Hesperidin induces anticancer effects on human prostate cancer cells via ROS-mediated necrosis like cell death

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Summary

Purpose: Hesperidin, a plant-based molecule, has been shown to exhibit anticancer effects against the human prostate cancer cells. However, its mechanism of action is still unclear. This study was undertaken to investigate the mechanism underlying the anticancer effects of hesperidin against prostate cancer cells.

Methods: The CCK-8 kit-based proliferation analysis was performed to find out the effect of hesperidin administration on prostate cancer cell growth, in vitro. Apoptosis of cancer cells was studied with dual Annexin V-FITC/propidium iodide (PI) staining combined with flow cytometry. The latter was also used for the analysis of cancer cell mitotic cell cycle. The intracellular levels of reactive oxygen species (ROS) were determined with ROS-detection kit. Fluorescent probing was used for determination of mitochondrial membrane potential (MMP) of prostate cancer cells. The migration and invasion of cancer cells was studied using transwell assay.

Results: The in vitro treatment of prostate cancer cells led to significant decrease of cell growth and viability in a dose-dependent manner. The decline in the growth of cancer cells was shown to be resulting from initiation of cell cycle arrest and necrosis-like apoptotic cell death. The latter was shown to be triggered by intracellular accumulation of ROS molecules and reduction of MMP. Moreover, the hesperidin administration significantly reduced the cancer cell migration and invasion.

Conclusion: Hesperidin was shown to selectively inhibit the growth of prostate cancer cells through apoptosis triggered by ROS generation. The results support its potential to act as lead molecule in anticancer drug design.

Key words: prostate cancer, hesperidin, apoptosis, ROS, migration, invasion

Introduction

The human prostate cancer ranks second to only the lung cancer in men, worldwide, in terms of incidence [1]. Besides, it is considered as the fifth most aggressive type of specific neoplastic malignancy in males at global level [2]. Worryingly, the mortality rates make prostate cancer the sixth most fatal cancer of men throughout the world [3]. Prostate cancer is currently being treated using radical prostatectomy which is often combined with radio or chemotherapy along with androgen deprivation therapy (ADT) [4]. The treatment procedures are seen to offer little success for the patients as often they show disease relapse. Furthermore, the management of prostate cancer becomes challenging at advanced disease stages which are often linked with metastasis to the bone. With this backdrop, the scientific efforts are rigorously being aimed at exploring more effective treatment modalities.
against prostate cancer. The dietary intake of flavanones, which are almost exclusively present in citrus fruits, has been shown to produce health beneficial effects on human body and reported to reduce the chances of cardiovascular, neurodegenerative disorders and also of other chronic disorders [5]. The flavanones form an inimitable subclass of plant flavonoids and comprise glycoside derivatives of aglycones, namely eriodictyol, hesperidin and naringen [6]. Hesperidin (3',5,7-Trihydroxy 4’-methoxyflavanone 7-rutinoside) is mainly present in oranges and lemons [7]. It has been reported to exhibit antioxidant, antiinflammatory, neuro and cardioprotective roles [8].

The anticancer effects of hesperidin have been established against human cancers like lung cancer, ovarian cancer, endometrial cancer and many others [9-11]. Previously, the in vitro administration of hesperidin was shown to inhibit the growth and proliferation of androgen-dependent prostate cancer cells and it was hypothesized to act through androgen receptors [12]. However, its mechanism of action against prostate cancer is largely unknown and its anticancer role has not been ascertained against hormone-independent prostate cancer cells. This study was designed to investigate the effects of hesperidin against human prostate cancer cells and to evaluate the underlying mechanisms.

Methods

Cell line maintenance and proliferation

The androgen-independent prostate cancer cell line DU145 together the normal prostate epithelial cell line PNT1A were recruited from American Type Culture Collection (ATCC, USA). A humidified incubator was used for the cell maintenance with 5% CO2, at 37°C. Both the cell lines were cultured utilizing RPMI-1640 medium (GIBCO, Waltham, Massachusetts, USA). 10% fetal bovine serum (FBS, Hyclone) along with penicillin-streptomycin (100U/mL, Hyclone, Logan, UT) was added to RPMI-1640 medium (Hyclone, Logan, UT) for in vitro cultivating the cell lines at 37°C.

