinman *et al*., 1981; Alitalo *et al*., 1982; Vasiliev, 1985).

In conclusion, the results of this study show that cisplatin induced the transformation of Vero cells, with the cells growing in multilayers and forming cellular aggregates indicative of a loss of contact inhibition during growth. Exposure to cisplatin resulted in intracellular changes that affected the chromosome number, mitotic index, polyploidy frequency and cell morphology and adhesion, all of which are characteristic of a neoplastic phenotype. These findings indicate that cisplatin is a cell-transforming agent and that it may be a potential human carcinogen.

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Figure legends

Figure 1. Modal chromosome number of control (VC) and transformed (VT) Vero cells. The modal chromosome number was determined by counting the chromosomes in 100 metaphases of intact control and transformed Vero cells, in the range of 50-60 chromosomes.

Figure 2. Representative metaphase of control Vero cells (1000X).

Figure 3. Representative metaphase of transformed Vero cells (1000X).

Figure 4. Immunocytochemical reaction for fibronectin. A to D: control Vero cells; E to H: transformed Vero cells. A: 600X; B to H: 1000X.

Figure 5. Adhesion assay using control (VC) and transformed (VT) Vero cells. Incubation times: 10, 20, 30, 40 and 60 min. Absorbance: λ = 550 nm. The results are expressed as the mean ± standard deviation (bars). *****$p<0.05$ compared to control Vero cells.

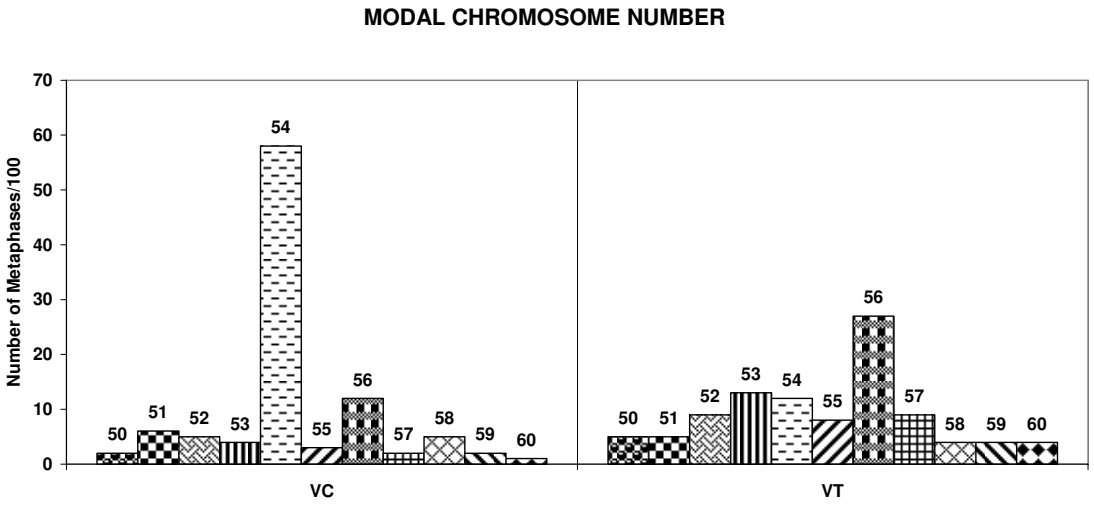

MODAL CHROMOSOME NUMBER
Number of Metaphases/100
70
60
50
40
30
20
10
0
50
51
52
53
54
55
56
57
58
59
60
VC
VT

Fig. 1

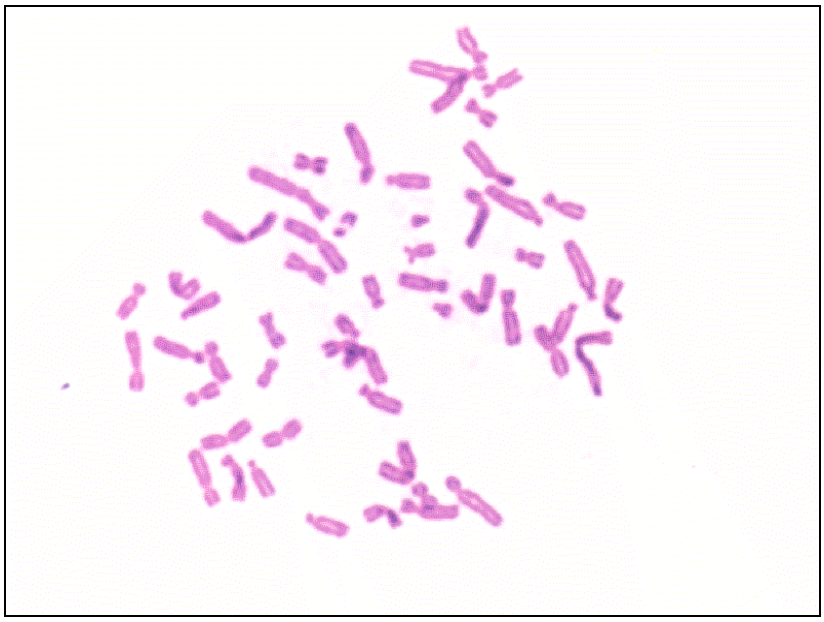


Fig. 2

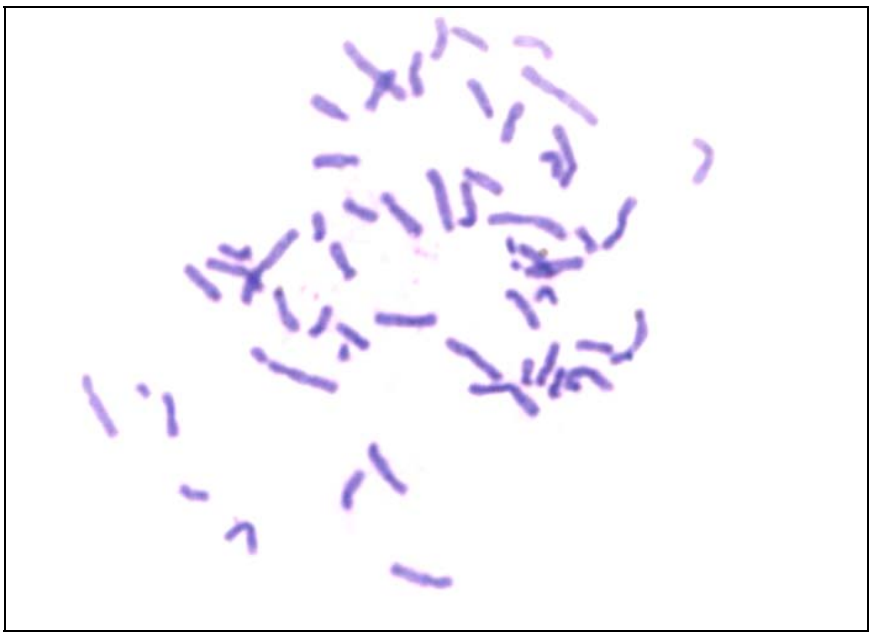


Fig. 3

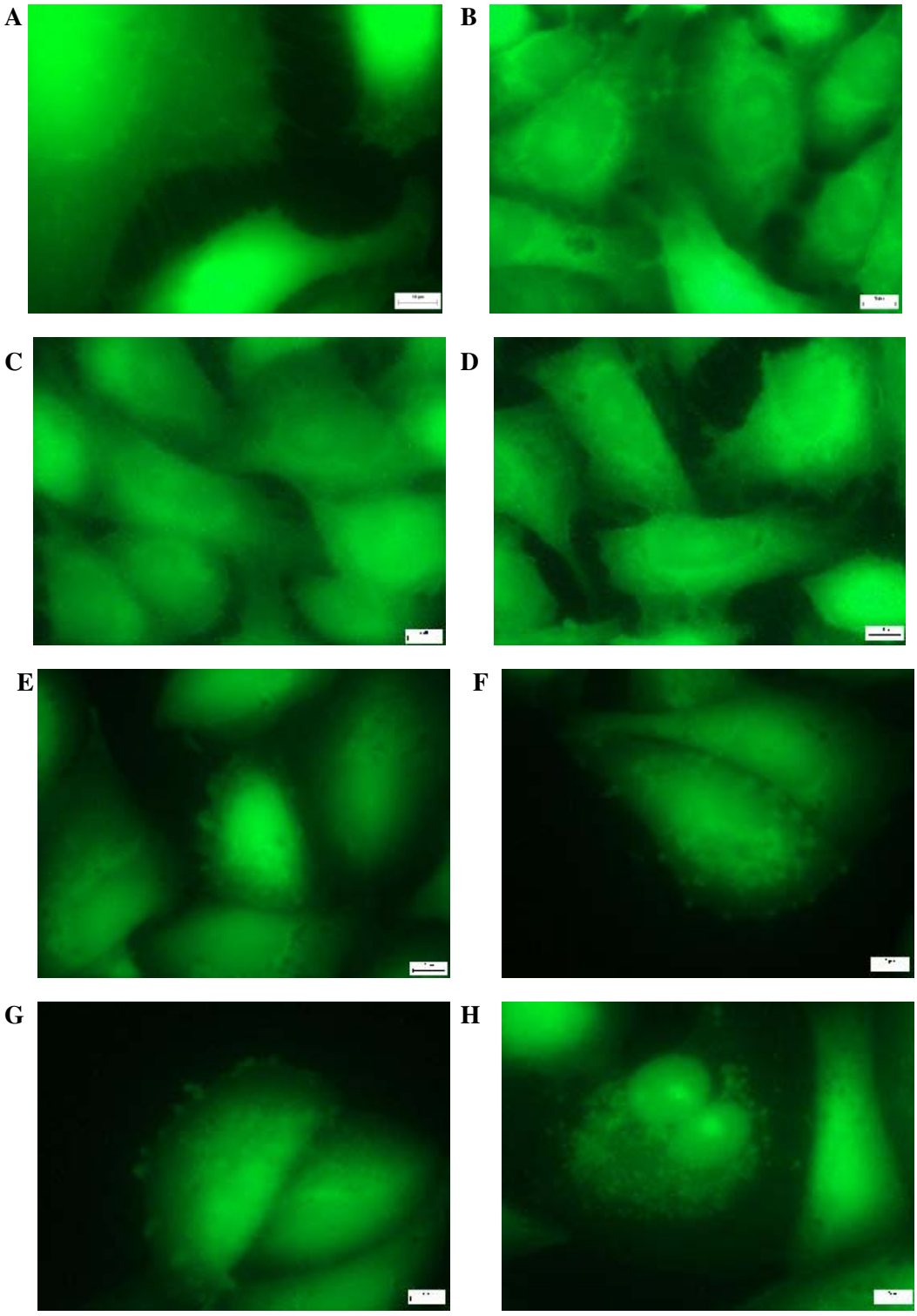
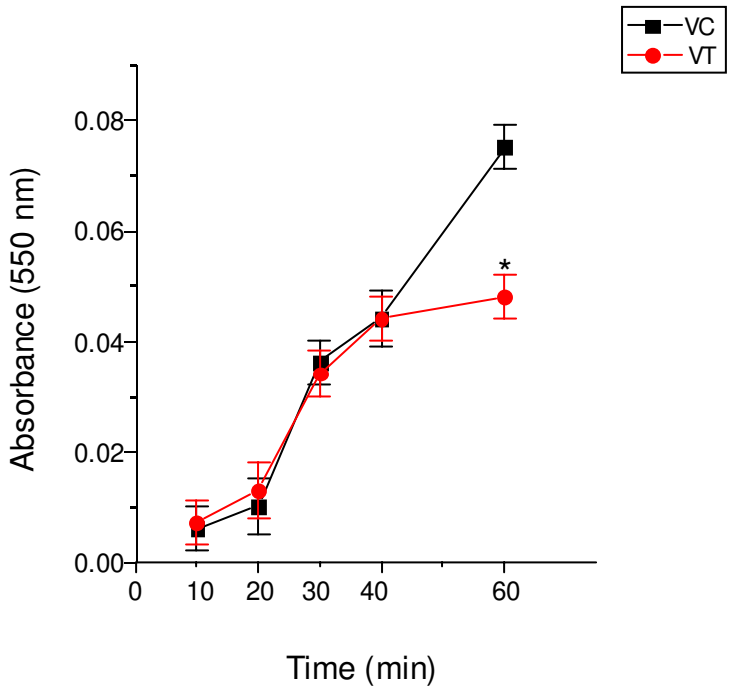


Fig. 4


VC
VT
Absorbance (550 nm)
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30
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60
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Time (min)

Fig. 5

VI. CAPÍTULO 4

Cisplatin-induced cytogenetic alterations in V79 cells.

Artigo a ser submetido para publicação.

Cisplatin-induced cytogenetic alterations in V79 cells

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Summary

Cisplatin is an antineoplastic agent used to treat solid malignancies, such as ovarian, testicular and bladder tumors. Studies have been shown that cisplatin induces genotoxic effects and chromosomal alterations that can result in genetic and chromosomal instability. In this work, we investigated the effects of cisplatin on the cytogenetic traits of cultured V79 cells based on the modal chromosome number, mitotic index, frequency of polyploidy and number of aneuploid metaphases. Cisplatin-treated V79 cells showed an altered chromosome number distribution, as well as an enhanced mitotic index, frequency of polyploidy and number of aneuploid metaphases. These alterations were probably related to the genetic instability produced by cisplatin. In addition, these cells showed characteristics associated with neoplastic development that corresponded to neoplastic processes related to the use of cisplatin in chemotherapy.

Key words: Cisplatin, Cytogenetic analyses, V79 cells.

Introduction

The antineoplastic activity of cisplatin was described in the late 1960s (Rosenberg *et al*. 1969) and resulted in its widespread use as a chemotherapeutic agent to treat a wide variety of neoplasias. Cisplatin is highly effective in the treatment of several types of tumors, including testicular, ovarian and bladder carcinomas, as well as head and neck, uterine cervix, esophageal and lung cancers (Giacone 2000). Since the introduction of cisplatin in oncological practice, there have been considerable advances in our understanding of the molecular mechanism of action of this drug´s antitumoral effects. The biochemical mechanism of cisplatin cytotoxicity involves the binding of cisplatin to DNA and other cell components, with the subsequent induction of cell death by apoptosis, necrosis or both of these mechanisms (González *et al*. 2001).

