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Is it safe to utilize in vitro reconstituted human oral epithelium? An oncogenetic pathway study

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Abstract Cell therapy is a therapeutic strategy used to replace or repair damaged tissue. The epithelium transplantation of cultivated keratinocytes has been applied to several modalities of reconstruction, like oral, urethra and ocular surface. Life and death signals work coordinately to ensure cellular quality control and the viability of an organism. The aim of this study is to verify that culture conditions did not induce genetic mutations through the analysis of the key genes: pAKT, Pten, p53 and MDM2 and investigate the presence of the related proteins in human oral keratinocytes obtained by primary culture and in vitro cultivated. Formalin fixed and paraffin embedded tissues from the oral cavity were utilized as control for normal expression of the related markers and two oral squamous cell carcinoma cell lines provided the expression pattern of the proposed markers in the event of cellular transformation. Akt, PTEN, p53 and MDM2 immunohistochemistry and Western-Blotting analyzes were performed. The results showed the expression levels and intracellular localizations of the four proteins evaluated. These analyzes confirmed that the produced in vitro epithelium is bio-compatible for its utilization as reconstruction and reparatory tissue, however further analyses and additional research on other biomarkers should be performed to analyse the long term engraftment of transplantable primary culture of oral keratinocytes and the long term resistance to cellular transformation.

Keywords Cell culture · Cell signaling · Keratinocytes culture · Normal human oral keratinocytes · Normal human mucosa · Signaling pathways

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Introduction

Cell therapy is an emerging therapeutic strategy that aims to replace or repair severely damaged tissues with cultured cells (Gage 1998). Since 1980, epithelium transplantation has been used as definitive procedure (De Luca and Cancedda 1992). Transplantation of cultivated oral epithelium is not restricted only to iso-type transplant. A transplantable cultivated epithelial sheet is a state-of-the-art therapeutic modality for various ocular surface disorders (Kinoshita et al. 2004). Oral mucosal grafts are also known to be the most promising substitute for urethra epithelium. Among several advantages in using oral

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mucosa is the easy accessibility of donor tissues besides its compatibility with wet environment as well resistance to infection. A thick epithelium and a thin connective tissue allow early inosculation with good medium-term results which are comparable to skin grafts results (Bhargava et al. 2008).

Cells under culture conditions may accumulate genomic mutations related to adaptation to the foreign extracellular environment. This process could cause a genomic instability leading to cellular transformation and aggressive phenotype. Therefore is essential to investigate the expression levels of key genes commonly modulated during cellular transformation before the clinical application of this technique.

Life and death cell signals work coordinately to ensure cellular quality control and organism viability. Oncoproteins and tumor-suppressor proteins regulate cell growth and viability (Mayo and Donner 2002). AKT is a serine–threonine kinase downstream of PTEN/PI3 K that is ubiquitously expressed, however its levels are variable depending upon the tissue type (Datta et al. 1999). AKT is phosphorylated and activated by growth factors, including insulin and insulin growth factor-1, and plays an important role promoting cell survival (Downward 2004). PTEN was identified in 1997 as a tumor suppressor gene that is often altered by somatic mutations in various types of cancer. Introduction of PTEN gene into tumor cells lacking the wild-type gene has revealed that PTEN takes part in the modulation of a number of cellular processes such as cell growth, survival, morphology and motility (Davies et al. 1998).

The tumor suppressor protein, p53, is considered to be one of the major indicators of stress-induced signaling in cells. In normal unstressed cells, p53 is continuously synthesized and is highly unstable, and its levels are kept low by ubiquitin-dependent degradation. Under stress, p53 activates signaling pathways involved in cell cycle arrest, DNA repair or apoptosis, hence preventing the propagation of defective genes or mutations that could have a potential role in tumorigenesis (Vousden and Lane 2007). The *mdm2* is an oncogene whose major role is to control of p53 degradation. The encoded protein binds to the transcription activation domain of p53, blocking the recruitment of additional factors necessary for induction of gene expression (Momand et al. 2000). MDM2 contains nuclear localization and nuclear export sequences and shuttles across the nuclear membrane in both directions (Juven-Gershon and Oren 1999). Nuclear export is an important mechanism through which MDM2 promotes p53 degradation (Mayo and Donner 2002).

