Study of the effects of a hypoxic microenvironment on human keratinocytes in vitro and its correlation with microenvironmental alterations in oral lichen planus pathology

Estudio de los efectos de un microambiente hipóxico en queratinocitos humanos in vitro y su correlato con alteraciones del microambiente en la patología de liquen plano oral

Estudo dos efeitos de um microambiente hipóxico em queratinócitos humanos in vitro e sua correlação com alterações microambientais na patologia do líquen plano oral

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Abstract

Hypoxia is a fundamental factor in the process of tumor genesis, as well as in precursor pathologies of cancer, such as Oral Lichen Planus (OLP).

Objective: To determine if it is possible to establish a correlation between the alterations that normal keratinocytes suffer in a hypoxic microenvironment in vitro and alterations that appear in the keratinocytes in the epithelium of the oral mucosa in the context of OLP pathology.

Methods: Morphological changes were studied by phase contrast microscopy, and the detection of markers associated with hypoxia of human keratinocytes (HaCaT), as an oral cell model, in a hypoxic microenvironment generated by the variant of the method "Hypoxia induced by coverslips".

Results: Using confocal microscopy, the presence of hypoxia markers GLUT-1 and Hipoxyprobe was observed in HaCaT cell cultures exposed to a hypoxic microenvironment. In addition, the presence of the GLUT-1 marker was observed by immunohistochemistry in human epithelial tissue derived from biopsies of OLP pathology.

Conclusions: A correlation was established between the alterations detected in human keratinocytes induced in a hypoxic microenvironment in vitro and the alterations detected in vivo in epithelial tissue of the oral mucosa.

Keywords: Hypoxia, Oral Lichen Planus, cancer, keratinocytes

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Resumen

La hipoxia es un factor fundamental en el proceso de génesis tumoral, así como en patologías precursoras de cáncer, como es el Liquen Plano Oral (LPO).

Objetivo: Determinar si es posible establecer una correlación entre las alteraciones que sufren queratinocitos normales en un microambiente hipóxico *in vitro* y alteraciones que aparecen en los queratinocitos en el epitelio de la mucosa oral en el contexto de la patología LPO.

Métodos: Se estudiaron los cambios morfológicos mediante microscopía de contraste de fases, y la detección de marcadores asociados a hipoxia de queratinocitos humanos (HaCaT), como modelo celular oral, en un microambiente hipóxico generado por la variante del método "Hipoxia inducida por cubreobjetos".

Resultados: Mediante microscopía confocal se observó la presencia de los marcadores de hipoxia GLUT-1 y aductos de pimonidazol (Hipoxyprobe) en los cultivos celulares de HaCaT expuestos a un microambiente hipóxico. Además, se observó la presencia del marcador GLUT-1 mediante inmunohistoquímica en tejido epitelial humano derivado de biopsias de la patología LPO.

Conclusiones: Se estableció una correlación entre las alteraciones detectadas en queratinocitos humanos inducidos a un microambiente hipóxico in vitro y las alteraciones detectadas in vivo en tejido epitelial de la mucosa oral.

Resumo

A hipóxia é um fator fundamental no processo de gênese tumoral, bem como em patologias precursoras do câncer, como o Líquen Plano Oral (LPO).

Objetivo: Determinar se é possível estabelecer uma correlação entre as alterações que os queratinócitos normais sofrem em um microambiente hipóxico *in vitro* e as alterações que aparecem nos queratinócitos no epitélio da mucosa oral no contexto da patologia do LPO.

Métodos: As alterações morfológicas foram estudadas por microscopia de contraste de fase e a detecção de marcadores associados à hipóxia de queratinócitos humanos (HaCaT), como modelo de célula oral, em um microambiente hipóxico gerado pela variante do método "Hipóxia induzida por lamínulas".

Resultados: Por microscopia confocal, observou-se a presença dos marcadores de hipóxia GLUT-1 e Hipoxyprobe em culturas de células HaCaT expostas a um microambiente hipóxico. Além disso, a presença do marcador GLUT-1 foi observada por imuno-histoquímica em tecido epitelial humano derivado de biópsias de patologia de LPO.

Conclusões: Foi estabelecida uma correlação entre as alterações detectadas em queratinócitos humanos induzidas em um microambiente hipóxico in vitro e as alterações detectadas in vivo no tecido epitelial da mucosa oral.

Palabras clave: Hipoxia, Liquen Plano Oral, cáncer, queratinocitos.

Palavras-chave: Hipoxia, Líquen Plano Oral, cáncer, queratinócitos.

