Potential therapy with the inhibitor of TGF-β receptors LY2109761 for oral squamous cell carcinoma

Terapia potencial com o inibidor dos receptores TGF-β LY2109761 para carcinoma de células escamosas oral

Terapia potencial con el inhibidor de los receptores de TGF-β LY2109761 para el carcinoma oral de células escamosas

Abstract
One way of trying to control oral squamous cell carcinoma is to invest in new therapies focused on the molecular biology of receptors and their intracellular signaling pathways. This study aimed to evaluate the effect of LY2109761 (an inhibitor of TGF-β receptors) on cell migration in oral squamous cell carcinoma in vitro. Actin cytoskeleton of SCC-4 cells control and LY2109761 (1, 5 and 10 μM) treated on three-dimensional Matrigel were analysed by using confocal laser microscopy. Control and LY2109761 (1, 5 and 10 μM) treated cells that migrated through the membrane of three-dimensional cell migration assays were counted, significance was p<0.05. Control cells were seen with voluminous cytoplasm, cell cortex preserved and actin cytoskeleton well developed with well distributed actin filaments. Regardless of concentration, cells treated showed: rounded morphology and small size, scanty cytoplasm, cortical F-actin less clear than the control cells, and disruption of actin filaments. The migratory cells were inhibited by treatment with LY2109761 [F (3, 11) = 3742, p<0.0001], in a dose-dependent manner. These results suggest that LY2109761 exerts an inhibitory effect on the actin cytoskeleton and cell migration on SCC-4 cells, therefore, it is a promising therapeutic option for oral squamous cell carcinoma.

Keywords: Cell migration; Cytoskeleton; LY2109761; Oral squamous cell carcinoma; SCC-4.

Resumo
Uma forma de tentar controlar o carcinoma de células escamosas oral é investir em novas terapias voltadas para a biologia molecular dos receptores e suas vias de sinalização intracelular. Este estudo teve como objetivo avaliar o efeito do LY2109761 (um inibidor dos receptores TGF-β) na migração celular no carcinoma epidermóide oral in vitro. Citoesqueleto de actina de controle de células SCC-4 e LY2109761 (1, 5 e 10 μM) tratado em Matrigel tridimensional foram analisados usando microscopia confocal a laser. Controle e células tratadas com LY2109761 (1, 5 e 10 μM) que migraram através da membrana de ensaios de migração de células tridimensionais foram contadas, a significância foi p <0,05. Células controle foram observadas com citoplasma volumoso, córtex celular preservado e citoesqueleto de actina bem desenvolvido com filamentos de actina bem distribuídos. Independentemente da concentração, as células tratadas apresentaram: morfologia arredondada e tamanho pequeno, citoplasma escasso, F-actina cortical menos clara que as células de controle e rompimento dos filamentos de actina. As células migratórias foram inibidas pelo
treatment with LY2109761 [F (3, 11) = 3742, p <0.0001], of a form dependent on dose. These results suggest that LY2109761 exerts an inhibitory effect on actin cytoskeleton and cell migration in oral squamous carcinoma cells. LY2109761 (1, 5 and 10 µM) treated cells on Matrigel tridimensional analysis using laser confocal microscopy. Cell counts in the control and LY2109761 (1, 5 and 10 µM) treated cells migrated through the membrane of the assays for cell migration in the oral squamous cell carcinoma cells SCC-4, demonstrating a significant difference p <0.05. Control cells showed voluminous cytoplasm with preserved cell cortex and actin cytoskeleton well developed with actin filaments well distributed. In cells treated with LY2109761, the actin cortex was less clear than the control cells and actin filaments broke down. These results suggest that LY2109761 exerts an inhibitory effect on cell migration in the oral squamous cell carcinoma cells SCC-4, thus being an option for therapeutic treatment for oral squamous cell carcinoma.

