Microbial Pathogenesis 112 (2017) 15–19

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Orlistat treatment induces apoptosis and arrests cell cycle in HSC-3 oral cancer cells

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ARTICLE INFO

Article history:
Received 9 August 2017
Received in revised form
31 August 2017
Accepted 1 September 2017
Available online 4 September 2017

Keywords:
Cyclin
Phosphorylation
Apoptosis
Cell cycle
Mitosis

ABSTRACT

The present study was aimed to investigate the effect of orlistat on an oral squamous cancer line HSC-3 as well as the underlying mechanism. Cell Counting Kit-8 (CCK-8) (Dojindo, Shanghai, China) was used for the analysis of proliferation, Annexin V-FITC and propidium iodide staining for apoptosis and flow cytometry for cell cycle distribution. Western blot assay was used to determine the alteration in the expression of cyclin D1, B1, E and CDK1. The results revealed a concentration and time-dependent decrease in the proliferation of HSC-3 cells by orlistat. The viability of HSC-3 cells was reduced to 23.4 ± 2.5 and 15.7 ± 1.6% at 40 and 50 μM concentration of orlistat after 48 h. Treatment of HSC-3 cells with orlistat resulted induction of apoptosis significantly ($p < 0.05$). Orlistat treatment led to the increase in proportion of apoptotic cells to 38.6 ± 2.5% after 48 h compared to 0.85 ± 0.34% in the control. Analysis of cell cycle showed that population of cells in G2/M phase in the cultures treated with orlistat for 48 h increased to 59.7 ± 5% compared to 10.2 ± 1.2% in the control. However, the population of cells in the G0/G1 and S phases was subsequently decreased. The expression of cyclin D1 and E was decreased and phosphorylation of CDK1 was increased by orlistat treatment in HSC-3 cells. Thus, orlistat induces apoptosis and cell cycle arrest in G2/M phase in HSC-3 cells through decrease in expression of cyclin D1 and E and increase in phosphorylation of CDK1. Therefore, orlistat can be used for the treatment of oral squamous cancer.

1. Introduction

Oral cancer represents a carcinoma tissue growing in the oral cavity and is considered one of the types of head and neck cancers [1]. It may originate either as a primary lesion in mouth or by the metastasis of cancer cells from distant organs bearing cancer [1]. Oral squamous cell carcinoma constitutes the 6th most commonly diagnosed cancer with 263,000 new cases detected every year throughout the globe [2,3]. It has been observed that among oral cancer patients more than 85% suffer from squamous cell carcinoma present in the mouth and lip tissue lining [4]. At present the oral squamous cell cancer is treated by using chemotherapy, radiation therapy and surgery either alone or in combination. The five-year survival rate of the patients with oral squamous cancer is still less than 50% [4]. This demands for the development of new techniques for diagnosis of oral cancer at initial stage and efficient treatment strategies for its treatment. Studies have demonstrated that natural products obtained from diverse sources play an important role as anticancer agents [5]. The mechanism of action of the natural products may involve induction of apoptosis, necrosis, cell cycle arrest, etc [5].

Orlistat is an anti-obesity drug having very low oral bioavailability and this drug was approved by the US Food and Drug Administration [6]. It shows promising inhibitory effect on cell proliferation as well as inhibits tumor growth in prostate and breast cancer cells [6]. The activity of orlistat has been found to be because of its FASN blocking potential [6,7]. Mechanism of the action of orlistat involves induction of apoptosis, arrest of cell cycle and reduction in the promoter activity of Her2/neu gene [8]. In the present study effect of orlistat on an oral squamous cancer line, HSC-3 as well as the underlying mechanism was investigated.

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https://doi.org/10.1016/j.micpath.2017.09.001
0882-4010/

2. Materials and methods

2.1. Cell line and culture

The human oral squamous cancer cell line, HSC-3 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown at 37 °C in a humidified chamber containing 5% CO_2 in Dulbecco's-modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (Hyclone, USA), penicillin (107 U/l) and streptomycin (10 mg/l).

2.2. Chemicals

Orlistat was supplied by Sigma-Aldrich (Sigma, St. Louis, MO, USA) and its stock solution prepared by dissolving in dimethyl sulfoxide (DMSO) and then diluted using culture medium.

2.3. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) (Dojindo, Shanghai, China) was used for analysis of the effect of orlistat on proliferation of HSC-3 cells according to the manufacturer's instructions. Briefly, 2×10^5 cells were seeded per well of the 96-well plates and incubated with orlistat (10, 20, 30, 40, 50 and 100 μM) in the culture medium for 24, 48 or 72 h. The cells in the control plates were incubated with DMSO (0.1%) alone. After incubation for required time, 10 μl of CCK-8 was added to each well of the plate and the plates were incubated again for 2 h at 37 °C. Following incubation, supernatant from each well was decanted and subjected to absorbance measurement at 450 nm using MK3 microplate reader (Thermo).

