Changes of the Expressions of Orphan and gon-ADAMTS in Chondrosarcoma Cells

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ABSTRACT

Some of A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes have been suggested to facilitate invasion and metastasis in cancer. ADAMTS20 is called gon-ADAMTS and ADAMTS10 and -17 are called orphan ADAMTSs. ADAMTS20 degrades versican and aggrecan in extracellular matrix. We aimed to investigate the effects of insulin on ADAMTS10,-17 and -20 in OUMS-27 chondrosarcoma cells. OUMS-27 cells were cultured in Dulbecco's modified Eagle' medium (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 ADAMTS10,-17 and -20 were estimated by qRT-PCR using appropriate primers. ADAMTS10 mRNA expression gradually decreased within 7 days after insulin induction compared to control group. There was a significant difference between control and Day 7 groups (p=0.021) as well as Day 1 and Day 7 groups (p=0.028). ADAMTS17 mRNA expression increased right after insulin induction at day 1 compared to control group and protected its high levels throughout insulin application. The most evident and statistically significant increase in mRNA concentration was observed at day 7 after insulin induction (p= 0.014). Our results demonstrated that ADAMTS10,-17 and -20 might have a role in cancer progression. Although functions of ADAMTS10 and -17 are not known, their expression levels have changed in chondrosarcoma cell line. Further studies are needed to characterize chondrosarcoma cells because of the possible association between cancer progression and ADAMTS proteins.

Keywords: ADAMTS, Chondrosarcoma, Insulin, qRT-PCR, OUMS-27

ÖZET

Kondrosarkom Hücrelerinde Orfan ve gon-ADAMTS'ların Ekspresyonundaki Değişimler

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzimlerinden bazılarının kanserde invazyon ve metastazı kolaylaştıracağı öngörülmektedir. ADAMTS20, gon-ADAMTS olarak ve ADAMTS10 ile -17, orfan ADAMTS olarak adlandırılır. Bunlardan ADAMTS20 hücre dışı matrikste versikan ve agrekanı parçalayan enzim olarak bilinir. Bu çalışmada OUMS-27 hücrelerinde insülinin ADAMTS10, -17 ve -20 üzerine etkilerinin araştırılması amaçlandı. OUMS-27 hücreleri 10 µg/ml insülin içeren Dulbecco's Modified Eagle Medium (DMEM) ortamında kültüre edildiler. Medyum 11. güne kadar iki günde bir değiştirildi. Hücreler 1, 3, 7 ve 11. günlerde toplandı ve her birinde aynı gün RNA izolasyonları gerçekleştirildi. ADAMTS10, -17 ve -20'nin RNA ekspresyon düzeyleri uygun primerler kullanılarak qRT-PCR ile hesaplandı. Kontrol grubuyla karşılaştırıldığında, ADAMTS10 mRNA ekspresyonu insülin indüksiyonundan sonra 7 gün içinde gittikçe azalmıştır. Kontrol ile 7. gün grubu arasında (p=0.021) ve 1. gün ve 7. gün grubu (p=0.028) arasında anlamlı farklılıklar vardı. Kontrol grubuyla karşılaştırıldığında ADAMTS17 mRNA ekspresyonu insülin indüksiyonundan hemen sonra 1. günde yükselmiş ve yüksek seviyelerini insülin uygulaması boyunca korumuştur. En belirgin ve istatistiksel olarak anlamlı mRNA konsantrasyon artışı insülin indüksiyonundan 7 gün sonra görülmüştür. Çalışma sonuçlarımız ADAMTS10, -17, ve -20'nin kanserin yayılmasında rolü olabileceğini göstermiştir. Her ne kadar ADAMTS10 ve -17'nin işlevleri bilinmese de, bunların kondrosarkom hücrelerinde ekspresyon düzeylerinin değiştiği bulunmuştur. ADAMTS proteinleri ile kanser progresyonu arasındaki olası ilişki nedeniyle kondrosarkom hücrelerini tanımlayacak daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: ADAMTS, Kondrosarkom, İnsulin, qRT-PCR, OUMS-27

INTRODUCTION

Chondrosarcoma, one of the high-incidence bone tumors (0.1/100 000 per year), is mostly common between 30 and 60 years of age.[1] These tumors, mostly arising from diametaphyseal region of long bones, have several subtypes.[1] The most common subtype is conventional (frequency is 85%), and the others are mesenchymal, dedifferentiated, periosteal and clear cell chondrosarcomas.[2] Low grade (grade 1 and 2) tumors are unlikely to metastasize, so its prognosis is satisfactory. The most effective treatment option is surgery.[3] Adjuvant therapies may also be required. In high-grade chondrosarcomas, surgical treatment with wide margins is necessary. These tumors are relatively resistant to chemo and radiotherapies.[4] Chondrosarcoma cells are well-differentiated tumors and produce chondroidal matrix.

