

Thrombomodulin-mediated Cell Adhesion

INVOLVEMENT OF ITS LECTIN-LIKE DOMAIN*

Received for publication, May 19, 2003, and in revised form, August 27, 2003
Published, JBC Papers in Press, September 1, 2003, DOI 10.1074/jbc.M305216200

Huey-Chun Huang^{‡§¶}, Guey-Yueh Shi^{‡§}, Shinn-Jong Jiang^{‡§}, Chung-Sheng Shi^{‡§}, Chun-Mei Wu[‡],
Hsi-Yuan Yang^{||}, and Hua-Lin Wu^{‡§**}

From the [‡]Department of Biochemistry and [§]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan 701, Republic of China, the [¶]Department of Medical Technology, China Medical College, Taichung, Taiwan 404, Republic of China, and ^{||}Institute of Molecular and Cell Biology, National Taiwan University, Taipei, Taiwan 107, Republic of China

Thrombomodulin (TM) is an integral membrane glycoprotein that is a potent anticoagulant factor. TM may also possess functions distinct from its anticoagulant activity. Here the influence of TM on cell adhesion was studied in TM-negative melanoma A2058 cells transfected with green fluorescent protein-tagged TM (TMG) or lectin domain-deleted TM (TMG(Δ L)). Confocal microscopy demonstrated that both TMG and TMG(Δ L) were distributed in the plasma membrane. TMG-expressed cells grew as closely clustered colonies, with TM localized prominently in the intercellular boundaries. TMG(Δ L)-expressed cells grew singly. Overexpression of TMG, but not TMG(Δ L), decreased monolayer permeability *in vitro* and tumor growth *in vivo*. The cell-to-cell adhesion in TMG-expressed cells was Ca²⁺-dependent and was inhibited by monoclonal antibody against the lectin-like domain of TM. The effects of TM-mediated cell adhesion were abolished by the addition of mannose, chondroitin sulfate A, or chondroitin sulfate C. In addition, anti-lectin-like domain antibody disrupted the close clustering of the endogenous TM-expressed keratinocyte HaCaT cell line derived from normal human epidermis. Double-labeling immunofluorescence staining revealed similar distributions of TM and actin filament in the cortex region of the TMG-expressed cells. Thus, TM can function as a Ca²⁺-dependent cell-to-cell adhesion molecule. Binding of specific carbohydrates to the lectin-like domain is essential for this specific function.

be one of the factors that localizes the coagulation cascade to sites of vascular injury (2).

The observations from a number of studies support the contention that TM may also play a role in other extravascular activities (3). Ablation of the TM gene causes early postimplantation embryonic lethality that precedes the establishment of a functional cardiovascular system (4). TM may also have anti-scarring properties, by virtue of the modulation of early collagen deposition of normal epidermis (5). Complete or nearly complete TM deficiency has not been reported in humans (6), which is consistent with the view that a severe reduction of TM function may have more dire consequences than the defects in coagulant or anticoagulant factors. An inverse correlation between TM expression and tumor progression is evident clinically (7–9). It was demonstrated that TM exerted a growth-suppressing effect independent of its anticoagulant activity but dependent on the lectin-like domain (10).

The myriad and diverse possible functions of TM may reflect the glycoprotein structure. TM consists of 557 amino acid residues arranged in five distinct domains: an NH₂-terminal lectin-like domain, a domain with six epidermal growth factor (EGF)-like structures that contain thrombin binding sites, an O-glycosylation site-rich domain, a transmembrane domain, and a cytoplasmic tail (11). The NH₂-terminal lectin-like domain has two modules. The first 155-amino acid module, is homologous to Ca²⁺-dependent lectin (12). The second module, adjacent to the EGF-like domain, is a hydrophobic region of 70 amino acid residues. These lectin-like domains exist in other proteins, where they participate in a wide variety of cell biological processes, including inflammation and cell-to-cell recognition processes (13–15). The TM lectin-like domain is not required for cofactor activity for activating protein C, and its biological function remains mostly unclear.

It has been reported that many null mutations in adhesion genes are lethal during embryonic development (16–18) and that TM is necessary for embryonic development (4). The lectin-like activity may be influential in a cell-to-cell adhesive interaction (19). It is conceivable that TM may function as an additional cellular adhesive molecule. Immunocytochemical studies have localized the TM antigen principally to the intercellular bridges between keratinocytes in stratified squamous epithelium of skin and in various benign or malignant squamous cell carcinomas (7, 20–22). Indeed, the levels of both E-cadherin and TM are decreased in metastases of squamous cell carcinoma (7, 23). It is well known that E-cadherin-dependent cell-to-cell adhesion is important for the maintenance of epithelial structural integrity and that the loss of E-cadherin expression is correlated with increased invasive potential of both carcinoma cell lines and tumor samples (24).

Thrombomodulin (TM)¹ is a membrane-intercalated glycoprotein, which functions in anticoagulation by virtue of complexation with thrombin. The complex can effectively activate protein C, which in turn catalyzes the proteolytic inactivation of blood coagulation factors Va and VIIIa, leading to down-regulation of the blood coagulation cascade (1, 2). TM is constitutively expressed on endothelial cells (1). As such, it might

* This work was supported by Ministry of Education Program for Promoting Academic Excellence of Universities Grant 91-B-FA09-2-4 and National Science Council, Republic of China, Grants NSC-89-2320-B006-151 and NSC-89-2316-B006-009-M42. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Tel.: 886-6-2353535-5541; Fax: 886-6-3028037; E-mail: halnwu@mail.ncku.edu.tw.

¹ The abbreviations used are: TM, thrombomodulin; GFP, green fluorescence protein; TMG, TM-GFP fusion protein; TMG(Δ L), lectin-like domain-deleted form of TM-GFP fusion protein; EGF, epidermal growth factor; PBS, phosphate-buffered saline.

The parallel relationship of the expression levels of E-cadherin and TM in tumor progression prompted us to test the adhesion and morphoregulatory activities of TM in comparison with E-cadherin.

Although the direct participation of TM in cell-to-cell adhesion is suspected, no supportive experimental evidence has been provided. The present study sought such evidence, through the testing of the hypothesis that TM functions as a cell-to-cell adhesion molecule, and, if so, elucidation of the roles of the participating TM domains. Clones of A2058 melanoma cells that stably expressed green fluorescent protein (GFP)-tagged full-length or lectin-like domain-truncated TM were generated. We report here on our observations that the lectin-like domain of TM prompted the clustering of cells in close proximity with one another by enhancing cell-to-cell adhesiveness through a Ca^{2+} -dependent interaction of TM molecules. This interaction could be involved in limiting cell growth.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture dishes and plastic ware were purchased from Corning Life Sciences (Corning, Inc.). Lipofectin and cell culture reagents were from Gibco-BRL (Gaithersburg, MD). Restriction enzymes used in DNA manipulation were purchased from New England Biolabs (Beverly, MA) or Promega Corp. (Madison, WI). The pEGFPN₁ vector was from BD Biosciences Clontech (Palo Alto, CA). Monoclonal mouse antibody to the EGF₅-EGF₆ domain of TM IgG₁ antibody and Chromozym PCa were purchased from American Diagnostica Inc. (Greenwich, CT). Human protein C, antithrombin III, G418 (neomycin), and anti-human E-cadherin antibody (clone HECD-1) were from Calbiochem-Novabiochem. Monoclonal anti-lectin-like domain antibody (clone D-3), isotype control IgG antibody, tetramethylrhodamine-conjugated phalloidin, and goat anti-human keratin antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Tetramethylrhodamine goat anti-mouse IgG and tetramethylrhodamine rabbit anti-goat antibody were purchased from Molecular Probes, Inc. (Eugene, OR). Supersignal enhanced chemiluminescence reagent was obtained from Pierce. D-Mannose, D-galactose, D-lactose, D-glucose, D-xylose, heparin, low molecular weight heparin, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C were from Sigma. All other chemicals were of the highest grade commercially available.