Objective

The aim of the present study is to evaluate the presence of pAkt, PTEN, p53 and MDM2 in the in vitro cultivated human oral keratinocytes obtained by primary culture and compare the results with those found in human oral epithelial tissue and oral squamous cell carcinoma (OSCC) cell lines.

Materials and methods

Primary culture

The primary culture of oral keratinocytes was established from human oral epithelial tissue from a volunteer undergoing ulectomy. The tissue sample was washed and disinfected before minced into small pieces to establish the primary culture and obtain the keratinocytes (Klingbeil et al. 2009). The keratinocytes were cultivated in a culture cell medium constituted of Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York, NY, USA), F-12 Nutrient Mixture (HAM, Gibco, New York, NY, USA) (2:1), supplemented with 10% Bovine Serum Product, Fetal Clone III (Hyclone, Logan, Utah, USA), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), amphotericin B (2.5 µg/ml), glutamine (4 mM), adenine (0.18 mM) (Sigma- Aldrich, St. Louis, MO, USA), insulin (5 µg/ml) (Sigma–Aldrich, St. Louis, MO, USA), hydrocortisone (0.4 µg/ml) (Sigma–Aldrich, St. Louis, MO, USA), cholera toxin (0.1 nM) (Sigma–Aldrich, St. Louis, MO, USA), triiodotyronine (20 pM) (Sigma–Aldrich, St. Louis, MO, USA) and Epidermal Growth Factor (EGF) (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) (Benassi et al. 1993).

These cells (6×10^3 cells/cm^2) were seeded on a feeder-layer 3T3-Swiss albino, a murine fibroblasts lineage (ATCC/catalogue number CCL-92) and kept in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 (Klingbeil et al. 2009).

Epithelium production

The keratinocytes (12×10^3) were seeded in a 1.98 cm^2 deep dish on a feeder-layer. Cell culture medium was initially changed every 2 days and then every day, after the cells reached the confluence. After the first confluent day, the formed epithelium was removed through dispase enzyme action (0.25%, Neutral Protease—grade II, Boehringer–Manheim, Germany) during 1 h at 37°C. The epithelium was stored in a 10% formaldehyde solution.

Cell lines and culture conditions

The cell lines utilized in this study were: SCC-9 (ATCC) and HN-6, both OSCC cell lines from base of tongue. The cells were seeded in a concentration of 6×10^3 cells/cm^2 in cell culture flasks and cultivated with Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York, NY, USA), with 10% Bovine Serum Product, (Bovine Fetal Serum, Cultilab, Campinas, SP, Brazil), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml) and glutamine (4 mM). For the SCC-9 cell line it was also added F-12 Nutrient Mixture (HAM, Gibco, New York, NY, USA) in a ratio of 1:1. These cells were kept in an incubator at 37°C in a humidified atmosphere containing 5% CO_2.

Normal oral mucosa fragment

The oral mucosa fragment used as control came from the biopsy of a volunteer undergoing ulectomy. The fragment was fixed in 10% buffered formalin and embedded in paraffin. This tissue came from the same oral region as the sample used for primary culture, however from different patient.

Immunohistochemistry and hematoxylin-eosin staining of the in vitro epithelium and normal oral mucosa