In cancers, cell-cell interactions and cell-extracellular matrix (ECM) interactions have critical roles on invasion and metastasis.[5] Cell invasion and metastasis are complex processes, which are associated with the migration of cells within cancer's ECM in response to biochemical signals.[6] For invasion and metastasis, tumor cells must cross the basement membrane and degrade the ECM. For this purpose, they secrete a lot of proteolytic enzymes including matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). In addition to degradation of ECM, angiogenesis should also occur in tumor tissues in which cells secrete diffusible factors to facilitate tumor growth.[7] MMP family, MMP2 and -9 have major roles in degradation of ECM during tumor invasion.[8] MMPs such as gelatinase and stromelysin, which are secreted into ECM, are inactivated by α2-macroglobulin or tissue inhibitor of metalloprotease (TIMP).[9] There is a tight balance between MMPs and TIMP in ECM formation. MMPs are closely associated with invasion and metastasis in cancers.[10] Relatively new members of MMP, ADAMTSs, are also associated with cancer formation and progression. ADAMTSs having 19 family members are secreted into ECM. These enzymes have roles in physiological and pathological conditions. In this family, ADAMTS1, -4, -5, -8, -9, -15 and -16 also called the aggrecanases are associated with cleavage of aggrecan; ADAMTS1 and -8 are associated with anti-angiogenesis; ADAMTS2, -3 and 14 also called the procollagen N-proteinases are associated with cleavage of procollagen N-proteinase and ADAMTS13 is associated with cleavage of von Willebrand factor. 11 Among them, ADAMTS20 is called gon-ADAMTS, and the others, ADAMTS10 and -17 are called orphan ADAMTSs.[11]

Chondrocytes have specialized extracellular matrix (ECM), including proteoglycans (aggrecan), perlecan, cartilage oligomeric matrix protein and collagen (Type ll, lX and Xl).[12] One of the chondrosarcoma cell line, OUMS-27, was firstly described by Kunisada in 1998 in Japan.[13] This cell line was taken from a patient who had grade 3 chondrosarcoma.[13] OUMS-27 cell line does not show contact inhibition and grows rapidly in multiple layers and is very useful model for the investigation of chondrosarcoma cell behaviors.[3] This cell line is also a very good model for studies, which investigate the behavior of chondrocytes.[14-16] It was shown that chondrosarcoma chondrocytes secrete intercellular matrix molecules in rats.[17] The growth of cancer cells depends on insulin, insulin-like growth factor, somatomedin, multiplication stimulating activity (MSA), and other molecules.[17,18] Insulin causes increase in ECM members, such as collagen and hyaluronic acid.[17] On the other hand, the effect of insulin on cartilage occurs via its receptors on cartilage.[19]

The essential functional role of insulin in cancer has not been clarified in details so far. There are growing evidence that abnormal insulin levels can lead to cancer development, progression and metastasis.[20,21] It has been shown that insulin may also affect the synthesis of some ADAMTS proteins through undefined mechanism(s).[22] We hypothesized that insulin might affect expression of orphan ADAMTSs (ADAMTS10 and 17) and gon-ADAMTS (ADAMTS20) in chondrosarcoma cells. Thus, we conducted the present study to determine the effect of insulin on above-mentioned ADAMTS proteins in OUMS-27 cell lines.

MATERIALS AND METHODS

Cell culture

OUMS-27 human chondrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin at 37^{0}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.

