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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

The effects of insulin on the expression levels of ADAMTS6 & 19 in OUMS-27 cell

İnsülinin OUMS-27 hücrelerinde ADAMTS6 ve 19 ekspresyon düzeylerine etkileri

Veli Uğurcu[1], Sümeyya Akyol[2], Aynur Altuntaş[3], Rıdvan Fırat[4], S. Fatih Kurşunlu[5], Özlem Çakmak[6], Kadir Demircan[2]

ABSTRACT

Objective: A Disintegrin-like Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteins are kind of matrix metalloproteinase enzymes that primarily founds in the extracellular matrix (ECM). Insulin is an important anabolic hormone, which acts on many tissues. The aim of this study is to evaluate the time-dependent effects of insulin on the two functionally unknown enzyme expressions (ADAMTS6 & 19) in OUMS-27 human chondrosarcoma cell line.

Methods: OUMS-27 cells were cultured in Dulbecco's modified Eagle' medium (DMEM) alone and DMEM containing 10 μg/mL insulin. The medium was changed every other day up to 11th day. Cells were harvested at 1, 3, 7, and 11th days and RNA isolation was performed at appropriate times according to study setup. The levels of RNA expression of ADAMTS6 and 19 were estimated by qRT-PCR using appropriate primers.

Results: According to qRT-PCR analysis, ADAMTS6 mRNA expression was found to be decreased as early as one day after insulin application and continued up to day 11, the last day of insulin induction (p=0.008). The ratio of ADAMTS6 in insulin-applied groups was changed between 1/2 and 1/4 of control values. The changes in ADAMTS19 mRNA levels in insulin-applied cells were not statistically significant compared to control cell group.

Conclusion: Our results demonstrated that insulin has a potential effect on alleviation of loss of extracellular matrix compounds by diminishing ADAMTS6 levels. To test this hypothesis and finding, more investigations are needed to recognize the real functions of orphan ADAMTS proteins.

Key words: Insulin, ADAMTS6, ADAMTS19, chondrosarcoma, OUMS-27, RNA

ÖZET

Amaç: A Disintegrin-like Metalloproteinase with Thrombospondin Motifs (ADAMTS) proteinleri ekstraselüler matrikste (ECM) bulunan bir çeşit matriks metalloproteinaz enzimidir. İnsulin birçok dokuda etki gösteren önemli bir anabolik hormondur. Bu çalışmanın amacı OUMS-27 insan kondrosarkom hücre kültüründe insülinin, fonksiyonları tam olarak bilinmeyen ADAMTS enzimlerinden ADAMTS6 ve 19 ekspresyonu üzerine zamana bağımlı olarak etkilerini değerlendirmek ve insülinin anabolik etkilerinden dolayı ADAMTS ekspresyonunu azaltma hipotezini test etmektir.

Yöntemler: OUMS-27 hücrelerinin, 10 μg/mL insulin içeren ve içermeyen 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 RNA izolasyonu yapıldı. ADAMTS6 ve 19 RNA ekspresyonları uygun primerler kullanılarak qRT-PCR yöntemi ile ölçüldü.

Bulgular: qRT-PCR cihazının verilerine göre, insülin uygulanmasından 1 gün sonra başlamak üzere deneyin son günü olan 11. güne kadar ADAMTS6'nın mRNA düzeyinde ciddi bir azalma meydana geldi (p=0.008). İnsülin uygulanan gruplarda ADAMTS6 oranı kontrol grubuna kıyasla 1/2 ve 1/4 aralığında değişti. İnsülin verilen hücre grubunda ADAMTS19 mRNA düzeyindeki değişiklikler ise kontrol grubuna kıyasla istatistiksel olarak anlamsızdı.

Sonuç: Sonuçlarımız, insülinin ADAMTS6 düzeylerini azaltarak ekstrasellüler matriks bileşenlerinin kaybının telafi edilmesinde potansiyel bir etkisinin olabileceğini gösterdi. Bu hipotez ve bulgunun anlaşılması için yetim ADAMTS proteinlerinin gerçek fonksiyonlarının inceleneceği çok sayıda araştırma yapılmasına ihtiyaç vardır.