However, cisplatin can cause genotoxic effects, chromosomical alterations, and mutations (Srb and Prochazkova 1983, Tandon and Sodhi 1985, Turnbull *et al*. 1979, Wiencke *et al*. 1979). These findings, and the observation that patients who survive cancer frequently develop a second malignancy after chemotherapy, suggest that cisplatin can exert a tumorigenic effect after its use in chemotherapy (Chambers *et al*. 1989, Mead *et al*. 1983, Van Imhoff *et al*. 1986). In view of this evidence that cisplatin can induce carcinogenesis in animal cells, tissues and organs, it is important to understand the effects of this drug in different models.

V79 cells, a diploid fibroblast line obtained from Chinese hamster (*Cricetulus griseus*) lung cells, is an excellent model for cytogenetic assays of its very stable karyotype and cytogenetic characteristics (Bradley *et al*. 1981). Although this cell line has been immortalized, the cells show a stable chromosome number, and a uniform size, shape and

growth pattern. This stability and the very well defined properties of V79 cells mean that subtle alterations induced by chemicals are easily detected as cytogenetically.

In this work, we investigated the cytogenetic traits of cisplatin-treated V79 cells by determining the modal chromosome number, the mitotic index, the frequency of polyploidy and the number of aneuploid metaphases compared to control V79 cells.

Materials and Methods

Control Culture Conditions

Chinese hamster V79 fibroblast cells obtained from the Adolfo Lutz Institute (São Paulo, SP, Brazil) at passage number 54 were maintained in control culture conditions in Ham F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS-Nutricell Nutrientes Celulares, Campinas, SP, Brazil), at 37°C.

Treatment with Cisplatin

Monolayers of V79 cells grown in culture flasks were treated with 50 μg of cisplatin (Sigma)/ml, prepared in complete medium at the desired concentration (Allavena *et al.* 1990). After 24 h, the culture medium containing cisplatin was removed and the treated cells were then maintained in control culture conditions (without cisplatin) during 10 successive subcultures. This assay was done in triplicate using non-treated V79 cells as a control.

Cytogenetic Analysis

Control and cisplatin-treated V79 cells were arrested in metaphase by adding 16 μg of colchicine (Sigma)/ml followed by incubation for 4 h at 37^{o}C. The cells were then harvested using trypsin-EDTA (Nutricell) and isolated by centrifugation at 1000 rpm for 10 min. Chromosomal preparations were obtained by swelling the cells in 0.075 M KCl (Merck KgaA, Darmstadt, Germany) followed by fixation in methanol:acetic acid (3:1, v/v)

(Merck). Slides were prepared according to standard techniques and stained with 5% Giemsa (Sigma) solution.

The modal chromosome number was determined by counting the chromosomes in 100 metaphases of intact control and cisplatin-treated V79 cells (Genari and Wada, 2003), in a range of 18-23 chromosomes. The mitotic index (MI) and polyploidy frequency (PF) were determined according to Deitch and Sawicki (1979) and Gilvarry *et al*. (1990), respectively. The MI was calculated by counting the metaphases in 1000 cells and then dividing the number of metaphases by the total number of cells analyzed (MI [%] = number of metaphases/1000 x 100). The PF was determined by counting the polyploid metaphases (number of chromosomes $\geq$ 42) in 1000 metaphases and then dividing the number of polyploid metaphases by the total number of metaphases analyzed (PF [%] = number of polyploid metaphases/1000 x 100). The number of aneuploid cells was determined by counting the aneuploid metaphases (range of 24-41 chromosomes) in 1000 metaphases. The chromosome number distribution in aneuploid cells was determined by counting the chromosomes in 33 aneuploid metaphases of cisplatin-treated V79 cells.

Results and Discussion

Cisplatin-treated V79 cells showed an altered chromosome number distribution: 59% of control V79 cells had 21 chromosomes (range of 18-23, n=100), whereas 42% of cisplatin-treated V79 had 21 chromosomes and 38% had 20 chromosomes (range of 18-23, n=100; Figure 1). Representative metaphases of control and cisplatin-treated V79 cells are shown in Figure 2A and 2B, respectively.

The normal diploid chromosome number in V79 cells is 22, with a range of 20-23 chromosomes, according to the American Type Culture Collection. In this study, control and cisplatin-treated V79 cells had a modal chromosome number of 21, although 38% of the cisplatin-treated V79 cells had 20 chromosomes, indicative of genetic instability when compared to control V79 cells. Compounds that induce genetic or chromosomal instability in cells can produce multiple genetic alterations. Various studies have indicated that genetic or chromosomal instability induced by radiation or chemical compounds is an important mechanism in carcinogenesis (Chang and Little 1992, Harper *et al*. 1997, Little 1999).

Although it is not clear how and when the cisplatin-treated V79 cells acquired this instability, it is likely that the alteration occurred immediately after treatment and that it persisted for many generations. The induction of genetic or chromosomal instability may be a common pathway to carcinogenesis, but little is understood of the underlying mechanisms. One possible mechanism is that a mutation in a gene or genes that contribute to genomic or chromosomal stability is responsible for the change in phenotype (Ohshima 2003).

The MI and PF were enhanced in cisplatin-treated V79 cells. The MI was 18.1% in control V79 cells and 22.8% in cisplatin-treated V79 cells. The higher MI in cisplatin-

treated V79 cells was related to altered cell proliferation and growth in culture, both of which are characteristic of cells transformed *in vitro* (Genari and Wada 1995, Genari *et al*. 1996, Hadnagy and Seemayer 1988). The PF (metaphases with ≥ 42 chromosomes) in control and cisplatin-treated V79 cells was 1.6% and 2.2%, respectively. Higher polyploidy frequencies have also been associated with cellular transformation *in vitro* (Genari and Wada 2000), and the presence of a large number of polyploid cells in tissue culture is characteristic of transformation and/or malignancy (Borenfreund *et al.* 1989, Gilvarry *et al*. 1990, Lothschutz *et al*. 2002).

Polyploid cells arise during a variety of pathological conditions. Genetic instability in polyploid cells may provide a route to aneuploidy and contribute to the development of cancer (Storchova and Pellman 2004). The number of aneuploid metaphases was increased in cisplatin-treated V79 cells; whereas control V79 cells contained 4 aneuploid metaphases (range of 24-41 chromosomes) per 1000 metaphases (0.4% of cells counted), cisplatin-treated V79 cells contained 33 aneuploid metaphases/1000 metaphases (3.3% of cells counted). Figure 3 shows the chromosome number distribution in aneuploid metaphases of cisplatin-treated V79 cells.

Although the basic causes of the cellular alterations involved in neoplastic transformations are not fully known, chromosomal changes probably play a major role in the emergence of transformed lineages. Several alterations are observed early after exposure to a chemical carcinogen and may be accompained by numerical and/or structural chromosomal changes (Barrett 1985). Malignancy is a multistep process in which cells acquire multiple genetic alterations followed by selective clonal expansion that results in the neoplastic phenotype. Hence, neoplastic transformation *in vitro* is a progressive,

multiple-stage process in which genetic alterations are obligatory (Zhu and Gooderham 2002).

The mechanisms of the cisplatin-induced changes in the cytogenetic characteristics of V79 cells were not investigated here. However, the toxicity of cisplatin in chemotherapeutic procedures is a consequence of DNA damage caused by the formation of platinum-DNA adducts, and this injury is apparently responsible for the induction of cell death (Reedjik 1987). However, DNA-adduct-forming agents may induce chromosomal breaks that can result in chromosomal gains via fusion or unrepaired breaks, with subsequent nondisjunction or loss of the defective chromosomes during mitosis, leading to a change in DNA content. Hence, cells with an abnormal DNA content will not be effectively excluded from the cell cycle and may continue to divide. These cells will be subject to asymmetric chromosomal segregation every time they divide (Holliday 1989).

In conclusion, the results of this study show that the incubation of V79 cells with a single concentration of cisplatin altered the chromosome number distribution, mitotix index, poplyploidy frequency and number of aneuploid metaphases of these cells. These alterations were probably related to the genetic instability induced by cisplatin treatment. The altered characteristics of these cells were similar to those associated with neoplastic processes *in vivo* following the chemotherapeutic use of cisplatin.

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Figure legends

Figure 1. Modal chromosome number of V79 control (V79 C) and cisplatin-treated (V79 T) cells. The modal chromosome number was determined by counting the chromosomes in 100 metaphases of intact control and cisplatin-treated V79 cells, in the range of 18-23 chromosomes.

Figure 2. Representative metaphases of control (A) and cisplatin-treated (B) V79 cells (1000X).

Figure 3. Chromosome number distribution in aneuploid cells in a total of 33 cisplatin-treated V79 cells, in the range of 24-41 chromosomes.

Figures

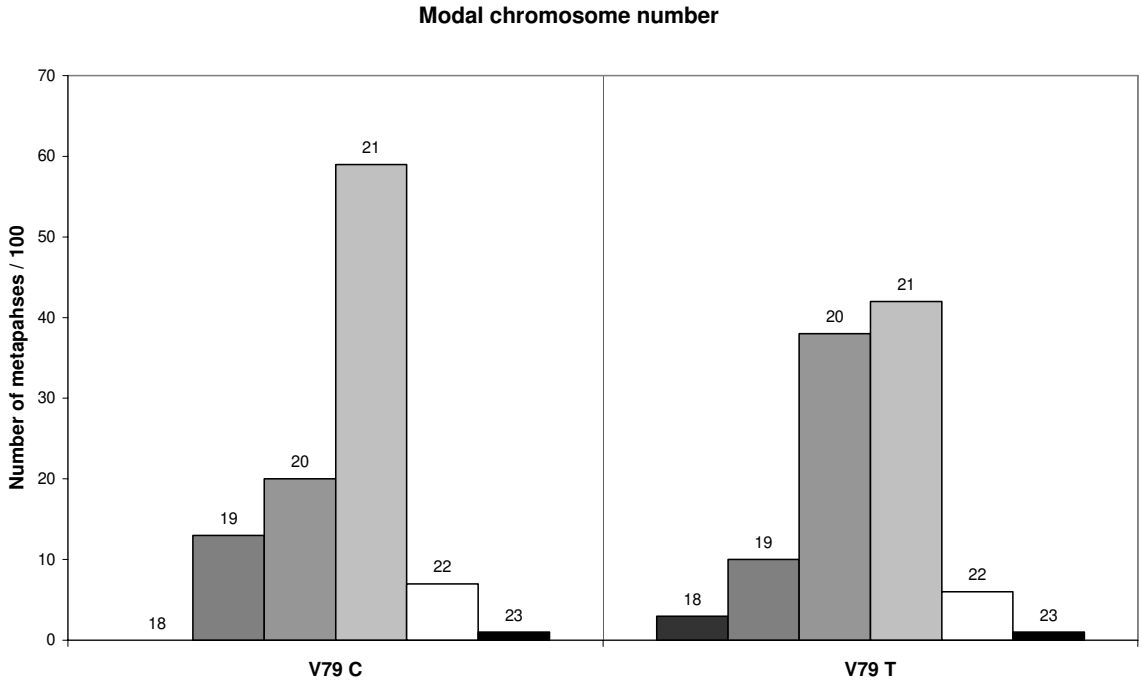
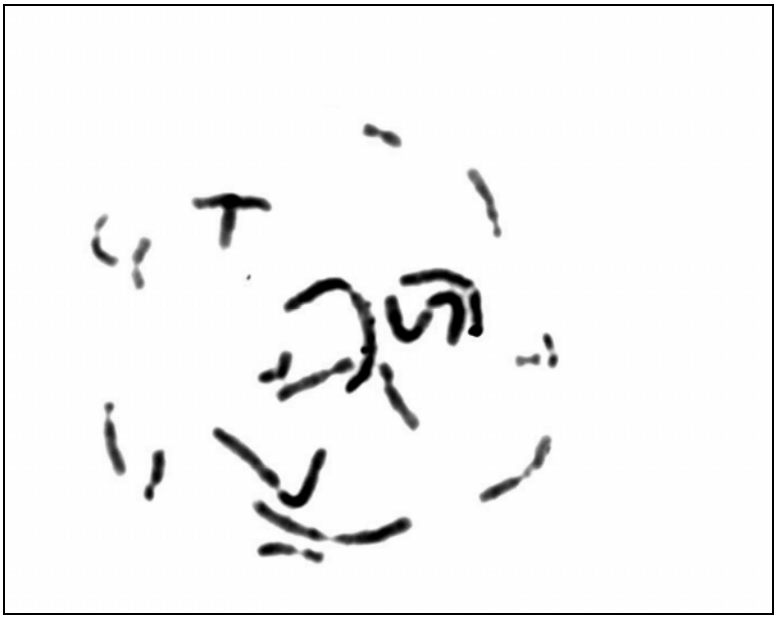


Figure 1

A



B

Figure 2

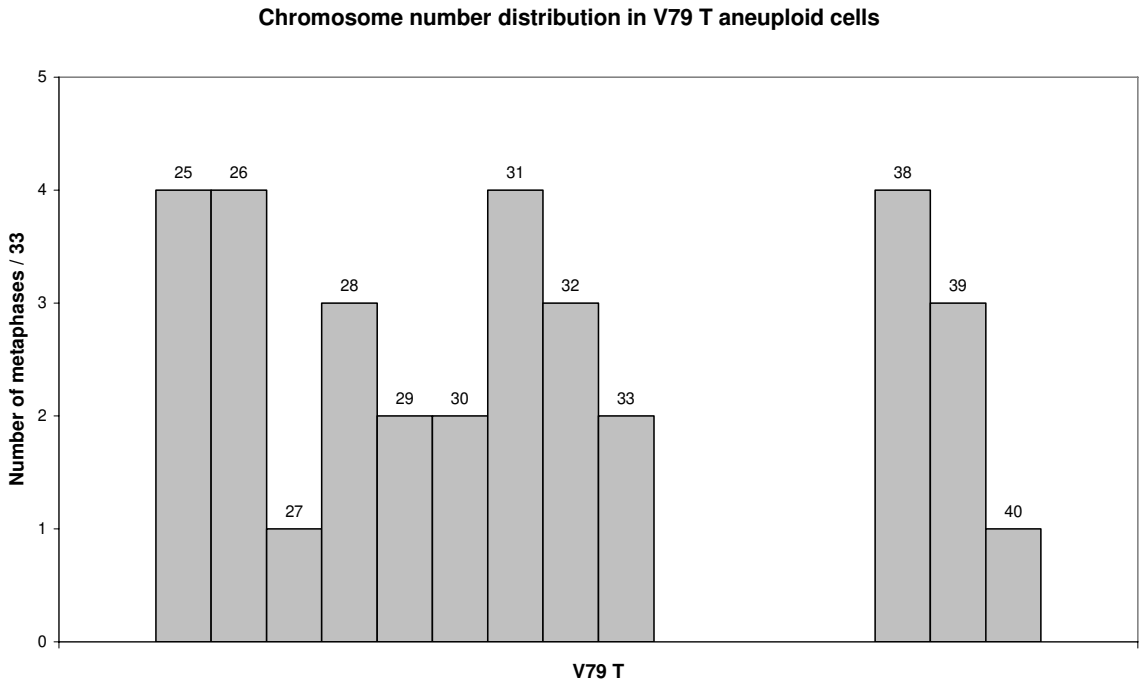

Chromosome number distribution in V79 T aneuploid cells
Number of metaphases / 33
0
1
2
3
4
5
25
26
27
28
29
30
31
32
33
38
39
40
V79 T

Figure 3

VII. CAPÍTULO 5 - CONCLUSÕES GERAIS

No primeiro estudo, foram utilizadas células imortalizadas da linhagem Vero como modelo para investigar o papel da cisplatina na transformação celular *in vitro*. Os resultados observados, como o crescimento em múltiplas camadas, a formação de grumos ou agregados celulares, as alterações no citoesqueleto de actina e nas características morfológicas e de crescimento das células transformadas, indicaram relações com as múltiplas etapas que são características do processo de desenvolvimento neoplásico. Assim, uma vez que as células Vero transformadas apresentaram características associadas ao crescimento neoplásico, as mesmas poderiam representar um modelo útil para estudos de células tumorais *in vitro*.

