JCEI /
Journal of Clinical and Experimental Investigations

2014; 5 (2): 226-232
doi: 10.5799/ahinjs.01.2014.02.0393

ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

ADAMTS13 expression in human chondrosarcoma cells induced by insulin

Insulin tarafından indüklenmiş insan kondrosarkom hücrelerinde ADAMTS13 ekspresyonu

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ABSTRACT

Objective: A Disintegrin-like Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteins is a proteinase enzyme group that primarily located in the extracellular matrix (ECM). Insulin has been known to stimulate proteoglycan biosynthesis in chondrosarcoma chondrocytes and thereby the levels of ADAMTS proteins. The aim of this study is to evaluate the time-dependent effects of insulin on the ADAMTS13 expression in OUMS-27 human chondrosarcoma cell line to test the hypothesis that insulin diminishes ADAMTS13 expression because of its anabolic effects.

Methods: To test this hypothesis OUMS-27 cells were cultured in Dulbecco's modified Eagle' medium (DMEM) containing 10µg/mL insulin. The medium containing insulin was changed every other day up to 11th day. Cells were harvested at 1, 3, 7, and 11th days and protein and RNA isolations were performed at the proper times. The levels of RNA expression of ADAMTS13 was quantified by qRT-PCR using appropriate primers while protein levels was detected by Western blot technique using anti-ADAMTS13 antibody.

Results: Although there was a decrease in both RNA and protein levels in insulin-applied groups compared to the control cells, it was not statistically significant.

Conclusion: Under the light of our findings, it is suggested that insulin does not participate in regulation of ADAMTS13 in OUMS-27 chondrosarcoma cells. *J Clin Exp Invest 2014; 5 (2): 226-232*

Key words: Insulin; ADAMTS13, chondrosarcoma, OUMS-27, RNA

ÖZET

Amaç: A Disintegrin-like Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteinleri esas olarak ekstraselüler matrikste (ECM) bulunan bir ptoteinaz enzim grubudur. İnsülinin kondrosarkom kondrositlerinde proteoglikan sentezini stimüle ettiği uzun yıllardır bilinmekteydi. Dolayısıyla bu çalışmanın amacı insülinin, OUMS-27 insan kondrosarkom hücre kültüründeki zaman bağımlı etkilerini değerlendirmek ve insülinin anabolik etkilerinden dolayı ADAMTS13 ekspresyonunu azaltma ihtimali hipotezini test etmektir.

Yöntemler: Bu hipotezi test etmek için OUMS-27 hücrelerinin, 10µg/mL insulin içeren Dulbecco's modified Eagle besiyerinde (DMEM) kültürü yapıldı. 11. güne kadar iki günde bir besiyeri ile birlikte insülin takviyesi yapıldı. Hücreler 1, 3, 7 ve 11. günlerde harvest edilerek uygun zamanlarda protein ve RNA izolasyonu yapıldı. ADAMTS13 RNA ekspresyonu uygun primerler kullanılarak qRT-PCR yöntemi ile ölçüldü ve anti-ADAMTS13 antikorları kullanılarak Western blot yöntemi ile protein miktarı ölçüldü.

Bulgular: Kontrol grubu ile karşılaştırıldığında insülin uygulanan hücre gruplarında, hem RNA hem de protein miktarı azaldı ancak bu istatistiksel olarak anlamlı değildi.

Sonuç: Bu bulgular ışığında insülinin OUMS-27 kondrosarkom hücrelerinde ADAMTS13 regülasyonuna pozitif ya da negative herhangi bir etki yapmadığı sonucu ortaya çıkmıştır.

Anahtar kelimeler: İnsulin, ADAMTS13, Kondrosarkom, OUMS-27, RNA

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Received: 13.05.2014, Accepted: 16.06.2014

INTRODUCTION

Chondrosarcoma is a cancer composed of cells that produce cartilage. It is the third frequent primary cancer of bone. Most of the time these tumors are not aggressive and metastasize is not common, and they have good prognosis after surgery [1]. Its incidence increases after fourth decade. They synthesize and secrete some of A Disintegrin-like Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteins. This proteinase enzyme group is primarily located in the extracellular matrix (ECM). It is a member of Matrix Metalloproteinase (MMP) family in the ECM that breaks substrates such as agrecan, versican, brevican, nidogen, and procollagen. It has 19 members and different functions have been identified for each member. They are involved in many physiological processes including ECM turnover, coagulation, angiogenesis and ovulation, as well as pathological processes such as arthritis, atherosclerosis, and cancer.

