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Original article

Oleanolic acid inhibits cell growth and induces apoptosis in A375 melanoma cells

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ABSTRACT

Melanoma is a life threatening condition, which mostly effects cocassions despite the advancements in current chemotherapeutic techniques. The aim of present study is to investigate the apoptotic inducing potential of oleanolic acid (OA) in A375 human melanoma cells. The anti-proliferative effects of OA (12.5–200 μM) were assessed by cell growth and XTT assay. The morphological and nuclear damage studies were carried out by Wright-Giemsa and DAPI staining, respectively. Further, the apoptotic inducing potential of OA in A375 cells were measured by DNA fragmentation ELISA. The results showed a dose-responsive effect of OA by inhibiting the cell growth significantly ($P < 0.05$) at 24 and 48 h with a decrease in cell viability (XTT data). The significant morphological changes included cellular annihilation, which was observed in A375 cells when compared to the control cells. Quantitative dose-dependent increase in apoptotic-DNA fragments in ELISA and nuclear fragments in DAPI results, further demonstrated the potential of this triterpenoid to induce apoptotic cell death at a concentration, particularly higher than 50 μM. Thus, we conclude that OA has wielded both anti-proliferative and apoptotic inducing potentials against A375 melanoma cells and can be a better choice for its progression.

1. Introduction

Skin cancer accounts for 5% of deaths worldwide, but the empirical data shows an exponential increase in the number of people suffering from different types of skin cancer [1]. Metastatic melanoma is the most deadly form of skin cancer, developed by the proliferation of transformed melanocytes from the basal region of the epidermis. Melanoma causes 75% of deaths when compared to non-melanoma skin cancers [2]. Hence, there is an urgent need for better therapeutic agents including ethno-based compounds.

The induction of apoptosis has gained attention in cancer chemotherapy, which has prompted researchers to isolate plant-based derivatives that have the potential to induce cell death in cancer cells by activating various cell death signalling pathways. Plant compounds, like vincristine, vinblastine, vindesine, vinorelbine, etc. have already been used successfully in treating cancer either alone or in combinations [3]. Hence, the present research strategies continue to focus more to identify a potent template from natural resources that can be used to treat various cancers.

In the present study, we investigated the anti-proliferative and apoptotic potentials of a pentacyclic triterpenoid (Fig. 1), oleanolic acid (OA) in A375 melanoma cells. OA has been reported for its promising anti-cancer activities in several cancer cell lines [4–6]. Though OA has been reported for its cytotoxic effects [7], to the best of our knowledge, its apoptotic activity against human melanoma A375 cells has not been investigated. In addition, our previous study [8] showed significant effects of OA on human keratinocyte (HaCaT) cells, which further prompted us to extend our investigation in A375 melanoma cells due to a close and important functional association between keratinocytes and melanocytes [9].

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM) with (4.5 g/L of glucose and L-glutamine), Dulbecco's phosphate buffered saline (PBS) (Ca^{2+}/Mg^{2+} free), Phenazine methosulfate (PMS) (also known as *N*-methylphenazonium methosulfate), Giemsa stain were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide}, DAPI (4,6-diamidino-2-phenylindole dihydrochloride) and OA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Apoptotic kit, Cellular DNA fragmentation ELISA (# 11 585 045 001) was obtained from Roche Diagnostics,

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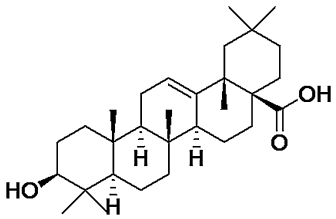

Fig. 1. The structure of OA.

Germany. The remaining chemicals and solvents used were of standard analytical grade.

2.2. Drug preparation

Stock solutions of OA were prepared at 25.77 mM in 100% dimethyl sulfoxide (DMSO) and the final concentration never exceeded 1% DMSO (v/v).

2.3. Cell culture

A375 (human melanoma) cells were obtained from National Centre for Cell Science (NCCS, Pune, India). The cells were propagated in DMEM media supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were maintained at the above-mentioned culture conditions for all the experiments and confluent cells between the second and the sixth passages were used for all the experiments.

