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Effect of insulin on the mRNA expression of procollagen N-proteinases in chondrosarcoma OUMS-27 cells

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Abstract. Chondrosarcoma is one of the most common bone tumors, and at present, there is no non-invasive treatment option for this cancer. The chondrosarcoma OUMS-27 cell line produces proteoglycan and type II, IX, and XI collagens, which constitutes cartilage tissue. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases are a group of secreted proteases, which include the procollagen N-proteinases ADAMTS-2, -3 and -14. These procollagen N-proteinases perform a role in the processing of procollagens to collagen and the maturation of type I collagen. The present study aimed to improve the understanding of the causes of metastasis, local invasion and resistance to chemo- and radiotherapy in chondrosarcoma, as well as the effect of insulin on cancer cells. The present study was designed to reveal the effects of insulin on procollagen N-proteinases in chondrosarcoma OUMS-27 cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) alone or in DMEM containing 10 μg/ml insulin. The medium was changed every other day for 11 days. The cells were harvested on days 1, 3, 7 and 11, and total RNA isolation was performed immediately following harvesting. The expression levels of ADAMTS2, ADAMTS3 and ADAMTS14 mRNA were estimated by reverse transcription-quantitative polymerase chain reaction using appropriate primers. ADAMTS2 mRNA expression was found to be decreased on day 7 (P=0.028) and increased at day 11 compared with the control group (P=0.016). The increase in mRNA concentration at day 11 was significantly different compared to the concentrations on days 3 (P=0.047) and 7 (P=0.008). The expression of ADAMTS3 mRNA decreased immediately subsequent to insulin induction on day 1 compared with the control group (P=0.008). The most evident decrease in mRNA concentration was seen at day 7 subsequent to insulin induction (P=0.008). The present results demonstrated that ADAMTS2 and ADAMTS3 may perform a role in the invasion and metastasis of tumors, and may also possess proteolytic activity that results in the breakdown of the extracellular matrix (ECM). Insulin itself can modulate the biosynthesis of ECM macromolecules that are altered in diabetes through various pathways.

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Introduction

Chondrosarcoma is one of the most common bone tumors and originates from hyaline cartilage. Although this cancer affects individuals of all ages, chondrosarcoma most frequently occurs in the elderly. The most notable symptom is pain, but pathological bone fractures may also occur. The pelvic bone, shoulder and small bones in the hands and feet are the most commonly affected (1). In chondrosarcoma, surgical resection is the best treatment option, as the tumor is resistant to chemotherapy and radiotherapy. While low-grade tumors spread locally, high-grade tumors undergo metastasis, most commonly to the lung. These tumors resemble normal cartilage and produce a dense cartilaginous extracellular matrix (ECM) (2). No non-invasive option is available for the treatment of chondrosarcoma at present (3). To treat chondrosarcoma, the inhibition of matrix metalloproteinases and angiogenic activities are particularly used (4).

The OUMS-27 cell line produces proteoglycan and type II, IX and XI collagen and is derived from chondrosarcoma cells, which produce cartilage (5). This cell line

is a useful model for studies investigating the association between chondrocytes and the ECM, and the development, differentiation and treatment of chondrosarcoma (6-9). Studies performed using these cells have revealed that recombinant chondromodulin is an anti-angiogenic factor, while vascular endothelial growth factor (VEGF) and fibroblastic growth factor (FGF) are angiogenic factors (4). However, the etiology of chondrosarcoma tumor formation has not been completely elucidated. The tumor development depends on several mechanisms, including angiogenesis and remodeling of the ECM. During the process of local invasion and metastasis of tumors, the association between the tumor cells and ECM is crucial (10).

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases are a group of secreted proteases that contains 19 members in humans (11). These proteins perform important tasks in the protection of the structure and function of cells in humans, particularly in the turnover and remodeling of the ECM. In addition, these proteins are involved in coagulation, angiogenesis, inflammation and fertility (12). ADAMTS proteases are also associated with diseases that include Alzheimer's disease, arthritis, tumors, atherosclerosis, tendinopathy, stroke and Ehler-Danlos syndrome (EDS) (13). As a unique ADAMTS family, ADAMTS2, ADAMTS3 and ADAMTS14 are also termed procollagen N-proteinases, and are involved in the processing of procollagens to collagen and maturation of type I collagen (14). Mutations in the ADAMTS2 gene, located on the long arm of chromosome 5, causes Ehlers-Danlos Syndrome (EDS) (15). In EDS, there is a dysfunction in the processing of type I procollagen into collagen. ADAMTS3 is involved in pathologies of the skin, cartilage, lung and aorta, whereas ADAMTS2 gene knockdown in mice results in the development of a lung disease. ADAMTS3 is essential in collagen-I-rich tissues and cartilage (16,17). ADAMTS14, located on chromosome 10, is associated with knee osteoarthritis (18).