Three-μm thick tissue sections were deparaffinized with xylol and rehydrated with a graded ethanol series washes. One slide was stained with Hematoxylin and Eosin. Sections were subjected to antigen retrieval: pAkt, PTEN and p53 ethylenediaminetetraacetic acid (EDTA) (10 mM, pH 8.0) and MDM2 citric acid (10 mM, pH 6.0) followed by endogenous peroxidase activity blockage with hydrogen peroxide. After primary antibody incubation with: pAkt (Santa Cruz, Rabbit sc-7985-r, 1:50), PTEN (Zymed, Rabbit 18-0256, 1:50), p53 (DAKO, Mouse M7001, 1:75) and MDM2 (DAKO, Mouse M0639, 1:40) the sections were exposed to the avidin–biotin complex (LSAB peroxidase kit, DAKO Auto Staining system). Antibody complexes were revealed after the addition of buffered diaminobenzidine, followed by counterstaining with Mayer's hematoxylin. The samples utilized for the external positive controls were: oral squamous cell carcinoma (pAkt) (Amornphimoltham et al. 2004), prostate epithelium (PTEN) (Abbott et al. 2003), colon carcinoma (p53) (Burkhard et al. 1997) and osteosarcoma (MDM2) (Longhi et al. 2001). The samples utilized for the external negative controls were: normal salivary glands (pAkt, p53 and MDM2) (Marques et al. 2008) and PC-3 prostate cancer cell line (PTEN) (Depowski et al. 2001). Negative controls of the reaction were obtained by omitting primary antibodies.

Western blotting

For Western blotting (WB) analysis, keratinocytes, SCC-9 and HN-6 were cultivated on 60.1 cm^2 dish until reaching the confluence and then washed three times with cold phosphate buffer solution (PBS). After that, cells were incubated on ice-cold lysis buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na3VO4, 0.5% NP40, 1% Triton X-100 and 1 mmol/l phenylmethylsulfonyl fluoride [pH 7.4]), with fresh protease inhibitor cocktail (Calbiochem, La Jolla, USA) over ice for 15 min. Cells were then scrapped and centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatant (total cell lysate) was collected, aliquoted and used on the day of preparation or immediately stored at −80°C. Twenty micrograms of protein were submitted to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After blocking the non-specific binding sites with non-fat dry milk or 1% BSA (phosphorylated proteins), membranes were incubated with anti-pAkt (1:100, Santa Cruz Biotechnology—sc7985), anti-PTEN (1:200, Santa Cruz Biotechnology—sc7974), anti-p53 (1:100, Santa Cruz Biotechnology—sc47698), anti-MDM2 (1:200, Santa Cruz

Biotechnology—sc5304), and anti-β-actin (1:5,000, Sigma–Aldrich—AC-15) overnight. The blots were then incubated with secondary antibody (goat anti-rabbit or goat anti-mouse, 1:3,000; Santa Cruz Biotechnology—sc2004 and sc2005, respectively). The bound antibodies were detected using the colorimetric detection kit Opti 4CN (Biorad Laboratories, Hercules, CA, USA). pAkt and PTEN proteins were chosen as positive controls for SCC-9 cell line, and MDM2 and p53, for HN-6 cell line (Towbin et al. 1979).

Results

Normal expression pattern of pAkt, PTEN, p53 and MDM2 in primary culture of keratinocytes

The Fig. 1 shows the morphologic aspect of the cultured keratinocytes in the culture dish in the moment that the cells became confluent (Fig. 1a). The normal human oral mucosa architecture can be observed in a histological section stained with Hematoxylin and Eosin (Fig. 1b).

The intracellular localization of pAkt, PTEN, p53 and MDM2 proteins in keratinocytes from cultured oral epithelium and normal oral mucosa utilized in this work as control can be observed at Fig. 2.

pAkt protein showed a strong and pronounced staining at the cell cytoplasm, as can be evidenced by the dark brown coloration, and less intense staining at the nucleus. It can also be observed that pAkt staining was slightly sharper at area that was in direct contact with the culture plate and was considered the basal layer of the cultured oral epithelium (Fig. 2a). At the oral mucosa, pAkt protein showed an intense staining at the cell nucleus from the different epithelial layers and at the cell cytoplasm as well. It is also possible to observe that various nuclei, especially at the squamous cell layer, were not stained (Fig. 2b).

The immunohistochemistry staining of PTEN was prominent at the cell cytoplasm of cultivated keratinocytes, however a less intense staining could also be observed at the cell nucleus (Fig. 2c). Confirming our findings, oral mucosa samples showed intense cytoplasmatic staining. Nuclei presented weaker staining and some of them were not stained (Fig. 2d).