Construction of Green Fluorescent Protein-tagged Thrombomodulin—Human TM gene in chromosomal DNA was amplified by PCR using a BamHI forward primer, TM719 (5'-CGGGATCCCGGAATGCTTGGGGTCTGGTCTTG-3') and an EcoRI reverse primer (5'-GGAATTCGGAGTCTCTGCGGCTCCGCT-3'). The 1.7-kb PCR product encoding amino acid residues 1–575 was digested with BamHI and EcoRI. The resulting fragment was ligated to the expression vector pEGFPN₁, which had been digested with BglII and EcoRI. This construct was named TMG.

The lectin-like module within the NH₂-terminal domain of TM was removed by recombinant PCR using the following method. Four primers were designed such that they overlapped and skipped the lectin-like module. Two oligonucleotide primers, TM719 and TM1480 (5'-CATTGCACGCGTGCAGCCGC-3'), flanked the region from nucleotide 719 to 1480. The other primers were 5'-CACGCTGCAGTCCCAAGCGCCACCCGGTGGCGCTC-3' and its reverse complement 5'-GAGCCGCAGCCGGTGGCGCTTGGGACTGCAGCGTG-3'. Each of the latter two primers was utilized with primer TM719 and TM1480 for PCR amplifications, respectively. The 92- and 66-bp PCR products were purified, denatured, annealed, and amplified using primers TM719 and TM1480. The recombinant 140-bp product was digested with BamHI and MluI and subcloned to replace the wild type NH₂-terminal domain of human TM in the expression vector pEGFPN₁. The final product was designated TMG(Δ L). Both constructs were confirmed by DNA sequencing.

Cell Culture and Transfection of Human Melanoma (A2058) Cells—A2058 cells (ATCC CRL-11147) or HaCaT cells (25) were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g/liter L-glutamine and 10% fetal bovine serum. A2058 cells grown until 40–60% confluence were transfected with TMG or TMG(Δ L) using Lipofectin reagent. To generate cell lines stably expressing the various constructs, cells were diluted and seeded 2 days after transfection and maintained in Dulbecco's modified Eagle's medium supplemented with 400 μ g/ml G418 (neomycin). Clonal expression was examined initially

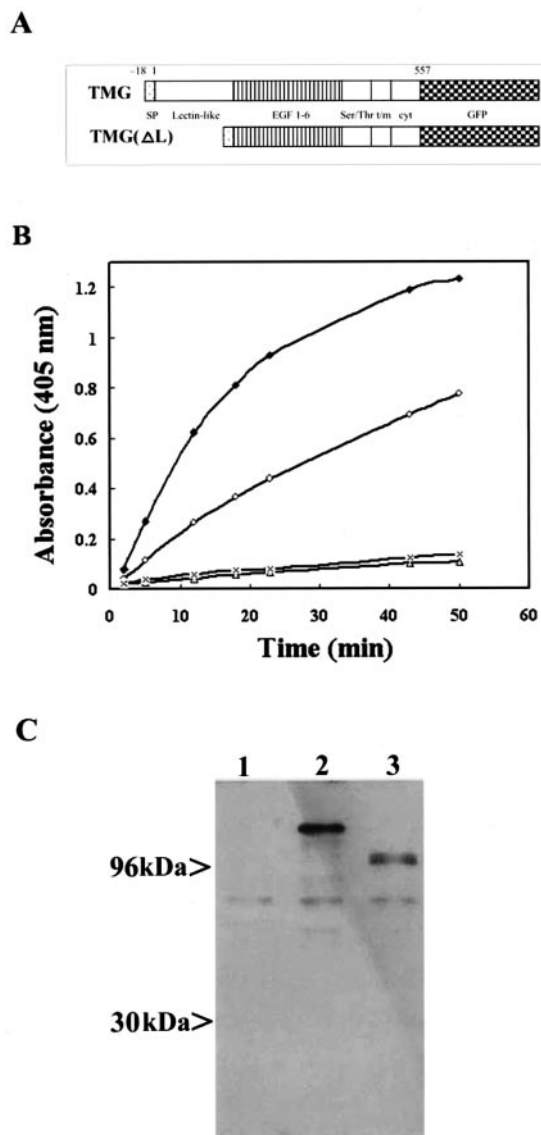


FIG. 1. A, schematic diagram illustrating TMG and TMG(Δ L) constructed in pEGFPN₁. Nascent human TM consists of 575 amino acid residues beginning with a putative 18-residue signal peptide (SP) at the N-terminal end. The signal peptide is followed by five structural domains: the NH₂-terminal lectin-like domain (including the hydrophobic region), six EGF-like repeats, the serine/threonine (Ser/Thr)-rich region, the transmembrane (tm) domain, and the cytoplasmic tail (cyt). cDNA constructs of the full-length or lectin-like domain-deleted TM were transfected into A2058 cells for expression of TMG or TMG(Δ L). B, TM activity assay of A2058 cells stably expressing TMG and TMG(Δ L). Confluent monolayers of A2058 cells (Δ), GFP control (\times), TMG (\circ) and TMG(Δ L) (\diamond) were assayed for TM-dependent protein C activation activity as described under "Experimental Procedures." The change in absorbance at 405 nm was monitored. C, characterization of TM proteins by mouse anti-human TM antiserum. 10 μ g of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto filters and blotting with mouse anti-human TM antiserum. Positions and apparent molecular mass standards (in kDa) are shown as indicated. Western blot results from A2058 cells transfected with GFP (lane 1), TMG (lane 2), and TMG(Δ L) (lane 3) are shown.

by fluorescence microscopy, and clones for further study were selected and expanded.

Thrombomodulin Activity Assay—Cells at a density of 2×10^4 /well were split into wells of a 96-well plate and allowed to reattach overnight. The cells were washed in a buffer containing 20 mM Tris (pH 7.4), 0.15 M NaCl, 2.5 mM CaCl_2 , and 5 mg/ml bovine serum albumin and incubated with 40 μ l of reaction mixture (37.5 nM thrombin and 5 μ g/ml protein C in the washing buffer) at 37 $^\circ\text{C}$ for 30 min. Protein C activation was terminated by adding 40 μ l of antithrombin III (6 IU/ml) and heparin (12 IU/ml). The enzymatic activity of activated protein C was

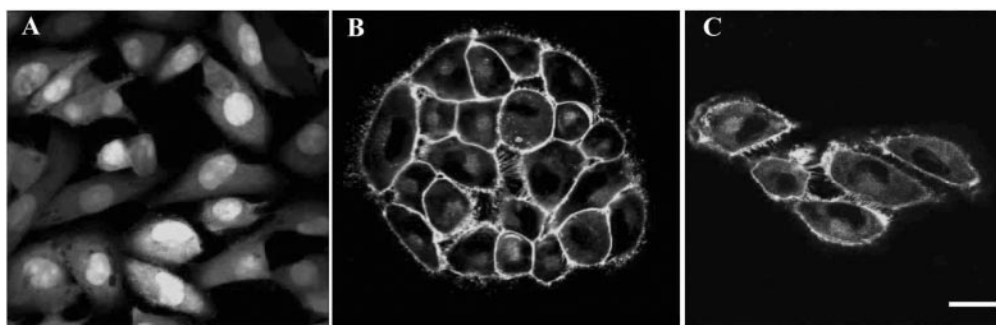


FIG. 2. Confocal microscopy examination of the subcellular distribution of TM proteins in A2058 cells and the cell morphology. Confocal microscopy was utilized to observe A2058 cells stably expressing GFP (A), TMG (B), and TMG(Δ L) (C). The cells were fixed and photographed to observe the localization of TM protein. Bar, 40 μ m.

