Synergistic Effects of Resistin and Visfatin as Adipocyte Derived Hormones on Telomerase Gene Expression in AGS Gastric Cancer Cell Line

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Abstract- Recently suggested that adipocytokines may play a role in pathogenesis and progression of certain cancers, especially in gastric cancer. The previous study showed Resistin and Visfatin, as adipocyte derived hormones, separately increases telomerase (hTERT) gene, the aim of this study is investigating synergic effects of Resistin and Visfatin on telomerase gene expression, in AGS gastric cancer cell line. In this study, human gastric cancer AGS cell line was selected. After stimulation with increasing concentrations of Resistin and Visfatin recombinant proteins for 24 and 48 hours, cell proliferation was assessed by XTT assay. In order to investigate the telomerase gene expression affected by these proteins, total RNA was extracted, cDNA was synthesized, and expression of hTERT mRNA was carried out by real-time reverse transcription polymerase chain reaction. After Resistin and Visfatin, recombinant proteins treatment was increased the gastric cell line proliferation and expression of Human Telomerase Reverse Transcriptase (hTERT), but co-stimulation with Resistin and Visfatin showed greater inducible effects on cell proliferation and telomerase gene expression in comparison with the stimulatory effect of the individual hormone. This study has shown Resistin, and Visfatin synergistically increased gastric cancer cell proliferation and enhanced the telomerase gene expression. These data showed that these two hormones in gastric cancer tissue could cooperatively accelerate cancer cell growth via enhancing the telomerase expression as a cancer gene.

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Keywords: Visfatin; Resistin; Gastric cancer; AGS cell line

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death worldwide (1), with a wide variation in incidence rates across different geographical areas (2).

Gastric cancer is a multi-factorial disease and develops as a result of continuous cell damage caused by lifelong exposure to different carcinogens. Endogenous and host factors, including those related to male gender, and several genetic backgrounds are known risk factors to a lesser extent (3). The intestinal histological subtype of gastric adenocarcinoma, as the most common form of gastric cancer, develops in an inflammatory background induced by *H. pylori* related chronic gastritis and progresses to atrophic gastritis, intestinal metaplasia, glandular dysplasia and eventually adenocarcinoma (4). Many environmental factors, including smoking, high salt intake and a diet with an insufficient level of antioxidants are involved in the pathogenesis of gastric cancer (5). Several epidemiological studies have investigated the effect of increasing body weight as well as metabolic syndrome (MS), body mass index (BMI) and other anthropometric measurements on the risk of gastric cancer (6-8). Obesity is closely linked to an increase adipose tissue. Adipocytes secrete a number of different factors that are commonly referred to as 'adipokines' (adipocyte-derived cytokines). Adipokines are involved in a variety of biological functions, including the regulation of energy balance, glucose homeostasis, lipid metabolism, and inflammation (9). In the last two decades, many researchers have tried to discover the possible role of adipocytokines in the regulation of angiogenesis and tumor growth (10). In the previous study in gastric cancer has shown that Resistin and Visfatin increased more than other adipocytokines and

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may be good biomarkers of gastric cancer (11).

There are many factors that contribute to gastric carcinogenesis (12-17). Currently, telomerase has been a major focus (18,19). Telomerase activation is associated with an early stage of stomach carcinogenesis (20). Telomerase is an RNA dependent DNA polymerase that synthesizes telomeric DNA sequences (TTAGGG), and almost universally provides the molecular basis for unlimited proliferate potential. Telomerase consists of two essential components: one is the functional RNA component (in humans called hTR or hTERC), which serves as a template for telomeric DNA synthesis; the other is a catalytic protein (hTERT) with reverse transcriptase activity. hTER is highly expressed in all tissues regardless of telomerase activity, with cancer cells generally having fivefold-higher expression than normal cells (21).

In this study, we try to investigate molecular mechanisms of obesity in gastric cancer with investigating of Resistin and Visfatin together effects on proliferation cancer cells and their effects on telomerase gene expression.

