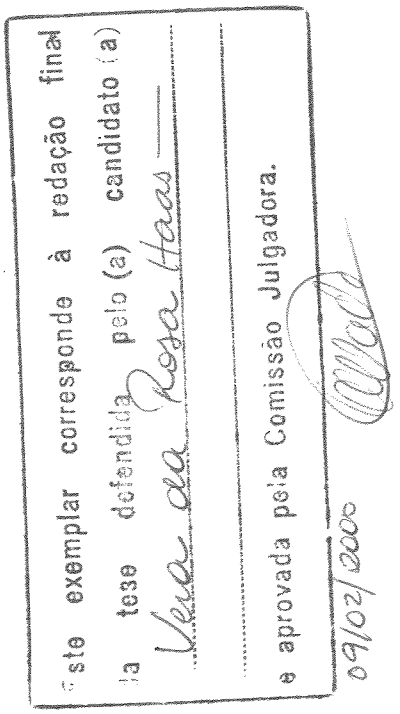
UNIVERSIDADE ESTADUAL DE CAMPINAS

VERA DA ROSA HAAS

ANÁLISE DO COMPORTAMENTO DE CÉLULAS VERO EM GÉIS DE COLÁGENO TIPO I UTILIZANDO A TÉCNICA DE CULTIVO EM SANDUÍCHE



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DEDICATÓRIA

Dedico este trabalho a minha família, que em todos os momentos esteve presente, apoiando, incentivando e que não mediu esforços para a realização deste sonho.

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RESUMO

As células Vero, uma linhagem estabelecida a partir de células renais de macaco verde africano (*Cercopithecus aethiops*), foram mantidas utilizando a técnica de cultivo em sanduíche. Dois tipos de sanduíche foram utilizados vidro/colágeno e colágeno/colágeno, com 10% e 20% de soro fetal bovino (SFB), com o objetivo de se verificar o comportamento das células Vero neste método de cultivo. As células foram avaliadas quanto a morfologia utilizando-se a coloração com hematoxilina-eosina (HE), citoquimicamente por meio de azul de toluidina pH 4,0 (AT) e xylidine Ponceau pH 2,5 (XP), e imunocitoquimicamente com anticorpos anti-colágeno IV e anti-laminina.

As células cultivadas durante um período de 7 dias, no sanduíche vidro-colágeno com 10% e 20% de SFB cresceram em ambos os substratos. As células que permaneceram sobre o vidro apresentaram morfologia variada com predomínio de células alongadas, sendo que em vários locais as lamínulas não apresentavam células mostrando que as mesmas foram capazes de migrar contra a força da gravidade em direção à matriz colagênica. Essas células que migraram para o colágeno I apresentaram formato alongado ou arredondado, sendo que este último predominou quando houve infiltração para o interior do gel colagênico. Observou-se também a contração do mesmo, ou seja, a redução do tamanho e a formação de dobras no gel de colágeno. Nas regiões onde as células não infiltraram, estas cresceram formando uma lâmina basal. No cultivo com a técnica de sanduíche colágeno/colágeno as células aderiram, proliferaram e infiltraram no colágeno existente abaixo e sobre as células, apresentando alterações na forma celular, caracterizando um processo de diferenciação induzido pelo substrato. O colágeno

apresentou contração nas duas camadas do sanduíche. Nas regiões em que não houve infiltração das células Vero para o interior do gel, houve a formação de um tecido com características semelhantes as de um epitélio, com produção de uma lâmina basal, sendo este resultado igual ao observado no sanduíche vidro/colágeno.

Na análise citoquímica, as células Vero, em ambos os sanduíches, quando coradas com AT mostraram núcleos, nucléolos e citoplasma basófilos e em algumas regiões evidenciou-se grânulos basófilos no meio extracelular. Quando as amostras foram coradas com XP , as células Vero apresentaram intensa coloração vermelho-alaranjado, onde pôde ser visualizada uma região acelular mais intensamente corada, entre as células e o colágeno, representando uma lâmina basal, que em algumas regiões era evidente e em outras não. Nesta região, os resultados imunocitoquímicos confirmaram a presença de uma membrana basal rica em colágeno IV e laminina.

Tendo por base estes resultados, conclui-se que as células Vero apresentam comportamento similar quando cultivados sobre o colágeno ou quando cultivadas pela técnica de sanduíche. As células em contato com o colágeno diferenciaram-se e passaram a exibir características de um tecido epitelial, inclusive com a formação de uma lâmina basal, ou se infiltraram para o interior do gel colagênico. O comportamento celular é similar quando se usa 10% ou 20% de SFB no meio de cultivo, sendo que neste último caso, a formação de uma membrana basal foi mais evidente e ocorreu menor infiltração das células para o interior do gel.

ABSTRACT

Vero cells were cultured using the sandwich technique. Two sandwich types, the glass/collagen and collagen/collagen variations, were used with 10% and 20% fetal calf serum (FCS), intending to define Vero cell behavior in these conditions. The cells were morphologically analyzed with hematoxilin-eosin staining, while cytochemical information was obtained with Toluidine blue at pH 4.0 (TB) and xylidine Ponceau at pH 2.5 (XP), and immunocytochemical data with anti-collagen IV and anti-laminin antibodies.

Cells cultured in the glass/collagen sandwich with 10% and 20% FCS grew on both substrata after 7 days. The cells on the glass surface had varied shapes but were predominantly elongated. In some regions of the coverslip, there were no cells, showing that these were able to migrate against gravity into the collagen covering. The migrated cells were elongated or rounded, with a greater number of rounded cells as they moved into the collagen I gel. The contraction, seen as folding and shrinkage of the collagen gel, was observed in this case. In the regions where cells did not penetrate the collagen layer, a basal lamina was produced. During cultivation in the collagen/collagen sandwich, the cells adhered, proliferated and infiltrated into the upper and lower gels, resulting in altered shapes, typical of substrate induced differentiation. Both collagen substrata contracted. In regions where cells did not penetrate the collagen layer, they formed a separating basal lamina as observed in the glass/collagen sandwich.

Cytochemical analysis in both types of sandwich, with TB staining showed basophilic nuclei, nucleoli, and cytoplasm. In some regions, there were also extracellular

basophilic granules. When the samples were XP stained, the Vero cells were stained strongly orange-red, bordered by a more intensely staining, acellular basal lamina, although this layer was not always present. In the basal lamina, the presence of collagen IV and laminin was immunocytochemically confirmed.

With these results, it can be concluded that Vero cells behave similarly when cultured on collagen or with the sandwich technique. Vero cells in contact with collagen undergo differentiation, expressing epithelial cell characteristics, including the formation of a basal lamina, or they may infiltrate into the collagen gel. Cell behavior is similar when 10% or 20% FCS is added to the culture medium, although the basal lamina is more evident, and there is less cellular penetration into the collagen in the higher serum concentration.

I-INTRODUÇÃO

A maioria das células dos diferentes tecidos do organismo está em constante interação com o ambiente extracelular, que nos organismos pluricelulares é preenchido por uma rede tridimensional denominada matriz extracelular (MEC). A matriz extracelular é composta por fibras colagênicas, proteoglicanos, glicosamonoglicanos, glicoproteínas de adesão, além de uma grande variedade de proteínas não colagênicas. Todos esses componentes se agregam de forma especial de modo a conferir as propriedades mecânicas e fisiológicas dos tecidos encontrados nos organismos superiores (VIDAL, 1986).