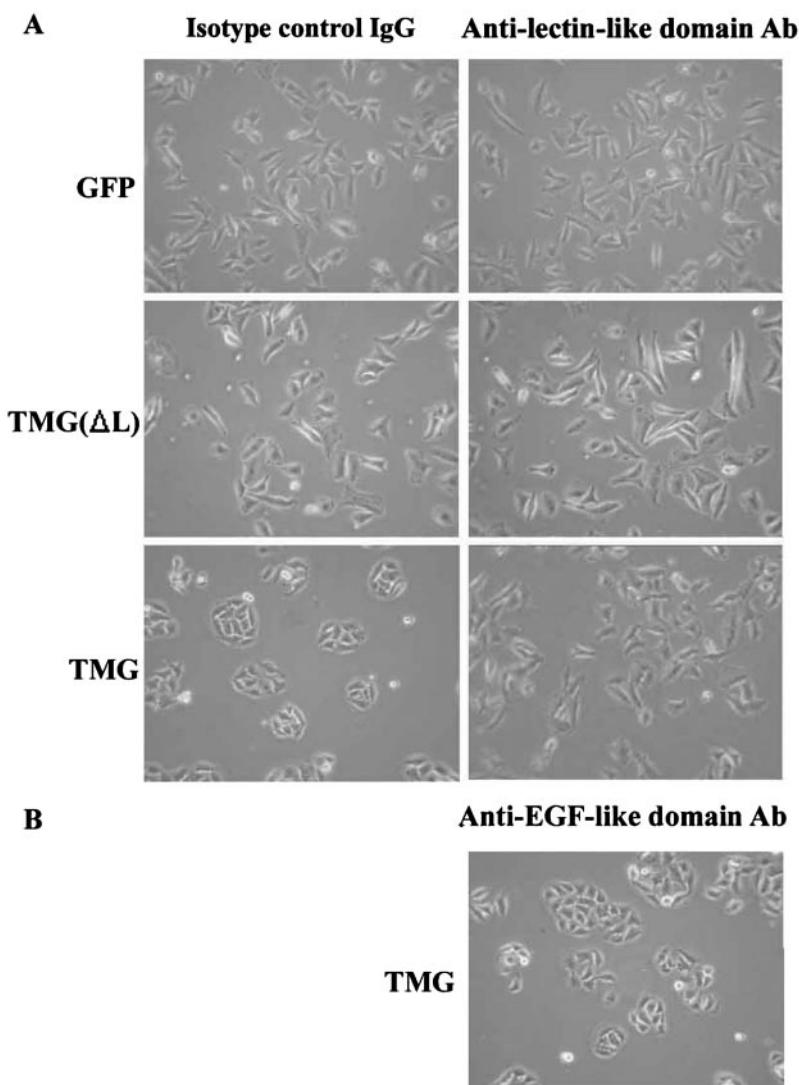


FIG. 3. Effects of anti-TM antibodies on the organization of cell-cell adhesion junctions in TMG-expressed A2058 cells. Anti-lectin-like domain antibody (10 μ g/ml) or isotype-matched control IgG (10 μ g/ml) was added to cultured monolayers for 24 h (A), and the cell morphology was examined using a Leica inverted microscope. A comparative morphology of the TMG-expressed cells treated with anti-EGF-like domain antibody (10 μ g/ml) was photographed (B).

measured with the peptide substrate H-D-Lys-Z-Pro-Arg-4-nitroanilide-diacetate (Chromozym PCA; 0.5 mM in 20 mM Tris, pH 7.4, 0.15 M NaCl, and 5 mg/ml bovine serum albumin) at 37 °C. The absorbance change at 405 nm was measured with a Thermomax Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). Controls containing thrombin and protein C in the absence of cells were treated similarly.

Electrophoresis and Immunoblot Analyses—TM-expressing cells were washed twice with cold phosphate-buffered saline (PBS), lysed in PBS containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 5 μ g/ml aprotinin, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, and 1 mM EDTA at 4 °C for 20 min and then disrupted with a needle. Total lysates were quantified using a micro-BCA kit (Pierce). Proteins (10 μ g) were resolved by SDS-poly-

acrylamide gel electrophoresis and transferred electrophoretically to a nylon filter. The nylon filter was blocked for 1 h in 5% (v/v) fat-free milk in PBST buffer (PBS with 0.05% Tween 20). After a brief wash in the buffer, the nylon filter was incubated overnight at 4 °C with mouse anti-human TM antiserum diluted in PBST buffer. The antiserum was prepared in our laboratory from BALB/c mice immunized with recombinant TM protein purified from the *Pichia pastoris* expression system. The primary antibody was removed, and the filter was washed four times in PBST buffer. Subsequent incubation with horseradish peroxidase-labeled goat anti-mouse antibody proceeded at room temperature for 2 h. The filter was washed four times in PBST buffer to remove the secondary antibody, and the blot was visualized with enhanced chemiluminescence reagent.

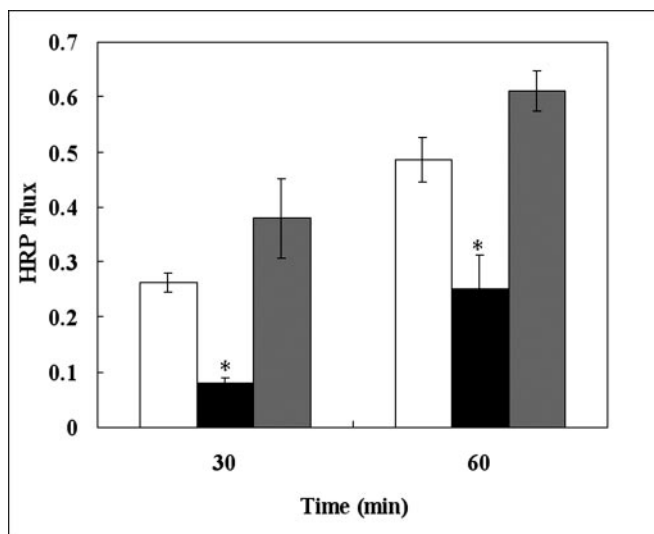


FIG. 4. **Overexpression of TMG decreases the A2058 cell monolayer permeability.** Monolayer cell permeability was decreased in A2058 cells overexpressing TMG as determined by horseradish peroxidase flux across confluent monolayer. Statistical evaluation of quadruplicate determinations showed a statistically significant between TMG (black bars) and TMG(Δ L) (gray bars) or GFP-expressed control (open bars). *, $p < 0.05$ versus GFP-expressed cells. An unpaired Student's t test was used. The experiment was repeated three times.

Confocal Microscopy—To examine the distribution of TM, transfected cells were grown on polylysine-coated coverslips overnight. The coverslips were washed three times with cold PBS, and the cells were fixed with a 3.7% (v/v) formaldehyde solution in PBS and mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Cells were observed using a laser-scanning confocal microscope (Leica model TCS2) with a Leica Mellis-Griot $\times 63$ numerical aperture oil immersion objective, pinhole of 1.5, and electronic zoom 1.5 or 2. GFP was excited using a 488-nm argon/krypton laser and detected with a 515–540-nm band pass filter. Tetramethylrhodamine was excited using a 543-nm argon/krypton laser and detected with a 550–620-nm band pass filter. The images were manipulated with a Leica TCS NT scanner.

Immunofluorescence Staining—For immunofluorescence staining, cells were grown on glass coverslips at 37 °C. After being fixed in 3.7% (v/v) formaldehyde in PBS, cells were permeabilized with 0.2% (v/v) Triton X-100 and blocked with 10% fetal bovine serum in PBS.

Tetramethylrhodamine-phalloidin, anti-keratin antibody, anti-lectin-like domain antibody, or anti-human TM EGF-like domain antibody was applied to the samples. After three PBS washes, cells were incubated for 1 h at room temperature with tetramethylrhodamine-labeled secondary antibodies. Glass coverslips were washed three times with PBS, mounted, and examined using a confocal microscope.

Calcium Switch Methods—Cells were grown overnight on glass coverslips at a constant density (5×10^4 cells/well) in 24-well culture plates. The cells were serum-starved for 8 h, and Ca^{2+} was removed by incubation with Dulbecco's modified Eagle's medium containing 4 mM EGTA and 1 mM MgCl_2 at 37 °C. After 1 h, Dulbecco's modified Eagle's medium containing 1.8 mM Ca^{2+} was added to replace the Ca^{2+} -free medium. In control experiments, cells received fresh medium in the absence of EGTA. In selected experiments, 10 $\mu\text{g}/\text{ml}$ of anti-TM lectin-like antibody, 10 $\mu\text{g}/\text{ml}$ isotype-specific control antibody or anti-E-cadherin antibody (20 $\mu\text{g}/\text{ml}$) was added to the Ca^{2+} -containing medium.

Determination of Carbohydrate Specificity for TM-mediated Cell Adhesion—A variety of simple carbohydrates (D-mannose, D-galactose, D-lactose, D-glucose, D-xylose), heparin, low molecular weight heparin, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C were tested to determine their ability to block cell-cell adhesion of the TMG cells. In these experiments, the carbohydrate to be tested was added to the cell monolayer to compare its ability to compete with the TMG. The plates were then incubated at 37 °C overnight and examined by light microscopy (Leica model DM IL).