The cultivated oral epithelium exhibited strong nuclear staining of p53 protein that was evidenced by the dark brown coloration. Cell cytoplasm also exhibit p53 staining, however it was weaker when compared to the nuclear staining (Fig. 2e). The oral mucosa presented just a few nuclei dark brown stained at the basal layer what indicates that this protein is not highly expressed in this tissue. At the squamous cell layer it is possible to observe slightly brown cytoplasm coloration at one cell (Fig. 2f).

MDM2 protein exhibited weak staining restricted to the nucleus of the cultivated keratinocytes (Fig. 2g) as well as the oral mucosa (Fig. 2h).

Culture techniques of human oral keratinocytes for transplantation does not induce cellular transformation

The results obtained by the Western Blot method showing expression levels of the evaluated proteins in

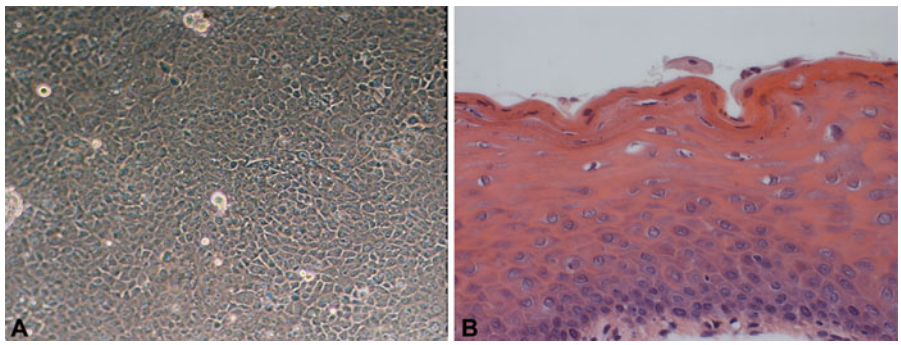

Fig. 1 Aspect of the keratinocytes culture and the oral normal epithelium. **a** shows the keratinocytes in the culture dish on the day that they became confluent (×400). **b** shows the histological architecture of the fragment of the human normal oral mucosa stained with Hematoxylin/Eosin (×400)