Cell proliferation and colony formation assays

The proliferation of untreated DU145 cancer cells and PNT1A normal prostate cells and those administered with varying hesperidin doses (0.78 µM, 1.56 µM, 3.12 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM or 200 µM) 10 mM stock solution of hesperidin (Sigma-Aldrich, St. Louis, Missouri, United States) was made by using DMSO as solvent) for 24 h at 37°C in 96-well plates, which was determined using Cell Counting Kit-8 (Djingo, Tokyo, Japan). Briefly, about 2000 cells were added per well of 96-well plates and incubated with indicated hesperidin doses for 24 h at 37°C. Afterwards, proliferation estimation was performed using the optical density (OD450) values as direct measure of cell proliferation. The proliferation/viability of respective untreated cells was taken as 100% and relative the percent viability was estimated accordingly.

The cancer cells DU145 were suspended in RPMI-1640 media, administered with different hesperidin doses (0 µM, 5 µM, 10 µM or 20 µM) and were seeded into 6-well plates at 250 cells per well. The cells were cultured for 12 days at 37°C. Afterwards, the colonies formed were analyzed and manually counted after being ethanol-fixed and stained using 0.1 % crystal violet solution.

Flow cytometric study of cell cycle and apoptosis

For the study of their mitotic cell cycle, the DU145 cells were cultured for 24 h at 37°C with or without hesperidin (5 µM, 10 µM or 20 µM) in 12-well plates at 5000 cells per well. Following 24-h incubation at 37°C, the cells were collected, trypsinized in PBS and ethanol-fixed. The cells were subsequently stained with PI solution and subsequently processed for flow cytometry (BD Biosciences, New Jersey, United States) to determine the relative count of cancer cells with different mitotic cell cycle phases.

The DU145 cells were also analyzed for their apoptotic study after being treated with different concentrations of hesperidin (0 µM, 5 µM, 10 µM or 20 µM) for 24 h at 37°C. Herein, the hesperidin untreated or treated cells in 12-well plates were harvested through centrifugation and washed with ice-cold PBS. The cells were re-suspended in 250 µl binding buffer (from Annexin V-FITC/PI kit). Afterwards, the cells were subsequently stained with Annexin V-FITC and PI solutions. Finally, the cell apoptosis was studied using flow cytometry.

Estimation of LDH, ROS and MMP

For determining the relative release of lactate dehydrogenase (LDH) from DU145 cancer cells treated for 24 h at 37°C with varying hesperidin concentrations (0 µM, 5 µM, 10 µM or 20 µM), approximately 105 cells were added into each of 12-well plates. The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corp, Madison, Wisconsin, United States) was then used to determine the relative LDH release, following the manufacturer guidelines. Spectrophotometer was finally used to analyze the LDH activity by measuring the optical density (OD) for each sample at 490 nm. The DU145 prostate cancer cells with or without hesperidin administration (5 µM, 10 µM or 20 µM) were processed for intracellular ROS estimation using Reactive Oxygen Species (ROS) Detection Assay Kit (PromoCell, Heidelberg Germany), as per the manufacturer method. The indicated cells were re-suspended in 10 µM DCFH-DA solution and incubated at 37°C for 20 min. The cells were finally collected at full confluence and their fluorescence intensity was measured using microplate reader. The fluorescence intensity, proportional to intracellular ROS level, was used for determination of relative ROS count.

