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Research paper

Potential chemotherapeutic effects of diosgenin, zoledronic acid and epigallocatechin-3-gallate on PE/CA-PJ15 oral squamous cancer cell line

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ABSTRACT

Objective: To study the potential chemotherapeutic effects of Diosgenin, zoledronic acid and Epigallocatechin-3-gallate on oral squamous cell cancer (OSCC).
Materials and methods: Cell viability, migration, apoptosis and cell cycle evaluation assays were performed in order to assess the effects of different doses of Diosgenin, zoledronic acid and Epigallocatechin-3-gallate on the PE/CA-PJ15 cell line.
Results: Doses of 100 μM of diosgenin or zoledronic acid reduced cell viability significantly after 72 h ($p < 0.001$), as well as increasing apoptosis ($p < 0.05$ and $p < 0.01$ respectively). All three agents reduced cell migration and altered the cell cycle, each at a different phase of the cycle.
Conclusion: while DG and ZA reduced cell viability, increased apoptosis, inhibited cell migration and modified the cell cycle in different ways, EGCG only modified the cell cycle and reduced cell migration. These agents present a potential chemotherapeutic effect on PE/CA-PJ15 OSSC cell line, which have to be further studied.

1. Introduction

Oral squamous cell cancer (OSCC) is the most frequent cancer of the oral cavity (Handschel et al., 2012). Surgical excision is the benchmark treatment for OSCC patients, but it is not effective in the advanced phases of the disease. Other options are chemotherapy and radiotherapy in combination with surgery, although the mortality ratio is approximately 50% after 5 years, due to the difficulty of preventing metastasis and reappearance of the tumor (Kaneko et al., 2016; Malik, Zarina, & Pennington, 2016; Nagler, 2009; Ralhan, 2007).

Oral squamous cell cancer (OSCC) can frequently invade bone of the mandible causing severe bone destruction, thus presenting problems not only in function but also in prognosis (Fives et al., 2016; Rao et al., 2004). During bone resorption, local osteoclasts erode bone allowing tumor cells to invade adjacent tissue. Thus, antiresorptive and anticarcinogenic agents could be a promising therapy on OSCC.

Zoledronic acid (ZA; 2-(Imidazol-1-yl)-1-hydroxyethane-1,1-diphosphonic Acid) belongs to a group of agents denominated bisphosphonates, which are pyrophosphate analogues. It acts on the bone remodeling process as a powerful antiresorptive agent, due to its osteoclast-inhibiting action (Nagaoka, Kajiya, Ozeki, Ikebe, & Okabe, 2015). For this reason, this group of drugs has been used to treat various diseases related to bone metabolism (Baykan et al., 2014; Tella & Gallagher, 2014; Wu, Ma, Bao, & Guan, 2015) as well as to prevent bone metastasis (Barrett-Lee et al., 2014). It has also shown good results in vitro against lung (Xie, Li, Gong, Zhang, & Ma, 2015), prostate (Fragni et al., 2016) and cervical cancer (Wang, Chou, & Lin, 2014), among others. Some research suggests that ZA may be effective against oral cancer (Lopez Jornet, Susana, Rosario, & Alvaro, 2015; Martin et al., 2010; Tamura et al., 2011) although the mechanisms whereby this takes place have not been studied in depth.

Polyphenol epigallocatechin-3-gallate (EGCG) represents the major catechin found in green tea (Camellia sinensis). EGCG, which exhibits a wide variety of properties that may offer health benefits, has been shown to have different chemotherapeutic effects; Inhibition of tumor angiogenesis in tumor-associated endothelial cells and endothelial progenitor cells (Ohga et al., 2009); apoptosis enhancement and cell viability reduction on fibrosarcoma cell line (Lee, Han, Hyon, & Park, 2011); as well as antiresorptive properties (Lee et al., 2010; Lin et al., 2009). Nevertheless, its effect on oral cancer has not been studied in depth and the results are not consistent (Chen, Chu et al., 2011; Ho, Yang, Peng, Chou, & Chang, 2007; Lee et al., 2015)

Diosgenin (DG; 3-hydroxy-5-spirostene) is a steroidal saponin found in numerous plants, such as Fenugreek (*Trigonella foenum graecum*) and

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Cheilocostus speciosus. It has proven several chemotherapeutic properties on different studies; inhibits cell viability with cell cycle arrest and induces apoptosis on different squamous cell carcinoma lines (Das et al., 2012), as well as in laryngocarcinoma and melanoma cell lines (Corbiere, Liagre, Terro, & Beneytout, 2004); reduces migration and invasion on a prostate cancer cell line (Chen, Chu et al., 2011). It also presents an antiresorptive effect mediated by osteoclasts (Qu et al., 2014; Shishodia & Aggarwal, 2006). However, its role in oral cancer has not been investigated.