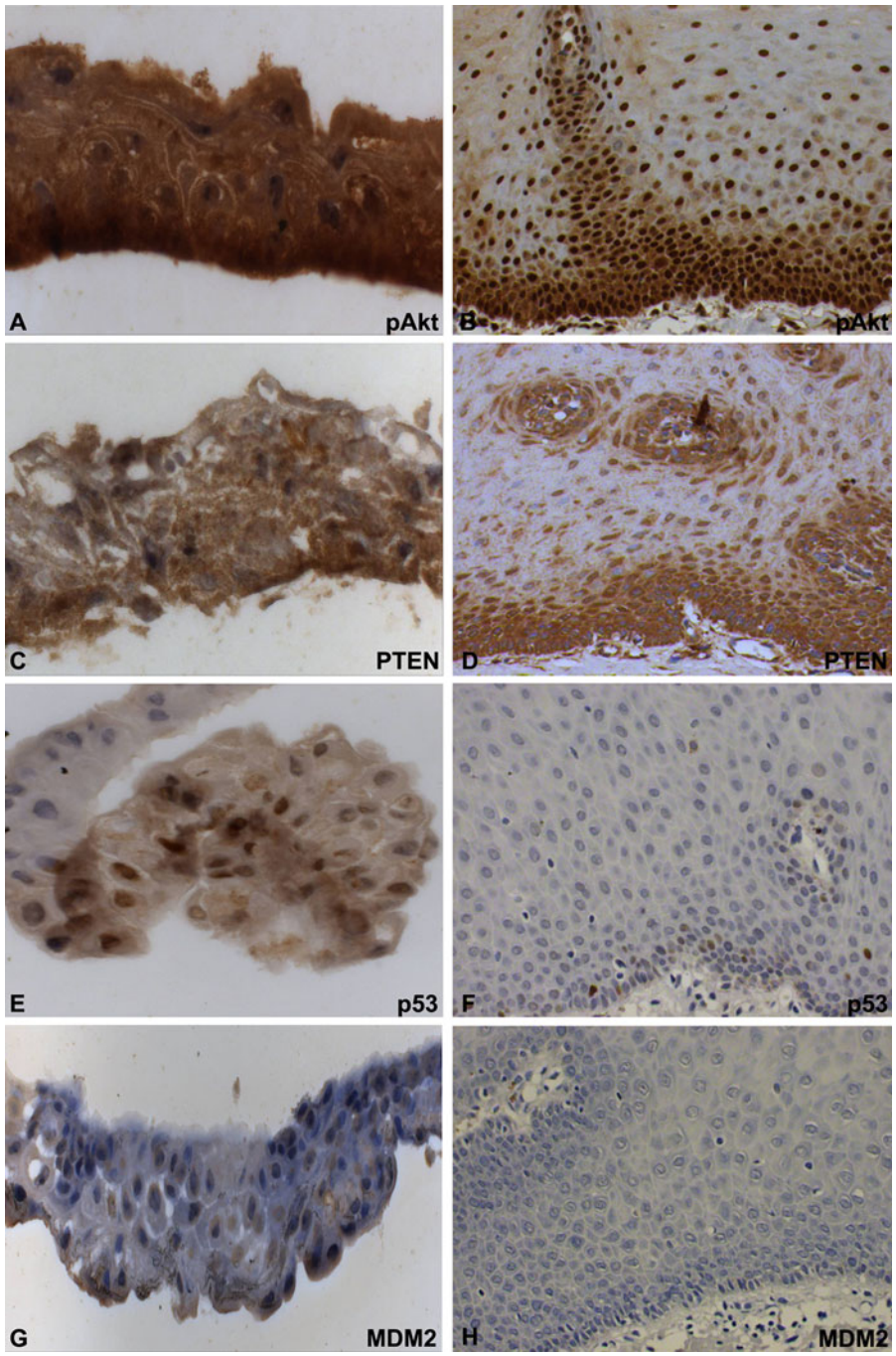

Fig. 2 Intracellular localization of pAkt, PTEN, p53 and MDM2 proteins at the cultured oral epithelium and normal oral mucosa. **a** shows strong cytoplasmatic and the nuclear expression of the pAkt in the in vitro cultivated epithelium. **b** shows the cytoplasmatic and nuclear staining of the pAkt at normal oral mucosa. **c** shows the cytoplasmatic and some nuclear expression of PTEN in the cultivated epithelium and the **d** shows the same expression level, with some nuclei without any staining in the oral mucosa. **e** and **f** show the strong nuclear and light cytoplasmatic signaling of p53 in both, cultivated epithelium and oral mucosa respectively. **g** shows a very weak expression of the nuclear MDM2 protein at cultivated epithelium and the same expression level can be seeing at oral mucosa (**h**). (×400)

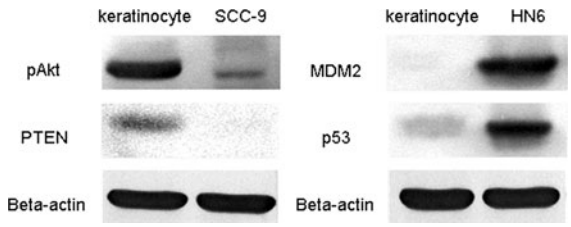

Fig. 3 Protein levels analysis of pAKT, PTEN, MDM2 and p53 based on Western blot results of the SCC-9 and HN-6; OSCC cell lines

cultured oral keratinocytes and the previously established OSCC cell lines, SCC-9 and HN-6, are presented at Fig. 3. β-actin was utilized to control total volume of each sample.

Cultivated oral keratinocytes showed higher expression levels of pAkt and PTEN when compared to the OSCC cell line SCC-9. When the same analysis was performed for the p53 and MDM2 proteins, it was observed that p53 showed low expression, while MDM2 was not expressed. These results were compared to the protein amount from the control cell line HN-6 according to the same methods. These data are in accordance to the results from the immunohistochemistry analyses.