No segundo estudo, onde também foram utilizadas células Vero em ensaio de transformação celular *in vitro* induzida pela cisplatina, as modificações observadas nas células transformadas após o tratamento, como alterações citogenéticas, morfológicas e no padrão de adesão e distribuição de fibronectina, indicaram que a cisplatina parece ser um agente de transformação celular que atua como indutor, não apenas através de um processo de seleção. Desta forma, a cisplatina poderia representar um carcinógeno humano em potencial, como descrito em diferentes estudos realizados *in vivo*.

No terceiro estudo, foram utilizadas células imortalizadas da linhagem V79 como modelo para investigar o efeito da cisplatina sobre parâmetros citogenéticos. As alterações que foram observadas nas células V79 relacionam-se com a instabilidade genética induzida pelo tratamento com cisplatina. Estas células apresentaram características associadas ao desenvolvimento neoplásico, provavelmente ligadas ao processo neoplásico *in vivo* que tem sido associado ao uso da cisplatina em procedimentos quimioterápicos.

VIII. CAPÍTULO 6 - REFERÊNCIAS BIBLIOGRÁFICAS

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IX. ANEXOS

SUMÁRIO

Artigo I

Morphological characterization of a human glioma cell line.

Artigo publicado no periódico *Cancer Cell International*.

Cancer Cell International

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Primary research

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Morphological characterization of a human glioma cell line

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Abstract

A human malignant continuous cell line, named NG97, was recently established in our laboratory. This cell line has been serially subcultured over 100 times in standard culture media presenting no sign of cell senescence. The NG97 cell line has a doubling time of about 24 h. Immunocytochemical analysis of glial markers demonstrated that cells are positive for glial fibrillary acidic protein (GFAP) and S-100 protein, and negative for vimentin. Under phase-contrast microscope, cultures of NG97 showed cells with variable morphological features, such as small rounded cells, fusiform cells (fibroblastic-like cells), and dendritic-like cells. However, at confluence just small rounded and fusiform cells can be observed. At scanning electron microscopy (SEM) small rounded cells showed heterogeneous microextentions, including blebs and filopodia. Dendritic-like cells were flat and presented extensive prolongations, making several contacts with small rounded cells, while fusiform cells presented their surfaces dominated by microvilli.

We believe that the knowledge about NG97 cell line may be useful for a deeper understanding of biological and immunological characteristics of gliomas.

Background

Malignant gliomas are the most common type of brain tumor in adults. These tumors are highly invasive and despite multi-modality treatment the mean survival of patients is still less than 1 year.

Cultures of malignant cells represent an excellent and permanent material for studying the biology of these tumors as, for example, specific antigens characterization, bioactive factors produced, determination of cellular proliferation, and heterogeneity of genotypic and phenotypic characteristics (Pohl et al. 1993; Tsujino et al. 1997; Bodmer et al. 1989; Di Tomaso et al. 2000; Halfter et al. 1998; Bigner et al. 1981).

Recently, we have established a human glioma cell line from tissue obtained from a patient diagnosed with glioblastoma multiforme of the right temporal lobe. Histological examination revealed a grade III astrocytoma according to the WHO classification. This cell line, called

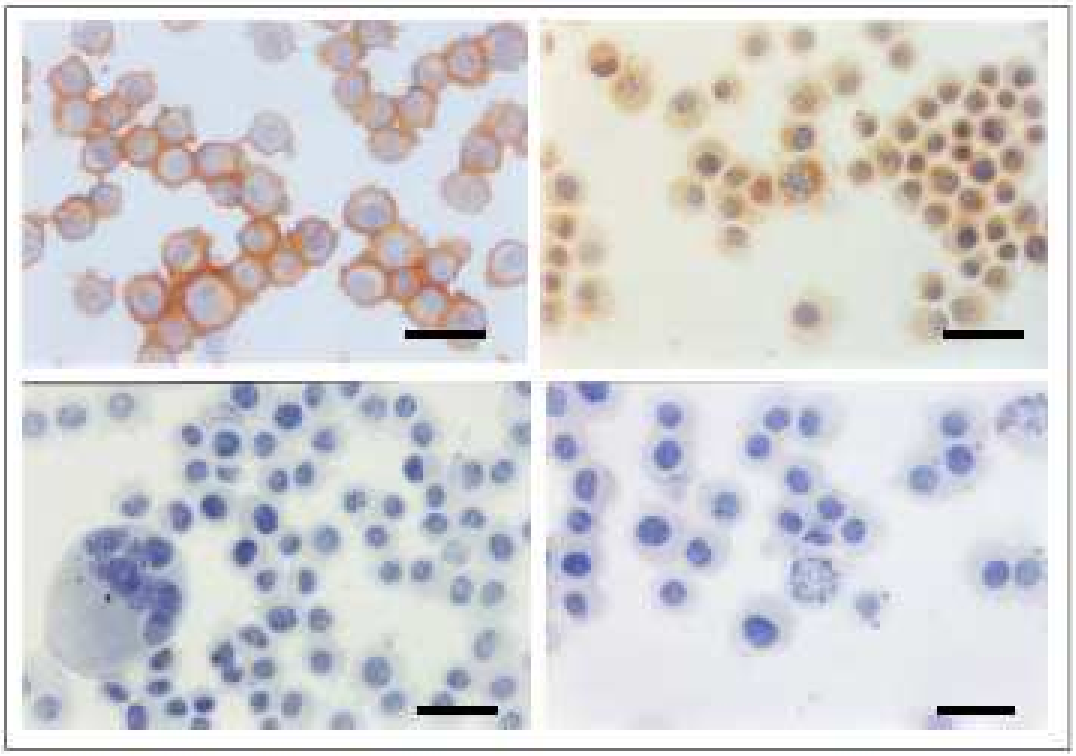

Figure 1
Immunocytochemical staining in NG97 cell line at passage 15. Note the immunopositive staining for GFAP (A) and S100 protein (B). On the other hand, cells were vimentin-negative stained (C). A negative control is also showed (D). Scale bar = 25 μm

NG97, has been sub-cultured in standard culture media without feeder layer or collagen coatings. The injection of NG97 cells into congenitally athymic mice induce the formation of solid tumor masses that can be retransplanted every 4 weeks. These tumors present features of malignant gliomas characterized by cell pleomorphism, necrosis and aggressive growth (Grippo et al. 2001).

The present work was undertaken to study growth kinetics, expression of marker proteins and morphological characteristics of early passaged cells present in the NG97 cell line.

Results

Markers

Immunocytochemical analysis of glial markers in the NG97 cells demonstrated that a large number of cells were positive for GFAP and S100 protein (Figure 1A and 1B, respectively). GFAP presents a diffuse perinuclear condensation, and S-100 protein is uniformly observed in the cytoplasm and irregularly observed in the nucleus of some cells. On the other hand, vimentin was undetectable in this cell line (Figure 1C). Figure 1D shows a representative control of all immunocytochemical experiments.

Microscopy Studies

Initially, NG97 cells formed mainly floating aggregates in the culture flasks and only small, rounded cells were seen (Figure 2A). At the 13th passage dendritic-like cells appear in the culture (Figure 2B). These cells present extensive prolongations making several contacts with small rounded cells and showed extra numerary nucleous (Figure 2C). As the cultures became dense, a third cellular type

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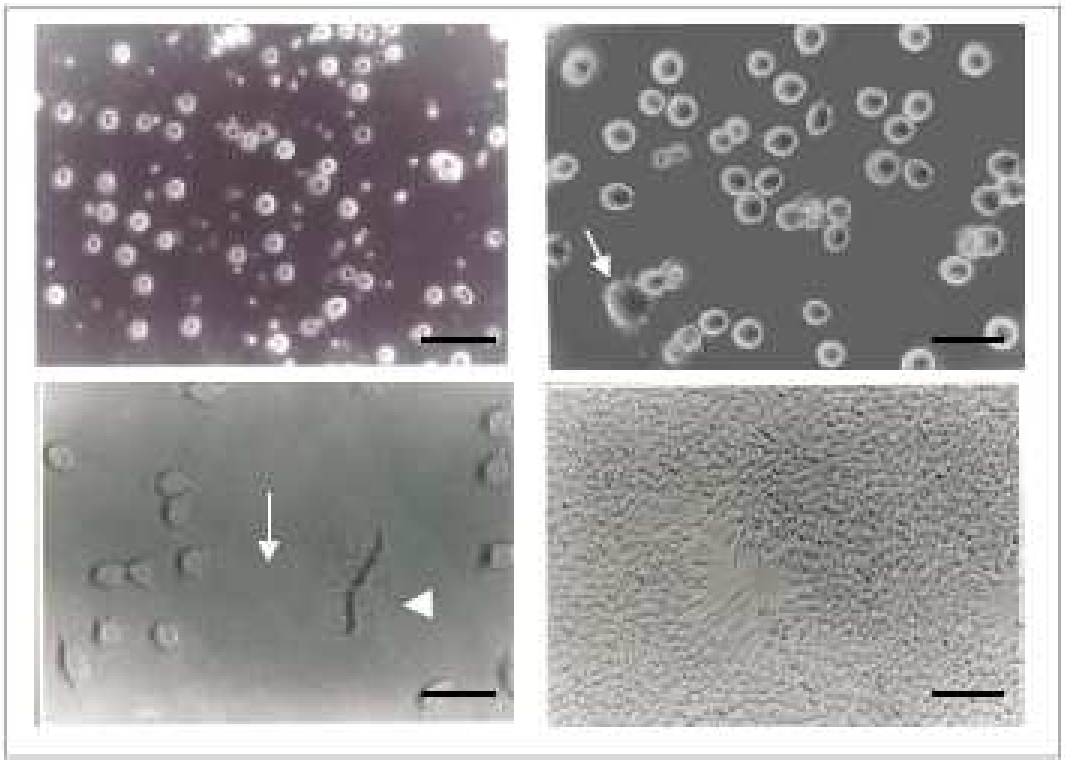

Figure 2
Phase contrast micrographs of NG97 cells. (A) small rounded cells growing as floating aggregates; (B) dendritic-like cells appear in the culture (→); (C) a dendritic-like cell with an extensive cytoplasmatic prolongation (→) and extra numerary nucleous (►); (D) confluent monolayer with small rounded and fibroblastic-like cells. Scale bar = 50 μm (A and D); 25 μm (B and C).

appears presenting a fusiform morphology (fibroblastic-like cells). At confluence, just small and fusiform cells can be observed in the culture (Figure 2D).

Scanning electron microscopy of small rounded cells showed heterogeneity of cytoplasmatic prolongation, including blebs and filopodia (Figure 3A and 3B). Dendritic-like cells are illustrated in Figures 3C through 3F. These cells presented high degree of cellular flattening, absence of blebs and, numerous and extensive cytoplasmatic prolongations. They were attached to the substrate making contact with small rounded cells. The third morphologically distinct cell type is presented in Figures 3G and 3H. These fusiform cells presented numerous microvilli on surfaces.

Growth kinetics

Until the 13th passage, when just small rounded cells were seen in the culture, a slow growth rate was observed (data not shown). At 13th passage, when the two other cell types appeared in the culture, the cells entered into an exponential growth phase. The population doubling time of NG97 cell line was about 24 h at 37°C and the saturation cell density was reached at 10×10^5 cells/cm^2 (Figure 4). The high growth rate was observed for the successive passages

Discussion

In this study basic characteristics of NG97 cell line are described. The investigated cell line was within passage 13 to 15. Our results show that NG97 cell line retains the expression of GFAP, which is a reliable marker of

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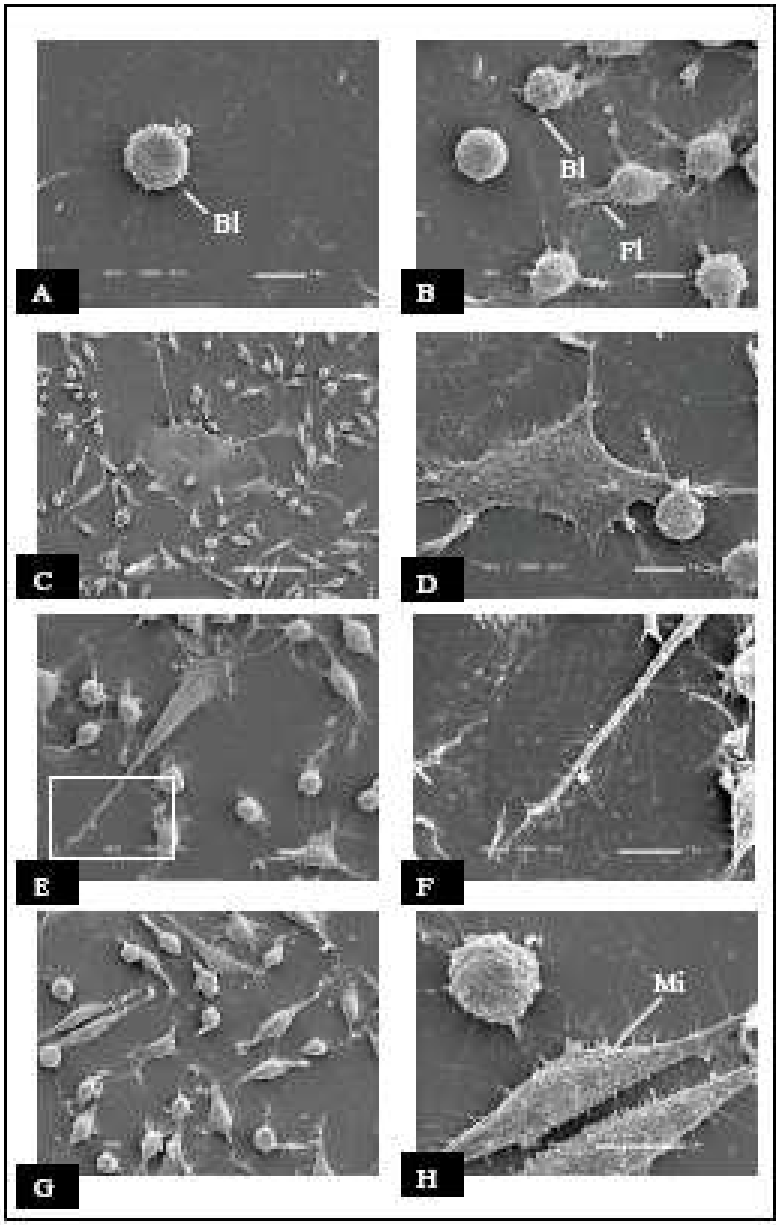

Figure 3
Scanning electron micrograph of NG97 cell line. A, B: small rounded cells presenting blebs (Bl) and filopodia (Fi) on their surfaces; C, D, E: dendritic-like cells with extensive cytoplasmatic prolongations. The area in the rectangle is shown at higher magnification in F. G: culture with two morphologic distinct cellular types: H: fibroblastic-like cells presencing microvilli (Mi) on the membrane surface.

