Molecular Cloning of a Gene Encoding a New Type of Metalloproteinase-disintegrin Family Protein with Thrombospondin Motifs as an Inflammation Associated Gene*

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A cellular disintegrin and metalloproteinase (ADAM) is a new family of genes with structural homology to the snake venom metalloproteinases and disintegrins. We screened genes which were selectively expressed in the cachexigenic colon 26 adenocarcinoma subline in vivo. It was found that one novel cDNA clone, identified as a cachexigenic tumor selective gene, encodes a cysteine-rich protein which shows a sequence similarity to that of both the snake venom metalloproteinases and thrombospondins. We named this cDNA clone A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1). ADAMTS1 consists of six domains, 1) a pro- and 2) a metalloproteinase, 3) a disintegrin-like, 4) a thrombospondin (TSP) homologous domain containing TSP type I motif, 5) a spacer region, and 6) COOH-terminal TSP submotifs. Unlike other ADAMs, ADAMTS-1 does not possess a transmembrane domain and is a putative secretory protein. Therefore, ADAMTS-1 is a new type of ADAM family protein with TSP type I motifs. We demonstrated that the TSP homologous domain containing the TSP type I motif of ADAMTS-1 is functional for binding to heparin. ADAMTS-1 mRNA could be induced by stimulating colon 26 cells with an inflammatory cytokine, interleukin-1, in vitro. Moreover, intravenous administration of lipopolysaccharide in mice selectively induced ADAMTS-1 mRNA in kidney and heart. These data suggest that ADAM-TS-1 may be a gene whose expression is associated with various inflammatory processes as well as development of cancer cachexia.

A disintegrin and metalloproteinase (ADAM) represents a new family of genes that show a significant sequence similarity to snake venom metalloproteinase (1) and disintegrin (2). Snake venom disintegrins are a family of anticoagulant peptides with a high cysteine content (3). At this moment, 11–13 ADAM genes, including fertilin, epididymal apical protein I, cyritestin, MDC, melitin, MS2, and metargidin, have been identified (4–10). Typical ADAMs are cell surface proteins which consist of pro-, metalloprotease-like, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane and cytoplasmic domains. Fertilin, the first ADAM described, has been implicated in integrin-mediated sperm-egg binding (4). Similarly, melitin has been shown to be required for myotube formation (8). Therefore, this family is thought to function in cell-cell interaction. On the other hand, the other members of the ADAM family, MDC is a candidate for tumor suppressor in human breast cancer and MS2 is a macrophage surface antigen (7, 9). However, the physiological role of these ADAMs has not been established yet.

Cachexia is frequently associated with cancer as well as bacterial, viral, and protozoan diseases (11). Cancer cachexia is a complexed pathological state characterized by progressive weight loss, anorexia, asthenia, anemia, and altered metabolic blood parameters, e.g. hypertriglyceridemia and hypoglycemia (12–14). Weight loss results from catabolism of host body compartments, particularly muscle and adipose tissue. In addition to several mediators including toxohormone-L (15) and proteolytic factor (16), lipid mobilizing factor (17), leukemia inhibitory factor (18), and interferon-γ (19), the involvement of inflammatory cytokines, such as tumor necrosis factor (20–22), IL-1 (23, 24), and IL-6 (25–27) has been suggested in various cancer cachexia models. In the mouse colon 26 adenocarcinoma model, the involvement of IL-6 in the induction of cachexia has been suggested (25, 26). Concomitant with the elevation of the serum IL-6 level, an increase in serum acute phase proteins was observed, suggesting that systemic inflammation occurs in mice bearing colon 26 cachexigenic tumor (26).

In this study, to elucidate the complexed pathological state during development of cancer cachexia, we have searched for genes which were selectively expressed on colon 26 cachexigenic sublines under in vivo tumor bearing states by means of differential display methods (28). The result enabled us to identify a new gene which is selectively expressed in the cachexigenic tumor line. This gene, named ADAMTS-1, encodes a novel ADAM family protein with thrombospondin (TSP) motifs. We also demonstrated that the expression of the ADAMTS-1 gene is closely associated with acute inflammation.