ADAMTS13, popularly known as von Willebrand factor cleaving protease (vWFCP), does not belong to any other sub-groups and is one member of the ADAMTS family [2]. ADAMTS13 is primarily produced and released from hepatic stellate cells [3-5], and endothelial cells [6,7].

ADAMTS13 is an important protein/enzyme and has a therapeutic interest because it is the target gene in thrombotic thrombocytopenic purpura (TTP) [8]. Pathological processes such as angiogenesis is also a topic of interest. Because ADAMTS13 is expressed in endothelial cells it is thought it has an important role in angiogenesis. In various studies, it has been suggested that ADAMTS13 inhibit vascular endothelial growth factor (VEGF) mediated angiogenesis [9], and ADAMTS13 activity is decreased at malignant patients [10]. Additionally, abnormal ADAMTS13 production is shown in obese patients [11]. In another study, it is concluded that, ADAMTS13 might be associated with diabetic nephropathy [12]. Hugenholtz et al. showed ADAMTS13 levels were decreased at acute liver failure [13]. Studies have demonstrated that reduced plasma ADAMTS13 activity and increased plasma VWF are risk factors for the occurrence of myocardial infarction [14-16], ischemic stroke [17-19], and preeclampsia [20].

Insulin is an anabolic hormone that stimulates the uptake of glucose to the muscle and fat cells, boots the conversion of glucose to glycogen or fat for storage, inhibits glucose production by the liver, prompts protein synthesis, and inhibits protein breakdown [21]. Metabolic effects of insulin are well known but the molecular mechanism of insulin action in many cells is not fully understood [22,23]. For its action, binding of insulin to specific receptors in the plasma membrane is accepted as the first step. Using anti-insulin-receptor antibodies, it was found that insulin stimulates proteoglycan synthesis via the insulin receptor [24]. One of preceding papers also demonstrated that insulin stimulates proteoglycan biosynthesis in rat chondrosarcoma chondrocytes [25].

Generally, ADAMTS show a proteoglycan degrading effect. Because ADAMTS13 is a member of ADAMTS family, we hypothesized that insulin induction to chondrocytes might decrease the RNA and protein amount of ADAMTS13. Therefore, the goal of this study was to evaluate the time-dependent effects of insulin on the chondrogenic ADAMTS13 expression to test the hypothesis that insulin act in a prohibitive manner to prevent ADAMTS13 expression.

METHODS

OUMS-27 cell culture: OUMS-27 chondrosarcoma cells were kindly provided by Dr. T. Kunisada (Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured at split ratios of 1:2-1:4 using trypsin plus EDTA every 7-10 days. Cells were used at passages 7-14 for all experiments. The medium was changed every other day with either control media or control media supplemented with 10 µg/mL insulin for a total of 11 days. Four groups of cells (two of each group, one for qRT-PCR and one for Western blot analysis) were subjected to insulin: For 1st day experiment, $2x10^5$ cells, for 3rd day experiment $1x10^5$ cells, for 7th day experiment $5x10^4$ cells, and $3x10^4$ cells were plated in 20-mm dishes and exposed to the different concentrations of insulin at the days indicated. After the experiment, cells were harvested and total RNA and protein isolation were made.

Total RNA isolation

Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Two microgram RNA were reverse transcribed with Rever Tra Ace (Thermo) and random hexamers (Thermo) with random primers according to the manufactur-

er's instruction (Table 1). Human GAPDH was amplified as a control for the PCR reaction. Samples lacking reverse transcriptase were amplified as a control for genomic DNA contamination. RNase-free water was used to elute total RNA from each sample. UV spectrophotometry was used to quantify and determine the purity of each sample. Samples required a 260/280 ratio of 2.0 and a 260/230 ratio of 1.7 for adequate purity.

Real-time PCR

qRT-PCR was performed on cDNA samples obtained (Qiagen) as described in our previous report [26]. Total RNA RT-PCR section uses the intercalating dye SYBR green (Qiagen) in the presence of primer pairs. The PCR mixture consisted of SYBR Green PCR Master Mix, which includes DNA polymerase, SYBR Green I Dye, dNTPs including dUTP, PCR buffer, 10 pmol forward and reverse primers and cDNA of samples in a total volume of 20 µL. The amplification of a housekeeping gene, GAPDH, was used for normalizing the efficiency of cDNA synthesis and the amount of RNA applied. PCR was performed with initial denaturation at 95 °C for 5min, followed by amplification for 40 cycles, each cycle consisting of denaturation at 95 °C for 10 s, annealing at 57 °C for 30 s, polymerization at 72 °C for 30 s and, the last stage, polymerization at 72 °C for 5 min. The results pertaining to ADAMTS13 were represented as graphics. The bars and error bars represent mean and standard deviation, respectively.

