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Primary cell culture of head and neck cancer: a challenge

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Abstract

Introduction: Head and neck cancer presents a high rate of recurrence and mortality, considering the sites affected. The use of primary culture allows pre-clinical trials that would not be possible in humans or would require a long time until the initial tests were approved. **Objective:** To establish primary culture of carcinomas and the disease-free surgical margin of individuals affected by neck cancer. **Methods:** Fragments of 6 cases of oral cavity carcinoma and 2 cases of non-malignant tissue (surgical margin) of patients with oral cancer were collected immediately after surgical resection. These specimens were packed in complete DMEM (Dulbecco Modified Eagle's Medium, SIGMA®) supplemented with 10% inactivated Bovine Fetal Serum (BFSi) and 5% antibiotic / antimycotic - and kept on ice for transportation to the Molecular Marker Laboratory And Cancer Cell Signaling in FCFRP-USP. Processing was carried out in a biosafety booth in a cell culture room, 2 hours after collection maximum. All specimens collected were advanced tumors of the oral cavity. **Results:** From the 6 cases collected and kept in culture, only 2 presented uncontrollable bacterial contamination and were discarded. Two other cases released fibroblasts in the first 3 to 5 days and the observation of neoplastic cells (keratinocytes) was only possible after seven to ten days. Both cell types exhibited monolayer expansion. **Conclusion:** The use of explants to establish the initial stages of primary culture of head and neck cancer is a viable and easily reproducible alternative. The effective success rate is achieved in 20-30% of the cases and the control of the contamination presents itself as one of the biggest obstacles to be surpassed in the initial stages of cultivation.

Keywords: Carcinoma; Squamous cell; Primary cell culture; Head and neck neoplasms.

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Introduction

Head and neck cancer presents high recurrence and mortality rates considering the affected areas. Thus, the biology study of head and neck cancer presents great relevance. The utilization of primary culture allows the accomplishment of pre-clinic trials that would not be possible in humans or would demand a long time until the primary test approval. The current pre-clinic trials limit in the utilization of cellular lineage available commercially[1], however, none derives from a Brazilian individual[2]. Therefore, the development of a cellular lineage of head and neck carcinoma originally national represents great relevance for the medical-scientific community. Beyond that, the primary culture preserves morpho-functional characteristics in a certain degree, preserving part of the complexity of the derivative organ microenvironment[3]. Thereby, derivative cells

of primary culture represent an excellent model for study and development of new therapies for cancer and helps the investigation for a better answer to chemotherapy in a personalized manner for the patient[4].

The objective is to establish primary culture of carcinomas and the freed disease surgical border of patients affected by head and neck cancer.

Methods

Obtainment of the surgical specimens

Fragments of six carcinoma cases of the oral cavity and two cases of non-malignant tissue (surgical border) of patients with oral cancer were collected immediately after the resection. All cases were from the *Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo/USP-RP.* These specimens were conditioned in DMEM (Dulbecco Modified Eagle's Medium – code D5648, SIGMA®) complete – supplemented with 10% of Fetal Bovine Serum inactivated (FBSi) and 5% of antibiotics/antimycotic (code A5955, SIGMA®) - and kept in ice for transportation until the *Marcadores Moleculares e Sinalização Celular no Câncer*'s laboratory, in FCFRP-USP. The processing was performed in a biosecurity cabin in the cell culture room, two hours after the collection. All the specimens collected, neoplastic or not, were advanced tumors of the oral cavity (Table 1).

Fragmentation of the specimens and maintenance in culture

Inside the biosecurity cabin, each fragment was washed three times in complete DMEM. Posteriorly, each fragment was conditioned in a sterile tube containing enough culture mean to cover them completely. The fragments were immediately transferred to the culture board of 100 mm (CORNING®, code 353003), and with the help of sterile scissors and tweezers were carefully pricked into fragments from 1 to 3 mm. This stage was performed maintaining the fragment humid in complete DMEM. After fragmentation, the resultant specimens were allocated in a new culture board of 100 mm and maintained dry for three minutes to permit adhesion to the board. After this period to initial adhesion, complete culture mean with ciprofloxacin added (20 µg/mL) was gently dripped on the fragments until observed a fine pellicle of the solution coating

Table 1. Epidemiological data of the patients.