Introduction

Hypoxia occurs when oxygen is not available in sufficient amounts to carry out the metabolic processes required by the cells. Oxygen level is one of the essential parameters in the microenvironment surrounding cells, both in vitro and at the tissue level. Therefore, an oxygen decrease can lead to conditions that impact cell physiology and tissue homeostasis significantly. At the molecular level, a reduction in oxygen availability for cells activates transcription factors known as "hypoxia-inducible factors" (HIFs). In particular, HIF-1 is a protein complex formed by two subunits: HIF-1α and HIF-1β.[1] This heterodimer is inactive when oxygen is abundant but activated under hypoxia.[2] Its function as a transcription

factor is to increase the transcription of specific genes that code for proteins involved in various cellular processes such as cell proliferation and energy metabolism. In this way, HIF-1 allows cells to adapt to adverse conditions, as in the case of cells altered by inflammatory pathologies and tumor formation.[3] In turn, it has been shown that hypoxia, mainly through the expression of HIFs transcription factors, plays a significant role in keratinocyte terminal differentiation, regulating the keratinization of epidermal epithelial cells.[4] At the tissue level, hypoxia is frequently associated with inflammatory processes and can be either a trigger or a consequence of inflammation.[2] It has been shown to be essential in tumor genesis and development. In various types of cancer, such as oral cancer, it has been observed that hypoxia markers increase as the degree of oral mucosa dysplasia increases until an oral carcinoma appears.[5] Hypoxia also leads to the metabolic reprogramming of tumor cells. They acquire a more glycolytic phenotype, secreting lactate and thus causing marked acidification of the extracellular medium, which has also been detected in oral cancer.[6]

It is necessary to understand the composition of the oral mucosa to understand the process by which it can lead to oral cancer precursor lesions and to cancer itself. The oral mucosa is formed by epithelial and connective tissue and is differentiated and classified according to its functions. Therefore, the oral cavity has three types of mucosa: the masticatory mucosa, the specialized mucosa, and the lining mucosa. The first covers the palate and gums, the second covers the dorsal surface of the tongue, and the lining mucosa covers the inner surface of the cheeks, the ventral surface of the tongue, the floor of the mouth, and the inside lining of the lips. The epithelial tissue of the oral mucosa, like the epidermis, is a stratified squamous epithelium with a basal layer and a spinous layer, and is formed by keratinocytes.[7] Oral lichen planus (OLP) is an inflammatory, autoimmune condition that affects the epithelial

tissue of the oral mucosa. It is a chronic condition with great clinical relevance. OLP is estimated to affect up to 4% of the world's population.[1] The etiology is not yet well defined, and there is evidence that OPL may include an autoimmune attack by cytotoxic T lymphocytes on the epithelial cells of the oral mucosa.[8] Clinically, it presents oral mucosa lesions such as raised, scaly white patches, edematous red patches, and open sores, which may cause burning or pain. These lesions can appear on the mucosa lining the cheeks—the most frequent location—and on the inside of the lips, gums, palate, and tongue.[8] Histologically, OPL presents a dense lymphocytic infiltrate in the oral epithelium, an increase in the superficial layers of terminally differentiated keratinocytes, and keratinocyte degeneration in the basal layer of the oral epithelium.[9] There is evidence of its connection with cancer, specifically oral squamous cell carcinoma (OSCC). Several studies have shown that patients diagnosed with OLP have developed OSCC.[1] Even patients with oral carcinoma developed from OLP are at higher risk than those without an OLP diagnosis because of increased metastasis rates.[1] For these reasons, the WHO has classified this condition as a precursor to oral carcinoma and recognized it as an oral potentially malignant disorder (OPMD).[1] According to research on the transformation mechanism, chronic inflammation in OLP is involved in tumorigenesis and tumor development. Furthermore, evidence suggests that OLP also presents a hypoxic microenvironment.[1] However, the presence of hypoxia markers in this pathology has not yet been studied in depth.

This work used the HaCaT cell line, formed by nontransformed human keratinocytes derived from epidermal epithelial tissue. This cell line is widely used in epithelial tissue research, given its high differentiation and proliferation potential in in vitro cell cultures.[10] The epidermis is very similar to the epithelial tissue of the oral mucosa, so the HaCaT cell line has been frequently used as an oral keratinocyte model in oral cell bio-

logy in vitro studies.[11] Given the evidence of a hypoxic microenvironment in OLP, this work reviews the hypoxic microenvironment in the HaCaT cell line using a variant of coverslip hypoxia.[12] This variant was designed by Arocena et al.[13] and consists in covering a small culture dish with an acrylic coverslip with a well in the center, like the original method, but with a perforated co-verslip in the center (Figure 1), so that oxygen diffusion to all the cells in the well only occurs through the hole in the center of the dish. This shows the differences between the cells located in the center of the dish, the area where the only oxygen source is located, and those far from the oxygen sources, the peripheral areas of the dish.[13]