Insulin, which is produced by the β cells of the pancreas, regulates carbohydrate and fat metabolism in organisms. Insulin receptors are present in the majority of tissues in the body, including chondrocytes. In mouse chondrosarcoma, it was demonstrated that insulin upregulates the synthesis of the ECM, which consists of collagen, hyaluronic acid and proteoglycan, in chondrocytes (19,20). The insulin receptor in these cells stimulates proteoglycan synthesis, and tunicamycin inhibits this process by binding to the insulin receptor (21,22).

In order to achieve an improved understanding of the reasons for metastasis, local invasion, and the resistance to chemotherapy and radiotherapy in chondrosarcoma, in addition to the effect of insulin on the cancer cells, a cell culture method was designed in the present study to determine the effects of insulin on the procollagen N-proteinases ADAMTS2, ADAMTS3 and ADAMTS14.

Materials and methods

Cell culture. The OUMS-27 chondrosarcoma cells used in the present study were kindly provided by Dr Kunisada from Okayama University Graduate School of Medicine and Dentistry (Okayama, Japan). Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum and 10,000 U/ml penicillin/10,000 μg/ml streptomycin (Hyclone SV30010; GE Healthcare Life Sciences, Logan, UT, USA), was used to culture the chondrosarcoma cells at 37˚C in a humidified atmosphere of 5% CO_2 in air. The cells were sub-cultured every 7-10 days with trypsin plus EDTA at split ratios of 1:2-1:4. The medium was changed every other day, with the cells being cultured in either control media or control media supplemented with 10 μg/ml insulin for 11 days in total. Four groups of cells were treated with insulin: $2x10^5$ cells for the experiment on day 1; $1x10^5$ cells for the experiment on day 3; $5x10^4$ cells for the experiment on day 7; and $3x10^4$ cells for the experiment on day 11 were plated in 20-mm dishes and treated with the same concentration of insulin on the indicated days. Following the experiment, the cells were harvested and total RNA measurements were performed.

Isolation of total RNA. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In total, 2 μg of mRNA was reverse transcribed using ReverTra Ace (Thermo Fisher Scientific, Waltham, MA, USA) with random hexamers (Thermo Fisher Scientific) and random primers, according to the manufacturer's instructions (Table I). Human GAPDH was amplified to act as a control for the polymerase chain reaction (PCR). Those samples lacking reverse transcriptase were amplified as a control for genomic DNA contamination. RNase-free water was utilized for the elution of total RNA from each sample. Ultraviolet spectrophotometry was utilized to quantify and determine the purity of each sample.

Quantitative PCR (qPCR). qPCR was conducted on obtained cDNA samples (Rotor-Gene Q; Qiagen, Venlo, Limburg, Netherlands) as previously described (12). The intercalating dye SYBR green (Maxima SYBR Green/ROX qPCR Mater Mix, Thermo Fisher Scientific) was used for qPCR of the total RNA in the presence of primer pairs. The PCR mixture consisted of SYBR Green PCR Master Mix, which consists of DNA polymerase, SYBR Green I dye, deoxynucleotide triphosphates, including deoxyuridine triphosphate, PCR buffer, 10 pmol forward and reverse primers and cDNA of samples in a total volume of 20 μl. The amplification of the housekeeping gene GAPDH was utilized for normalizing the efficiency of cDNA synthesis and the amount of mRNA applied. PCR was performed with an initial denaturation at 95˚C for 5 min, followed by amplification for 40 cycles, each cycle consisting of denaturation at 95˚C for 10s, annealing at 57˚C for 30s and polymerization at 72˚C for 30s. The final stage consisted of polymerization at 72˚C for 5 min. The results for the PCR performed for ADAMTS2, ADAMTS3 and ADAMTS14 were represented as graphic charts. The bars and error bars represent the mean and standard deviation of the mean, respectively.

Statistical analyses. SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) was utilized for the statistical assessment of the data, and the non-parametric Kruskal Wallis test was used. The association between the variables was assessed using the Mann-Whitney U test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. The forward and reverse primers used in the real-time polymerase chain reaction analyses for ADAMTS2, ADAMTS3, ADAMTS14, and GAPDH.

