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MINIREVIEW

Implication of GnT-V in cancer metastasis: A glycomic approach for identification of a target protein and its unique function as an angiogenic cofactor

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Abbreviations: **GnT-V, UDP-GlcNAc, α-mannoside β1,6-*N*-acetylglucosaminyltransferase; GlcNAc, *N*-acetylglucosamine; L_4-PHA, leukoagglutinating phytohemagglutinin.**

Introduction

It is known that sugar chains have a variety of functions and play a key role in development, differentiation, cell adhesion and cell growth in cells [1,2]. Our group has focused on the underlying mechanism of the biosynthesis of *N*-glycan branchings in glycoproteins, and reported on various *N*-glycan branching enzymes that play a pivotal role in various steps [3].

A possible link between β1-6 branched structures of *N*-glycans and cancer metastasis has been proposed by several authors [4–6]. However before the cloning of the UDP-GlcNAc α-mannoside β1-6-*N*-acetylglucosaminyltransferase (GnT-V) enzyme which catalyzes the formation of β1-6 branching was carried out, it was not clear whether or not β1-6 branching is actually related to the metastatic potential of cancer cells. The enzyme was independently purified from rat kidney [7] by Pierce's group and from human cancer cells [8] by our group, and both groups subsequently cloned their cDNAs [9,10]. Using the cloned genes several studies have presented evidence for the presence of strong association between β1-6 branching and cancer metastasis [11–13]. Knock out mice developed by Dennis's group confirmed that the gene is highly linked to cancer development [14]. However, in the case of glycosyltransferase gene targeting mice or gene transfected cell lines, phenotypic changes are not necessarily directly associated with gene effects because phenotypic changes can not be solely explained by the deletion of a single gene. The reason for this is that an alternation in glycosylation may affect several target endogenous glycoproteins and these target proteins secondarily affect some pathophysiological changes. Therefore even if one could delete a specific glycosyltransferase gene or over-express a specified gene, an explanation for the phenotypic changes would be difficult unless target protein(s) had been identified or the same human disease due to the lack of these genes had been previously identified. Our goal was to identify the target protein(s) of the glycosyltransferase gene, in order to elucidate the pathophysiological significance of sugar chains, namely, in order to identify the actual function of sugar chains, a functional glycomic approach represents one of the most important issues in the field of glycobiology [15]. In this review we described that some studies on GnT-V in which one of its target was identified and also reported on a new function of GnT-V was identified.

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Identification of a target protein of GnT-V which enhances invasion and metastasis

Structural changes in *N*-glycans are one of the critical steps in cellular transformation and malignant transformation. β1-6 GlcNAc-branching on *N*-glycans is a product of GnT-V, which is liked to tumor metastasis and malignant transformation. Two

groups (including ours), independently purified GnT-V from human cancer cell lines and rat kidney, and cloned their cDNAs. Gene transcription of GnT-V is regulated by proto-oncogenes such as the Ets family [16,17], src [18] and erbB2 [19]. The 5′ flanking region of the GnT-V gene contained the functional binding sites for the Ets family.

Using GnT-V knock out mice, the expression of GnT-V was shown to be essential for tumor growth and metastasis [14] and GnT-V suppressed membrane ruffling by the activation of phosphatidylinositol 3 kinase and protein kinase B. The modification of specific glycoproteins which contain β1-6 GlcNAc-branching, however, has not yet been clarified. A high level of expression of the GnT-V gene in human colorectal and breast cancer is correlated with distant or lymph node metastasis with a poor prognosis [5,11,20]. On the other hand, the expression of GnT-V is an early event in rat hepatocarcinogenesis, and the level of GnT-V expression in patients with hepatoma did not correlate with the prognosis of the patient after an operation [12,21]. In fact, using athymic mice experiments, hepatoma cells with high levels of GnT-V, such as Huh7 and HepG2 cells, showed no metastasis. This discrepancy between colon cancer and hepatoma could be attributed to by difference in target glycoproteins of GnT-V in various tissues. Even though limited information is available at present concerning the structural nature of glycoprotein(s) which are glycosylated by GnT-V, such as integrins and LAMP-2 (lysosomal associated membrane protein 2), functional changes in those proteins by glycosylation are not clear [6].