Figure 1: Diagram of the coverslip hypoxia variant taken from Arocena et al., 2019[13]
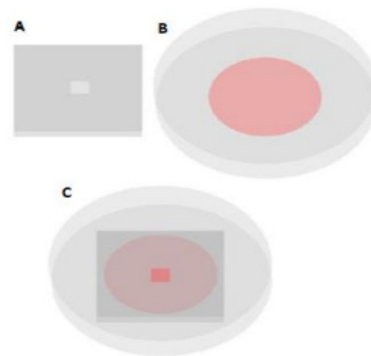
Materials and methods

Study cell line

The cell line used was HaCaT, formed by spontaneously immortalized, non-transformed human keratinocytes. These were maintained in low-glucose DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS and antibiotics (penicillin, streptomycin, and amphotericin) incubated at 37ºC and 5% CO2. Cells were passaged by trypsinization each time the cells reached confluence.

Method for inducing a hypoxic microenvironment in vitro with the coverslip variant

The method used to create a hypoxic microenvironment for keratinocytes is a variant of the "coverslip hypoxia" model, in which cell culture dishes are covered with acrylic coverslips with a perforation in the center. The perforated acrylic coverslip was placed 24 hours after transferring the cells to the dishes. One dish was used as a control, so no coverslip was added to avoid inducing a hypoxic microenvironment. These dishes containing the cell cultures, with and without coverslips, were incubated at 37ºC and 5% CO2 for 48 hours.

Observation of cell morphology by phase contrast microscopy

Cells incubated with and without the coverslip were observed by phase contrast microscopy after 48 hours. A Nikon Diaphot inverted microscope and 10X and 20X phase contrast objectives provided by the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE) were used.

Observation of cell morphological changes by confocal microscopy

Labeling was performed with the fluorescent actin filament detection probe Alexa Fluor 488 Phalloidin to observe morphological changes in the cytoskeleton of the cells. For this, HaCaT cells were fixed with 4% PFA coverslips, incubated for 10 minutes at room temperature, and after 10 minutes, washed with 1X PBS. After the last wash, cells were stained with Alexa Fluor 488 Phalloidin (Invitrogen, Waltham, MA) at a dilution of 1:200 and left to incubate for 30 minutes. Three washes were performed with 500 μL 1X PBS for 5 minutes each. After discarding the PBS from the last wash, DAPI (Invitrogen) was

added at a 1:1000 dilution to stain the nuclei and incubated for 15 minutes in the dark at room temperature. Finally, the cells were observed by fluorescence microscopy under a Zeiss LSM 800 confocal microscope at the IIBCE.

Immunocytochemistry with the Hypoxyprobe kit in vitro

Immunocytochemistry was used using a hypoxia detection kit called Hypoxyprobe (Hypoxyprobe, Burlington, MA) to assess the presence of induced hypoxia in the study cells. For this, HaCaT cells were incubated with coverslips for 48 hours, and then pimonidazole was added. This is a nitroimidazole that forms drug-protein adducts at low intracellular oxygen levels. It was added at a final concentration of 200 µM for 2 hours. Cells were then fixed with 4% PFA and incubated for 10 minutes at room temperature. After 10 minutes, they were washed with 1X PBS. The dishes were permeabilized with 200 µL 0.1% Triton for 5 minutes. Then, 200 µL BSA was added as a blocking agent, and the dishes were incubated for 30 minutes at room temperature. Finally, 2 µL of an antibody that recognizes pimonidazole protein adducts, conjugated to a fluorophore (Hypoxyprobe Red Mab Dylight 549 Mab), was added, and the dish was incubated overnight at 4°C. After discarding the PBS from the last wash, DAPI (Invitrogen) was added at a 1:1000 dilution to stain the nuclei and incubated for 15 minutes in the dark at room temperature. Finally, the cells were observed by fluorescence microscopy using a Zeiss LSM 800 confocal microscope.

Immunocytochemistry with GLUT-1 in vitro

The presence of hypoxia marker GLUT-1 was assessed in vitro by immunocytochemistry. For this, HaCaT cells were fixed with 4% PAF coverslips and incubated for 10 minutes at room temperature. Washes were performed with 1X PBS, and cells were permeabilized with 200 µL 0.1% Triton for 5 minutes. Then, they were blocked with 200 µL BSA, and the dishes were incubated for 30 minutes at room temperature. Finally, the primary anti-GLUT-1 antibody (mouse monoclo-

nal antibody, Santa Cruz) was added at a dilution of 1:200, and the dish was left to incubate overnight at 4°C. The primary antibody was discarded, and three washes were performed with PBS. After the last wash, 200 µL of secondary antibody (Alexa Fluor 488-conjugated anti-mouse antibody, from Invitrogen) diluted in BSA (1:1000 dilution - 0.5 µL secondary antibody in 450 µL BSA) was added and left to incubate for one hour at room temperature in the dark. After discarding the antibody, 3 washes were performed with 500 µL 1X PBS for 5 minutes each. After discarding the PBS from the last wash, DAPI (Invitrogen) was added at a 1:1000 dilution to stain the nuclei and then incubated for 15 minutes in the dark at room temperature. Finally, the cells were observed by fluorescence microscopy using a Zeiss LSM 800 confocal microscope.