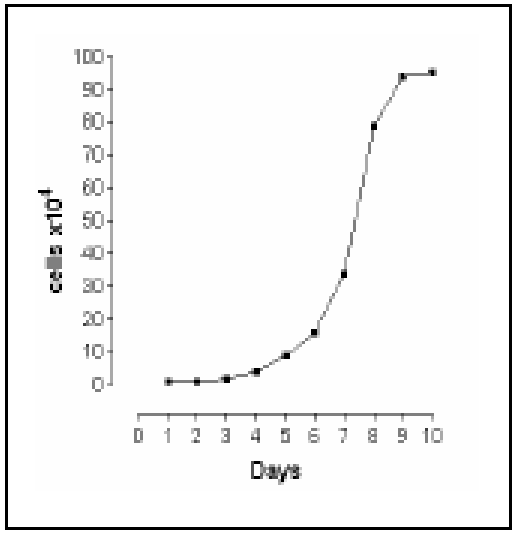

Figure 4
Growth curve of NG97 cell line.

astrocytic cells, and S100 protein that was originally identified as brain specific (Moore, 1965). Literature has shown, by and large, a negative correlation between the degree of malignancy and expression of GFAP and S100 protein in the majority of human gliomas (Jacques et al., 1981; Duffy et al., 1982). However, NG97 cells are tumorigenic in nude mice, indicating that cells are neoplastic and malignant. Besides, these two biochemical markers are present in the xenografts of NG97 cells in nude mice (Grippo et al., 2001). Interestingly, the vimentin that has been identified in some human glioma cell lines (Roessmann et al. 1983; Rutka et al. 1988) was not detectable in NG97 cells. Hedberg and Chen (1986) found that a human adrenal tumor cell line, named SW-13, expressed vimentin filaments and clones derived from these cells were characterized as lacking any detectable cytoplasmic intermediate filaments (vim-). Later, Sarria et al. (1994) demonstrated that the nuclei of the SW-13 vim- cells often appeared to be highly folded, forming prominent lobes and clefts. However, the authors also showed that the effect of vimentin filaments on the invaginations or folding in the nucleus is not an absolute, and raise the possibility that this nuclear configuration could be an indirect effect of a metabolic difference between cells that contain or lack organized vimentin filaments. To all appearances, our results indicate that in NG97 cell line the absence of an organized vimentin filament network does not affect the shape of the nucleus.

Heterogeneous cell types can be found in NG97 cultures. At early passages, cultures grew slower and presented only the small, rounded cells. At 13th passage, dendritic-like cells appear in the culture. It is not clearly for us the exact events that lead to the appearance of this cell type in the culture. We hypothesize that one small, rounded cell accumulates unbalanced divisions forming an extra numerary nucleous cell that secretes some products capable to induce alterations on the other cells. More elaborate experiments would test this possibility. In addition, dendritic-like cells present numerous and extensive cytoplasmatic prolongations, which may be associated with communication between this cell and the small ones. It seems also that dendritic-like cells provide an anchorage to the small rounded cells, which in turn present an increase in the filopodias to ameliorate the substrate connections. Of note, when dendritic-like cells appear in the culture we have noted an increase of the cellular growth rate. Future analyses should test if dendritic-like cells are able to modulate cell growth.

Fusiform cells appear when the culture becomes dense. These cells are majority in confluent monolayer cultures and present a large number of microvilli on the surface that propitiates an intimate contact with the environment. In the same way, further studies of this cell will help to unveil more NG97 cell line secrets.

Conclusion

NG97 cells grow *in vitro* as three sub populations with distinct morphological appearance and, undoubtedly, constitute a glial-committed cell line since are positive for GFAP and S-100 protein. Until 13th passage only small rounded cells were seen in culture and the growth kinetics was very slow. From this point, two other cell types presenting dendritic and fibroblastic characteristics could be observed and results were evident for overgrowth of cells. The possibility that these cells are able to modulate cell growth can not be discarded and are now under investigation in our laboratory.

This cell line may prove useful for cellular and molecular studies as well as in studies of gliomas treatment.

Methods

Glioma Culture

NG97 cells were grown in plastic flasks (25 cm^2) with RPMI 1640 medium (Sigma Chemical Co., St Louis, MO), supplemented with 50 μM 2-ME, 2 mM L- glutamine, 100 μg/mL garamycin and 20% inactivated fetal bovine serum (complete medium). The cultures were incubated at 37°C in an atmosphere containing 95% air and 5% CO_2. The

medium was changed after intervals of 48 hs and when the culture reached confluence, the subculture was performed by treatment with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).

Immunocytochemistry

Immunocytochemical analysis of glial markers (GFAP, vimentin and S-100 protein) was performed by using specific antibodies purchased from Dako Envision+ Systems/HRP (Dako Corporation, Carpinteria, CA). Briefly, cultured NG97 cells were harvested (at passage 15), washed using low speed centrifugation (150 × g, 10 minutes) and ressuspended in complete medium. Then, cells were cyto-centrifugated on glass slides, dried at room temperature for 15 minutes and fixed in cold acetone for 15 minutes at -20°C. After a thorough wash with 0.5% BSA in PBS, the cells were treated with polyclonal rabbit anti-GFAP, monoclonal anti-vimentin and polyclonal rabbit anti-S100 antibodies according to the manufactures' instructions. The bound primary antibody was detected using peroxidase labeled polymer conjugated to either mouse or rabbit secondary antibodies. Subsequently, the slides were incubated with a substrate mixture of 3,3-diaminobenzidine (DAB) and 0.02% H_2O_2. Cells were then counterstained with haematoxilin and eosin (HE). Control slides that stain positively for the specific antigens were used to assure correct staining and stability of reagents used. Negative controls included the omission of the primary antibody.

Phase Contrast Microscopy

Growing cells on cover slips were observed with a phase-contrast microscope (Olympus IX50 with a PMC35Dx photo micrographic system).

Scanning Electron Microscopy

NG97 cells were grown to sub confluence on 13 mm round coverslip in complete medium. The cells were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (pH 7.4) for 1 hour at room temperature. Then, the cells were post-fixed in 1.0% osmium tetroxide (OsO_4) for 10 minutes, washed in 0.1 M phosphate buffer (pH 7.2) and dehydrated in a grade series of ethanol. Cover slips were critically point dried using liquid CO_2 as transition fluid. The specimens were cold sputter coated with gold and observed in a JEOL JMS 5800 LV scanning electron microscope (SEM) accelerating voltage of 10 kV.

Growth Curve

NG97 cells were collected from 13th passage for determination of growth curves. Briefly, semi confluent cultures were trypsinized and cells resuspended in complete medium for counting. Cells (1×10^4) were plated into each well of a 12-well plate and counts from triplicate wells were made daily for 10 days. Trypsinized cells were counted in hemacytometer chamber and numbers were averaged for each time interval. Cell population doubling time was calculated from the linear phase of the growth curve, and the saturation density was the plateau point on the growth curve after the linear growth phase.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

This work is part of a Master's Dissertation by Camila M.L. Machado

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Artigo II

TEL, a plant lectin, induces apoptosis in cultured mammalian cell line.

Artigo submetido para publicação no periódico *Cell and Tissue Research*.

TEL, A PLANT LECTIN, INDUCES APOPTOSIS IN CULTURED MAMMALIAN CELL LINE

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Abstract

TEL, a novel lectin from *Talisia esculenta* seeds, has recently been purified and characterized. In this work, the cytotoxic effects of TEL on Vero cells, a non-tumourigenic mammalian cell line, were investigated. Cell viability was assessed using the neutral red uptake assay. The morphological and apoptotic effects were examined using microscopy techniques as well as by the terminal dUTP nick-end-labeling (TUNEL) method. TEL caused a time and concentration-dependent cell death. Morphological changes (rounding, cellular shrinkage and chromatin condensation) consistent with apoptotic cell death could be distinguished as early as 3h after TEL treatment. In addition, Vero cells incubated with TEL exhibited disruption of the actin cytoskeleton. DNA fragmentation was detected by TUNEL labeling and was confirmed through agarose gel electrophoresis. This results show that the lectin isolated from *Talisia esculenta* seeds can induce apoptosis on mammalian cells in culture.

1. Introduction

Lectins are carbohydrate-binding proteins that bind glycans of glycoproteins, glycolipids, or polysaccharides with high affinity (Goldstein et al., 1980). They are widespread in the biosphere and occur in almost every living organism. A rich source of lectins are plants, in particular their storage organs as for example seeds, tubers, bulbs, rhizomes or bark (Rüdiger, 1998). Their physiological role within the plant remains speculative. However, their most likely function is believed to be defense against different kinds of plant-eating organisms (Peumans and Van Damme, 1995; Rüdiger, 1998). A number of studies have shown that lectin binding to cell surface carbohydrates triggers various biological effects. This property of lectins has been widely used for analysis of the cell carbohydrates of normal and malignant cells (Dennis, 1992; Ohba et al., 2004; Stanley, 1980). It has been reported that lectin from *Saraca indica* and Viscum album agglutinin-I (VAA-I) stimulate lymphocytes proliferation and lymphokine production (Ghosh et al., 1999; Hajtó et al., 2003). Some lectins, such as wheat germ agglutinin (WGA), ricin, as well as Con A are high cytotoxic and are able to kill normal or malignant cells (Gastman et al., 2004; Kulkarni and McCulloch, 1995; Ohba et al., 2004). Numerous plant lectins present toxic properties toward different insect species (Macedo et al., 2002; Harper et al., 1995) and animals (Grant et al., 1993), but the exacts mechanisms by which these proteins exert their toxic activity is poorly known. Recently, some lectins have been shown to induce apoptosis, which whould explain their cytotoxicity (Gastman et al., 2004; Hajtó et al., 2003; Ohba et al., 2004). It was reported that lectins isolated from mistletoe induce apoptosis in several cells lines (Yoon et al., 1999; Valentiner et al., 2002).

Programmed cell death (PCD) is important for sculpting tissues and destroying harmful cells such as autoreactive immune cells and tumor cells (Mattson, 2000). Apoptosis is a modality of PCD that is dependent on the activity of caspase protease and is accompanied by highly conserved morphological changes including chromatin compaction, cell shrinkage and DNA fragmentation (Abraham and Shahan, 2004; Green et al., 2003). Its has been the most investigated and characterized form of cell death, in which cells display activation of caspases, the effectors of cell suicide. The relationship between caspase activation and the morphological changes that accompany apoptosis remains ill defined (Abraham and Shahan, 2004).

Talisia esculenta (St. Hil.) Radlk., locally know as pitomba, belongs to the family Sapindaceae and occurs in northern and northeastern Brazil. The fruit of *T. esculenta* is consumed by humans and also by birds. Popular information mentions that chickens die after ingesting the fruit. A novel lectin from *Talisia esculenta* seeds (TEL) has recently purified and characterized (Freire et al., 2002). TEL showed two protein bands in SDS-PAGE (Mr 20 kDa and 40 kDa) and agglutinated human and animal erythrocytes. Previous studies reported that this lectin possesses fungitoxic (Freire et al., 2002) and anti-insect (Macedo et al., 2000) properties but its cytotoxic activities have not been studied. The present study was designed to investigate the ability of TEL to induce cytotoxic effects in Vero cells, an adherent and non-tumourigenic fibroblastic cell line.

2.0 - Materials and Methods

2.1 – TEL purification

T. esculenta lectin (TEL) was prepared according to Freire et al. (2002). Dehulled *T. esculenta* seeds were finely ground and extracted with 150mM NaCl (1:5 meal to buffer ratio) for 24 h at 4°C and then centrifuged at 10,000g for 30 min at the same temperature. The clear supernatant (crude extract) was used to determine the protein content and hemagglutinating activity. The extract wasdiluted in 150 mM NaCl and applied to a SephadexG-100 column (2.5 × 80 cm) equilibrated with the same solution. The lectin-rich fraction was recovered and applied to a chitin column (20 ml) equilibrated with 50 mM phosphate buffer, pH 7.6, and eluted with 100 mM HCl. The hemagglutinating activity was monitored during TEL preparation. The purity of TEL was checked by SDS-PAGE (Laemmli, 1970). The purified lectin was extensively dialyzed and lyophilized.

2.2 - Cell culture

Vero (African green monkey kidney) cell line obtained from the Laboratory of Cell Culture, DMI, UNICAMP, Brazil, was grown in Eagle´s minimal essential medium (EMEM) with Earle´s salts (Nutricell, Campinas, SP, Brazil), supplemmented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), penicillin (1000 U/ml) and streptomycin (250 µg/ml). The cells were incubated in a 5 % CO2 atmosphere at 37 °C.