Powdered insulin was dissolved within 0.01N HCl solution. The stock solution had 2 mg/mL concentration within 0.01N HCl and working solution had 10 μg/mL within medium. Four groups of cells 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 for 11th day experiment, 3x10^4 cells were plated in 20 mm dishes and exposed to same concentrations of insulin at the days indicated. Briefly, cells were incubated with insulin within medium for 1 day, 3 days, 7, and 11 days. For each condition, there were cells in 5 dishes. After the experiment, cells were harvested and RNA isolations were performed.

Total RNA Isolation

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA, Cat#15596-018) according to the manufacturer's instructions. Two microgram RNA were reverse transcribed with RevertAid M-Mulv Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) and random hexamers (Thermo Scientific, Waltham, MA, USA, Cat# EP0441) 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.

Real-time PCR

qRT-PCR was performed on cDNA samples obtained (Qiagen Rotor-Gene Q RT-PCR, Limburg, Netherlands) as described in our previous reports.[23,24] Total RNA RT-PCR section uses the intercalating dye SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix, Waltham, MA, USA, Cat#K0221) 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^{0}C for 5 min, followed by amplification for 40 cycles, each cycle consisting of denaturation at 95^{0}C for 10 s, annealing at 57^{0}C for 30 s, polymerization at 72^{0}C for 30 s and, the last stage, polymerization at 72^{0}C for 5 min. The results pertaining to ADAMTS 10, 17, and 20 were represented as graphics. The bars and error bars represent means and standard error of means of

Table 1. The forward and reverse primers used in the qRT-PCR analyses for ADAMTS10, 17, 20, and GAPDH

ADAMTS10	Forward	GAGTCTGGGAAGCACCGTTA	111 bp product
	Reverse	CAGAAGCTGTCCAGGGACTT	
ADAMTS17	Forward	CCAAGCTTGTCCTGCTACGA	137 bp product
	Reverse	GGGAACCTGGTTATTGCCGA	
ADAMTS20	Forward	TGCAACAATCTACAAAGATCCAAGT	193 bp product
	Reverse	AGCAGTGTCATGGTGGGAAG	
GAPDH	Forward	CCTGCACCACCAACTGCTTA	108 bp product
	Reverse	TCTTCTGGGTGGCAGTGATG	

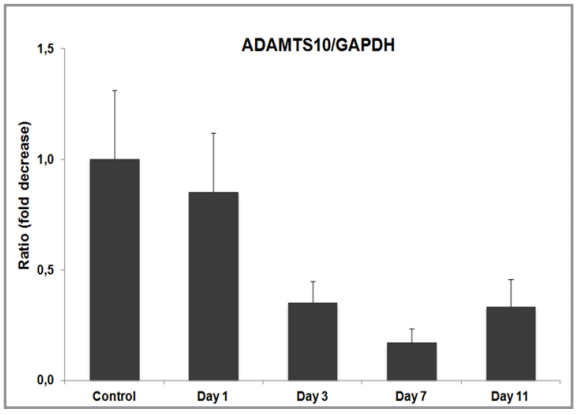

Figure 1. The results of ADAMTS10 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS10 to GAPDH. There were statistically significant differences between Control-Day 7 (p=0.021) and Day 1-Day 7 (p= 0.028) values.

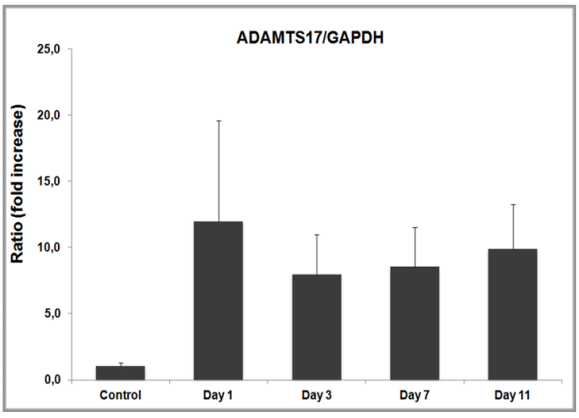

Figure 2. The results of ADAMT17 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS17 to GAPDH. There is statistically significant difference between Control-Day 7 (p= 0.014).

amplicon concentrations obtained by PCR reaction (pg per μl of volume) after dividing the values of study groups to the control values to acquire ratios (to get fold increases/decreases compared to control).