Gene	Direction	Primer sequence	Product size, bp
ADAMTS2	Forward	ACATCAACGTGGTCCTGGTG	148
	Reverse	TATTCATCGTGGCCCGTGTC	
ADAMTS3	Forward	ACCATGATGAGTCCCTCGGA	128
	Reverse	GCGACACACATTCTCCAAGC	
ADAMTS14	Forward	TGAGTCCCTGGGGGTTCATA	180
	Reverse	ACAACGTGGTCATGGTGCT	
GAPDH	Forward	CCTGCACCACCAACTGCTTA	108
	Reverse	TCTTCTGGGTGGCAGTGATG	

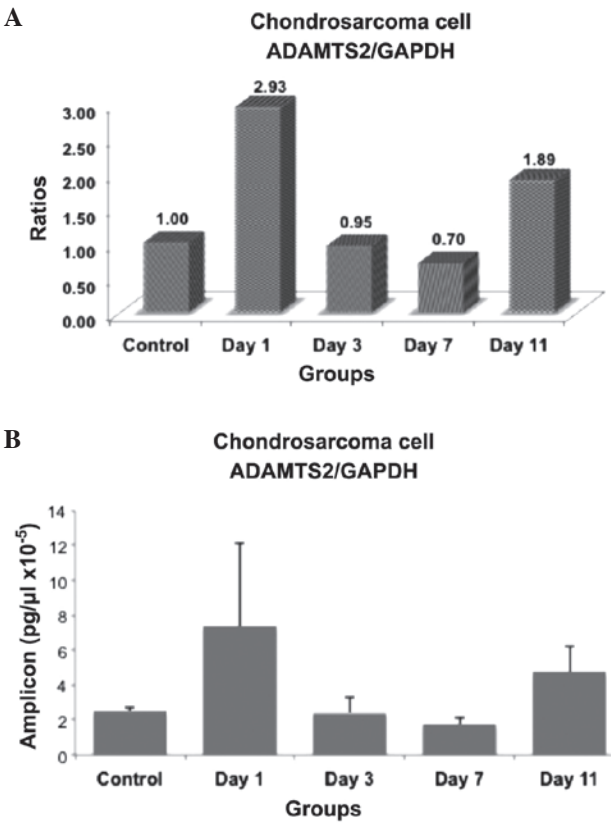

Figure 1. (A and B) Quantitated results of reverse transcription-quantitative polymerase chain reaction for ADAMTS2 in five experiments. The values were standardized by normalizing ADAMTS2 expression against GAPDH expression. Statistically significant differences were identified between the control and day 7 (P=0.028), control and day 11 (P=0.016), day 1 and day 7 (P=0.016), day 3 and day 11 (P=0.047), and day 7 and day 11 (P=0.008). ADAMTS2, A disintegrin and metalloproteinase with thrombospondin

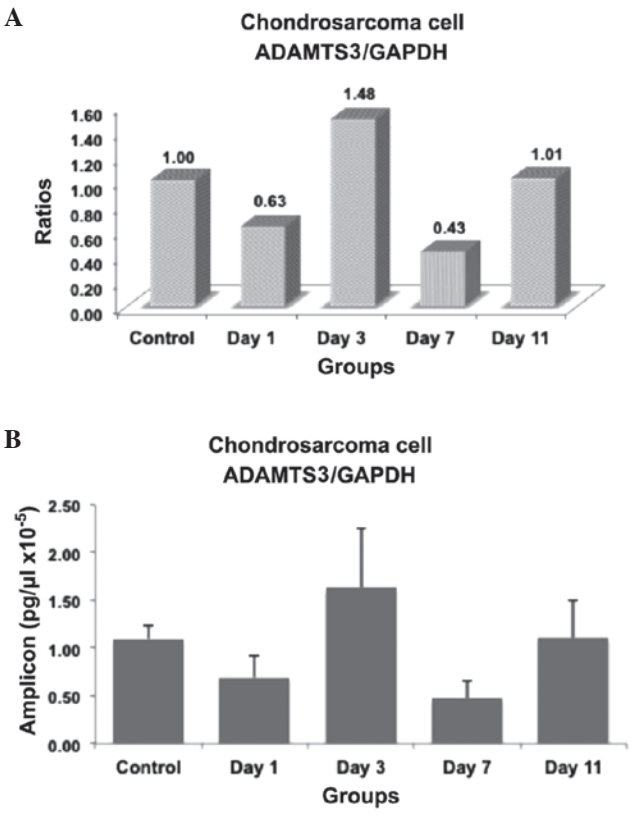

Figure 2. (A and B) Quantitated results of reverse transcription-quantitative polymerase chain reaction for ADAMTS3 in five experiments. The values were standardized by normalizing ADAMTS3 expression against GAPDH expression. Statistically significant differences were identified between the control and day 1 (P=0.008), the control and day 7 (P=0.008), day 1 and day 3 (P=0.016), day 1 and day 7 (P=0.016), day 3 and day 7 (P=0.008), and day 7 and day 11 (P=0.008). ADAMTS3, A disintegrin and metalloproteinase with thrombospondin motifs 3.