Nile Red fluorescent probe labeling in vitro

For lipid detection, labeling was performed with the fluorescent probe Nile Red (Invitrogen, Waltham, MA), which stains intracellular lipid droplets. For this purpose, HaCaT cells were fixed with 4% PAF coverslips and incubated for 10 min at room temperature. After 10 minutes, they were washed with 1X PBS. Cells were then stained with the Nile Red probe at 0.1 µg/ml and left to incubate for 15 minutes. Three washes were performed with 1X PBS for 5 minutes each. After discarding the PBS from the last wash, DAPI (Invitrogen) was added at a 1:1000 dilution to stain the nuclei and incubated for 15 minutes in the dark at room temperature. Finally, the cells were observed by fluorescence microscopy using a Zeiss LSM 800 confocal microscope. Nile Red has different emission/excitation peaks, depending on whether it binds to neutral lipids or phospholipids, emitting green for neutral lipids and orange/red for phospholipids. Nile Red fluorescence was detected with excitation/emission of 510/582 nm to detect neutral lipids and 540/624 nm to detect phospholipids.[13,14]

Immunohistochemistry with GLUT-1 in vivo

The expression of hypoxia marker GLUT-1 in

the oral epithelium was evaluated by immunohistochemistry using histological sections from biopsies of patients with OLP and fibrous hyperplasia; the latter was used as a positive control. The samples were provided by the Molecular Pathology Department of the School of Dentistry. Six slides were used, three from OLP biopsies and three from fibrous hyperplasia biopsies. The preparations were first deparaffinized in an oven at 60°C for 60 minutes and then in Xylol for 5 minutes. Next, the preparations were placed on the hydration train, performing 15 baths for each one from 100% Xylol to distilled water. The preparations were then unmasked in a pressure cooker. They were left to cool for eight minutes after pressure release and then left to stand for ten minutes in running water. After that, three washes were performed with distilled water. The preparations were left in 0.9% hydrogen peroxide for 5 minutes, and then 3 more washes were performed with distilled water. The slides were mounted in the corresponding racks and coverplates, washed with 1X PBS for 5 minutes, and 2 drops of peroxidase blocker (Mouse/Rabbit ImmunoDetector Peroxidase Blocker) were added to each slide and left to act for 5 minutes. After several washes, 100 μL of the primary antibody GLUT-1—used for in vitro immunocytochemistry—was added to each slide (1:100 dilution with Da Vinci Green Diluent) and incubated for 1 hour. It was washed with PBS for 5 minutes, then 2 drops were added to each slide of the anti-mouse secondary antibody with biotin and left to incubate for 30 minutes at room temperature. After 30 minutes, another wash was performed with 1X PBS for 5 minutes, and then 2 drops of Mouse/Rabbit ImmunoDetector HRP Brown was added to each slide, which were left to incubate for 30 minutes. A further wash was performed with 1X PBS for 5 minutes, the slides were removed from the coverplates, and approximately 5 mL of DAB (diaminobenzidine) developer was added and left to incubate for 5 minutes. The slides were then rinsed with distilled and tap water for 5 minutes. After 5 minutes, the slides were placed in hematoxylin for 2 minutes, rinsed again in distilled water and tap water for approximately 3 minutes, and dehydrated (15 baths from distilled water to 100% Xylol alcohol). The slides were left to dry and then mounted with a drop of Entellan on each slide and a coverslip. Finally, the slides were observed under the Nikon Eclipse Ci microscope provided by the Molecular Pathology Department of the School of Dentistry, Universidad de la República, equipped with a Nikon Ds-Fi2 digital camera.

Results

Observation of morphological changes in HaCaT cells subjected to coverslip hypoxia

Phase contrast microscopy was used to observe the morphological differences of control cells, which did not contain coverslips, and the morphology of cells under coverslip hypoxia. Phase contrast microscopy showed morphological differences regarding the location of cells on the dish (Figure 2). The cells found in the peripheral regions (Figures 2C and 2D) have a more elongated and stretched shape than those in the center, where the oxygen source is located (Figure 2B), and, above all, compared to the control cells, which were not induced with hypoxia (Figure 2A).