# case	Gender	Age	Color	Location
1	F	65	White	Oral cavity
2	M	63	White	Oral cavity
3	M	-	-	Oral cavity
4	F	78	White	Oral cavity
5	F	66	White	Oral cavity
6	M	62	Black	Oral cavity

all the board surface. An identic procedure was executed for the non-neoplastic tissue fragments. Subsequently, the boards were cautiously transferred into an incubator at 37 °C with 5% CO_2 and controlled humidity. The boards were maintained in the incubator for three days. Thereafter, 50% of the culture mean was substituted by an equal volume of fresh mean. Seven days after the boarding of the fragments, the boards were analyzed and photographed with a photographic camera coupled to an inversed optic microscope (AxioVert 40 CFL, software AxioVision Rel. 4.8, Zeiss®, Germany). From this analysis it was possible to observe the fibroblasts liberation from some fragments. After the first partial trade of the culture mean performed three days after the boarding, 50% of the culture mean was replaced every other day. Figure 1 shows the evolution of the primary culture during five weeks.

This project was approved by the Ethics Committee of Humans Research of the Pharmaceutical Sciences Faculty of Ribeirão Preto and the Medicine Faculty of the São Paulo University, campus Ribeirão Preto (CEP/FCFRP n° 342). All the participants consented the participation in the research by signing the Consented Form.

Results

Establishment of primary culture of head and neck cancer. From the six cases collected and maintained in culture, two presented uncontrollable bacterial contamination and were discarded (Table 2). Other two released fibroblasts in the first three to five days and the observation of neoplastic cells (keratinocytes) was only possible seven to ten days later. Both cell types presented expansion in monolayer. It was observed that the neoplastic cells showed cytoplasm abundantly vacuolized and hard spots inside its nucleus. The keratinocytes frequently also presented long cytoplasmatic projections that anastomosed. These neoplastic cells expanded, preserving its main morph-histological characteristic already described for this kind of tissue, that is, with juxtaposed cells between themselves. The fibroblasts also presented with its typical morphological characteristic. However, the fibroblasts showed a

Table 2. Characterization of primary culture.

#case	Technique	Cultivation time (weeks)	Contamination	Success
1	Explant	4 weeks	No	No
2	Explant	12 – present	Yes	Yes*
3	Explant	3	Yes	No
4	Explant	8 – present	No	Yes
5	Explant	4 – present	No	Yes
6	Explant	3 – present	No	Yes

*This case was divided into two culture boards and only one of them presented contamination and was discarded. The culture of the other board remains in cultivation and without contamination.