Anahtar kelimeler: İnsulin, ADAMTS6, ADAMTS19, kondrosarkom, OUMS-27, RNA

[1] *Private Bilecik Orhangazi Dialysis Center, Bilecik, Türkiye*
[2] *Department of Medical Biology, Faculty of Medicine, Turgut Özal University, Ankara, Türkiye*
[3] *Department of Chemistry, Ankara Regional Office of Council of Forensic Medicine, Ankara, Türkiye*
[4] *Division of Medical Biochemistry Laboratory, Gölbaşı Hasvak State Hospital, Ankara, Türkiye*
[5] *Department of Periodontology, Faculty of Dentistry, Adnan Menderes University, Aydın, Türkiye*
[6] *Department of Biology, Faculty of Education, Gazi University, Ankara, Türkiye*

Yazışma Adresi /Correspondence: *Veli Uğurcu,*
Private Bilecik Orhangazi Dialysis Center, Bilecik, Turkey Email:veliugurcu@yahoo.com

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INTRODUCTION

Articular cartilage is a specialized connective tissue found in many areas of the body such as joints, ear, nose, intervertebral disk etc. Cartilage matrix comprises of collagen type 2, 9, and 11, aggrecan, cartilage oligomeric matrix protein and other compounds [1]. Some members of matrix metalloproteinase (MMP) and A Disintegrin-like Metalloproteinase with Trombospondin Motifs (ADAMTS) have been implicated in cartilage matrix destruction [2].

ADAMTS proteins are a kind of matrix metalloproteinase and have 19 members [3]. They take part in many physiological mechanisms such as blood coagulation, ovulation, extracellular matrix turnover, and have many important roles at pathological processes such as angiogenesis and cancer [4]. These proteins perform their functions by breakdown structural matrix proteins such as aggrecan, versican, and breavican. ADAMTS proteins are classified according to their functions, while some ADAMTS proteins are classified as "orphan ADAMTS" (including ADAMTS 6, 19, and etc.) because their functions are not known clearly [5].

Chondrosarcomas are heterogeneous tumors that produce cartilage [6], which are the third most common primary bone malignancy following myeloma and osteosarcoma [7]. These tumors grow slowly and have low potential of metastasis. They produce and secrete some types of ADAMTS proteins. OUMS-27 cell line is one of the most known human chondrosarcoma cell lines that are used in cell culture experiments.

Insulin is an anabolic hormone that produced at β cells of Langerhans islets in response to high glucose levels. Many functions are described such as glucose to glycogen conversation, inhibiting glucose production at liver. Its metabolic effects begin with binding of insulin to a specific receptor at plasma membrane [8]. Regardless of its mechanism of action, a role of insulin in the metabolism of proteoglycans in the extracellular matrix of cartilage may have considerable biological implications [9]. The retarded wound healing and bone fractures, long bones' susceptibility to fractures [10], the unsuccessful attempts for the maxillary sinus graft procedure, also referred to as maxillary sinus floor elevation [11] operations are currently main problems in patients with uncontrolled diabetes mellitus, especially in type 2 form. Therefore, this study was planned to evaluate the time-dependent effects of insulin on the chondrogenic ADAMTS 6 and ADAMTS 19 expressions in OUMS-27 chondrosarcoma cell line. Based on this, we also aimed to illuminate the regulatory role of insulin on these ADAMTS proteins and the function of ADAMTS 6 and 19 in OUMS-27 cells.

METHODS

OUMS-27 cell culture: OUMS-27 cell line is one of the most known human chondrosarcoma cell lines that are frequently used in cell culture experiments. 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% CO2 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 were subjected to insulin: For 1st day experiment, $2x10^5$ cells, for 3th 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 same concentrations of insulin at the days indicated. After the experiment, cells were harvested and total RNA measurements were performed.

Total RNA isolation: Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms RNA were reverse transcribed with Rever-Tra Ace (Thermo Scientific, Waltham, MA, USA) and random hexamers (Thermo Scientific, Waltham, MA, USA) with random primers according to the manufacturer's instruction (Table 1). Human glyceraldehyde-3-phosphate dehydrogenase (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.

Table 1. The forward and reverse primers used in the real-time polymerase chain reaction analyses for ADAMTS6, ADAMTS19, and GAPDH.

