Vimentin expression and the influence of Matrigel in cell lines of head and neck squamous cell carcinoma

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Abstract: Vimentin is a cytoeskeletal intermediate filament protein commonly observed in mesenchymal cells; however, it can also be found in malignant epithelial cells. It is demonstrated in several carcinomas, such as those of the cervix, breast and bladder, in which it is widely used as a marker of the epithelial to mesenchymal transition that takes place during embryogenesis and metastasis. Vimentin is associated with tumors that show a high degree of invasiveness, being detected in invasion front cells. Its expression seems to be influenced by the tumor microenvironment. The aim of this study was to evaluate vimentin expression in head and neck squamous cell carcinoma (HNSCC) cell lines, and to investigate the contribution of the microenvironment to its expression. HNSCC cell lines (HN6, HN30 and HN31) and an immortalized nontumorigenic cell line (HaCaT) were submitted to a three-dimensional assay with Matrigel. Cytoplasmatic staining of the HN6 cell line cultured without Matrigel and of the HN30 and HN31 cell lines cultured with Matrigel was demonstrated through immunohistochemistry. Western Blotting revealed a significant decrease in vimentin expression for the HN6 cell line and a significant increase for the HN30 and HN31 cell lines cultured with Matrigel. The results suggest that vimentin can be expressed in HNSCC cells and its presence is influenced by the microenvironment of a tumor.

Descriptors: Carcinoma, Squamous Cell; Vimentin; Extracellular Matrix.

Conflict of interest statement:
The authors declare that they have no conflict of interest.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. It represents 90% of all head and neck malignancies and causes more deaths than any other oral disease.[1,2]

It is known that cancer progression is marked by genetic changes that control the interaction between the cells and the extracellular matrix. Thus, some signaling pathways can be stimulated, or even suppressed, thereby inducing cell proliferation[3,4] and replacing the epithelial phenotype of intercellular tight junctions and polarity by a more mesenchymal phenotype with reduced cell-cell adhesions, altered shape, expression of mesenchymal cellular markers and enhanced cell motility.[5-7]

These morphological changes define the epithelial-mesenchymal transition (EMT), which is recognized as a hallmark of tumor progression, that characterizes highly invasive and metastatic carcinomas.[7,8]

Proteins related to invasion, cell proliferation and metastasis are being evaluated exhaustively. Vimentin is a type III intermediate filament protein, present in most mesenchymal cells, that is expressed when tumor cells undergo EMT.[9-11] This protein contributes to the aggressive pattern of cancer cells. It is widely studied in carcinomas of the breast, cervix and bladder,[12,13] and has been suggested to be a predictor of the recurrence and invasive potential of prostate cancer cells.[14]

The extracellular matrix controls cell behavior and influences cell development, migration, proliferation, morphology and function, thereby creating an anchor for several other structures.[15] It is known that neoplastic cells have abnormal interactions with their environments.[16]

Matrigel is a reconstituted basement membrane that biologically mimics this environment and stimulates cell differentiation.[17] Although the two-dimensional model is the usual method used for *in vitro* cell culturing, and provides a convenient and fast means for research, it represents a limited tool for analyzing specific tissue functions and signaling pathways.[18]

Therefore, the purpose of the present investigation was to evaluate vimentin expression in HNSCC cell lines and to investigate the influence of Matrigel upon its expression.

Methodology

This study was approved by the Ethics Committee of the School of Dentistry of the University of São Paulo (protocol 134/05). All experiments were performed in triplicate.

Cell lines and culture conditions

The HNSCC cell lines [HN6 (base of tongue), HN30 (pharynx), HN31 (lymph node metastatic cells)][19] and HaCaT, an immortalized nontumorigenic human skin keratinocyte cell line, were cultured in DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA), and 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, USA). The cells were maintained in a 5% CO_2-humidified incubator.