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

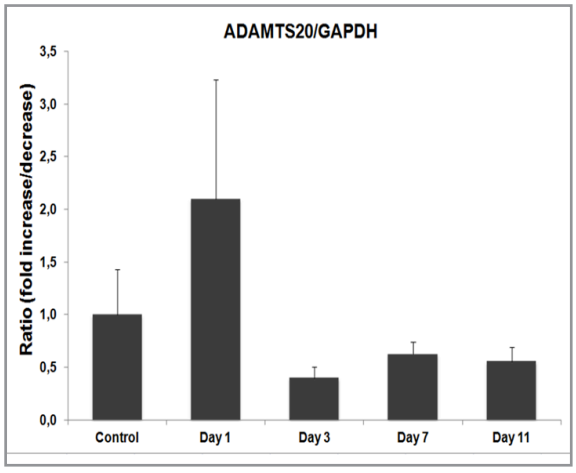

Figure 3. The results of ADAMTS20 qRT-PCR calculations of 5 different experiments. The values were standardized by division of ADAMTS20 to GAPDH. There is no statistically significant difference neither between control and insulin induction groups nor among insulin induction groups.

RESULTS

We examined whether the expression of the ADAMTS10, -17 and -20 genes are induced or suppressed by insulin in OUMS-27 chondrosarcoma cells. Results were summarized in figures. The ratios of insulin-induced cells compared to control cells were given in Figures 1, 2, and 3, respectively. In these figures, mRNA expression levels were shown as ratios within the study groups compared to control. qRT-PCR analyses have shown that ADAMTS10 mRNA expression had decreased gradually and significantly in 7 days after insulin induction compared to control group (p= 0.021 when compared Day 7 to control). At Day 11, ADAMTS10 levels increased compared to Day 7, but its expression was still lower and was not statistically significant compared to all other groups including control group. Additionally, there was a significant decrease in Day 7 group when compared to Day 1 group (p= 0.028). ADAMTS17 mRNA expression increased right after insulin induction at Day 1 compared to control group but it was not statistically significant because of high standard error of means value (broad difference between in-group values). ADAMTS17 level was unchanged during insulin application up to Days 7 and 11, giving a significantly important difference between Day 7 and control group (p= 0.014). Therefore the most evident increase in mRNA concentration was seen at Day 7 after insulin induc-

tion (p= 0.014). ADAMTS20 mRNA expression increased right after insulin induction but it was not consistent throughout further days. At Day 3, ADAMTS20 levels decreased and it gradually increased at Day 11. However, ADAMTS20 levels from Day 1 to Day 11 groups were not statistically significant when compared to the value of control group.

DISCUSSION

ADAMTS enzymes together with the other metalloproteases have been widely implicated in tissue remodeling events manifested in cancer development, progression and metastasis. There are limited researches on the role of specific ADAMTS proteins in cancer. Some of them has been investigated for their role in cancer development, angiogenesis, and metastatic progression.[25,26] This is the first study in the literature on expression levels of orphan and gon-ADAMTS in insulin-induced human chondrosarcoma cell line. Also, there have been no studies about the levels of ADAMTS proteinases (ADAMTS10, -17 and -20) in chondrosarcoma cells in literature so far. According to our findings, it was proved that there were differences in ADAMTS10, -17 and -20 mRNA concentrations in some extend. It was shown that these ADAMTS genes were differentially regulated.

OUMS-27 cell line was isolated from a patient who had a grade 3 chondrosarcoma.[13] This cell line is a very useful model for investigations for chondrosarcoma. It is also used for studies about cartilage, because it maintains chondrocytic phenotypes.[12] Since this cell line mimics normal cartilage tissue, it is not surprising that it produces aggrecan and collagen (type II, -III, -IX etc.).[12]

