The Investigation of ADAMTS16 in Insulin-Induced Human Chondrosarcoma Cells

Ozlem Cakmak,¹ Ismail Comertoglu,² Ridvan Firat,³ Hacı Kemal Erdemli,⁴ S. Fatih Kursunlu,⁵ Sumeyya Akyol,⁶ Veli Ugurcu,⁷ Aynur Altuntas,⁸ Bahattin Adam,⁹ and Kadir Demircan⁶

Abstract

Objectives: A disintegrin-like metalloproteinase with thrombospondin motifs (ADAMTS) is a group of proteins that have enzymatic activity secreted by cells to the outside extracellular matrix. Insulin induces proteoglycan biosynthesis in chondrosarcoma chondrocytes. The purpose of the present *in vitro* study is to assess the time course effects of insulin on ADAMTS16 expression in OUMS-27 (human chondrosarcoma) cell line to examine whether insulin regulates ADAMTS16 expression as well as proteoglycan biosynthesis with multifaceted properties or not.

Methods: Chondrosarcoma cells were cultured in Dulbecco's modified Eagle's medium having either 10 $\mu\text{g/mL}$ insulin or not. While the experiment was going on, the medium containing insulin had been changed every other day. Cells were harvested at 1st, 3rd, 7th, and 11th days; subsequently, RNA and proteins were isolated in every experimental group according to their time interval. RNA expression of ADAMTS was estimated by quantitative real-time polymerase chain reaction (qRT-PCR) by using primers. Immunoreactive protein levels were encountered by the western blot protein detection technique by using proper anti-ADAMTS16 antibodies.

Results: ADAMTS16 mRNA expression level of chondrosarcoma cells was found to be insignificantly decreased in chondrosarcoma cells induced by insulin detected by the qRT-PCR instrument. On the other hand, there was a gradual decrease in immune-reactant ADAMTS16 protein amount by the time course in insulin-treated cell groups when compared with control cells.

Conclusion: It has been suggested that insulin might possibly regulate ADAMTS16 levels/activities in OUMS-27 chondrosarcoma cells taking a role in extracellular matrix turnover.

Key words: ADAMTS16, chondrosarcoma, insulin, OUMS-27, protein, RNA

Introduction

Chondrosarcomas are a heterogeneous tumor group, which show benign and malign behaviors. Chondrosarcoma, which produces cartilaginous neoplastic tissue (type II and IX, aggrecan), is a common bone malignant tumor.¹ This tumor grows slowly and rarely metastasizes. It has a good prognosis due to low growth rate and poor vascularity.² Prognosis of chondrosarcoma is well and is

associated with histological grade, anatomic location of tumor, and its size.³ The most common subtype of chondrosarcoma is conventional chondrosarcoma (~85% of all chondrosarcomas).⁴ Other subtypes are dedifferentiated chondrosarcoma, mesenchymal chondrosarcoma, and clear cell chondrosarcoma.⁵ It has low metastatic potential. There are a few treatment options: surgical resection, radiotherapy, and chemotherapy.⁶ Surgical treatment is the best treatment option and chemotherapy and radiotherapy

¹Department of Biology Educations, Faculty of Education, Gazi University, Ankara, Turkey.

²Department of Medical Genetics, Faculty of Medicine, Mevlana University, Konya, Turkey.

³Division of Medical Biochemistry Laboratory, Golbasi State Hospital, Ankara, Turkey.

⁴Department of Biochemistry Laboratory, Corum Training and Research Hospital, Corum, Turkey.

⁵Department of Periodontology, Faculty of Dentistry, Adnan Menderes University, Aydın, Turkey.

⁶Department of Medical Biology, Faculty of Medicine, Turgut Ozal University, Ankara, Turkey.

⁷Department of Medical Biochemistry, Dumlupinar University Medical Faculty, Kutahya, Turkey.

⁸Division of Chemistry, Ankara Regional Office of Council of Forensic Medicine, Ankara, Turkey.

⁹University of California Davis Medical School, Department of Biochemistry and Molecular Medicine, Sacramento, California.