Protein Extraction, Western Blot Analysis, and Antibodies

After stimulation, the cells were washed once with PBS and then scraped from the plates. Cells were solubilized in 300 µL of CelLytic TMM (Sigma) with a protease inhibitor mixture. After incubation in a rotator at 4 °C for 15 min, the samples were centrifuged, and the supernatants were collected. The protein concentration of the cell extracts was determined using a protein assay kit (Thermo Scientific Bradford Assay Kit) and standard bovine serum albumin. Protein samples were boiled at 95 °C within Laemli Sample buffer and β-merkaptoethanol for 8 min. Ten micrograms of total protein were used for Western blot analysis. Briefly, 10 µL of each sample including protein marker (BioRad Precision Plus Protein Western C Standard) were loaded to Western blot gel (BioRad Mini-PROTEAN TGX Stain-Free Gels, 4–15%, 15-well comb, 15 µL) within BioRad 1X Tris/Glycerine/SDS running buffer and run at 250 V for 20 min. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (BioRad Trans-Blot Turbo Transfer Pack, 0.22 µM PVDF) by using transfer system (BioRad Trans-Blot Turbo Transfer System, Singapore). Membranes were blocked for 1 h in 2.5% nonfat dried skim milk in TBS containing 0.05% Tween 20 (TBST). The membranes were incubated overnight (16 hours) with primary anti-ADAMTS13 and anti-GAPDH antibodies (Table 2) diluted in blocking buffer. After stringent washing with TBST three times for 8 min each at room temperature, the membranes were incubated 1 hour with the appropriate secondary antibodies (Table 2). Following three successive washes with TBST, immunoreactive bands were visualized using the enhanced chemiluminescence system (BioRad Immun-Star Western C kit) for 90 second. Signals were detected with BioRad ChemiDoc MP Imaging System (Singapore), and the densitometry was performed with Image J software (W. Rasband, Research Services Branch, NIMH, National Institutes of Health, Bethesda,MD) and normalized to the signal intensity of GAPDH for equal protein loading control of each sample in each experiment. This quantification was performed with the linear range of the standard curve defined by the standard sample, GAPDH, for all densitometry analysis.

Table 1. The forward and reverse primers used in the real-time polymerase chain reaction analyses for ADAMTS13 and GAPDH

ADAMTS13	Forward	TGCACCTGGTGAAGATGGTC	120 bp product
	Reverse	CGTCCTCAGGGTTGATGGTC	
GAPDH	Forward	CCTGCACCACCAACTGCTTA	108 bp product
	Reverse	TCTTCTGGGTGGCAGTGATG	

Table 2. Primary and secondary antibodies used for Western blot techniques

Primary Ab	Primary Ab concentration	Secondary Ab	Secondary Ab concentration	Reaction	kDa
ADAMTS13	1/1000	Goat	1/4000	Mouse, rat, human	85-110
GAPDH	1/10000-1/50000	Rabbit	1/4000	Human	36

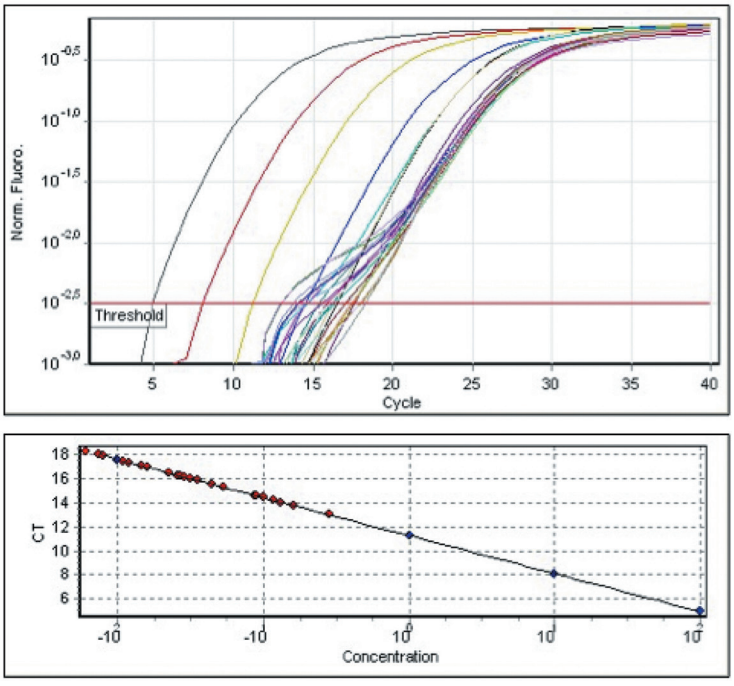

Figure 1. qRT-PCR cycle quantitation and standard concentration graphics of ADAMTS13