2.4. Cell growth assay

Cell growth assay was performed accordingly with minor modifications as described earlier [10]. Cells (1×10^5) were initially seeded in 6 well culture plate at time 0 h. Cells were then exposed to OA concentrations (12.5–200 μM) for 24 and 48 h, respectively. Live cell quantification was achieved by trypsinization of the cultured cells and its consequent counting using a hemocytometer with trypan blue (0.4%) staining. The experiment was performed in triplicate.

2.5. Cytotoxicity analysis: XTT assay

The effect of OA in A375 cells was tested by the method of XTT–formazan dye formation [11]. Then, 1×10^4 cells were seeded in a 96-well plate and 200 μL of the culture medium was added to the cell suspension in the micro wells and incubated at 37 °C for a period of 24 h. The media were then replaced with 200 μL of the fresh media containing varying concentrations of OA, and subsequently, re-incubated for an additional 24 h. Drug medium was then substituted by 200 μL of the fresh medium. About 50 μL of XTT reagent, prepared in the medium (0.6 mg/mL) containing 25 μM of PMS was then added to all the wells and the plate was incubated under humidified conditions in the dark at 37 °C for 4 h. After incubation, the orange coloured complex formed was read at 450 nm using a Dynex Opsys MRTM Microplate Reader (Dynex Technologies, VA, USA) with a 630 nm reference filter. Wells containing cells without the OA served as the control and wells containing only culture medium and XTT reagent served as the blank. The percentage cytotoxicity of the extracts was calculated by using the formula:

$$\%\,\text{Cytotoxicity} = \frac{(\text{OD of control} - \text{OD of treated cells})}{\text{OD of control}} \times (100)$$

2.6. Wright-Geimsa staining

A375 cells were allowed to grow till 70% confluence and treated with different concentrations of OA for a period of 24 h. The cells were then washed in PBS and kept in PBS/methanol (1:1) for 2 min. Cell fixation was done by incubating cells in methanol for 10 min. After removing the methanol, the cells were stained with Wright-Giemsa stain for 2 min and observed under inverted phase-contrast microscope.

2.7. DAPI staining

A375 cells were grown on cover slips to attain 70% confluence. Cells were treated by OA at 277.5 μM (IC_{50} value from XTT assay) for 24 h followed by washing with PBS for two times. The cells were fixed with 4% paraformaldehyde for 15 min and then washed with PBS. DAPI (1 μg/mL) staining was then performed as described previously [12] and observed for nuclear fragments under fluorescence microscopy at a magnification of 100×.

2.8. Apoptotic detection – cellular DNA fragmentation ELISA

The potential of OA to induce apoptosis was studied using cellular DNA fragmentation ELISA kit as per the supplier's instructions. Briefly, A375 cells were labelled with 10 μM BrdU at 1×10^5 cells/mL density. Then, 100 μL of these BrdU-labelled cells in culture medium were treated with varying concentrations of OA for a period of 4 h. The cells were then lysed and the apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min and subjected to ELISA. About 100 μL of this obtained sample was transferred to an anti-DNA coated 96-well, flat-bottom microplates (MTPs). The plates were incubated for 90 min at 15–25 °C. DNA was then denatured by microwave irradiation (500 W for 5 min) followed by the addition of 100 μl anti-BrdU-POD conjugate solution. The plates were further incubated for 90 min and were washed 3 times with wash buffer (1×). Then, 100 μL substrate (TMB) solution was then added for a blue colour development. The absorbance was read at 450 nm after the addition of 25 μL of stop solution.

2.9. Statistical data analysis

All the analytical experiments were carried out in triplicate ($n = 3$). Data was expressed as mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA. MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Significant differences between groups were determined at $P < 0.05$.

3. Results

3.1. OA inhibited cell growth

A375 cells were treated with OA for a period of 24 and 48 h at different concentrations and analysed by trypan blue assay to assess cell viability. OA exhibited significant reduction in cell numbers when compared to control cells in a concentration-dependent manner. Longer exposure time (48 h) of A375 cells to OA markedly inhibited cell growth when compared to the exposure time of 24 h (Fig. 2).

3.2. Cytotoxic effects of OA

Cytotoxicity of OA on A375 cells were analyzed by XTT assay after 24 h treatment. XTT is metabolically reduced by mitochondrial dehydrogenase enzyme in viable cells to a water-soluble