Materials and Methods

Cell lines and culture conditions

Human gastric adenocarcinoma is consisting of mucus-secreting epithelial cells and were purchased from the Institute pastor cell bank in Iran. The cells were incubated in Ham's F-12 (Sigma-Aldrich/USA) culture medium containing 100 ng/ml of penicillin, 100 ng/ml of streptomycin, 15 mm Hepes, 1.2 g/l sodium bicarbonate, and 10% fetal bovine serum (Biochorom/Belin). The cell monolayer in a 25 cm^2 flask was subcultured at 1:5 ratios every 3 days by treatment with 0.1% trypsin and 0.03% EDTA. The flask was maintained in incubator at 37° C in a humidified atmosphere with 5% CO_2.

Cell proliferation assay

Growing cells (7×10^3 cells/well) were seeded in 96-well plates after 24 hours incubation at 37° C, incubated with various concentrations of recombinant human Visfatin, Resistin separately and together (0, 5, 10, 50, 100, 200 ng/ml) for 12, 24 and 48 hours. Cell number and viability were determined using a hemocytometer after staining with trypan blue. Cell proliferation was analyzed using the XTT assay kit (BIOTIUM, Inc) according to the manufacturer's instructions. Briefly, add 50 µl of the activated XTT solution (Mix 25 µl activation reagent with 5 ml XTT solution to derive activated XTT solution) to each well and were incubated for 4 hours. Measure the absorbance of the samples with a spectrophotometer (ELISA reader) at a wavelength of 470 nm wavelength and a reference wavelength of 630 nm.

Total RNA extraction

For gene expression assay, 7×10 5 cells/ well were seeded in 25-T culture flasks and incubated overnight. The total medium was then replaced with serum-free medium for 24 hours for cell cycle synchronization. The cells were treated with the most effective dose of Visfatin and Resistin and Resistin/Visfatin on cell proliferation and incubated for 6, 12 and 24 hours.

Before RNA extraction AGS cells were cut out by harvesting medium for each flask. Adherent cells were washed twice with PBS and trypsinized; the cell pellets were collected by centrifugation at 1000 g for 10 min at 4° C. Total RNA was extracted from each cell culture flask using the guanidine isothiocyanate based RNX-plus solution (Sinna Gen INC, IRI) according to the manufacturer protocol. Briefly, 1 ml of RNX plus reagent in a clean RNase-free tube and was incubated for 5 min at room temperature. After incubation, 200 µl chloroform was added, shaken rigorously for 15 seconds, and incubated for another 5 min. The mixture was centrifuged at 12000 g for 15 minutes. The aqueous phase was transferred to a clean RNase-free tube. The total RNA was precipitated by adding 0.5 ml isopropyl alcohol and incubating for 15 minutes at room temperature. The pellet including total RNA was washed using 75% ethanol and centrifuged at 7500 g for 8 minutes. After drying the ethanol, the RNA pellet was dissolved in TE buffer. The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm. The absence of degradation of the RNA was confirmed by RNA electrophoresis on a 1.5% agarose gel containing ethidium bromide.

Quantitative real-time RT-PCR

Levels of hTERT RNA molecules were determined by quantitative real-time RT-PCR technique using the Syber Green-I (Roche, Germany) by the Rotor-Gene™ 6000 system (Corbett Research, Australia) according to the manufacturer's instructions with specific primers: forward sequence 5′-CCGCCTGAGCTGTACTTTGT-3′ and reverse sequence 5′-CAGGTGAGCCACGAACTGT-3′. Alternative spliced variants of hTERT mRNA were not measured because they do not reconstitute telomerase activity (22,23). The GAPDH mRNA measured as the internal control by specific primers: forward sequence 5′-

CAAGGTCATCCATGACAACTTTG-3′ and reverse sequence 5′-GTCCACCACCCTGTTGCTGTAG-3′. The program for real-time PCR reaction was as follows: initial denaturation at 95° C for 10 minutes, followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 30 seconds. Finally, amplicons were assessed by melting curve analysis of 70° C to 95° C. The quantity of PCR product generated from amplification of the hTERT gene was standardized using the quantity of GAPDH product for each sample to obtain a relative level of gene expression. After quantitation, results were analyzed by $2^{-\Delta\Delta Ct}$ method. All data were derived from at least three independent experiments, and statistical analyses were performed using independent t-test. Values were presented as Mean±SEM P<0.05.