A MEC apresenta uma grande variedade de funções que extrapolam o mero suporte mecânico, podendo agir como uma barreira física ou filtro seletivo a moléculas solúveis (ADAMS & WATT, 1993) e atuando na modulação de processos biológicos complexos que requerem interações da célula com o ambiente extracelular, como a adesão e a migração celular, os processos de cicatrização, a invasão tumoral e a formação de metástases, o crescimento e a diferenciação celular (WADA & VIDAL, 1991; ADAMS & WATT, 1993).

Uma vez que a MEC é capaz de influenciar o comportamento das células nos diferentes tecidos *in vivo*, resultados semelhantes podem também ser observados *in vitro*. Alterações morfológicas em vários tipos celulares podem ser observadas quando estas são postas em contato com diferentes componentes da MEC em condições de cultura apropriada (KLEINMAN *et al.*, 1981). O uso de componentes da MEC em cultura de células ou tecidos, freqüentemente resulta em um processo de diferenciação destes, o que possibilita a reconstrução de sistemas que mostram comportamento semelhante àqueles observados *in*

vivo. Estes sistemas podem ser úteis para estudos básicos da fisiologia, metabolismo e morfogênese dos tecidos, além de propiciarem estudos com aplicações em farmacologia, toxicologia, etc. (BERTHIAUME *et al.*, 1996).

O colágeno é o componente encontrado em maior quantidade na MEC, e é também uma das proteínas mais abundantes nos vertebrados (HAY, 1981; PIEZ & REDDI, 1984; ALBERTS *et al.*, 1997), existindo até o momento dezoito tipos diferentes de colágeno reconhecidos. Todas as proteínas agrupadas como pertencentes à família do colágeno possuem uma seqüência de aminoácidos *gly-x-y*, sendo que em *x* há uma grande proporção de prolina e em *y* há uma grande proporção de hidroxiprolina, e apresentam estrutura terciária em tripla hélice. O colágeno mostra uma grande diversidade de funções, as quais variam de acordo com o seu tipo , sua distribuição, sua concentração e o grau de agregação desta proteína em um dado tecido (LISENMAYER, 1991; VAN DER REST & GARRONE, 1991).

Vários trabalhos mostram que o colágeno, quando utilizado como substrato para o crescimento celular, pode modular o comportamento das células em cultura. Quando células são cultivadas em colágeno I elas são capazes de manter a configuração original apresentada *in vivo*, e tendem a manter suas características originais de diferenciação (GOSPODAROWICZ *et al.*, 1978). Células isoladas de ácinos pancreáticos, por exemplo, quando cultivadas em géis tridimensionais (géis 3D) de colágeno I, são capazes de se reassociarem assumindo uma polaridade que se assemelha à natural (BENDAYAN *et al.*, 1986). Por outro lado, ácinos isolados têm a habilidade de permanecer com sua morfologia característica normal por mais de um mês quando cultivados em géis tridimensionais (YAUN *et al.*, 1997).

Alguns tipos celulares quando cultivados em géis de colágeno I induzem a contração do substrato e este, por sua vez, interfere na morfologia celular. STOPAK & HARRIS (1982) demonstraram a ocorrência da contração do gel de colágeno cultivando fibroblastos obtidos a partir de embriões de galinha, em géis tridimensionais. Após três dias de cultura, a matriz de colágeno e as células dentro dela, que originalmente ocupavam todo o recipiente, sofreram um processo de contração de modo a formar uma superfície com tamanho de 1/6 da área original. O fenótipo do fibroblasto pode diferir drasticamente, dependendo do tipo de cultura utilizada que pode ser: géis 3D de colágeno tipo I flutuantes ou ancorados. Gel ancorado é a denominação dada ao substrato colagênico que está preso à superfície da placa ou frasco de cultura, enquanto que gel flutuante é o termo normalmente utilizado para quando o substrato colagênico não mais se encontra preso ao frasco de cultura, permanecendo livre no meio de cultivo. A contração de géis flutuantes de colágeno origina um tecido mecanicamente relaxado cujas células tem características morfológicas e de proliferação semelhantes às da derme, as quais são fusiformes, ao passo que matrizes ancoradas desenvolvidas em um tecido tensionado assemelham-se ao tecido de granulação, modificando a morfologia da célula de fusiforme para esférica. Medições de força ou de tensão demonstram que a força exercida pelos fibroblastos em matrizes colagênicas ancoradas é comparável com aquela gerada em ferimentos onde a pele é contraída durante o processo de regeneração ou ainda durante a erupção dental (DELVOYE *et al.*, 1991). Células cultivadas em matrizes colagênicas ancoradas não mostram uma parada em seu crescimento, mas continuam a sintetizar DNA e a proliferar, aumentando o número de células e preenchendo o gel (NISHIYAMA *et al.*, 1989).

Experimentos realizados com fibroblastos demonstraram que quando estes são

cultivados dentro de géis flutuantes de colágeno I, fazem com que os mesmos exerçam força de tração sobre a matriz que os rodeia resultando em uma reorganização e condensação da matriz colagênica. Quando mudanças estruturais são induzidas no colágeno, as células cultivadas no interior deste podem apresentar alterações em sua morfologia, que freqüentemente passa de alongada para esférica (Mc CARTHY *et al.*, 1996).

O fenômeno de contração do gel de colágeno até hoje não é bem compreendido, mas sabe-se que essa contração está intimamente relacionada à atividade funcional do citoesqueleto das células que são cultivadas sobre ele (BELL *et al.*, 1979). Sabe-se também que esta contração é estimulada pelo soro fetal bovino e/ou por produtos secretados pelas células cultivadas na presença de soro (GUIDRY & GRINNELL, 1985).

A contração do gel de colágeno envolve a reorganização das fibrilas de colágeno e condensação da MEC. De importância óbvia na remodelagem do colágeno I estão as interações das células com elementos da MEC, que se processa através de receptores. Destes receptores se destaca a classe das integrinas (ALBERTS *et al.*, 1997; GULBERG *et al.*, 1990. *Apud* WATSON *et al.*, 1998). As integrinas são proteínas integrais da membrana plasmática, compostas por duas subunidades denominadas α e β. Essas subunidades apresentam várias isoformas sendo que a associação diferencial dessas isoformas origina a especificidade dos diferentes receptores aos vários componentes da MEC (ALBERTS *et al.*, 1997). Muitos estudos têm indicado que o complexo integrina $\alpha_2\beta_1$ exerce importante papel na contração do gel de colágeno promovido por células musculares lisas oriundas de vasos sangüíneos e por células osteogênicas (SCHIRO *et al.*, 1991). Estudos realizados por

POZZI *et al.* (1998) com fibroblastos dérmicos de rato comprovaram que a integrina $\alpha_1\beta_1$ tem sítio para ligação ao colágeno, controla a proliferação de fibroblastos dérmicos tanto *in vivo* quanto *in vitro*, e que a sobrevida das células e a sua proliferação no substrato colagênico, são mediados especificamente e unicamente por esta proteína.

