RESISTIN EFFECT ON TELOMERASE GENE EXPRESSION IN GASTRIC CANCER CELL LINE AGS

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Abstract

Background. Resistin, as an adipokine, has been shown to be increased in serum plasma of gastric cancer patients and suggested to be a major factor in gastric carcinogenesis. However, it is still not clear how Resistin influences gastric cancer progression. The aim of this study was to evaluate Resistin effect on cell proliferation and expression of telomerase gene in gastric cancer cell line (AGS).

Methods. In this study, the proliferating activity of AGS cells stimulated with Resistin was also evaluated by using 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay and trypan blue staining method. To investigate telomerase gene expression affected by Resistin, total RNA was extracted, cDNA was synthesized and expression of hTERT mRNA was carried out by real-time reverse transcription polymerase chain reaction.

Results. Exogenous Resistin has induced gastric cancer cells proliferation in a dose-dependent manner and could improve cell viability. Also the expression of Human Telomerase Reverse Transcriptase (hTERT) was upregulated in 24 hours, after Resistin treatment.

Conclusions. This study has shown Resistin induces exogenously gastric cancer cell proliferation and increases hTERT gene expression. These findings may clarify the role of Resistin in gastric carcinogenesis. Therefore blocking Resistin signaling and limiting its secretion may be valuable for the treatment of gastric cancer.

Key words: Gastric cancer, Resistin, Telomerase, hTERT, Adipokine.

INTRODUCTION

Gastric adenocarcinoma (GC) is the fourth most common cancer worldwide but the second leading cause of cancer death (1). One of the most important risk factors of gastric cancer is obesity whose prevalence is

increasing especially among children and young adults (2, 3).

Since obesity is accompanied by alteration of serum levels of adipocytokines, hormones derived from adipose tissue, there are many documents focused on associations between malignancies and the hormones. These hormones have been linked to several mechanisms of carcinogenesis, including angiogenesis, cell proliferation, metastasis, and alteration in sex-steroid hormone levels (4-6). One of these adipokines which has recently received a great deal of attention is Resistin. Resistin is a member of family of cysteine-rich proteins called "Resistin-like molecules" (RELMs). Its gene, called Retn mapping to the p13.3 band of chromosome 19 (7), encoded a 114-amino acid polypeptide (8) which is secreted as a disulphide linked homo-dimer (9) and circulates in two distinct assembly states: an abundant high-molecular weight hexamer and a less abundant, but more bioactive, trimer (10). Few studies, in human, investigated the association of resistin and RELMs with gastric cancer. In a previous study Zheng *et al.* 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 [zheng]. Also Nakajima and *et al.* showed that Resistin and Visfatin as adipocyte derived hormones increased more than other adipocytokines and may be good biomarkers of gastric cancer (11). But in a previous study we showed that Resistin is not expressed endogenously in AGS human gastric cancer cells. Therefore Resistin could not be a good biomarker (12, 13).

On the other hand, there are many factors that contribute to gastric carcinogenesis such as telomerase activity that is associated with an early

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stage of stomach carcinogenesis (14-23). Telomerase is a ribonucleoprotein polymerase that adds telomeric sequences, G-rich hexameric sequences (TTAGGG), onto the ends of chromosomes to compensate for the DNA end-replication problem (24). Telomerase activity in humans has been detected in germline and tumor tissues (25). In normal somatic cells, the absence or low expression of telomerase is thought to result in progressive telomeric shortening with each cell division. Therefore, it has been proposed that reactivation of telomerase is a critical step in tumor genesis.

Telomerase is composed of human telomerase RNA component (hTR), human telomerase-associated protein (TEP1), and human telomerase catalytic subunit (hTERT) (26-28). Recent studies have demonstrated a close correlation between telomerase activity and hTERT expression (29, 30). Those have shown that enhanced expression of human telomerase reverse transcriptase (hTERT) is a direct cause of telomerase activity in cancers (31). Therefore, numerous studies have focused on the cancer-specific regulation of hTERT and its application of tumor diagnosis and treatment. Based on these studies, we postulate that Resistin may up-regulate the expression of hTERT and this may mediate the role of Resistin in gastric cancer progression by induction of cancer cell proliferation.

