A disintegrin-like metalloproteinase with thrombospondin motif 8 expression analysis in OUMS-27 chondrosarcoma cells before and after insulin administration

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A disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) family, comprising 19 members, is zinc-dependent Matrix Metalloproteinases (MMPs) localized in the extracellular matrix (ECM). These enzymes have multidomain structures and are associated with many functions, including cleavage of aggrecan (aggrecanases; ADAMTS1, 4, 5, 8, 9, and 15) cleavage of pro-collagen (pro-collagen N-proteinases; ADAMTS2, 3, and 14) and cleavage of von Willebrand factor (vWF), (vWF cleaving protease; ADAMTS13) in the ECM. However, there is no detailed information about some ADAMTSs which are called the orphan ADAMTSs (ADAMTS6, 7, 10, 12 and 16–19). Besides the above mentioned functions, ADAMTSs also catalyze the cleavage of proteoglycans, brevican, and versican which are associated with angiogenesis, ovulation, and tissue morphogenesis in humans and are linked to numerous diseases (osteoarthritis, rheumatoid arthritis, Alzheimer’s disease, stroke, tendinopathy, Ehler–Danlos syndrome, etc.) including cancers.

ADAMTS8 is a secreted protease. It has been proven that ADAMTS8 and ADAMTS1 is an anti-angiogenic proteinase, which inhibits vascular endothelial growth factor (VEGF) induced angiogenesis, endothelial cell proliferation and fibroblast growth factor-2 (FGF-2) induced vascularization. Previous studies have demonstrated that ADAMTS8 is downregulated in cancers. Furthermore, ADAMTS8 is more effective...
than ADAMTS1 and endostatin in anti-angiogenesis activity and is also a member of the aggrecanases family and a target protein for tumor suppression.

Chondrosarcoma which produces cartilage matrix is the third most common primary malignancy of bone and is known to develop slowly. This form of cancer is frequently observed in appendicular and axial skeleton and usually appears in patients aged between 30-60 years. The most effective treatment of chondrosarcoma is surgery; radiotherapy and chemotherapy are other palliative treatment options. Chondrosarcoma cells have low cell division rate and poor vascularity, hence they are resistant to chemo/radiotherapy. Insulin is a major anabolic hormone, which affects numerous tissues including cartilage. In animal models, chondrosarcoma cells were found to be insulin dependent and the physiologic concentrations of insulin were observed to stimulate proteoglycan synthesis.

Angiogenesis and ECM degradation are known to be the key events in tumor progression. It has been reported that the OUMS-27 cell line from a patient with grade III chondrosarcoma could be a useful model for the investigations of chondrosarcoma. The OUMS-27 cells grow rapidly, have no contact inhibition and show normal chondrocyte functions (expressing proteoglycan and collagen). This cell line is a relatively useful model for understanding the nature and treatment of chondrosarcoma.

In the present study, we investigated the expression levels of ADAMTS8 in chondrosarcoma cells to elucidate the effect of insulin on the tumor cells in terms of ADAMTS production. Effect of insulin on chondrosarcoma cells in terms of ADAMTS8 expression has not been reported earlier.

**Materials and Methods**

The OUMS-27 human chondrosarcoma cells were donated by Dr. T Kunisada (Okayama University, Graduate School of Medicine and Dentistry, Okayama, Japan). The cell line has firstly been established by Kunisada et al. in 1998. The 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. Subsequently, the cells were sub-cultured at split ratios of 1:2–1:4 using trypsin plus EDTA every 7–10 days. The cells were used at passages 7–14 for all the experiments. The medium was changed every other day with either control medium or control medium supplemented with 10 µg/mL insulin for a total of 11 days.

Powdered insulin was dissolved in 0.01 N HCl solution. The stock solution contained 2 mg/mL insulin within 0.01 N HCl and the working solution contained 10 µg/mL insulin in the medium. Four groups of cells were subjected to different concentrations of insulin on different days. For the 1st, 3rd, 7th and 11th day experiments, 2 × 10⁵, 1 × 10⁵, 5 × 10⁴ and 3 × 10⁴ cells were plated onto 20 mm dishes and exposed to different concentrations of insulin (total dose) on the days indicated respectively. Total 1 × 10⁵ cells were plated onto 20 mm dishes for control group. Briefly, the cells were incubated with a specific concentration of insulin in the medium for 1, 3, 7 and 11 days. Five replicates were employed for each experiment. After each experiments, the cells were harvested and the proteins were isolated.

