



Isolation and characterization of myofibroblast cell lines from oral squamous cell carcinoma

LAYS MARTIN SOBRAL, KARINA GOTTARDELLO ZECCHIN, SIBELE NASCIMENTO DE AQUINO, MARCIO AJUDARTE LOPES, EDGARD GRANER and RICARDO D. COLETTA

Department of Oral Diagnosis, School of Dentistry, State University of Campinas, Piracicaba, São Paulo, Brazil

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Abstract. Oral squamous cell carcinoma (OSCC) invasion is followed by several stromal events such as inflammatory and immune cell infiltration, neo-vascularization, fibroblast activation and occasionally myofibroblast emergence. Our previous studies demonstrated that myofibroblasts in the stroma of OSCC are associated with a more aggressive behavior, leading to shorter patient overall survival. Therefore, we evaluated whether OSCC-associated myofibroblasts have different characteristics compared to OSCC-associated fibroblasts. OSCC myofibroblast cell lines were isolated, cultured and characterized on the basis of the expression of specific isoform α of smooth muscle actin (α -SMA) and of the excessive production of type I collagen. To assess the proliferative potential of the cell lines, growth curves were constructed, whereas the production and activity of matrix metalloproteinases (MMP) were analyzed by ELISA and enzymography, respectively. Myofibroblast clones were positive for α -SMA and vimentin, and negative for pan-cytokeratin and CD34. In long time cultures, Western blotting, flow cytometry and ELISA analysis revealed constant α -SMA expression and elevated production of type I collagen. There were no differences on proliferative potential between fibroblast and myofibroblast clones, but myofibroblast cells secreted significantly higher levels of MMP-1, -2, -9 and -13. Furthermore, MMP-2 gelatinolytic activity was significantly higher in myofibroblast clones. The results of this study suggest that myofibroblasts may contribute to OSCC invasion through elevation of MMP synthesis.

Introduction

Oral squamous cell carcinomas (OSCC) are tumors formed by neoplastic epithelial cells surrounded by a biologically complex stroma composed of various types of host cells and

extracellular matrix (ECM) molecules, both of which create a unique tumor microenvironment (1). For many years the research focus have been in the epithelial cells or more specifically, on genetic changes that occur in the epithelial cells as they progress from normal to malignant. However, the stroma has recently received increasing attention because of its recognized participation on tumor development, including invasion and metastasis, and of its influence on therapeutic response (2-5). Tumor-associated cells comprise immuno-competent and inflammatory cells, blood and lymph endothelial cells, fibroblasts, and eventually myofibroblasts. Evidence demonstrated that all of them may critically influence the processes of tumorigenesis (6-8), although the role of the myofibroblasts in oral cancer has not been fully elucidated.

Myofibroblasts were first described in skin wounds where they are co-opted by the remodeling tissues, facilitating wound healings (9,10). Morphologically they are large spindle-shaped mesenchymal cells that share some characteristics with smooth muscle cells and fibroblasts (11). Myofibroblasts are characterized by expression of the specific isoform α of the smooth muscle actin (α -SMA) and by the excessive synthesis of collagen (12). Through cell-cell contacts and through secretion of an extensive repertoire of molecules, including cytokines, growth factors, chemokines, hormones, neurotransmitters, inflammatory mediators, adhesion proteins, and ECM proteins, myofibroblasts promote effects in both physiological and pathological conditions (13-15). Those cells also secrete enzymes responsible for matrix remodeling, including matrix metalloproteinases (MMP) (16,17). Presence of myofibroblasts in the stroma of several cancers, including those of the oral cavity, is correlated with a worse prognosis (18,19). In oral squamous cell carcinomas, increased amount of myofibroblasts was significantly correlated with lymph node metastasis, vascular, lymphatic and perineural invasion of the tumor cells, and patient's shorter overall survival (18). Moreover, mutual interactions between OSCC cells and myofibroblasts exist and are dependent on multiple invasive growth-promoting factors via paracrine signals (20,21).

The aim of this study was to establish myofibroblast cell lines from OSCC stroma and to compare their proliferative potential and production of MMPs with OSCC-stromal fibroblast cell lines.

Correspondence to: Dr Ricardo D. Coletta, Department of Oral Diagnosis, School of Dentistry, State University of Campinas, CP 52, CEP 13414-018, Piracicaba, São Paulo, Brazil
E-mail: coletta@fop.unicamp.br

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Materials and methods

Tissue samples. Tissue fragments from 3 patients with tongue SCC were removed during tumor biopsy and divided into two parts: one was fixed in formalin and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry with α -SMA antibodies (22), while the other was washed with phosphate-buffered saline (PBS) and incubated in 500 μ l of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum (CS) and antibiotics for 16 h at 4°C.

