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Expression Pattern of Human ADAMTS-2 (A disintegrin and metalloproteinase with thrombospondin motif, 2) in Different Cell Lines

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Abstract

The ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a family of metalloproteases that are found in mammals and invertebrates. This family is known to influence development, angiogenesis, coagulation and progression of arthritis. ADAMTS-2 plays an essential role in the processing of the fibrillar collagen precursors into mature collagen molecules by cleaving of the amino terminus of the type I, II, III and V procollagen molecules. Collagens are the most abundant and important components of the extracellular matrix. Mutations in the ADAMTS-2 gene cause Ehler Danlos Syndrome Type VIIC in humans. Expression analysis of the ADAMTS-2 in some human tissues have been investigated so far. There is restricted studies on the expression pattern of ADAMTS-2 gene in cell lines. In this study, ADAMTS-2 mRNA expression levels were determined by semiquantitative RT-PCR analysis in MG-63, Saos-2, DU-145, PC-3, HUVEC, HeLa, HL-60 and Isichawa cell lines. ADAMTS-2 protein was visualised in Saos-2 osteosarcoma cell model by immunofluorescence for the first time. This preliminary studies about ADAMTS-2 gene expression levels will be beneficial for further ADAMTS-2 gene regulation studies in different cancer models.

Keywords: MMP, ADAMTS-2, collagen, sqRT-PCR, Immunofluorescence

INTRODUCTION

Extracellular matrices (ECM) are secreted molecules that provide structural and biochemical support to many tissues such as basement membrane, bone and cartilage. Glycoproteins, collagens, glycosaminoglycans and proteoglycans are the components of the ECM. For the function of the cells, they must be properly supported, having contacts with neighbouring cells and/or the extracellular matrix (ECM). Cell movement and tissue remodelling are important in both physiological and pathological processes. In order for these processes to occur, the ECM must be degraded to allow the free movement of cells or the processing and deposition of new matrix. These processes are accomplished by proteases and also heparanase [1, 2]. There are two well-known families of proteases that are involved in the biology of the ECM, the matrix metalloproteinase (MMP) and the A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) families. [1, 3].

ADAMTSs are extracellular, multidomain metalloproteinases that first described in mice by Kuno and his colleagues in 1997 and have subsequently been identified in mammals and *Caenorhabditis elegans* [4, 5]. 19 members of this family have been identified in humans [6]. ADAMTSs have significant roles in processing amino terminus of the procollagen molecules, cleavage of the matrix proteoglycans aggrecan, versican and brevican; inhibition of angiogenesis; and blood coagulation homoeostasis as the von Willebrand identified [11]. The expression of ADAMTS-2 mRNA has been demonstrated in a number of tissues such as aorta, bone, skin, tendon, bladder, retina, kidney, lung, intestine, liver and skeletal muscle in the body [12]. Until now, only limited data are available on the expression of ADAMTS-2 in the cell lines. In this study, we determined ADAMTS-2 mRNA expression levels in different cell lines by semiquantitative RT-PCR analysis. We also visualised ADAMT-2 protein in Saos-2 cells by immunofluorescence. This studies would be beneficial further transcriptional regulation studies on ADAMTS-2 gene in different cancer cells.

MATERIAL AND METHODS

Cell Culture

Saos-2 (sarcoma osteogenic) cells were provided from Dr. Deborah Mason (Cardiff, School of Biosciences, Cardiff UK), MG-63 (human osteosarcoma) cells were provided from Dr. Kenneth Wan (Cardiff, School of Biosciences, Cardiff UK), HeLa (human cervical cancer) cells were provided from Dr.Dipak Ramji (Cardiff, School of Biosciences, Cardiff, UK), HUVEC (human umblical vein endothelial cells) were provided from Dr. Ayşe BegümTEKİNAY (National Nanotechnology Research Center, Bilkent University, UNAM), PC-3 and DU-145 (human prostate cancer) were provided from Prof. Dr. Kemal S. Korkmaz (Ege University, Faculty of Bioengineering, İzmir, TURKEY), HL-60 (human myeloid leukemia) and

HT-29 (human colon adenocarcinoma) cells were provided from Prof. Dr. İsmet D. GÜRHAN (Ege University, Faculty of Bioengineering, İzmir, TURKEY). Ishikawa (human endometrial adenocarcinoma) cells were provided from Prof. Dr. Ayhan Bilir (Çapa Medical Faculty, Istanbul, TURKEY). The adherent cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (1 g/L glucose) and HL60 suspension cells were cultured in RPMI (Roswell Park Memorial Institute medium) (Both from Invitrogen) supplemented with 10% heat inactivated (56°C for 1 h) fetal calf serum (Sigma) and 2 mM of L-Glutamine (Hyclone). The cultures were maintained at 37°C in a humidified incubator containing 5% (v/v) CO_2 in air.