The mitochondrial membrane potential (MMP) of DU145 cancer cells treated with different hesperidin concentrations (0 µM or 10 µM) was determined through fluorescent probing using MitoTracker™ Red CMXRos and MitoTracker™ Green FM (both from Thermo Fisher.
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Hesperidin selectively inhibited prostate cancer cell proliferation

The chemical structure of hesperidin is shown in Figure 1A. The DU145 prostate cancer cells and PNT1A normal prostate cells were incubated with varying doses of hesperidin (0 µM, 0.78 µM, 1.56 µM, 3.12 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM or 200 µM) for 24 h at 37°C. CCK-8 kit was used to estimate the relative cell proliferation. The proliferation of cancer cells was shown to be significantly inhibited with an estimated IC$_{50}$ value of 10 µM (Figure 1B). The normal prostate cells, on the other hand, displayed little proliferative decline and the hesperidin IC$_{50}$ value was significantly higher, around 150 µM, which indicated the selective inhibitory role of hesperidin against the prostate cancer cells. The colony formation from prostate cancer cells was also shown to be markedly inhibited by hesperidin and followed the dose-dependence pattern, i.e., the higher the hesperidin dose, the lower the colony formation and vice versa (Figure 1C). Thus, hesperidin showed growth inhibitory potential against the prostate cancer cells in vitro.

G2/M cell cycle arrest and necrosis-like cell death was induced by hesperidin in cancer cells

Flow cytometry was used to find out the underlying mechanism behind the growth inhibitory effects of hesperidin. The analytical investigation confirmed that with the increase in the concentration of hesperidin, the percentage of G2/M phase DU145 prostate cancer cells increased proportionally and significantly (Figure 2A). Besides, the proportion of late apoptotic prostate cancer cells also increased remarkably in linear proportion with the increasing hesperidin doses (Figure 2B). The levels of LDH release were shown to be also increasing significantly in a dose-dependent manner under hesperidin administration (Figure 2C). The latter suggested the onset of necrosis of prostate cancer cells by hesperidin. Together, the findings are supportive of induction of G2/M phase cell cycle ar-

Figure 1. Prostate cancer cell growth is selectively inhibited by hesperidin. A: chemical structure of hesperidin (3’,5,7-Trihydroxy 4’-methoxyflavanone 7-rutinoside). B: analysis of viability of PC-3 cancer cells and PNT1A prostate epithelial cells administered with varying hesperidin doses (0 µM, 0.78 µM, 1.56 µM, 3.12 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM or 200 µM) for 24 h at 37°C. C: analysis of colony formation from PC-3 cancer cells treated with 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
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Hesperidin administration led to ROS generation and MMP reduction

To further understand the mechanism of action of hesperidin, the intracellular levels of reactive oxygen species (ROS) were analyzed under varying concentrations of hesperidin (5 µM, 10 µM and 20 µM) with respect to control DU145 cells (untreated). The ROS levels were shown to be significantly higher under all hesperidin doses applied (Figure 3A). The ROS percentage was almost 2-fold higher when DU145 cells were treated with 20 µM hesperidin concentration. Analysis of mitochondrial membrane potential (MMP) showed that MMP of DU145 cancer cells was significantly declined in relation to hesperidin concentration (Figure 3B). The loss of MMP is suggestive of loss of membrane integrity which was confirmed from the mitotracker probe based fluorescent analysis (Figure 3C). The results indicate that hesperidin administration led to intracellular ROS generation as a consequence of MMP decline in prostate cancer cells, signifying the onset of cellular necrosis modulating the decrease in cell proliferation.

Cancer cell migration and invasion was restricted by hesperidin

The migration and invasion of hesperidin-treated DU145 prostate cancer cells (10 µM hesperidin) were studied using transwell assay. It was shown that the hesperidin-treated prostate cancer cells (10 µM) significantly inhibited their migration in vitro (Figure 4A). The migration declined

rest and necrosis-like death of prostate cancer cells by hesperidin which was manifested as decline in their growth and proliferation.