** MATERIALS AND METHODS **

Mice—Pathogen-free, 8–10-week-old BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). Animals were quarantined for 1 week under pathogen-free conditions at the Animal Research Center of Kanazawa University to confirm the absence of disease before use. Cell Culture and Inoculation of Tumors—The murine colon 26 adenocarcinoma cell line, clones 20 and 5, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Subconfluent cells were treated with trypsin and resuspended at the concentration of 5 × 10^6 cells/ml in phosphate-buffered saline. Two hundred μl of the cell suspension (1 × 10^6 cells) was inoculated into the footpad of each mouse. Twelve days after inoculation of the tumor cells, tumors formed...
in the footpad were removed and total RNA was immediately prepared by RNAzol B (Biotex Laboratory, Houston, TX).

In some experiments subconfluent colon 26 cells were incubated in the presence or absence of 10 ng/ml recombinant mouse IL-1α (29) for the indicated time period. Total RNA was prepared by RNAzol B.

**Differential Display Analysis**—For the differential display assay, total RNA was treated with Message Clean kit (GenHunter, MA) to remove the chromosomal DNA contamination. Differential display analysis was performed as described (28) using RNA map kits (GenHunter). Two hundred ng of the total RNA was incubated in a 20-μl reverse transcription reaction with T<sub>7</sub>MN primer at 37 °C, followed by PCR reaction with various combinations of T<sub>7</sub>MN and AP primers in the presence of [γ<sup>32</sup>P]dCTP (Amersham). The cycling parameters were as follows: 94 °C for 30 s, 40 °C for 2 min, 72 °C for 30 s for 40 cycles followed by 72 °C for 5 min. The amplified products were separated on a 6% DNA sequencing gel and analyzed by autoradiography.

**Reamplification of PCR Product and Cloning of cDNAs**—cDNA bands of interest were excised from the DNA sequencing gel and DNA was eluted by heating at 95 °C for 15 min and recovered by ethanol precipitation. cDNAs were reamplified by a PCR reaction with oligo(dT)12 BamHI and AP primers anchoring the EcoRI site. Amplified cDNA fragments were digested by EcoRI and BamHI and cloned into the pCR1 vector (Invitrogen).

**Northern Blot Analysis**—Sixteen μg of total RNA was separated by a 1% denaturing agarose gel and transferred onto a nylon membrane (Amersham, United Kingdom). The blots were hybridized to probes prepared by labeling isolated fragments from the cDNA clones with [γ<sup>32</sup>P]dCTP as described previously (24). Hybridization with an 18 S rRNA probe confirmed that equal amounts of RNA were loaded in each lane.

**Construction of cDNA Library and Isolation of a Full-length 151C (ADAMTS-1) cDNA**—A murine colon 26 clone 20 tumor cDNA library was constructed by AZAP cDNA synthesis kit (Stratagene). This library contained 8 × 10<sup>6</sup> independent clones, and the average insert size was about 1.5 kb. After screening 10<sup>6</sup> phages with a 151C partial cDNA fragment, 6 positive clones with different size were identified and converted into phagemids according to the *in vitro* excising protocol (Stratagene). The longest phagemid clone, pBL151C-24, contained a 4.6-kb cDNA of 151C gene, but lacked the NH<sub>2</sub>-terminal region.

5'-Rapid amplification of cDNA ends reaction was performed to isolate the 5'-end of 151C cDNA by means of Marathon cDNA amplification kit (Clontech). In brief, 1 μg of clone 20 tumor-derived poly(A) RNA was reverse-transcribed with a specific primer (primer 7) of 151C gene, followed by second-strand cDNA synthesis. After ligation of the adapter with T<sub>4</sub> ligase, the first PCR amplification was performed using the primer 7 and the primer within the adapter. The second PCR reaction was performed using first PCR products as a template with the internal primer (primer 8) of the 151C gene and another primer within the adapter. PCR products were digested with appropriate restriction enzymes and cloned into pGEM vector (Promega).