Monolayer Permeability Assay—Horseradish peroxidase flux across A2058 cell monolayers was measured using Transwell cell culture chambers (0.4- μm pore polycarbonate filters; Corning) as previously

described (26). Briefly, A2058 cells (7.5×10^4) were cultured for 2–3 days in Transwell units (Corning). After reaching confluence, cells were washed, and the medium was replaced with serum-free medium (1.5-ml upper chamber and 2.6-ml lower chamber). Type IV-A horseradish peroxidase (0.1 μM) was added to the upper chamber and incubated at 37 °C. At the indicated time, medium in the lower chambers was assayed for horseradish peroxidase activity using a 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate system according to the manufacturer's instructions (Sigma).

Tumor Growth in Vivo—To study the *in vivo* growth of A2058 cells, BALB/c SCID male mice were used. Cells (10^6) in 100 μl of PBS were injected subcutaneously into 6–8-week-old male mice. Tumor sizes were recorded every 7 days by measuring the two largest diameters.

RESULTS

Expression of TMG and TMG(Δ L) Proteins in A2058 Cells—The cDNA encoding either the full-length human TM or the lectin-like domain truncated TM was cloned from human DNA and ligated to the GFP gene in the mammalian expression vector pEGFPN1 (Fig. 1A). Each recombinant gene was transfected into A2058 cells. Several stable clones expressing TM-GFP fusion proteins were initially screened by the presence of GFP autofluorescence on the cell membrane. These clones (TMG and TMG(Δ L)) were maintained for the experiments described subsequently.

The thrombin-interacting domain of TM extended to the outer surface of the cells (as expected in native TM) because the cells expressing TMG or TMG(Δ L) proteins activated protein C in conjunction with thrombin, whereas clones that expressed GFP alone showed no thrombin-dependent protein C activation (Fig. 1B). The TMG and TMG(Δ L) proteins had molecular masses of 110 and 94 kDa, respectively, which were close to the calculated values (Fig. 1C).

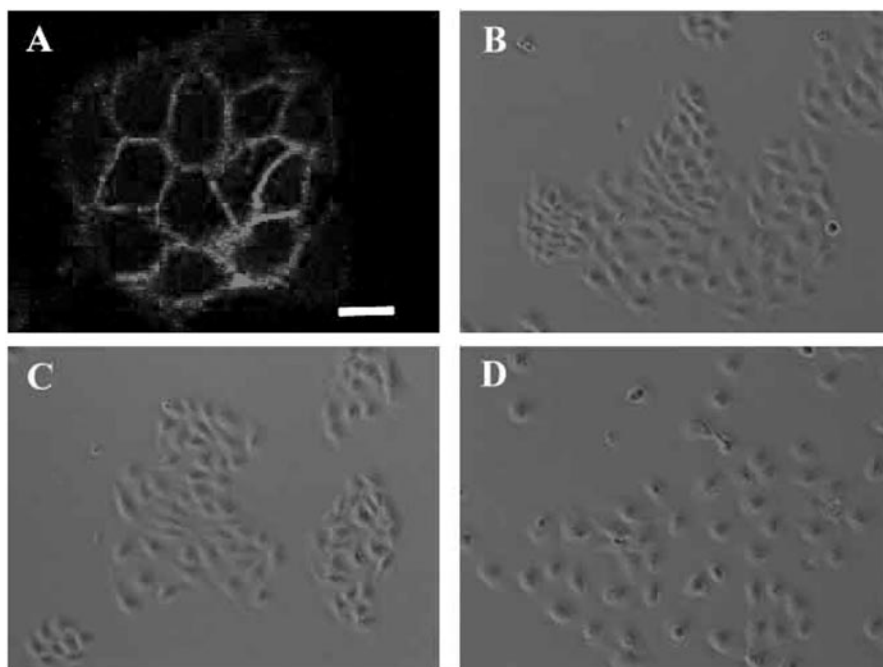
TM Localization and Cell Morphology—The cell morphology and subcellular localization of GFP-tagged TM protein in A2058 cells were monitored by confocal microscopy. The green fluorescence of the GFP-expressed cells was evident in the cytoplasm, with a higher concentration in the nuclear region (Fig. 2A). Conversely, the green fluorescence of TMG proteins was distributed near the cell surface, particularly at regions of cell-to-cell contact (Fig. 2B). The fluorescence of TMG(Δ L) was evenly distributed on the cell membrane (Fig. 2C).

The cells expressing TMG clustered closely together, with strong cell-to-cell adhesion that was distinctly different from parental A2058 cells and cells expressing GFP or TMG(Δ L) (Fig. 2). Phase-contrast images also showed that cells expressing TMG produced compact cell colonies, and cells at the edges of colonies rarely extended membrane protrusions onto the surrounding cell-free surface (Fig. 3A). In contrast, both the clones of the GFP- or TMG(Δ L)-expressed cells were poorly compacted and had a more fibroblastic morphology than TMG-expressed cells (Fig. 3A). Similar results were observed in five stable clones of TMG and seven clones of TMG(Δ L).

Lectin-like Domain-mediated Cell-to-cell Adhesion—Because accumulation of TM proteins in the cell-to-cell adhesion sites led to the establishment of the compact clustering morphology, we further explored whether the lectin-like domain of TM played a critical role in cell-to-cell contacts. A monoclonal antibody (clone D-3) directed against the lectin-like domain was used to block the function of lectin-like domain and to test its effect on cell morphology.

The antibody bound specifically to TM protein in the TMG cell lysates rather than any proteins in the control or TMG(Δ L) cell lysates, as shown by Western blotting results. When the TMG-expressed cells were incubated with a monoclonal antibody directed against the lectin-like domain, the cell-to-cell contacts were completely inhibited (Fig. 3A). On the other hand, the antibody specific for the EGF-like domain of TM did not cause the TMG-expressed cells to assume the dispersed

FIG. 5. Localization of TM protein and effects of anti-TM antibodies on the cell-cell adhesion of HaCaT cells. HaCaT cells were incubated with the anti-lectin-like domain of the TM antibody and detected with tetramethylrhodamine-conjugated anti-mouse antibody. Cells were photographed by confocal microscopy. *A*, bar, 40 μm . HaCaT cells were treated with 10 $\mu\text{g}/\text{ml}$ of isotype-control IgG (*B*), 10 $\mu\text{g}/\text{ml}$ anti-EGF-like domain antibody (*C*), or 10 $\mu\text{g}/\text{ml}$ anti-lectin-like domain antibody (*D*) for 24 h. Cells were photographed by a Leica inverted microscope. The results shown are representative of three independent experiments.



type colony (Fig. 3*B*). These results were consistent with a more critical role of the lectin-like domain *versus* the EGF-like domain in promoting the formation of close cell-to-cell contacts in the cultures of TMG-expressed cells.

To further investigate the effect of TM expression on the permeability of the cell-to-cell junction, the infiltration ability of horseradish peroxidase through a monolayer of the cell cultures on the polycarbonate membrane was measured using a Transwell assay system. The permeability of the monolayer of TMG cell cultures was significantly lower compared with the TMG(Δ L) and control cells (Fig. 4).

Previous reports have demonstrated that human keratinocytes express TM, which appears to be predominantly localized to the cell membrane and the intercellular bridges (20). To further verify the potential physiologic importance of TM in the cell-to-cell adhesion junction, a keratinocyte cell line derived from normal human epidermis (HaCaT) (25), which expressed TM endogenously, was used to observe the morphologic changes in the presence of a monoclonal antibody directed against the lectin-like domain of TM. Immunofluorescence microscopy using an anti-TM lectin-like domain antibody showed that a high concentration of TM protein was localized at the intercellular boundary of HaCaT cells (Fig. 5*A*). The cultured HaCaT cells showed typical compacted sheet-forming colonies. However, HaCaT cells were almost completely dissociated when cultured in the medium containing the anti-lectin-like domain antibody for 24 h. The anti-EGF domain antibody or the isotype control IgG failed to dissociate the compact colonies (Fig. 5, *B–D*).