ADAMTS6	Forward	GTGGCCCGCTTAATTGTTCTC	71bp product
	Reverse	AGGGACTTGTCTGCATGGTG	
ADAMTS19	Forward	CAGTCTGAGTGTGCAGGTCA	137 bp product
	Reverse	TGCCAAATTCTTCATGTTGCCA	
GAPDH	Forward	CCTGCACCACCAACTGCTTA	108 bp product
	Reverse	TCTTCTGGGTGGCAGTGATG	

Real-time PCR: qRT-PCR was performed on cDNA samples obtained (Qiagen Rotor-Gene Q RT-PCR, Limburg, Netherlands) as described in our previous report [1]. Total RNA RT-PCR section uses the intercalating dye SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix) 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 30s and, the last stage, polymerization at 72°C for 5min. The results pertaining to ADAMTS6 and ADAMTS19 were represented as graphic charts. The bars and error bars represent mean and standard deviation, respectively.

Statistical Analyses

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

RESULTS

We first examined whether the expressions of the ADAMTS6 and 19 genes are induced or suppressed

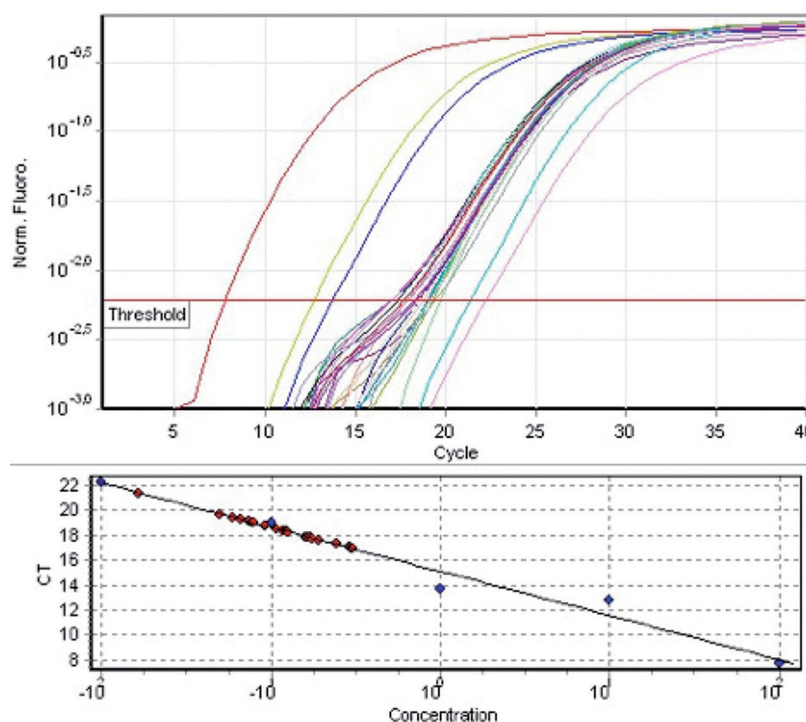

Figure 1. qRT-PCR cycle quantitation and standard concentration graphics of ADAMTS6

upon insulin application in OUMS-27 cells. As shown in Figure 2, according to qRT-PCR analyses, ADAMTS6 mRNA expression significantly decreased as early as 1st day after insulin induction compared to control group (p=0.008). At day 3, ADAMTS6 level was the same as seen in day 1 when compared to control group (p=0.008). The decreases in ADAMTS6 mRNA levels were continued to decrease in day 7 up to 1/4 ratio compared to control group's level (p=0.008). ADAMTS6 mRNA level was 1/2 of control values in day 11 again as seen in days 1 and 3 (p=0.027). There were statistically significant differences between the insulin-applied groups such as D1-D7 (p=0.047), D3-D7 (p=0.008), and D7-D11 (p=0.014). As shown in Figure 4, according to qRT-PCR analyses, ADAMTS19 mRNA expression increased in day 1, decreased in days 3 and 7, and again increased at day 11 when compared to control group but the differences were not statistically significant (p>0.05).