Resumen
Una forma de intentar controlar el carcinoma oral de células escamosas es invertir en nuevas terapias centradas en la biología molecular de los receptores y sus vías de señalización intracelular. Este estudio tuvo como objetivo evaluar el efecto de LY2109761 (un inhibidor de los receptores de TGF-β) sobre la migración celular en el carcinoma oral de células escamosas in vitro. El citosqueleto de activa del control de las células SCC-4 y LY2109761 (1, 5 y 10 µM) tratados en Matrigel tridimensional se analizaron utilizando microscopía láser confocal. Se contaron las células de control y tratadas con LY2109761 (1, 5 y 10 µM) que migraron a través de la membrana de los ensayos de migración celular tridimensional, la significancia fue p <0.05. Se observaron células de control con citoplasma voluminoso, corteza celular conservada y citosqueleto de actina bien desarrollado con filamentos de actina bien distribuidos. Independientemente de la concentración, las células tratadas mostraron: morfología redondeada y tamaño pequeño, citoplasma escaso, F-actina cortical menos clara que las células de control y rotura de los filamentos de actina. Las células migratorias se inhibieron mediante el tratamiento con LY2109761 [F (3, 11) = 3742, p <0.0001], de una manera dependiente de la dosis. Estos resultados sugieren que LY2109761 ejerce un efecto inhibidor sobre el citosqueleto de actina y la migración celular en las células SCC-4, por lo tanto, es una opción terapéutica prometedora para el carcinoma oral de células escamosas.

1. Introduction

Among head and neck tumors, oral squamous cell carcinomas account for 90% of cases, whose primary lesions may present as symptoms like difficulty in swallowing and hoarseness, and the prognosis for early cases is promising, unlike advanced cases, where regional and/or distance metastasis are common (Sanderson & Ironside, 2002). During the development of metastasis, the displacement of cells within primary site tissues, cell migration, occurs to target distant organs at secondary sites (Krakhmal et al., 2015); (Albini & Noonan, 2010; Kramer et al., 2013), when cell migration occurs (Albini & Noonan, 2010). It is important the development of new therapeutics (Capece et al., 2017), the blockade of the TGF-β signaling pathway is a promising therapeutic option (Neuzillet et al., 2015). The LY2109761 is a dihydropyrrolopyrazole inhibitor which has oral bioactivity and a potential of inhibition in vivo greater than 50% (Li et al., 2008) and acts on the intracellular domain of the TGF-β type I receptor (TβRI), through competition for the catalytic site of ATP blocking phosphorylation of the receptor and activation of the intracellular signaling pathway (Connolly et al., 2012).

The TGF-β signaling is one of the pathways involved in tumor progression (Loomans & Andl, 2014), acting in the processes of invasion and cellular migration (Drabsch & ten Dijke, 2012), regulation of growth, differentiation, proliferation and apoptosis (Zhang et al., 2014), therefore, the blockade of the TGF-β signaling pathway is a promising therapeutic option (Neuzillet et al., 2015). The LY2109761 is a dihydropyrrolopyrazole inhibitor which has oral bioactivity and a potential of inhibition in vivo greater than 50% (Li et al., 2008) and acts on the intracellular domain of the TGF-β type I receptor (TβRI), through competition for the catalytic site of ATP blocking phosphorylation of the receptor and activation of the intracellular signaling pathway (Connolly et al., 2012).

This study aimed to evaluate the effect of LY2109761 treatment, by using three-dimensional assays, on the regulation of the actin cytoskeleton and cell migration in oral squamous cell carcinoma in vitro.

2. Methodology

This is a prospective, experimental, quantitative and analytical study (Pereira et al, 2018). This research was dismissed from ethical evaluation by the Research Ethics Committee of the Federal University of Triângulo Mineiro for using commercially available cell lines. This study the SCC-4 cell line, originating from moderately differentiated human oral
squamous cell carcinoma, was used (American Type Culture Collection - ATCC®). Cell culture was done in a humid incubator with 5% CO2/95% atmosphere at 37 °C in Dulbecco's Modified Eagle's Medium F12 (Sigma-Aldrich, St. Louis, MO) containing fetal bovine serum (FBS) 10% (Cultilab, Campinas, SP, Brasil) 100 μg/ml penicillin, 100 U/ml streptomycin (Sigma-Aldrich, St. Louis, MO), 400 ng/ml hydrocortisone (Ariston, São Paulo). Analysis of the effect of LY2109761 on three-dimensional actin cytoskeleton and cell migration in oral squamous cell carcinoma was performed in biological triplicates and experimental duplicates.