Figure 2: Phase contrast microscopy observation of HaCaT cells
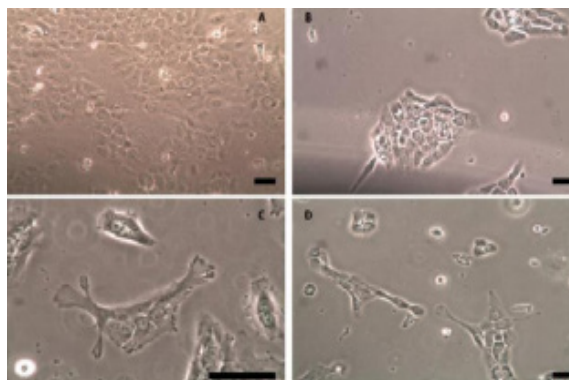

A) Culture of control cells in a normal microenvironment without hypoxia induction. B) Cells subjected to a hypoxic microenvironment via coverslip hypoxia and located in the center of the dish. C and D) Cells subjected to a hypoxic microenvironment via coverslip hypoxia and located in the periphery of the dish.

Based on the morphological changes observed via phase contrast microscopy in the hypoxia-induced condition, cell morphology was further studied by visualizing the actin cytoskeleton using the Faloidin probe, which detects filamentous actin. This probe is conjugated to a fluorophore (Alexa Fluor 488). Therefore, confocal microscopy showed the labeled cells and their morphological changes. This labeling showed that periphery cells have a more intense signal for actin filaments (Figure 3B) than cells near the oxygen source in the center of the dish (Figure 3A). In turn, these actin filaments appear in prominent stress fibers in the periphery cells (Figure 3B) compared to those in the center of the dish (Figure 3A).

Figure 3
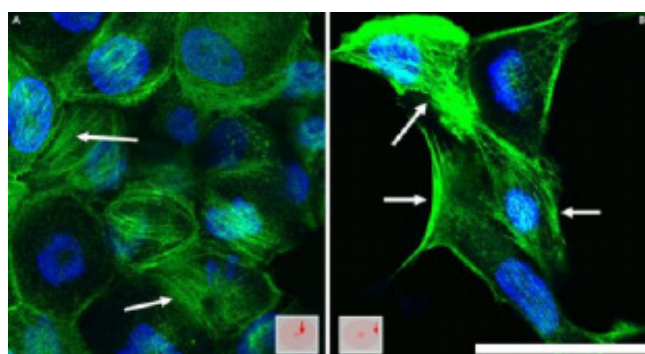
Observation by confocal microscopy of human keratinocytes cultured with the coverslip hypoxia method variant, which were labeled with an actin marker (green) and stained with DAPI (blue). A) HaCaT cells in the center of the dish, near the oxygen source. B) HaCaT cells in the periphery of the dish, far from the oxygen source.

Increased keratinization or cornification of the cells farther away from the oxygen source was detected due to the induced hypoxic microenvironment. Cells with cornified envelopes were also detected (Figure 4). Several studies indicate that the formation of these cornified envelopes is typical of and very important in the terminal differentiation of keratinocytes.[15] Cornification elements were observed by phase contrast microscopy throughout the study, 48 hours after placing the coverslip. These structures increased over time. This characteristic detected in cells in a more hypoxic microenvironment was also found with the fluorescent lipid marker Nile Red (see below, Figure 7).

Figure 4
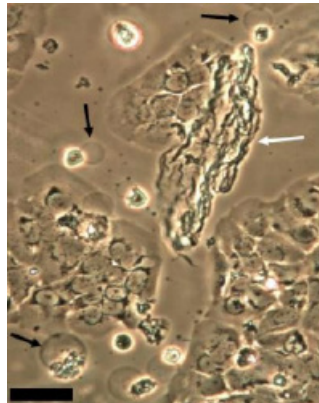
Phase contrast microscopy observation of HaCaT cells. Cells subjected to a hypoxic microenvironment via coverslip hypoxia and located in the periphery of the dish, where a fully keratinized cell appears in the center of the field, as well as terminal differentiation bubbles (indicated by arrows).

Detection of hypoxia using pimonidazole in vitro in HaCaT cells subjected to coverslip hypoxia

Immunocytochemistry was performed with the pimonidazole probe to detect hypoxia in HaCaT cells induced in a hypoxic microenvironment. It forms protein adducts, mainly with proteins with thiol groups, when oxygen levels are very low (pO2<10mmHg). These adducts are recognized using an antibody conjugated to a fluorophore and can be observed with confocal microscopy. [16] Thus, as cells move away from the center of the dish, they receive lower oxygen levels. This is evidenced by a higher pimonidazole signal toward the peripheral areas (Figure 5C) and a lower signal in regions away from the periphery (Figures 5A and 5B).