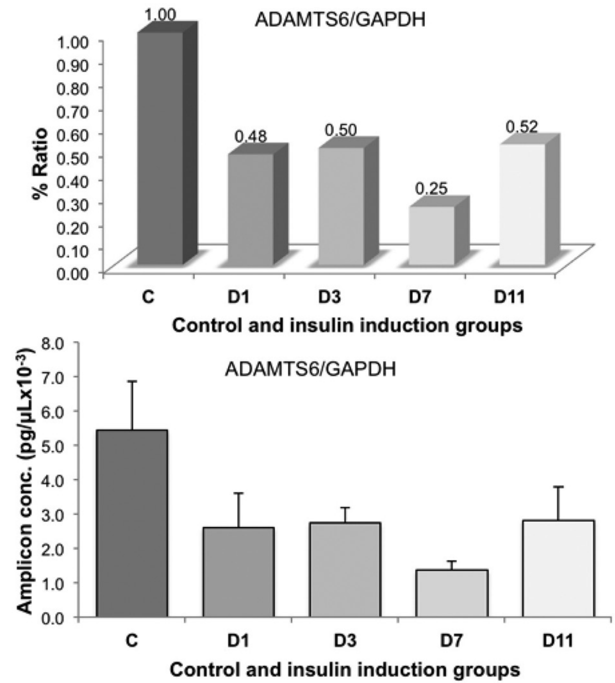

Figure 2. The results of ADAMTS6 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS6 to GAPDH. There is statistically significant differences between C-D1 (p=0.008), C-D3 (p=0.008), C-D7 (p=0.008), C-D11 (p=0.027), D1-D7 (p=0.047), D3-D7 (p=0.008), and D7-D11 (p=0.014)

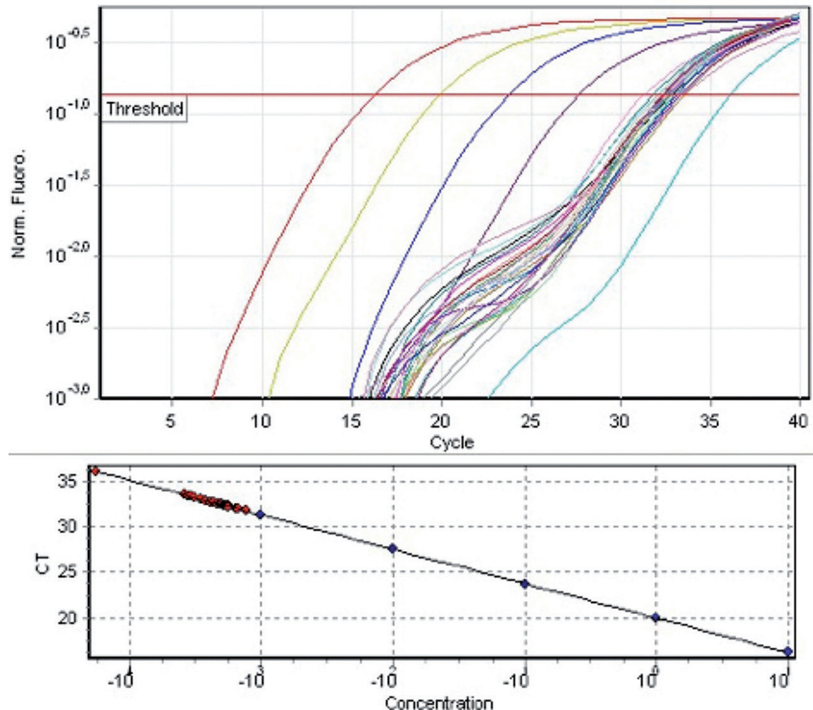

Figure 3. qRT-PCR cycle quantitation and standard concentration graphics of ADAMTS19

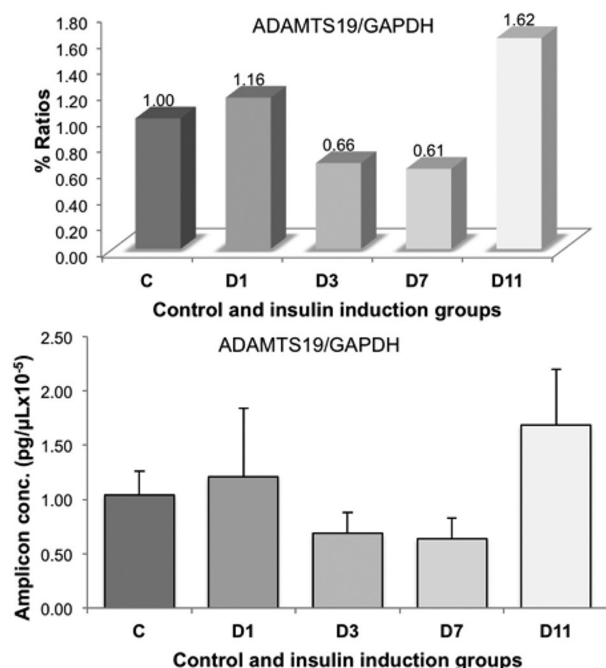

Figure 4. The results of ADAMTS19 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS19 to GAPDH. There is no statistically significant difference neither between control and insulin induction groups nor among insulin induction groups