Results

The present study examined whether the expression of the ADAMTS2, ADAMTS3 and ADAMTS14 genes are upregulated or downregulated by insulin in chondrosarcoma OUMS-27 cells. The results were summarized in Figs. 1-3. The ratios and amplicon concentrations of the insulin-induced cells compared with the control cells are shown in Figs. 1-3. In these figures, the mRNA expression levels were expressed as ratios within the groups (Figs. 1A, 2A and 3A) and the PCR product concentration was expressed as pg/ml (Figs. 1B, 2B and 3B). Reverse transcription (RT)-qPCR analyses demonstrated that ADAMTS2 mRNA expression had increased compared with the control group on day 1 subsequent to insulin induction, but the expression was not significantly different due to the large range of individual results (P=0.095). On day 3, the ADAMTS2 levels were not significantly different compared with the control group (P>0.05), whereas a significant decrease

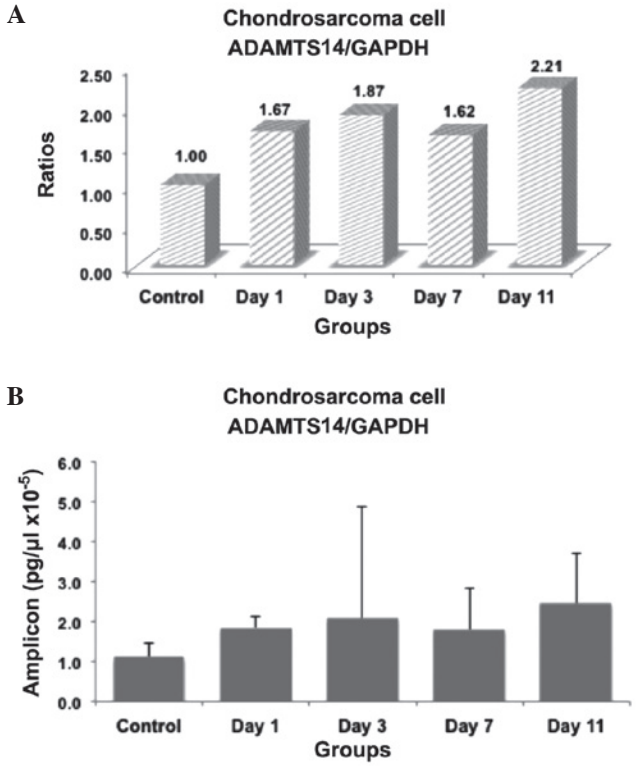

Figure 3. (A and B) Quantitated results of reverse transcription-quantitative polymerase chain reaction for ADAMTS14 in five experiments. The values were standardized by normalizing ADAMTS14 against GAPDH. No statistically significant differences were identified between the control and insulin-induced groups, or between the four insulin-induced groups. ADAMTS14, A disintegrin and metalloproteinase with thrombospondin

in ADAMTS2 expression was observed on day 7 (P=0.028) and an increase was also observed on day 11 in the cells induced with insulin compared with the control group (P=0.016). The increase in mRNA concentration observed on day 11 was significantly different from the mRNA concentration on days 3 (P=0.047) and 7 (P=0.008). ADAMTS3 mRNA expression decreased immediately subsequent to insulin induction on day 1 compared with the control group (P=0.008). The most evident decrease in mRNA concentration was observed on day 7 subsequent to insulin induction (P=0.008). No significant difference was identified between the amplicon concentration in insulin-induced cells on days 3 and 11 compared with the control cells. Significant differences were detected between the concentrations observed in the insulin-induced cells on days 1 and 3 (P=0.016), days 1 and 7 (P=0.047), days 3 and 7 (P=0.008) and days 7 and 11 (P=0.008). No statistically significant differences were identified between the ADAMTS14 mRNA concentrations in the control and insulin-induced groups.

Discussion

There are a limited number of studies in the literature that assess the effects of insulin on chondrosarcoma OUMS-27 cells (23,24). In addition, to the best of our knowledge, no studies have investigated the expression levels of procollagen N-proteinases in an insulin-induced human chondrosarcoma cell line, or the role of the procollagen N-proteinases ADAMTS2, ADAMTS3 and ADAMTS14 in chondrosarcoma. According to the present study, there were significant differences in the ADAMTS2 and ADAMTS3 mRNA concentrations between the control and insulin-induced groups, but no difference in the concentration of ADAMTS14 mRNA. As a notable finding, ADAMTS2 and ADAMTS3 have been revealed to be modulated by insulin in a time-dependent manner.