Statistical analysis

Results were analyzed using SPSS 15. Student's t-test was used for comparisons between two groups. The difference in mRNA levels of telomerase between control and treated cells was assessed by ANOVA. P of less than 0.05 was considered to be statistically significant.

Results

Resistin and visfatin proliferate AGS cell line

To explore stimulatory effects of Resistin and Visfatin (separately and together) for 12, 24 and 48 hours, XTT assay performed and results showed that these two adipokines stimulated gastric cancer cell proliferation in a dose-dependent manner in which the most effective dosage Resistin, Visfatin, and Resistin/Visfatin was 10, 5 and 5 ng/ml. Also, cell counts showed that in these concentrations had maximum cell viability (P<0.05) (Figure 1).

Results showed that co-stimulation with Resistin and Visfatin had greater inducible effects on cell proliferation and had a synergistic effect on proliferation AGS cell line in the concentration of 5 ng/ml with maximum cell viability and cell count.

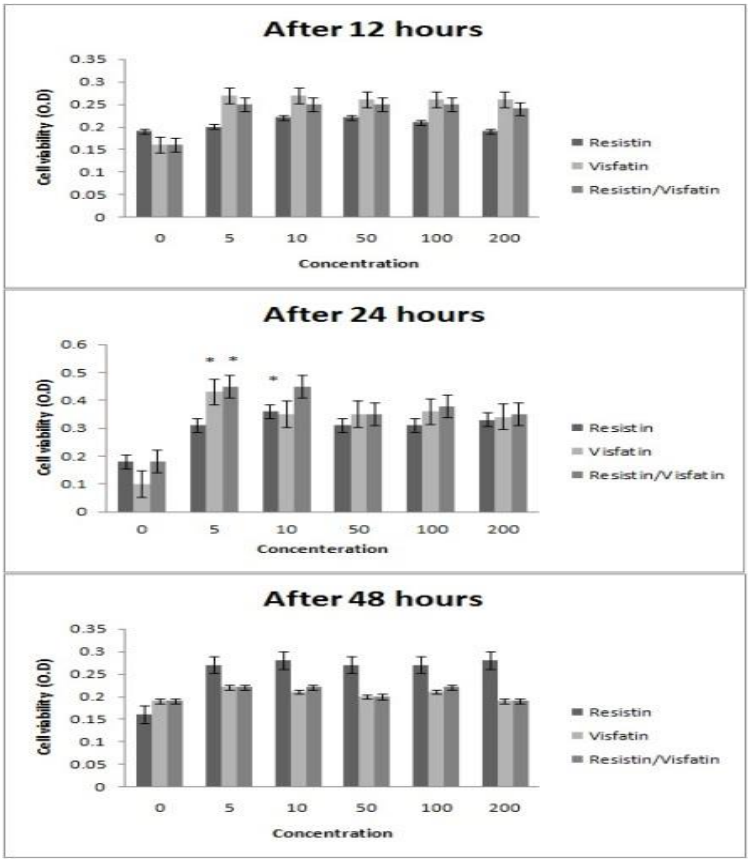

Figure 1. The effect of Resistin, Visfatin, and Resistin/Visfatin on AGS cell proliferation after 12, 24 and 48 hours. * The effect of Resistin, Visfatin and Resistin/Visfatin on AGS cell proliferation after 24 hours at 10, 5 and 5ng/ml (respectively) are significantly higher than other groups. (P<0.05)

Resistin and visfatin up regulate expression of hTERT mRNA

Since telomerase activity is tightly associated with the expression of hTERT, the catalytic reverse transcriptase of telomerase, we evaluated the levels of hTERT mRNA in AGS cells by real-time RT-PCR. Results showed that telomerase gene expression significantly increased in treated AGS cells in comparison with control cells in a time-dependent fashion (Figure 2). After 24 hours mRNA levels of hTERT in treated AGS cells with Resistin were increased about 1.6 folds, Visfatin was increased about 1.2 folds, and Resistin/Visfatin were increased about 2.15 folds (P<0.05) that was shown Visfatin and Resistin had synergic effect on proliferation AGS cell line. Data showed that Resistin, Visfatin and Resistin/Visfatin had an inhibitory effect on hTERT expression at 6 and 12 hours compared to controls, but stimulates hTERT expression at 24 hours that may be because these two adipokines in early time has an inhibitory effect on hTERT expression and after 24 hours starts its stimulatory effect on hTERT expression and need more study in this field.