Address correspondence to: Ozlem Cakmak; Department of Biology Education, Faculty of Education, Gazi University; 06500 Besevler, Ankara, Turkey

E-mail: ocakmak21@gmail.com

are palliative treatment options.⁷ These tumors are relatively resistant to radiotherapy and chemotherapy.⁶

Matrix metalloproteinases (MMPs) have been known as zinc-dependent proteinases in human extracellular matrix (ECM). They have 23 members and digest ECM macromolecules (collagen, gelatin, elastin, fibronectin, laminin, etc.) and some molecules such as growth factors and cytokines, which are not found in ECM.⁸ A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) also belongs to the proteinases family and has 19 members (1–10, 12–20).⁹ ADAMTS, which is secreted into ECM, is blocked by the tissue inhibitors of metalloproteinases.¹⁰ They have lots of substrates in ECM, including procollagen, cartilage oligomeric matrix protein, neurocan, versican, aggrecan, brevican, and von Willebrand factor.¹⁰ They have functions of angiogenesis (ADAMTS1 and ADAMTS8), cleavage of aggrecan (ADAMTS1, -4, -5, -8, -9, and -15), cleavage of the procollagen N-proteinases (ADAMTS2, -3, and -14), and cleavage of von Willebrand factor (ADAMTS13).¹¹

ADAMTS members individually have functions in cancer formation and development. Some members of ADAMTS (ADAMTS1, -3, -5, -8, -9, 10, and -18) are downregulated in cancers and some of them are upregulated (ADAMTS4, -6, and -14).¹² ADAMTS16, which is expressed in the lung, kidney, brain, ovaries, and gonads, is associated with a lot of pathologic conditions, including osteoarthritis, cancers, hypertension, and psychiatric disorders.¹³

The OUMS-27 cell line, which has chondrocytic properties, is developed from grade III human chondrosarcoma by Kunisada et al.¹⁴ This cell culture, which expresses collagen (type II, IX, and XI) and proteoglycan, is useful for etiology and treatment of chondrosarcoma.¹⁵ Produced by β cells of pancreas, insulin is the major anabolic hormone, which regulates the metabolism of carbohydrates and fats. In rats, growth of chondrosarcoma cells depends on insulin.¹⁶ In physiological concentrations, insulin increases proteoglycan, collagen, and hyaluronic acid.¹⁶ Insulin stimulates anabolic responses in cartilage.¹⁷

There is no study about ADAMTS16 on chondrosarcoma cells until today. The authors have no information about the effect of insulin on chondrosarcoma and ADAMTS proteinases as well. There are no *in vivo* or *in vitro* studies about chondrosarcoma and MMP/ADAMTS proteinases, so the authors have designed this study to enlighten the subject. The purpose of this prospective investigation is to show the time course effects of insulin on ADAMTS16 expression in OUMS-27 human cell lines to examine whether insulin affects ADAMTS16 expression because of multifaceted properties or not.

Materials and Methods

Cell culture

The chondrosarcoma cell line was gently supplied by Dr. T. Kunisada from Okayama University, Japan. Cell cultures were practiced within Dulbecco's modified Eagle's medium containing penicillin/streptomycin and fetal bovine serum (10%) at 37°C in a humidified atmosphere, including CO₂ (5%). They were subcultured every 7–10 days at a split ratio of 1:2–1:4 by using EDTA and trypsin. The cells utilized in the experiments were passaged at 7–14. The cell media used as control or insulin-applied groups (10 μ g/mL insulin, totally 11 days) were replaced every other day. Cells (total

four groups, two of each group, one for western blot test, and one for quantitative real-time polymerase chain reaction [qRT-PCR] test) were applied with insulin: For the 1st day group, 2×10^5 cells, for 3rd day group, 1×10^5 cells, for 7th day group, 5×10^4 cells, and for 11th day group, 3×10^4 cells were plated in dishes (20 mm). Cells were harvested at the end of experiments to isolate total RNA and protein.

Total RNA isolation

TRIzol (Invitrogen) commercial kits were used to extract total RNA by following official instructions. RNA (2 μ g) was used for reverse transcription using RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific) and random hexamers (Thermo Scientific) with random primers according to the official instruction sheet (Table 1). The human housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as control for PCRs. To prevent genomic DNA contamination, reverse transcriptase-deficient samples were amplified as controls. Elution of total RNA from samples was accomplished by using RNase-free water. To determine RNA concentration and to check the purity of samples, UV spectrophotometry was used. For that purpose, the purity was decided from samples that required 260/280 ratio of 2.0 and 260/230 ratio of 1.7.