Discussion

Cell therapy is an emerging therapeutic strategy aiming to replace or repair severely damaged tissues with cultured cells (Gage 1998). The technique to grow keratinocytes in vitro and generate cohesive sheets of stratified epithelium that maintains the characteristics of authentic epidermis was developed by Rheinwald and Green in 1975 (Rheinwald and Green 1975) and that is the most commonly used technology for producing graftable epithelia (Ronfard et al. 2000).

With the goal of evaluating the quality of the in vitro cultivated oral epithelium, the presented work compared the cellular localization of the proteins pAkt, PTEN, p53 and MDM2 in the in vitro cultivated oral epithelium to localization at the oral mucosa. Expression levels of these proteins were also compared to the levels at the OSCC cell lines, HN-6 and SCC-9.

Several growth factors were added to the culture medium, which could explain the high levels of pAkt expression observed in the immunohistochemistry and western blotting analyzes.

It has been shown that the addition of hydrocortisone to the cell culture can stimulate cell proliferation (Bradley et al. 1994). Cholera toxin, another factor added to the cell culture medium, increases the levels of cyclic adenosine monophosphate (c-AMP). The c-AMP is an intracellular mediator directly related to cell cycle. It responds to different extracellular signal, like hormones, growth factors and neurotransmitters. Thus c-AMP regulates some biological events, such as cell proliferation and differentiation (Adachi et al. 1982). Through c-AMP, cholera toxin impairs keratinocytes tendency to increase in size, thus delaying their terminal differentiation (Pullar and Isseroff 2005). EGF (epidermal growth factor) stimulates the duplication and migration of keratinocytes and consequently increases the longevity and number of cells (Rheinwald and Green 1977). EGF seems to antagonize terminal differentiation and increases cell division rate by a mechanism distinct from that exercised by the addition of the cholera toxin. Together: hydrocortisone, EGF and cholera toxin increase the clonogenic fraction, the growth population in culture and life (life span). Thyroid hormones regulate cellular metabolic activity and co-regulate cell proliferation, apoptosis, and differentiation (Puzianowska-Kuznicka et al. 2006). All these may corroborate with the obtained results.

Keratinocytes growth is regulated by the balance between molecules controlling cell survival and death (Hussein et al. 2004).

Cell growth and viability are regulated by different tumor-suppressors and oncoproteins. The proteins analyzed in this study are expressed in different tumor types as well as in normal tissues and they are involved in PI3 K/Akt signalling pathway. Activation of PI3 K occurs in response to the engagement of antigen-receptors and numerous growth factors, such as EGF and insulin (Kandel and Hay 1999). PI3 K signaling pathway promotes cellular proliferation and survival, increases resistance to apoptosis, and enhances adhesion and migration. PI3 K is regulated by PTEN, a multi-functional phosphatase whose lipid phosphatase activity is associated with tumour suppression. The major substrate of PTEN is PIP (Maehama and Dixon 1998) a lipid molecule generated by the action of PI3 K. Actually, PI3 K phosphorylates PtdIns P2 (PIP2) yielding PtdIns P3

(PIP3), which serves as an anchoring for intracellular proteins, including Akt. Membrane-bound Akt is phosphorylated in a threonine residue in the catalytic domain by the kinase PDK1, which also binds PIP3; this results in partial activation of Akt, which becomes fully activated only after the subsequent phosphorylation of a key serine residue in the regulatory domain (Stokoe et al. 1997; Stephens et al. 1998). As shown in this work, the presence of activated/phosphorylated form of Akt was found in cytoplasm and nucleus from the cultivated epithelium and normal oral mucosa. According to Cichy et al. (Cichy et al. 1998) the presence of nuclear pAkt can be related to insulin like growth factor binding protein 1 (IGFBP1), but until now there is no consensus about the real role of pAkt in the nucleus in the literature (Cichy et al. 1998).

By dephosphorylating the D3 position of PIP3, PTEN negatively regulates the PI3 K pathway and Akt activation, and thus tumorigenesis. PTEN is thus an important regulator of normal development and spontaneous and chemically induced oncogenesis in the skin (Suzuki et al. 2004) Akt activation can be necessary and sufficient for cell survival. Akt promotes survival by phosphorylating substrates that decrease the activity of pro-apopototic or increase the activity of anti-apoptotic proteins (Mayo and Donner 2002).

