Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Gallic acid modulates phenotypic behavior and gene expression in oral squamous cell carcinoma cells by interfering with leptin pathway

Eliane Macedo Sobrinho Santos[a,b,d], Rogério Gonçalves da Rocha[a,d], Hércules Otacílio Santos[c,d], Talita Antunes Guimarães[a,d], Carlos Alberto de Carvalho Fraga[c,d], Luiz Henrique da Silveira[a,d], Paulo Ricardo Batista[e,d], Paulo Sérgio Lopes de Oliveira[f,d], Geraldo Aclécio Melo[g,d], Sérgio Henrique Santos[h,d], Alfredo Maurício Batista de Paula[a,d], André Luiz Sena Guimarães[a,d,1], Lucyana Conceição Farias[a,d,*,1]

[a] Department of Dentistry, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
[b] Instituto Federal do Norte de Minas Gerais – Campus Araçuaí, Minas Gerais, Brazil
[c] Instituto Federal do Norte de Minas Gerais – Campus Salinas, Minas Gerais, Brazil
[d] Universidade Federal de Alagoas, Arapiraca, Alagoas, Brazil
[e] Programa de Computação Científica – Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
[f] National Biosciences Laboratory (LNBio), Campinas, São Paulo, Brazil
[g] Department of Biology, Universidade Estadual de Montes Claros, Minas Gerais, Brazil
[h] Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Leptin
Gallic acid
Oral squamous cell carcinoma
Cell proliferation
Cell migration
Angiogenesis

ABSTRACT

Gallic acid is a polyphenolic compost appointed to interfere with neoplastic cells behavior. Evidence suggests an important role of leptin in carcinogenesis pathways, inducing a proliferative phenotype. We investigated the potential of gallic acid to modulate leptin-induced cell proliferation and migration of oral squamous cell carcinoma cell lines. The gallic acid effect on leptin secretion by oral squamous cell carcinoma cells, as well as the underlying molecular mechanisms, was also assessed. For this, we performed proliferation, migration, immunocytochemical and qPCR assays. The expression levels of cell migration-related genes (MMP2, MMP9, Col1A1, and E-cadherin), angiogenesis (HIF-1α, mir210), leptin signaling (LepR, p44/42 MAPK), apoptosis (casp-3), and secreted leptin levels by oral squamous cell carcinoma cells were also measured. Gallic acid decreased proliferation and migration of leptin-treated oral squamous cell carcinoma cells, and reduced mRNA expression of MMP2, MMP9, Col1A1, mir210, but did not change HIF-1α. Gallic acid decreased levels of leptin secreted by oral squamous cell carcinoma cells, accordingly with downregulation of p44/42 MAPK expression. Thus, gallic acid appears to break down neoplastic phenotype of oral squamous cell carcinoma cells by interfering with leptin pathway.

1. Introduction

Leptin (Lep), a hormone secreted by adipose tissue, is known to be a component of the homeostatic loop of body weight regulation [1,2]. This hormone signaling can lead to the metabolic features associated with cancer malignancy, such as switching in cell energy balance from mitochondrial β-oxidation to the aerobic glycolytic pathway [3,4]. Furthermore, Lep provides the tumor microenvironment, mainly through its ability to potentiate both endothelial cells migration and angiogenesis and to sustain the recruitment of macrophages and monocytes, which in turn secrete vascular endothelial growth factor and proinflammatory cytokines [5]. Lep has been associated with increased risk of several cancers, as well as proliferative and anti-apoptotic effects on cancer cells [6,7].

Despite the scarcity of studies, Lep pathway has emerged as an important target, possibly involved in oral carcinogenesis. Surveys were conducted to assess serum Lep level in oral squamous cell carcinoma patients, suggesting that Lep can contribute to oral cancer-induced loss of body mass [8–10]. Besides, gene polymorphisms of Lep and its receptor LepR revealed an increased risk to oral carcinogenesis [11].

* Corresponding author at: Universidade Estadual de Montes Claros, Hospital Universitário Clemente de Faria, Laboratório de Pesquisa em Saúde, Avenida Cula Mangabeira, 562, Montes Claros, Minas Gerais, Zip code: 39401-001, Brazil.
E-mail address: lucyanacfarias@gmail.com (L.C. Farias).
[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.prp.2017.11.022
Received 28 February 2017; Received in revised form 16 November 2017; Accepted 28 November 2017

Therefore, Lep can be pointed as a possible target for new therapeutic investigations. Current strategies to inhibit Lep pathway, such as soluble Lep receptors (LRs), synthetic Lep-antagonists, and anti-LR monoclonal antibodies (anti-LR mAbs) [12] can be limited due to toxicity, as well as low efficacy.

Currently, there is substantial interest in understanding the therapeutic potential of herbal extracts in cancer. Several extracts have demonstrated activity against different kinds of cancer [13]. Gallic acid (3,4,5-trihydroxybenzoic acid; GA) is a polyphenol found widely in fruits and plants; it has been shown to have anticancer effects in human leukemia HL-60RG [14], lung cancer [15], stomach cancer, colon cancer [16], prostate cancer [17], melanoma [18] and esophageal cancer [19], pheochromocytoma [20], mouse leukemia WEHI-3 cells [21] and oral cancer [22,23]. In oral carcinogenesis, the real effect of GA on neoplastic cells is not well understood.

