The Antimetastatic Role of Thrombomodulin Expression in Islet Cell–Derived Tumors and Its Diagnostic Value

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ABSTRACT

Islet cell tumors, endocrine neoplasm arising from pancreatic islets of Langerhans, are histologically difficult to diagnose as benign or malignant. Molecular markers are associated with the clinical characteristics that most of insulinoma are usually benign tumors, whereas other islet cell tumors are malignant but have not been identified. In this context, we newly found that an endothelial anticoagulant thrombomodulin was expressed in the normal islet B cells and insulinoma, but not of other islet components or non-insulinoma islet cell tumors. Clinically, all of the subjects (n = 15) of the insulinoma group showed no metastasis together with thrombomodulin expression in the lesions, whereas the other islet cell tumor groups showed a high incidence of metastasis (82%) and a low expression rate of thrombomodulin (6%). To examine the functional role of thrombomodulin, especially regarding the clinical characteristics of islet cell tumors, we tested the effect of exogenous thrombomodulin overexpression on cell adhesiveness and proliferation using MIN6 insulinoma cell line. In cell-based experiments, thrombomodulin overexpression reduced cell proliferation and enhanced Ca2+-independent cell aggregation, possibly through direct interaction with neural cell adhesion molecule. Taken together, these results are suggesting that thrombomodulin may act as antimetastatic molecule of insulinomas. In addition, thrombomodulin is a clinically useful molecular marker not only for identifying β-cell–origin islet cell tumors (i.e., insulinomas) but also for predicting disease prognosis of islet cell tumors.

INTRODUCTION

Neuroendocrine neoplasms arising primarily from the pancreatic islets of Langerhans (also termed islet cell tumors) are quite rare, with an overall incidence of only 1 to 1.5 per 100,000 in the general population (1, 2). Up to 50% of all of the islet cell tumors secrete biologically active peptides, causing their systemic clinical symptoms; thus, such tumors are categorized as “functional” islet cell tumors (i.e., insulinoma, glucagonoma, gastrinoma, somatostatinoma and VIPoma). By contrast, other islet cell tumors showing no substantial secretion of the peptide hormones are categorized as “nonfunctional” islet cell tumor. Clinically, it is very difficult to diagnose islet cell tumors as “benign or malignant” by using histologic examination alone. To determine their malignant potentials, clinical features of islet cell tumors (i.e., lymph node metastasis or distant metastasis) must also be taken into consideration. According to the past studies concerning the metastatic capacities of islet cell tumors, more than 90% of insulinomas were clinically benign, whereas 40 to 60% of other functional islet cell tumors (1–4) and 80 to 90% of nonfunctional islet cell tumors (2, 5) were malignant. Thus, most insulinoma cases are usually considered to be benign tumors in the clinical situations. Nevertheless, molecular markers clinically associated with the metastatic potentials of islet cell tumors have not yet been identified.

Thrombomodulin has been identified as an endothelial membrane protein (6), and the thrombomodulin–protein C pathway has become well recognized for its essential anticoagulant/antithrombotic properties. The association of thrombin and thrombomodulin on the endothelial surface blocks the procoagulant properties of thrombin and redirects substrate specificity toward the activation of plasma protein C (6–12) as well as toward the inhibitor of fibrinolysis, thrombin-activatable fibrinolysis inhibitor (13, 14). Activated protein C exerts additional anticoagulant effects by inactivating coagulant cofactors Va and VIIIa (7, 8, 12). Thrombomodulin is also expressed at extra-vascular sites, such as in syncytiotrophoblasts in the placenta, in the epithelial tissues of gingiva, in the skin, lungs and digestive organs, and in the synovial lining cells (15–19). However, the functional role of thrombomodulin in the extra-vascular space, apart from the possible limitation of thrombin generation at these sites, remains uncertain. Recent studies have shown that thrombomodulin also attenuates inflammatory responses (20–22) and acts as an antimetastatic molecule against malignant tumor progression (23–27) beyond its anticoagulant activities. In the context of the antimetastatic properties of thrombomodulin against tumor progression, we considered a possible link between thrombomodulin and the benign character of insulinomas. In the present study, we first show the evidence that...
thrombomodulin is expressed dominantly in cases of insulinoma as well as in normal β cells in the pancreatic islets, which negatively correlates with the clinical incidence of tumor metastasis. Furthermore, the results shown herein also demonstrate for the first time that thrombomodulin functions as a regulator for cell adhesion and proliferation in vitro.