Immunohistochemistry and hematoxylin-eosin staining

For two-dimensional assay, the cell lines were cultured in 75 cm² culture flasks until reaching 80% confluence. Then, they were removed (trypsinization - Trypsin Tryple Express - Gibco, Grand Island, USA), fixed in 4% paraformaldehyde (Synth, Diadema, Brazil) and dehydrated in a graded ethanol series (Synth, Diadema, Brazil). HistoGel™ (Richard-Allan Scientific, Kalamazoo, USA) was added and the samples were incubated in a graded ethanol series, ethanol/xylene (1:1), xylene (Synth, Diadema, Brazil) and paraffin, to obtain cell blocks.

In terms of a three-dimensional cell culture model, the cell lines were seeded in 75 cm² culture flasks, cultivated until reaching 80% confluence, removed (trypsinization - Trypsin Tryple Express - Gibco, Grand Island, USA) and resuspended in 1:3 Matrigel (BD Matrigel™ Matrix - cat. Number 356231 - Two Oak Park, Bedford, USA) for 72 hours. Then, the samples were fixed following the same protocol as in the two-dimensional assay.

Three-µm thick tissue sections were deparaffinized and re-hydrated in a graded ethanol series (Synth, Diadema, Brazil). One slide was stained with Hematoxylin and Eosin (Synth, Diadema, Brazil). Vimentin expression was detected by the avidin-biotin peroxidase method. Sections were deparaffinized and, for antigen retrieval, were treated with citric acid (10 mM, pH 6.0 - Sigma-Aldrich, St. Louis, USA) by heating at 95 °C for 30 min. Endogenous peroxidase was then blocked with hydrogen peroxide and sections were incubated with anti-vimentin (cloneV9, concentration of 1:200, Dako, Carpinteria, USA). Diaminobenzidine (Dako, Carpinteria, USA) was used as the chromogen, followed by counterstaining with Mayer's hematoxylin (Synth, Diadema, Brazil). Negative controls were obtained by omitting primary antibodies.

Western blotting

The cells cultured without Matrigel were harvested in 58 cm² culture dishes, cultivated until reaching 80% confluence and washed three times with cold 1X PBS (Sigma-Aldrich, St. Louis, USA). They were lysed with lysis buffer [50 mmol/L Tris–HCl

(pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS (Sigma-Aldrich, St. Louis, USA) with freshly added protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA)] at 4 °C for 20 min and scraped so the lysate could be collected in a microfuge tube. The sample was cleared by centrifugation at 13,000 rpm for 25 min at 4 ºC, and the supernatant was collected and stored at −80 ºC for posterior quantification. Protein concentrations of all samples were determined using the Pierce BCA method (Pierce Biotechnology, Rockford, USA), following the manufacturer's protocol.

In the case of the three-dimensional cell culture model, the cell lines were cultivated in 75 cm^2 culture flasks until reaching 80% confluence, removed (trypsinization - Trypsin Tryple Express - Gibco, Grand Island, USA) and resuspended in 1:3 Matrigel for 72 hours. Cold 1X PBS (Sigma-Aldrich, St. Louis, USA) was added, the sample was cleared by centrifugation at 13,000 rpm for 25 min at 4 ºC and the supernatant was discarded. This step was repeated thrice. The cell pellet was incubated in an ice-cold lysis buffer for 20 min at 4 °C and clarified by centrifugation at 13,000 rpm for 25 min at 4 ºC. Supernatants (total cell lysate) were collected and stored at −80 ºC for posterior quantification, as previously stated.