A contração do gel colágeno *in vitro* depende do soro fetal bovino, um dos constituintes do meio de cultura. O soro fetal bovino é rico em vários fatores que podem estimular o crescimento e a adesão de células em cultura. Entre esses fatores, os que mais se destacam são: fibronectina, vitronectina e trombospondina. Fibroblastos de pele humana quando cultivados sobre géis de colágeno I em meio suplementado com soro fetal bovino, onde vários fatores de adesão foram removidos, demonstraram que a contração do gel é dependente desses componentes séricos. A supressão da contração foi observada quando a fibronectina foi removida do soro, mostrando que esta proteína é fundamental para este processo. Quando os mesmos fibroblastos foram cultivados em géis colagênicos em um meio sem soro, totalmente desprovido de fibronectina, a contração do substrato também não ocorreu. Se, no entanto, a fibronectina fosse adicionada ao meio, a contração do gel de colágeno poderia ser observada. Esses resultados demonstram que a contração do gel de colágeno em cultura de fibroblastos de pele humana é dependente de fibronectina e que outros fatores protéicos sintetizados pelas células ou contidos no soro são também necessários (GILLERY *et al.*, 1986). Fibronectina é uma proteína multifuncional de interação celular, sendo um dímero composto de duas subunidades muito grandes unidas por um par de pontes de bissulfeto próximos às suas extremidades C-terminais. Cada subunidade é dobrada em uma série de domínios funcionais diferentes, em forma de bastão,

separados por regiões de cadeias flexíveis. Os domínios por sua vez, consistem de módulos menores, cada um repetido em série e normalmente codificado por diferentes exons. A fibronectina apresenta domínios de ligação compostos por Arg-Gly-Asp (RGD), que podem ligar-se ao colágeno, a heparina e também a superfície de vários tipos celulares. Os receptores para fibronectina nas membranas plasmáticas são as integrinas. A fibronectina é produzida por uma grande variedade de tipos celulares, exercendo diversas atividades entre elas adesão e migração (YAMADA, 1991; ALBERTS *et al.*, 1997).

Além do cultivo de células em géis de colágeno tridimensionais, alguns trabalhos mostram que é possível o cultivo das células entre duas camadas distintas de géis de colágeno I. Esse sistema de cultura ficou conhecido como "*configuração em sanduíche*". Células endoteliais e vários tipos de células epiteliais, após terem formado uma camada única sobre uma superfície simples de um substrato de gel de colágeno, reorganizam-se em estruturas acinares quando são recobertas com uma segunda camada de matriz extracelular, especialmente de colágeno I (BERTHIAUME *et al.*, 1996). Hepatócitos isolados colocados em sanduíche de colágeno I exibem um aumento gradual na expressão da função hepática específica durante a primeira semana de cultura. Reciprocamente, as mesmas células colocadas sobre uma superfície bidimensional do colágeno I progressivamente param de expressar estas funções e perdem a viabilidade. Após a primeira semana de cultivo a expressão das funções hepático-específicas de hepatócitos cultivados em sanduíche de colágeno tipo I fica estável, permanecendo assim por várias semanas (BERTHIAUME *et al.*, 1996).

As células Vero, uma linhagem celular estabelecida a partir de células renais do macaco verde africano (*Cercopithecus aethiops*), tem padrão morfológico e de crescimento

característico em cultura. Esta linhagem quando cultivada em substrato de vidro ou plástico apresenta células semelhantes a fibroblastos, as quais crescem em monocamada até atingirem a confluência (LEE & ENGELBARDT, 1977). Uma vez atingida a confluência, essas células podem entrar em senescência e degeneração se não for efetuado o subcultivo, pois apresentam inibição por contato (GENARI & WADA, 1995; GENARI *et al.*, 1996).

WADA & VIDAL (1991), observaram que células Vero cultivadas em esponjas de colágeno tipo I liofilizado sofreram alterações morfológicas, passando de uma forma poligonal para uma alongada, com vários prolongamentos. Essas células foram capazes de alterar a matriz colagênica sob a qual cresceram, provavelmente através de secreção de colagenase. Esses mesmos autores descreveram que as células Vero crescem e formam arranjos tridimensionais que, segundo eles, demonstraria a tendência das células à reconstrução do tecido do qual se originaram. Essas células quando cultivadas no gel de colágeno I adquirem um padrão de crescimento diferenciado quando comparado ao crescimento celular em lamínulas. No substrato colagênico essas células migram para o interior do gel (MARIA, 1994) ou formam múltiplas camadas celulares de acordo com variações nas condições de cultura (SANTOS, 1996). Santos demonstrou que o crescimento para o interior do gel era dependente da produção de colagenase, pois estas células crescendo na presença de inibidores desta enzima (dexametasona e altas concentrações de soro fetal bovino) não penetravam no gel de colágeno e formavam uma estrutura com várias camadas celulares, semelhante a um epitélio estratificado, com a produção de lâmina basal. Quando mantidas na superfície do substrato as células em contato com o colágeno exibem um aspecto poligonal e as células mais superficiais apresentam aspecto achatado (SANTOS, 1996). Foi observado também que essas células promovem a reorientação das

fibras colagênicas, com conseqüente contração do gel, com o decorrer do tempo de cultivo (SANTOS, 1996; MARIA & WADA, 1997).

Uma vez que as células Vero se comportam de maneira diferente em função das alterações nas condições de cultivo, esse trabalho se propõe a analisar a resposta das células Vero quando submetidas ao cultivo pela técnica de sanduíche de colágeno.

II – OBJETIVOS

O objetivo geral deste trabalho foi verificar o comportamento das células Vero submetidas à duas técnica de cultivo em Sanduíche: vidro-colágeno tipo I e colágeno tipo I – colágeno tipo I.

Os objetivos específicos foram:

1. Verificar alterações morfológicas induzidas nas células;
2. Verificar alterações fisiológicas através de técnicas citoquímicas e imunocitoquímicas
3. Observar o efeito da concentração de soro fetal bovino (SFB) nas alterações morfológicas e fisiológicas celulares.

III - TRABALHO A SER SUBMETIDO À PUBLICAÇÃO

BEHAVIOR OF VERO CELLS CULTURED IN COLLAGEN I WITH THE SANDWICH TECHNIQUE

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Running title: Vero cells cultured in collagen I sandwich

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Abstract.

Vero cells, a fibroblastic lineage derived from fibroblastic kidney cells of African green monkey, were cultivated with the sandwich technique: glass coverslip/collagen and collagen/collagen with varied fetal calf serum concentrations in the culture medium. When cultured on coverslips, then receiving a type I collagen gel layer on top, the cells migrated from the coverslip to the collagen layer. However, when the cells were cultivated on collagen followed by a covering of type I collagen gel, the cells migrated into both collagen layers. Celullar morphology was very similar, independent of the type of sandwich and serum concentration used. Cells in contact with collagen either migrated into the layer or formed a basal lamina separating them from the collagen matrix. The formation of a basal lamina with laminin and collagen IV deposition was most noticeable when 20% fetal calf serum (FCS) was used in the culture medium. Cellular infiltration into the collagen gel was most evident with the use of 10% FCS. The gel contraction was similar for the two serum concentrations employed.

Introduction

The cell-extracellular matrix interactions play a critical role in cell differentiation and morphogenesis that occurs in the developing embryo (Lin and Bissel 1993; Hay 1993, 1995). Signals from the extracellular matrix (ECM) can modulate many parameters of cell behavior such as growth, proliferation, migration and synthesis of new ECM molecules (Schaefer *et al.* 1996). Collagen, because of its abundance, is the ECM element that has been studied in greatest detail (Hay 1993, 1995).