ECM, which is crucial component in tumor tissues, has several proteins including collagen, elastin, laminin, fibronectin, aggrecan, brevican, and versican. These components maintain tissue integrity.[27] In cancer, interactions between neoplastic cells and ECM are essential for invasion and metastasis. Proteolytic enzymes, which are secreted into ECM, have important roles in tumor formation and progression.[28] A great deal of MMPs and ADAMTS have played role in these processes. MMPs degrade collagen and elastin in ECM and degradation of collagen is one of the most important events in invasion and metastasis.[29] ADAMTS proteases also exist in tumor formation. There are 19 members of the family. Each member of ADAMTS has different roles in physiological and pathological events. ADAMTS1, -4, -5, -8, -9, -15 and -16 have aggrecanase activity in ECM. They cleave aggrecan that is one of the most important proteoglycans in ECM. ADAMTS2, -3 and -14 have roles in collagen synthesis. ADAMTS1 and -8 have also anti-angiogenic properties.[11] However, functions of some members of ADAMTS in ECM are not known clearly by now. ADAMTS10 and -17 are examples of these ADAMTSs. ADAMTS10 and -17 are also called orphan ADAMTSs, which means no known function or substrate yet. ADAMTS20, also known as gon-ADAMTS, is relatively a newly found ADAMTS's member.

ADAMTS10, which has five thrombospondin type 1 repeats and one cysteine-rich PLAC (protease and lacunin) domain, is localized on chromosome 19 in humans.[30] ADAMTS10 that is expressed in cartilage, skin, lung, liver, hearth and kidney is closely associated with ADAMTS6.[31] Mutations in ADAMTS10 and Fibrillin-1 (FBN-1) cause Weill-Marchesani syndrome, an inherited connective tissue disorder. Coexistence of ADAMTS10 and FBN-1 in this disease suggest that there may be a functional interaction between them.[32] Kutz et al.[33] showed that there was a direct interaction between ADAMTS10 and fibrillin-1 in ECM. Like other ADAMTS, ADAMTS10 mutations may change biomechanical features of ECM in animals.[34] In our study, after insulin application, ADAMTS10 levels decreased in OUMS-27. Gruber et al.[35] showed that ADAMTS10 was decreased in intervertebral disk degeneration. Up to now, there was no study about cancer and ADAMTS10. Our study is the first one showing ADAMTS10 levels on insulin-applied chondrosarcoma cells.

ADAMTS17 is present in lung, brain, prostate, cartilage and liver.[36] Specific function of ADAMTS17, another orphan ADAMTS, has not been known so far. Mutations in ADAMTS17 cause familial spherophakia, which is an uncommon ocular condition.[37] ADAMTS17 has a role in pediatric stroke pathogenesis as well.[38] Van Duyvenvoorde et al.[39] showed that ADAMTS17 was associated with

short stature and growth disorders. Mutation in ADAMTS17 is associated with Weil-Marchesani syndrome like ADAMTS10.[40,41] Our study is the first study to investigate relations of cancer and ADAMTS17 as well. According to our findings, ADAMTS17 levels were increased after insulin application in chondrosarcoma.

A member of ADAMTS, also called gon-1, has important roles in reproduction of nematode Caenorhabditis Elegans. It may degrade basement membrane for cell migration. While ADAMTS9, -20 and gon-1 have similar domain organization and exon structure; they are supposed to be a subfamily of gon-1 related ADMTS family in humans.[42] Unlike the other ADAMTS, these ADAMTS do not have a zinc binding active site.[42]

ADAMTS9 and ADAMTS20 are involved in related subfamilies.[43] ADAMTS9 and -20 also belong to aggrecanases and degrade versican and aggrecan in ECM.[44] ADAMTS20 is over-expressed in brain, colon and breast carcinomas.[41] In our study, ADAMTS20 has increased just after insulin application, but after that, it has decreased in day 3 and protected its low level throughout the study interval. Baine et al.[45] showed that ADAMTS20 could distinguish pancreatic cancer patients from healthy controls and ADAMTS20 may be potential diagnostic marker in pancreas cancers. In the present study, ADAMTS20 was found to be decreased gradually in chondrosarcoma cell line. Similarly, ADAMTS20 and 9 were reported to have roles in melanoma formation.[46]

Our results demonstrated that ADAMTS10, -17 and -20 might have roles in cancer formation. Although functions of ADAMTS10 and -17 have been not known yet, their expression levels have been changed in chondrosarcoma cell line. In a long-term observation, insulin led to decrease in ADAMTS20 mRNA levels, although it led to over-expression at the first day. This finding on cancer cells did not verify previous studies in which ADAMTS20 was found to be over-expressed. Further studies are needed to characterize chondrosarcoma cells because of the possible association of cancer progression and ADAMTS proteins.

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