RNA Isolation and Semi-quantitative RT-PCR (sq RT-PCR)

Total RNA was extracted from cultured cells using the GeneJET™ RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) using Revert Aid Reverse Transcriptase (200 U) and oligo(dT) as primer (100 pmol), (Both from Fermentas). PCR was performed 50 µl final volume using 1µl of cDNA and 1.25 U of Taq DNA Polymerase (Fermentas). ADAMTS-2 forward: 5'-CTG TGG CGA CGA GGT GCG-3', ADAMTS-2 reverse 5'-GGT GCA CAC ATA GTC CCG TCC-3' primers were used for PCR amplification for ADAMTS-2 and H-β-2 forward: 5'-TTT CTG GCC TGG AGG CTA TC-3' and H-β-2 reverse: 5'-CAT GTC TCC ATC CCA CTT AAC T-3' primers were used to amplify H-β-2 microglobulin genes. Appropriate number of cycles was determined by testing the different cycles of 18, 20 22, 30 and 35 for ADAMTS-2 and human β-2 microglobulin genes. Cycling conditions were optimised for human ADAMTS-2. The reaction conditions for human ADAMTS-2 were 94°C for 3 min, (94 °C for 45 s, 58 °C for 45 s, 72 °C for 45 s) 35 cycles and 72C for 10 min final step. For human β -2-microglobulin, 55°C annealing temperature and 22 cycles was performed. These conditions were in the exponential phase of amplification and therefore provided a direct correlation between the amount of products and RNA template abundance in the samples [13]. The PCR products were size-fractionated on a 1% (w/v) agarose gel, photographed using a Gel Doc documentation system. The intensity of ethidium-bromide luminescence images was quantified densitometric analysis using ImageJ image-processing software package (ImageJ: National Institutes of Health), and normalized in respect to the corresponding fragment concentration of the ubiquitously expressed genes human β-2 microglobulin.

Immunofluorescence

Saos-2 cells were cultured on the 12 mm coverslips in a 24-well plate. Cells were fixed with 4% paraformaldehyde in PBS at 30 min. Permeabilization were performed using 0.3% triton X-100. Cells were incubated in 1% BSA in PBS to block unspecific binding of the antibodies. Rabbit polyclonal anti-Adamts-2 antibody (ab125226) was used as primary antibody (Abcam) and Alexa Flour 488 conjugated goat anti rabbit IgG (H+L) was used as secondary antibody. Cells were stained with DAPI (2-(4-amidinophenyl)-1H -indole-6-carboxamidine) for nucleus determination. Labeled cell preparations were then examined with Olympus BX51 ED200 fluorescence microscope and images were captured Olympus DP72 CCD camera. Images were photoshop CS2 programme was used.

RESULTS

Analysis of the ADAMTS-2 mRNA Levels in Different Cell Lines

The expression of ADAMTS-2 mRNA levels were demonstrated in a number of cell lines namely adherent MG-63, Saos-2, PC-3, DU-145, HT-29, HUVEC, Ishikawa and suspension HL-60 cells. After cells were cultured as described material and methods 1µg of RNA reverse transcribed into cDNA. 1µl of the cDNA was used in PCR amplifications. ADAMTS-2 and human β-2 microglobulin were amplified with using gene specific expression primers. PCR conditions were performed as stated before. H-β-2 was used as an internal control. Densitometric analysis was performed using Image J (NIH) programme and normalised to h-β-2. Maximum ADAMTS-2 mRNA level was determined in MG-63 (bone osteosarcoma) cells that have fibroblastic character (~0.8 fold) and respectively in PC-3 (0.151 fold), Ishikawa (0.15 fold), Saos-2 (0.139 fold), HT-29 (0.10 fold), HeLa (0.08 fold), DU-145 (0.06 fold), HL-60 (0.06 fold) and HUVEC (0.012 fold) cells as can be seen in Figure 1.

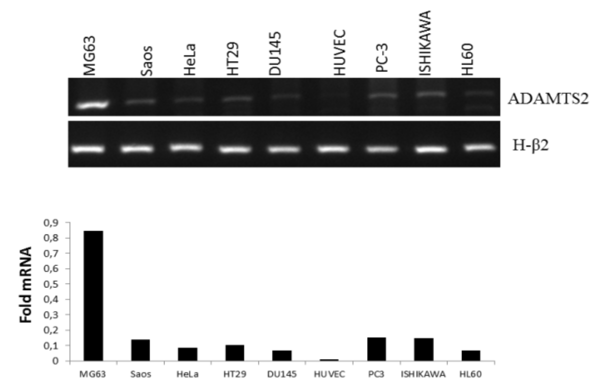

Figure 1. ADAMTS-2 mRNA expression pattern in human cell lines analyzed by semiquantitative RT-PCR. Cells were cultured as described material and methods. Total RNA was isolated according to manufacturer's instructions. 1µg of total RNA was reverse transcribed into cDNA. ADAMTS-2 was amplified using specific expression primers. Human β-2 microglobulin was used as an internal control. Image was analysed densitometrically with Image J (NIH) programme. The values shows fold ADAMTS-2 mRNA levels.