Ca²⁺ Involvement in TM-mediated Cell-to-cell Adhesion—The NH₂ terminus of the TM molecule contains a C-type lectin domain, to which the binding of potential ligand is Ca²⁺-dependent. The Ca²⁺ switch method was utilized to investigate whether TM-mediated adhesion junction assembly is Ca²⁺-dependent. The cell-to-cell contacts of the A2058TMG (Fig. 6*A*) or HaCaT (Fig. 6*F*) cells were disrupted when the culture medium was changed to the EGTA-containing medium for 40 min (Fig. 6, *B* and *G*). The cell-to-cell adhesion junction was restored in the Ca²⁺-containing medium for 1 h with the presence of 10 $\mu\text{g}/\text{ml}$ control IgG (Fig. 6, *C* and *H*).

To further verify the TM domain that was directly involved

in the Ca²⁺-modulated adhesion, the functional antibody against the lectin-like domain of TM was added to the Ca²⁺-containing medium, following the EGTA dissociation of cell-to-cell adhesion. No restoration of cell-to-cell adhesion occurred in the presence of a 10 $\mu\text{g}/\text{ml}$ concentration of the antibody (Fig. 6, *D* and *I*). Furthermore, TM localization became more uniformly distributed rather than concentrated at the intercellular region (Fig. 6*I*). Anti-E-cadherin antibody (20 $\mu\text{g}/\text{ml}$) was able to inhibit Ca²⁺-dependent cell-cell adhesion in HaCaT cells (Fig. 6*J*) but not in TMG cells (Fig. 6*E*).

TMG Colocalized with Actin Filaments at the Submembrane Cortex—The intracellular domains of adhesion molecules, including cadherins and integrins, interact with the cytoskeleton actin filaments or intermediate filaments through adaptor proteins inside the cell. These interactions provide mechanical continuity from cell to cell (27). We examined the colocalization of the TMG proteins and these cytoskeletal elements in TMG-expressed cells by confocal microscopy. The actin and intermediate filaments in the cultured cells were immunohistochemically stained with tetramethylrhodamine-labeled phalloidin or anti-human keratin antibody, respectively. The surface TM molecules and actin filaments were colocalized at the cortex region in cell-cell adhesion sites (Fig. 7*A*). In contrast, the keratin filaments localized in the cytoplasmic region, and there was little overlap in distribution between the TM and intermediate filaments (Fig. 7*B*).

Influence of Mannose, Chondroitin Sulfate A, or Chondroitin Sulfate C on the Cell-cell Adhesion in TMG Cells—Based on the observation that TMG-expressed A2058 cells formed close clustering colonies, we proposed that the lectin-like domain of TM might mediate the cell-cell adhesion by binding to specific carbohydrate moieties of the neighboring cells. To test the hypothesis, different carbohydrates, including D-mannose, D-galactose, D-glucose, D-xylose, D-lactose, chondroitin sulfate A, B, and C, heparin, and low molecular weight heparin, were tested for their ability to disperse the close clustering morphology of the TMG culture cells. Among these monosaccharides, only mannose was found to be effective in inducing of cell dispersion. Among the sulfate-containing polysaccharides, chondroitin sulfate A (chondroitin 4-sulfate) and chondroitin sulfate C (chondroitin 6-sulfate) also could induce cell disper-

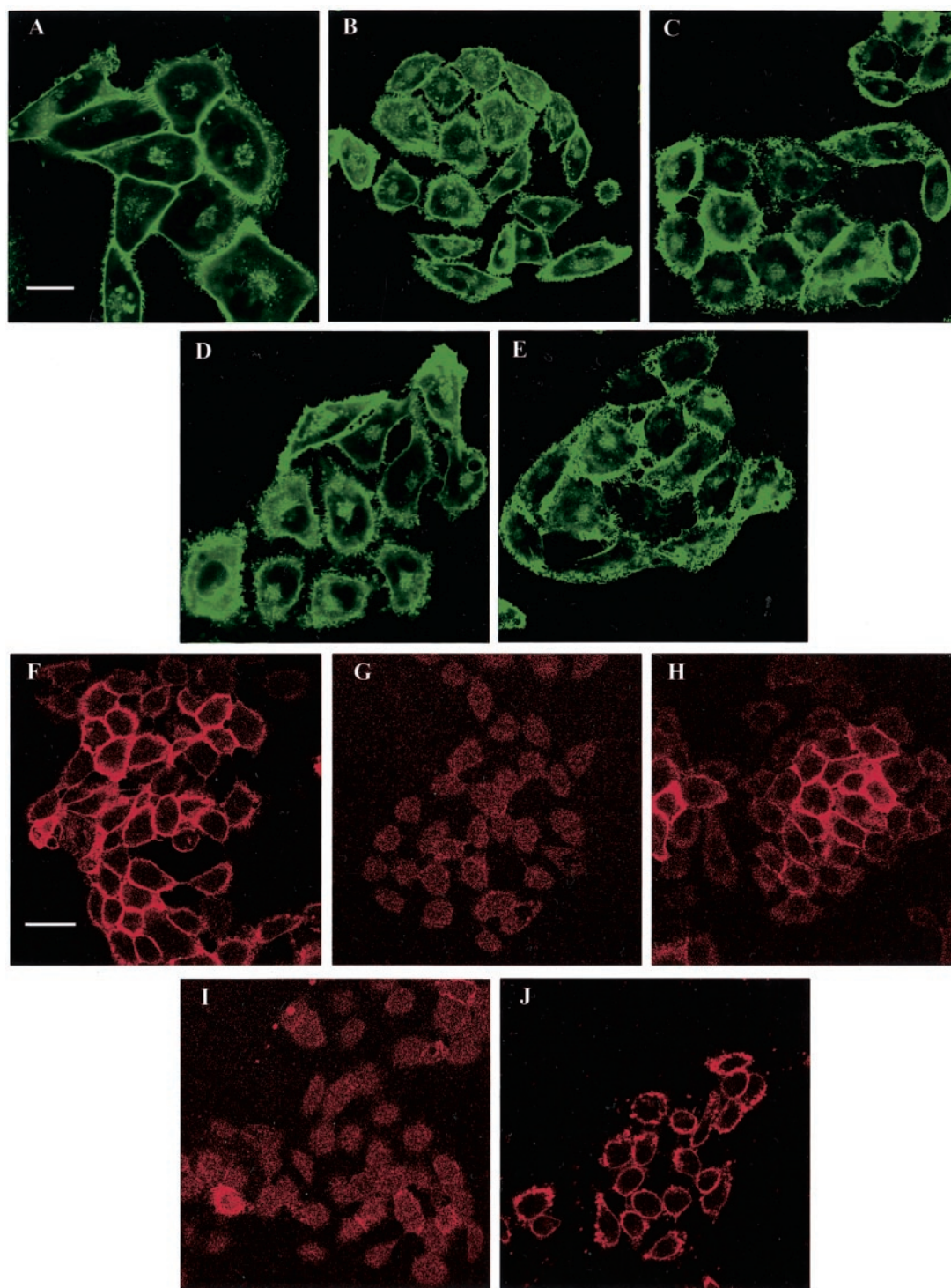


FIG. 6. TM participates in Ca^{2+} -dependent cell-cell adhesion. A2058TMG (A–E) and HaCaT cells (F–J) were left untreated (A and F) or treated with 4 mM EGTA for 40 min (B–E and G–J). The EGTA-containing medium was then replaced with Ca^{2+} -containing medium in the presence of 10 $\mu\text{g}/\text{ml}$ control IgG (C and H) for 1 h. After treatment with EGTA, cells were incubated for 1 h in medium containing 1.8 mM Ca^{2+} and 10 $\mu\text{g}/\text{ml}$ function-blocking anti-lectin-like antibody (D and I) or anti-E-cadherin antibody (E and J). Green fluorescence shown in A–E is the GFP-tagged TM proteins, whereas F–J indicate the TM distribution by anti-EGF-like domain antibody detected with tetramethylrhodamine-conjugated anti-mouse antibody. Bar, 40 μm .

sion. Heparin showed a minor inhibitory effect on the cell adhesion. On the other hand, chondroitin sulfate B (dermatan sulfate) showed no effect (Fig. 8). A similar inhibition of the cell-cell adhesion by mannose or chondroitin sulfate C was observed in HaCaT cells, whereas the other sugars had no effects.