Cell culture. Cells were established using tissue explants as described previously (23). Briefly, specimens were washed 3 times with PBS, minced, placed in 25-cm² cell culture flasks containing 1 ml of DMEM plus 10% CS and antibiotics, and incubated at 37°C in a humidified atmosphere of 5% CO₂. The cell growth was monitored every day and the medium was replaced every 2-3 days. When the cells growing out from the explant reached confluence, they were trypsinized and plated in very low density to select individual clones with cloning rings. Individual clones were propagated, and characterized as fibroblast or myofibroblast cell lines by immunocytochemistry, flow cytometry, and Western blot analysis. As marker of myofibroblast activity, type I collagen production was evaluated by enzyme-linked immunosorbent assay (ELISA).

Immunocytochemistry. Ten thousand cells were plated in each well of an 8-well culture chamber slide (Lab Tek, Nunc, Naperville, IL, USA) and incubated at 37°C in humidified air containing 5% CO₂ for 24 h. Following incubation, cells were fixed in 10% formalin, permeabilized with 0.5% Triton X-100 in PBS, treated with 3% H₂O₂ for endogenous peroxidase inactivation, and thus incubated with primary antibodies for 16 h at 4°C. The primary antibodies used were as follows: anti- α -SMA diluted 1:100, anti-vimentin diluted 1:200; anti-pan-cytokeratin diluted 1:200 and anti-CD34 1:50. All antibodies were purchased from Dako Co. (Carpenteria, CA, USA). Subsequent incubations were with biotinylated IgG followed by streptavidin-biotin peroxidase complex (LSAB+ System-HRP, Dako Co.). Reactions were developed with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) containing 0.01% H₂O₂ and counterstained with Mayer's haematoxylin. Omission of the primary antibodies was used as negative controls.

Flow cytometry. After released from culture flasks, single-cell suspensions were fixed with 70% ethanol and stained with anti- α -SMA antibody followed by goat anti-mouse IgG conjugated with fluorescein (Vector Labs, Burlingame, CA, USA). Cells were washed, resuspended in PBS, and analyzed on a FACScalibur flow cytometer equipped with an argon laser (Becton-Dickinson, San Jose, CA, USA). A minimum of 10,000 events was collected on each sample, and only cells with forward and orthogonal light scatter characteristics similar to whole and intact cells were included in the analysis. Quantitative flow cytometric analysis was performed with

the aid of CellQuest software (Becton-Dickinson), measuring the percentage of α -SMA-positive cells.

Western blot analysis. Cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40%, 1% deoxycholic acid, 0.5% sodium dodecyl sulphate, 1 mM phenylmethylsulphonyl fluoride, 1 mM N-ethylmaleimide, 1 mM dithiothreitol, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) for detection of α -SMA. After centrifugation, protein concentrations were measured using a protein assay according to the manufacturer's instructions (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Thirty micrograms of total protein per sample was resolved in a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes (GE Healthcare, Vienna, Austria). The membranes were blocked overnight with 10% non-fat dry milk in PBS containing 0.1% Tween-20, rinsed in the same buffer, and incubated for 2 h with a of the follow antibodies: anti- α -SMA (Dako Corp.) and anti- α -actin (Sigma-Aldrich). Reactions were developed using a chemiluminescent Western blot system (Enhanced chemiluminescent Western blot kit, GE Healthcare).

Cell growth assay. Cells in 2 ml of DMEM containing 10% CS were allowed to settle in triplicate wells of 24-well culture plate (Corning Corp. Cambridge, MA, USA) for 24 h. After washing with PBS, the medium was replaced by serum-free DMEM for 24 h to reach cellular synchronism. Cells were then cultured in DMEM supplemented by 10% CS and the number of cells was determined at days 3, 5, 7, 9 and 11 with the toluidine-blue stain method. Briefly, cells were fixed with 10% formalin for 15 min, washed in PBS and stain with a 1% toluidine blue in 1% borax solution for 5 min. After washing and solubilization of the toluidine blue with 1% SDS, absorbance was determined using an automated microplate photometer at 650 nm. In parallel, a standard curve with crescent number of cells (4,000-128,000 cells/well) was constructed.