Our recent findings clearly showed a novel pathway of GnT-V-mediated metastasis *via* the up-regulation of matriptase [22,23] an epithelial-derived, integral membrane serine protease. This proteinase actually activates two important cancer invasion effectors, the membrane-bound activator of urokinase-type plasminogen activator (uPA) and hepatocyte growth factor (HGF), also designated as a scatter factor on the surface of cancer cells [24,25].

Protease secreted by GnT-V transfected MKN45 cells is a metal ion dependent serine proteinase, matriptase

In order to understand the underlying mechanisms that control the increases in the metastatic potential of GnT-V transfectants, gelatinolytic activity in the conditioned medium of mock and GnT-V transfectants was assayed by gelatin zymography and an increase in gelatinase activity of an approximately 80 kDa protease was found in the GnT-V transfectants. The intensity of this band in the GnT-V transfectants was significantly higher than that of a Mock transfected gastric cancer cell line, MKN45 cells. The addition of EDTA eliminated the gelatinolytic activities of all proteases but added Matrix metalloproteinase (MMP) specific inhibitor BE16627B [26] did not. However, the gelatinolytic activities of the approximately 92 and 72 kDa proteins in the conditioned medium were completely inhibited by BE16627B suggesting that the 92 and 72 kDa proteases corresponded to MMP9 and MMP2, respectively. The proteolytic activity of the 80 kDa protease in the conditioned medium was completely blocked by aprotinin, a serine protease inhibitor. These results indicate that the 80 kDa protease is a metal-dependent (divalent cation-dependent) serine protease.

A search of the literature indicated that such a protease was secreted from human breast cancer cells, T47D [27]. This protease was purified and shown to be matriptase [22], and independently cloned as the membrane-type serine protease-1 (MT-SP1) by another group [23]. Matriptase is a type 2, integral membrane, trypsin-like serine protease, which may be involved in tissue remodeling, cell growth and cancer metastasis [28,29]. Western blot analysis using the anti-matriptase antibody mAb21-9 showed dramatic increases in both the cleaved 80 kDa (non-complexed) and 95 and 110 kDa (complexed) forms of matriptase with fragments of hepatocyte growth factor activator inhibitor-1 (HAI-1) in the conditioned media of GnT-V transfectants, compared to that of mock cells. Three forms of matriptase were observed in the cell lysate, including a cleaved (non-complexed) 80 kDa, full-length 90 kDa, and 125 kDa forms, complexed with the 55 kDa full-length HAI-1. Interestingly, the activated form of matriptase/HAI-1 complexes was observed only in the conditioned media of GnT-V transfectants, as judged by western blot analysis using anti-(total) matriptase mAb 21-9 or anti-two-chain matriptase mAb M69. Immunodepletion using an anti-matripatase antibody resulted in the disappearance of gelatinolytic activity.

The mRNA expressions of matriptase in the mock and GnT-V transfectants remained unchanged, indicating that an enhanced expression of matriptase did not occur at the transcriptional level.

Lectin blot analysis showed that a large number of glycoproteins were modified by GnT-V transfection. Matriptase contains 4 potential sites for Asn-linked oligosaccharides [23]. To determine addition of β1-6 blanching on the oligosaccharide contained by matriptase, we performed two-dimensional electrophoresis, followed by lectin blot using L_4-PHA. As shown Figure 1A, combinational analysis of immunoblot and L_4-PHA lectin blot indicated that immunochemically stained band with an anti-matriptase IgG coincided with a stained with L_4-PHA in GnT-V transfectants (Figure 1A, arrow). Furthermore, L_4-PHA precipitation followed by western blot of matriptase, revealed the strong binding of matriptase to L_4-PHA lectin precipitation pellets in GnT-V transfectants (Figure 1B). These data indicate that *N*-glycans contained by matriptase were glycosylated by GnT-V.