Influence of the Overexpression of TM on Tumor Cell Growth in Vivo—To assess the functional consequences of TM-mediated adhesion, we next investigated whether the observed effects of TM-mediated adhesion could also affect the growth of A2058 tumor cell lines. TMG-, TMG(Δ L)-, or GFP-expressed cells were used to initiate tumors in SCID mice. The tumors

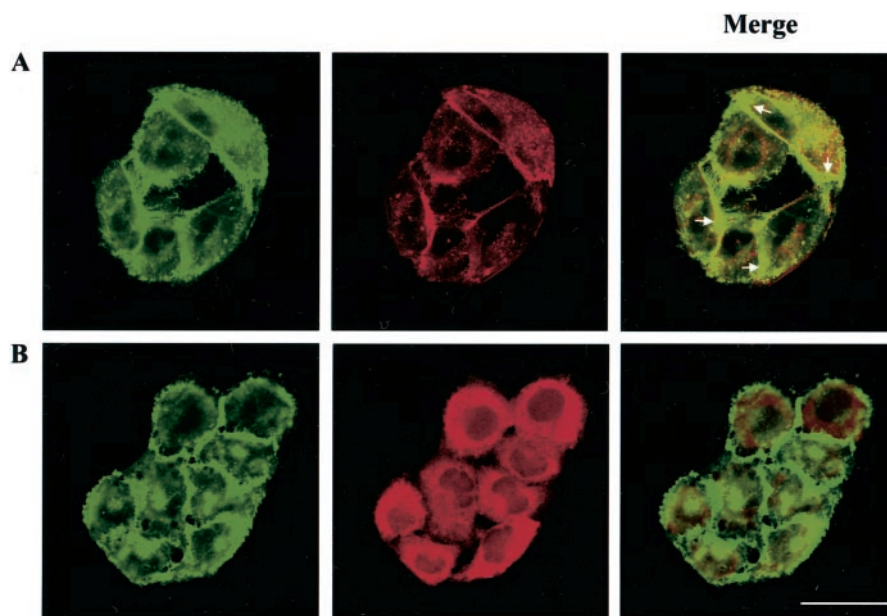


FIG. 7. Colocalization of TM (green) and the actin (red) in TMG-expressed cells. TMG-expressed cells were fixed, permeabilized, and immunolocalized using tetramethylrhodamine-conjugated phalloidin (A) or a monoclonal antibody recognizing human keratin (B). The Merge panel shows the superimposed images from TMG and tetramethylrhodamine fluorophore. Three separate experiments produced similar results. The arrows denote the colocalization of TM and actin filaments. Bar, 20 μm .

induced by inoculation with TMG(Δ L)-expressed cells had an about 1.4-fold increased size, relative to the tumors induced by the GFP-expressed cells (Fig. 9). The tumors induced by the TMG-expressed cells have the smallest size in comparison with other transfected cells.

DISCUSSION

TM, which is a well known anticoagulation factor, may function as a cell adhesion molecule, given that the glycoprotein is present in the junction of different epithelial cells (7, 8, 19). This role for TM was investigated in the present study. We utilized the A2058 cell line, which has no endogenous expression of TM or E-cadherin. A2058 cells were transfected with different constructs of TM genes to investigate the functions of TM and its domains in cell-cell adhesion and cell morphology. A2058 cells without TM assumed a fibroblastic-like cell morphology and were dispersed as single cells in cultures of non-confluent cell densities. In the clones of TMG, the green fluorescence-tagged TM was located at the cell surface, especially near the cell-cell junctions. Interestingly, the transfected cells assumed an epithelial-like morphology and formed sheet-like colonies with obvious cell-to-cell adhesion in the culture (Fig. 2). In contrast, the clones of TMG(Δ L)-expressed cells displayed fibroblastic-like morphology with less adhesion observed. The results indicate that TM acts as a cell-cell adhesion molecule. Adhesion molecules, in particular E-cadherin, have also been reported to cause morphological transition, for example, from the fibroblastic cell type to the epithelial cell type of several cell lines (23, 28, 29). In the TMG-transfected A2058 cells, E-cadherin could not be detected by immunohistochemical staining (data not shown). In this study, TM appears to assume the function typically attributed to E-cadherin.

Lectins represent a diverse category of carbohydrate binding proteins (including C, P, and I types). Among these distinct types of lectins, the C-type lectins are distinguished by their requirement for Ca^{2+} for sugar binding (15). Biological functions of the lectin-like domain unrelated to the anticoagulant activity of TM that have been proposed include internalization of the TM-thrombin complex (30), regulation of cell proliferation (10), and interference with the adhesion of polymorphonuclear leukocytes to activated endothelial cells (31).

Presently, we provide several lines of evidence to demonstrate that the TMG lectin-like domain may be directly involved in cell-to-cell interaction. First, only the culture of TMG-transfected A2058 cells formed close cell-cell contacts, not the culture of parental or TMG(Δ L) cells (Figs. 2 and 3). Second, the cell-cell adhesion in TMG-expressed cells is Ca^{2+} -dependent, since cell association could be blocked by EGTA and was restored by Ca^{2+} (Fig. 6). The observation reveals that the lectin-like domain of TM is a C-type lectin. Furthermore, this restoration of cell-cell adhesion can be blocked by anti-lectin-like domain antibody, consistent with the proposed function of TM. Third, anti-lectin-like domain antibody, not the anti-EGF-like domain antibody, is able to block cell-to-cell contacts and inhibit the close clustering morphology in TMG cells (Fig. 3). In the A2058 cell line, no E-cadherin was expressed, and anti-E-cadherin antibody could not block the cell-cell adhesion (Fig. 6E). Fourth, it is noteworthy that the infiltration rate of protein molecules has been decreased to 40–50% in the TMG cell monolayer (Fig. 4). Such a shift in protein permeability suggests that TM might be either a mediator of cell-cell contacts or a molecule directing the establishment and the maintenance of the cell-cell adhesion. Finally, analysis of possible carbohydrate ligands showed that mannose, or chondroitin sulfate (including A and C), blocked the cell-to-cell adhesion in TMG cells. The results provided more detailed evidence concerning the function of lectin-like domain in the TM-mediated cell-cell adhesion. Taken together, these observations prompt us to propose that the lectin-like domain in TMG-transfected A2058 cells mainly mediates cell-cell adhesion by Ca^{2+} -dependent binding to its specific carbohydrate ligands on the neighboring cells.

Adherens junction is a specialized form of cadherin-based adhesive contacts required for epidermal sheet organization. E-cadherin is expressed throughout the epidermis and has been identified as one of the major adherens junction molecules mediating keratinocyte-keratinocyte interaction. Specific antibodies inhibiting its function cause severe perturbations in normal skin structure (32). Similarly, TM antigen was reported to be lost in blistering dermatoses, implying that TM may also participate in cell-cell adhesion in epidermis (33).