ELISA. Type I collagen production was determined according the method described for Sobral *et al* (12). For MMP quantification, culture mediums were collected, whereas the cells were fixed and used for cell count by toluidine blue stain method. In essence, microtite plate wells were coated with 100 μ l of the culture medium for 2 h at room temperature. The wells were then washed 3 times with 400 μ l of 1% Tween-20 in PBS and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, anti-MMP-1 (diluted 1:10,000, Calbiochem-Merck KGaA, Darmstadt, Germany), anti-MMP-2 (diluted 1:3,500, Calbiochem), anti-MMP-9 (diluted 1:2,000, Calbiochem) and anti-MMP-13 (1:2,000, Calbiochem) antibodies in PBS were added to the wells and incubated for 2 h. After another washing step, goat anti-mouse peroxidase-conjugated IgG (Vector Labs) diluted 1:1,000 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenylenediamine (Sigma-Aldrich) in 0.5 M citric buffer pH 5.5 containing 0.01% H₂O₂ for 20 min. After terminating the reaction with 50 μ l of 2 N

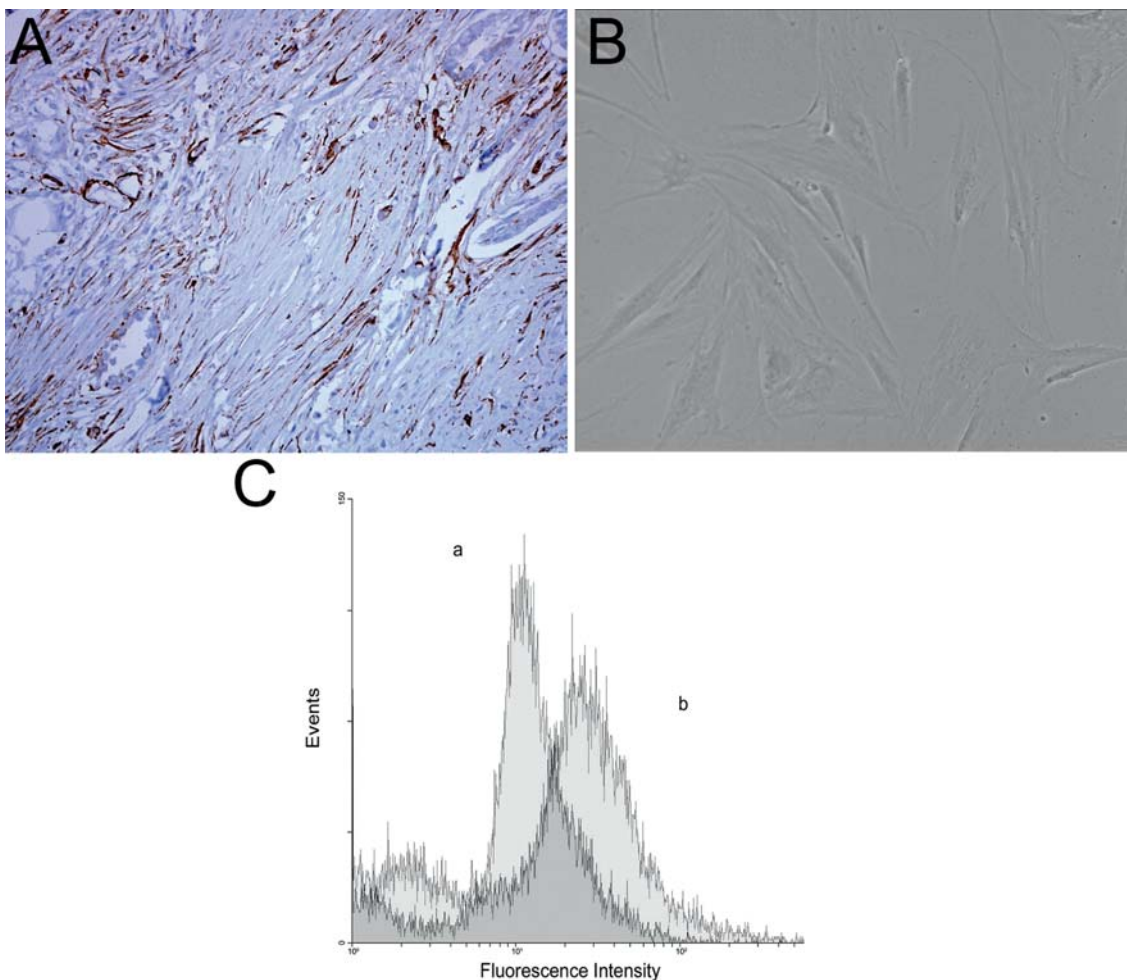
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Figure 1. (A) Immunohistochemical expression of α -SMA in one of the tumor samples of this study. Myofibroblasts were found immediately adjacent to the islands of tumor cells (original magnification, $\times 100$). (B) Phase-contrast microscopic features of cells cultured from one of the tumors. Note the typical spindle characteristics with a central spherical nucleus and typical cytoplasmic prolongations. (C) Flow cytometric analysis revealed a mix population of mesenchymal cells formed by both α -SMA-negative (a) and α -SMA-positive cells (b).