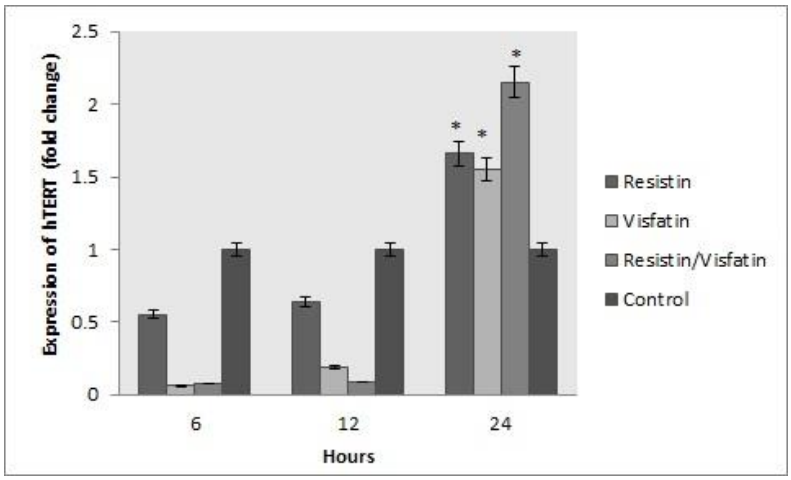

Figure 2. The effect of Resistin, Visfatin, and Resistin/Visfatin on hTERT gene expression. *The effect of Resistin, Visfatin, and Resistin/Visfatin on hTERT gene expression after 24 hours are significantly higher than other groups. (P<0.05)

Discussion

Since it has identified that adipose tissue displays characteristics of an active endocrine organ, adipocytokines have become subjects of extensive research (24,25) particularly, in the last two decades, many researchers have tried to discover the possible role of adipocytokines in the regulation of angiogenesis and tumor growth (10). A previous study of gastric cancer has shown that Rsistin and Visfatin increased more than other adipocytokines and their levels gradually increased with stage progression and suggested may be good biomarkers of gastric cancer (11).

In a previous study, we demonstrated that Resistin doesn't express in gastric cancer cells (26) and increased Resistin serum level in gastric cancer patient is due to other cells infiltrate in to cancer tissue and according to the previous study Resistin is a molecule accumulating at the site of inflammation and supports the inflammatory process by triggering cytokine production and NF-κB activation while simultaneously up-regulating its own expression from inflammation cells (27). Therefore Resistin could not be a good biomarker. But Visfatin (Nampt) is expressed endogenously in gastric cancer AGS cell line and increase level in cancer cells as well as serum concentration of Visfatin in patients and correlate significantly with stage progression (28). Therefore is suggested that expression of visfatin in real samples could be a biomarker for gastric cancer and could be used as a potential diagnostic and prognostic tool.

For investigation of Resistin and Visfatin effects on cell viability was performed XTT assay. Exactly, recombinant proteins of Resistin and Visfatin, separately as well as together, caused a proliferation in different concentration on AGS cell line. The maximum effect of Resistin on cell proliferation was 10 ng/ml, but Visfatin and Resistin/Visfatin together was 5 ng/ml. The findings show that Resistin needs to more concentration for effects on a proliferation than Visfatin and Resistin/Visfatin together. In fact, due to Resistin isn't expressed in cell line and it needs to more concentration for apply proliferation effects.