MATERIALS AND METHODS

Tissue Samples. Surgical specimens were obtained from 31 patients (14 men and 17 women) with islet cell tumor, who underwent tumor resection in the Department of Surgical Oncology and Digestive Surgery (Kagoshima University Graduate School of Medicine and Dental Science) between 1977 and 2003. The age of the patients ranged from 18 to 79 years, with an average age of 42 years. Type of tumor were as follows: (a) 15 patients were diagnosed with insulinoma; (b) 10 patients were diagnosed with nonfunctional islet cell tumor; (c) 1 patient was diagnosed with glucagonoma; (d) 2 patients were diagnosed with gastrinoma; (e) 2 patients were diagnosed with somatostatinoma; and (f) 1 patient was diagnosed with VIPoma. Of the 31 patients, 7 showed liver metastasis and 6 had lymph node metastasis (Table 1).

Immunohistochemistry. Immunohistochemical staining was performed using an ABC kit (Vector Laboratories, Burlingame, CA), as described previously (17, 28). Briefly, paraffin-embedded tissue samples were reacted with each antibody, as indicated in the presence of 1% BSA for 12 hours at 4°C. Biotinylated IgG was used as a second antibody.

Reagents and Antibodies. Recombinant human soluble thrombomodulin spanning the extracellular domain of the protein was generously provided by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Recombinant neural cell adhesion molecule-IgG-Fc fusion protein and its control IgG were purchased from R&D Systems Inc. (Minneapolis, MN). We also purchased antistomatostatin rabbit IgG (Biomedora Corporation, Hayward, CA), antinsulin-guinea pig IgG (DakoCytomation Inc., Carpinteria, CA), antihuman-neural cell adhesion molecule mouse IgG (Zymed Laboratories, Inc., San Francisco, CA), antismall cell neural cell adhesion molecule rat IgG (Chemicon International, Inc., Temecula, CA), rabbit anti-pan–cadherin whole antisemur (Sigma Chemical Co., St. Louis, MO), antihuman-E-cadherin mouse IgG (TaKaRa Holdings Inc., Shiga, Japan), and 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Dojindo Laboratory, Kumamoto, Japan). Rabbit antiserum – Case Meta

Table 1 ICT cases used in the present study

<table>
<thead>
<tr>
<th>Case</th>
<th>Case</th>
<th>Meta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinoma</td>
<td>15</td>
<td>0/15</td>
</tr>
<tr>
<td>NFICT</td>
<td>10</td>
<td>9/10</td>
</tr>
<tr>
<td>Glucagonoma</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td>Gastrinoma</td>
<td>2</td>
<td>2/2</td>
</tr>
<tr>
<td>Somatostatinoma</td>
<td>2</td>
<td>2/2</td>
</tr>
<tr>
<td>VIPoma</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td>Total patients</td>
<td>31</td>
<td>13/31</td>
</tr>
</tbody>
</table>

NFICT, nonfunctional islet cell tumor.

Cells and Cell Cultures. Dr. Susumu Seino (Chiba University, Chiba, Japan) kindly donated the islet β cell-derived MIN6 line (29, 30). Cells were maintained in DMEM (high glucose; Life Technologies, Inc., Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 0.5% 2-mercaptoethanol at 37°C (5% CO2). To obtain thrombomodulin-overexpressing MIN6, we produced pTracer-CMV/Bsd Vector (Invitrogen Corp., Carlsbad, CA) containing cDNA of the entire precursor of thrombomodulin (gift from Asahi Chemical Industry Co., Ltd.). MIN6 was transfected with the vector construct using LipofectAMINE Reagent (Invitrogen).

Islet Isolation. Male ICR mice were housed in the pathogen-free facility of the Animal Resource Center, Kagoshima University. All experiments involving animals were conducted following the guidelines of the NIH and with the approval of the Institutional Animal Care and Use Committee. The islets were isolated from 8-week-old male ICR mice as described previously (31).

FACS Analysis. Briefly, cell suspension was incubated with rabbit antiserum against thrombomodulin (1:50) for 30 minutes. The samples were subsequently washed and reacted with fluorescence-conjugated antirabbit IgG antibody for 30 minutes. Fluorescent measurements were then performed using FACS (EPICS Profile, Beckman Coulter Inc., Fullerton, CA).