The total RNA was extracted with TRIzol (Invitrogen, Cat#15596-018, Carlsbad, CA, USA) according to the manufacturer’s instructions. A total of 2 µg of RNA were reverse transcribed with RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific, Cat # EP0441, Waltham, MA, USA) and random hexamers (Thermo Scientific, Waltham, MA, USA) with random primers according to the manufacturer’s instructions (Table 1). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as the control for the PCR. Samples lacking reverse transcriptase were amplified as the control for genomic DNA contamination. RNase-free water was used to elute the total RNA from each sample. UV spectrophotometry was used to quantify and determine the purity of each sample. The cDNA samples obtained were subjected to qRT-PCR (Qiagen Rotor-Gene Q RT-PCR, Limburg, Netherlands) as described in a previous report. The total RNA RT-PCR was conducted with the intercalating dye SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) Cat#K0221, Waltham, MA, USA) and exposed to different concentrations of insulin within 0.01 N HCl and the working solution contained 10 µg/mL insulin in the medium. Four groups of cells were subjected to different concentrations of insulin on different days. For the 1st, 3rd, 7th and 11th day experiments, 2 × 10⁵, 1 × 10⁵, 5 × 10⁴ and 3 × 10⁴ cells were plated onto 20 mm dishes and exposed to different concentrations of insulin (total dose) on the days indicated respectively.

**Table 1 — Forward and reverse primers used in the qRT-PCR analyses for ADAMTS8 and GAPDH**

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<td>TCCTTGCGGTTGCGTTGAT</td>
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Results
We examined whether the expression of the ADAMTS8 gene was induced or suppressed by insulin treatment in OUMS-27 chondrosarcoma cells. The results pertaining to ADAMTS8 were represented as graphs. The bars and error bars indicate the mean and standard deviations of the amplicon concentrations obtained by PCR respectively. A comparison of the ratios and amplicon concentrations of the insulin-induced cells with those of the control cells is given in Fig 1A and B. The mRNA expression levels have been shown as ratios within the groups (Fig 1A) and the PCR product concentration has been indicated as pg per mL (Figure 1B). The qRT-PCR analyses revealed that the ADAMTS8 mRNA expression decreased on day 1 after insulin induction when compared with that in the control group. This decrease was statistically significant because of the large ranges of individual results ($P = 0.008$). Furthermore, the decrease in the mRNA concentration on day 7 ($P = 0.047$) and day 11 ($P = 0.008$) was significantly different. On day 3, the ADAMTS8 levels did not change when compared with that in the control group ($P > 0.05$).

Discussion
The present study is the first to perform a detailed investigation of the ADAMTS8 gene expression in insulin-induced human chondrosarcoma cells. Significant differences in the ADAMTS8 mRNA concentrations between the control and insulin-treated groups were noted. The ADAMTS8 expression in OUMS-27 cells decreased with the administration of insulin.