Figure 5
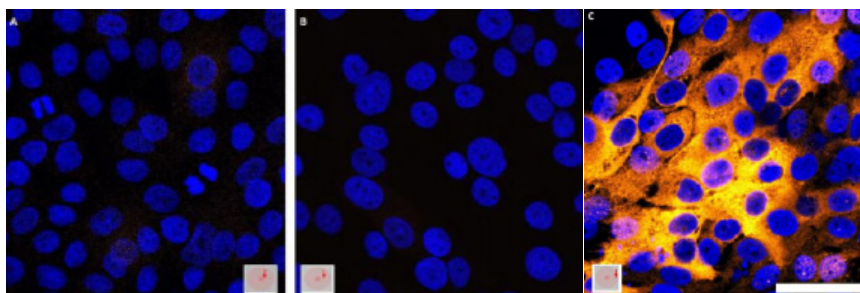
Confocal microscopic observation of human keratinocytes cultured under coverslip hypoxia conditions with the hypoxia detection probe Hypoxyprobe (orange) and stained with DAPI (blue). A) HaCaT cells in the center of the dish, where only the nuclei labeled with DAPI are observed. B) HaCaT cells in an intermediate region of the dish, where only the nuclei labeled with DAPI are observed. C) HaCaT cells in the periphery of the dish, where nuclei labeled with DAPI and a noticeable pimonidazole signal are observed.

GLUT-1 detection in vitro in HaCaT cells subjected to coverslip hypoxia

Immunocytochemistry was performed with hypoxia marker GLUT-1 after describing the hypoxia induction model and identifying hypoxia in areas away from the oxygen source. Immunofluorescence showed a strong GLUT-1 signal in the periphery of the dish (Figure 6B) but not in the center (Figure 6A) when the cells were observed with a confocal microscope.
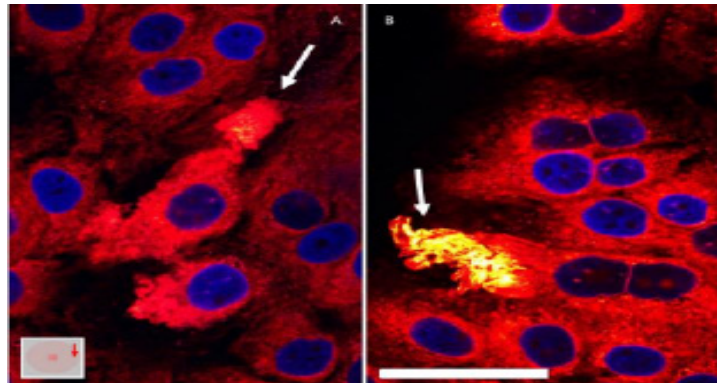
Figure 6
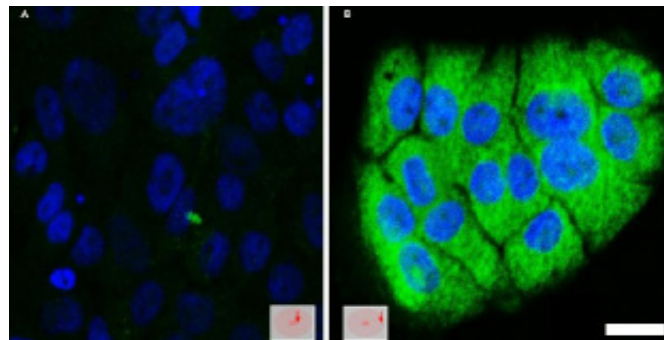
Confocal microscopy observation of HaCaT cells cultured with coverslip hypoxia. Immunofluorescence with GLUT-1 (green) was performed, and the cells were stained with DAPI (blue). A) HaCaT cells in the center of the dish, close to the only oxygen source, where only DAPI-labeled nuclei are observed. B) HaCaT cells in the periphery of the dish, away from the oxygen source, showing DAPI-labeled nuclei, in blue and a strong GLUT-1 expression.

Lipid detection with the Nile Red marker in vitro in HaCaT cells subjected to hypoxia

The detection of Nile Red, a membrane lipid marker, was studied by confocal microscopy after detecting increased keratinocyte cornification via phase contrast microscopy in the peripheral areas of the coverslip dish. This probe was detected with an excitation/emission of 510/582 nm (neutral lipids) in green, and 540/624 nm for phospholipids, in red. Marked signs of cell keratinization were observed in the periphery of the dish, showing the beginning of cornification and a lipid accumulation (Figure 7A) and more advanced cornification (Figure 7B). This is aligned with what was observed by phase contrast microscopy. Cells at the periphery (Figures 7 and 8B) show a more intense membrane lipid signal than HaCaT cells in the center of the dish labeled with Nile Red (Figure 8A). An increase in the accumulation of neutral lipid droplets in the peripheral areas was also observed (Figure 8B), as in previous studies.[13] Neutral and membrane lipids also presented an increased signal intensity.