ADAMTS are secreted enzymes that are involved in ECM degradation and turnover (25). ADAMTS2, ADAMTS3 and ADAMTS14 are associated with collagen synthesis. Collagen is synthesized from procollagen by specific proteinases. These proteinases are bone morphogenetic protein-1 (BMP-1) and ADAMTS2, ADAMTS3 and ADAMTS14, also termed procollagen N-proteinases, which are a novel family of ECM proteases (26). Procollagen N-proteinases remove N-terminal peptides from procollagen to synthesize collagen (27). This event is of considerable importance for normal and pathological conditions. The degradation of the ECM occurs normally during development, growth and tissue repair (28). However, excessive degradation of the ECM is observed in several pathological conditions, including osteoarthritis and rheumatoid arthritis (29). Tumor invasion, metastasis and tumor angiogenesis require the participation of MMP, the expression of which increases in association with tumorigenesis (30); however, there is an exception. The conversion of procollagen to collagen by ADAMTS2, ADAMTS3 and ADAMTS14 was hypothesized to trigger the effects that protect tissues from tumor invasion, as it may strengthen the associated tissues through collagen synthesis (30).

ADAMTS2 is associated with type I and type II procollagen (28). The ADAMTS2 gene is located at 5q23-q24 (31), and mutations in this gene cause EDS and animal dermatosparaxis, which particularly affects the skin (15,25,32). This disease results in fragile skin, while other collagen-rich tissues, including bone and tendons, are not affected (33). Matullo *et al* (34) revealed that matrix metalloproteinases and ADAMTS2 influence survival in malignant pleural mesothelioma. These effects were attributed to the metalloendopeptidase and metallopeptidase activities of matrix metalloproteinases and ADAMTS2 (34).

In the present study, ADAMTS2 was upregulated on day 11, which may reflect increased collagen synthesis associated with tissue repair. Based on our findings, ADAMTS2 has proteolytic activity and may be involved in cancer progression, similar to other matrix metalloproteinases. Association between ADAMTS2 and the ECM causes the invasion and metastasis of cancer cells, and ADAMTS2 may be involved in this process (35). It has previously been revealed that ADAMTS1, ADAMTS9, ADAMTS12, ADAMTS15 and ADAMTS18 are associated with cancer (10). These proteases possess proteolytic activity and break down the ECM, in addition to being involved in angiogenesis in cancer (10). ADAMTS2 is a procollagen N-proteinase that may be involved in cancer development and progression.

ADAMTS3 is located on 4q21 (31) and is also involved in collagen synthesis. Similar to other procollagen N-proteinases, ADAMTS3 performs an important role during wound healing (36). ADAMTS3 is expressed in bone, cartilage and musculo-tendinous tissues (25). In human breast

carcinoma, Porter *et al* (37) revealed that ADAMTS3 was consistently downregulated, ADAMTS14 was upregulated and ADAMTS2 was not upregulated or downregulated. In the present study, ADAMTS2 mRNA expression decreased immediately subsequent to insulin induction on day 1 and the most evident decrease was observed on day 7. This finding is similar to that reported in the study by Porter *et al* (14) and may reflect the decrease in ADAMTS2 levels in cancer. However, this finding requires further investigation by additional studies.

ADAMTS14, first described in 2001, is located in 10q21on (38). Extremely few studies that investigated ADAMTS14 revealed an association between multiple sclerosis and osteoarthritis (39,40). Al Nakouzi *et al* (41) identified that the expression of ADAMTS14 was increased in metastatic prostate cancer. ADAMTS14 has also been revealed to be upregulated in human breast carcinoma (37). In the present study, no difference in the mRNA levels of ADAMTS14 mRNA was found in the OUMS-27 cells, compared with the previous studies, described above.

Extremely little is known about the role of these enzymes in cancers, particularly in chondrosarcoma. The present study provides a novel insight into the process of cartilage metabolism in cancer and the effect of insulin on this complex process. The present study also indirectly aimed to identify the effect of diabetes mellitus on the chondrosarcoma-associated outcome. It was indicated that changes associated with the application of insulin mediate crucial effects on chondrosarcoma progression, in which matrix metalloproteinases may play a critical role. In conclusion, these experiments suggest that the application of insulin is able to modulate the biosynthesis of ECM macromolecules altered in diabetes by various pathways and mechanisms. Additional studies in which other ADAMTS proteins are investigated in addition to the presently studied ADAMTS proteases are required in order to achieve more accurate information and data on the exact role of insulin in ECM metabolism in chondrosarcoma.

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