Western Blotting. Western blotting analysis was performed as described previously (32). Briefly, lysates were separated by 8% SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane. The membranes were incubated for 1 hour in blocking solution (1% BSA and 5% skimmed milk in 25 mmol/L Tris-HCl buffer saline containing 0.02% Tween 20) followed by reaction with each first antibody (1:500) for 1 hour. Horseradish peroxidase-conjugated IgG was used as a second antibody. The chemiluminescence of horse-radish peroxidase was detected using an ECL system (Amer sham Bioscience, Inc., Buckinghamshire, United Kingdom).

Reverse Transcription-PCR. The cDNA converted from total RNA was synthesized using the cDNA Synthesis System (Life Technologies, Inc.), and reverse transcription-PCR was performed using the TaKaRa Ex Taq system.

Cell Proliferation Assay. MIN6 cells at a density of 1 × 105 cells/ml were suspended in culture medium and seeded in a 24-well culture plate. Cell viability was measured by MTT assay at 24 hours, 48 hours, and 72 hours after seeding.

Cell Adhesion Assay. MIN6 cells at a density of 5 × 105 cells/ml were suspended in HEPES buffer [5.4 mmol/L KCl, 136.9 mmol/L NaCl, 0.34 mmol/L Na2HPO4, 12H2O, 5.6 mmol/L glucose, 9.7 mmol/L (pH 7.4) and 1 mmol/L CaCl2] and seeded in type IV collagen coated, type I collagen coated, or plastic surface 24-well culture plates as indicated. Adherent cells (%) were measured at 1 hour after seeding using a hemocytometer.

Cell Aggregation Assay. MIN6 cells at a density of 5 × 105 cells/ml were suspended in HEPES buffer [5.4 mmol/L KCl, 136.9 mmol/L NaCl, 0.34 mmol/L Na2HPO4, 12H2O, 5.6 mmol/L glucose, and 9.7 mmol/L (pH 7.4)] in the presence or
absence of 1 mmol/L CaCl₂. Subsequently, cells (1 ml of suspension) were incubated in a 24-well culture dish at 37°C and shaken (80 rpm/minute) on a rotary shaker (EYELA Multi Shaker MMS, Tokyo Rikakikai Co., Tokyo, Japan). Aliquots of samples were taken at 0 minutes, 5 minutes, 10 minutes, and 15 minutes for a cell count assay, and the total number of single cells was calculated to examine the levels of cell aggregation [i.e., cell aggregation (%) = (total cell number - single cells)/(total number of cells) × 100 (%)].

Assays to Assess the Protein-Protein Interactions. To examine the protein-protein interaction between neural cell adhesion molecule and thrombomodulin, we used immunoprecipitation.

Fig. 1 Immunohistochemical patterns of thrombomodulin expression in normal islets. A, H&E; B, mouse control IgG; C, antiglucagon IgG; D, anti-insulin IgG; E, antisomatostatin IgG; F, antithrombomodulin IgG; G, anti-pan-cadherin IgG; and H, antineural cell adhesion molecule IgG. Note that thrombomodulin-positive cells and insulin-positive β cells are located at the central part of the islet (D and F), whereas glucagon-positive α cells and somatostatin-positive δ cells are located at the peripheral part of the islet. The expressions of pan-cadherin and neural cell adhesion molecule do not differ between β and non-β cells.
method and pull-down assay. In cell-free pull-down assay, protein G beads conjugated with neural cell adhesion molecule-immunoglobulin [i.e., the pellet obtained after the coincubation of 50 ng neural cell adhesion molecule-immunoglobulin protein (R&D) and 100 μL protein G agarose beads (Bio-Rad, Tokyo, Japan)] were coincubated with 10 ng/100 μL recombinant human soluble-thrombomodulin in immunoprecipitation buffer [1% NP40, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, and 25 mmol/L Tris-HCl (pH 7.5)] for 1 hour, and then the beads were washed three times with immunoprecipitation buffer. The amount of bound thrombomodulin protein to the beaded neural cell adhesion molecule-immunoglobulin was assayed by the Western blotting method. To determine whether thrombomodulin could interact with neural cell adhesion molecule on the surface of MIN6, thrombomodulin-overexpressing or mock-transfected MIN6 cells (10⁶ cells per pellet each) were lysed in immunoprecipitation buffer and centrifuged (10,000 rpm for 10 minutes at 4°C). Then the supernatant samples were used for immunoprecipitation of neural cell adhesion molecule protein expressed on MIN6. In the immunoprecipitation procedure, the samples were incubated with protein G beads and antihuman-neural cell adhesion molecule mouse IgG for 1 hour. After washing the beads, the levels of bound thrombomodulin protein (through the binding to neural cell adhesion molecule-antibody complex) were assayed by the Western blotting method.