2.4. Apoptosis

Apoptosis induction in HSC-3 cells was determined by using Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Jose, CA, USA). The cells were cultured for 24 h in 6-well plates at a density of 2×10^6 cells per well and subsequently incubated with various concentrations of orlistat. Following incubation, the cells were washed with cold PBS two times and then re-suspended in 100 μl 1× binding buffer. To each of the well was added 3 μl Annexin V-FITC (BD Bioscience) and 10 μl propidium iodide (PI; BD Bioscience) and the plates were incubated for 20 min. The flow cytometry (Becton-Dickinson-San Jose CA, USA) was used for the determination of fluorescent intensities. The experiments were performed in triplicates.

2.5. Cell cycle analysis using flow cytometery

HSC-3 cells after treatment with orlistat (30, 40 and 50 μM) or untreated (control) were harvested, rinsed twice with PBS and then suspended (2×10^6 per ml) in 2 ml hypotonic fluorochromic solution [50 mg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.1% Triton X-100] for 2 h at 50 °C under dark conditions. The FACS caliber flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with ModFIT cell cycle analysis software version 2.01.2 (BD Biosciences) was used for the analysis of PI fluorescence. Under each condition the experiment was performed in triplicates.

2.6. Western blot assay

HSC-3 cells were treated with orlistat for 48 h, harvested and then lysed using lysis buffer [1% Nonidet P-40, 0.1% Triton X-100, 30 mM sodium phosphate (pH 7.4) containing 1 mM sodium orthovanadate, 2.5 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 μg/ml of leupeptin, aprotinin] for the extraction of total proteins. The concentration of the proteins was determined using the Bio-Rad protein assay kit (Bio-Rad, Shanghai, China). Protein samples were isolated by electrophoresis on 12% SDS-PAGE and subsequently transferred onto PVDF membranes (GE Healthcare Life Science, Milan, Italy). The membranes after washing with Tris-buffered saline and Tween (TBST; Probe Co. Ltd., Guangzhou, China) were subjected to incubation for 1 h with blocking buffer (5% dried milk in PBS) at 37 °C. Incubation of the membranes was performed at 4 °C overnight with primary antibodies. The primary antibodies used were against cyclin D1, cyclin E, β-actin, cyclin dependent kinase 1 (CDK1) and phospho-CDK1 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). All the monoclonal antibodies were used at a dilution of 1000-fold. The membranes were then washed with TBST thrice followed by incubation secondary antibodies for 1 h. The enhanced chemiluminescence detection system (Amersham) was used for the visualization of the blots. The β-actin expression was used as the normalization control for protein loading.

2.7. Statistical analysis

All the data presented are the means ± standard deviation obtained from three experiments performed independently. The GraphPad Prism 3 Software (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical evaluation of the results. The one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test were used for the analysis of differences between controls and treatment cells. Statistically significant differences were considered at $p < 0.05$.

3. Results

3.1. Orlistat treatment inhibits proliferation of HSC-3 cells

In HSC-3 cells orlistat induced concentration and time-dependent decrease in the viability (Fig. 1). Treatment with orlistat at 50 μM significantly ($p < 0.05$) reduced the viability of HSC-3 cells after 48 h. Effect of orlistat on the cell viability was analyzed after treatment for 12, 24, 48 and 72 h. The concentrations of orlistat used were 10, 20, 30, 40, 50 and 100 μM. The cell viability was reduced to 23.4 ± 2.5 and 15.7 ± 1.6% at 40 and 50 μM concentration of orlistat after 48 h (Fig. 1).

3.2. Induction of apoptosis in HSC-3 cells by orlistat

The apoptosis induction in HSC-3 cells by orlistat was analyzed by flow cytometry using FITC-conjugated Annexin V PI staining (PI). The results showed that orlistat treatment of HSC-3 cells for 48 h at 50 μM concentration caused a significant ($p < 0.05$) increase in the proportion of apoptotic cells (Fig. 2). Orlistat treatment led to the increase in proportion of apoptotic cells to 38.6 ± 2.5% after 48 h compared to 0.85 ± 0.34% in the control (Fig. 2).

3.3. Alterations in the cell cycle in HSC-3 cells by orlistat

The results from flow cytometry showed that orlistat significantly ($p < 0.005$) increased the population of HSC-3 cells in the G2/M phase of cell cycle. However, the population of cells in the G0/G1 and S phases was subsequently decreased in HSC-3 cells on treatment with 50 μM concentration of orlistat for 48 h (Fig. 3). The population of cells in G2/M phase in the cultures treated with orlistat for 48 h increased to 59.7 ± 5% compared to 10.2 ± 1.2% in the control (Fig. 3).