The Effect of Irisin on Proliferation, Apoptosis, and Expression of Metastasis Markers in Prostate Cancer Cell Lines

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ABSTRACT

Introduction: Irisin is a newly discovered myokine released from skeletal muscle during exercise. The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that play a key role in the metastatic process via degrading extracellular matrix. The aim of this study was to investigate the effect of irisin on expression of metastatic markers MMP2 and MMP9 and induced apoptosis in human prostate cancer cells.

Methods: In this study, we examined the effect of different concentrations of irisin on induced apoptosis and cell viability of two cell lines, LNCaP and DU-145, by using flow cytometry and MTT assay, respectively. The expression of MMP2 and MMP9 genes was also analyzed by real-time PCR after irisin treatment. Data were analyzed using the comparative cycle threshold $2^{-\Delta\Delta C_t}$ method.

Results: Cell viability was reduced in both LNCaP and DU-145 cell lines at different concentrations of irisin. However, this decreased cell viability was strongly significant ($p < 0.05$) only at 5 and 10 nM concentrations of irisin in the LNCaP cell line. Furthermore, irisin could induce apoptosis in both cell lines at a concentration of 10 nM compared to 5 nM. Real-time PCR results also demonstrated a decreased expression in MMP2 and MMP9 genes in a concentration-dependent manner in both cell lines.

Conclusion: These results showed the anti-cancer effects of irisin on cell viability of both LNCaP and DU-145 cell lines and also on the expression of MMP2 and MMP9 genes occurred in a dose- and time-dependent manner.

Keywords: Prostate cancer; Irisin; Matrix metalloproteinase; Metastasis; Cell line
INTRODUCTION

Prostate cancer (PCa) is the second most common cancers in men globally and seventh leading cause of death of men in Iran [1, 2]. Prostate cancer arises from epithelial cells and androgens are the main stimulants of cell division and cell proliferation in the prostate epithelium. Although early stages of prostate tumors are mediated by androgen secretion, promotion of tumor metastasis is generally androgen independent [3]. Epithelial-to-mesenchymal transition (EMT) is a significant aspect in prostate cancer progression. During cancer development, tumor cells promote their ability to invade and metastasize through the EMT process by some alterations in the extracellular matrix (ECM). The matrix metalloproteinase (MMPs) are a large family of Zn\(^{2+}\)-dependent endopeptidases involved in degradation and remodeling of the ECM [4–6]. MMP-2 and MMP-9, two members of the MMPs family, are suggested to have a key role in facilitating prostate cancer progression, invasion, and metastasis by breaking down connective tissue barriers [7, 8]. Tissue inhibitors of metalloproteinases (TIMPs) play a major role in the homeostasis of the ECM by strongly regulating the gene expression of MMPs at the transcriptional and protein level. Altered expression of various MMPs including MMP2 and MMP9 genes has been reported in various breast, colorectal, and lung cancers [9, 10]. Several pieces of evidence have also revealed the elevated value of MMP-9 expression in human prostate tissues and prostate cancer cell lines [11, 12]. In prostate cancer, an imbalance in expression of MMPs and TIMPs, as their specific inhibitors, affects the connective tissue homeostasis which leads to degradation of the extracellular membrane [13] and promotes EMT and cell proliferation [14]. Previous studies have demonstrated that high levels of MMP-2 were associated with the promotion of metastasis to the lymph nodes in prostate cancer. In vitro and animal experiments have also provided some evidence that overexpression of MMP-2 and MMP-9 is associated with enhanced risk for metastasis in prostate cancer [15, 16].

Irisin is a newly identified myokine that is secreted from skeletal muscle during exercise. It is produced by cleavage of the transmembrane-bound protein FNDC5 [17]. Although irisin was first found in skeletal muscle and adipose tissue involved in energy homeostasis [18], it was recently also identified in the liver, pancreas, spleen, stomach, brain [19], heart [20], breast [21], and skin [22]. Irisin directly modulates lipid metabolism through browning of white adipose tissue [23] and is also indirectly associated with fundamental processes of tumor growth and development [24–26]. The suppressive effects of irisin on cell proliferation and metastasis [27] by inhibiting of EMT via various signaling pathways including PI3K/AKT [28], STAT3/SNAI, AMPK-mTOR [29], and NF-κB [30] have been confirmed in multiple tumors. Thus, irisin can be considered as a future therapeutic biomarker for prostate cancer treatment. The aim of this study was to evaluate the anticancer effect of irisin against two human prostate cancer cell lines, LNCaP and DU-145, by studying the process of proliferation, apoptosis, and expression of metastatic markers (MMPs).
METHODS

Compliance with Ethics Guidelines

Our research was approved by the local ethical committee of Kashan University of Medical Sciences, Kashan, Iran, under reference no. 2012-15.