Figure 2. Hesperidin induces necrotic-like cell death in prostate cancer cells. A: Flow cytometry study of phase distribution of PC-3 prostate cancer cell mitosis under 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. B: analysis of apoptosis of PC-3 prostate cancer cells administered with 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. C: relative percentage of LDH leakage from PC-3 prostate cancer cells administered with 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Figure 3. Hesperidin reduces prostate cancer cell MMP through ROS-accumulation. A: Relative percentage of intracellular ROS levels from PC-3 prostate cancer cells administered with 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. B: relative percentage of MMP from PC-3 prostate cancer cells administered with 0 µM, 5 µM, 10 µM or 20 µM hesperidin for 24 h at 37°C. C: fluorescent probing of mitochondria of PC-3 prostate cancer cells administered with 0 µM or 10 µM hesperidin for 24 h at 37°C. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Figure 4. Hesperidin restricts the migration and invasion of prostate cancer cells. Analysis of migration and invasion of PC-3 prostate cancer cells administered with 0 µM or 10 µM hesperidin for 24 h at 37°C. The figure shows that hesperidin inhibits the migration and invasion of PC-3 cells. The experiments were performed in triplicate.
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by 61% with respect to untreated cells. The invasion of prostate cancer cells was also minimized significantly by hesperidin (10 µM) and the cancer cells showed 67% loss of their in vitro invasiveness (Figure 4B). Such results are indicative of the therapeutic potential of hesperidin to control prostate cancer metastasis but need in vivo confirmation.

Discussion

Hesperidin, a flavanone largely extracted from the citrus fruits, has been reported to offer considerable health promoting effects on human body [7,13]. Both the in vitro and in vivo clinical investigations have shown that hesperidin has considerable antioxidant, anti-inflammatory and neuroprotective properties [8]. Besides, it is therapeutically active against cardiovascular and psychiatric disorders [14]. The anticancer role of hesperidin is also well recognized. It has been confirmed to exhibit tumor-suppressive effects against different types of human cancers [9-11]. The in vitro treatment of hesperidin effectively diminished the growth and proliferation of androgen-dependent prostate cancer cells, as reported by a previous study [12]. Nevertheless, its anticancer role could not be established against hormone-independent prostate cancer cells and its mechanism of action of its anticancer role against prostate cancer is by far elusive. In the present study, the anticancer effects of hesperidin were investigated against the hormone-independent prostate cancer cell line DU145. Astonishingly, the prostate cancer cells were shown to proliferate with much reduced capacities when subjected to hesperidin treatment, while the effects were minimal against the normal prostate epithelial cells. This selective antiproliferative role of hesperidin suggests its suitability to be tried in systemic therapy against prostate cancer. The selective growth inhibitory effects of hesperidin have also been reported for other human cancers [10,11]. Hesperidin was shown to induce apoptotic cell death and G0/G1 cell cycle arrest in A549 non-small lung cancer cells to inhibit their growth and viability [9]. Similar mechanism possibly mediated the growth-inhibitory effects of hesperidin against prostate cancer cells, and, therefore, studies on their cell cycle and apoptosis were made. The prostate cancer cells were shown with cell cycle arrest but at G2/M stage. The higher number of late apoptotic cells and increase in LDH leakage under hesperidin administration were suggestive of necrotic cell death. Loss of mitochondrial membrane potential (MMP) was shown to be one of the possible mechanisms of anticancer role of hesperidin against the human hepatocellular carcinoma cells without any significant alteration of ROS-production [15]. However, we found that prostate cancer exhibited both reduction of their MMP and increase in ROS-generation through which the anticancer effects of hesperidin were exerted. The migration and invasion are the crucial activities of cancer cells and are directly linked with the process of metastasis and hence the cancer aggressiveness [16]. The migration and invasion of prostate cancer cells was greatly restricted by hesperidin as has been previously shown for non-small lung cancer cells [17]. Summing up, the results suggest a sizeable anticancer role of hesperidin against the human prostate cancer cells but this needs to be tested in in vivo systems.

Conclusions

Hesperidin selectively inhibited the growth of prostate cancer cells. The growth inhibitory effects of hesperidin were mediated through ROS-driven cell necrosis. Migration and invasion of cancer cells were also substantially reduced, making hesperidin a possible candidate for anticancer drug. The semi-synthetic chemistry approaches might enhance the anticancer role of hesperidin and this might also be supported from in vivo studies.

Conflict of interests

The authors declare no conflict of interests.

References

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