2.3 - Cytotoxicity assays (phase contrast microscopy)

To determine cytotoxic activity, 2 X 10^5 cells/well were seeded in flat-bottomed 96-well plates (Costar, Cambridge, MA, U.S.A). After 24 h, culture medium was aspired and 75 µl of fresh EMEM without FBS, and 25 µl of purified TEL (300 µg/ml final concentration) were added to each well. The plates were incubated at 37 °C in a 5% CO2 atmosphere and morphological changes of untreated and TEL treated cultures were photographed using 10X objective with an inverted phase-contrast microscope (Olympus IX50 with a PMC35Dx photomicrograph system) at different time intervals.

2.4 – Cell viability assay: Neutral red test

The neutral red assay is based on incorporation of the supravital dye neutral red into the lysossomes of viable cells. Cells were incubated in the presence of the TEL (different concentrations) for 24 or 48h. Neutral red stock solution (0.4%) was prepared in distilled water and stored at room temperature. Before staining, a fresh 1:80 dilution of the dye (50 µg/ml final concentration) in the medium was prepared. In accordance with the test of Borenfreund and Puerner (1984), 200 µl of medium containing neutral red were added to each well, and the microplates were incubated in a 5 % CO2 atmosphere at 37 °C for 3 h. Media were removed and the cells washed with a mixture of 4% formaldehyde / 1% calcium chloride solution to remove unincorporated neutral red. Ethanol-acetic acid solution (200 µl/well) was added and the microplates were shaken for 15 min to extract the dye from the viable cells. The color reaction was measured with ELISA plate reader (Multiskan Bichromatic) at 540 nm. The CD50, was defined as the minimum amount of

TEL required to kill 50 % of cells (1 CD50), and was determined from the lectin concentration resulting in 50% neutral red absorbance compared to control cells as 100%.

2.5 - Characterization of the morphological alterations induced by TEL in Vero cell.

2.5.1 – Light microscope

Light Microscopic observations were performed on confluent monolayers cultures of untreated and treated Vero cells grown on coverslips in 24-well plates. Cells were treated with TEL (1.5 CD50) for 3 and 6 h at 37 °C in a 5% CO2. Untreated and TEL treated cells were washed with PBS, fixed in 1% paraformaldehyde in PBS for 10 min, then washed with distilled water and stained with 0.025 % toluidine blue in McIlvaine buffer at pH 4.0 (Mello and Vidal, 1980). In another series of experiments, nuclear alterations were examined after the Feulgen reaction; in this case, the cells were hydrolysed in 4 N HCl for 75 min, washed and incubated with Schiff´s reagent for 40 min. The coverslips were then mounted on glass slides using Entelan.

2.5.2 – Scanning electron microscopy (SME)

Vero cells grown on coverslips in 24-well plates were treated with TEL (1.5 CD50) for 3 and 6 h at 37 °C in a 5% CO2. After incubation, medium was removed and cells fixed with 0.7 glutaraldehyde in 50 mM sodium cacodylate buffer and post-fixed with 1 % OsO4. Cell specimen were critical point-dried, sputtered with 5 nm palladium-gold and viewed in an electron microscope (JEOL JSM 5800 LV).

2.6 - Actin cytoskeleton labeling

Cells were plated on coverslips and treated by TEL (1.5 CD50) for 3 and 6 h. Untreated and treated cells were fixed with paraformaldehyde in PBS for 10 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. After three washes in PBS, coverslips were incubated with a 5 µg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma Chemical Co.) in PBS for 20 min to specifically stain filamentous actin (Clerc and Sansonetti, 1987). Coverslips were washed with PBS, mounted in glycerol / PBS (9:1) and examined by fluorescence microscopy (Nikon Eclipse E800).

2.7 – DNA fragmentation Assay

DNA fragmentation, in untreated and TEL treated (1.5 CD50) Vero cells, was investigated in situ by the terminal dUTP nick-end-labeling (TUNEL) method using an In situ Apoptosis detection ApopTag Kit (Roche, Mannheim, Germany). Experiments were performed according to the manufacturer's instructions.

2.8 - Quantification of changes in nuclear morphology

The percentage of altered nuclear morphology of TEL treated cells for 1, 3 and 6 h was determined after Feulgen reaction by counting ten optical fields selected at random from three preparations.

2.9 - DNA extraction and agarose gel electrophoresis

Vero cells cultured in 24-well plates at a density of 2 X 10 6 were treted with TEL (1.5 CD50) for 3 and 6 h and then lysed in 500 µl of cold TNE buffer (10mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA) containing 0.5 % SDS. Cell lysates were incubated at 56°C for 3 h in the presence of 100 µg/ml proteinase K (Sigma Chemical Co.). After digestion, DNA was purified by successive phenol-chloroform extractions and the resultant aqueous phase was mixed with 3M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at – 20 °C overnight and then centrifuged at 13 000 x g for 10 min. The purified DNA was washed with 70 % (v/v) ethanol, resuspended in TE buffer (10 mM Tris-HCl and 1 Mm EDTA pH 7.5) and treated with 0.1 % Dnase-free Rnase A (Sigma Chemical Co.) for 1 h. Finally, samples were resuspended in loading buffer (0.1 ml of 0.25 % Bromophenol Blue, 0.25 % xylone cyanol and 30% glycerol) and resolved on a 1.5 % agarose gel.

2.10 - Statistical analysis

Data are expressed as mean ± S.E.M. of three observations. Differences between data sets assessed by one-way analysis of variance (ANOVA) followed by Student's unpaired t-testes. Results with $P < 0.05$ was accepted as statistically significant.

3.0 – Results

3.1 - TEL-induced cytotoxicity

We first examined whether Vero cells exhibited susceptibility to TEL. In this study, we choose initially an aleatory concentration of TEL (300 μg/ml). TEL treated cells demonstrated retraction and a complete rounding up of cells (Fig. 1), which were seen as early as 3 h (Fig. 1C) and continued up to 6 h (Fig. 1D). In addition, after 6h of TEL treatment, cells showed a loss of attachment to the substrate. Untreated (Fig. 1A) and 1 h TEL treated (Fig. 1B) cells maintained their normal fibroblastic morphology and were well spread on the plastic plate. The morphological changes occurred at random and were asynchronous. As demonstrated in Fig. 2, TEL induced cell death in a concentration and time-dependent manner in Vero cell culture. A concentration of about 100 μg/ml was the threshold concentration of TEL induced toxicity. The minimal concentration of TEL that caused 50 % cell death after 24 h exposure period was about 200 μg/ml. Thus, the killing of the Vero cells in the presence of TEL reflects the general cytotoxicity of this lectin.

3.2 - Effects of TEL on Vero Cell Morphology

When we examined the effects of TEL on the morphology of Vero cells we detected cytoplasm and nucleu alterations (Fig 3 and 4). Cells treated with TEL for 3 h displayed cellular shrinking (Fig. 3, F-H), chromatin condensation (Fig. 3B) and nuclei with different levels of compactation (Fig. 4 A and C); additionally, incubation with TEL triggered significant vacuolating within the cytoplasm (Fig. 3C). Nuclear fragmentation (Fig. 3D), formation of the apoptotic bodies (Fig. 3, G and H), pycnotic nuclei (Fig. 4 C) and a highly disorganized cytoplasm (Fig. 3D) were observed after 5h of TEL treatment. Control cells

demonstred nuclei with dispersed chromatin (Fig. 3A and 4B), nucleoli well defined (Fig. 3A) and it's maintained to integrity of the cellular monolayer (Fig. 3E). Collectively, these microscopic observations demonstrated morphologic alterations classically described for apoptotic cells. Figure 4D, shows that nuclei undergoing apoptosis (identified by the nuclear phenotype after Feulgen-staining) were more frequent after 6 h TEL treatment; about 46% of the cells showed altered nuclei.

3.3 - TEL induces actin cytoskeleton alterations

Fluoresce microscopy revealed dramatic changes in the arrangement of the filamentous actin on Vero cells (Fig. 5). After 3h of TEL treatment, the filamentous actin arrays was poorly defined and intense spots of actin fluorescence were seen dispersed in cytoplasm (Fig.5, B and D). Filamentous actin collapsed around the nucleus was observed after 6h of TEL treatment (Fig. 5C)**.** Control cells displays organized actin filamentous (Fig. 5A).

3.4 - DNA Fragmentation in Vero cells treated with TEL

DNA fragmentation was detected by TUNEL assay, which clearly revealed a distinct pattern of nuclear staining (Fig. 6). There was a marked labeling in 3 h (Fig. 6C) and 6 h (Fig. 6A and B) TEL treated Vero cells. Untreated cells exhibited only weak or non-labeling (Fig. 6D). Consistent with light microscopic observations, nuclear fragmentation was also observed after TUNEL staining (Fig. 6C). In addition, the agarose gel electrophoresis revealed a characteristic ladder resulting from the internucleossomal cleavage of DNA (Fig 6, G and H).

4.0 – Discussion

Lectin binding to cell surface carbohydrates elicits various effects on cell processes, such as proliferation and apoptosis (Hajtó et al., 2003; Gastman et al., 2004). The toxic effects of lectins on cell survival are a widespread phenomenon observed in a number of cells lines and involve different mechanisms (Gastman et al., 2004; Leist and Wendel, 1996; Ohba et al, 2004). Since lectins have been shown to induce cell death in normal and tumor cells (Collins et al, 1997; Prime et al, 2004; Valentiner et al., 2002), we decide to available whether TEL, a novel lectin belonging to Sapindaceae family, would induce cytotoxic effects on cells in culture. To the best of our knowledge, this is the first report of the use TEL against mammalian cells. Like other plant lectins, our studies showed that TEL exerted cytotoxic effects on mammalian cell line. Analysis of the morphological alterations (Fig. 1 and 3) and of DNA content (Fig. 4 and 6) demonstred that TEL had a cytotoxic effect on the Vero cells by inducing apoptosis. It has been reported that a galactose-specific lectin from mistletoe induced apoptosis in normal and tumor cells (Ververken et al., 2000; Valentiner et al., 2002). Recently, Ohba et al. (2004) also showed that abrin-a, a plant lectin belongs *Abrus precatorius* seeds, induces cell death by apoptosis in several cell lines.

Cells dying by apoptosis, in most situations, display a very similar pattern of morphological changes (Majno and Joris, 1995). Regardless of which situation is investigated or which agent is used to induce apoptosis experimentally, the appearance of cell death at least has some similar features, which are often exactly the same (Häcker, 2000; Green et al., 2003). The major finding of this study is that TEL treatment of Vero cells resulted in significant morphological alterations (Fig. 1, 3 and 4), which are characteristic of cell death by apoptosis. Identical observations have been reported for

lectins from Korean mistletoe (Yoon et al., 1999) and *Canavalia ensiformes* (Con A) (Kulkarni and MacCulloch, 1995) that induced chromatin condensation and formation of apoptotic bodies in Jukart and 3T3 fibroblactic cells, respectively. Moreover, our results demonstrated that apoptotic surface morphology was very similar for ricin induction on mammalian cell type (Collins et al, 1997). This is consistent with the well-know highly conserved nature of this process. Indeed, apoptosis is heralded by cell rounding, presumably through loss of substrate adhesion, cellular shrinkage, chromatin condensation and fragmentation of nuclear DNA (Abraham and Shahan, 2004; Häcker, 2000; Majno and Joris, 1995). Furthermore, in our study, the dying cells exhibited cleavage of nuclear DNA, a major feature of apoptosis, as they showed oligonucleossomal DNA fragments (apoptotic ladder), labeling of free 3'OH termini of DNA by terminal deoxynucleotidyl transferase which catalyses the addition of deoxynucleotides to these termini (TUNEL) (Collins et all., 1997). A significant increase in the number of TUNEL-stained profiles associated with morphological alterations observed in Vero cells treated with TEL confirms a classic apoptotic cell death.

The apoptotic effect of TEL is time–dependent and in this action the disruption of actin filamentous is involved, at least in part. The actin cytoskeleton plays a key role in cell adhesion and cell morphogenesis (Martry-Detraves et al., 2004); therefore, we investigate the possibility that the morphological alterations caused by TEL are correlated with a rearrangement of the actin cytoskeleton. Various actin-containing structures involved in cell substratum interactions have been described, including focal adhesion, point contacts and filipodia (Adams, 1997; Geiger et al., 2001). Besides morphological alterations, disruptions in the integrity of the actin cytoskeleton interfere with vesicular transport of protein precursors and, consequentially, contribute to cell death (Hesketh and Pryme,

1991). We found that actin is a target for TEL, which caused rearrangement of actin filamentous (Fig. 5). These results are similar to observations by Kulkarni and MacCulloch (1995) who noted that Con A lectin induces a disruption of actin cytoskeleton in human gingival fibroblast (HGF). Previous studies have shown that mediators of apoptosis are known to be responsible by the degradation of an increasing number of proteins. Among them, some are cytoskeletal proteins such vimentin and actin (Brown et al., 1997; Lavastre et al., 2002).

Binding of lectins to specific oligossaccharides on cell membrane is an important step in lectin-mediated cell killing (Dennis, 1992; Stanley, 1980). Furthermore, internalization of some lectins also is considered to be required for apoptosis induction (Ohba et al., 2004; Shin et al., 2001). However, it is not known whether TEL is internalized to cause apoptosis in Vero cells. Macedo et al (2004) showed that the insecticidal activity of TEL involves a specific carbohydrate-lectin interaction with glyconconjugates on the surface of digestive tract epithelial cells. Freire et al. (2002) isolated TEL lectin and demonstrated that its hemagglutination was inhibited by mannose. Since several lectins mannose specific can bind to the carbohydrate site on the cell surface (Kulkarni and MacCulloch, 1995; Leist and Wendel, 1996), it is likely that TEL binds to the cellular membrane in a mannose-specific site and exerts the cytotoxic effect. However, lectins other than Con A and TEL, with similar carbohydrate specificity and affinity did not induce cytotoxicity (Leist and Wendel, 1996), so biochemical events secondary to the similar carbohydrate specificity seem to be involved in lectins- induced cytotoxicity.

In conclusion, our results demonstrated that TEL has apoptosis inducing activity on mammalian cell line. Thus, the exact mechanism of TEL – induced cytotoxicity and the striking morphological alterations described in this paper remain to be elicited.

Acknowledgements

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Figure Legends

Fig. 1 - Phase contrast photomicrographs of Vero cells. Untreated (A) and 1 h TEL treated cells (B) showing a typical confluent monolayer. Vero cells after 3 h (C) and 6 h (D) of TEL treatment demonstred retraction and rounding up of cells. Described alterations are indicated with arrows. Bars: 50 µm.

Fig. 2 - Effect of TEL treatment on Vero cells viability. Vero cells were treated with different concentrations of TEL for 12 or 48 h, and cell viability was determined by neutral red assay**.** The data are representative of three independent experiments and mean values, ± S.D., are shown.