**Three-dimensional actin cytoskeleton assays**

Was prepared Matrigel™ (BD Biosciences, Bedford, MA, USA) 1:2 medium and applied in glass coverslipes put in six well plates. The plates were placed in humidified incubator for 30 minutes to form a gel. It was seeded 1x10⁴ SCC-4 cells/well and incubated for 30 minutes, then medium/well were added. After 24 hours of incubation, it was performed the treatment with LY2109761 (Cayman Chemical, Ann Arbor, MI, USA), at concentrations of 1, 5 and 10 μM, and for control cells medium DMSO was added. After 24 hours of LY treatment, cells were washed with Dulbecco’s Phosphate Buffered Saline (D-PBS) with calcium and magnesium for 5 minutes. The fixation was performed with 4% paraformaldehyde for 1 hour. After two washes with D-PBS for 5 minutes permeabilization was performed with triton X-100 (Sigma-Aldrich, St Louis, Missouri, USA) 0.2 % for 5 minutes. The blocking of nonspecific sites was done with albumin solution from bovine serum (BSA) (Sigma-Aldrich, St Louis, Missouri, USA) 1% for 5 minutes.

During 30 minutes the f-actin was stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA) 1:100 DPBS. The evidenciation of the nuclei was made for 5 minutes with 4’6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO): 100 D-PBS. The assembly of the slides was performed with Vectashield® (Vector Laboratories, Burlingame, California, USA). The images of actin citoskeleton experiment were obtained in confocal laser scanning microscope LSM 510 Meta (Zeiss®, Goettingen, Germany) and the entire length of the blades were analyzed by two independent observers.

**Three-dimensional cell migration assays**

In the upper chambers of the cell migration plate (BD BioCoat™ 24-well plate, 8.0 μm, BD Bioscience, Bedford, MA), 1x10⁵ SCC-4 cells were seeded in medium with/without addition of treatment. To the middle of the control cells was added DMSOv; and to the medium of the treated cells, LY2109761 at concentrations of 1, 5 and 10 μM. 500 μl of medium containing 20% FBS was added in the lower chambers of the plate. The migration plates were kept in a humid incubator with 5% CO2/95% atmosphere for 24 hours

After the treatment period, cells that did not migrate on the upper surface were carefully removed with swab. The fixation of the cells that migrated through the membrane was performed with 30% methanol for 30 seconds and, staining as per manufacturer's instructions, with Instant Prov Newprov® (Pinhais, PR, Brazil). The control and treated cells that migrated through the membrane were counted in ten random fields, using a 20x objective inverted microscope (Axio Vert.A1, Zeiss ®) by one observer.

**Statistical analysis**

Results were analyzed using the IBM SPSS® 20.0 (Chicago, IL, USA) and graphs with Graphpad Prism (San Diego, CA, USA). The Levene variance test, the analysis of variance (ANOVA) and Tukey's post-test were used, considering p<0.05.
3. Results

LY2109761 treatment affected the actin cytoskeleton of OSCC \textit{in vitro}. Control cells were seen with morphology: polarized, voluminous cytoplasm, eccentric spherical nuclei, cell cortex preserved and actin cytoskeleton well developed with well distributed actin filaments. Filopodia and lamellipodia were seen only in control cells (Figure 1A). After treatment with LY2109761, regardless of concentration, the cells showed: rounded morphology and small size, scanty cytoplasm, spherical nuclei and central, cortical F-actin less clear that the control cells, and disruption of actin filaments (Figure 1B-D).

\textbf{Figure 1.} Effect of treatment with LY2109761 on actin cytoskeleton of SCC-4 cells. Confocal Analysis of F-actin seen three planes. SCC-4 cell cultured in three-dimensional assay in Matrigel\textsuperscript{TM} for 24 h. Nuclei stained with DAPI (blue) and F-actin with rhodamine phalloidin (red). Control cells (A), LY2109761 treatment: 1 µM (B), 5 µM (C) and 10 µM (D).
LY2109761 treatment inhibited cell migration of oscc in vitro.

There was a significant difference between the number of migratory cells inhibited by treatment with LY2109761 [F (3, 11) = 3742, p<0.0001], and compared to the control cells (475.70±1.30 cells), the amount of treated cells was smaller in the three concentrations: 1 μM (470.97±1.09 cells, p<0.001), 5 μM (450.43±0.66 cells, p<0.0001) e 10 μM (399.97±0.70 cells, p<0.0001). The percentage of reduction of migrating cells treated with LY2109761 relative to control cells was: 1.06% (1 μM), 5.27% (5 μM) and 16% reduction (10 μM). The inhibitory effect of treatment with LY2109761 between the treated cell groups and control cells occurred in a dose-dependent manner, the difference between the number of migratory cells treated with the inhibitor LY2109761 was statistically significant: 1 μM vs 5 μM (p<0.0001), 1 μM vs 10 μM (p<0.0001), and 5 μM vs 10 μM (p<0.0001) (Figure 2).