Real-time polymerase chain reaction

cDNA samples obtained were used for qRT-PCR (Qiagen Rotor-Gene Q RT-PCR).¹⁸ Total RNA was amplified by using SYBR green intercalating dye (Thermo Scientific Maxima SYBR[®] Green/ROX qPCR Master Mix) in the existence of primer pairs in RT-PCR. There were SYBR Green PCR Master Mix, SYBR Green I Dye, DNA polymerase, dNTPs, PCR buffer, forward and reverse primers (10 pmol each), and cDNA of samples in a total volume of 20 μ L of PCR mixture. The amplified GAPDH, housekeeping gene, was used to balance the competence of cDNA synthesis and the load of RNA applied. PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, pursued by amplification for 40 cycles, each cycle consisting of denaturation at 95°C for 10 seconds, annealing at 57°C for 30 seconds, polymerization at 72°C for 30 seconds, and polymerization at 72°C for 5 minutes. The findings related to ADAMTS16 are presented as graphics, in which the bars represent means of measurements and error bars represent standard error of means (SEM).

TABLE 1. THE FORWARD AND REVERSE PRIMERS USED IN THE REAL-TIME POLYMERASE CHAIN REACTION ANALYSES FOR ADAMTS16 AND GAPDH

ADAMTS16		
Forward (45801)	GGATGGAAAAGGGCGAAT	75 bp product
Reverse (45802)	TTTCATGGGACACGTAATCG	
GAPDH		
Forward (544445)	CCTGCACCACCAACTGCTTA	108 bp product
Reverse (524446)	TCTTCTGGGTGGCAGTGATG	

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE 2. PRIMARY AND SECONDARY ANTIBODIES USED FOR WESTERN BLOT TECHNIQUES

Primary Ab	Primary Ab concentration	Secondary Ab	Secondary Ab concentration	Reaction	kDa
ADAMTS16	1/1000	Rabbit	1/4000	Mouse, rat, human	40
GAPDH	1/10,000–1/50,000	Rabbit	1/4000	Human	36

Protein isolation and western blot analysis

At the end of experiments, media were wiped and cells were gently washed by using phosphate-buffered saline and subsequently inscribed from the plates. They were dissolved in 300 μ L of CellLytic TMM (Sigma), including protease inhibitor. Cells were incubated in a rotator at 4°C for 15 minutes and centrifuged, and the upper part of mixture (supernatant) was aspirated to another tube. The Thermo Scientific Bradford Assay Kit was used to measure the protein amount of the cell supernatant using bovine serum albumin as standard.

Afterward, samples were boiled for 8 minutes at 95°C within Laemmli sample buffer plus β -mercaptoethanol. Western blot analyses were performed by using 10 μ g of total protein after adjustment of samples. A total of 10 μ L samples, including BioRad Precision Plus Protein Western C Standard (protein marker), were run to western blot gel (BioRad Mini-PROTEAN TGX Stain-Free 4%–15% Gels, 15-well comb) within running buffer (BioRad 1 $\times$ Tris/Glycerine/SDS) and run at 250 V for 20 minutes. By the time electrophoresis ended, proteins were transferred onto 0.22- μ M PVDF membranes (BioRad, Trans-Blot Turbo Transfer Pack) by using the trapped protein transfer system (BioRad Trans-Blot Turbo Transfer System). Total of 2.5% nonfat dried skim milk in TBS containing 0.05% Tween 20 (TBST) was used for the blockade of membranes (total 1 hour).

Afterward, membranes were incubated with primary anti-GAPDH and anti-ADAMTS16 antibodies (Table 2) dissolved in blocking buffer (overnight, 16 hours). The membranes were incubated for 1 hour with the proper second antibodies (Table 2) after forceful washing with TBST (thrice) for 8 minutes at room temperature. Following washing with TBST (thrice), exposure of membranes with enhanced chemiluminescence system (BioRad Immun-Star Western C kit) for 90 seconds let the immunoreactive bands be visible. The BioRad ChemiDoc MP Imaging System detected the signal spreading by the chemiluminescence reaction. ImageJ software (W. Rasband, Research Services Branch, NIMH, National Institutes of Health) was used to measure density of bands, and then they were normalized by comparing signal intensity of GAPDH for equal protein loading control of each experiment. The linear range of standard curve determined by the standard sample, GAPDH, was used to perform the quantification for all densitometry analyses.