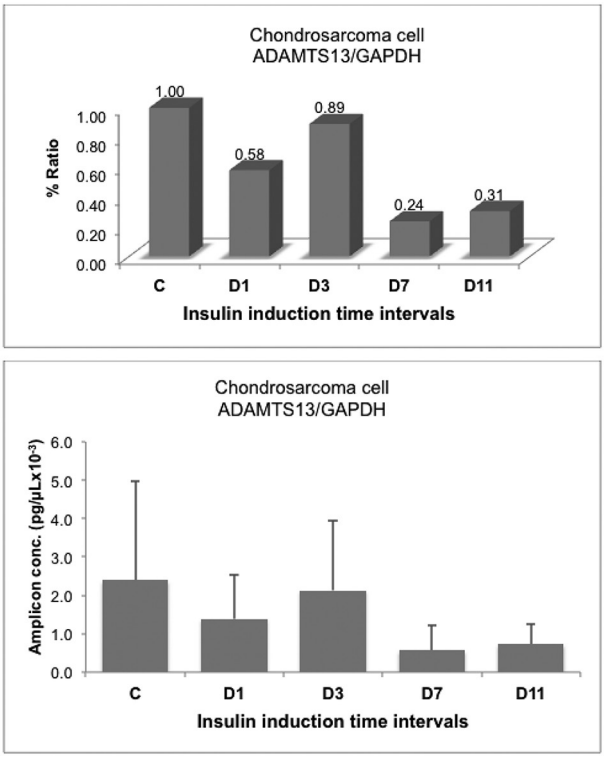

Figure 2. The results of ADAMTS13 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS13 to GAPDH. There is no statistically significant difference between the groups.

Statistical analyses

Statistical Package for Social Studies version 16.0 was used for all statistical tests. Nonparametric Kruskal Wallis test were applied. The relationships between the variables were tested by Mann-Whitney U test. $p<0.05$ was accepted as significant.

RESULTS

Expression levels of ADAMTS13 in insulin-induced OUMS-27 chondrosarcoma cell lines were analyzed by using qRT-PCR and Western blot techniques. The results were represented as graphics. In qRT-PCR results, the bars in the graphics represent the means of ADAMTS13 expression (as ADAMTS13/GAPDH ratio) and the error bars represent the standard deviation of means. qRT-PCR cycle quantitation and standard concentration graphics of ADAMTS13 can be shown in Figure 1. There was difference between control cells and insulin-induced cells in terms of ADAMTS13/GAPDH ratio (Figure 2) but it was not statistically significant. Two protein bands for ADAMTS13 were detected in Western blot analysis: 115 kDa and 50 kDa (Figure 3). According to the first band, there was a sharp decrease (approximately 2-fold) in protein amount of ADAMTS13 at the day 1 after insulin induction. We can see this decrease in band 2 as well (about %25 decrease in protein levels). When the protein levels in con-

trol group was adjusted to the value '1', the protein values were found to be 0.83, 0.75, and 0.83 in the days of insulin induction 3, 7, and 11, respectively, in the first band (115 kDa) detected. According to the second 50 kDa band, there was a sharp decrease at 1st day of insulin induction (about 25%), but after that, there was no decrease at 3th day, but a gradual increase up to 50% at 11th day.