Cell types of mesenchymal origin when cultured on three-dimensional collagen gels are capable of invading the collagen matrix (Schor 1980; Hay 1993, 1995). Some authors have previously reported that fibroblastic cells are capable of migrating into collagen gels and of forming structures with characteristics similar to a loose connective tissue (Stopak and Harris 1982; Maria and Wada 1997). Utilizing some special culture conditions, it is also possible to obtain structures similar to a dense and modelled connective tissue (Stopak and Harris 1982). On the other hand, epithelial cells cultured on collagen substrata stay on the surface, as a stable epithelia, normally with the production of a basement membrane (Hay 1993, 1995). Thus, the culture of cells on/in collagen matrices have been used to create *in vitro* three-dimensional structures that could simulate some characteristics of many tissues for cytotoxicity and pharmacological tests, or to make prothesis for experimental orthopedic repair (Taguchi *et al.*, 1997; Sirica and Gainey, 1997; Young *et al.*, 1998; O'Connor, 1999; Schoop *et al.*, 1999).

The cell-ECM interactions, the expression of new ECM components or receptors to these elements, are modulated by a variety of physiological factors, such as hormones and

growth factors. In culture systems, the serum is an agent commonly used and capable of promoting changes in cell growth patterns. The fetal calf serum (FCS) is a heterogeneous mixture, rich in hormones and growth factors, which is necessary and normally utilized to induce the increase of cell division rate on *in vitro* assays (Pledger *et al.* 1984). Variations of serum concentration could induce changes in the behavior of cell cultured on collagen. For example, the capacity of cells to promote collagen gel contraction is proportional to serum concentration. The serum addition in cell culture on collagen matrices promotes a kind of cell-ECM interaction that results in an increase of cell synthesis capacity (Schor 1980; Andujar *et al.*, 1992; Shin *et al.*, 1999). On the other hand, when FCS is not used, there is a decrease in cell proliferation and, in some cases, cell death (Andujar *et al.*, 1992).

The objective of the present study is to evaluate the behavior of Vero cells cultured in collagen I gel using the sandwich technique and with variations in FCS concentration.

Materials and methods

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were used. The cells were cultured in Ham-F10 medium (Sigma Chemical Company, St. Louis, MO, USA) containing 10% FCS (Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are a lineage that is an international standard recommended for studies of

cytotoxicity and for cell-substratum interactions in biomaterial research (Kirkpatrick, 1992).

Preparation of substratum and cell culture on collagen I gel with the sandwich technique

The type I collagen gel was prepared from 3 month old rat tail tendons by a modification of the method described by Schor (1980). The concentration was adjusted to 4.0-4.5mg/ml. Three-dimensional gels were prepared in a 24 wells cell culture plate (Corning glass works) rapidly mixing 0.72 ml of collagen solution with 0.4ml of 10 times concentrated Ham-F10 medium and 0.4 ml of 4.4% $NaHCO_3$. The solution was incubated at 37°C for 2 h. After detachment with trypsin-EDTA (Sigma), 1.2 x 10^6 cell/ml were inoculated on the collagen I gel in Ham-F10 medium with 10% of FCS and on the glass coverslip for 6hs. After this incubation time, the samples were washed with Ham F-10 medium and then were cultured in Ham F-10 with 10% or with 20% FCS. Samples of cell growth on coverlips and on collagen I gels were harvested after 48hs of culture and fixed in Karnovsky's fixative. The glass coverslips were washed, and air dried. Some samples cultured in collagen gels were dehydrated through a graded ethanol series, cleared in xylene, and embedded in Paraplast from which 4μm thick sections were obtained for morphologycal, cytochemical and immunocytochemical analysis. Other samples received another layer of collagen gel prepared as described. This technique resulted in two types of sandwich: glass coverslip/collagen and collagen/collagen. These materials were incubated for 5 days at 37° and then fixed and imbedded in the same conditions described above.

Morphological, cytochemical and immunocytochemical analysis

For morphological analysis the samples were stained with hematoxilin-eosin (HE). For the cytochemical study we used toluidine blue at pH 4.0 (TB, by Sigma) and xylidine Ponceau pH 2.5 (XP by Sigma). In the immunocytochemical test we used monoclonal primary anti-collagen IV (Sigma: clone COL-94, dilution 1:500) obtained from mouse ascidic fluid and anti-laminin antibodies (Sigma: L-9393, dilution 1:30). Secondary rabbit anti-mouse IgG peroxidase conjugate antibodies (Sigma: dilution 1:200) were also used. After washing in 0.1M PBS, pH 7.4, at 37°C, the endogenous peroxidase was removed with 0.3% H_2O_2 in 100% methanol, for 15min. The material was preincubated for 10min in 1% bovine serum albumin (BSA, by Sigma) in PBS to block nonspecific staining. The preparations were then incubated overnight in a moist chamber at 4°C with monoclonal anti-collagen IV or anti-laminin. After washing in PBS at 37°C, the antigenic sites were observed using indirect marking with peroxidase-conjugated anti-mouse IgG and diaminobenzidine (Sigma) treatment (diaminobenzidine, in 0.06 M phosphate buffer at pH 7.2 and 5µl of 30% H_2O_2). Control experiments were performed with the omission of primary antibodies.

Results

Morphological analysis

The cells maintained on glass coverslips for two days were predominantly polygonal and shape but also presented rounded and star-shaped morphology. The monolayer was

subconfluent and dividing cells were observed. The morphology of cells cultivated in 10 and 20% FCS was the same (fig. 1a, e). With the highest concentration, the cell monolayer was confluent.

Cells cultured over collagen for two days were more elongated, but polygonal cells were predominant. Some regions in which the cells grew in a single layer were found, while in others there were double or triple layers (fig. 1b, f). In some regions cells were seen within the collagen gel. The collagen surface showed many irregular pleats or folds, which are characteristic of contracted collagen gels. This was similar for gels in both FCS concentrations. Although the cells that migrated into the collagen matrix were not quantified, fewer cells could be seen in the gels cultivated with 20% FCS.

The cells cultivated with the sandwich coverslip/collagen technique, for seven days in 10% FCS were analysed in relation to two aspects: the cells that remained on the coverslip and those that migrated to the type I collagen matrix. The cells found on the glass coverslip had different shapes, although they were predominantly elongated. There were some regions without cells in spite of the seven day culture period, indicating that migration had occurred into the collagen gel placed above them. The cells that migrated to this layer were elongated and arranged themselves in a layer covering the collagen (fig. 1c, g) but still in contact with the cell monolayer on the coverslip.

When the cells migrated into the gel they appeared rounded, as also happened in regions where the cells formed two or more layers. The collagen surface in contact with the cells was contracted.

In the culture medium containing 20% fetal calf serum, the analysis of cells on the coverslip or of those which migrated into the collagen showed that they are very similar to the same cells cultured in medium with 10% serum (fig. 1g).

The analysis of the Vero cells cultured by the collagen/collagen sandwich tecnique for seven days in medium with 10 or 20 % fetal calf serum showed cells organized in one or more layers (fig. 1d, h). The cells in monolayers were more rounded. The same cell characteristics were observed in the layer that covered the upper collagen gel and in the cells in the lower collagen layer. Cell migration ocurred between the two layers and the collagen was strongly contracted in both layers. There was no marked difference in relation to the morphology and gel contraction in the two different concentrations of serum used.