To verify the participation of TM in cell adhesion, we inves-

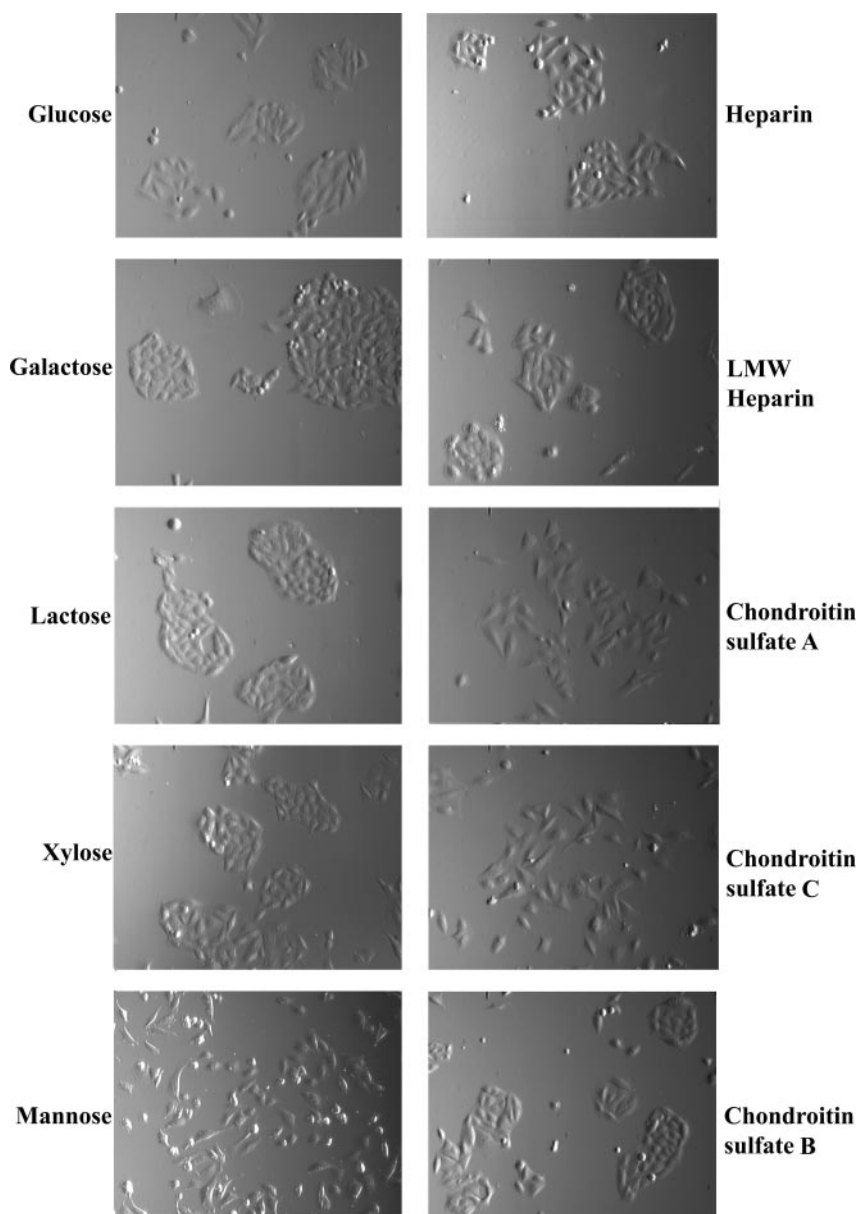


FIG. 8. Effect of different carbohydrates on the cell adhesion of TMG cells. TMG-expressed A2058 cells were challenged with 20 mg/ml monosaccharides (including D-glucose, D-galactose, D-lactose, D-xylose, and D-mannose), 20 mg/ml chondroitin sulfate A, B, C, or 15 mg/ml heparin or low molecular weight (LMW) heparin. Following incubation at 37 °C overnight, images were obtained. Each image is representative of the observations from three separate experiments.

tigated the perturbing effect on the cell-cell adhesion of HaCaT by incubating the cells with anti-TM and anti-E-cadherin antibodies. In this spontaneously transformed keratinocyte cell culture, both TM and E-cadherin are detected on the cell membrane, especially with a high concentration at cell-cell junctions. As shown in Fig. 6, either the anti-lectin-like domain antibody or anti-E-cadherin antibody could inhibit Ca^{2+} -induced cell-cell readhesion of EGTA-treated HaCaT cells. It appears that both E-cadherin and TM are necessary for the formation of cell junctions in HaCaT cell lines. We were able to further evidence this hypothesis through the characterization of TM distribution during cell-cell adhesion disruption. When HaCaT cells were treated with EGTA, TM became clearly disorganized and intracellularly localized (Fig. 6I), consistent with the involvement of TM in the scaffolding of adhesion molecules at the lateral membrane during events of cell-cell adhesion formation or disruption. Furthermore, we have noted similar staining patterns between TM and E-cadherin on HaCaT cells (data not shown). Blockage of the function of either one of these two potential adhesive molecules will cause the cells incapable to form cell-cell attachment at least in the time period. A

functional hierarchy in the assembling of cell-cell adhesion by these two adhesion molecules is being investigated.

It is important to identify the physiological ligands of the C-type lectin-like domain of TM to fully understand the functional significance and mechanism of TM in the cell-cell adhesion. Adhesion molecules such as cadherins prefer to bind themselves through homotypic interactions, whereas molecules of the immunoglobulin-cell adhesion molecule family bind cell surface proteins via heterotypic interactions (34). The ligands of some endogenous lectins have been recognized, including sialyl-Lewis^x, sialyl-Lewis^a, sulfated polysaccharides, and the mannose 6-phosphate-containing polysaccharides (35–37). However, the ligand of lectin-like domain of TM has never been identified. The present study reveals that the lectin-like domain of TM can bind carbohydrates. The candidate carbohydrates contain mannose residue, chondroitin sulfate A, or chondroitin sulfate C moieties, although mannose showed the highest dispersing effect. Although previous studies reported that TM contained mainly chondroitin sulfate A (chondroitin 4-sulfate) (38), we found that the same concentration of chondroitin sulfate C (chondroitin 6-sulfate) also dispersed the clus-

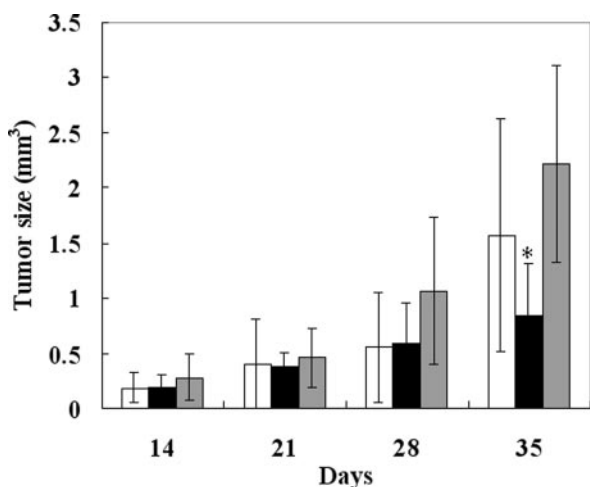


FIG. 9. *In vivo* tumor growth assay of TM-expressed A2058 melanoma cells. A total of 1×10^6 TMG (black bars)-, TMG(Δ L) (gray bars)-, or GFP (open bars)-expressed cells was injected subcutaneously into SCID mice. Tumor size was recorded every 7 days by measuring the two largest diameters. Data show mean \pm S.D. of five tumors in each group, and the experiment was repeated three times independently. *, $p < 0.05$ versus GFP-expressed cells. An unpaired Student's *t* test was used.

tering colonies. Unlike chondroitin sulfate, mannose was broadly existed in carbohydrate moiety of glycoproteins or glycolipids (39). This result suggests that mannose could be one of the ligands of the lectin-like domain. However, we cannot rule out the possibilities that the carbohydrate ligands of TM lectin-like domain belong to the glycoconjugated molecules on cell surfaces other than TM. The structure of the carbohydrate ligands still remained to be identified.

The interaction of cytoplasmic domain of adhesion molecules and cytoskeleton also has been demonstrated to be essential for cell-cell adhesion. These interactions could provide adhesion strength in endothelial or epithelial sheets, allowing them to resist mechanical disruption (40). Presently, we observed that the TMG protein and actin molecules were colocalized in the cortex region of the cells (Fig. 7). The culture cells with cytoplasmic domain-truncated TM did not form close-clustering colonies.² The results indicate that the cytoplasmic domain of TM is necessary in forming stable cell-to-cell adhesion. One of the possible functions of the cytoplasmic domain is that it may associate with actin through uncharacterized linkage proteins that regulate TM distribution on the cell surface as cadherin (27). However, transgenic mice lacking of TM NH₂-terminal lectin-like domain (31) or cytoplasmic domain of TM (41) were found to have normal fetal development. It is possible that in animal tissues, other potential molecules such as cadherins may compensate the loss of adhesive function of the truncated TM domains (23). The physiological significance of TM-mediated cell-cell adhesion remained to be further investigated *in vivo*.

The down-regulation of E-cadherin expression has been documented in the cells of poorly differentiated tumors, indicating that a decrease in cell-cell adhesion may be associated with undifferentiated phenotype and aberrant growth of tumors (24). Similarly, the inverse relationship of TM expression and cell proliferation rate has been reported in hepatocellular carcinoma, ovarian carcinoma, and esophageal squamous carcinoma (7, 8, 42). The parallel correlation between E-cadherin and TM in tumor progress implies that less cell-cell adhesion may be involved in tumor progression and the onset of an

invasive phenotype of carcinomas.