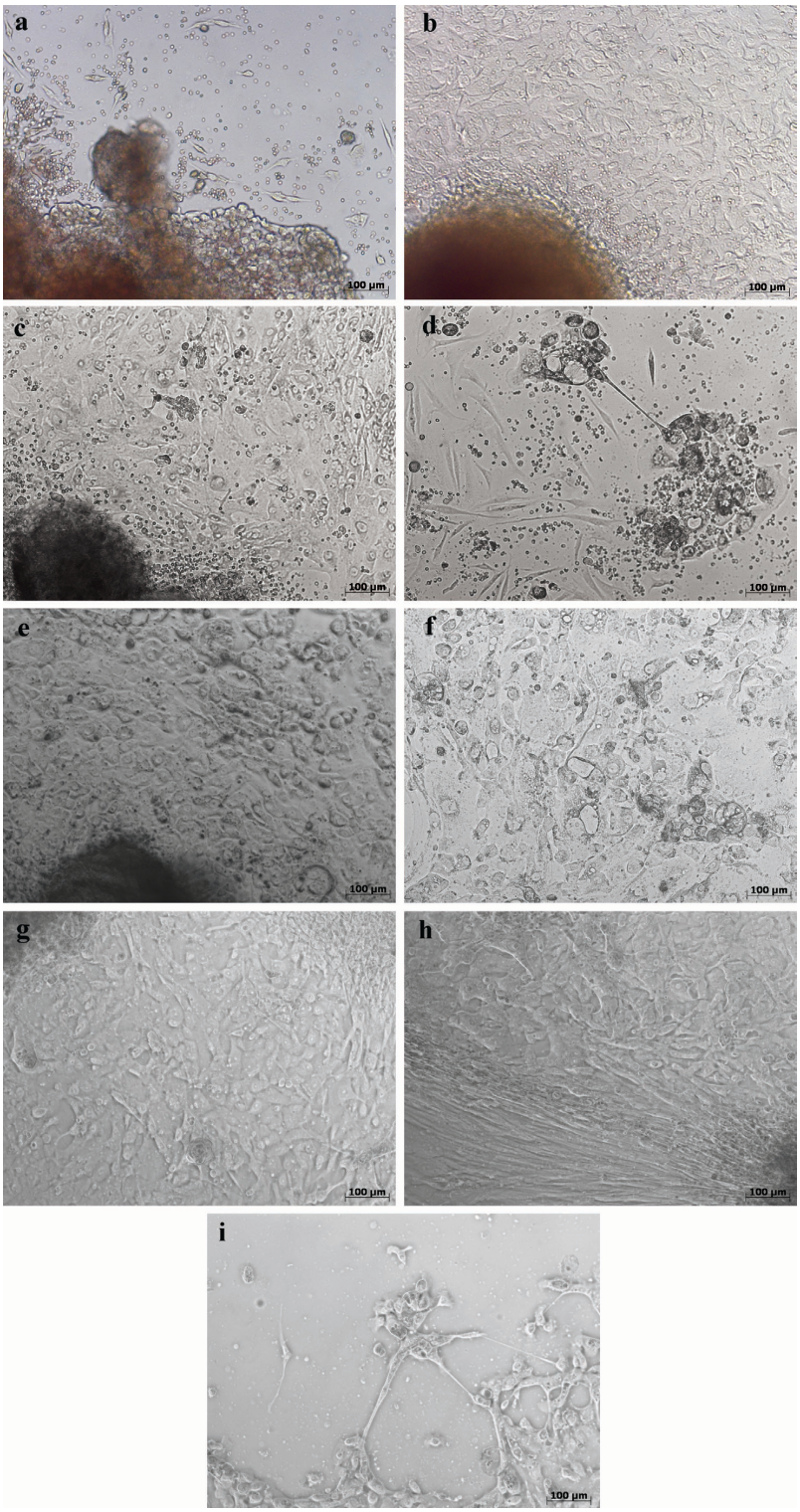

Figure 1. Representative photomicrograph of one of the primary cultures over five weeks of culture. **A and B** - the first week of cultivation. Initial monolayer formation of fibroblasts and keratinocytes occurring on opposite sides of the same fragment is observed. **C and D** - second week of cultivation; neoplastic cells with highly vacuolized cytoplasm and occurrence of cytoplasmic projections. **E and F** - third week of cultivation. **G, H and I** - fifth week of culture. There is intense progression of the monolayer of both cell types and the circumscription of neoplastic cells by fibroblast bundle. In I, the cellular anastomosis of keratinocytes.

different organizational distribution from the keratinocytes: diffuse elongated bundles, spaced between themselves, and in variated directions. Moreover, the fibroblasts presented higher proliferation rate when compared to the keratinocytes (neoplastic cells). This unbridled fibroblast growth limited the neoplastic cells expansion and directed the expansion of the monolayer of these in opposite direction of those. When it was observed that the board was almost all dominated by fibroblasts and, consequently the decrease of the neoplastic cells expansion due to shortage of adhesion available area, it was performed the differential trypsinization[5]. This technique consists in the enzymatic removal from the great part of the fibroblasts using trypsin-EDTA (code T4174, SIGMA®) for one to three minutes. This is possible due to the proteolytic action of the trypsin-EDTA on the adhesion molecules of the fibroblasts, which in a short period does not affect cells of the keratinocyte kind in a considerable way.

For the samples of normal tissue, high liberation of non-plastic cells (keratinocytes) and expressive growth of the monolayer in only 14 days of culture were observed. Only two non-plastic tissue fragments presented fibroblasts liberation, however, in numbers the quantity is insignificant (ten fibroblasts per board maximum). In a general way, the normal tissue culture presented itself fibroblasts free. That shows that there is a different behavior of the two cells types, fibroblasts and keratinocytes, in the tumoral microenvironment with significative modification of the fibroblasts[6].