H_2SO_4 , absorbance was read at 450 nm. The values were expressed as MMPs/cell.

Enzymography and densitometry. Enzymographic analysis was performed as previously described (23). Same amounts of protein per sample were mixed with non-reducing sample buffer and resolved in 10% SDS-PAGE copolymerized with 1.6 mg/ml of gelatin (Bio-Rad Lab, Hercules, CA, USA) as substrate. After electrophoresis, the gels were washed for 1 h in 2% Triton X-100 and incubated for 16 h in activation buffer (10 mM Tris-HCl pH 8.0, 5 mM CaCl_2) at 37°C . Gelatinolytic activity was visualized after staining with Coomassie blue R-250 (Bio-Rad). The intensities of the negative bands were determined using a GS-700 imaging densitometer (Bio-Rad). To confirm the identity of the enzymes, 1 mM of 1,10-phenanthroline (Sigma), a specific inhibitor of MMP activity by chelating Zn^{++} ions of their catalytic domain, was utilized.

Statistical analysis. The Kruskal-Wallis multiple comparison test was used to test group effects at 5% significance ($p < 0.05$). All assays were performed at least three times in triplicates or quadruplicates.

Results

Characterization of fibroblast and myofibroblast clones isolated from OSCC. Immunohistochemical analysis of the tumor tissues used for explant cultures demonstrated a significant proportion of α -SMA-positive cells (myofibroblasts) in close contact to tumor epithelial cells, but fibroblasts (α -SMA-negative fusiform cells) were also observed in all samples (Fig. 1A). After 2 weeks of culture, fusiform cells extended from almost all explant fragments, and by day 30, cells were confluent and ready to be trypsinized and sub-cultured (Fig. 1B). Since flow cytometric analysis confirmed that all cultures were formed by both α -SMA-negative and α -SMA-positive cells (Fig. 1C), individual clones were selected and screened for α -SMA expression and type I collagen production.

To assess α -SMA a number of modalities were employed that included immunocytochemistry, flow cytometry and Western blot analysis. Immunocytochemical analysis demonstrated a strong cytoplasmatic staining for α -SMA in the myofibroblast clones, which was not observed in the fibroblast clones (Fig. 2). Immunoreactivity for vimentin was detected in almost 100% of both fibroblast and myofibroblast

