AENSI Journals

Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/aeb.html

Visfatin Proliferative Effect on Hct-116 Colorectal Cancer Cell Line

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ARTICLE INFO

Article history:
Article History
Received 25 March 2014
Received in revised form 20 April 2014
Accepted 15 May 2014
Available online 5 June 2014

Key words:
Visfatin, Telomerase gene expression, colorectal cancer

ABSTRACT

Objective(s): Visfatin, a recently discovered adipokine, has been shown to be increased in serum plasma of colorectal cancer patients and suggested to be a major factor in colorectal carcinogenesis. It has also been demonstrated that Visfatin has a higher expression in cancerous tissue than normal. However, it is still not clear how Visfatin influences colorectal cancer progression. The aim of this study was to evaluate Visfatin effect on cell proliferation and expression of telomerase gene in colorectal cancer cell line (HCT-116). Materials and Methods: HCT-116 cells were treated with escalating dosage of Visfatin over the duration of 24 and 48 hours. Then in order to determine the cell proliferation rate, XTT assay was performed. After treating the cancer cells with Visfatin, telomerase gene expression alteration was assessed by Real-Time PCR, in order to investigate the fundamental mechanism of Visfatin effect on cancer cell proliferation. Results: Visfatin significantly induced colorectal cancer cell proliferation and viability in a dose-dependent manner ($p< 0.05$). Telomerase gene expression was increased in Vifatin treated cancer cells after 24 hours. Conclusion: These data revealed that in colorectal cancer tissue, Visfatin can promote cancer cell proliferation via enhancing the cancer gene, telomerase, expression in an autocrine manner. Therefore Visfatin signaling blockage and its secretion restriction seem to be useful in treatment of colorectal cancer with elevated Visfatin levels.

To Cite This Article: Sara Ghaemmaghami, Mehdi Hedayati, Seyed Mojtaba Mohaddes, Masumeh Gorgian Mohammadi, Mohammad Rahmati, Nosratollah Zarghami, Visfatin proliferative effect on HCT-116 colorectal cancer cell line. ***Adv. Environ. Biol., 8(9),*** *55-60, 2014*

INTRODUCTION

Globally, colorectal cancer (CRC) is the third most common malignancies and the third most common cause of cancer death after prostate and lung cancer among men and after breast and lung cancer among women. Yet this type of cancer is still known as one of the most difficult medical challenges to manage. [1]

One of the most important risk factors of CRC is obesity whose prevalence is increasing especially among children and young adults.[2]

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. One of these adipokines which has recently received a great deal of attention is Visfatin/PBEF/NAMPT. This protein is described as a 52-kDa secreted molecule termed pre-B cell enhancing factor (PBEF) which enhanced the effect of IL-7 and stem cell factor on pre-B cell colony formation.[3] It is also called NAMPT because of its significant sequence and functional homology with nicotinamide phosphoribosyl transferase (NAMPRTase), a rate-limiting enzyme involved in nicotinamide adenine dinucleotide (NAD) biosynthesis from nicotinamide.[4] Previous studies have reflected high visfatin expression in several malignancies including breast, prostate, endometrium, glioblastoma and gastric.[5-9] . Recent investigation has shown that Visfatin plasma levels are increased in colorectal cancer patients independently from BMI (Body Mass Index) and are significantly correlated with cancer stage progression.[10-12]

Adipose tissue and peripheral blood lymphocytes are not the only sources of Visfatin expression but bone marrow neutrophils, peripheral blood granulocytes (PBG), liver, muscle tissue, brain, kidney, lung, myometrium, placenta, all layers of human fetal membrane and human amniotic epithelial cells also can secrete this hormone.[13] Visfatin can also be expressed by gastric and colorectal cancer cells and participates in

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colorectal carcinogenesis predominantly in an autocrine manner.[14, 15] However, its possible effects on colorectal cancer progression and the underlying mechanism are still unknown.