RESULTS

Thrombomodulin Expression by Islet β-Cell and β-Cell Tumors. Islets of Langerhans are composed primarily of endocrine α cells, β cells, δ cells, and pancreatic polypeptide cells,
secretion of glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. In immunohistochemical analysis of pancreas serial sections as shown in Fig. 1, insulin-positive β cells, which appeared as major components of islets, were located at the central part of the islet, surrounded by non-β cells (i.e., glucagon-positive α cells and somatostatin-positive δ cells). Compared with endocrine markers, thrombomodulin expression was dominantly localized on insulin-positive β cells (Fig. 1, D and F), whereas the expressions of cadherin and neural cell adhesion molecule, currently considered as islet adhesion molecules (33, 34), did not differ between β and non-β cells (Fig. 1, G and H). These results suggested that thrombomodulin expression might be a good identifier of β cells and also led us to postulate that thrombomodulin might be an applicable marker for the histologic diagnosis of insulinoma. To confirm this, we conducted immunohistochemical examination of 31 islet cell tumors [15 insulinoma and 16 noninsulinoma (1 glucagonoma, 2 gastrinoma, 2 somatostatinoma, 1 VIPoma, and 10 nonfunctional islet cell tumors)]. Expectedly, all of the insulinoma cases (n = 15) were positive for thrombomodulin staining. In contrast, 15 of the 16 noninsulinoma cases (94%) were thrombomodulin negative. This immunohistochemical evidence was additionally confirmed by Western blot analysis (representative data are shown in Fig. 2B). On the basis of these results, the sensitivity, specificity, and accuracy of thrombomodulin staining for the diagnosis of β-cell tumors (i.e., insulinoma) were 100%, 93%, and 96%, respectively. Furthermore, even in a case of insulinoma showing partial insulin production, thrombomodulin was strongly expressed in whole tumor lesion (Fig. 2, A–b). Thus, we conclude that thrombomodulin could be a useful molecular marker for identifying β cell-derived tumors.

**Table 2. Characteristic profile of ICTs**

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor type</th>
<th>TM (primary)</th>
<th>Meta</th>
<th>TM (meta)</th>
<th>E-cadherin</th>
<th>N-CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>Insulinoma</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>Insulinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>Insulinoma (invasive type)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>NFICT</td>
<td>+</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>NFICT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>NFICT</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>19</td>
<td>NFICT</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>25</td>
<td>NFICT</td>
<td>–</td>
<td>LN</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>26</td>
<td>Glucagonoma</td>
<td>–</td>
<td>26</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>27</td>
<td>Gastrinoma</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>28</td>
<td>Gastrinoma</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>29</td>
<td>Somatostatinoma</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>30</td>
<td>Somatostatinoma</td>
<td>–</td>
<td>Liver</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>31</td>
<td>VIPoma</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: TM, thrombomodulin; N-CAM, neural cell adhesion molecule; NFICT, nonfunctional islet cell tumor; LN, lymph node.
modulin expression by islet cell tumors is negatively correlated with the clinical incidence of tumor metastasis, at least in the cases of insulinoma. Although a case of noninsulinoma non-functional islet cell tumor (Case 16 in Table 2) was complicated with thrombomodulin-negative metastatic lesion in liver, its immunohistochemical feature showed a focal thrombomodulin expression pattern in the primary lesion (Fig. 2, A-c).