For Western blotting analysis, 20 µg of protein were loaded onto 10% polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (PVDF membrane) (Biorad Laboratories, Hercules, USA). Non-specific binding sites on the membrane were blocked by incubation in a blocking buffer (5% non-fat dry milk, 1% Tween-20 in 20 mmol/L Tris buffered saline (pH 7.6 - Sigma-Aldrich, St. Louis, USA) for 2 hours. Blots were probed overnight with primary antibody vimentin (clone V9, concentration of 1:200, Sigma-Aldrich, St. Louis, USA), or for 2 hours with primary antibody beta-actin (the ACTB gene is one of six different actin isoforms which have been identified in humans) (Sigma-Aldrich, St. Louis, USA), and for 90 minutes with peroxidase-conjugated secondary antibody (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, USA). A bound antibody was detected by a colorimetric method using an Opti 4CN kit (Biorad Laboratories, Hercules, USA). Relative band intensities were determined using densitometry software (NIH, Image J 1.42, Bethesda, USA). Statistical analyses, to compare each group, were estimated using Student's *t*-test. Data were considered significant if the *P* value was < 0.05.

Results

Histological findings

The HaCaT cell line, cultured either with or without Matrigel, demonstrated a non-pleomorphic, monotonous appearance (Figures 1A and 1B). On the other hand, the HNSCC cell lines cultured without Matrigel exhibited nuclear pleomorphism, discrete cell size variation and absence of mitosis (Figure 1C). The same morphological characteristics were found when these cell lines were cultivated with Matrigel, although they were more evident and some mitotic cells could be identified (Figure 1D).

Vimentin immunohistochemical expression

Vimentin expression was not observed in the HaCaT, HN30 and HN31 cell lines cultured without Matrigel. In contrast, scattered cells from the HN6 cell line showed cytoplasmic positivity (Figure 1E). When cultured with Matrigel, the HaCaT and HN6 cell lines demonstrated no vimentin expression while the HN30 and HN31 cell lines showed immunopositivity (Figures 1F and 1G).

Vimentin intracellular levels

Western blotting confirmed the results shown by immunohistochemistry. A significant decrease in vimentin expression levels for the HN6 cell line ($p = 0.022$), and a significant increase for the HN30 ($p = 0.027$) and HN31 ($p = 0.030$) cell lines cultured with Matrigel, were noted. HaCaT showed no expression when cultured with Matrigel. Additionally, only the HN6 cell line showed evident vimentin expression levels when cultured without Matrigel (Figure 2).

Discussion

Many biological processes such as motility, invasion, cancer progression and epithelial cell differentiation may be better mimicked in three-dimensional