Fig. 3 - Toluidine blue staining (A-D) and SME (E-H) of Vero cells. After 3 h of TEL treatment, cells showed chromatin condensation (B), cellular shrinking (F) and cytoplasmic vacuolization (C). 6h TEL treated cells exhibited nuclear fragmentation (D) and apoptotic bodies (G and H). Control cells demonstred normal appearance of the nucleu, which showed nucleoli well defined (A) and typical confluent monolayer (E). Described alterations are indicated with arrows. Light microscopy, bars: 5 µm. SME, (E): 2,500X; (F): 1,500X; (G): 3,000X; (H): 3,500X.

Fig. 4 - Feulgen reaction (A-C) and percentage of Vero cells underwent apoptosis (identified after Feulgen reaction) after TEL treatment (D). 3 h TEL treated cells showed nuclei with different levels of compactation (A). After 6 h of TEL treatment, cells exhibited pycnotic nuclei (C). Control cells showed nuclei with dispersed chromatin (B). Described

alterations are indicated with arrows. The data are representative of three independent experiments and mean values, ± S.D., are shown (D). Bars: 5 μm.

Fig. 5 - Actin cytoskeleton labeling of Vero cells. After 3 h of TEL treatment, cells exhibited intense spots of actin fluorescence dispersed in cytoplasm (B and D). 6 h TEL treated cells showed filamentous actin collapsed around the nuclei (C). Control cells demonstred the well-defined actin cytoskeleton (A). Described alterations are indicated with arrows. Bars: 5 μm.

Fig. 6 - DNA fragmentation of TEL treated Vero cells was detected by TUNEL method (I) and agararose gel electrophoresis (II). Cells showed intense labeling after TEL treatment for 3 h (C) or 6 h (A and B). Nuclear fragmentation can also to be seen after 6h TEL treatment (B). Described alterations are indicated with arrows. A typical DNA ladder was detected after 3 h (G) or 6 h (H). DNA content of Untreated (D and E) and 1h TEL treatment (F) did not show alterations. Bars: 10 μm.

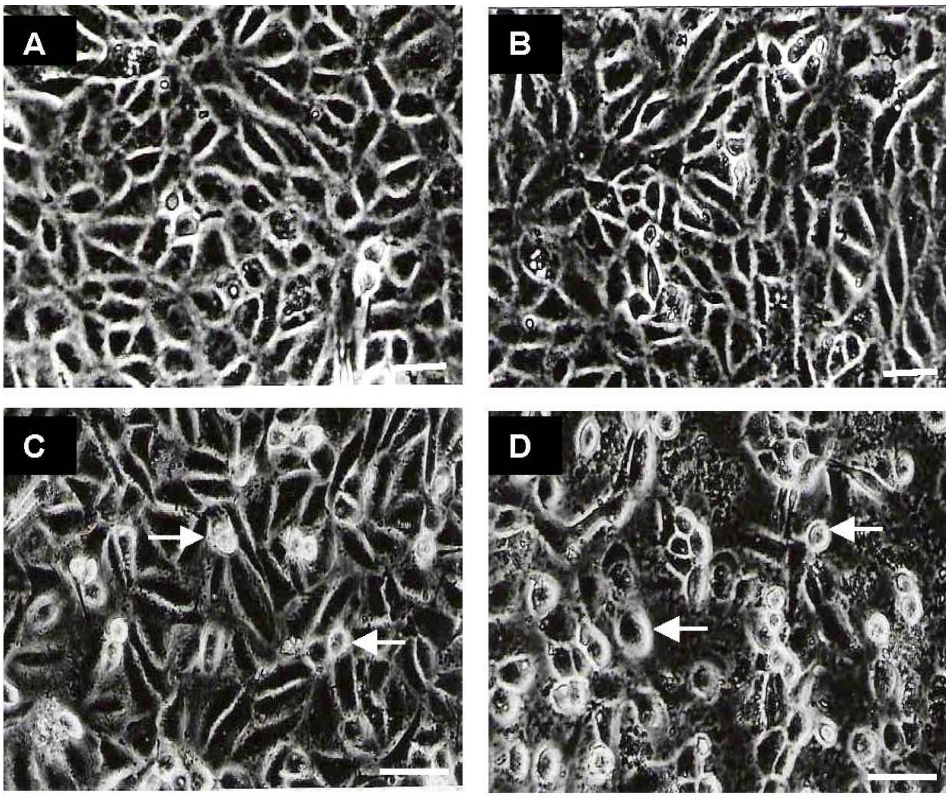

Fig. 1

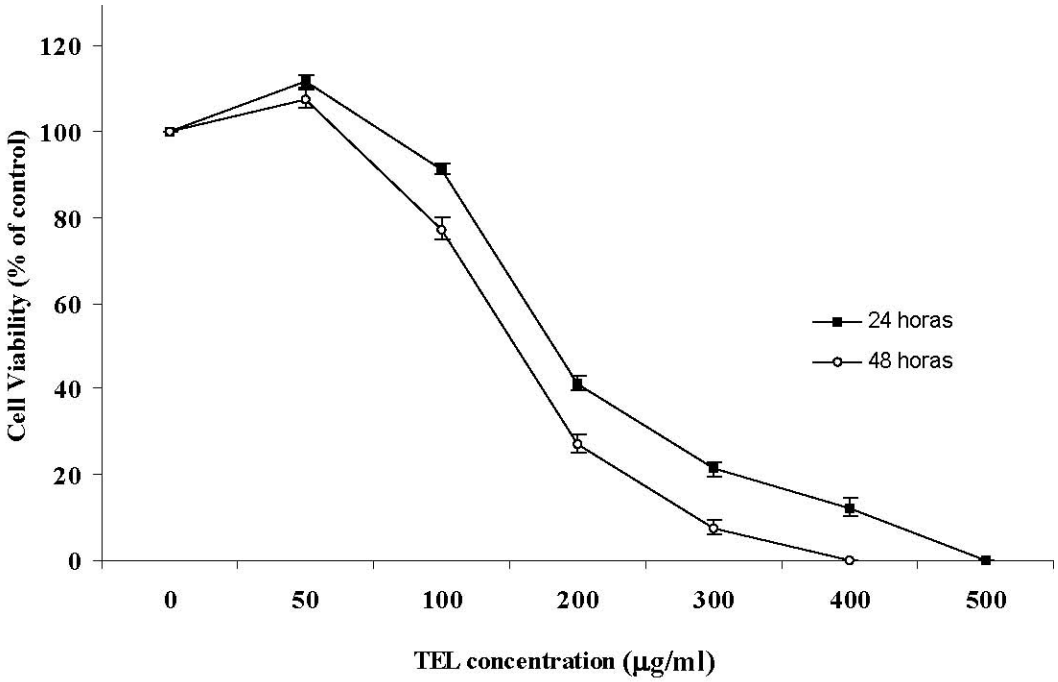
120
100
80
60
40
20
0
Cell Viability (% of control)
0
50
100
200
300
400
500
TEL concentration (µg/ml)
24 horas
48 horas