Molecular mechanism underlying the enhanced expression of matriptase in GnT-V transfectants

The degradation of matriptase was dramatically delayed in cell lysates in 100 mM Tris HCl (PH7.5) and 1% Triton-X 100 buffer from the GnT-V transfectants. Even after 300 minutes, approximately 80% of the matriptase remained in the undegraded state, in the case of GnT-V transfectants. Interestingly, the matriptase

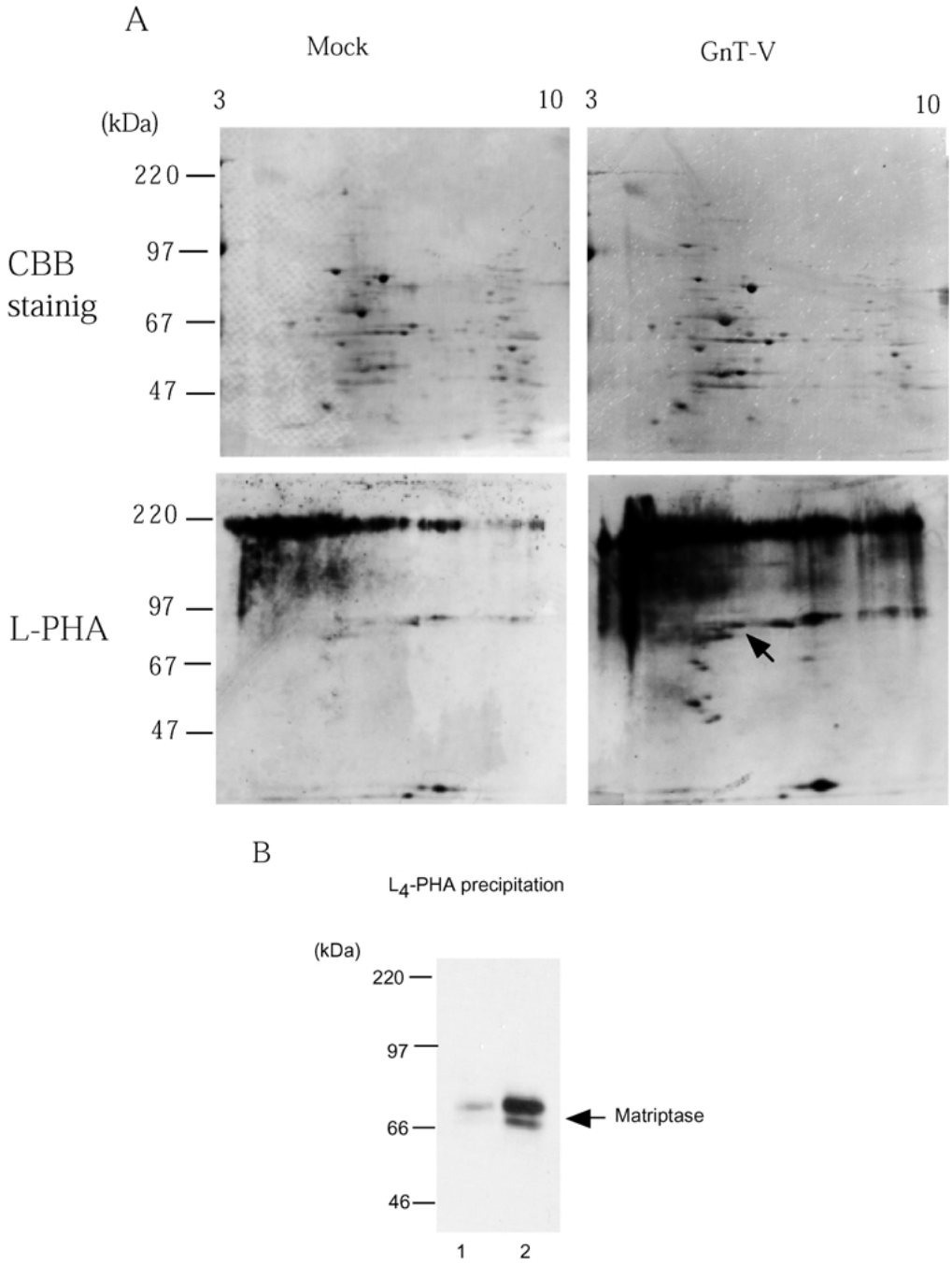

Figure 1. Detection of β1-6 GlcNAc branching on matriptase A, two-dimensional electrophoresis followed by L_4-PHA lectin blot. 200 μg of total cellular proteins extracted from mock and GnT-V transfectants were subjected two-dimensional electrophoresis. Upper panel indicates Commassie brilliant blue staining, lower panels indicated L_4-PHA lectin blot staining. An arrow indicated matriptase stained with L_4-PHA. B, L_4-PHA precipitation followed by western blot of matriptase. Both pellets of L_4-PHA precipitation (lane 1 indicates mock transfectant and lane 2 indicates GnT-V transfectant) were incubated in 1$\times$ SDS sample buffer in the absence of reducing agents at room temperature for 5 min prior to SDS-PAGE, and then subjected to western blot analysis using anti-matriptase antibody, (mAb21-9). See in ref. [50].