In the present study, we attempted to evaluate the effect of Resistin on the cell viability in AGS cells, and also evaluate the expression of hTERT in response to Resistin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human gastric adenocarcinoma consisting of mucus-secreting epithelial cells was obtained 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, 100ng/mL of streptomycin, 15mM Hepes, 1.2g/L sodium bicarbonate, and 10% fetal bovine serum (Biochorom/Belin). The cells were seeded at the concentration of 7×10^3 cells/well in 96-well culture plates and allowed to attach in an incubator overnight. The complete serum was then replaced with serum-free medium for 24 h to allow for cell cycle synchronization. The medium was then replaced with serum-free medium containing different doses of human recombinant Resistin protein (Syd Labs, Japan) and cells were incubated for 12h, 24h and 48h for cell proliferation assay. For gene expression assay, 7×10^5 cells in 25-T culture flasks were seeded

and incubated overnight. The flask was maintained in incubator at 37°C in a humidified atmosphere with 5% CO₂.

XTT assay

Growing cells were seeded in 96-well plates after 24h incubation at 37°C, incubated with various concentrations of recombinant human Resistin (0, 5, 10, 50, 100, 200ng/mL) for 12h, 24h and 48h. Cell viability was analyzed using the XTT assay kit (BIOTIUM, Inc) according to the manufacturer's instructions. Briefly, add 50µL of the activated XTT solution (Mix 25µL activation reagent with 5mL XTT solution to derive activated XTT solution) to each well and then incubated for 4 hours, 37°C, 5% CO₂. Formazan production by viable cells was assessed at 470 nm with a 96-well plate reader Sunrise (TECAN, Austria).

Cell number counting

After seeded in six-well plates and treated with recombinant human Resistin, (0, 5, 10, 50, 100, 200ng/mL) the cells were harvested at the indicated time points (for 12h, 24h and 48h) and stained with 0.5% Trypan blue. Subsequently, the number of viable cells was counted under a light microscope.

Total RNA extraction

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 was poured in a clean RNase-free tube and was incubated for 5 min at room temperature. After incubation, 200µl 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 incubated 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 260nm. The purity of the RNA was checked by measuring the ratio of the absorbance at 260 and 280nm. 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-GeneTM 6000 system (Corbett Research, Australia) according to the manufacturer's instructions. After cDNA synthesis, specific primers: forward primer 5'-CCGCTGAGCTGTACTTTGT-3', reverse primer 5'-CAGGTGAGCCACGAACTGT-3', used to amplify hTERT mRNA. Alternative spliced variants of hTERT mRNA were not measured because they do not reconstitute telomerase activity (32, 33). The GAPDH mRNA measured as the internal control by specific primers (forward sequence 5'-CAAGGTCATCCATGACAACCTTG-3', GAPDH 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- $\Delta\Delta C_t$ 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 $\pm$ SEM P value <0.05.

Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM) and analyzed using SPSS 20. Differences among groups were analyzed using one-way ANOVA with Dunnett's multiple comparison tests. Student's t-test was used for comparisons between two groups. P value of less than 0.05 was considered to be statistically significant.

RESULTS

Resistin proliferate AGS cell line

After AGS cell incubation with different amount of Resistin for 12, 24 and 48 hours, XTT assay showed that Resistin stimulated gastric cancer cell proliferation in a dose-dependent manner in which the

most effective dosage was 10ng/mL. Also cell count showed that Resistin in this concentration (10ng/mL) had maximum cell viability ($p < 0.05$) (Fig. 1).

Figure 1. Resistin effect on the cell viability. Cell viability performed by XTT assay, after triplicate cell plating, treatment, and incubation. The most effective dose of cell viability of AGS cell line was 10 ng/ml. Data are shown at the mean $\pm$ SEM.