Impact of Thrombomodulin-Overexpression on Tumor Cell Activities Concerning Metastatic Capacity. To understand the mechanism of insulinoma-specific characteristics (i.e., lower metastatic capacity), we examined the functional role of thrombomodulin expression by insulinoma cells. For this purpose, we used a mouse immortalized β-cell line, MIN6, as an in vitro model of β-cell islet cell tumors. As shown in Fig. 3, A and B, analyses of reverse transcription-PCR and Western blotting revealed that MIN6 expressed thrombomodulin at both mRNA and protein levels. However, the protein level of thrombomodulin expression by MIN6 was substantially less than that in normal mouse pancreatic islets (Fig. 3B), leading to consider that MIN6 was a suitable model of low-thrombomodulin expression (i.e., almost close to thrombomodulin-negative islet cell tumor cells, characteristically) to test the effects of thrombomodulin overexpression. To obtain thrombomodulin-overexpressing MIN6 (thrombomodulin-MIN6) as a thrombomodulin-positive islet cell tumor model, MIN6 was transiently transfected with thrombomodulin-expression vector construct (Fig. 3, B and C). Thrombomodulin-MIN6 had a less proliferative character compared with control mock-transfectant (Fig. 3D), showing increased cell aggregation, morphologically (data not shown). Thus, we speculated that thrombomodulin might act as an antiproliferative regulator through modulating the functions of certain islet adhesion molecules, such as neural cell adhesion molecule, E-cadherin, and N-cadherin (33, 34). Indeed, MIN6 expressed both cadherin and neural cell adhesion molecule at a protein level (Fig. 4A), which was not affected by thrombomodulin overexpression (data not shown). In this con-
text, neural cell adhesion molecule, an immunoglobulin-like adhesion molecule acting independently of Ca\(^{2+}\), can increase \(\beta_1\)-integrin activities (35), whereas cadherin is a Ca\(^{2+}\)-dependent adhesion molecule. As shown in Fig. 4B, although no effects in the Ca\(^{2+}\)-present condition, thrombomodulin overexpression substantially increased cell aggregation by MIN6 in the absence of Ca\(^{2+}\) (Fig. 4B). Furthermore, thrombomodulin overexpression also up-regulated the ability of firm adhesion by MIN6 to basement membrane matrix type IV collagen but not to type I collagen or to the plastic surface in the presence of Ca\(^{2+}\). D, \(\beta_1\)-integrin expression level was not different between thrombomodulin+MIN6 and Mock+MIN6. Bars, ±SD. (N-CAM, neural cell adhesion molecule; TM, thrombomodulin)

***Physiologic Interaction between Thrombomodulin and Neural Cell Adhesion Molecule.*** To validate the hypothesis, we examined whether thrombomodulin could directly bind to neural cell adhesion molecule. A cell-free pull-down assay of neural cell adhesion molecule using recombinant proteins (i.e., recombinant thrombomodulin and neural cell adhesion molecule-immunoglobulin proteins) revealed a molecular interaction between thrombomodulin and neural cell adhesion molecule (Fig. 5A). The molecular interaction was competitively inhibited by the addition of excessive recombinant protein spanning NH\(_2\)-terminal lectin-like domain (Fig. 5A) but not COOH-terminal epidermal growth factor-like domain of thrombomodulin protein (data not shown). These additionally implied that thrombomodulin could interact with neural cell adhesion molecule through the site of its lectin-like domain. Furthermore, the interaction between thrombomodulin and neural cell adhesion molecule was also evidenced physiologically on the cell surface of thrombomodulin-overexpressing MIN6 by the immunoprecipitate procedure of endogenous neural cell adhesion molecule protein (Fig. 5B), suggesting that thrombomodulin might function as a counter-ligand or an associate molecule of neural cell adhesion molecule to regulate the function of cell adhesion and

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**Fig. 4** Impacts of thrombomodulin expression on the functions of islet cell adhesion molecules. A, endogenous expressions of cadherin and neural cell adhesion molecule by MIN6 cells. MIN6 cells were analyzed for the expression levels of cadherin and neural cell adhesion molecule by Western blotting using anti-pan-cadherin antibody or antimouse-neural cell adhesion molecule antibody. Mouse brain tissue was used as a positive control for the expressions of cadherin and neural cell adhesion molecule. Note that MIN6 showed only a M\(_r\) 140,000 isoform of neural cell adhesion molecule, neither showing the expressions of M\(_r\) 120,000 or M\(_r\) 180,000 isoforms. B, cell aggregation assay (i.e., assay to assess “cell to cell” adhesion). To assess the cell-to-cell adhesion, transfecnt cells were incubated as described in Materials and Methods for the indicated time in the presence (left) or absence (right) of 1 mmol/L Ca\(^{2+}\). C, cell adhesion assay (i.e., assay to assess “cell to matrix” adhesion). Note that thrombomodulin overexpression substantially increased firm cell adhesion to basement membrane matrix type IV collagen but not to type I collagen or to the plastic surface without affecting the protein level of cellular \(\beta_1\)-integrin (Fig. 4, C and D), suggesting that thrombomodulin might increase the function of \(\beta_1\)-integrin in MIN6. Thus, these led us to hypothesize that thrombomodulin might regulate neural cell adhesion molecule function in the mechanism of thrombomodulin-mediated cellular events.
proliferation, which may play an important role in the benign and less-metastatic character of β-cell tumors (i.e., insulinoma).