Telomerase gene which is responsible for telomere elongation and consequently cellular immortalization, has been reported to be highly expressed in many cancers namely CRC and increased along with cancer progression. [16, 17] It has been also indicated that enhanced human telomerase reverse transcriptase (hTERT) expression is a direct determinant of telomerase activity.[18] Therefore, numerous studies are focused on the cancer-specific regulation of hTERT and its application in tumor diagnosis and treatment. Based on these studies, we postulate that Visfatin may upregulate the expression of hTERT and this may mediate the role of Visfatin in colorectal cancer progression by induction of cancer cell proliferation.

MATERIALS AND METHODS

Cell culture, reagents and treatments: Human HCT-116 colorectal cancer epithelial cells were purchased from National cell bank of Iran (NCBI) and maintained in high glucose DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Biochem, Germany), penicillin/streptomycin (100U/ml and 100mg/ml, respectively) at 37˚C, with 5% CO_2 atmosphere. Cells were seeded at 5×10^3 cell/well in a 96 well culture plates and incubated overnight in order to adhere to the bottom of culture plate in an incubator. Then to achieve cell cycle synchronization, the complete serum was replaced with serum-free medium for 24 hours. The medium was then changed with serum-free medium containing various doses of human recombinant Visfatin (Syd Labs, Japan) keeping the pathophysiologic range of Visfatin levels,[10, 19, 20] 5, 10, 50, 100, and 200ng/ml, with incubation over the period of 24 and 48 hours.

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 on cell proliferation and incubated for 6, 12 and 24 hours.

Cell Proliferation Assay:

HCT-116 colorectal cancer cell proliferation was evaluated in triplicate by a colorimetric assay which measures the metabolic conversion by mitochondrial dehydrogenases of a water-soluble tetrazolium salt, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Biotium, USA) into formazan, an orange product that is soluble in cell culture media. The amount of formazan produced is directly proportional to the number of live cells. After seeding and growing cells in 96-well culture plates and treating with aforementioned doses of human recombinant Visfatin for 24 and 48 hours, the assays were performed according to the manufacture's instruction by adding 50µl activated XTT reagent and Phenazine Methosulfate (PMS) as electron coupling reagent per well. Then cells were incubated for 4 hours at 37˚C, under 5% CO_2 atmosphere. Foramazan produced by viable cells was measured at 510 nm with a 96-well plate reader Sunrise (TECAN, Austria). Each experimental set was performed for three times, n=3.

RNA Extraction, CDNA Synthesis:

Total RNA was isolated from the cultured cells using RNX plus solution (Cinna Gen INC, IRI). Shortly, after harvesting the cells by treatment of trypsin/ EDTA and centrifugation at 1000g for 10min at 4°C, 1 ml ice cold the guanidine/ phenol solution (RNX-Plus) was added to a 2ml tube containing cell pellet and incubated at room temperature for 5 minutes. Then, 200µl of chloroform was added and shaked vigorously for 15 seconds and incubated on ice for 5min. The mixture was centrifuged at 12000 g for 15 minutes. The aqueous phase was transferred to a sterile RNase-free tube. The total RNA was precipitated by adding 0.5ml isopropyl alcohol and incubating for 15 minutes at room temperature. The pellet including total RNA was washed using 75% ethanol and centrifuged at 7500g for 8 minutes. After the ethanol was dried, the RNA pellet was dissolved in DEPC treated water. Yield and purity of RNA were assessed by spectrophotometric analysis. To confirm the absence of RNA degradation, RNA electrophoresis was done on a 1.5% agarose gel containing ethidium bromide.

Total RNA (3µg) from each sample was subjected to reverse transcription by the First strand cDNA synthesis kit (Fermentas, Lithuania) on the basis of the manufacture's instruction using random hexamer primers.