Cell Culture

Prostate cancer cell lines LNCaP and DU-145 were purchased from the National Cell Bank, Pasteur Institute of Iran. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO₂. Trypsin-EDTA solution was used to remove cells from the bottom of the flask after their density reached 70–80% [4, 5]. Then the number of cells was counted and cells were stored in an incubator (Memmert GmbH, Germany) (5% CO₂) at 37 °C for subsequent experiments.

MTT Assay

The MTT assay was used to access cell survival and proliferation in LNCaP and DU-145 cells treated with irisin. In order to obtain a growth curve, cells first were seeded in 96-well plates in 200 μL of DMEM and were incubated for 24 h at 37 °C. Cells were then treated with different concentrations of irisin (5, 10, 20, and 40 ng/mL) for 24, 48, 72, and 96 h, respectively. Experiments were repeated three times for each concentration. Then, 20 μL of MTT solution (5 mg/mL) was added to each well and cells were incubated (37 °C, 5% CO₂) for an additional 4 h. After that, the optical density of the cells was read in an ELISA reader at 570 nm wavelength. Finally, the cell viability percentage was calculated in the treated group compared to the control group.

RNA Isolation and cDNA Synthesis

For total RNA extraction from LNCaP and DU-145, we used the RNA Extraction Kit (RiboX), according to the manufacturer’s protocol. Isolated RNA was kept frozen at −80 °C until further use. RNA concentration and purity were measured using a Nano drop ND-1000 spectrophotometer and agarose gel electrophoresis (1%), respectively. Furthermore, cDNA Synthesis kit (Takara Co., Japan) was used for cDNA synthesis from 500 ng of total RNA, following the manufacturer’s instructions. The reactions were incubated at 37 °C for 15 min, then at 85 °C for 5 min in order to inactivate the reverse transcriptase. The cDNA was stored at −20 °C until qPCR.

Real-Time PCR and Gene Expression

We measured the gene expression of MMP2 and MMP9 in PCa cells by real-time PCR. The primers used for the assay were designed using Allele ID Version 7.5, and BLAST web sites. The

Annexin V/Propidium Iodide (PI) Staining

We performed the annexing V/PI staining and flow cytometry to detect cell death caused by apoptosis and necrosis by analyzing phosphatidylserine attached to Annexin V–FITC on the outer surface of apoptotic cell membranes. For this purpose, LNCaP and DU-145 cells were seeded in 6-well plates (1 × 10⁵ cells/well) overnight, then treated with IC₅₀ irisin for 48 h. After that, the cells were trypsinized and washed twice in PBS, then centrifuged at 1200 rpm for 6 min. Apoptotic cells were distinguished from necrosis cells using an Annexin V–FITC/PI apoptosis detection kit (Sigma, BD Bioscience), according to the manufacturer’s instructions. Finally, 1 × 10⁵ treated cells were incubated with Annexin V–FITC and PI and analyzed using flow cytometry. Typically, cells exhibiting the Annexin V−/PI−, Annexin V+/PI−, Annexin V+/PI+, and Annexin V−/PI− phenotype, respectively, were identified as healthy cells, early apoptosis cells, late apoptosis cells, and necrotic cells.
primers used in this study were as follows: 
MMP2 (261 bp) F: 5’ TGGAGATA-CAATGAGGTGAAGAAG 3’; MMP2 R: 5’ GAAGGCAGTGAGAGGAAG 3’; MMP9 (239 bp) F: 5’ TGACAGCGACAAGAAGTGG 3’; MMP9 R: 5’ GTGTGGTGTTGGTTGGAG 3’.

Real-time PCR was performed in a 10-μL reaction volume containing 5 μL of cDNA, 5 μL of SYBER Green/ master mix (BioFact, Korea), 0.5 μL of each primer, and 2 μL of DNase-free water. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Negative controls were used to confirm that no genomic DNA contamination existed. Data were normalized to GAPDH (housekeeping gene) as an internal reference gene. We also used (1%) agarose gel to reveal the accuracy of amplification products.

Statistical Analysis

Data was analyzed by using SPSS Statistics software (version 19; IBM SPSS). The data are expressed as the mean ± standard deviation (SD). Student’s t tests, one-way ANOVA, and GraphPad Prism 5.0 software were used to compare two control and experimental groups. p < 0.05 was considered to be statistically significant. REST 2009 Software was applied for gene expression analysis. The PCR amplification specificities for each set of primers were confirmed by melting curves. The comparative 2−ΔΔCt method was used to calculate relative fold changes in expression of the target genes. Ct values of each target gene were compared to another and normalized to GAPDH as the reference gene.