Cancers are one of the most important diseases in the world which lead to mortality. Despite the relatively high prevalence the etiology of cancers is not well known. However, remodeling of ECM, apoptosis, angiogenesis and cell cycle control are known to be very important events in tumor formation and development. Although numerous tumors persist without neovascularization for a long duration, cancer cells switch to an angiogenic state in which tumor cells grow rapidly. Angiogenesis is regulated by positive (VEGF and bFGF) and negative (angiostatin, endostatin, vasostatin etc.) angiogenic regulators. VEGF and FGF-β have very important roles in vasculogenesis and angiogenesis. Angiogenesis is crucial for tumor growth, invasion and metastasis and angiogenesis inhibitors are used for cancer treatment. For example bevacizumab is used for glioblastoma, breast cancer, pancreatic cancer and colorectal cancers, in addition sunitinib, sorafenib, temsirolimus and everolimus are also used for this purpose. MMPs can have both pro-angiogenic and anti-angiogenic roles and provide a balance between the two states.
MMPs are zinc-dependent endopeptidases in the ECM and have numerous functions including normal development, wound healing and in a wide variety of pathological processes such as cancer formation and development. The ADAMTS family belongs to this group and comprises 19 members (1–10, 12–20). The ADAMTS proteinases secreted into the ECM are inhibited by the tissue inhibitor of metalloproteinase (TIMP). The ADAMTSs are known to have broad catalytic activities against a range of substrates including proteoglycan (aggrecan), brevican, versican, cartilage oligomeric matrix protein (COMP) etc., and play a role in the remodeling of ECM, which is essential for numerous important events including cancers. The ADAMTS family is associated with colorectal cancer, esophagus cancer, breast cancer, non-small-cell cancer, head and neck cancer etc. It has been reported that ADAMTS.8, which is also known as metalloprotease and thrombospondin domains (METH)-2 is located at 11q25. ADAMTS8 has greater anti-angiogenic activity than ADAMTS1 or endostatin, and this effect is exhibited by inhibiting angiogenesis and vascularization. Thus, ADAMTS8 has roles in tumor invasion and metastasis.

In the present study we found that the expression of ADAMTS8 was decreased in insulin-treated OUMS-27 cells. Several studies on cancers and ADAMTS revealed that ADAMTS8 decreases in cancer cells. In the OUMS-27 cell line, Demircan et al. showed that IL-1β did not increase the mRNA levels of ADAMTS8 and concluded that ADAMTS9 is an IL-1β- and TNFα-inducible gene that appears to be more responsive to these proinflammatory cytokines than other aggrecanases including ADAMTS8. In the present study we applied insulin to OUMS-27 cells and observed that ADAMTS8 was decreased when compared with that noted in the control group. In another study on head and neck squamous cell carcinoma (HNSCC), Stokes et al. showed that ADAMTS8 was reduced in the HNSCC group when compared with the control group, and indicated that this enzyme has a role in tumor progression. They concluded that ADAMTS8 and other MMPs might be used as a diagnostic, predictive and prognostic molecular marker for these malignant tumors in the future. In a recent study, in the control groups of chondrosarcoma ADAMTS8 was not found to decrease. In another study on breast carcinoma and ADAMTS8, Sarah et al. showed that seven ADAMTS genes including ADAMTS8 gene, were downregulated. In particular they emphasized the downregulation of ADAMTS1 and ADAMTS8 because of their high anti-angiogenic activity and concluded that cancer progression might be associated with the suppression or abrogation of antiangiogenic molecules. In the present study, after insulin treatment ADAMTS8 was decreased indicating that insulin may be associated with cancer progression in chondrosarcoma. In another study, ADAMTS8 was upregulated in malignant thyroid cancers. Furthermore, in a study on breast carcinoma, Ocak et al. investigated the effect of certain concentrations of hypericin on MCF7 cells and found that hyperican increased the expression of ADAMTS9 but did not affect the expression of ADAMTS8. Dunn et al. showed that ADAMTS8 was downregulated in brain tumors when compared with that in normal brain tissue, however, the reduction was not tumor type specific (metastasis, glioma, meningioma, medulloblastoma, or hemangioblastoma). In another study, ADAMTS8 was downregulated in non-small-cell lung carcinomas (NSCLCs), whereas a study on the role of ADAMTS8 and ADAMTS1 in pancreatic cancer did not reveal any relation between ADAMTS8 and pancreatic cancer.

Based on the above-mentioned findings, it can be concluded that the expression of ADAMTS8 is usually downregulated in tumors. However, it has been reported that in chondrosarcoma cells the expression of ADAMTS8 did not change. This finding may be associated with the fact that chondrosarcoma is a relatively less aggressive tumor when compared with the other tumors studied. However, upon insulin treatment, the expression of ADAMTS8 was downregulated which suggested that insulin may be a negative effector on tumor formation and metastasis. Further studies are needed to establish the cause–effect relationship between insulin-treated chondrosarcoma cells and ADAMTS8. In addition, the effects of insulin on other tumor cells should also be investigated.

References
4. Guo F, Lai Y, Tian Q, Lin EA, Kong L & Liu C, Granulin-epithelin precursor binds directly to ADAMTS-7 and