Real-Time PCR:

The cDNA of the treated cells was amplified by Real-Time PCR using Maxima SYBR Green/ROXqpcr Master Mix (Fermentas, Vilnius, Lithuania) by the Rotor-GeneTM 6000 system (Corbett Research, Australia) according to the manufacturer's instructions and follow primers: forward 5′-CCGCCTGAGCTGTACTTTGT-3′, reverse 5′-CAGGTGAGCCACGAACTGT-3′ for hTERT gene and forward 5′-CAAGGTCATCCATGACAACTTTG-3′ and reverse primer 5′-GTCCACCACCCTGTTGCTGTAG-3′ for GAPDH primer as house keeping gene. The Real-Time PCR

reactions (25µl total volume, containing 5pmol primer, 12.5µl SYBR green master mix and 2.5µl c DNA) were performed under the following conditions: initial denaturation 95°C for 10 minuets, denaturation 95°C for 15 seconds, annealing 60°C for 30 seconds and extension 72°C for 30seconds for about 40 cycles. Finally, amplicons were assessed by melting curve analysis of 70°C to 95°C. The quality of Real-Time PCR reactions was controlled by running standard samples as triplicate.

Statistical Analysis:

All data are presented as mean±SD. The differences among the groups were analyzed using one-way ANOVA with Dennett's multiple comparison tests. Student t-test was used for comparison between two groups. A p-value<0.05 was considered as significant. The SPSS 15.0 statistical software was used for the calculations.

Results:

Visfatin stimulates HCT-116 cell proliferation:

After HCT-116 cell incubation with different doses for period of 24 and 48 hours, XTT assay showed that Visfatin stimulated colorectal cancer cell proliferation in a dose-dependent manner in which the most effective dose was 10ng/ml ($p<0.05$) (Fig.1).

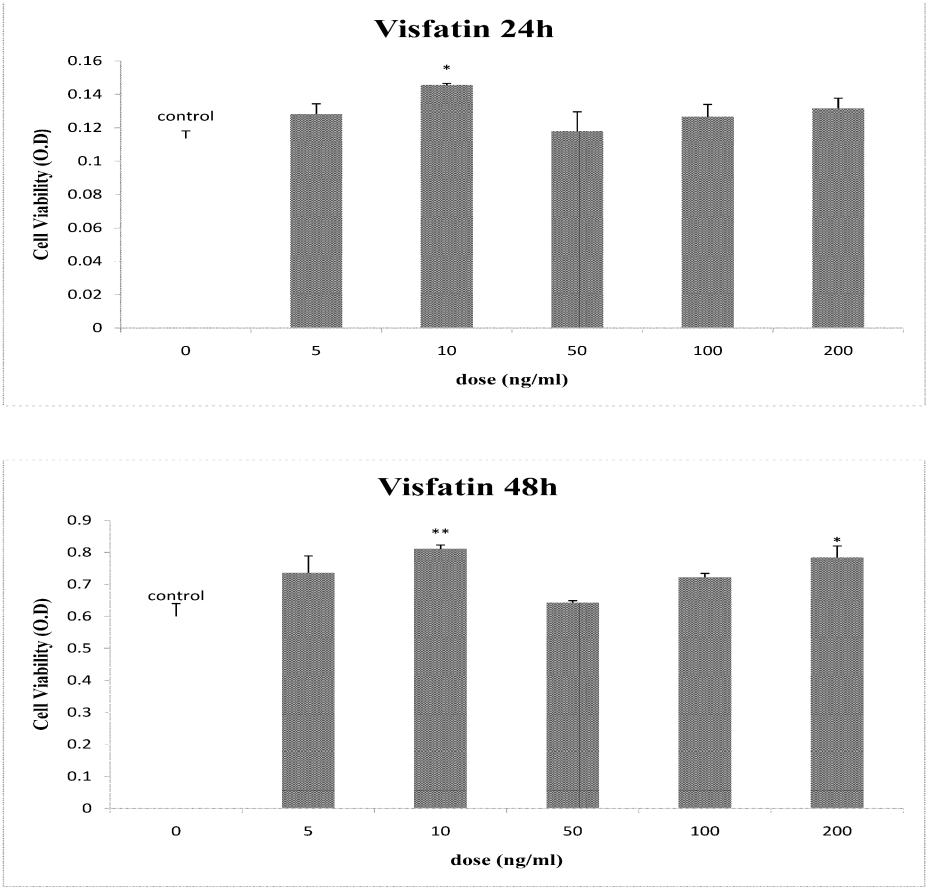

Fig. 1: Visfatin effect on colorectal cancer cell proliferation in HCT-116, all values are expressed as the mean±SEM of three wells per group. *P<0.05; **P<0.01 for Visfatin vs. control.