In this study, we analized the chemotherapeutic activity of ZA, EGCG and DG in PE/CA-PJ15 oral human squamous carcinoma cells. We studied the behavior of this agents on cell viability, cell cycle, migration and apoptosis

2. Materials and methods

2.1. Cell culture and reactive species

The human squamous carcinoma cell line PE/CA-PJ15 (European Collection of Cell Cultures) comes from a tongue tissue lesion of a 45 year old male patient with OSCC. It was cultured at conditions of 37 °C, 95% oxygen and 5% CO2 in Iscove's modified Dulbecco's modified Eagle medium (IDMEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin and 1% streptomycin. The medium (IMDM), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Epigallocatechin-3-gallate (EGCG) and Diosgenin (DG) were obtained from Sigma Co. (Madrid, Spain). Zoledronic acid was acquired from Intatrade Chemical GmbH (Muldestausee, Germany).

2.2. Agent preparation

EGCG, DG and ZA were prepared in 10 mM stock solutions and frozen at −20 °C until use. ZA was dissolved in phosphate-buffered saline (PBS), while EGCG and DG were diluted in ethanol. Ethanol and PBS were added as vehicle on control cells (PE/CA-PJ15 untreated cells).

2.3. Cell viability test (MTT)

Cell viability was measured using the MTT assay, as previously described (Atienzar, Camacho-Alonso, & Lopez-Jornet, 2014). Cells were cultivated at a density of 5000 cells/well in 96-well plates. After 24 h, cells were treated with different concentrations of the agents (5, 25, 50, or 100 μM) with nine replicates per treatment during 24, 48 and 72 h.

2.4. Apoptosis: DNA-associated histone fragments, enzyme-linked immunosorbent assay (ELISA)

The Cell Death Detection ELISA PLUS kit (Roche diagnostics, Madrid, Spain) was used to quantify apoptosis in the treated cells, following the manufacturer's instructions. Cells were seeded at a density of 5000 cells per well in quadruplicate in 96-well plates. Twenty-four, 48 and 72 h after treatment, of the cytosol extract were obtained and apoptosis measurement was made as previously described (Atienzar et al., 2014).

2.5. Cell-cycle analysis

Two hundred thousand cells were seeded in 25 cm^2 flasks and treated with 25 or 50 μM zoledronic acid, 50 or 100 μM EGCG, 10 or 25 μM Diosgenin for 24 or 48 h. Before reaching 80% confluence, cells were detached with trypsin and fixed in 70% ethanol in phosphate-buffered saline (PBS), incubated and stained with PI/RNase Staining Buffer (100 μg/ml) and propidium iodide (40 μg/ml). The stained cells were analyzed with a flow cytometer (BD FACSCalibur System, CA, USA) registering over 20,000 events. Data was processed using CELLQUEST software (BD Biosciences, San José, Ca, USA) and cell phases were analyzed using MODFIT (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were classified according to three phases of the cell cycle: G0-G1, G2-M or S. The assays were performed in duplicate in two independent assays and mean values were calculated.

2.6. Wound healing assay

Cells were seeded in 6-well plates until an adequate confluence as a monolayer was reached. Afterwards, a wound was made in the monolayer using a 200 μl sterile pipette and the medium was removed with the cells in suspension. Then, the new medium was added with the different concentrations of ZA, DG and EGCG and the distance of the wound was measured at 0, 4 and 8 h with a digital camera attached to a microscope (Nikon Eclipse TE2000-U, Nikon corp., Japan). Distances from the wound edge were obtained by counting image pixels, as previously (Lopez Jornet et al., 2015; Valster et al., 2005). The test was evaluated in triplicate in two independent assays.

2.7. Statistical analysis

Statistical analysis was performed using SPSS v 20.0 software (SPSS Inc., Chicago, IL USA). Student́s *t*-test was used to observe the associations between different quantitative variables for two independent samples. Data is expressed as Mean ± Standard deviation. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of EGCG, DG and ZA on PE/CA-PJ15 cell viability

3.1.1. ZA

ZA's action was time-dependent but not dose-dependent until 72 h, when the most noticeable effects were observed, reducing cell viability with statistically significant difference in comparison with the control group for all doses assayed at 72 h, the 100 μM dose being the most effective ($p < 0.001$) (Fig. 1C).

3.1.2. DG

DG's action was dose-dependent, causing a significant reduction in viability in comparison with the control group after 24 h, especially at 50 and 100 μM doses ($p < 0.001$). The most powerful effects were seen with the 100 μM dose at 72 h ($p < 0.001$.). At 5 μM, viability increased at all times, although only the 24-h time was statistically significant ($p < 0.01$) (Fig. 1A).

3.1.3. EGCG

With most of the doses assayed, cell viability increased at all times. The greatest increase in comparison with the control group was seen at 24 h at doses of 50 and 100 μM ($p < 0.001$) (Fig. 1B).

After this assay we decided only to choose only two concentrations for the rest of the experiments. We chose the higher doses of ZA and EGCG, and intermediate doses (25 and 50 μM) of diosgenin due to its high toxicity.

3.2. Effects of EGCG, DG and ZA on PE/CA-PJ15 cell apoptosis

As shown in Fig. 2, at 24 h, the most effective doses of DG were 25 and 50 μM ($p < 0.05$), while other doses showed similar effects to the control group. At 48 and 72 h, the most effective doses of ZA were 50 and 100 μM, with major differences in comparison with the others reaching their maximum effect at 72 h ($p < 0.01$). At almost all doses EGCG cell apoptosis was lower than in the control group, with significant difference at 72 h ($p < 0.01$).