Fig. 2

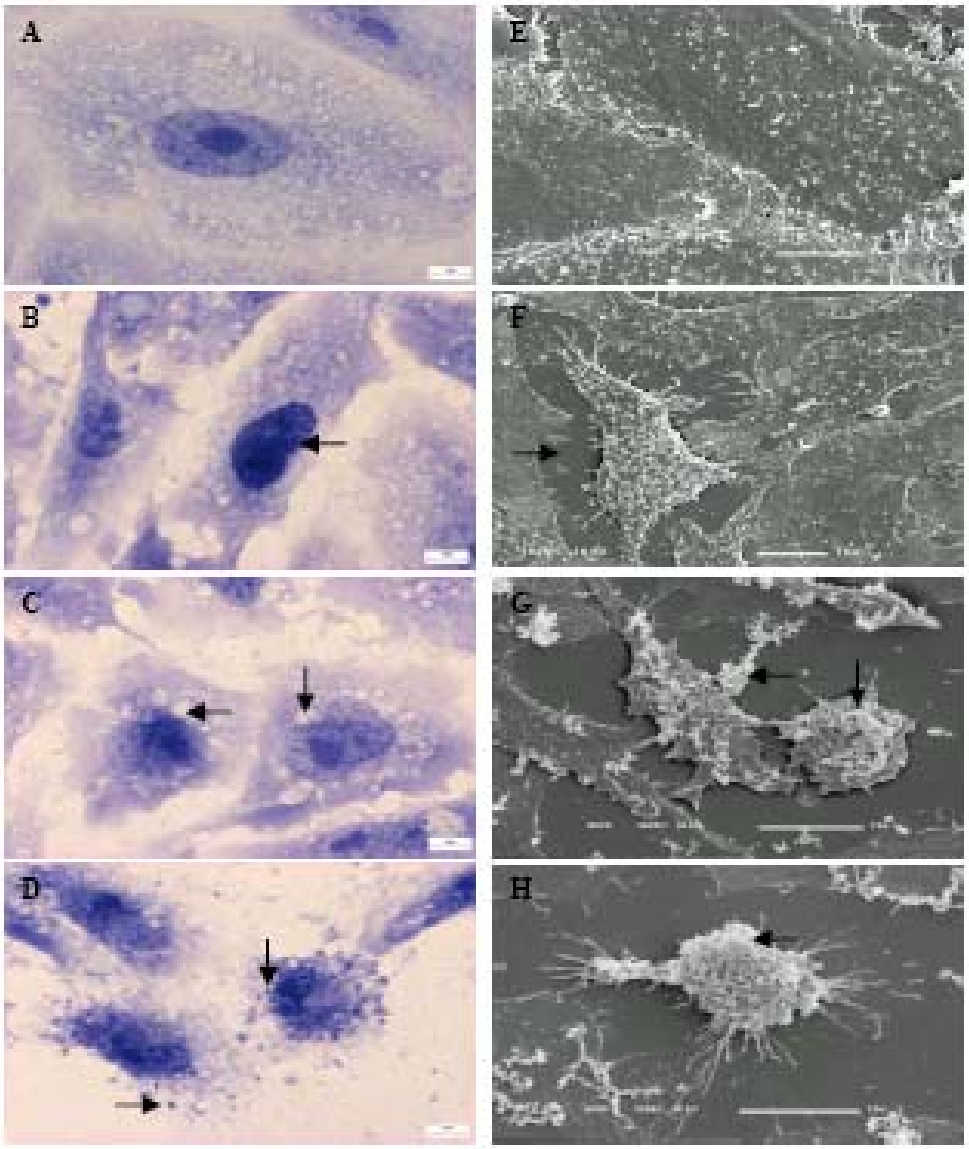

Fig. 3

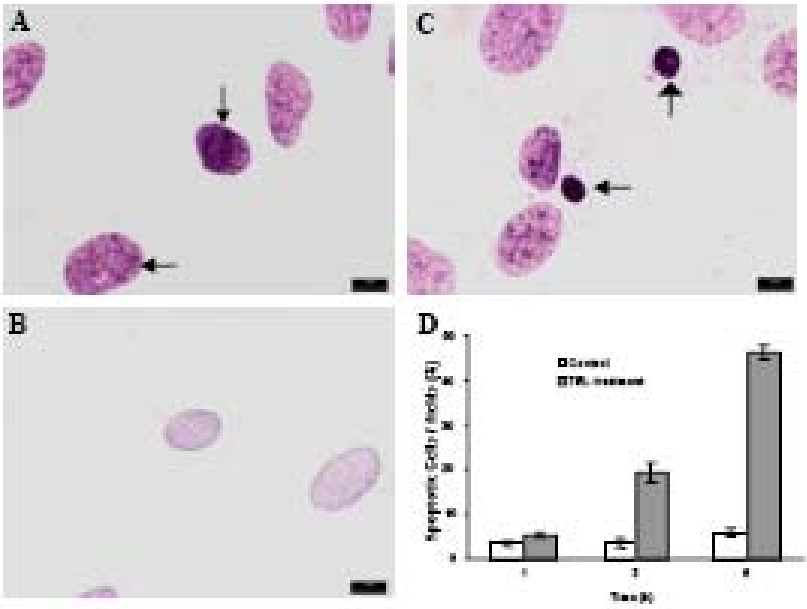

Fig. 4

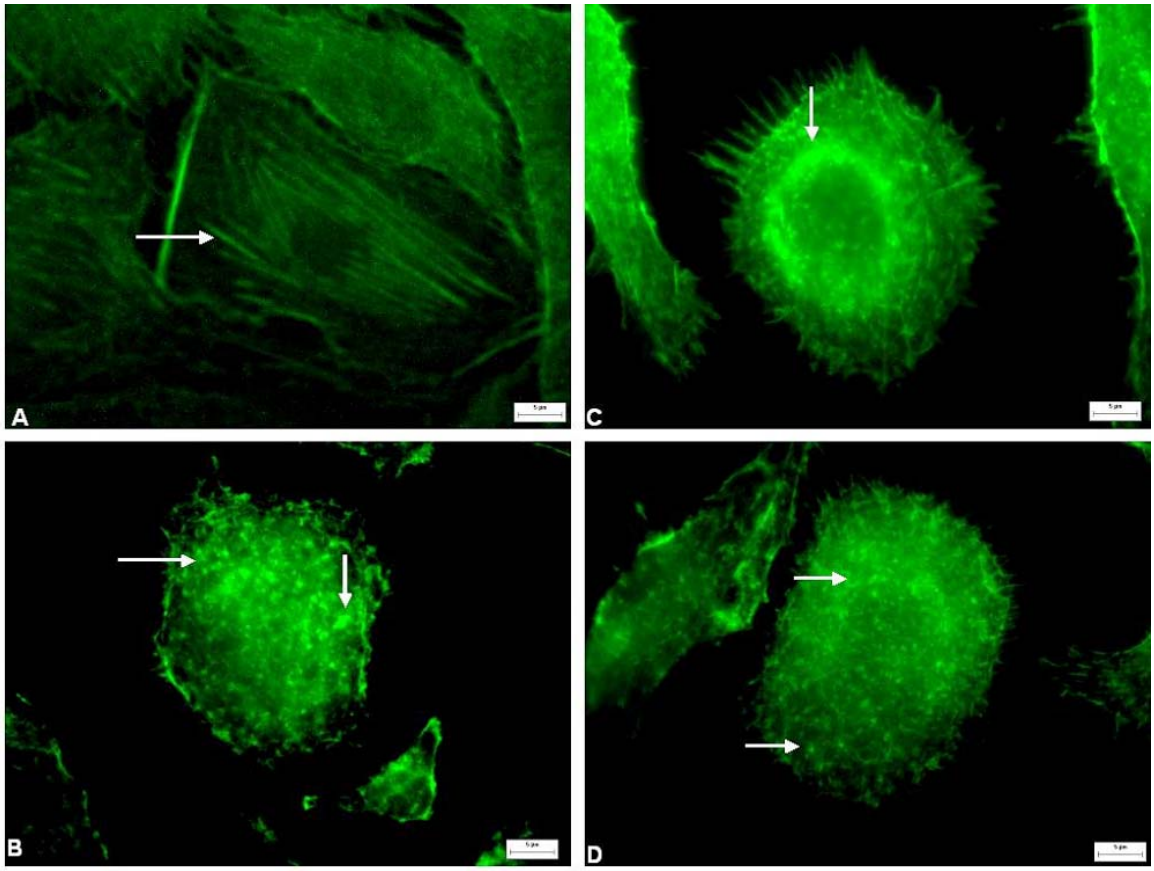

Fig. 5

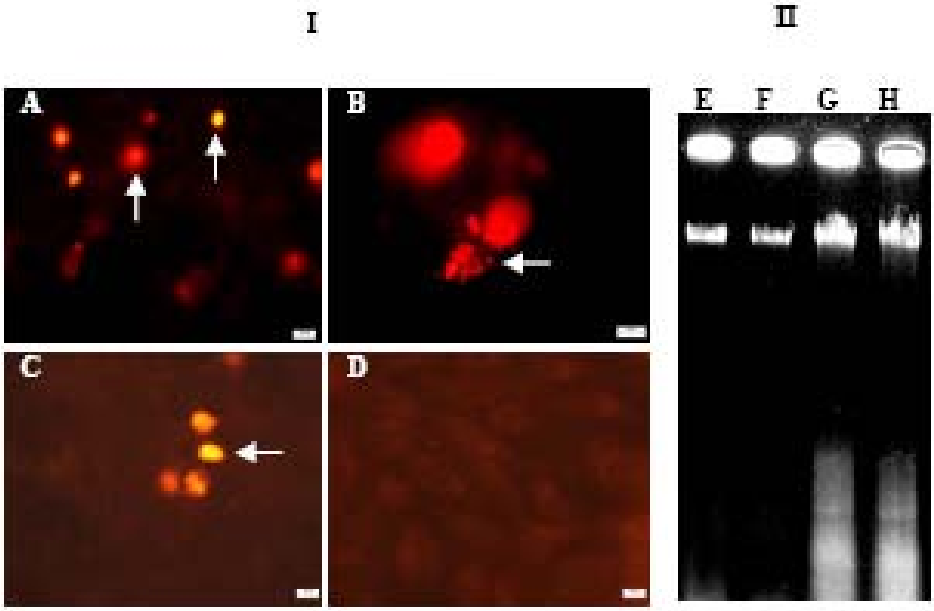

Fig. 6

Artigo III

Talisia esculenta lectin (TEL) induces cytogenetic and morphological alterations in Vero cells.

Versão preliminar de artigo a ser submetido para publicação.

Talisia esculenta lectin (TEL) induces morphologic and cytogenetic alterations in Vero cells

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Abstract

TEL, a novel lectin from *Talisia esculenta* seeds, has recently been purified and characterized. In this work, the effects of TEL on Vero cells were investigated. Cell viability was assessed using the MTT reduction assay. Cytogenetical and morphological effects of TEL were examined using cytogenetical and morphological analysis. Apoptotic cell death was detected by the terminal dUTP nick-end-labeling (TUNEL) method. TEL induced cell death in a concentration-dependent manner. 100 μg/ml was the threshold concentration of TEL-induced toxicity. The minimal concentration of TEL that caused 50% cell death after 24 h exposure period was 200 μg/ml. For the subsequent assays, were used the concentrations of 50 or 100 μg/ml of TEL. Cytogenetical alterations could be distinguished in both concentrations utilized. Morphological changes consistent with apoptotic cell death could be observed only in Vero cells treated with 100 μg/ml of TEL. In addition, these cells exhibited disruption of the actin cytoskeleton and DNA fragmentation was detected by TUNEL labeling. This result show that the lectin isolated from *Talisia esculenta* seeds induces cytogenetical and morphological alterations on Vero cells and the apoptotic cell death could be distinguished at threshold concentration of TEL-induced toxicity.

Keywords: *Talisia esculenta*, Vero cells, lectin, cytogenetical analysis, morphological alterations.

Introduction

Lectins form a large group of heterogeneous proteins that bind carbohydrates on the surface of responsive cells (Goldstein *et al*., 1980). Plant lectins can mimic endogenous mammalian lectins, and therefore, have been widely used to study physiopathological processes (Bento *et al*., 1993; Lima *et al*., 1999). Studies have been shown that lectins can bind to cell surface carbohydrates resulting in several biological effects, like lymphocytes proliferation, lymphokine production (Ghosh *et al*., 1999; Hajtó *et al*., 2003) and cytotoxicity on normal or malignant cells (Gastman *et al*., 2004; Kulkarni and McCulloch, 1995; Ohba *et al*., 2004).

Talisia esculenta (St. Hil.) Radlk., locally known as pitomba, belongs to the family Sapindaceae and occurs in northern and northeastern Brazil. The fruit of *T. esculenta* is consumed by humans and also by birds, the latter acting as dispersers for the seeds (Macedo *et al*., 2002). Recently was purified and characterized a novel lectin from *T. esculenta* seeds (TEL), the first of its kind from the family Sapindaceae. TEL showed two protein bands in SDS-Page (MW = 20,000 and 40,000 kDa) and agglutinated human and rat erythrocytes *in vitro* (Freire *et al*., 2001), and induced in vivo a typical inflammatory reaction in mice (Freire *et al*., 2003).

To investigate the effects of a lectin from *T. esculenta* (TEL) in cell culture, were analyzed viability, cytogenetical and morphological characteristics of Vero cells, an immortal non-tumorigenic fibroblastic cell line established from kidney cells of the African green monkey (*Cercopithecus aethiops*), exposed to TEL and compared to control Vero cells.

Materials and Methods

TEL Purification

T. esculenta lectin (TEL) was prepared according to Freire *et al*. (2002). Dehulled *T. esculenta* seeds were finely ground and extracted with 150 mM NaCl (1:5 meal to buffer ratio) for 24 h at 4°C and then centrifuged at 10,000 g for 30 min at the same temperature. The clear supernatant (crude extract) was used to determine the protein content and hemagglutinating activity. The extract was diluted in 150 mM NaCl and applied to a Sephadex G-100 column (2.5 × 80 cm) equilibrated with the same solution. The lectin-rich fraction was recovered and applied to a chitin column (20 ml) equilibrated with 50 mM phosphate buffer, pH 7.6, and eluted with 100 mM HCl. The hemagglutinating activity was monitored during TEL preparation. The purity of TEL was checked by SDS-PAGE (Laemmli, 1970). The purified lectin was extensively dialyzed and lyophilized.

Cell Culture

Vero (African green monkey kidney) cell line obtained from the Laboratory of Cell Culture, DMI, UNICAMP, Brazil, was grown in Eagle's minimal essential medium (EMEM) with Earle´s salts (Nutricell, Campinas, SP, Brazil), supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), penicillin (1000 U/ml) and streptomycin (250 µg/ml). The cells were incubated in a 5% CO_2 atmosphere at 37°C.

Cell Viability Assay

Vero cells adherent in culture flasks were treated by TEL with 50, 75, 100 or 200 µg/ml for 24 h, at 37°C in 5% CO_2. After incubation, the culture medium containing TEL

was removed and cells were washed with PBS, pH 7.4 at 37°C, followed by the addition of 100 µl of MTT (Sigma) (5 µg/ml in Ham F-10 medium) and incubation for 2 h at 37°C. The cells were then treated with isopropanol (Isofar, Jacaré, RJ, Brazil). The color reaction was measured with ELISA plate reader (Multiskan Bichromatic) at 540 nm. In some samples, TEL was dissolved in a solution of D-mannose (Man) before incubation, at concentration of 50 mM to investigate the effect of this carbohydrate on the cytotoxical activity of TEL.

Cytogenetical Analysis

Vero cells were treated by TEL with 50 (T50) or 100 (T100) µg/ml for 24 h, at 37°C in 5% CO_2. TEL-treated and control Vero cells were arrested in metaphase by the addition of 16 µg/ml of colchicine (Sigma) followed by a 4 h incubation period. Cells were harvested and chromosome preparations were made according to routine procedures. Cells were collected with trypsin-EDTA (Nutricell) and isolated by centrifugation at 1000 RPM for 10 minutes. KCl (Merck) 0.075M was used for swelling and methanol/acetic acid (Merck) 3:1 (v/v) for fixation. Slides were made according to standard techniques and stained with 5% Giemsa (Sigma) solution.

The modal chromosome number was determined by counting the chromosomes in 100 metaphases of integral cells for each cell population (Genari and Wada, 2003). The mitotic index was obtained according to Deitch and Sawicki (1979), by counting the metaphases in 1000 cells. Results were obtained dividing the number of metaphases by the total of cells analyzed (MI [%] = number of metaphases/1000 x 100).

Morphological Analysis

Light Microscopy

Light microscopic observations were performed on confluent monolayer cultures of untreated and treated Vero cells grown on coverslips in 24-well plates. Cells were treated by TEL with 50 (T50) or 100 (T100) µg/ml for 24 h, at 37°C in 5% CO_2. Untreated and TEL-treated cells were washed with PBS, fixed in methanol/acetic acid (Merck) 3:1 (v/v) for 10 min, washed with distilled water and stained with 0.25% violet cresyl (Merck). The coverslips were then mounted on glass slides using Entellan.

Scanning Electron Microscopy (SME)

Vero cells grown on coverslips in 24-well plates were treated by TEL with 50 (T50) or 100 (T100) µg/ml for 24 h, at 37°C in 5% CO_2. After incubation, medium was removed and cells fixed with 2.5% glutaraldehyde (Sigma) and post-fixed in 1% osmium tetroxide (Sigma), dehydrated in a graded ethanol series (Labsynth), critical point dried (Balzers CDT 030) and coated with 5 nm palladium-gold in a sputter coater (Balzers CDT 050). The cells were examined with a JEOL JSM 5800 LV scanning electron microscope.

Actin Cytoskeleton Labeling

Cells were plated on coverslips and treated by TEL with 50 (T50) or 100 (T100) µg/ml for 24 h, at 37°C in 5% CO_2. Untreated and treated cells were fixed with paraformaldehyde in PBS for 60 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. After three washes in PBS, coverslips were incubated with a 5 µg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma Chemical Co.) in PBS for 20 min to

specifically stain filamentous actin (Clerc and Sansonetti, 1987). Coverslips were washed with PBS, mounted in glycerol / PBS (9:1) and examined by fluorescence microscopy (Nikon Eclipse E800).

DNA Fragmentation Assay

DNA fragmentation, in control and TEL-treated Vero cells (with 50 (T50) or 100 (T100) µg/ml for 24 h), was investigated *in situ* by the terminal dUTP nick-end-labeling (TUNEL) method, using an *In situ* Apoptosis detection ApopTag Kit (Roche, Mannheim, Germany). Experiments were performed according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as mean ± S.E.M. of three observations. Differences between data sets assessed by one-way analysis of variance (ANOVA) followed by Student's unpaired t-testes. Results with $p < 0.05$ was accepted as statistically significant.

Results

Cell Viability Assay

TEL induced cell death in a concentration-dependent manner. 100 μg/ml was the threshold concentration of TEL induced toxicity. The minimal concentration of TEL that caused 50% cell death after 24 h exposure period was 200 μg/ml (CD_{50}). The cytotoxical activity of TEL was inhibited by addition of D-mannose (Figure 1). These results suggested that the cytotoxical activity of TEL probably involved a specific carbohydrate-lectin interaction.

Cytogenetical Analysis

TEL-treated cells showed altered chromosome numbers. Control Vero cells had 58% of cells with 54 chromosomes (range 50-60, n=100), whereas T50 Vero cells presented 35% of cells with 54 chromosomes and 22% with 55 chromosomes (range 48-57, n=100). T100 Vero cells presented 23% of cells analyzed with 54 chromosomes, 14% with 53 chromosomes and 13% of cells, presented 55 chromosomes (range 43-61, n=100). The modal chromosome numbers are represented in Figures 2A to 2C (A: control cells; B: T50 cells; C: T100 cells). Mitotic index was also altered in TEL-treated Vero cells. Control Vero cells showed a mitotic index of 3.1%, whereas T50 cells presented a mitotic index of 6.1% (proliferative effect) and T100 Vero cells had a mitotic index of 1.7% (antiproliferative effect).

Morphological Analysis

Light microscopy and Scanning electron microscopy (SME)

The control and treated Vero cells were stained for cell morphology with violet cresyl. The morphology at light microscopy and at scanning electron microscopy, was very similar for control and T50 Vero cells (Figures 3A and 3F: control cells; Figures 3B and 3F: T50 cells), whereas T100 cells presented some morphological alterations consistent with apoptotic cell death (Figures 3C, 3D and 3G).

Actin cytoskeleton labeling

The actin cytoskeleton labeling was similar for control and T50 Vero cells (Figures 4A and 4B, respectively). Vero T100 cells exhibited disruption of the actin cytoskeleton organization (Figure 4C).

DNA Fragmentation Assay

The DNA fragmentation was detected by TUNEL labeling only in T100 Vero cells (Figures 5C and 5D), whereas control and T50 cells were not TUNEL positive (Figures 5A and 5B, respectively).

Legends of figures

Figure 1. Cell viability assay (MTT reduction test). Data are expressed as mean ± S.E.M. of three observations. Cell numbers are expressed as counting cells x 1000 / well. TEL concentrations: 0 (Control), 50, 75, 100 or 200 μg/ml. Samples with (+) or without (-) 50 mM of D-mannose (Man).

Figure 2. Cytogenetical analysis: modal chromosome number, determined by counting the chromosomes in 100 metaphases of integral cells (n=100). VC: Control Vero cells; T50: TEL-treated Vero cells (50 μg/ml); TEL100: TEL-treated Vero cells (100 μg/ml). VC: range 50-60 chromosomes; TEL50: range 48-57 chromosomes; TEL100: range 43-61 chromosomes.

Figure 3. Light microscopy (0.25% cresyl violet) and scanning electron microscopy (SEM) of control (A and E), T50 (B and F) and T100 (C, D and G) Vero cells. Magnification: 600 X (A and C); 1000 X (B and D); 1500 X (E and F); 2200 X (G).