Cytochemical analysis

In samples harvested after 48h of culture, the cells cultured on glass coverslips showed basoplilic staining of the citoplasm, nucleus, and nucleolus (fig. 2a, e). The cells cultured over collagen in medium containing 10% FCS formed a layer of basophilic cells on the collagen substratum stained by TB (fig 2b, c, d thin arrow). The collagen gel did not stain. TB staining showed basophilic cells invading the collagen gel in the sandwich glass coverslip/collagen and collagen/collagen. We also found in those samples some basophilic granulations surrounding the cells in the collagen gel (fig. 2b, c, d thin arrow). We obtained similar results when cells was cultured with 20% FCS (fig. 2, g, h). In this cases, there was a decrease in cell migration into the substrata. In these samples, we did not find deposition of extracellular granulations in the collagen substratum, but we could see an acellular

material between the cells and the collagen gel. When stained with XP this layer was stained, indicating that it was a proteinaceous material (fig. 3b, c, d).

This proteinaceous layer was not found in all the experimental conditions tested in this study. In the medium with 20% fetal calf serum it was more intense and continuous (fig. 3f, g). In the 10% fetal calf serum cultures, the layer appeared interrupted in many regions, exactly the regions in which cells migrated into the gel (fig. 3b, c, d).

Immunocytochemical analysis

During the first 48hs culture, we found a diffuse laminin band between the cell layer and the collagen type I gel in two techniques used in this work (fig. 4) but we found a collagen IV band between the cell layer and the collagen I gel (fig. 5). This band was not constant along the contact layer between cells and collagen matrix when the cells were cultivated with 10% of fetal calf serum (FCS). When we used 20% FCS and the cell invasion was blocked, we could see a continuous layer rich in laminin (fig.4d, e, f) and in collagen IV (fig. 5d, e, f, g) between the cells and collagen matrix.

Discussion

In the present work the Vero cells cultured on the glass coverslips/collagen I and collagen I/collagen I gel sandwich techniques showed a decrease in proliferation rate when we compared the cells grown on collagen for 2 days or for 7 days. This result can be explained by the fact that collagen matrices induce the cells to differentiate (Stopak and Harris, 1982; Hay, 1993, 1995; Maria and Wada, 1997; O'Connor, 1999) and there is an inverse relation

of cell differentiation and proliferation. This assay did not show that the cell proliferation is proportional to the FCS concentration. This result was expected by us because collagen induces cell differentiation as well as fetal calf serum used in the culture medium to promote cell proliferation for almost all cell types (Pledger *et al.*, 1984; Freshney, 1994).

Even before cell adhesion was completed, the cells were capable of interacting with the substratum which can promote alteration of cell growth and their differentiation pattern. The cells that grew in medium with 10% FCS were not only capable of adhesion to the substrate but also of migrating into the collagen matrix. In the collagen gels, the cells were capable of secretion and deposition of many extracellular granules. These granulations were stained by TB. The TB can stain PO_4^-, SO_3^- and COO^- anions and at pH 4.0, the PO_4^- anions are found only in DNA or RNA, and the SO_3^- and/or COO^- groups only in glycosaminoglycans (Lison, 1960; Mello, 1997). This indicated that the basophilic extracellular granulations observed in collagen I gel, are probably due to deposition of glycosaminoglycans. Extracellular granulations in the collagen gels are also stained with Xylidine Ponceau pH 2.5. At this pH, XP stains all protein NH_3^+ groups, indicating that these granulations are proteinaceous. Apparently, the samples cultured with 10% of FCS showed more granulations than samples cultured with 20% FCS. By comparing these findings we conclude that cells in the collagen matrix synthetize ECM glycosaminoglycans and/or proteoglycans.

The capacity of Vero cells to secrete proteoglycans and/or glycosaminoglycans in collagen gel was previously described by Maria and Wada (1997). In this work, our results corroborate with findings obtained by them . This suggestion makes sense because the serum addition in cell culture on collagen matrices promotes a kind of cell-ECM interaction

that results in an increase in cell proliferation and synthesis capacity (Schor 1980; Andujar *et al.*, 1992; Shin *et al.*, 1999). When FCS is not used, there is a decrease of cell synthesis and proliferation which, in some cases, could lead to cell death (Andujar *et al.*, 1992). But the *in vitro* synthesis and deposition of ECM components by fibroblastic cells in the absence of serum has also been reported (Golombick *et al.*, 1995). With the serum supplementation in culture medium, human embryonic skin fibroblast cells, were still capable of synthesis of glycosaminoglycans and proteoglycans *in vitro* (Coster *et al.*, 1979).

The cells cultured with high serum concentration, showed a different behavior on the collagen I gel. In these cases, the cells were eneabled to invade the collagen matrix, and we found the resulting many-layered cell structures, with round cells in the basal layer and, in some cases, flattened cells in higher layers. The cell layers were rich in anionic groups, as shown by TB, and in proteinaceous material, as indicated by XP staining. We did not find extracellular granulations in the collagen I gel, but we found a band between the cells and the substratum. This cytochemical data suggests the formation of a structure similar to a basement membrane. Our suggestions were corroborated by the immunocytochemical detection of collagen IV and laminin in this region. Fibroblastic cells in culture have the capacity to invade the collagen gel. On the other hand, when epithelial cells are cultured in the same substrata, they do not migrate into the collagen, staying on the surface of the gel (Greenburg & Hay, 1982; Hay, 1995; Pilcher *et al.*,1997). The presence of a basement membrane has great importance in the induction and maintenance of a differentiated epithelial cell phenotype (Hay, 1993, 1995; Schoop *et al.*, 1999). Thus, our results show that when Vero cells are cultured with the sandwich technique, in site of their fibroblastic

lineage, they show some epithelial characteristics, forming a basal lamina between the cells and the collagen gel on which they were originally deposited.

In our experiments, we also observed that the Vero cells cultured on collagen, in the coverslip/collagen or with the collagen/collagen sandwich technique were able to contract the collagen gel, considerably reducing their diameter. This fact was also observed by Santos (1996) and Maria & Wada (1997) with Vero cells.

After 2 days in culture, collagen gel contraction was observed and resulted in a folded gel surface and a tighter arrangement of collagen fibers. The collagen gels in a conventional culture plate remain attached to the culture plate surface, but detachment and floating of this gel in medium was observed and occurs due to a contraction process that was demonstrated by the reduction in diameter of the gel surface. The cells reorganize collagen as they attempt to move throught the matrix, a process called tractional remodeling (Harris *et al.*,1981).

The contraction phenomenon is seen as a model system that mirrors the contraction observed during developement and repair (Bell *et al.*, 1979; Ehrlich, 1987; Guidry & Grinnell, 1985; Tingstrom *el al.*, 1992). However, contration in culture depends on the gel type, its concentration and mainly the cell type employed (Bell *et al.*,1979).

Reorganization and alignment of collagen fibrils is believed to occur as a result of the tension exerted by cells during their migration and infiltration into the gels. Bell *et al.* (1979) associated similar results to the patching of collagen fibrils after water loss. According to Guidry and Grinnell (1985) collagen gel contraction by fibroblasts involves the synthesis and degradation of collagen, without enzymatic alteration or natural collagen cross-linking. According to the authors the contraction will only occur on a matrix capable

of propagating the contractile strength that fibroblasts generate, which makes the collagen gel an excellent substrate for culture tests on contraction. The formation of a dermal-like structure after substrate contraction has been described (Bell *et al*, 1979).

Contracted collagen substrates with fibroblasts were transplanted and reintegrated as dermic equivalents with success (Hull *et al.*,1983). Guidry and Hook (1991) reported that colagen substrate contraction by fibroblasts, besides being a model system for the research of fibroblasts *in vitro,* is also an interesting model system for research of developing connective tissue, wound healing, and fibrosis.