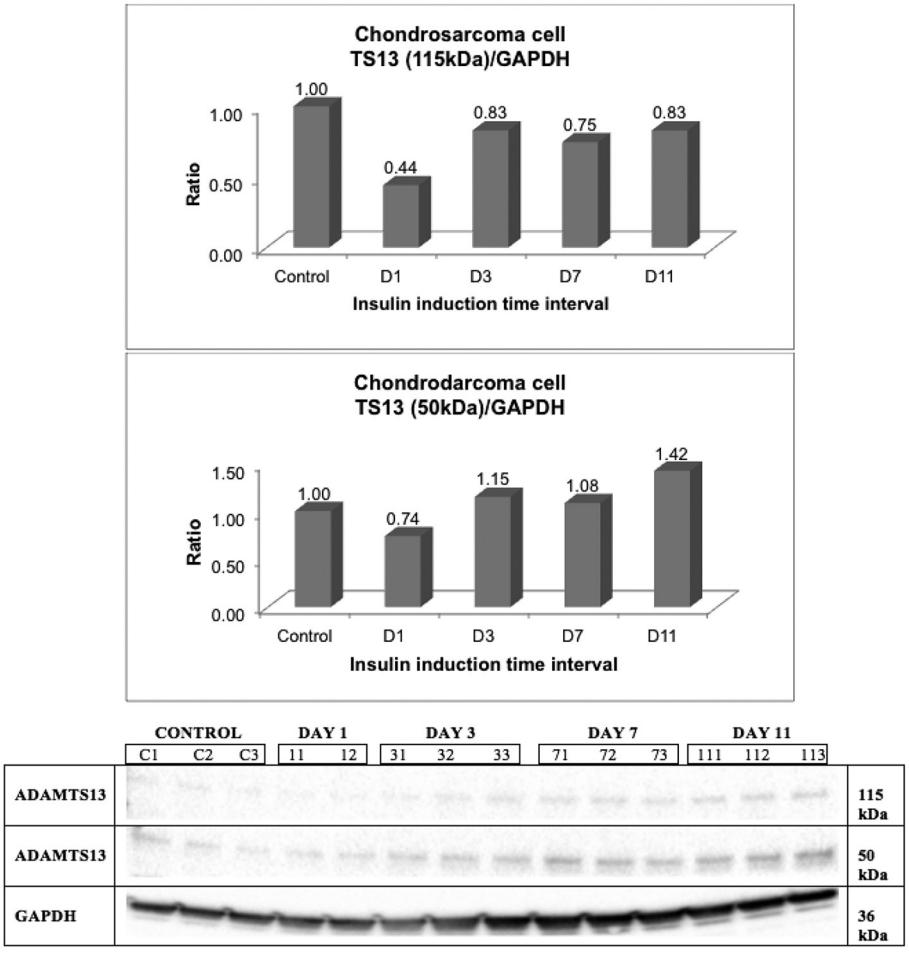

Figure 3. Immunoreactive protein bands detected by Western blot technique of ADAMTS13 and GAPDH. The bar graphics of calculated band densities of both two ADAMTS13 bands and GAPDH. The obtained values were standardized by division of ADAMTS13 to GAPDH

DISCUSSION

There were only a few studies in the literature about the effect of insulin on the human chondrosarcoma cell lines. Moreover, there has been no study on the expression levels of ADAMTS13 in insulin-induced OUMS-27 chondrosarcoma cells, even in normal chondrocytes to detect the RNA and/or protein levels of this enzyme up to now. This report is the first one that shows the expression of ADAMTS13 in both chondrocytes from human chondrosarcoma and insulin-induced chondrosarcoma cells. According to the results of the present study, there was no significant difference between control chondrosarcoma cells and insulin-induced chondrosarcoma cells in terms of RNA and protein levels of ADAMTS13.

The studies on ADAMTS13 were focused mostly on the processing of von Willebrant factor by this ADAMTS enzyme. At the first time, vascular endothelial-produced von Willebrant factor was found to be cleaved and degraded into smaller parts by a specific plasma protease [27]. Severe deficiency of plasma ADAMTS13 activity results in thrombotic thrombocytopenic purpura while mild to moderate deficiencies of plasma ADAMTS13 activity are emerging risk factor for developing myocardial and cerebral infarction, pre-eclampsia, and malignant malaria [28]. Lately, systemic administration of ADAMTS13 (20 min after surgery, 100 µg per 10 gram body weight) was found to reduce neuronal injury after experimental subarachnoid hemorrhage (SAH) by reducing microthrombosis formation and neuronal inflammation, thereby providing a new option for

mitigating the severity of neuronal injury after SAH [29]. There are several studies focused on the effect of ADAMTS13 in organ injuries/deficiencies rather than blood coagulation disorders. ADAMTS13 was found to have a critical role in the central nervous system, particularly after neuronal injuries, because of the fact that ADAMTS13 was expressed in cultured astrocytes and microglia but not in neurons [30]. ADAMTS13 deficiency due to mutation in the gene encoding for ADAMTS13 was identified as the cause of history of recurrent acute renal failure associated with microangiopathic hemolytic anemia and thrombocytopenia [31]. ADAMTS13 deficiency was suggested to be a cause of chronic kidney disease when features of thrombotic microangiopathy are present on renal biopsy [32]. Lately, decreased ADAMTS13 was found to be associated with a poor prognosis in sepsis-induced organ failure [33, 34]. ADAMTS13(-/-) mice develop more severe inflammatory responses, leading to increased ischemia reperfusion injury and formation of atherosclerosis [28].

In conclusion, the present study has reported that insulin does not participate in the regulation of ADAMTS13 level and/or activity in OUMS-27 chondrosarcoma cells. This study will provide a new insight for understanding action of insulin on chondrogenesis, chondrocyte action, and the levels of ADAMTS proteins and RNA in cellular level.

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