Resistin up-regulate expression of hTERT mRNA

Telomerase (hTERT) gene expression was investigated in order to examine the possible molecular link between Resistin and AGS cell proliferation induction. Real-Time PCR was performed after cell treating with 10ng/mL Resistin for 6, 12 and 24 hours. Telomerase gene expression significantly increased in Resistin-treated AGS cells in comparison with control cells in a time- dependent fashion (Fig.2). After 24 hours mRNA levels of hTERT were increased about 1.6 folds ($p < 0.05$).

Figure 2. Resistin effect on hTERT mRNA in AGS gastric cancer cell line. AGS cells were treated with Resistin at 10 ng/mL and incubated for 6, 12 and 24h to examine telomerase gene expression by real time PCR. Data are represented as the mean $\pm$ SEM.

Data was shown that resistin 10 ng/mL had inhibitory effect on hTERT expression at 6 and 12 hours compared to control, but stimulates hTERT expression at 24 hours that maybe because resistin in early time has inhibitory effect on hTERT expression and after 24 hours its stimulatory effect on hTERT expression starts and need more study in this field.

DISCUSSION

Many studies have shown the relationship between obesity and many malignancies such as gastric cancer. There are different mechanisms considered to mediate the effect of increased BMI and the risk of gastric cancer. The roles of some adipokines which are increased with fat accumulation in carcinogenesis have recently drawn many researchers' attention. Resistin is an adipokine which has been shown to be highly expressed in serum plasma gastric cancer patient and had been suggested to be good biomarker but, in a recent study we showed that Resistin is not expressed endogenously in AGS human gastric cancer cells. Therefore, Resistin could not be a good biomarker (12, 13). Although the mechanisms of association between increased levels of serum Resistin and lack of its expression in gastric cancer cells are not clear, there are some suggestions such as, this increase may be due to the presence of other sources of Resistin such as leukocytes and macrophages and molecules of the RELM family that were found in the inflamed tissues (34).

In fact Resistin accumulates at the site of inflammation and supports the inflammatory process by triggering cytokine production and NF- κ B activation

while simultaneously up-regulating its own expression. Also pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) increase the expression of Resistin in human PBMC in humans peripheral blood mononuclear cells (PBMC) that seem to be a major source of Resistin (35).

Several studies have shown that resistin has proliferation effects. For example, Kolosova *et al.* have shown resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways (36). Kolosova *et al.* have shown Resistin-Like Molecule α stimulates proliferation of mesenchymal stem cells (37). Kim *et al.* have shown resistin induces prostate cancer cell proliferation through PI3K/Akt signaling pathways (38). Few studies, in human, investigated the association of resistin and RELMs with gastric cancer. In a previous study performed by Zheng *et al.*, a higher expression of RELMb was recorded 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 (39). About resistin, in a recent study performed by Nakajima *et al.*, it was found to correlate significantly with stage progression. In this study, we showed that resistin has a proliferation effect.

There is little evidence about Resistin effect on telomerase expression. Therefore, we evaluated hTERT mRNA level in AGS cells after treating with resistin for 6, 12, and 24 h and observed that this adipokine could enhance hTERT expression after 24 h. This result suggests that enhancement of telomerase gene expression might be the molecular linkage between Resistin and gastric cancer cell progression and malignant phenotype. However, more *in vivo* investigations are necessary to confirm the Resistin role in gastric cancer.

On the other hand, recently, several studies suggest 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 (40). For example, hTERT regulates the expression of cyclinD1 (an important cell cycle protein) and vascular endothelial growth factor (VEGF, a key angiogenic factor). With this finding and our study results, we could conclude that Resistin could directly up-regulate hTERT transcription and may promote cellular survival molecules and pathways. Therefore locking Resistin signaling and limiting Resistin secretion may be valuable for the treatment of gastric cancer with elevated Resistin levels.

Conflict of interest

The authors declare no conflict of interests.

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