**DISCUSSION**

Our study on the diagnostic value of thrombomodulin in the identification of β-cell tumors strongly indicated that thrombomodulin might be a clinically useful marker to define the origin of islet cell tumors. In addition, the present study also demonstrated the clinical relationship between thrombomodulin expression and a low metastatic potential of insulinoma, the mechanism of which might be explained by the thrombomodulin-mediated various cellular events, including the induction of aggregation and the reduction of proliferation. Consistent with these conclusions, our previous studies have revealed that thrombomodulin-negative squamous cell carcinomas showed a substantially higher rate of metastasis than thrombomodulin-positive squamous cell carcinomas, which also substantially affected disease prognosis (26, 27). Additionally, all of the metastatic lesions shown in some cases of thrombomodulin-positive esophageal cancer (i.e., pathologically diagnosed as squamous cell carcinomas) were observed as thrombomodulin-negative or less thrombomodulin-expressed lesions than the primary lesions (26). As with squamous cell carcinomas, the study using a melanoma line also showed a less invasive and proliferative characteristics of high-thrombomodulin expressors (23, 24). These evidences also support our conclusions that thrombomodulin-positive islet cell tumors are likely to be clinically benign tumors in character and hypothesis concerning the diagnostic value of thrombomodulin expression in islet cell tumors as a clinical predictor of disease prognosis.

Interestingly, thrombomodulin rather than neural cell adhesion molecule, currently recognized as an antimetastatic adhesion molecule in β-cell tumor (36), seemed to negatively correlate with the clinical incidence of islet cell tumor metastasis. For example, in our study, thrombomodulin was almost specifically expressed on only β-cell tumors (i.e., insulinoma), whereas neural cell adhesion molecule was expressed not only on β-cell tumors, but also on other types of islet cell tumors. With respect to the mechanism of antimetastasis, thrombomodulin may regulate the function of neural cell adhesion molecule through the molecular interaction at the site of its lectin-like domain. In this context, the NH2-terminal lectin-like domain of thrombomodulin is structurally similar to human phagocytic C1q receptor, which acts as an adhesion molecule for innate immune host defense. The evidence is also suggesting that the lectin-like domain of thrombomodulin may have an important role for cell-cell interaction (37, 38). Thus, we strongly put forth the hypothesis that thrombomodulin may be an essential antimetastatic factor in β-cell tumors, acting as a counter-ligand or associate molecule for neural cell adhesion molecule. Regarding the more physiologic role, thrombomodulin may have a role for organizing islet structure, such as a central core of β-cell assembly surrounded by the three other endocrine cell types (i.e., classified as non-β cells) in the procedure of islet development (39). In this context, it has been suggested that only differences of intensity of cell adhesion molecules direct the sorting-out of intermixed embryonic cells and the spreading of the less cohesive cell population over the surface of cohesive cells (40). Neural cell adhesion molecule has been reported to play an important role in islet construction (33), despite its expression pattern (i.e., also expressed by non-β cells as well as by β cells). Therefore, regulation of neural cell adhesion molecule function by thrombomodulin protein may contribute to the development of islets.

Likewise, an anticoagulant thrombomodulin, a coagulant tissue factor, was also expressed on islets, which might trigger the activation of an extrinsic blood coagulation cascade by the binding between factor VII and tissue factor (41). The tissue factor-mediated activation of the coagulation system leads to thrombin generation and results in the triggering of both coagulation and inflammatory response (42). In additional to its physiologic role, thrombomodulin as an anticoagulant molecule on normal islets might play a protective role for inhibiting coagulation cascade (43), maintaining islet homeostasis through the microcirculation of islets.

In conclusion, the present study has demonstrated the first
evidence of thrombomodulin expression by β cells and β-cell tumors, suggesting its potential clinical diagnostic value. Our data also introduce a new antimitostatic mechanism through thrombomodulin-mediated neural cell adhesion molecule activation.

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The Antimetastatic Role of Thrombomodulin Expression in Islet Cell-Derived Tumors and Its Diagnostic Value

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