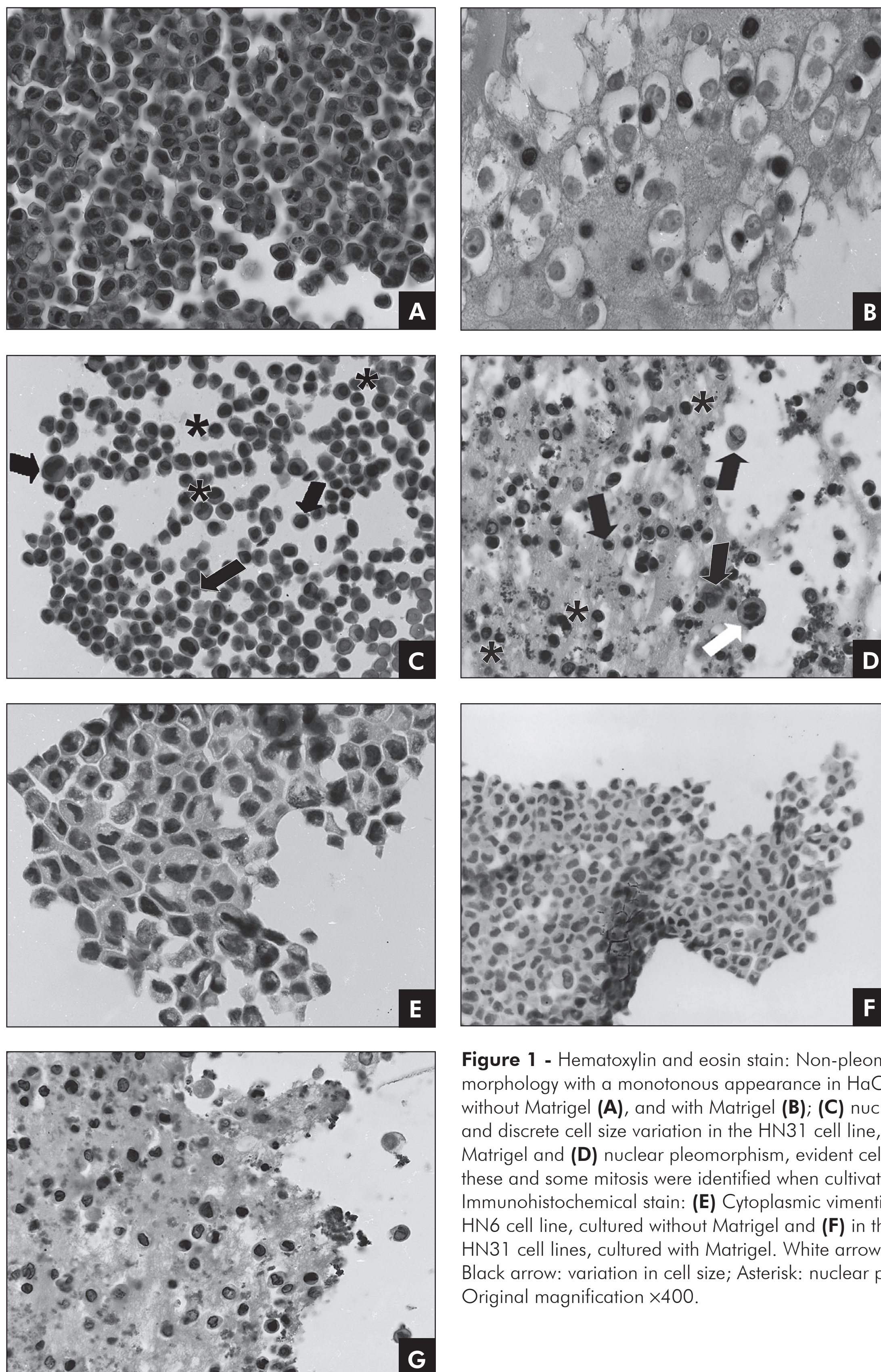

Figure 1 - Hematoxylin and eosin stain: Non-pleomorphic cell morphology with a monotonous appearance in HaCaT cell line cultured without Matrigel **(A)**, and with Matrigel **(B)**; **(C)** nuclear pleomorphism and discrete cell size variation in the HN31 cell line, cultured without Matrigel and **(D)** nuclear pleomorphism, evident cell size variation: these and some mitosis were identified when cultivated with Matrigel. Immunohistochemical stain: **(E)** Cytoplasmic vimentin expression in the HN6 cell line, cultured without Matrigel and **(F)** in the HN30 and **(G)** HN31 cell lines, cultured with Matrigel. White arrow: mitosis figure; Black arrow: variation in cell size; Asterisk: nuclear pleomorphism. Original magnification ×400.