For the sequencing of the 151C (ADAMTS-1) gene, a restriction enzyme map was generated and DNA fragments were subcloned into the pGEM vector. DNA sequencing analysis was performed using a PCR procedure employing fluorescent deoxyxynucleotides and a model 373A automated sequencer (Applied Biosystem).

**Preparation of GST Fusion Protein and Binding to Heparin-Sepharose**—A cDNA corresponding to the TSP homologous domain of ADAMTS-1 (amino acids 535–615) was amplified by PCR and subcloned into GST fusion protein expression vector (Pharmacia). GST fusion protein was expressed as described in Ref. 30.

**GST Fusion Protein**—GST fusion protein (30 μg) was incubated with 50 μl of heparin-Sepharose beads (Pharmacia) in the binding buffer (20 mm sodium phosphate (pH 7.5), 1% Triton X-100, 50 mm NaCl) in batch. After washing the beads, samples were eluted stepwise with the same buffer containing 0.15, 0.25, 0.5, 0.75, 1, and 2 mm NaCl.

**In Vitro Transcription and Translation**—ADAMTS-1 cDNA was subcloned into Bluescript SK+ (Stratagene) and transcribed by T7 polymerase, and subsequently translated in a rabbit reticuolyte lysate (Promega Biotech) in the presence of [35S]methionine (Amersham).

**LPS Treatment**—LPS (Escherichia coli 055:B5) purchased from Difco (Detroit, MI), was resuspended in sterile pyrogen-free saline solution and injected intravenously at a dose of 10 μg/mouse. Five h after treatment, mice were sacrificed and total RNA was quickly extracted from several organs with RNAzol B.
ing the selective expression of 151C gene in cachexigenic tumor in vivo. Isolation of full-length 151C cDNA—Short cDNA fragments obtained by differential display analysis are most probably derived from the 3'-noncoding region. In order to determine the complete structure of 151C gene, we isolated a full-length cDNA of 151C. A mouse colon 26 tumor (clone 20) cDNA library prepared from clone 20 tumor poly(A) RNA was screened with the 151C cDNA fragment as a probe. The largest phagemid clone contained a 4-kb 151C cDNA fragment, but this was smaller than the mRNA size (4.6 kb) as determined by Northern blot analysis. Therefore, the 5'-end region of 151C cDNA was isolated by means of 5'-rapid amplification of cDNA end PCR reaction with specific primers. In 5'-rapid amplification of cDNA end reactions using a clone 20 tumor poly(A) RNA, we obtained an 800-base pair PCR product, a part of which overlapped with the sequence of the phagemid clone. Utilizing this fragment, we obtained a 4.6-kb full-length cDNA of the 151C gene.

151C (ADAMTS-1) Gene Encodes a Novel Metalloproteinase-disintegrin Protein with TSP Motifs—Sequencing analysis showed that 151C cDNA clone contained a single open reading frame encoding 951 amino acids. The deduced amino acid sequence of 151C protein is shown in Fig. 2. The NH2-terminus of 151C protein contains the hydrophobic stretch for a predicted signal peptide (Fig. 2). The hydropathy plot (Fig. 3) and the TMPA prediction derived from the World-Wide Web service (33) showed that 151C protein does not include the transmembrane region. There are many cysteine residues, especially from residues 346 to 950, and four putative N-glycosylation sites in the protein. These results indicate that the 151C gene product is a putative cysteine-rich secretory glycoprotein. To confirm the open reading frame determined by DNA sequencing, RNA transcript was synthesized in vitro using the 151C cDNA as a template and translated in a rabbit reticulocyte lysate. In vitro translation yielded a polypeptide of about 100 kDa (Fig. 4).