Subculture and contamination control. After one week of cultivation, it was performed the fibroblasts subculture associated to the tumor. They were removed from the board that contained the fragments by enzymatic dissociation with trypsin-EDTA and transferred to another culture board of 100 mm. The fibroblasts were maintained in 10mL of complete culture mean, yet without ciprofloxacin. After 5 days, the fibroblasts confluency was approximately of 80% and then it was performed the freezing of these fibroblasts in FBSi with 5% of DMSO (Dimethyl sulfoxide, SIGMA®, code D2438) and aliquots packaging in a freezer at -80 °C. The neoplastic cells subculture was performed three weeks after fibroblasts removal. For this procedure a similar protocol used for the fibroblasts was applied, however the trypsin action was permitted for longer time (6 to 7 minutes). In these cases, as it was expected, there was dissociation of both fibroblasts and neoplastic cells, and both were transferred to the 6 wells board. The tumoral cells were maintained in complete DMEM without ciprofloxacin and with final volume of 2 mL per well. The fragments remained in culture with 2 mL of complete DMEM associated to 20 µg/mL of ciprofloxacin. Two days after explants subculture, it was performed the partial substitution of the culture mean and it was observed cellular adhesion of both tumoral cells as fibroblasts. However, two days after the first partial trade of the culture mean, it was observed uncontrollable bacterial contamination of the subculture. Hence it was decided to discard these boards with the subculture. Until now it was not performed new subculture of none of the other cases that remain in expansion.

Discussion

The establishment of primary culture is a challenge observed in many labs. In this study the delicate initial stages of primary culture of malignant and non-malignant cells of oral cavity tissues obtained from mouth cancer patients were described. Usually, the development of primary culture presents itself with cellular composition initially heterogeneous[3]. In this study, lots of debris and keratin fragments present in the first weeks of culture were observed. The predominant fibroblasts and keratinocytes presence was also observed. However, yet in the first weeks the culture presented high quantity of cell types morphologically similar to the immune system cells (Figure 1). The pertinent literature has reported the importance of the tumoral microenvironment for progression and maintenance of the neoplasms[6]. Going to in vitro culture, it can be proposed that the presence of fibroblasts e the probable cytokines and chemokines liberated by them, contribute to the initial stage of tumoral cells expansion in the primary culture. The causes of unsuccess in this study were always related to uncontrollable bacterial contamination, senescence of the cells after a certain cultivation time or even by absence of liberation of any cellular kind from the fragments. However, in all success cases, it was observed the usually intense presence of fibroblasts, which suggests that in the presence and maintenance of the fibroblasts in the initial stages is important for the establishment of the keratinocyte in culture.

The intense bacterial contamination reported for some cases is understandable and even expected because of the origin of the samples. The oral cavity presents delicate balance of its complex and numerous microbiota. Although incipient and discreet, the interest in the bacterial performance in the oral carcinogenesis has increased. Some studies reported the considerable proliferation of aerobic and anaerobic micro-organisms in the biofilm present in oral carcinoma in humans[7]. This study observed that the bacterial contamination in the explants were relatively controlled with success, however, when there is an exacerbated bacterial proliferation, the keratinocytes expansion stops. This uncontrolled contamination also shows to be resistant to some antibiotics, once the concentration of 5% of antibiotics (10.000 U de penicillin, 10 mg de streptomycin e 25 µg de amphotericin B/ mL) associated to ciprofloxacin (20 µg/mL) was not capable to stop it. There are reports in the literature of establishment and obtention of cellular lineages of head and neck carcinomas, but during the obtention of the lineages, many cultures were lost due to contamination by bacteria or the inability of the cells to survive in culture (tumor intrinsic), indicating that the strategy still needs to be improved and the bacterial contamination eliminated[5].

Conclusion

The utilization of explants for establishment of the initial stages of primary culture of head and neck cancer is a viable alternative and of easy reproducibility. The effective success rate has achieved in 20-30% of the cases and the contamination control presents as one of the biggest obstacles to be surpassed in the initial stages of cultivation.

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