Figure 2. Effect of treatment with LY2109761 on migration of SCC-4 cells from oral squamous cell carcinoma. Migration cells counted in the cell migration assay: control and treated with LY2109761: 1, 5 and 10 μM. ANOVA, p<0.001 and Tukey’s posttest. **p<0.01 and *** p<0.0001.

4. Discussion

This study demonstrated through three-dimensional assays that treatment with LY2109761, a TβRI inhibitor, affected the actin cytoskeleton and inhibited cell migration in OSCC. Among the steps involved in the formation of metastasis, the reconfiguration of the actin cytoskeleton, the main responsible for cell migration, plays a crucial role in cellular motility (Sun et al., 2015), which makes the LY2109761 a promising target for antimetastatic drugs.

Metastatic formation begins in the primary tumor with loss of cell-cell adhesion, motility, and interactions of tumor cells with extracellular matrix and basement membrane, invading the stroma, entering blood and/or lymphatic vessels, reaching the target organ, extravasate and expand the tumor at a distant site (Howell & Grandis, 2005). In this study treatment with the specific inhibitor of TβRI, LY2109761, has affected biological processes that occur in the formation of metastasis.

The cell surface initiates cell movement by means of structural rearrangements in the actin cytoskeleton, such as the formation of filopodia and lamelipodia (Yamaguchi & Condeelis, 2007). The filopodia has the ability to probe the environment and direct the migration (Lehtimäki et al., 2016), and the lamelipodia are responsible for the attachment of the cell to the migration site and generation of the necessary force for the movement (Yamaguchi & Condeelis, 2007). LY2109761 reduced
the amount of filopodia and lamelipodia in SCC-4 cells.

Tyrosine kinase receptors (RTKs) are transmembrane proteins located on the cell surface and are important regulators of several key processes such as proliferation, differentiation, migration, survival (Lemmon & Schlessinger, 2010) and TGF-β receptors are RTKs (Chen et al., 2020). The increase of RTK’s activation is an important cancer characteristic (Lemmon & Schlessinger, 2010) leading to higher oncogenic mutation (Blume-Jensen & Hunter, 2001). This excessive activation and numerous mutations of tumors made RTKs an important target of new cancer treatments because of the direct effects on tumor cells (Hunter, 2014). In tumors TGF-β signaling pathway plays a progression role, stimulating cell motility and consequently metastasis (Drabsch & ten Dijke, 2012), promoting changes in the cytoskeletal architecture of tumor cells (Yang, 2010). LY2109761 exerted an inhibitory effect on the cytoskeleton, demonstrating that of this pathway may play a meaningful role in the cellular motility in OSCC.

In cells from the Tca8113 line of OSCC, treated with TGF-β for 24 hours, there was a phenotypic changes from epithelial cells to elongated fibroblast-like cells (Bu & Chen, 2017). This study demonstrated that control cells exhibited elongated morphology and reorganization of the actin cytoskeleton, while the LY2109761, TGFβ inhibitor, treatment leads cells to became smaller, rounded and with fewer organized actin filaments.

There are different forms of cell migration, the main ones being ameboid and mesenchymal. Elongated cells are associated with mesenchymal migration, while rounded cells are related to ameboid migration. It is known that cells have the ability to modify the morphology and mode of migration according to external conditions and intracellular regulation, and such adaptations facilitate the formation of metastasis (Clark & Vignjevic, 2015). LY2109761 seems to have an anti-metastatic potential as demonstrated in cell studies with glioblastoma (Joseph et al., 2013) and in colorectal cancer cells applied in mice (Zhang et al., 2010). In this study, the treatment with LY2109761 lead to a reduction of cell migration rate, and it could be indicated a relation with the anti-metastatic potential of this inhibitor.

5. Conclusion

The results of this study showed that control SCC-4 cells were elongated, and cells treated with LY2109761 were rounded, and the rate of cell migration reduced. Therefore, the inhibitor LY2109761 is a promising therapeutic option for OSCC since it exerts an inhibitory effect on the actin cytoskeleton and cell migration in SCC-4 cells of OSCC by blocking the TGF-β signaling pathway. Further studies such as evaluation of processes such as apoptosis and cell proliferation should be performed to analyze the effects of LY2109761, as well as preclinical and possibly clinical studies for this inhibitor to be used in the treatment of OSCC.

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