RESULTS

The study results indicated the effects of irisin on the LNCaP cell line in a dose- and time-dependent manner with IC50 of 5, 2.5, and 1.25 μg/ml after 48, 72, and 96 h, respectively. Whereas IC50 was approximately 5 nM for the DU-145 cell line. As shown in Fig. 1, these results clearly showed the significant decreasing effect of irisin on the cell proliferation and viability of both LNCaP and DU-145 cells, which was obviously time- and dose-dependent only in the LNCaP cell line (Fig. 2).

Furthermore, the effect of irisin at concentrations of 5 and 10 on gene expression of MMP2 and MMP9 in the two different cell lines was evaluated by real-time PCR. These findings demonstrated that the altered expression was dose-dependent in both genes and in both cell lines. As shown in Fig. 3, the analysis of data showed that expression level of MMP9 gene was decreased after 48 h of irisin treatment in both

Fig. 1 Results of LNCaP cell viability after treatment with irisin (mean ± SD; *p < 0.05, p < 0.01; PRISM)
cell lines compared to the control group. This decreased gene expression was significantly different only at 5 and 10 nM concentrations in both treated cell lines compared to the control group. However, it was higher with the 10 nM dose compared to 5 nM and also in LNCaP than in DU-145, showing a more significant difference in both lines (Fig. 3).

We got the nearly same results for MMP2 expression in both cell lines (Fig. 4). MMP2 expression was also decreased in treated LNCaP lines compared to untreated controls within 48 h. This decrease in gene expression was more
significantly different at 10 nM than at 5 nM. Also, the effect of irisin in the treated DU-145 line was statistically significant only at a concentration of 10 nM compared to the control group.

Flow cytometry test using Annexing V/PI staining was used to evaluate apoptosis and necrosis rate in LNCaP and DU-145 cell lines after treatment with irisin. Based on the flow cytometry data, the apoptotic cell percentage increased at a concentration of 10 nM of irisin in both cell lines compared with control cells after 48 h. Although the percentage of apoptotic cells in both cell lines at a concentration of 5 nM irisin was also higher than in the control cells, this apoptotic effect at this concentration was not statistically significant. These results indicated that irisin can induce apoptosis only at the concentration of 10 nM in both treated cell lines (Fig. 5).

DISCUSSION

Prostate cancer has the highest mortality rates in elderly men worldwide. Prostate cells usually metastasize to lymph, adrenal gland, and bone. The progression and survival of prostate tumor depends on the androgen. For this reason, the relapse of prostate cancers after surgical therapy in men is often a result of producing a high percentage of testosterone at each site. Although androgen deprivation therapies have been used for earlier stages of treatment for prostate cancer, it rarely cures the disease itself. Chemotherapy, surgery, and radiotherapy are the most common types of treatment for prostate cancer. However, they were not effective in the advanced stage of the disease.

Irisin is a newly discovered myokine which is released from the skeletal muscles during physical activity and exercise [15]. As a precursor to irisin, FNDC5 consists of signal peptide, hydrophobic C-terminal domain, and fibrin III domain, and is highly expressed in muscle tissues [31]. Recent studies revealed irisin’s function in energy homeostasis and metabolism, and it also has a suppressive effect on various cancer cells and tissues. In this research we investigated the inhibiting effect of irisin on proliferation and metastasis of two prostate cancer cell lines, LNCaP and DU-145, by using the MTT assay and flow cytometry. Firstly, we found that irisin at all tested concentrations (5, 10, 20, 40 nM) after 24, 48, 72, and 96 h decreased cell viability and proliferation in both treated cell lines compared to the control cells. Interestingly, the longer the time and the higher dose of irisin treatment obviously resulted in the lower cell viability and proliferation.
in LNCaP cell lines. Moreover, a concentrations of 10 nM irisin statistically significantly induced apoptosis ($p < 0.05$, $p < 0.01$) in both PCa cell lines compared to control group. We concluded that the antiproliferation property of irisin on both cell lines occurred in a dose-dependent manner. These results agreed with the findings of Aktas, indicating that the inhibiting
effect of irisin on the proliferation of prostate cancer cells occurred in a dose-dependent manner after treating two cell lines, LNCaP and DU-145, with different concentrations (0, 0.1, 1, 10, and 100 nM) of irisin for 24 h. That study also revealed that irisin exerts this antiproliferation effect by affecting the EMT AMPK pathways [32].