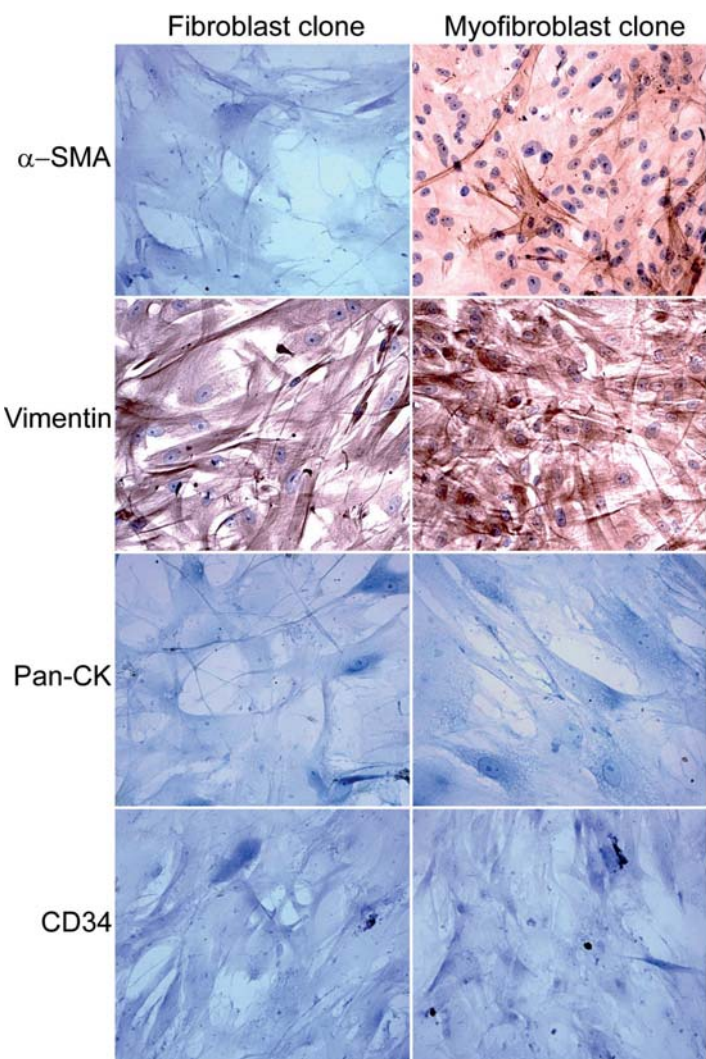


Figure 2. Immunocytochemistry analysis of markers of cell differentiation. Myofibroblast clones demonstrated a vivid cytoplasmatic staining for α -SMA, which was not observed in the fibroblast clones. Immunoreactivity for vimentin was detected in almost 100% of both fibroblast and myofibroblast clones. Both fibroblast and myofibroblast clones were negative to pan-cytokeratin (pan-CK) and CD34.

clones, whereas cells were negative for pan-cytokeratin and CD34 marker (Fig. 2). Western blot analyses of cell homogenates revealed a significant difference in the production of α -SMA between clones, without detection in the fibroblast ones (Fig. 3). All 3 myofibroblast clones expressed high levels of α -SMA. The expression of α -SMA in fibroblast and myofibroblast clones was also confirmed by flow cytometric assay (Fig. 4). Since one of the major features of myofibroblasts is the elevate production of type I collagen (24), we further characterized myofibroblast phenotype by quantification of type I collagen. As expected, myofibroblast clones shown a significantly higher production of type I collagen production compared with fibroblast cells (Fig. 5). Most importantly, myofibroblast clones retained their morphology, α -SMA expression and higher type I collagen production for at least 20 passages.

Proliferation and production of MMPs of fibroblast and myofibroblast clones. Growth curves of fibroblast and myofibroblast clones are depicted in Fig. 6. No differences on proliferative potential were observed between groups,

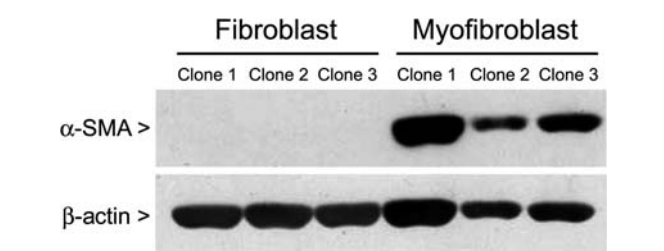


Figure 3. Western blot analysis performed with total cellular proteins from fibroblast and myofibroblast clones. Similar amount of protein of each sample was separated on SDS-PAGE, transferred to nitrocellulose membranes, and α -SMA production was analyzed utilizing anti- α -SMA antibodies. Blots were stripped and reprobbed with anti- α -actin antibodies. Note that myofibroblast clones expressed high levels of α -SMA, whereas fibroblast clones lacked expression.

although myofibroblast clone 3 showed a slight higher proliferation. Our results also showed that myofibroblast cell lines can be maintained in a serum-free medium without cellular growth, and all clones demonstrated a contact inhi-