Statistical analyses

Statistical tests were performed by using Statistical Package for Social Studies version 16.0. While nonparametric Kruskal–Wallis test was used for general statistics, the relationships between the variables were performed by Mann–Whitney *U* test. A *p*-value <0.05 was accepted to be significant.

Results

The authors analyzed whether there is any regulatory impact of insulin on ADAMTS16 gene expression and protein levels in OUMS-27 chondrosarcoma cells. The ratios of ADAMTS16 RNA of insulin-induced cells compared with control cells are given in Figure 1. In this figure, mRNA expression levels of ADAMTS16 (ratio $\pm$ SEM) are shown as ratios within the groups after correction with GAPDH. The culture of OUMS-27 cells was done in the presence of insulin and subsequently total RNA was extracted at 1, 3, 7, and 11 days. qRT-PCR analyses have shown that ADAMTS16 mRNA expression decreased in days 1, 7, and 11 after insulin induction in comparison with the control group, but they were not significant (*p*>0.05). At day 3, ADAMTS16 levels increased in comparison with the control group, but they were not significant (*p*>0.05).

FIG. 1. The findings of ADAMTS16 qRT-PCR tests of five different experiment groups after insulin application in OUMS-27 cells. By dividing ADAMTS16 to GAPDH, the results were standardized. The bars represent means and error bars represent SEM. There are insignificant differences between control, D1, D3, D7, and D11 groups (*p*>0.05). ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; D, day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of means; qRT-PCR, quantitative real-time polymerase chain reaction.

FIG. 2. The findings of western blot protein analyses of ADAMTS16. The graphic was acquired by calculating the intensity of protein bands of ADAMTS16 and GAPDH and dividing them by each other.

The authors examined ADAMTS16 protein levels in insulin-induced OUMS-27 cells. Protein extraction was made from cells with or without insulin induction ($n=3$ independent experiments, respectively), and western blot analysis was applied using anti-ADAMTS16 polyclonal antibodies. The best band image was selected and the densities of this image were calculated among three experiments. A powerful band of 40 kDa, which represents an active form of ADAMTS16, was clearly notified (Fig. 2).

As a general evaluation, ADAMTS16, one of the aggrecanase family members, was found to be expressed in OUMS-27 cells. Western blot analysis exhibited that ADAMTS16 protein was gradually decreased in insulin-induced OUMS-27 cells up to the 11th day (Fig. 2). The ratios of ADAMTS16 protein levels in insulin-induced OUMS-27 cells in comparison with control cells were 1, 1.13, 0.74, 0.73, and 0.28 at D1, D3, D7, and D11, respectively. Immunoreactive protein bands with molecular mass of approximately 40 kDa for ADAMTS16 were detected.

According to this band, there was a gradual decrease in the protein amount of ADAMTS16 after insulin induction. All the bands for ADAMTS16 and GAPDH were detected in place where they were anticipated to be found (Fig. 2). After insulin was applied, in day 1, ADAMTS16 was slightly increased and the ADAMTS16/GAPDH ratio was 1.13. After the first day, ADAMTS16 levels have been decreased and the most prominent decrease in ADAMTS16 level was shown in day 11. The ADAMTS16/GAPDH ratio in this group was 0.28 compared with control value.

Discussion

This study is one of the first investigations of the ADAMTS16 gene in insulin-induced human chondrosarcoma. This study is the first ADAMTS16 investigation in chondrosarcoma. In the present work, the authors show that there were important changes in ADAMTS16 protein levels between the control and insulin-applied groups. Although chondrosarcoma is one of the most well-prognosis tumors, the only curative treatment option is surgical. Chemoradiotherapy is an adjuvant treatment option. Especially, in high-grade chondrosarcomas, there are no effective treatment

options. For this reason, it is now essentially recommended to establish therapeutic objectives for a better choice of accompanying treatment modalities for patients. In this study, the authors investigated the ADAMTS16 gene in chondrosarcoma. Western blot analysis showed that ADAMTS16 levels in OUMS-27 cells were gradually decreased with the use of insulin.