Comparison of the deduced amino acid sequence of 151C protein with the database (Human Genome Center, Institute of Medical Science, the University of Tokyo) revealed that the middle part (519–615 amino acids) of the 151C gene product showed about 40% homology with thrombospondin 1 (34) and 2 (35). This homologous domain of these proteins includes the TSP type I motif (Fig. 5A). Three tryptophans followed by six cysteine residues are well conserved in both 151C and thrombospondins. In addition, two repeats of the TSP submotif, showing a lesser homology to TSP-1, are found in the COOH terminus of 151C (Fig. 5B). Other functional domains of throm-
bospondin 1 and 2, such as Ca$^{2+}$ binding or procollagen homologous domain, are not found in the 151C protein.

The NH$_2$-terminal half-region of 151C protein shows homology with the snake venom metalloproteinase proteins such as hemorrhagic toxin a, b, c, d, and e (1, 36), and fibrolase (37) (Fig. 2 and data not shown). The sequence alignment of 151C with the snake venom proteins spans from the pro-region, through the protease domain, to the disintegrin domain. The 151C protein also shows homology with the mammalian genes, such as fertilin (4), meltrin (8), MS2 (9), MDC (7), epidermal apical protein I (5), and cyritestin (6), which belong to ADAM family (Fig. 2 and data not shown). Sequence comparison of these proteins revealed that the NH$_2$-terminal half-region of the 151C protein consists of three distinct structural domain, pro-region, zinc-dependent metalloproteinase, and disintegrin-like domains. Taken collectively, these data indicate that the 151C gene encodes a new type of the metalloproteinase-disintegrin protein with the TSP type I motifs. We named this cDNA clone a disintegrin and metalloproteinase with TSP motifs (ADAMTS-1).

ADAMTS-1 is an IL-1 Inducible Gene—In colon 26 adenocarcinoma cachexia model, IL-1 has been reported to be involved in the induction of IL-6 (25, 26). In order to examine whether or not ADAMTS-1 is an IL-1 inducible gene, colon 26 clone 20 cells were treated with IL-1 in vitro. As shown in Fig. 7, ADAMTS-1 mRNA level was significantly enhanced 2 h after IL-1 stimulation, indicating that ADAMTS-1 is an IL-1 inducible gene. In addition, the level of ADAMTS-1 transcript in non-activated clone 20 cells was very low compared with that of clone 20 tumor in vivo (data not shown). These results suggest the possibility that ADAMTS-1 transcript is not constitutively expressed in clone 20 cells but induced by an inflammatory cytokines, such as IL-1, in vivo tumor.

ADAMTS-1 mRNA Induction in Kidney and Heart following LPS Administration in Vivo—Tissue distribution of ADAMTS-1 mRNA was examined by Northern blot analysis. Very weak signal for ADAMTS-1 mRNA was detected in the heart and kidney, but ADAMTS-1 mRNA was not detected in other organs including lung, liver, brain, and muscle (data not shown). These results indicate that the level of ADAMTS-1 transcript is very low in normal tissues. Since the ADAMTS-1 gene was activated by IL-1 stimulation in vitro, LPS was administered intravenously into mice for induction of systemic inflammation, and the mRNA levels for ADAMTS-1 in several organs were evaluated by Northern blot analysis. As shown in Fig. 8, ADAMTS-1 mRNA was significantly enhanced in heart and kidney after LPS treatment, but not in other organs (data not shown). This result indicates that the ADAMTS-1 gene is an inflammation-associated gene.