DISCUSSION

This study was undertaken to compare the mRNA levels of ADAMTS6 and 19 in OUMS-27 cells in response to insulin hormone application. We have shown that insulin had potential positive effects on matrix metabolism in isolated human chondrosarcoma cell line. Insulin suppressed ADAMTS6 mRNA, and probably as a result, ADAMTS6 enzyme activity, which is a potential factor for removal of surrounding extracellular matrix. Therefore, we suggested that insulin inhibited matrix breakdown and stimulated matrix synthesis in OUMS-27 cells at least by decreasing ADAMTS6 levels even if its function is not known exactly. On the other hand, another orphan ADAMTS member, ADAMTS19, was not affected by insulin induction.

ADAMTS family members of matrix metalloproteinases are zinc-dependent endopeptidases known for their ability to degrade one or more extracellular matrix constituents, as well as non-matrix proteins [12]. A broad spectrum of several pathological conditions are associated with the metalloproteinases such as Alzheimer's disease, Parkinson's disease, cerebral aneurysm, neurodegenerative diseases, cancer, human periodontal diseases, rheumatoid arthritis, atrial cardiomyopathy, cardiac fibrosis, congestive heart disease, atherosclerosis, chronic obstructive pulmonary diseases, asthma, acute respiratory distress syndrome, liver fibrosis, and chronic ulcers [12]. Orphan ADAMTSs are such an ADAMTS group that their function of substrate cannot be shown to date. These are currently ADAMTS6, 7, 10, 12, 16, 17, 18, and 19. Although the exact mechanism is not known, ADAMTS16 has been shown to be dysregulated in osteoarthritis [13]. The chromosomal location of ADAMTS6 gene is 5q12 and ADAMTS19 gene 5q31 [5]. These two ADAMTS are very closely located on chromosome 5 and most probably, they show similar functioning pattern though it could not be enlightened to date.

Chondrocytes like OUMS-27 cells synthesize and secrete some macromolecules that constitute the extracellular matrix of cartilage. Glucose is a fundamental nutrient in cartilage tissues for both metabolic and structural needs. Because glucose also serves as a source for glucose-derived sugars such as glucosamine sulfate and vitamin C, which are of great importance in the maintenance, repair and remodeling of cartilage [14], insulin stands an important junction to moderate this anabolic/catabolic events. The studies in this field have demonstrated that chondrosarcoma chondrocytes is uniquely responsive *in vitro* to physiological concentrations of pig insulin, producing cartilage-like proteoglycans [15, 16], type II collagen [17], hyaluronic acid [18] and some other secretory proteins [19]. Glade et al. showed the modulatory effects of insulin and thyroid hormones on chondrocyte metabolism via multiple biosynthetic/receptor pathways [20]. It should be noted that our insulin application procedure was limited to 11 days because of our previous experience that the cells could not be appropriately manipulated after that time. Therefore, by adding supplementary materials to extend the viability time-course of cells, the experiments should be repeated in future to achieve more certain and clear results of long-term insulin induction effects on OUMS-27 cells.

The effects of insulin to ADAMTS expression has not been studied much. Recently at our study we found that insulin increases the expression of ADAMTS13 but this increase was not statistically significant [21]. Insulin and other ADAMTS relationships should be investigated in detailed later.

Our results demonstrated that insulin mitigated loss of extracellular matrix compounds and probably stimulated de novo synthesis of new molecules to replace lost ones. To test this hypothesis, future experiments investigating the extracellular matrix molecules such as versican, brevican, and aggrecan as well as their breakdown products together with other orphan ADAMTS members should be planned. These studies will also play an important role in determining the functions of orphan ADAMTS members, which has not been explained up to now, and contributing to the related literature.

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