bands which were resistant to degradation coincided with those that contained β1-6 GlcNAc-branching. Pulse-chase studies of matriptase showed that the half-life of matriptase remained unchanged in total cell lysates, but was markedly prolonged in the conditioned media of GnT-V transfectants. When we investigated the time-dependent accumulation of matriptase in the conditioned media, an additional accumulation of matriptase in the cell culture of the GnT-V transfectants was observed

after 60 hours of culture. These results strongly suggest that the up-regulation of matriptase in the GnT-V transfectants is due to the resistance of matriptase to degradation, as a result of β1-6 GlcNAc-branching. When matriptase transfectants were injected peritoneally into athymic mice, an increase in metastasis to the lymph nodes was observed, similar to the data obtained for the GnT-V transfectants. This indicates that glycosylated matriptase by GnT-V becomes resistant to degradation, we conclude one of the likely target proteins for GnT-V is matriptase.

A novel function of GnT-V as an inducer of angiogenesis and GnT-V transfectants induces hypervascularization in athymic mice

Angiogenesis is known to be a critical step in cancer progression [30,31]. Fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) are known to contribute to tumor growth. The production of these factors are controlled by the increased gene expression, posttranslational modifications and interactions with the extracellular matrix. Some of the growth factors and growth factor receptors are glycoproteins and function as a key role in tumor angiogenesis. Cell remodeling by glycosyltransferase genes have revealed that changes in the oligosaccharide structure of these receptors lead to alterations in intracellular signaling, thus resulting in cellular transformation.

Our group recently found that GnT-V transfectants induced a dramatic increase in angiogenic activity. The induction of tumor angiogenesis by GnT-V can be explained by the following mechanisms (1) increases in the expression/production of angiogenic factors, (2) changes in their function via the addition of β1-6 branching and (3) other unknown mechanisms. Our recent studies showed that a secreted type of GnT-V itself was able to induce angiogenesis with no detectable mediation in glycosylation. In addition, we also found that a basic domain found in GnT-V caused the direct release of FGF-2 from heparan sulfate proteoglycan (HSPG) on the cell surface and/or extracellular matrix. These data strongly suggest that GnT-V is a bifunctional protein and that a secreted type of GnT-V protein itself plays a pivotal role in tumor angiogenesis, acting as an angiogenic cofactor of FGF-2.

In order to confirm the hypothesis that GnT-V is implicated in angiogeneis, we established stable transfectants of it using the human colon cancer cell line WiDr, along with control transfectants of β1,4-*N*-acetylglucosaminyltransferase-III and α1,6-fucosyltransferase. WiDr cells express the above glycosyltransferases at very low or negligible levels. When these transfectants were transplanted into athymic mice, transplanted tumors of the GnT-V transfectants showed a dramatic hypervascularization, compared to the other transplants.

In order to confirm the induction of angiogenesis by the GnT-V transfectants, the chorioallantoic membrane of chick embryo (CAM assay) was employed [32,33]. The invasion of blood capillaries into a collagen sponge was increased only in the case of the GnT-V transfectants. This angiogenesis was also observed when the GnT-V gene was transiently expressed in WiDr, COS-1 and CHO cells. These data suggest that the induction of angiogenesis is a common effect of GnT-V gene transfection.

To evaluate the induction of angiogenesis in the GnT-V transfectants, we measured their effects on DNA synthesis in human umbilical vein epithelial cells (HUVEC). DNA synthesis of HUVEC was increased as the result of replacement with the conditioned medium from the GnT-V trasnfectants, while no effects were detectable when the conditioned medium from the other transfectants was used. These data indicate that the GnT-V transfectants secreted a growth-stimulating factor for HUVEC. The addition of fresh medium increased the HUVEC proliferation to a higher level than that of the conditioned medium from the GnT-V transfectants. This is probably due to a supply of growth-stimulating factors such as FGF-2 that are contained in fetal bovine serum.