Various reports in the literature show that collagen gel contractions related to the presence of fetal calf serum. Theoretically, based on the results of other authors (Allen & Schor,1983; Guidry & Grinnell,1985; Gillery *et al.*,1986; Maria & Wada, 1997), a greater gel contraction occurred when the cell culture was carried out in 20% FCS. Our results show that the collagen contraction was similar, independent of the serum concentration. It is possible that there is an optimum concentration for the FCS in relation to collagen contraction. If this really occurs, this optimum concentration should be around 10% FCS. There is no information to explain this behavior in previous study, since the effect of different concentrations of FCS, added to the culture medium, on collagen contraction has not been studied in relation to collagen substratum contraction. Only the contracting of collagen in relation to presence or absence of FCS has been studied (Allen & Schor, 1983; Guidry & Grinnell, 1985; Tomasek *et al.*,1992; Reed *et al.*,1994; Maria & Wada,1997). The only reference to a comparative analysis of increasing concentrations of FCS in relation to 3D collagen substrates was found in the work of Gillery *et al.* (1986). However, these authors did not use concentrations higher than 10% FCS in their culture mediums.

Therefore, we can conclude that Vero cells behave in a similar manner when cultivated over collagen or with the sandwich technique. Independent of the culture technique employed, the cells either infiltrate into the collagen layer, or deposit a laminin and collagen IV layer between themselves and the substrate. The deposition of these proteins is more evident in cultures using 20% FCS, while migration of cells into the collagen gels was more evident in the cultures with 10% FCS medium.

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Figure legends

Figure 1- Hematoxilin-Eosin staining. Figures b-d, f-h are transverse sections of gels. A = 400X.

a, e Cells cultured on glass coverslips for two days. The cells were polygonal in shape, the nuclei with loose chromatin and a clearly stained nucleolus. The morphology of cells cultured in 10 **(a)** or 20 % FCS **(e)** was the same.

b, f Cells cultured over collagen for two days. In some regions the cells grew in a single layer, while in others there were double or triple layers.Cells could be seen in the gels (*) cultivated with 10% FCS **(b);**fewer cells could be seen in gels (*) cultivated with 20% FCS **(f)**. The collagen surfaces showed many irregular pleats or folds, which are characteristic of contracted collagen gels.

c, g Cells cultivated with the coverslip/collagen sandwich technique for seven days. The cells that migrated to the type I collagen gels were elongated and arranged in a single layer. When the cells migrated into the gel (*) they appeared rounded. The cells cultivated with 10 **(c)** or 20% FCS **(g)** showed a similar morphology.

d, h Cells cultured with the collagen/collagen sandwich technique for seven days. The cells were organized in one or more layers. The cells in monolayers were more elongated. Round cells also were seen. The same characteristics were observed for in two layers of collagen gels. There was no marked difference in relation to the morphology and gel contraction in the two different concentrations of serum used. **d)** 10% FCS; **h)** 20% FCS.

b, c, d Cells cultured with collagen in medium containing 10% FCS. In some regions, a clear XP positive separation between cells and the adjacent collagen can be observed, while in other regions, XP positive granulations can be seen (arrow). This aspect was similar in the two culture conditions. **b)** Cells on collagen; **c)** Coverslip/collagen sandwich technique; **d)**Collagen/collagen sandwich technique.

f, g, h Cells cultured over collagen in medium containing 20% FCS. Results were very similar to those described above. With more continuous regions of XP positive bands between the cells and the collagen. **f)** Cells grown on collagen; **g)** Coverslip/collagen sandwich technique; **h)** Collagen/collagen sandwich technique.Collagen gel is represented by (*).

Figure 4- Immunocytochemistry for laminin. A = 400X

a, b, c Cells cultured in 10% fetal calf serum (FCS). The reaction for laminin is diffuse and does not form a continuous layer between the cells and the type I collagen gel, in all experimental conditions (arrow). **a)** collagen substratum, 2 days culture; **b)** Glass coverslip/collagen sandwich technique, 7 days culture; **c)** Collagen/collagen sandwich technique, 7 days culture.Collagen gel (*).

d, e, f Cells cultured in medium containing 20% FCS. The reaction for laminin is more evident, marking a clear band (arrow), interrupted at a few intervals, which correspond to regions of cell migration into the collagen gel (*). This staining was similar for all experimental conditions. **d)** Collagen substratum, 2 days culture; **e)**

Glass coverslip/ collagen sandwich, 7 days culture; **f)** Collagen/collagen sandwich. Collagen gel is represented by (*).

Figure 5- Immunocytochemistry for type IV collagen. A = 400X.

a, b, c Cells cultivated in 10% FCS. A layer of collagen IV positive material (arrow) was deposited between the cells and the collagen gel. In these culture conditions, this layer is less evident than with 20% FCS (described below). **a)** Collagen substratum, 2 days culture; **b)** Coverslip/collagen sandwich technique, 7 days culture; **c)** Collagen/ collagen sandwich technique, 7 days culture.

d, e, f, g Cells cultivated in 20% FCS. A clear collagen IV positive layer (arrow) is present and appears in all culture conditions. **d)** Collagen substrate, 2 days culture; **e)** Coverslip/collagen sandwich technique, 7 days culture; **f)** and **g)** Collagen/collagen sandwich technique, 7 days culture. Figure **f** A = 100X. Collagen gel is represented by (*) in all pictures.