In other previous studies, has been shown Resistin have proliferation effects. For example, Kolosova and *et*

al., have shown Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways (29). Kolosova and *et al*., have shown Resistin-Like Molecule α stimulates proliferation of mesenchymal stem cells (30). Kim and *et al*., have shown Resistin induces prostate cancer cell proliferation through PI3K/Akt signaling pathways (31). Few studies, in human, investigated the association of Resistin and RELMs with gastric cancer. In the previous study performed by Zheng *et al*., was recorded a higher expression of RELMb in intestinal-type compared to diffuse-type gastric carcinomas. In addition, RELMb correlated positively with tumor differentiation and inversely with tumor infiltration, lymph node metastasis and heparanase expression (32).

Also, several studies have shown the role of Visfatin in different cancer (33-37). Recent studies have shown that possible role of Visfatin in the regulation of angiogenesis and tumor growth. In prostate cancer cells, exogenous Visfatin increased proliferate (38). In breast cancer cells, transcription of human Visfatin genes is regulated by hypoxia inducible factor-1, a key factor in malignant tumor progression (39). Also, Kim and *et al*., reported that Visfatin regulates proliferation of MCF-7 human breast cancer cells. Exogenous administration of recombinant Visfatin increased cell proliferation and DNA synthesis rate in MCF-7 cells. Furthermore, Visfatin activated G1-S phase cell cycle progression by upregulation of cyclin D1 and cdk2 expression. Visfatin also increased the expression of matrix metalloproteinases 2, matrix metalloproteinases 9, and vascular endothelial growth factor genes, suggesting that it may function in metastasis and angiogenesis of breast cancer (40). Visfatin stimulates vascular smooth muscle cell (VSMC) proliferation via NMN-mediated ERK1/2 and p38 signaling (41). Zhang and *et al*., reported that APO866, a potent inhibitor of NAMPT, is a potent growth inhibitor against glioblastoma through targeting NAMPT (42). About Visfatin in gastric cancer, a recent study was performed by Nakajima *et al*., was found to correlate significantly with stage progression.

According to the previous findings of the effects of Resistin and Visfatin on proliferation in different cells and the findings of this study, can say Resistin and Visfatin have proliferative effect on AGS cell line. For determination of molecular pathways involved in Resistin and Visfatin induced proliferation of gastric cancer cells, we focus on telomerase activation.

In fact, there are many factors that contribute to gastric carcinogenesis. Currently, telomerase has been a major focus. Telomerase activation is associated with an early stage of gastric carcinogenesis (43,44). Human telomerase reverse transcriptase (hTERT) has been identified as a catalytic subunit of human telomerase. Recent studies have demonstrated correlation between telomerase activity and hTERT expression (45,46).

Telomerase activity in humans has been detected in germline and tumor tissues (47). In somatic cells, the absence or low expression of telomerase is due to the telomeric shortening with each cell division (48). Therefore, reactivation of telomerase is a critical step in tumor growth. Recent studies have demonstrated correlation between telomerase activity and hTERT expression (49,50).

It has been shown that high expression of human telomerase reverse transcriptase (hTERT) is a direct cause of telomerase activity in cancers (51). Therefore, numerous studies have focused on the expression of hTERT in cancer and its application in tumor diagnosis and treatment. Recently, several studies have suggested that the function of hTERT is not limited to the maintenance of telomeres and telomerase activation.

Lee *et al*., reported that hTERT promoted cellular survival independent of telomerase activity (52). For example, hTERT regulates the expression of cyclin D1 (an important cell cycle protein) and vascular endothelial growth factor (VEGF, a key angiogenic factor).

Based on these studies, Visfatin and Resistin in gastric cancer tissue can cooperatively accelerate cancer cell growth via enhancing the telomerase expression as a cancer gene with time dependent manner and co-stimulation with Resistin and Visfatin showed greater inducible effects on cell proliferation and telomerase gene expression.

Our study demonstrates that Visfatin and Resistin induced exogenously gastric cancer cell proliferation and increased telomerase (hTERT) gene expression, as a cancer gene with time dependent manner and have synergistic effects. Therefore, blocking Visfatin and Resistin signaling and limiting Visfatin secretion may be valuable for the treatment of gastric cancer with elevated Visfatin and Resistin levels.

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