Visfatin Upregulates hTERT MRNA:

Telomerase (hTERT) gene expression was investigated in order to examine the possible molecular link between Visfatin and HCT-116 cell proliferation induction. Real-Time PCR was performed after cell treating with 10ng/ml Visfatin for 6, 12 and 24 hours. Telomerase gene expression significantly increased in Visfatin-treated HCT-116 cells in comparison with control cells in a time- dependent fashion (Fig.2). After 24 hours mRNA levels of hTERT were increased about 8 folds ($p<0.05$). However, enhancement of hTERT mRNA levels after 6 and 12 hours were not significant.

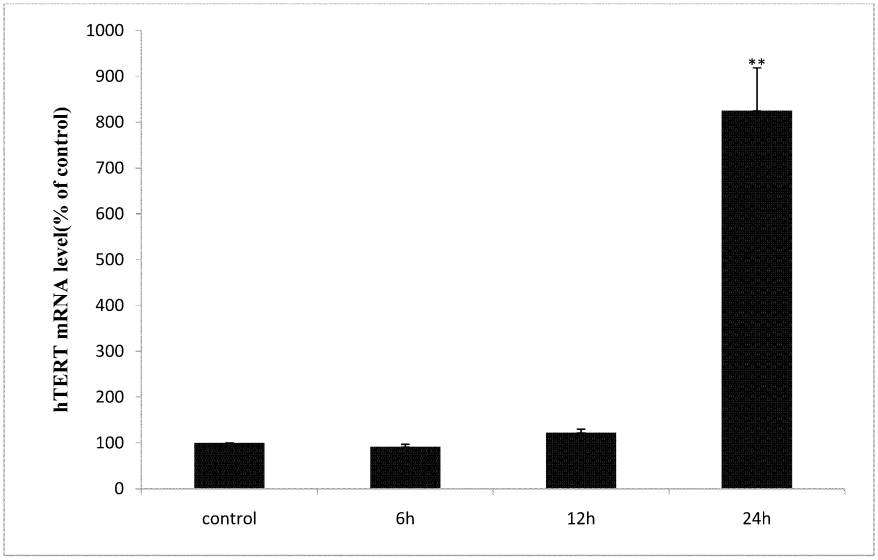

Fig. 2: Visfatin effect on telomerase gene expression in HCT-116 colorectal cancer cell line: Three independent experiments were done. The data are presented as the mean±SEM.

Discussion:

Many studies have shown the relationship between obesity and many malignancies such as colorectal cancer. There are different mechanisms considered to mediate the effect of increased BMI and the risk of colorectal cancer including insulin pathways, insulin-like growth factor (IGF) and phosphoinositide3-kinase/AKT pathways which are most known pathways altered in epithelial tumors.[11, 12, 21, 22] The roles of some adipokines which are increased with fat accumulation in carcinogenesis have recently drawn many researchers' attentions.[23-25] Visfatin is a relatively new identified adipokine which has been shown to be highly expressed in obesity.[26] It has also been well known to be involved in early B cell lines proliferation.[3] The biological role of Visfatin has not been entirely understood. However, several studies have indicated glucose lowering and insulin-mimicking effects of visfatin through binding to insulin receptor and downstream signaling kinase pathways. On the other hand, Visfatin has been shown to act as a growth factor and promote vascular smooth muscle cell maturation and proliferation through ERK1/2 and P38 signaling pathways mediated by NMN in a dose and time dependent manner.[4, 27, 28] Recent studies have revealed an association between Visfatin expression and many malignancies including breast, prostate, endometrium, glioblastoma and gastric cancer.[5-9] Moreover, there are documents demonstrating that Visfatin has a role in carcinogenesis of malignancies by tumor invasion and angiogenesis via elevation the matrix metalloproteinase 2/9 and VEGF expression.[29] Exogenous administration of Visfatin also stimulates cell proliferation in MCF-7 breast cancer cells and PC3 prostate cancer cells via ERK-1/2 and p38 activation.[7, 30] Recently Nakajima and his colleagues have reported a significant increase in Visfatin serum levels of CRC patients compared with the control group. Furthermore, HCT-116 colorectal and AGS gastric cancer cell lines have been found to secrete this hormone abundantly.[14, 15] Visfatin over expression in colorectal cancer tissue was first reported by Hufton and his colleagues in 1999.[31] All these earlier data suggest that Visfatin may be involved in CRC carcinogenesis. Conformable to aforementioned studies our results showed that Visfatin can promote colorectal cancer progression by increasing cancer cell proliferation.