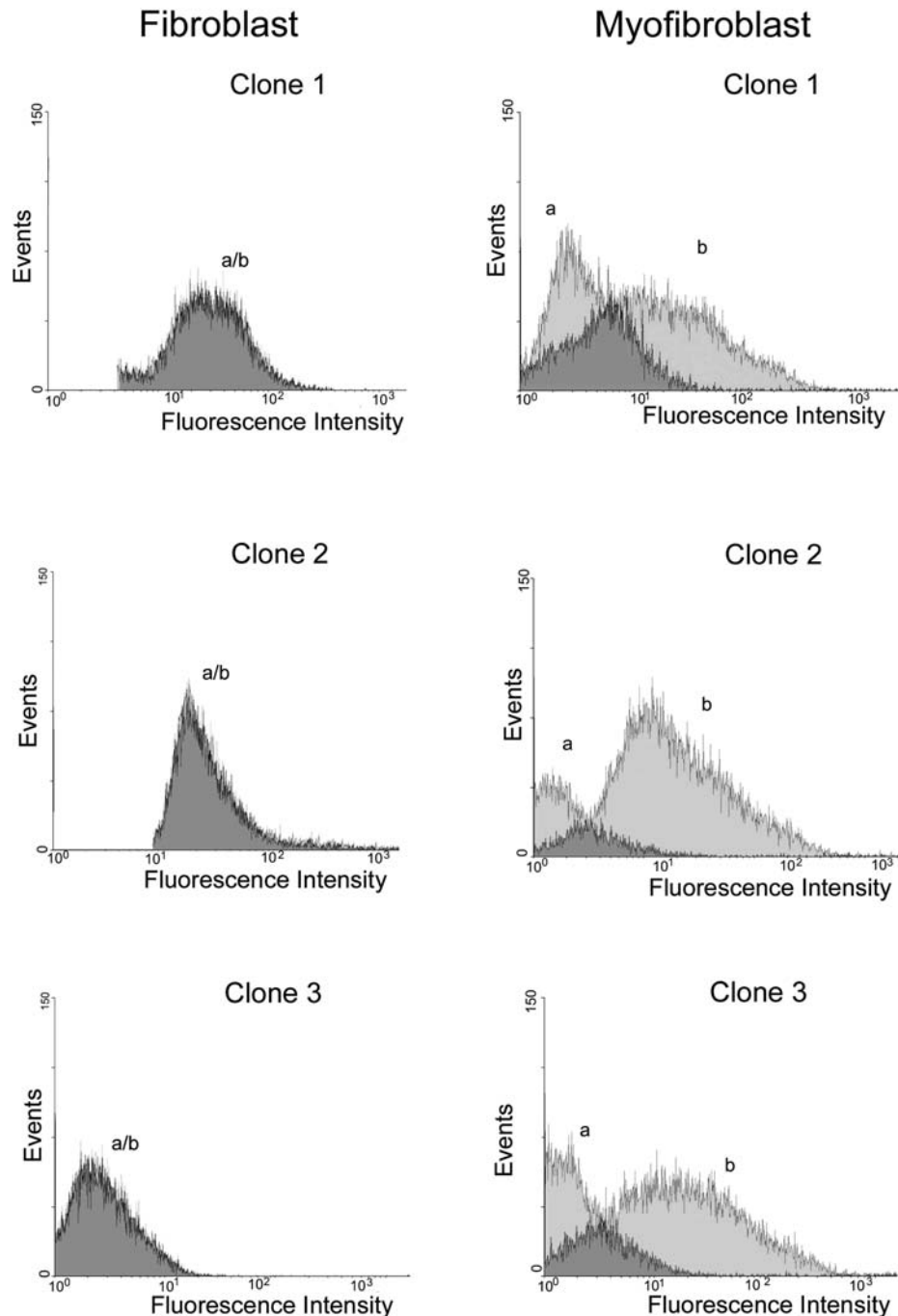


Figure 4. Flow cytometric analysis of fibroblast and myofibroblast clones with FITC anti- α -SMA antibodies. Representative histograms of each clone are depicted. On the left, fibroblast clones and on the right myofibroblasts. (a) control negative cells (FITC antibodies alone) and (b) FITC-anti- α -SMA stain. Myofibroblast clones were formed by almost 100% of α -SMA-positive cells, confirming the purity of the cell lines.

bition of growth (data not shown), arguing for the non-transformed nature of the clones. Next, we compared the production of MMPs between fibroblast and myofibroblast clones by ELISA (Fig. 7). The productions of MMP-1, -2, -9 and -13 were significantly higher in myofibroblasts compared with fibroblasts. Comparison of production of MMP-1, -2, -9 and -13 in fibroblast and myofibroblast clones indicated that in myofibroblasts, MMP-1 was increased approximately by 3.3-fold, MMP-2 by 4.7-fold, MMP-9 by 2.7-fold and MMP-13 by 3.1-fold of the value observed in corresponding normal control fibroblasts. One gelatinolytic band with ~70 kDa was detected in the supernatants of all studied clones (Fig. 8A).

This activity was attributed to the activated form of MMP-2, since it was completely inhibited by 1,10-phenanthroline. Densitometric analysis of 3 independent experiments demonstrated that MMP-2 production by myofibroblast cells was dramatically higher than that observed in fibroblast cell lines (Fig. 8B).