Figura 7

Confocal microscopy observation of HaCaT cells cultured with coverslip hypoxia. They were stained with Nile Red (red) and DAPI (blue). Both images show HaCaT cells in different peripheral areas of the dish, away from the oxygen source, with their nuclei labeled with DAPI, and a marked lipid signal. Cells with cornified envelopes were also detected.
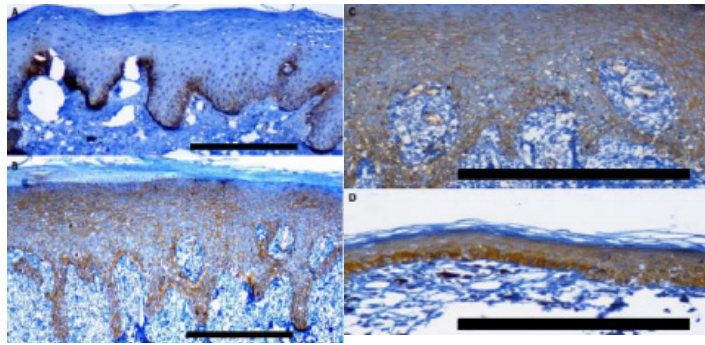
Figure 8
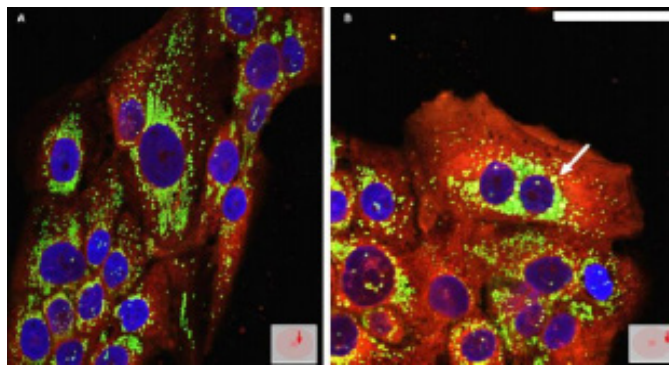
Confocal microscopy observation of HaCaT cells cultured with the coverslip hypoxia method variant, labeled with Nile Red, detected with excitation and emission at 510/582 nm (green) and excitation and emission at 540/624 nm (red), and staining with DAPI (blue). Membrane lipids are detected in red, neutral lipid droplets in green, and cell nuclei in blue. A) HaCaT cells in the center of the dish, near the oxygen source. B) HaCaT cells in the periphery of the dish, far from the oxygen source.

GLUT-1 detection in vitro in OLP preparations

Oral mucosa keratinocytes taken from OLP samples were studied in vivo with immunohistochemistry to detect GLUT1. Fibrous hyperplasia preparations were used as a positive control. Microscopic observation showed a marked expression of GLUT1 in all OLP preparations (Figures 9A, 9B, and 9C). A substantial increase in keratinization was also observed in the OLP oral epithelium. This agrees with the in vitro studies of human keratinocytes (Figure 10). Figure 10A shows an extensive stratum corneum, and Figure 10B shows an inner area of cornified epithelium.

Figure 9

Microscopic observation of the immunohistochemistry result with GLUT-1 performed on biopsy preparations of OLP and fibrous hyperplasia as a control. A) OLP at 10X. B) OLP at 20X. C) OLP at 40X. D) Fibrous hyperplasia at 20X.

Figure 10
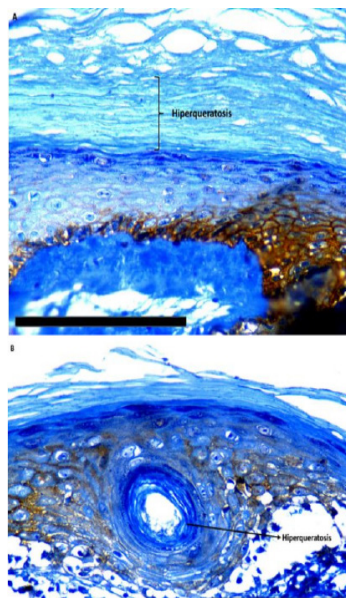
Microscopic observation of the immunohistochemistry results with GLUT-1 performed on OLP biopsy preparations shows a marked increase in keratinization. A) Hyperkeratinization in the stratum corneum is prominent. B) Increased keratinization in the central area of the epithelium.