Figure 4. Actin cytoskeleton labeling by fluorescein isothiocyanate-phalloidin of control (A), T50 (B) and T100 (C) Vero cells. Magnification: 600 X (A to C).

Figure 5. DNA fragmentation assay by TUNEL labeling of control (A), T50 (B) and T100 (C and D) Vero cells. Magnification: 400 X (A to D).

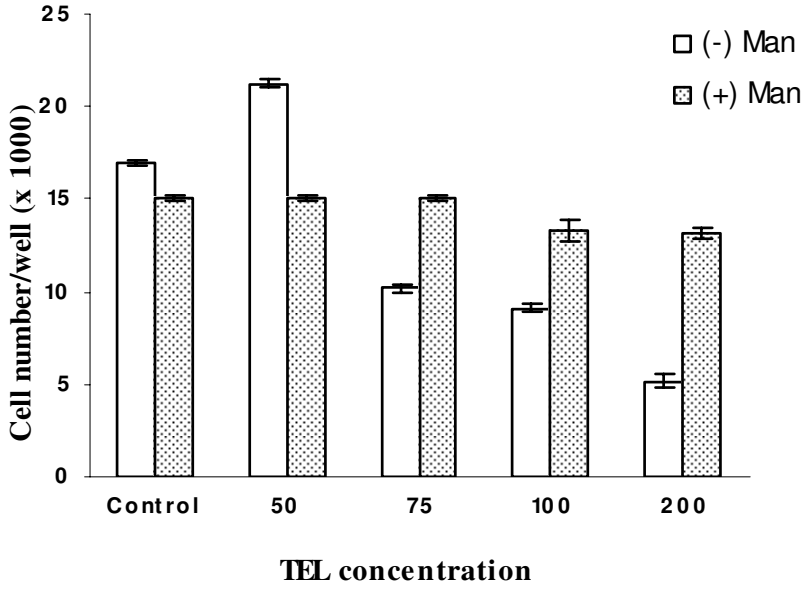

25
20
15
10
5
0
Cell number/well (x 1000)
(-) Man
(+) Man
Control
50
75
100
200
TEL concentration

Figure 1

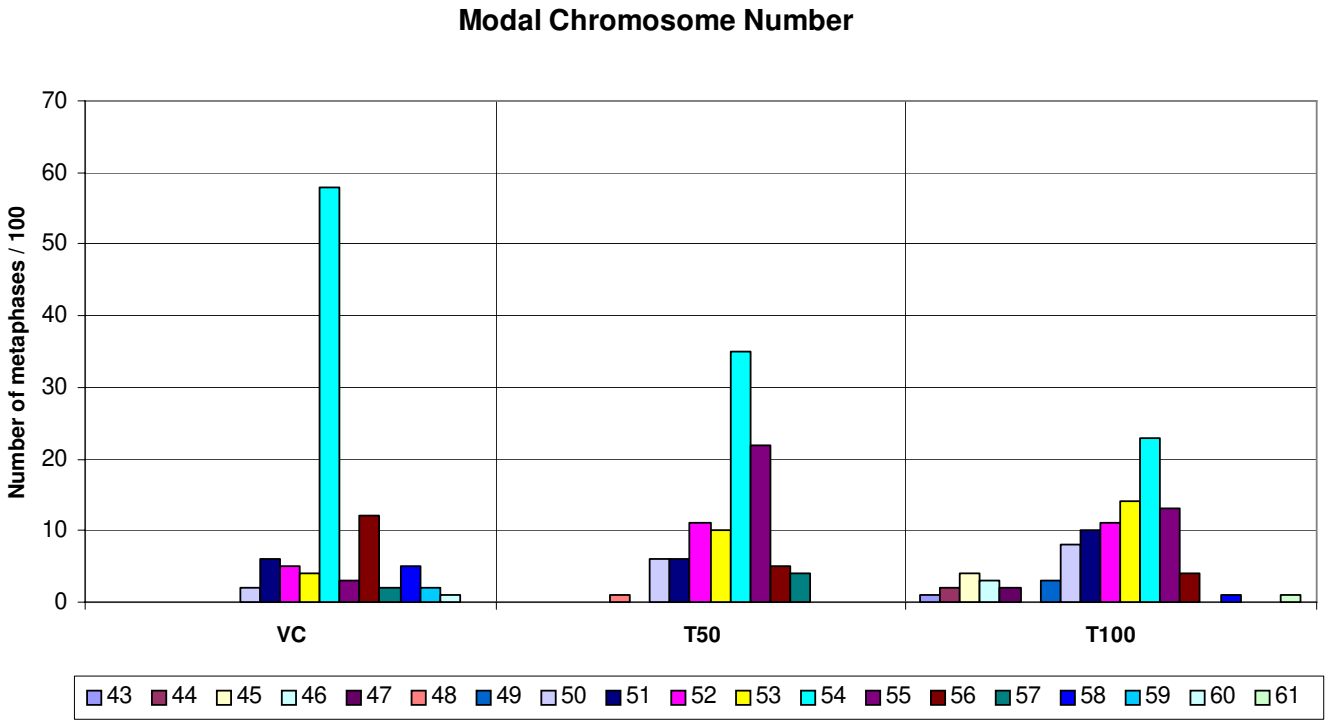

Modal Chromosome Number
Number of metaphases / 100
70
60
50
40
30
20
10
0
VC
T50
T100
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61

Figure 2

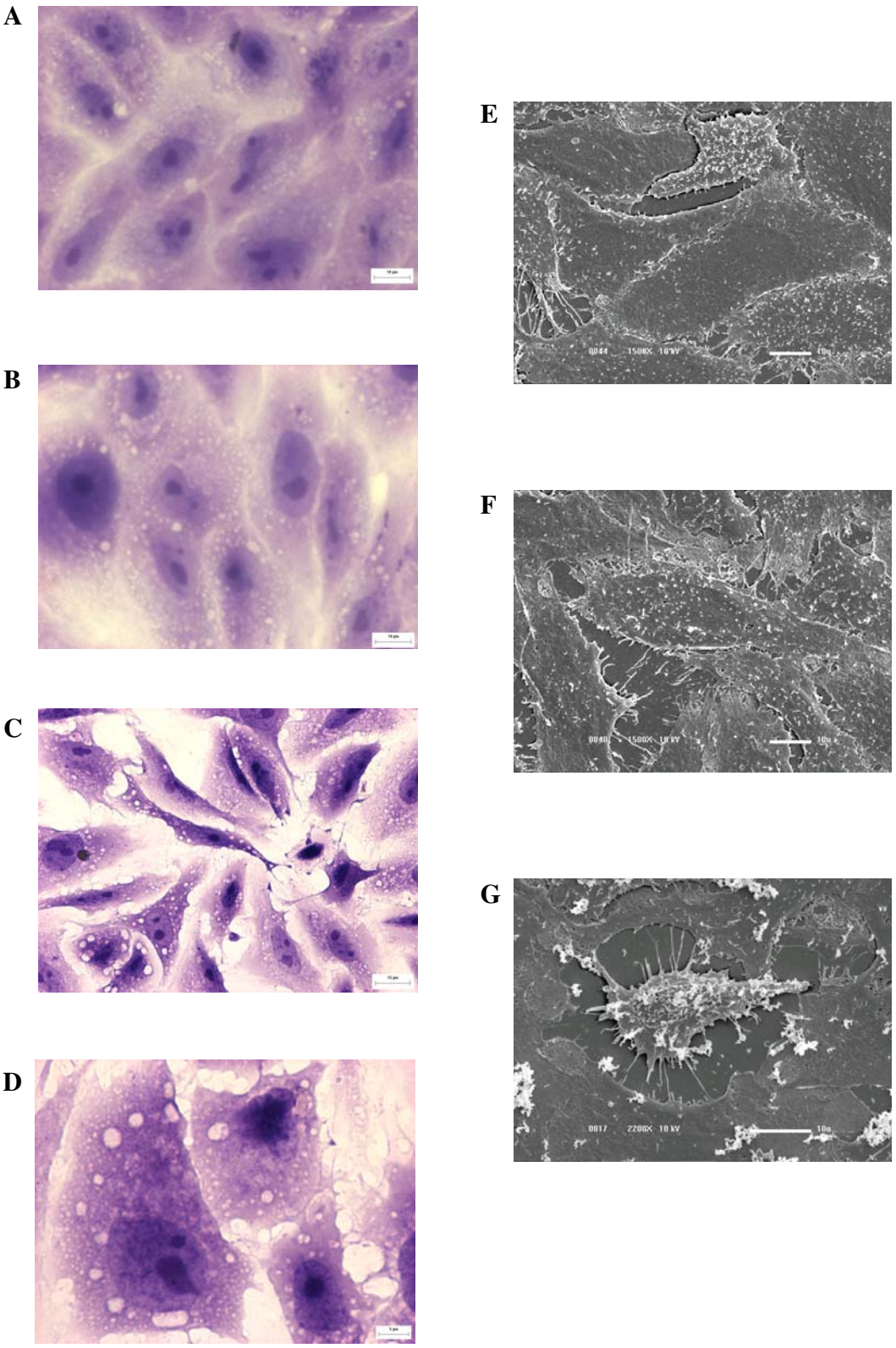
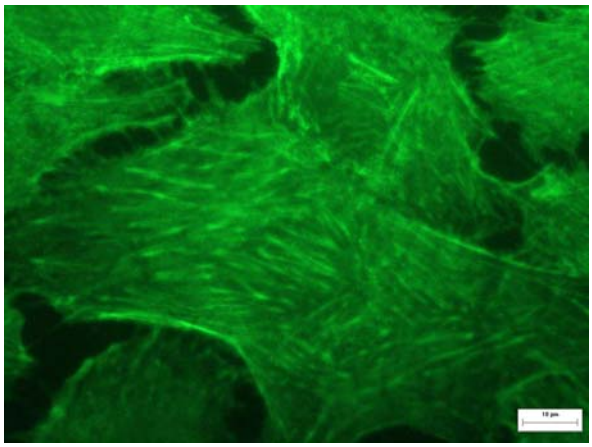
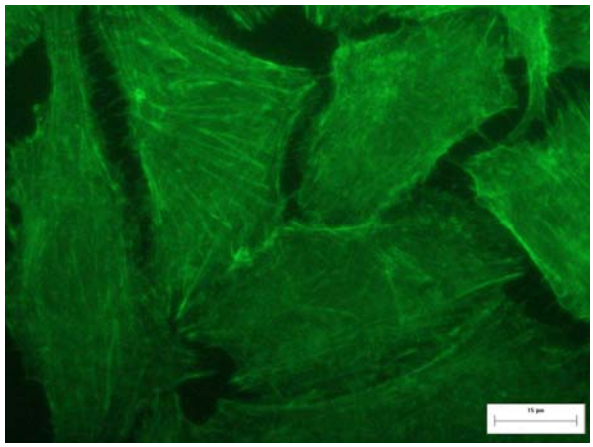


Figure 3

A



B



C

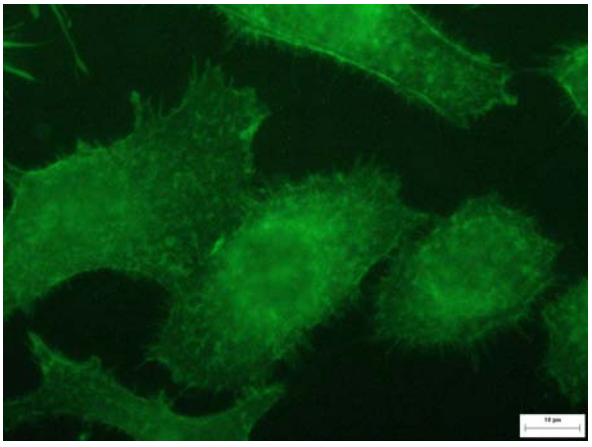


Figure 4

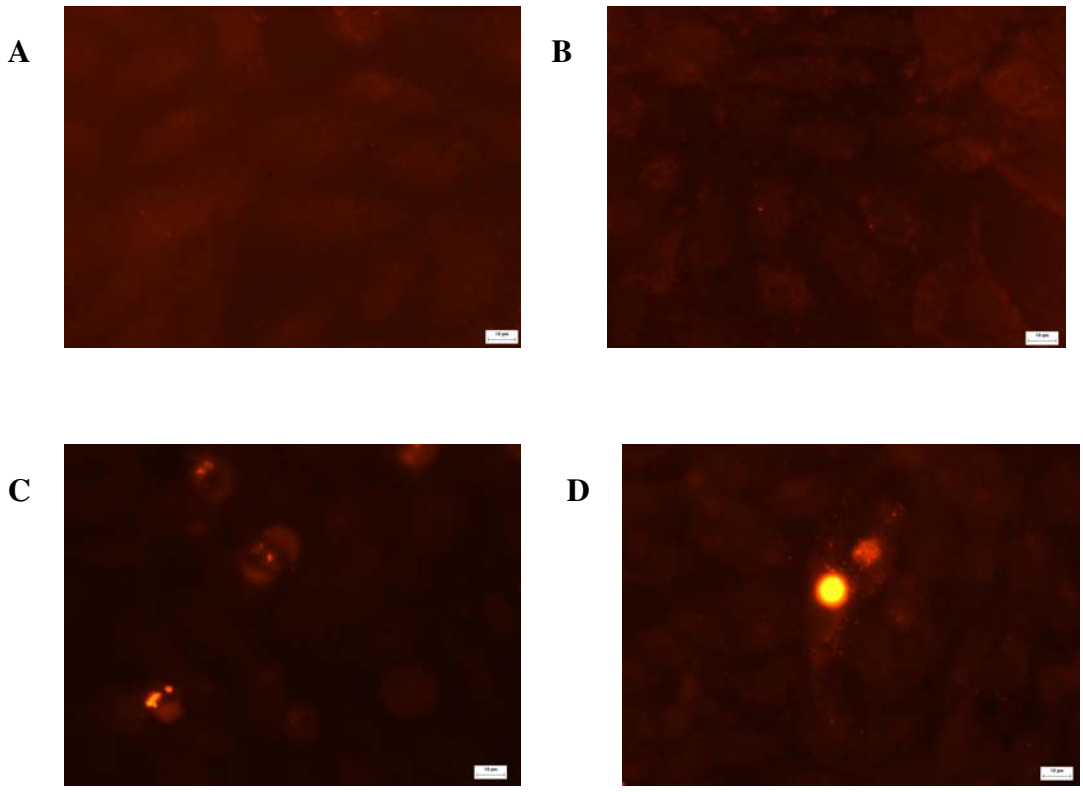


Figure 5