Effect of recombinant GnT-V on HUVEC proliferation

Angiogenic activity in the conditioned medium from the GnT-V transfectants was next characterized using column chromatography, in conjugation with monitoring the HUVEC proliferation-stimulating activity of eluted fractions. With heparin-affinity chromatography, a high activity fraction was eluted with 0.3 M NaCl. This characteristic is completely different from hitherto-known angiogenic factors (e.g. FGF-1, FGF-2, VEGF, placental growth factor (PlGF), and hepatocyte growth factor (HGF)), which are eluted with 0.8–1.5 M NaCl [34–38]. Western blotting analysis using an anti-GnT-V antibody confirmed that the HUVEC proliferation activity is due to GnT-V. It is known that GnT-V, as well as other glycosyltransferases, are secreted from tumor cells, although the physiological significance of this remains unknown. We hypothesized that a secreted type of GnT-V itself induces the proliferation of HUVEC and prepared a special type of recombinant GnT-V referred to as GnT-VD73, which lacks the transmembrane domain, but in which glycosyltransferase activity is retained [39]. HUVEC proliferation was increased as the result of the administration of GnT-VD73 in a dose-dependent manner. The utilized concentration appears to be within the physiological concentration range. The concentration of GnT-V in conditioned medium from the GnT-V transfectants was determined to be 140 ng/ml on the basis of the specific activity of GnT-VD73. Furthermore, conditioned medium from B16-F10 mouse melanoma cells, which have a high endogenous GnT-V activity, contained approximately 70 ng/ml GnT-V. B16-F10 cells also showed an angiogenic activity similar to the GnT-V transfectants in the CAM assay, suggesting that the GnT-V secreted from B16-F10 cells is able to stimulate angiogenesis in this assay system. These data clearly indicate that a secreted type of GnT-V within the physiological concentration range has growth-stimulating activity for HUVEC.

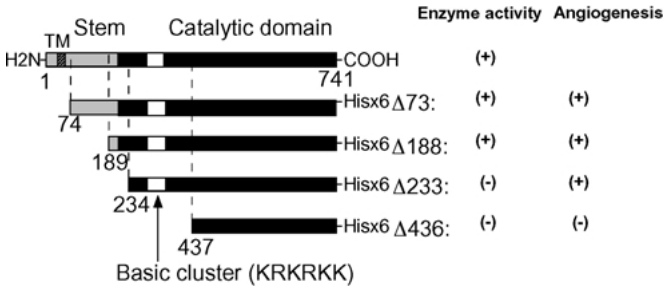

Figure 2. Constructs of varions deletion mutants of GnT-V. TM, the transmembrane domain, Stem, stem region of GnT-V and basic cluster indicates basic region containing heparin binding domain. Angiogenic activity is localized at the basic cluster of GnT-V but not associated with enzyme activity.

Domain analysis of GnT-V affecting HUVEC proliferation and the identification of a basic amino acid-clustered region of GnT-V that induces angiogenesis

To determine which domain of GnT-V contains the HUVEC growth-stimulating activity, we analyzed several types of deletion mutants of GnT-V as depicted in Figure 2 and found that the HUVEC growth-stimulating activity is located in the region corresponding to amino acids 234-436 of GnT-V, which does not encompass glycosyltransferase activity of the protein.

There is a markedly basic cluster, corresponding to amino acids 254-269, of human GnT-V, the sequence of which is, KSVRGKGKGQKRKRKK, and which is very similar to the sequence of amino acids 142-157 of VEGF189 [34]. In addition, the context of basic amino acids in this region is conserved in PlGF-2 and heparin binding type epidermal growth factor-like growth factor (HB-EGF) and serves as a heparin-binding motif [34]. Barillari et al. [40] reported that a basic peptide GRGKRR, derived from the sequence of PlGF-2, induced the growth of endothelial cells by releasing FGF-2 from heparan sulfate proteoglycans (HSPG) on the cell surface and/or extracellualr matrix. We synthesized a basic peptide, KRKRKK, which corresponds to amino acids 264-269 of GnT-V and a non-basic control peptide, FSGGPL (corresponding to amino acids 291-296 of GnT-V), and examined their effects on the growth of HUVEC. The amount of FGF-2 released from HSPG on HUVEC was measured after various truncated GnT-Vs and synthesized peptides were administrated to a culture medium of HUVEC at 4°C. GnT-VD73 and peptide KRKRKK induced the release of FGF-2, whereas GnT-VD436 and peptide FSGGPL had no effect. Both GnT-VD188 and D233, as well as GnT-VD73, also induced the release of FGF-2. Similarly, heparin, which is known to release HSPG-binding molecules by competing for their heparin-binding site, also induced the release of FGF-2. The peptide KRKRKK promoted the growth of HUVEC to an extent similar to GnT-VΔ73. This effect was completely suppressed by the co-addition of a neutralizing antibody against FGF-2. These results suggest that the KRKRKK region is sufficient for HUVEC growth-stimulating activity, and that the GnT-V protein stimulates angiogenesis by releasing FGF-2 from HSPG on endothelial cells via the action of the basic region of the protein.