ADAMTS-2 Protein Analysis in Saos-2 Cells by Immunofluorescence

Subcellular localisation of the proteins determine their functions. Subcellular localisation of the ADAMTS-2 protein analysed with immunofluorescence using specific antibodies. Because of large size of the nucleus of the Saos-2 cells its difficult to clearly identify cytoplasm region and therefore exact localization of the proteins with immunofluorescence. To investigate ADAMTS-2 protein localisation in Saos-2 cells they were cultured on cover slips in 24-well plates (125 X10^3 cells/well) and ADAMTS-2 protein was labelled as described material and methods. It can be suggested that in cells ADAMTS-2 protein, although observed in the cytoplasm, was present predominantly in the nucleus (Figure 2).

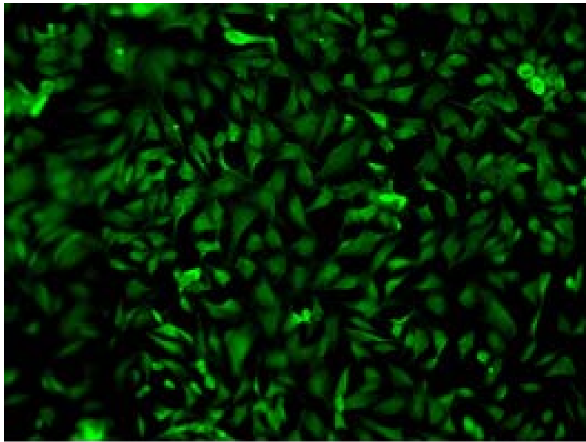

Figure 2. Immunofluorescence staining of the ADAMTS-2 protein (in green) in Saos-2 cell line.

DISCUSSIONS

ADAMTS-2 cleaves the amino terminus of the type I, II, III and type V fibrillar collagen precursors. It is the key regulator of procollagen maturation and collagen fibril formation. ADAMTS-2 is essential for maturation of type I collagen fibrils in the skin, and that neither ADAMTS-3 nor ADAMTS-14 compensate adequately for ADAMTS-2 deficiency in this tissue [5, 14]. Mutations in the ADAMTS-2 gene cause Ehler Danlos Syndrome type VIIC that an autosomal recessive disorder characterized by severe skin fragility, joint laxity (hyperextensibility) and characteristic facies (short stature, micrognathia, epicanthic folds and depressed nasal bridge) [5]. ADAMTS-2 also has important roles in male fertility and correct dentin formation [9, 10]. In a recent study anti-angiogenic and anti-tumoral functions of ADAMTS-2 have been demonstrated [11].

To date ADAMTS-2 expression levels in different tissues ,in the human body, have been determined [12]. There is restricted studies on the expression levels of ADAMTS-2 in different cancer cell lines. In these studies ADAMTS-2 expression was determined in HEK 293 EBNA (human embryonic kidney), MG-63 (human osteosarcoma), MM6 (mono Mac6), THP-1(acute monocytic leukemia), A549 (adenocarcinomic human alveolar basal epithelial cells), Calu-3 (lung adenocarcinoma), Colo320 (human colon adenocarcinoma), BT-20 (mammary gland/breast carcinoma), ARPE-19 (retinal pigment epithelial cells) and some fibroblastic cell models [15-17].

In this research ADAMTS-2 mRNA expression levels were determined by sq RT-PCR analysis. Among the investigated cell lines ADAMTS- 2 mRNA was found most abundant in MG-63 cell line. As a similar osteosarcoma model, Saos-2 has lower expression level than MG-63 as expected. Because MG-63 cells have fibroblastic origin whereas Saos-2 cells have epithelial origin. This indicates that MG-63 cells were immature cancer stage of bone osteosarcoma than Saos-2 cells. Among the investigated classical cell line of prostatic cancer, DU145 cells have moderate metastatic potential compared to PC-3 cells which have high metastatic potential [18]. ADAMTS-2 mRNA expression was more abundant in PC-3 cells than DU-145. PC-3, Ishikawa, Saos-2, HT-29, HeLa, DU-145, HL-60 and HUVEC cells have decreased ADAMTS-2 expression levels respectively. These findings would be useful for choosing cell model for ADAMTS-2 gene regulation studies in different cancers. Determining of the subcellular localization of proteins provides important information about its function. The other part of the study, ADAMTS-2 protein was visualized in Saos-2 cells by immunofluorescence. As can be seen in the Figure 2, ADAMTS-2 protein can be detected in the nucleus of the permeabilized Saos-2 cells as well as in the cytoplasm. This finding would be beneficial for further investigations on the specific functions of the ADAMTS-2 protein.

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