As mentioned**,** our results showed a strong expression of pAkt protein in cultivated keratinocytes. This result might be a consequence of the growth factors added to the cell growth medium that influence cell proliferation and life span in the cultivated epithelium. However, we believe that pAkt overexpression might be regulated by PTEN protein that is absent in OSCC cell line, as evidenced by western blotting results. Weng and cols in 2001, showed that the cell stimulation increased the overall levels of akt phosphorylation, but did not alter the effect of PTEN on Akt phosporylation, which corroborates our findings (Weng et al. 2001).

Recently, several reports have indicated that PTEN shuttles between the nuclear and cytoplasmic compartments. Nuclear PTEN is primarily found in normal cells and is directly correlated to cell differentiation (Gericke et al. 2006). Our results showed, through the immunohistochemistry method, a prominent citoplasmatic staining.

Cells normally go through the different stages of the cell cycle in a well-regulated manner. A number of different proteins involved in this regulation have been identified and the p53 protein is one of them (Nylander et al. 2000) This protein plays a major role regulating the response of mammalian cells to stresses and damage, in part through the transcriptional activation of genes involved in cell cycle control, DNA repair, senescence, angiogenesis and apoptosis (Levine 1997). Evidence that p53 is a tumor suppressor comes from genetic studies showing that mice null for the p53 gene develop tumors with higher frequency (Donehower et al. 1992) Several reports have documented p53 expression in normal tissues (Brys et al. 2000). Dippold et al. have reported p53 nuclear staining in proliferating and cytoplasmic in non-neoplastic cell lines (Dippold et al. 1981).

The p53 protein has a very short half-life and is not abundant in normal cells (Levine 1997), therefore, it can be hard to detect in normal tissue. However, the protein can remain in the tissue for longer periods for certain reasons, such as a mutation, a defect in the degradation pathway, or by binding to other proteins (Nylander et al. 2000). The literature confirms the results obtained in the present work which showed, by western blot, that p53 is overexpressed in OSCC cell lines but downregulated in cultured oral keratinocytes. The regulation of keratinocytes life time in cell culture is probably controlled by this protein expression. It was observed a range of about 20 days until the epithelia were totally degraded (data not shown) showing that cells reached the end of their culture life resulting in the senescense and apoptose process (Klingbeil et al. 2009). The p53 detection at the nucleus is probably related to its functional activity but not its degradation through the MDM2 protein, which shows light nuclear expression by the immunohistochemistry in the cultivated epithelium. These data were confirmed by the oral mucosa immunohistochemistry results.

Blanco-Aparicio et al. (2007), demonstrated that inducible activation of AKT results in growth arrest and a senescent phenotype in normal primary human epithelial cells. Hence, activation of AKT in primary cells may represent an antitumorigenic effect. In this context, it has been proposed that p53 was the effector of AKT induced senescence (Blanco-Aparicio et al. 2007).

The MDM2 (Murine double minute gene 2) proto-oncogene constitutes a powerful negative regulator of

p53. Our findings showed that, according to the immunohistochemistry results, MDM2 expression was not significant in either in vitro cultivated epithelium or normal oral mucosa. Results for the in vitro cultivated epithelium were confirmed by the quantitative protein analyzes using western blot methodology. It is possible that when the p53 levels are high and the balance between both, MDM2 and p53, shows no pathogenic role from the cultured epithelium. The overexpression of the MDM2 protein may result in uncontrolled cell growth and subsequent tumorigenesis (Marques et al. 2008). In this way, the negative MDM2 expression in our results indicates that is safe to say that this oncogenic pathway has been not accessed.

Conclusion

The production of oral epithelium based in the primary culture of keratinocytes is safe according to the in vitro results and the expression pattern of the selected markers are compatible to normal tissue discarding any change in protein expression that could indicate cellular transformation.

Ethics

This project was approved by the Research Ethics Committee of the Nuclear and Energetic Institute Research, under License Number 087/CEP-IPEN/SP.

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