Telomerase, a ribonucleotide complex, synthesizes DNA onto the ends of chromosomes and provides the genomic integrity and stability. The two essential genes constituting the cellular telomerase complex are including TERC and TERT. Interestingly only hTERT is sufficient to activate telomerase activity and bypass senescence and lead to cell immortalization. Therefore in many cancers including colorectal cancer, telomerase is up-regulated or reactivated and considered as a potential target for treatment of this disease.[32, 33] Furthermore, hTERT expression increases replicative potential of cancer cells and promotes cell growth in adverse conditions therefore it may act as an anti-apoptotic agent. Previous studies have shown that hTERT expression and/or telomerase activity is higher in CRCs than in adjacent noncancerous mucosa and increases along with cancer progression. More over, high level of hTERT has been recently shown as a prognostic marker of shorter overall survival independent of disease stage and Dukes' classification in patients with CRC.[34]

In a previous study, leptin as an adipokine related to obesity has been demonstrated to up regulate telomerase activity and hTERT mRNA in MCF-7 breast cancer cells,[35] while there is little evidence about Visfatin effect on telomerase expression. As shown in the Fig.2, this hormone enhanced hTERT expression after

24 hours. This result suggests that telomerase might be the molecular link between Visfatin and colorectal cancer cell progression and malignant phenotype. On the other hand, based on clinical studies on cancer patients, some primary malignancies and most metastatic tumors undergo a period of dormancy prior to entering the stage of progressive growth. While telomerase-negative cells may provide information on cell dormancy, telomerase-positive cells might predict early disease recurrence.[36] Therefore, it can be suggested that Visfatin may lead cancer cell progression from cell dormancy toward next pathologic stages.

Previous investigations have demonstrated that cells with Visfatin-overexpression are substantially more resistant to apoptosis induced by many chemotherapeutic factors including etoposide and methylmethane sulfonate (MMS). In addition, visfatin-knocked down cells are more sensitive to camptothecan and topoisomerase I inhibitor.[29] Moreover, many attempts have done to lower Visfatin level in over weight and obese people.[37]

Since the present study revealed the inducible role of Visftain in HCT-116 cancer cell line progression; targeting this adipokine may be an effective therapeutic strategy for CRC. There are two pharmacological Visfatin inhibitors including: FK866/APO866 and CHS828/GMX1777, which evaluated in broad variety of solid tumors such as renal cell carcinoma, human liver carcinoma, neuroendocrine tumors, midgut carcinoid, pancreatic carcinoid, and many lymphomas.[29] Further studies are required to evaluate the possible beneficial impacts of Visfatin inhibitors in CRC treatment.

In conclusion Visfatin can contribute to the colorectal cancer development by cell proliferation induction and elevating the telomerase gene expressions in cancer cells and promote them into more aggressive phenotype. However, more in vivo investigations are needed to verify the role of Visfatin in colorectal cancer. On the other hand, in the current study Visfatin effect was examined only on a cancer cell while its function might be cell specific and different on other cancer cells. Nevertheless, Visfatin signaling blockage and restriction of its secretion may be valuable in the therapy of colorectal cancer. Therefore, Visfatin specific inhibitors are recommended to be used for CRC treatments in order to block cell proliferation or as sensitizer for chemotherapy.

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