One of the most important features in cell metastasis is the ability of tumor cells to grow via degradation of the ECM mainly by EMT markers (MMPs) [33–35]. Some evidence has shown that EMT markers (MMPs) are associated with degradation of the ECM in the process of invasion and metastasis [36]. Irisin can inhibit EMT and metastasis processes in cancer cells by inhibiting STAT3 activity in the PI3K/AKT pathway via decreasing IL-6 and the EMT markers (MMP-2, MMP-7, and MMP-9) [37–39]. On the basis of previous findings, elevated levels of MMP-2 and MMP-9 in prostate cancer are associated with cancer progression [12, 15, 34]. To further explore the suppressive effect of irisin on metastasis in PCa, we measured the expression of MMP2 and MMP9 genes in LNCaP and DU-145 cell lines using qPCR after treatment with irisin at concentrations of 5 and 10 nM. According to our results, both cell lines exhibited decreased levels of MMP2 and MMP9 expression compared with the untreated group. However, a significantly decreased expression of MMP2 was detected only in the DU-145 line and at a concentration of 10 nM irisin, while MMP9 was significantly reduced in both cell lines at concentrations of 5 and 10 nM irisin. This finding suggested that irisin may affect the expression of EMT markers in a dose-dependent manner, leading to inhibiting invasion and metastasis in prostatic cells. Moreover, flow cytometry results showed that irisin induces apoptosis in PCa cell lines at an irisin concentration of 10 nM. These findings confirmed that irisin exerts its suppressive role on metastasis in prostate cancer via affecting MMP2 and MMP9 expression. Our results were similar to those of Lichtinghagen et al., who determined the increased expression of MMP9 gene at the mRNA level in prostate cancer tissue using RT-PCR [40]. Also, Simi et al. reported the enhanced MMP9 expression in lung cancer cells compared with control group [41]. In contrast to our results, Reis in 2012 reported reduced MMP2 expression in PCas, revealing that MMP2 is not associated with the promotion of metastasis in PCa [42]. TIMPs as MMPs inhibitors have a crucial role in progression and metastasis of tumor cells [8, 34]. An imbalance between MMPs and their inhibitors TIMPs can be a main contributing factor in proteolytic degradation of the matrix ECM and basement membrane, leading to invasion of tumor cells. In fact, TIMPs–MMP complex on the cell surface is a key factor in tumor metastasis that regulates matrix degradation of ECM [43–46]. Thus, it is suggested that expression level of MMPs may be a promising prognostic markers in PCa as a result of its effect on signaling pathways involved in cell proliferation and metastasis [4, 5]. Although the precise mechanism underlying the effect of irisin on metastasis of prostatic cells is unclear, previous studies have been revealed the anticancer effect of irisin through suppressing EMT via the PI3K/AKT/Snail pathway [28, 39]. Irisin inhibits STAT3 and Snail through the PI3K/AKT pathway as the major regulator of Snail [47]. Moreover, irisin prevents the EMT activity by regulating the expression of E cadherin, N cadherin, vimentin, fibronectin, MMP-2, MMP-7, and MMP-9 [27, 47–49]. Irisin also exerts its inhibitory effect via regulating growth inhibitors by targeting the AMPK-mTOR pathway [50, 51]. Results of multiple studies have also demonstrated that physical activity in patients with prostate cancer improved quality of life, reduced cardiovascular risk, and improved outcomes [52, 53]. Moreover, it may contribute to maintaining endothelium homeostasis by affecting endothelial cell angiogenesis via the ERK signaling pathway [54]. Further studies have illustrated that irisin can induce apoptosis in cancer cells by reducing inflammation and stimulate the activity of caspase 3 and 7 [55], AMPK phosphorylation, and acetyl coenzyme A carboxylase, inhibit the NF-κB pathway and proinflammatory factors, and modulate the PI3K/Akt pathway [56].

There were two main limitations to this study: first, we had limited time to do the project and we had to finish it at a defined time; second, there were financial problems. We did
not have financial support to conduct a prestigious project; and because of tough economic conditions in our country, we cannot provide different kinds of agents such as antibodies.

CONCLUSION

Our results indicated that irisin can inhibit tumor development and induce apoptosis by inhibition of EMT through various signaling pathways. Irisin also decreases the expression of MMP2 and MMP9 that may be potential cancer markers in the diagnosis of prostate cancer. However, the comparison of our results with previous findings revealed many contradictions, which may be due to experimental methods used or tissue or cell line properties. Therefore, further research is needed to support our results in the study of irisin. In addition, the mechanisms involved in irisin function, key factors, and associated signaling pathways need to be further clarified. These findings suggest the possibility of using of irisin as a new attractive and potential therapeutic target drug and the profile of MMPs as prognostic and diagnostic biomarker to treat prostate cancers in the future.

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Compliance with Ethics Guidelines. Our research was approved by the local ethical committee of Kashan University of Medical Sciences, Kashan. Iran, under reference no. 2012-15.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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