Discussion

It is well recognized that tumor progression is characterized not just for the acquisition of genetic alterations of the tumor cells, but also for crosstalk between malignant cells and the

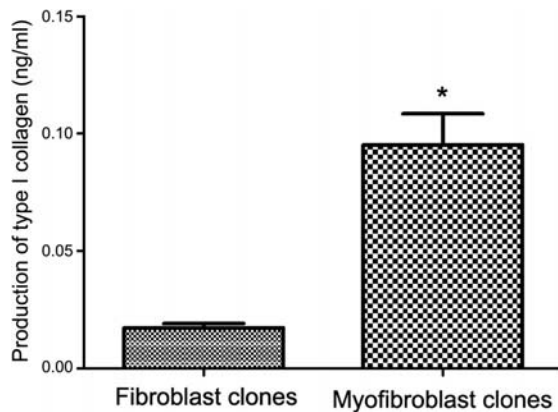


Figure 5. Myofibroblasts produce significantly higher levels of type I collagen than fibroblasts. Production of type I collagen by monolayers of cells as determined by ELISA. The values represent the mean of ng/cell of type I collagen production for triplicate wells \pm SD. * $p < 0.005$.

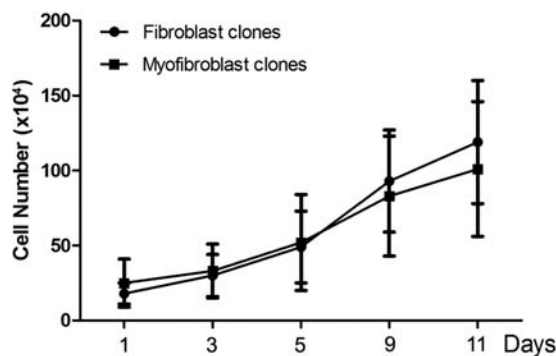


Figure 6. Growth rate of fibroblast and myofibroblast clones. Each time-point represent the mean of 3 counts in quadruplicate for each strain. From day 3 to 11, the growth rates of fibroblast and myofibroblast cell lines were quite similar.

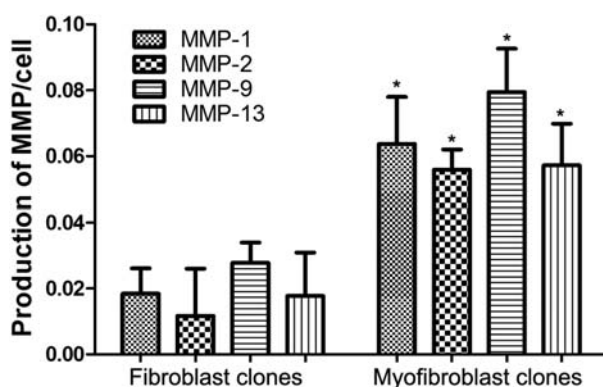


Figure 7. Production of MMPs by fibroblast and myofibroblast cell lines. Data are expressed as production of protein/cell. The amount of MMP-1, -2, -9 and -13 synthesized by myofibroblast clones was significantly higher than fibroblast cell lines ($p < 0.01$).

surrounded stroma (1,25,26). One of the characteristics of the tumor stroma is the emergence of a mesenchymal-specific cell called myofibroblast (27). Both clinical and experimental data support the hypothesis that myofibroblasts may

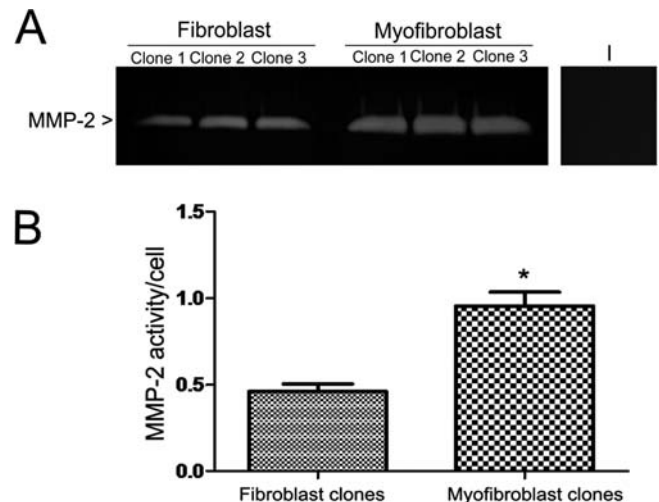


Figure 8. Activity of MMP-2 is significantly higher in myofibroblast clones. (A) Enzymographic analysis of the culture supernates of fibroblast and myofibroblast clones. Gelatinolytic activities were detected at ~70 kDa consistent with the presence of the active form of MMP-2. Lane I show the complete inhibition of gelatinolytic activity of MMP-2 with 0.5 mM of 1,10-phenanthroline. (B) Densitometric analysis of the MMP-2 band demonstrated a significant higher MMP-2 activity in the myofibroblast clones. * $p < 0.005$.