**DISCUSSION**

Colon 26 adenocarcinoma is a suitable model for cancer cachexia because weight loss is induced when the tumor burdens are relatively small (31). In addition to weight loss, colon 26 was found to cause hypoglycemia and disorders of hepatic functions (31). IL-6 has been proposed as a mediator of cancer cachexia in this model (25, 26), but IL-6 alone is not sufficient to induce cachexia (25, 26). Therefore, the precise mechanism required to induce cancer cachexia in the colon 26 model has not been elucidated. Fujimoto-Ouchi et al. (32) isolated cachexigenic (clone 20) and noncachexigenic (clone 5) clones of colon 26. These sublines of colon 26 carcinoma are a useful tool for
identification of genes which are up-regulated during development of cancer cachexia because the basal expression levels of a number of unrelated genes are presumed to be similar for each subline. In this study, we identified ADAMTS-1 as a cachexigenic tumor selective gene, which shows a sequence similarity to that of snake venom metalloproteinase and belongs to the ADAM family.

The precursor form of snake venom metalloproteinase, such as Ht-a, consists of four domains, pro-region, metalloproteinase, disintegrin, and cysteine-rich domain (1,36)(Fig. 9). Until now, 11 to 13 mammalian genes have been identified as members of this family, named ADAMs (1). The membrane-type ADAMs, such as fertilin, MS2, and meltrin, include the epidermal growth factor-like transmembrane and cytoplasmic domains in those COOH-terminal regions (Fig. 9). A non-membrane type gene, the MDC gene, contains the cysteine-rich domain following disintegrin domain (Fig. 9). In contrast, ADAMTS-1 does not include either an epidermal growth factor-like or transmembrane domain. Instead of these domains, the ADAMTS-1 protein has TSP type I motifs and a spacer region in its COOH-terminal half-region. Therefore, the ADAMTS-1 protein is a new type of ADAM family protein containing the TSP type I motifs.

It has been suggested that the catalytic site of the snake venom metalloproteinase is masked with the cysteine residue in the pro-domain and that the proteinase is activated by autolysis in a similar manner to matrix metalloproteinase, "cysteine switch mechanism," resulting in the removal of pro-domain (1,36,40). In addition, it was reported that the precursor form of the venom metalloproteinase is post-translationally processed to remove the disintegrin domain (36). Similarly, fertilin (PH30), which is suggested to be involved in sperm-egg fusion (41), undergoes processing during sperm maturation (42), resulting in the removal of proteinase domain. On the other hand, the ADAMTS-1 protein has a zinc-binding motif in metalloproteinase domain. Hence, it is possible that the pro-region and proteinase domain of ADAMTS-1 may be processed by appropriate stimulation in vivo, resulting in the exposure of a disintegrin-like domain and TSP type I motifs.

The TSP type I motif has been identified as a conserved repeated element between thrombospondin 1 and 2, which are known as multifunctional secretory glycoproteins to support blood coagulation, to regulate proliferation, adhesion, and migration of a number of normal and tumor cells (43,44) and to be a physiological inhibitor of angiogenesis (45). The biological activities of thrombospondins are attributed to their binding to
cell surfaces and many matrix macromolecules, including heparan sulfate, proteoglycans, fibronectin, laminin, and collagen (43, 44). Thrombospondins are composed of multiple discrete domains including a TSP type I motif, to mediate binding to various ligands. The TSP type I motif of thrombospondin 1 and 2 has been thought to be involved in binding to matrix molecules. In particular, the CS-(A/V)-TCG element within the TSP type I motif is an important determinant in binding to cells (39, 46). In addition, the W-(S/G)-X-W motif found in the NH2-terminal of TSP type I motif, was shown to promote the adhesion of melanoma cells through heparan sulfate (38). Both elements are conserved in the type I motif of ADAMTS-1. Here, we demonstrated that the TSP homologous domain of ADAMTS-1 containing TSP type I motif possess binding capacity to heparin. Since ADAMTS-1 is a putative secretory protein, it is possible that ADAMTS-1 may be incorporated into the extracellular matrix through interacting with sulfated glycoconjugates such as heparin and heparan sulfate.