Discussion

Observation of morphological changes in Ha-CaT cells subjected to coverslip hypoxia

The results of phase contrast microscopy observation agree with those expected, as the cells in the central region are located at the oxygen source, where the coverslip perforation is located. Therefore, these cells should not be under severe hypoxia. As the distance between the cells and the center of the dish increases, the oxygen level progressively decreases towards the peripheral areas. Therefore, the cells farthest away from the central area, in the periphery, receive less oxygen and should be in a more hypoxic environment, as observed (see Figure 5). Therefore, it can be assumed that this altered environment coincides with the morphological changes observed.[17] These are confirmed by observation of the cells by confocal microscopy to visualize the actin cytoskeleton. This agrees with previous observations of stress fiber accumulation in hypoxia.[17]

In turn, it has been observed that "bubbles" appear around the keratinized cells as part of keratinocyte terminal differentiation when the stratum corneum is formed (Figure 4, indicated with arrows). These bubbles are vesicles excreted from terminally differentiated cells. They contain cytoplasmic components that are secreted into the extracellular space, possibly to be reused by other cells.[15,18] Detecting these vesicles and cornified envelopes in cells exposed to a hypoxic environment suggests that hypoxia stimulates keratinocyte terminal differentiation, as previously observed.[4]

Detection of hypoxia using pimonidazole in vitro in HaCaT cells subjected to coverslip hypoxia

The expected results were obtained when observing HaCaT cells by confocal microscopy cells in a hypoxic microenvironment labeled with pimonidazole. This occurred because the cells far from the only oxygen source are in a state of hypoxia, unlike the cells in the center of the dish, thus validating the variant of the confocal microscopy method used for these cells.

Notably, this hypoxia detection probe, pimonidazole, detects exceptionally high levels of hypoxia. That is, it forms protein adducts at oxygen levels that are well below the normal range ($pO_2 < 10$ mmHg).[13,16] This means that in the intermediate areas of the dish when the distance from the center begins to increase, a hypoxic or pre-hypoxic microenvironment could already exist but not sufficiently hypoxic to be detected with the formation of protein-pimonidazole adducts.

GLUT-1 detection in vitro in HaCaT cells subjected to coverslip hypoxia

The expected results were obtained when observing HaCaT cells by confocal microscopy in a hypoxic microenvironment. These results agree with those obtained with pimonidazole marking. This confirms that the cells that are far from the only source of oxygen are in a state of hypoxia

and, therefore, with higher GLUT-1 expression.

Lipid detection with the Nile Red marker in vitro in HaCaT cells subjected to hypoxia

The results obtained when labeling the cells with Nile Red confirm the phase contrast findings regarding increased keratinization in a hypoxic environment. Additionally, the observed increase in the labeling intensity of lipid droplets containing neutral lipids in a hypoxic environment is similar to previous observations in other cell types subjected to hypoxia.[13,19]

GLUT-1 detection in vitro in OLP preparations

As mentioned above, OLP presents an altered, potentially hypoxic microenvironment, as does the microenvironment of tumor cells. In fact, it is a precursor pathology to oral carcinoma. This increase in GLUT1 expression is in line with previous studies reporting that hypoxia contributes to the pathogenesis of this disease.[20,21]

All these data suggest the existence of a microenvironment in OLP where GLUT-1 expression is induced and, in turn, increased keratinization accompanied by keratinocyte terminal differentiation. This is similar to what we observed in the hypoxic microenvironment in vitro. Such correlation suggests that the alterations in the oral epithelium of OLP could partly be due to a hypoxic microenvironment in vivo.

Conclusions

This study detected hypoxia markers—pimonidazole and GLUT-1—in human keratinocytes used as a model of the oral mucosa and subjected to a hypoxic microenvironment in vitro by applying a variant of the coverslip hypoxia method. In addition, marked morphological changes were detected in the cells in response to the induced hypoxic microenvironment and a significant presence of cornification as part of keratinocyte terminal differentiation. Marked expression of hypoxia marker GLUT-1 has also been detected in oral mucosa epithelial tissue in OLP in vivo.

Additionally, we observed a marked increase of cornification in the oral mucosa in OLP, not only in the outermost area (stratum corneum) but also in basal regions (ectopic keratinization). Our results suggest a correlation between the alterations detected in human keratinocytes induced to a hypoxic microenvironment in vitro and the alterations seen in vivo in oral mucosal epithelial tissue in OLP. This is shown through the expression of hypoxia marker GLUT1, both in vitro and in vivo, and by increased keratinization. These data are consistent with the potential presence of hypoxia in the microenvironment of OLP. Finally, our results show that the coverslip hypoxia variant used here is helpful in studying the response of oral mucosa keratinocytes subjected to a hypoxic microenvironment and correlating these observations to oral mucosa in vivo under pathological conditions.

Limitations of the study

Only six OLP preparations were used to study the detection of GLUT-1 protein in vivo as they were the only samples that could be obtained from the Anatomic Pathology Department of the School of Dentistry. Furthermore, these samples were the only ones that met the optimum conditions for conservation. The limited sample size yields preliminary results, and therefore, as in the case of the studies that reported rare low-frequency tumors, we can only speak of a trend. Statistical tests are not recommended with a small sample size. However, although there are few OLP—a limitation of the in vivo study—we consider that our preliminary in vitro results support the discussion in the article.

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