In vivo angiogenesis by GnT-V protein

The induction of angiogenesis was also observed in other *in vitro* angiogenic assays, such as the capillary-like tube formation [41] and migration assays [42] using HUVEC. In order to investigate the angiogenic activity of GnT-V *ex-vivo*, a CAM assay using GnT-VD73 protein was performed. GnT-VD73 induced angiogenesis in chick micro vessels as well as FGF-2. Moreover, the KRKRKK peptide even induced a similar angiogenesis, and the induction of angiogenesis by GnT-VD73 and peptide KRKRKK was inhibited by treatment with a neutralizing antibody against FGF-2. These results indicate that a secreted type of GnT-V and GnT-V derived peptide KRKRKK induce angiogenesis via the action of FGF-2. Considering the results relative to HUVEC proliferation, the basic region of GnT-V may cause the release of FGF-2 from HSPG on endothelial cells.

Discussion

The present review shows the identification of key molecules in cancer metastasis that are mediated by GnT-V. We found that the addition of β1-6 GlcNAc-branching on matriptase inhibited its degradation, resulting in the up-regulation of matriptase expression in the conditioned media and on the cell surface despite the fact that no changes in matriptase mRNA expression were observed. Moreover, this increase in matriptase expression is directly linked to tumor metastasis because the overexpression of matriptase in gastric cancer cells led to lymph node metastasis in athymic mice.

Recently, a large-peptide inhibitor of matriptase, ecotin [43], has been shown to retard the growth of PC-3 prostate tumors in nude mice. These data suggest that matriptase might be a central regulator of cell migration and cancer invasion. Coimmunohistochemical staining of GnT-V and matriptase is now underway using human colon cancer tissues. Our preliminary data suggested that expression of GnT-V and matriptase are parallel in some cases of those samples. These results would indicate that GnT-V enhances both *in vitro* and *in vivo* cancer metastasis through the upregulation of matriptase.

The present review also outlines a novel pathway of tumor metastasis through oligosaccharide modification, which could yield potential insights into diagnostic or therapeutic strategies.

Angiogenesis is one of the key steps essential for tumor malignancy. Several endogenous stimulators and inhibitors of angiogenesis have been identified and the net balance of these regulators represents the angiogenic phenotype of tumor cells. For instance, angiostatin and endostatin are produced by the proteolysis of plasminogen and type XVIII collagen, respectively [44,45]. We found that a secreted type of GnT-V protein induces a type of angiogenesis that is unrelated to the usual

glycosyltransferase activity of GnT-V. Although a number of previous studies have concluded that GnT-V is directly linked to tumor metastasis, the mechanistic details of its action at the molecular level remains unknown. Dennis's group reported that oligosaccharide structures which are modified by GnT-V on an integrin or T cell receptor affect cell-cell or cell-extracellular matrix interactions in the processes of tumor metastasis and the immune system. Our studies suggested an alternate mechanism for GnT-V-related tumor metastasis, which is not mediated by glycosylation. It appears that GnT-V is capable of acting as a bifunctional protein. GnT-V is a Golgi enzyme, but is also secreted by some cultivated cells [8,46]. The concentration of GnT-V sufficient to induce angiogenesis is within the range of concentration that is actually observed in the conditioned medium of B16-F10 cells. We propose that GnT-V secreted from cancer cells releases a deposited FGF-2 from HSPG at a competitive site, and the released FGF-2 subsequently approaches the FGF receptor, creating a signaling through the formation of the ternary complex of FGF-2, the FGF-2 receptor and heparan sulfate at another site. GnT-V may not directly act on HUVEC, because the receptor/binding protein against GnT-V was not detected on the HUVEC surface on the basis of a cross-linking analysis using [^{125}I]-labeled GnT-V. This hypothesis is supported by the report that when endothelial cells were pre-cultured with VEGF, the addition of a protein containing a basic amino acid cluster such as GnT-V induced the release of VEGF from HSPG [40]. Furthermore, the growth-stimulating effect of GnT-V was also observed in other cell lines, such as breast carcinoma cell lines MCF-7 and MDA-MB231, which are responsive to FGF-2 [47,48].

