A proposal of an in vitro model which mimics in situ areas of carcinoma

Elizabeth F. Martinez · Pollyanna T. Montaldi ·
Ney S. de Araújo · Albina Altemani · Vera C. de Araújo

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Abbreviations

PA	pleomorphic adenoma
DCS	donor calf serum
DMEM	Dulbecco's modified Eagle medium
FGF2	Fibroblast growth factor type 2

The curious situation of in situ carcinoma where there is a direct contact between malignant cells with normal or benign cells has led us to look for an in vitro model which mimics this condition. In ductal carcinoma of the breast, this interaction occurs between malignant cells (epithelial luminal) and normal cells (myoepithelial cells) and, in salivary gland carcinoma ex-pleomorphic adenoma between the malignant cells and benign myoepithelial cells.

This model raises the possibility of investigating the mechanism that controls cancer invasion. It was inspired by an in vivo study of in situ areas of carcinoma ex-pleomorphic adenoma where the modification of benign myoepithelial cells phenotype, provoked by epithelial cells malignant transformation was demonstrated, revealing that there was a cross-talking between them (Araújo et al. 2006; Martinez et al. 2012).

The purpose of this model is to allow future investigators to evaluate data that could contribute to answering the great question: How do the malignant cells win the suppressor effect of myoepithelial cells and finally invade the tissue?

The in vitro model

Benign myoepithelial cells were obtained from explants of pleomorphic adenoma (PA) tumours provided by surgery. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma, St Louis, MO, USA) supplemented by 1% antimycotic-antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per ml in 0.9% sodium chloride; Sigma), containing 10% of donor calf serum (DCS; GIBCO, Buffalo, NY), plated in 60-mm diameter plastic culture dishes and incubated under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO₂) following the used protocol for this cell lineage culture (Miguita et al. 2010). After the cells had reached confluence, they were detached with 0.05% trypsin and subculture at a density of 110 cells/mm² on the top of Fibronectin (Sigma) at 20 µg/ml.

It is considerably evident that culturing cells over an extracellular protein is crucial to sustaining the in vivo condition since it maintains the cell-cell and cell-extracellular matrix interactions (Campbell and Watson 2009). Inasmuch, the matrix component fibronectin is fundamental for malignant cell colonization accompanied by cell cluster formation (Martinez et al. 2012).

Squamous cell carcinoma cells (CAL27) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were used in the same density (110 cells/mm²) and

E. F. Martinez (✉) · P. T. Montaldi · N. S. de Araújo ·
V. C. de Araújo
Laboratory of Oral Pathology,
São Leopoldo Mandic Institute and Research Center,
R. José Rocha Junqueira, 13,
Campinas, SP, Brazil CEP. 13045-755
e-mail: efmartinez@ig.com.br

A. Altemani
Department of Pathology, State University of Campinas,
Campinas, SP, Brazil

medium (DMEM) described for myoepithelial cells. The medium of these cells was removed 48 h after plating and then benign myoepithelial cells from PA cultures in DMEM for 24 h were incubated with the non-filtered malignant conditioned medium for at least 4 days. Figure 1 synthesizes the protocol used. This condition was used because our previous study showed that in non-filtered malignant conditioned medium there are non-adherent viable malignant epithelial cells from squamous cell carcinoma capable of seeding in the plate where the benign myoepithelial cells are cultured. As a control, the analysis was carried out without malignant conditioned medium (i.e. DMEM).

With this methodology, it was possible to observe an *in situ* situation that modifies at different cell culture times, as observed after 4 d, 9 d and 16 d of cell culture (Fig. 2) by increasing the number of malignant cells and decreasing benign myoepithelial cells until their complete disappearance.

It is important to highlight that the difference in cell growth rate alone does not always trigger cell competition (de la Cova et al. 2004). In this context, it was demonstrated that the activity of malignant cells on fibronectin substrate induces the increase of FGF2 by the benign myoepithelial cells favouring malignant cell proliferation (Martinez et al. 2012). Recently, it has been shown that there are metabolic inter-dependencies between epithelial cancer cells and the cells of the stroma microenvironment which by mitochondrial oxidative stress drives lactate production promoting cancer tumor growth (Balliet et al. 2011).

Fig. 1 Flow chart synthesizing the protocol used

Fig. 2 Immunostaining for Vimentin (green) in myoepithelial cells (MC) and for AE1/AE3 (red) in carcinoma cells (CAL27) on fibronectin substrata after 4 d, 9 d and 16 d. At 4 days (a), it is possible to observe malignant epithelial cells surrounded by benign myoepithelial cells assuming a cluster forming. At 9 days (b) the number of carcinoma epithelial cells is more abundant. After 16 days of cell culture (c), there is malignancy predominance with the myoepithelial cell fading. Bar: a=50 µm; b,c=100 µm

Thus, this model can be useful in the future to facilitate the investigation of factors that influence the interaction among myoepithelial and cancer cells which is very important to control cancer invasion.

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