modulate growth, invasion and metastasis (28-33). The first evidence in favor of a role for myofibroblasts in tumor progression was obtained by Dimanche-Boitrel and collaborators (34). Those authors demonstrated that tumor cells isolated from rat colon cancers failed to invade *in vitro*, whereas suspensions containing tumor cells and tumor-associated stromal cells were invasive *in vitro* and *in vivo*. Similarly, it was demonstrated that myofibroblasts secrete high levels of HGF/SDF, which promotes OSCC invasion (20). Our previous data demonstrated that elevated amount of myofibroblasts is correlated with an infiltrative phenotype of the OSCC, as demonstrated by the presence of vascular, lymphatic and perineural invasion of the tumor cells and lymph node metastasis (18). We further demonstrated that myofibroblasts secrete tumor growth factors that stimulate OSCC cell proliferation (21), and its elevated production of MMP-2 contribute to the rupture of the cortical bone in ameloblastomas, one of the major prognostic markers of aggressiveness of this odontogenic tumor (35). The precise role of myofibroblasts in tumor-stroma interactions in OSCC has not been completely defined, in part because of the difficulty in obtaining primary tumor-associated myofibroblasts for analysis.

In this study we have established 3 myofibroblast cell lines derived from the stroma of tongue SCCs. There is no myofibroblast-specific immunocytochemical marker, but α -SMA is the most reliable one (36). In a recent review, De Wever and collaborators (16) suggested a minimum criterium for myofibroblast characterization, which included positivity for α -SMA, vimentin and type I collagen maturation enzyme prolyl-4 hydroxylase (P4H), and negativity for cytokeratin. Here, we demonstrated that myofibroblast cells expressed α -SMA and vimentin, but did not express pan-cytokeratin, the marker of epithelial cells, and CD34, the



endothelial cell marker. In addition, those cells great amounts of type I collagen. Consistently, myofibroblast cell lines demonstrated those features in a long culture period, without losing their untransformed characteristics such as growth factor dependency to proliferation and contact inhibition. Together, those features led us to conclude that those cells are myofibroblasts.

Myofibroblasts secrete several functional molecules, including growth factors, and express receptors for many of these ligands, allowing in both paracrine and autocrine manner the control of the growth, migration and invasion (16). Previous study demonstrated that OSCC-associated fibroblasts, which were α -SMA-positive, showed an elevated proliferation rate compared to normal oral mucosal fibroblasts (37). The current study did not find differences on the proliferative potential between OSCC stromal clones of fibroblasts and myofibroblasts. This lack of difference may be explained because during invasion tumor cell-released factors active receptors in both cell types allowing similar responsiveness to growth stimuli. However, myofibroblast cells produced significantly higher amounts of all analyzed MMPs (MMP-1, -2, -9 and -13) compared with fibroblast clones. MMP-2 activity was also significantly higher in myofibroblast cells, as revealed by gelatine enzymography. In accordance, Webber *et al* (38), by enzymographic analysis of secreted MMPs, showed a higher MMP-2 activity in WPMY-1 cells, an immortalized prostatic stromal myofibroblast cell line, compared to WPE1-10 epithelial cells.

MMPs are a large family of zinc-dependent extracellular matrix-degrading endoproteases with pathogenetic significance in a broad range of disorders (39). In cancer, they are considered major end-stage effectors for ECM degradation and tumor invasion (17,40). MMP production is dramatically increased in OSCC, and study in our laboratory have previously shown that patients with tumors showing elevated activity of MMP-2 and MMP-9 had shorter disease-free survival period after treatment than patients with tumors exhibiting low MMP activities (41). Interestingly, previous reports demonstrated myofibroblasts as the main source of MMPs in fibrotic diseases (42,43) and some types of cancers (44-46). Furthermore, the observation that myofibroblasts may enhance the ability of cancer cells to invade is supported by the fact that *in vitro* the presence of MMPs secreted by myofibroblasts increased the invasion of cancer cells (47,48).

In conclusion, differences between fibroblasts and myofibroblasts derived from OSCC stroma exist, in favor of a higher production and secretion of MMPs by myofibroblast cells. The myofibroblast cell lines may be a useful tool to study myofibroblast-epithelial cell interactions on tumorigenesis, mainly because it can provide new insights into the mechanism involved in tumor growth, invasion and metastasis.

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