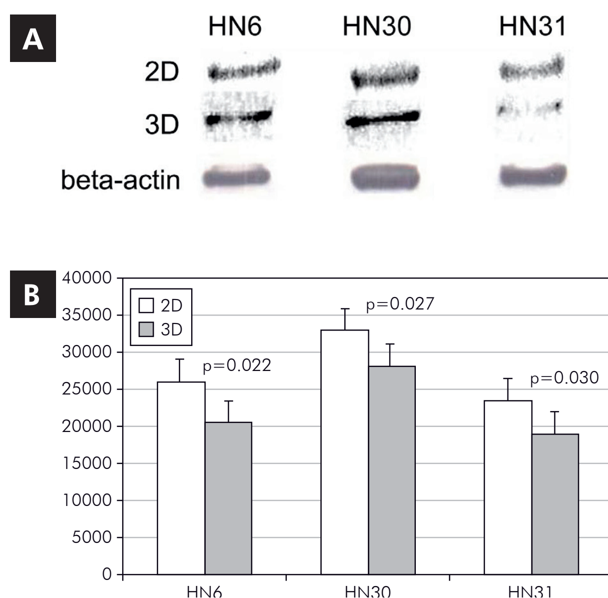

Figure 2 - (A) Bands demonstrated in Western blotting analyses of the HNSCC cell lines, for the antibody investigated (Vimentin). Beta-actin was used to control total volume of each sample. **(B)** Representative graph of mean values plus standard deviation. Statistically significant differences between each cell line are represented by distinct letters: (a) HN6 - p = 0.022; (b) HN30 - p = 0.027; (c) HN31 - p = 0.030.

microenvironments rather than on two-dimensional surfaces.[20] To mimic the extracellular matrix, a three-dimensional cell culture with Matrigel was used in this study. Matrigel contains components of the human extracellular matrix including laminin, type IV collagen, nidogen, entactin and heparan sulfate.[17]

The present study showed no spindle cells compatible with mesenchymal phenotype in the HNSCC cell lines cultivated with Matrigel. *In vivo*, this phenotype change is usually described in carcinoma cells at the tumor invasion front, and is associated with more aggressive behavior.[8] However, characteristics also related to a more aggressive cellular pattern, like evident nuclear pleomorphism, cell size variation and mitosis, were observed in the HNSCC cell lines cultivated with Matrigel. This suggests the ability of this substrate ability to simulate the *in vivo* microenvironment.[21]

Matrigel probably stimulates growth and retains the cells in an undifferentiated state, which represents an invasive phenotype.[22] This hypothesis explains the evident vimentin expression in the HN30 and HN31 cell lines after being cultured with Matrigel.

The HN6 cell line has a constitutively activated epidermal growth factor receptor.[19] Interestingly, among all the studied cell lines cultured without Matrigel, only the HN6 cell line expressed the vimentin protein, suggesting its more aggressive phenotype.

It is known that the vimentin protein contributes to the aggressive pattern of cancer cells because it regulates the interaction between cytoskeletal proteins with cell adhesion molecules. It thereby participates in cell adhesion, migration, invasion and cell signal transduction in tumor cells, tumor-associated endothelial cells and macrophages. Its highly dynamic balances between polymerization and depolymerization, and its complex phosphorylation may serve as the regulation mechanisms for tumor metastasis and cell-cell interactions. Therefore, vimentin is recognized as a hallmark of tumor progression that characterizes highly invasive and metastatic carcinomas.[14]

On the other hand, no immunohistochemical expression and significant decrease in vimentin intracellular levels were noted for the HN6 cell line cultured with Matrigel. Although this vimentin expression could have occurred subsequently, the time spent in the cell culture was probably not enough to promote it for this cell line. This time dependence can be an important factor to be considered when working with three-dimensional culture models. In this study, it was observed that Matrigel degraded after 3 days. Thus, if the cells remain in contact with the substrate for a longer time, the experiment would become 2D (conventional method of cultivation) and important data from the cell-environment interaction could be lost.

Another important finding demonstrated through immunohistochemistry was that, for the cell lines that did express vimentin (HN6 cultured without Matrigel; and HN30 and HN31 cultured with Matrigel), only part of these cells showed vimentin staining. This could be explained by the different degrees of cellular differentiation. In agreement, Gilles *et al.*[13] demonstrated that only part

of the epithelial mammary cells express vimentin, while the most differentiated cells do not. Furthermore, the lack of vimentin expression in the HaCaT cell line confirms its immortalized but nontumorigenic nature.[23]

Conclusions

Data from this study indicate differences between two and three-dimensional cell culture models. Therefore, the distinct expression of vimentin found for each of the cell lines studied, as well as its strict relation to the extracellular matrix, indicates that the behavior of the HNSCC cell lines can be influenced by both the extracellular microenvironment and the culture conditions.

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