Given the above, we hypothesized that GA interacts with components of Lep signaling pathways, and it can interfere with the neoplastic role of this hormone. So, the study purpose was to investigate the potential of GA to modulate Lep-induced proliferation and migration of OSCC cell lines and the underlying molecular mechanisms. We also explored GA effect on Lep secretion by OSCC cells.

2. Material and methods

2.1. Bioinformatics analysis and in silico docking experiments

The hypothesis that GA interacts with Lep was first explored by *in silico* analysis, aiming to conduct the *in vitro* functional assays.

For the molecular docking simulation, the atomic coordinates of the GA structure were taken from the Zinc Database [24] under the code ZINC0000104, in the mol2 format. The structures of the human Lep and LepR were taken from the Protein Data Bank (PDB) codes 1ax8 [25] and 3v6o [26], respectively. Before our docking simulations, the protein structures were prepared with the addition of hydrogens taking into account the most likely protonation state of each titrable residue (considering a pH of 7.2), using the PROPKA program [27], inside the pdb2pqr program [28,29]. All molecular docking calculations were performed using the Autodock Vina program [30], in a two-step approach: *i.* a blind docking procedure and *ii.*a pocket search method. The blind docking procedure consisted of searching the entire protein surface to determine the potential binding pocket(s). This was achieved using the grid center as the center of each protein, using a grid size big enough to cover the entire protein surface. After finding the binding pockets, we centered the grid center within the discovered binding pocket where the best-scored conformation is located, and performed a more accurate search (the pocket search procedure), using the following parameters: energy_range = 10, num_modes = 20 and exhaustiveness = 800.

Investigation of putative target genes of GA was obtained from STITCH 3.1 (http://stitch.embl.de/) [31]. Briefly, this server predicts interactions of chemical compounds with proteins based on known interactions from metabolic pathways, crystal structures, binding experiments, and drug–target relationships. It also allows the use of information from phenotypic effects, text mining and chemical structure similarity in order to predict relations between chemicals.

2.2. Cell culture

Two human OSCC cell lines, SCC9 and SCC4 (CRL-1629 and CRL1624, ATCC cell bank, USA), were cultured in DMEM/Ham's F-12 (Gibco, USA), supplemented with 10% fetal bovine serum and 0.4 µg/ml hydrocortisone (Gibco, South America). All experiments were performed in triplicate and at least three independent experimental times.

2.3. Cell proliferation assay

Cell proliferation assay was performed as described before [32] with necessary adaptations. A density of 2×10^5 OSCC cells was plated in 60 mm dish and incubated at 37 °C for approximately 24 h to establish adherent monolayers. Then, cells were treated with 100 ng/ml of human recombinant leptin (Invitrogen, USA) and 10 µg/ml of GA (Sigma-Aldrich, USA) for 72 h. The GA concentration was previously defined through a dose-response curve in another study of our group [32]. The comparing groups, as Lep-treated cells, GA-treated cells and cells cultivated only in culture medium were included in study design. Cell proliferation was determined by trypan-blue exclusion. The proliferation assay was performed under normoxia, and mimicking hypoxia by the addition of 100 µM $CoCl_2$ (Sigma-Aldrich, USA).

2.4. Cell dead/viability assay

Acridine orange/ethidium bromide (AO/EB) staining was used to visualize dead and viable cells [33]. A volume of 25 µl of cell suspension (2.0×10^6cells/ml) was incubated with 1.0 µl of a solution containing 1 part of l00 µg/ml acridine orange in PBS; (AO, Sigma, St. Louis, USA) and 1 part of 100 µg/ml ethidium bromide in PBS (EB, Sigma, St. Louis, USA). The cell suspension was placed onto a microscopic slide and covered with a glass coverslip. Cells were observed in a fluorescence microscope FSX100 (Olympus, Center Valley, PA, USA). Intense EB staining (Ex360-370, Em420-460, filter DM400) indicates cell death, while intense AO (Ex460-495, Em510-550, filter DM505) indicate live cells.

2.5. Migration assay

Cell migration was assayed by *wound healing* method [34]. Briefly, at the full confluence, OSCC cells were scraped away horizontally using a 200 µl tip. Culture medium was then replaced by serum-free medium, adding 100 ng/ml leptin and/or10 µg/ml of GA for 72 h. The migration characteristic of cells treated with Lep and/or GA was also evaluated under hypoxia condition. In order to measure the wound covered area by migrating cells, images of the wounded cell monolayers were taken using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA, USA) coupled to camera SC30 (Olympus, Center Valley, PA, USA) at 0 and 72 h after wounding.

2.6. Clonogenic assay

Treated or untreated OSCC cells were plated in 60 mm dish at a density of 1.0×10^2cells and maintained in culture for 14 days. Then, cells were fixed with 70% ethanol at 4 °C and stained for the counting of colonies formed. Colonies with over 50 cells were considered for analysis. Survival fraction (SF) was calculated as previously described [35].

2.7. Secreted leptin dosing

In all experimental groups, secreted Lep level by OSCC cells in culture medium, under normoxia condition, was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Leptin Human ELISA-LDN®). This ELISA kit shows a limit of quantification around 100 ng/ml. Secreted leptin dosing was performed as recommended by the commercial kit manufacturer. To this analysis, leptin dosage was adjusted by cell number in each group.

2.8. Immunocytochemical assay

Immunocytochemical was performed as described before [36] with necessary adaptations. A density of 2×10^4 OSCC cells was plated on coverslips and submitted to the experimental treatments. At the end of treatments, the cells were fixed with 70% ethanol for 30 min.