The angiogenic potential of GnT-V is related to a basic region, which is conserved in the heparin-binding domains of hitherto-known angiogenic growth factors. It is noteworthy in this respects that VEGF189 contains the same basic peptide KRKRKK as GnT-V. VEGF189 is known to exist on cell surfaces as a cell-associated form, distinct from the secretable isoforms of VEGF121 and VEGF165. In order to be associated with a cell surface, the basic region of the C-terminus of VEGF189, including the KRKRKK sequence, is required [49]. However, there is no evidence to show that VEGF189 induces the release of FGF-2 from HSPG. In contrast, a secretable factor PlGF-2, which contains the basic region, induces the release of FGF-2 [40]. It appears that a secretable factor that contains a basic amino acid cluster might effect the release of a FGF-2 associated with HSPG. Although FGF-2 was identified as a target of the secreted type of GnT-V in this study, other growth factors associated with HSPG also might be released by GnT-V.

In summary we could propose at least two possible pathways by which GnT-V is implicated in cancer invasion and metastasis (Figure 3). Namely, GnT-V is upregulated by a transcriptional factor Ets-1 or other transcriptional factor(s) in cancer cells with high metastatic potential. High activity of GnT-V added β1-6 branching of matriptase which resulted in constitutive active matriptase with high stability against certain proteinases. Stable

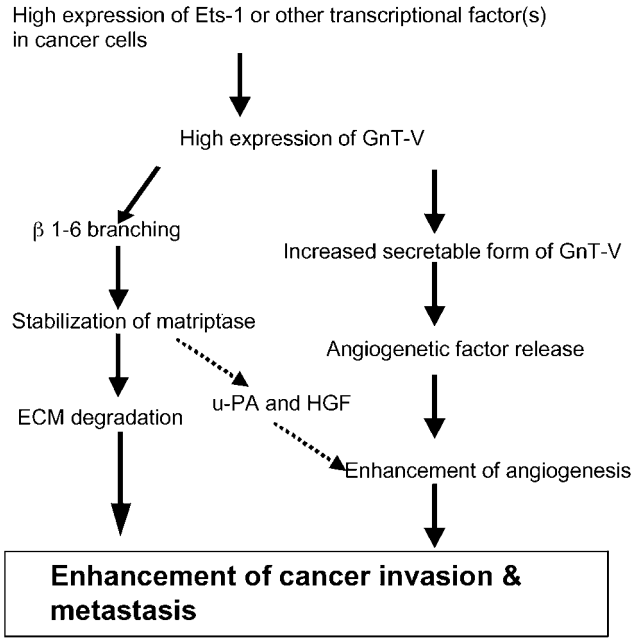

Figure 3. A proposed model for implication of GnT-V in cancer invasion and metastasis. ECM, extracellular matrix; u-PA, urokinase-type plasminogen activator; HGF, hepatocyte growth factor.

matriptase may activate urokinase-type Plasminogen activator (u-PA) as well as Hepatocyte Growth Factor (HGF) both of which are highly associated with angiogenesis. On the other hand, high expression of GnT-V produces also a large amount of secretable form of GnT-V with angiogenic factor releaser and releases angiogenic factor(s) to enhance cancer invasion and metastasis.

Future perspectives

A considerable body of data has accumulated to date concerning the involvement of GnT-V in cancer progression. Here we report on novel mechanisms through a target protein of GnT-V, matriptase which plays a critical role in tumor invasion and metastasis [50] and we also report on a unique function of a secreted type of GnT-V as an angiogenic cofactor of FGF-2 [51]. These two pathways would be implicated in the role of GnT-V in terms of cancer invasion and metastasis. Therefore, inhibiting the action of GnT-V in terms of the above functions represents a reasonable strategy for inhibiting cancer progression and metastasis. A specific inhibitor for the GnT-V functions may solve this problem.

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