In order to test the hypothesis that TM-induced cell-cell adhesion may affect the tumor growth rate *in vivo*, the tumor growth rates were measured in SCID mice following inoculation of TMG- or TMG(Δ L)-expressed A2058 cells. Tumor size was greatest in tumors arising from TMG(Δ L)-expressed cells, intermediate in vector, and lowest in TMG-expressed cells (Fig. 9). The results show that TMG without the lectin-like domain lack the restrain activity in cell proliferation. We therefore suggested that the increase of cell-to-cell adhesion upon TM expression may slow down tumor growth *in vivo*.

We conclude that TM might be present as a multifunctional molecule. It not only can function as an anticoagulant regulator in the vascular endothelial cells, it may also function as a cell-cell adhesion molecule through a Ca²⁺-dependent interaction of lectin-like domain.

REFERENCES

- Esmon, C. T., Esmon, N. L., and Harris, K. W. (1982) *J. Biol. Chem.* **257**, 7944–7947
- Esmon, C. T. (1995) *FASEB J.* **9**, 946–955
- Boffa, M. C., Burke, B., and Haudenschild, C. C. (1987) *J. Histochem. Cytochem.* **35**, 1267–1276
- Healy, A. M., Rayburn, H. B., Rosenberg, R. D., and Weiler, H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 850–854
- Raife, T. J., Lager, D. J., Peterson, J. J., Erger, R. A., and Lentz, S. R. (1998) *J. Invest. Med.* **46**, 127–133
- Lane, D. A., and Grant, P. J. (2000) *Blood* **95**, 1517–1532
- Tezuka, Y., Yonezawa, S., Maruyama, I., Matsushita, Y., Shimizu, T., Obama, H., Sagara, M., Shirao, K., Kusano, C., Natsugoe, S., Yoshinaka, H., Baba, M., Fukumoto, T., Aikou, T., and Sato, E. (1995) *Cancer Res.* **55**, 4196–4200
- Tabata, M., Sugihara, K., Yonezawa, S., Yamashita, S., and Maruyama, I. (1997) *J. Oral Pathol. Med.* **26**, 258–264
- Suehiro, T., Shimada, M., Matsumata, T., Taketomi, A., Yamamoto, K., and Sugimachi, K. (1995) *Hepatology* **21**, 1285–1290
- Zhang, Y., Weiler-Guettler, H., Chen, J., Wilhelm, O., Deng, Y., Qiu, F., Nakagawa, K., Klevesath, M., Wilhelm, S., Bohrer, H., Nakagawa, M., Graeff, H., Martin, E., Stern, D. M., Rosenberg, R. D., Ziegler, R., and Nawroth, P. P. (1998) *J. Clin. Invest.* **101**, 1301–1309
- Suzuki, K., Kusumoto, S., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S., and Horiguchi, S. (1987) *EMBO J.* **6**, 1891–1897
- Petersen, T. E. (1988) *FEBS Lett.* **231**, 51–53
- Chay, C. H., and Pienta, K. J. (2000) *J. Cell. Biochem. Suppl.* **35**, 123–129
- Gabius, H. (1997) *J. Cancer Invest.* **15**, 454–464
- Mody, R., Joshi, S., and Chaney, W. (1995) *J. Pharmacol. Toxicol. Methods* **33**, 1–10
- Larue, L., Ohsugi, M., Hirchenhain, J., and Kemler, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8263–8267
- Riethmacher, D., Brinkmann, V., and Birchmeier, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 855–859
- Hynes, R. O., and Wagner, D. D. (1996) *J. Clin. Invest.* **98**, 2193–2195
- Ogawa, H., Yonezawa, S., Maruyama, I., Matsushita, Y., Tezuka, Y., Toyoyama, H., Yanagi, M., Matsumoto, H., Nishijima, H., Shimotakahara, T., Aikou, T., and Sato, E. (2000) *Cancer Lett.* **149**, 95–103
- Raife, T. J., Lager, D. J., Madison, K. C., Piette, W. W., Howard, E. J., Sturm, M. T., Chen, Y., and Lentz, S. R. (1994) *J. Clin. Invest.* **93**, 1846–1851
- Matsushita, Y., Yoshiie, K., Imamura, Y., Ogawa, H., Imamura, H., Takao, S., Yonezawa, S., Aikou, T., Maruyama, I., and Sato, E. (1998) *Cancer Lett.* **127**, 195–201
- Larger, D. J., Callaghan, E. J., Worth, S. F., Raife, T. J., and Lentz, S. R. (1995) *Am. J. Pathol.* **146**, 933–943
- Takeichi, M. (1991) *Science* **251**, 1451–1455
- Conacci-Sorell, M., Zhurinsky, J., and Ben-Ze'ev, A. (2002) *J. Clin. Invest.* **109**, 987–991
- Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) *J. Cell Biol.* **106**, 761–771
- Herren, B., Garton, K. J., Coats, S., Bowen-Pope, D. F., Ross, R., and Raines, E. W. (2001) *Exp. Cell Res.* **271**, 152–160
- Kinch, M. S., Clark, G. J., Der, C. J., and Burridge, K. (1995) *J. Cell Biol.* **130**, 461–471
- Pignatelli, M., Liu, D., Nasim, M. M., Stamp, G. W., Hirano, S., and Takeichi, M. (1992) *Br. J. Cancer* **66**, 629–634
- Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., and Takeichi, M. (2002) *Neuron* **35**, 77–89
- Conway, E. M., Pollefeyt, S., Collen, D., and Steiner-Mosonyi, M. (1997) *Blood* **89**, 652–661
- Conway, E. M., Van de Wouwer, M., Pollefeyt, S., Jurk, K., Van Aken, H., De Vriese, A., Weitz, J. I., Weiler, H., Hellings, P. W., Schaeffer, P., Herbert, J. M., Collen, D., and Theilmeier, G. (2002) *J. Exp. Med.* **196**, 565–577
- Hirano, S., Nose, A., Hatta, K., Kawakami, A., and Takeichi, M. (1987) *J. Cell Biol.* **105**, 2501–2510
- Jackson, D. E., Mitchell, C. A., Mason, G., Salem, H. H., and Hayman, J. A. (1996) *Pathology* **28**, 225–228
- Hubbard, A. K., and Rothlein, R. (2000) *Free Radic. Biol. Med.* **28**, 1379–1386
- Jacob, G. S., Welply, J. K., Scudder, P. R., Kirmaier, C., Abbas, S. Z., Howard,

² H.-C. Huang, G.-Y. Shi, S.-J. Jiang, C.-S. Shi, C.-M. Wu, H.-Y. Yang, and H.-L. Wu, unpublished results.

- S. C., Keene, J. L., Schmuke, J. J., Broschat, K., and Steininger, C. (1995) *Adv. Exp. Med. Biol.* **376**, 283–290
36. Varki, A., and Varki, N. M. (2001) *Braz. J. Med. Biol. Res.* **34**, 711–717
37. Stahl, P. D. (1992) *Curr. Opin. Immunol.* **4**, 49–52
38. Nawa, K., Sakano, K., Fujiwara, H., Sato, Y., Sugiyama, N., Teruuchi, T., Iwamoto, M., and Marumoto, Y. (1990) *Biochem. Biophys. Res. Commun.* **171**, 729–737
39. Pollack, L., and Atkinson, P. H. (1983) *J. Cell Biol.* **97**, 293–300
40. Pokutta, S., and Weis, W. I. (2002) *Curr. Opin. Struct. Biol.* **12**, 255–262
41. Conway, E. M., Pollefeyt, S., Cornelissen, J., DeBaere, I., Steiner-Mosonyi, M., Weitz, J. I., Weiler-Guettler, H., Carmeliet, P., and Collen, D. (1999) *Blood* **93**, 3442–3450
42. Wilhelm, S., Schmitt, M., Parkinson, J., Kuhn, W., Graeff, H., and Wilhelm, O. G. (1998) *Int. J. Oncol.* **13**, 645–651