Disintegrin is a family of anti-coagulant peptides found in snake venoms, which are characterized by a high cysteine content (3). Disintegrin functions as an integrin ligand and has the ability to disrupt cell-matrix interaction (47). Recently, the disintegrin domain of fertilin expressed on sperm was shown to be involved in binding to the egg, through interaction with integrin (41). Sequence similarity between ADAMTS-1 and hemorrhagic toxin, Ht-e, extends to the disintegrin domain and seven cysteine residues are conserved in ADAMTS-1 (Fig. 2). However, the homology of ADAMTS-1 to Ht-e in the disintegrin domain is relatively low (21% identity) compared with those of other members of this family proteins, such as fertilin and MS2 (30–39% identity to Ht-e). We prepared disintegrin-like domain of ADAMTS-1 as GST fusion protein and checked the inhibitory activity against platelet aggregation. However, we found that the GST disintegrin-like domain of ADAMTS-1 (at 300 nm) had no effect on platelet aggregation which was induced by ADP. The function of disintegrin-like domains of ADAMTS-1 remains to be elucidated.

Our present study has identified the ADAMTS-1 gene as being selectively expressed in the cachexigenic tumor in vivo. Strassmann (25) proposed that IL-1, produced by infiltrated macrophages, potentiates IL-6 production by colon 26 tumor cells in vivo. Previously, we demonstrated the selective expression of the IL-6 gene in clone 20 tumor, but not in clone 5 tumor in vivo which was dependent upon the balance between IL-1 and IL-1 receptor antagonist (26). In fact, we have shown here that the ADAMTS-1 mRNA was enhanced by in vitro IL-1 stimulation, suggesting that selective expression of ADAMTS-1 in the cachexigenic clone 20 tumor may be due to the action of IL-1. In addition, we found that transcripts of a certain class of VL30 elements were strongly expressed in the cachexic clone 20 tumor in vivo, but not in the non-cachexic clone 5 tumor, indicating that the signaling pathway leading to the activation of the long terminal repeat of a certain class of VL30 is selectively activated in the cachexigenic tumor in vivo and may contribute to the induction of multiple genes during development of cancer cachexia.

Involvement of inflammatory cytokines, such as tumor necrosis factor, IL-1, and IL-6 has been demonstrated in various cancer cachexia model (21–27). Concomitant with the elevation of serum IL-6 level, haptoglobin in the serum is significantly increased in clone 20 tumor-bearing mice (26), suggesting that systemic inflammation occurs during induction of cancer cachexia. Interestingly, we found that ADAMTS-1 mRNA was markedly induced in heart and kidney by in vivo LPS administration. These results indicate that ADAMTS-1 gene expression is up-regulated by inflammatory mediators such as IL-1 during inflammation.

Further investigation is required to establish the role of the ADAMTS-1 gene product during inflammation and cancer cachexia. Of the other ADAM family proteins, MS2 is selectively expressed on monocyteic lineage and has been shown to be up-regulated by LPS (9). Therefore, it is likely that some ADAMs, such as ADAMTS-1 and MS2, may play a role in the

Fig. 8. Induction of ADAMTS-1 mRNA by LPS treatment in vivo. Mice were treated with LPS (10 μg, intravenously/mice). Total heart and kidney RNA was extracted at 5 h after LPS treatment and ADAMTS-1 mRNA was analyzed by Northern blotting. The lower panel shows the blot hybridized with an 18 S rRNA probe as a control.

Fig. 9. Schematic representation of the domain organization of ADAMTS-1 and other metalloproteinase-disintegrin family proteins. TM, transmembrane; TSP, thrombospondin type I motif; EGF, epidermal growth factor-like motif. Position of zinc binding motif is indicated by Zn.
inflammatory process. It has also been established that snake venom metalloproteinases degrade the proteins of basement membrane including collagen IV, nidogen, and laminin (48, 49). Similar activities can be expected for some ADAM family proteins, including ADAMTS-1, during inflammation. On the other hand, ADAMTS-1 is a unique member of this family because it possesses the TSP homologous domain containing TSP type I motifs. It is therefore presumed that ADAMTS-1, by mediating cell adhesion, may play a role in cell proliferation, migration, and angiogenesis.

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