FIGURE 1

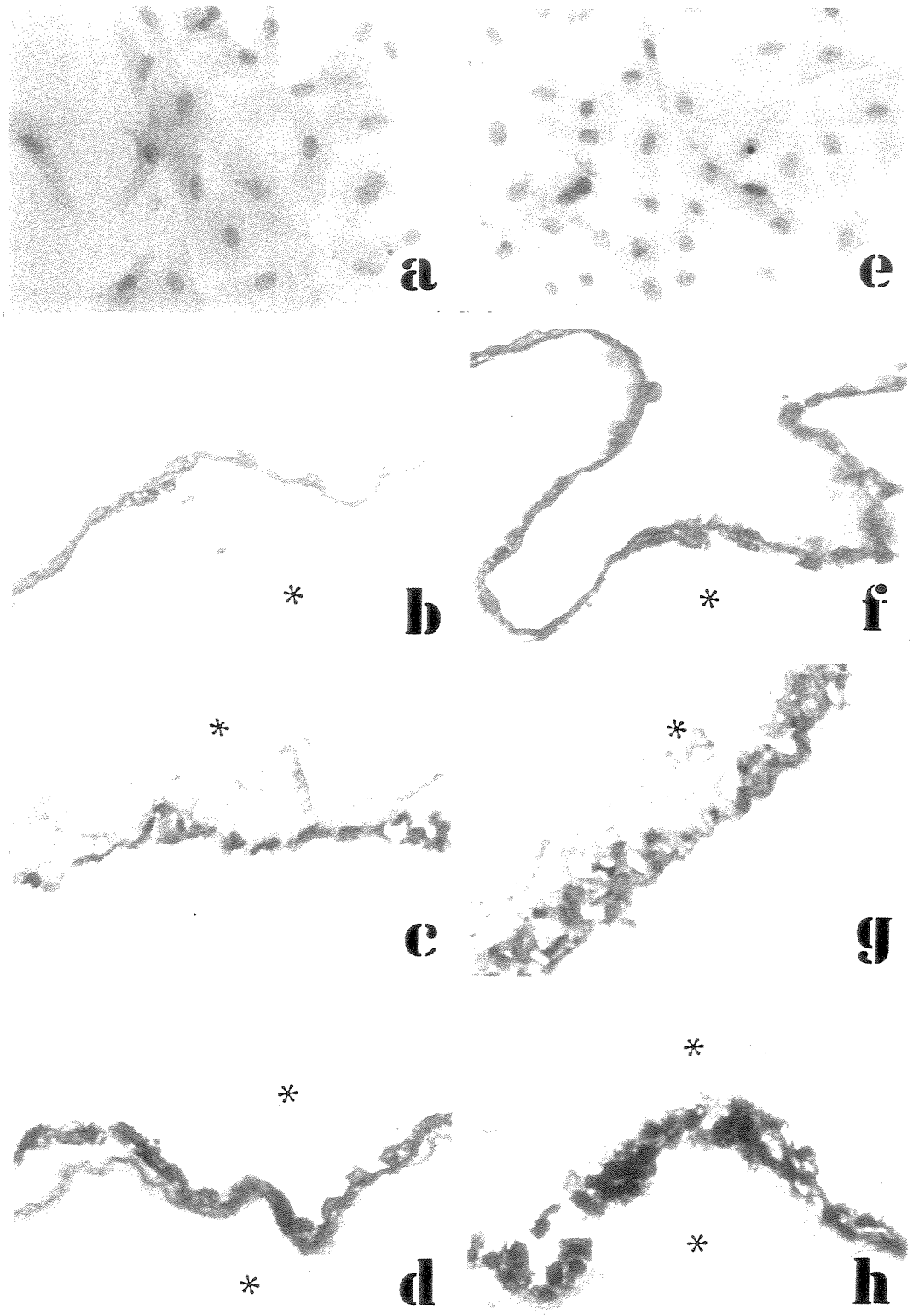


FIGURE 2

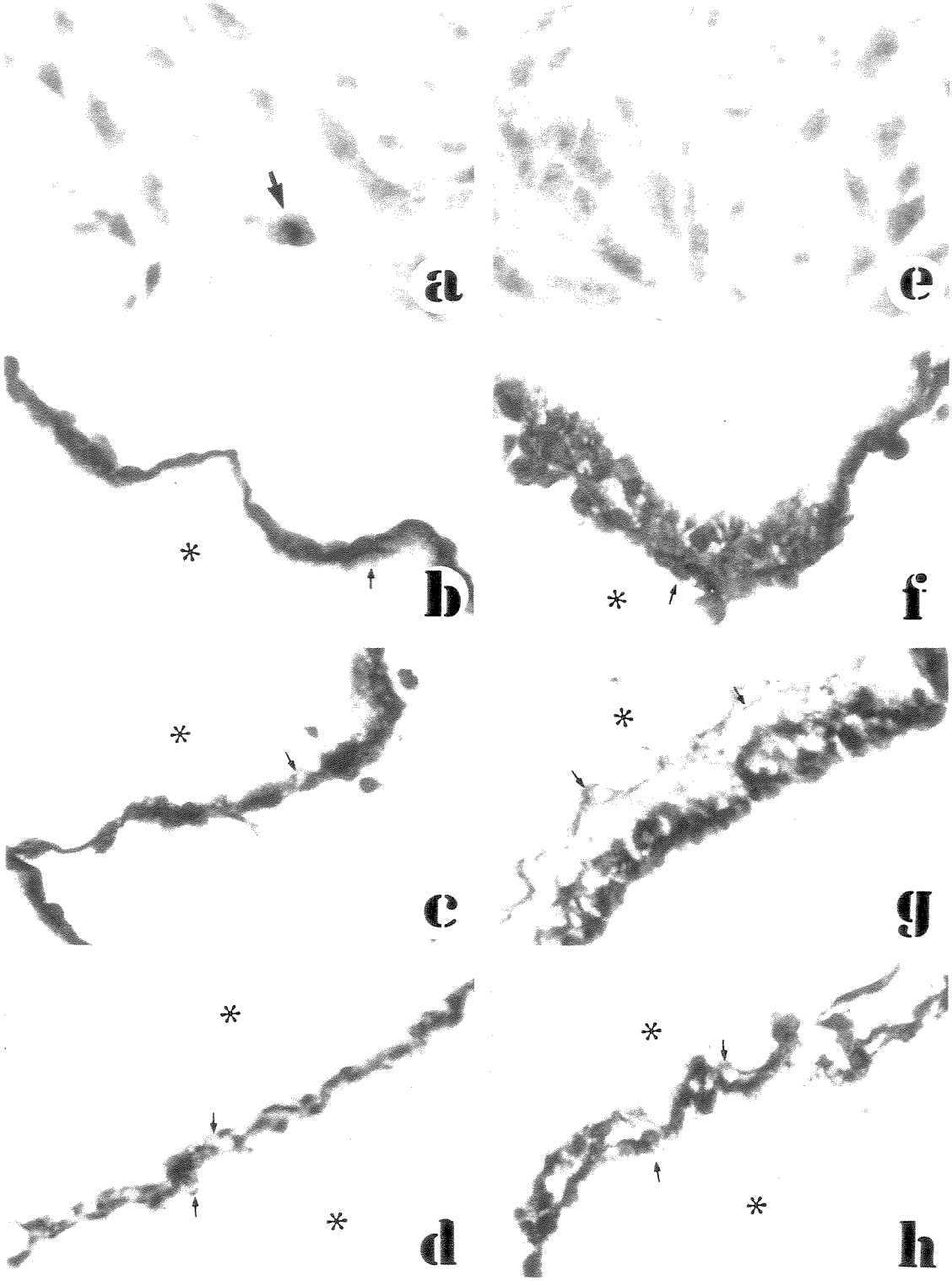


FIGURE 3

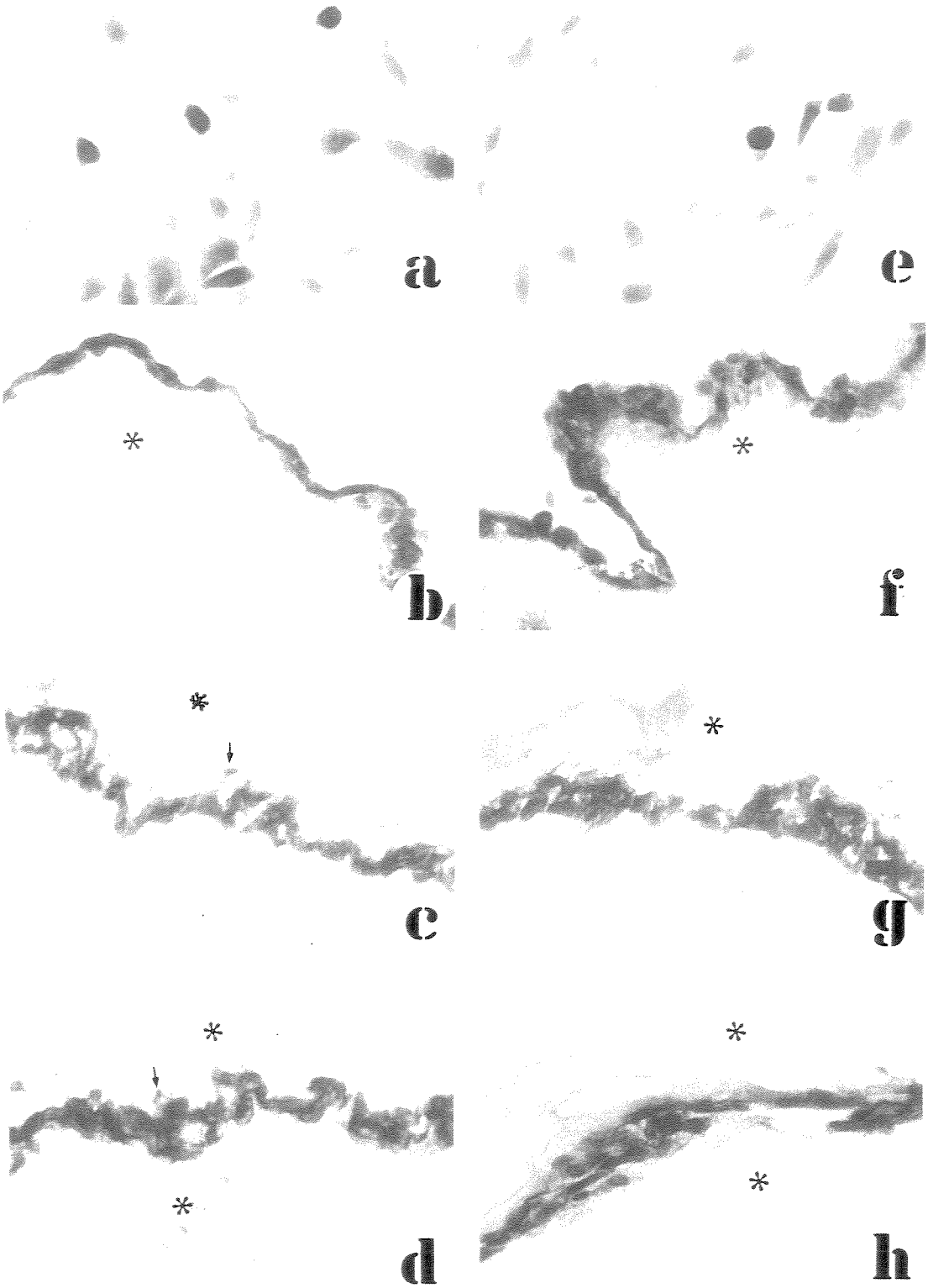
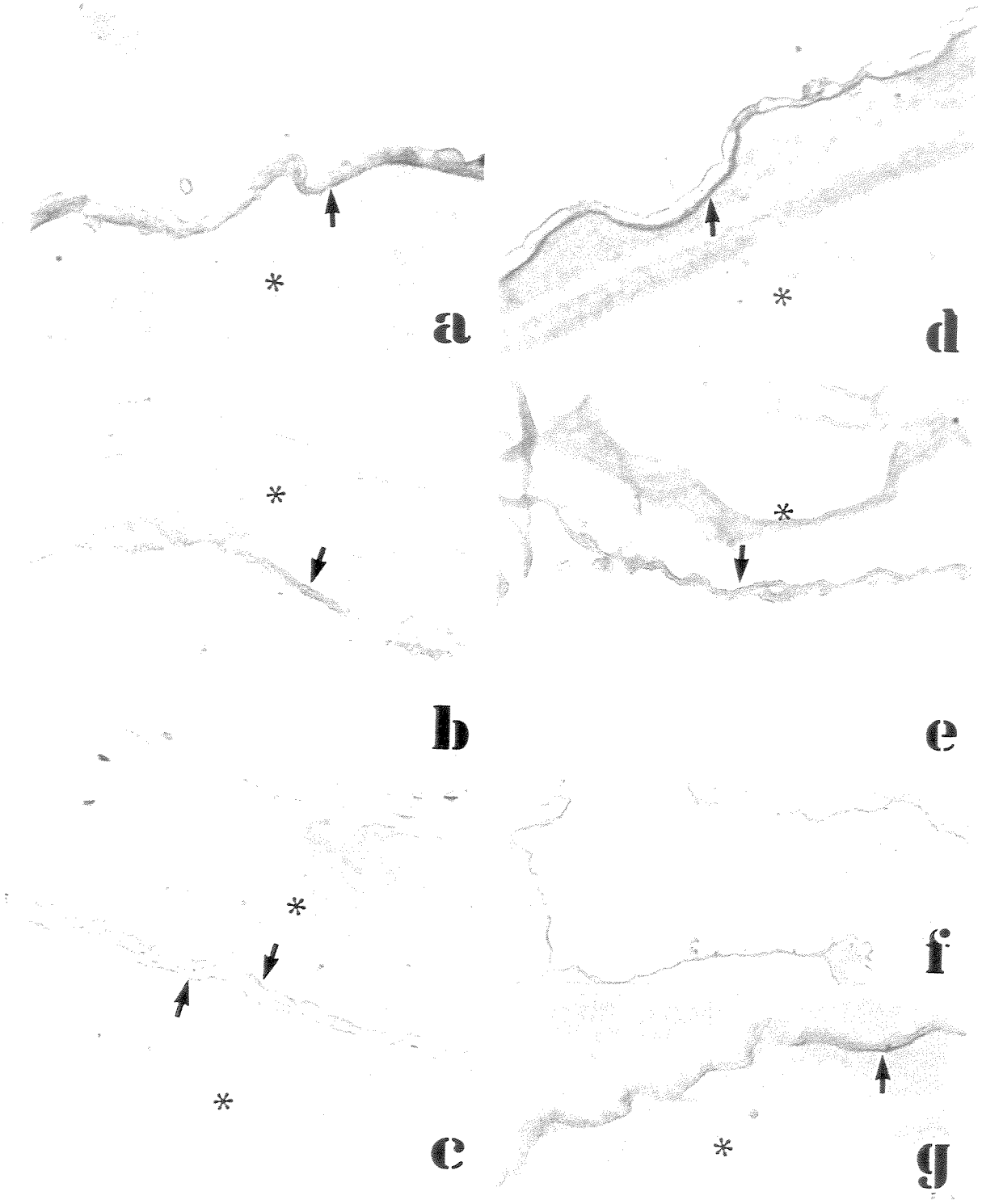


FIGURE 4

FIGURE 5



IV - CONCLUSÕES

1- As Células Vero apresentam comportamento similar quando cultivadas sobre o colágeno ou quando cultivadas pela técnica de sanduíche lamínula/colágeno ou colágeno /colágeno.

2- Quando em contato com o colágeno as células se diferenciam crescendo sobre o mesmo, formando um tecido com característica de tecido epitelial com uma ou mais camadas de células ou penetram no interior do gel, independente da técnica utilizada: cultivo sobre colágeno, sanduíche lamínula/colágeno ou sanduíche colágeno/colágeno.

3- Quando se utiliza 10% de soro fetal bovino no meio de cultivo esta formação tipo epitelial não é contínua pois se observa maior infiltração de células para o interior do gel. Nas regiões onde a infiltração não ocorre as células se diferenciam formando uma lâmina basal rica em colágeno tipo IV e laminina.

4- Quando se utiliza 20% de soro fetal bovino no meio de cultivo a infiltração de células para o interior do gel é diminuída e a lâmina basal formada se torna mais nítida.

5- A contração do gel de colágeno é similar quando se cultiva as células Vero sobre o colágeno ou quando se utiliza a técnica de cultivo em sanduíche.

V - REFERÊNCIAS BIBLIOGRÁFICAS

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