Cancer is one of the most serious health diseases in the world. Each year in the world, there are 7.6 million deaths because of the frightening disease.¹⁹ In cancer, mortality and morbidity mostly result from invasion and metastasis. Cancer cells and tumor's microenvironment play important roles in cancer formation and progression.²⁰ Cell-cell and cell-ECM interactions have crucial roles in formation and development of cancers.²¹ Tumor cells, which grow in an uncontrolled manner, have interactions with ECM, immune system cells, and vascular tissue.²² MMPs and other proteinases (ADAM and ADAMTS) have very important roles in cancer development and progression.

MMPs and ADAMTS are zinc-dependent endopeptidases, which have roles in cancer formation and progression.²³ These proteases have important roles in degradation of ECM and invading surrounding tissues in tumors.²² Like ADAM and MMPs, the ADAMTS family is associated with tumors. Some domains of ADAMTS proteases, which have the ability to bind cells and other ECM members, have proteolytic activities.²⁴ While the active form of metalloproteinase domain degrades ECM components, the cysteine-rich domain has functions of adhesion and migration of cells.²⁴

The OUMS-27 cell line has chondrocytic properties, having no contact inhibition, and grows rapidly. This cell line expresses type I, II, III, IX, and XI collagens and proteoglycans. These tumor cells have aggressive behaviors because of the synthesis of collagen type III.¹⁴ For these reasons, this cell line is a perfect model for investigation of chondrosarcoma. It is also used for investigations for rheumatoid arthritis and osteoarthritis.

Some of the ADAMTS members generally increase in tumors and some of them decrease. The same member of this family can increase in some tumors, but can decrease in other types of tumors.^{25,26} ADAMTS is associated with cell

proliferation, survival, invasion, metastasis, cleavage ECM molecules, and angiogenesis in tumors.²⁷

ADAMTS16 is a member of ADAMTS whose function is poorly understood. However, ADAMTS16 that can cleavage the proteinase inhibitor α 2-macroglobulin shows weak aggrecanase activity.²⁸ ADAMTS16, which is located in 5p15, is expressed in the human lung, kidney, brain, ovary, and cartilage.^{28,29} These proteases were found to be associated with hypertension,³⁰ premature ovarian failure,³¹ osteoarthritis³², and cancers (brain medulloblastoma, lung large cell carcinoma, endometrial adenocarcinoma, and retinoblastoma).³³ ADAMTS16 also has functions in the genitourinary system.¹³

Sakamoto et al. showed that ADAMTS16 was upregulated in esophageal squamous cell carcinoma.³⁴ They also said that ADAMTS16 might be arranged as a sensitive biomarker in this tumor. In contrast to this study, the authors found that in chondrosarcoma, expression of ADAMTS16 was gradually decreased. ADAMTS proteases show different behaviors in different cancers. For example, while expression of ADAMTS1 was decreased in lung, prostate, and pancreatic cancers, it was increased in chondrosarcoma.³⁵ The experimentally silencing of the *ADAMTS* gene was found to inhibit cell growth and invasion capability of the TE5 esophageal cancer cell line.³⁴ In this study, in the insulin-applied chondrosarcoma cell line, ADAMTS16 was gradually decreased. This situation may be associated with insulin that may have positive effects in tumor's invasion. However, in the recent study, insulin may have negative effects in this cell line (unpublished data). Further studies are needed to show insulin's clear role in cancers and chondrosarcoma.

Despite the downregulation of ADAMTS16 mRNA and protein seen in the majority of brain tumors, it was found that there was no clear correlation between protein and mRNA expression. These data may show the altering sensitivities of the western blot and RT-PCR techniques or propose that ADAMTS16 expression might be controlled through transcription as well as factors that have an impact on protein assurance/stability, with a sustained protein half-life in some cases. The mechanism of downregulation of ADAMTS16 by insulin in OUMS-27 cells is unknown, but it should be investigated whether there is a possible hypermethylation of the promoter region of the gene or not.

The authors have shown that there was a gradual decrease in protein amount of ADAMTS16 after insulin induction. This finding might be important because it has a potential impact to understand the behavior of cancer cells. Since ADAMTS16 is a member of aggrecanases, it might have a role in tumor cell's invasion and metastasis. The authors suggest that these implications can be regarded as quite early. To support these findings, especially, clinical human studies are needed. These original findings on chondrosarcoma and ADAMTS can be regarded as a pathfinder study for future studies.

Disclosure Statement

No competing financial interests exist.

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