Aberrant Glycosylation of E-cadherin Enhances Cell-Cell Binding to Suppress Metastasis*

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Introduction of the β1-4 N-acetylglucosaminyltransferase (GnT-III) gene was reported to suppress metastasis in highly metastatic B16-hm murine melanoma cells (Yoshimura, M., Nishikawa, A., Ihara, Y., Taniguchi, S., and Taniguchi, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8754-8758). In this study, the effect of GnT-III gene transfer on E-cadherin was studied, since E-cadherin acts as a suppressor of metastasis. E-cadherin expression at cell-cell contacts of B16-hm cells expressing high GnT-III activity was greater than controls without affecting transcription. Lectin blotting showed that E-cadherin from GnT-III transfectants was glycosylated by ectopically expressed GnT-III. The glycosylated E-cadherin exhibited the delayed turnover and the decreased release from cell surface, as compared with the native E-cadherin, resulting in the elevated expression at the cell-cell border of GnT-III transfectants. Furthermore, cell-cell aggregation was enhanced in GnT-III transfectants, indicating that the glycosylated E-cadherin is biologically functional. These results suggest that the glycosylated E-cadherin contributes to the suppression of metastasis by the introduction of GnT-III gene into melanoma cells.

The malignant phenotype, including metastatic potential, has been reported to be associated with the β1-6 branches of N-oligosaccharides, the product of β1-6 N-acetylglucosaminyltransferase (GnT-V, EC 2.4.1.155) in ras-transformed rat fibroblasts, rat mammary carcinoma cells, rat lymphoma cell line (1, 2), and human colon cancer cells (3). The composition of β1-6 branches is also reported to correlate with the progression and staging of human breast and colon neoplasia (4). Ectopic expression of GnT-V gene in mouse lung epithelial cell line resulted in loss of contact inhibition, progressive growth of tumors, and increase of lung metastasis after subcutaneous inoculation into nude mice (5). This report also supports that the β1-6 structure plays a causative role in tumorigenicity and metastasis.

As shown in Fig. 1, both β1-4 N-acetylglucosaminyltransferase (GnT-III, EC 2.4.1.144) and GnT-V use the biantennary structure of N-oligosaccharides as a substrate, and substrate specificity studies showed that GnT-V is not able to form any further triantennary structure in the presence of a bisecting GlcNAc residue (6, 7). On the basis of nuclear magnetic resonance data (8), the biantennary structure of a core mannose was twisted in the presence of bisecting GlcNAc. This conformational change renders the substrate inaccessible to GnT-V to form the β1-6 structure, which raised the possibility that β1-6 branch formation could be suppressed by the introduction of the GnT-III gene, thereby resulting in changes in the metastatic potential and of malignant phenotypes. Actually, in our investigation, the decrease of β1-6 structure by the competition between the endogeneous GnT-V and ectopically expressed GnT-III led to the suppression of lung metastasis by mouse melanoma cells (9). The metastatic process is a complex of many biological events, and many molecules are responsible for metastasis (10). As almost all of these molecules are glycoproteins, the alteration of enzyme activity, which catalyzes the formation of the sugar chain such as the introduction of the GnT-V gene (5) or the GnT-III gene (9), may change the structure and composition of N-glycans and may affect the physiological function of the molecules responsible for metastasis.

The present investigation was undertaken to examine whether the reduced metastatic potential of B16 murine melanoma cells expressing ectopic GnT-III was due to the altered biological function of E-cadherin that mediates homotypic cell-cell adhesion, since the E-cadherin expression correlates inversely to metastatic phenotype in many cancer cells (11-13). We found that the E-cadherin, which was glycosylated by GnT-III, was increased due to the prolonged turnover rate and the decreased release from cell surface. Furthermore, cell aggregation was increased in GnT-III transfectants, indicating that the glycosylated E-cadherin participates in the suppression of metastasis by GnT-III gene transfer.

EXPERIMENTAL PROCEDURES

Reagents—Biotinylated erythroagglutinating phytohemagglutinin (E-PHA) was obtained from Honen Corp. (Tokyo, Japan). Rat anti-mouse E-cadherin monoclonal antibody (ECCD-2) (12) was purchased from Vector (Burlingame, CA), and goat anti-rat IgG and FITC-conjugated goat anti-rat IgG was from Becton Dickinson.

Cell Lines—B16-hm displaying highly metastatic potential, GnT-III-transfected B16-hm cells displaying low metastatic potential, and mock-transfected B16-hm cells, which had been already established (9), were used in this investigation. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. Cells in exponential growth and between 4 and 10 passages were used throughout this investigation, and the results described here were independent of passage number.

Immunofluorescence Microscopy—B16-hm cells and GnT-III transfectants were seeded on 3.5-cm dishes with a coverglass on the bottom of the dish. Cells were washed once with phosphate-buffered saline...
Enhanced E-cadherin-mediated Adhesion by GnT-III

**FIG. 1. Reactions catalyzed by GnT-III and GnT-V.** GnT-V fails to catalyze the transfer of GlcNAc to form the β1–6 branch in the presence of bisecting GlcNAc.

(PBS), fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized with Tris-HCl-buffered saline, pH 7.4 (TBS) containing 0.5% Tween 20 at 10°C. The nonspecific binding sites were blocked by incubation for 1 h with 3% bovine serum albumin in TBS, cells were incubated with 20°C for 4 h with 3% bovine serum albumin in TBS, cells were incubated with ECCD-2 (10 μg/ml) or probed with ECCD-2 (10 μg/ml) for 4 h at 4°C. The fluorescence of the cells was visualized with a photomicroscope with epifluorescence (IIRS, Olympus, Japan) and photographed with a 20-s exposure. Cells were also observed and photographed with a microscope camera (ELWD 0.3, Nikon, J apan).

Lectin Blot and Immunoprecipitation—Cells were pelleted and lysed in the lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4) containing 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl₂, 1% (w/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 20 min on ice. Following clarification at 15,000 rpm for 20 min at 4°C, the protein concentration was measured using a BCA kit (Pierce). For immunoprecipitation, cell lysates (200 μg of protein) were precleared with normal rabbit serum (Vector) and protein G-Sepharose beads (Pharmacia, Uppsala, Sweden) and then incubated with ECCD-2 (10 μg/ml). Immunocomplexes were collected with protein G-Sepharose beads and were released by boiling in Laemmli’s sampling buffer, separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE), and electrotransferred onto nitrocellulose membranes (Schleicher and Schuell, Germany). The blots were blocked in TBS with 0.05% Tween 20, the blots were incubated for 1 h with 3% bovine serum albumin in TBS, cells were incubated with biotinylated anti-IgG (10 μg/ml) and then incubated with ECCD-2 (10 μg/ml) or probed with ECCD-2 (10 μg/ml) and then incubated with ECCD-2 (10 μg/ml) or probed with ECCD-2 (10 μg/ml) for 4 h at 4°C and then stained with FITC-conjugated anti-rat IgG antibody (10 μg/ml) for 1 h at 4°C. The fluorescence of the cells was visualized with a photomicroscope with epifluorescence (IIRS, Olympus, Japan) and photographed with a 20-s exposure.

**RESULTS**

Accumulation of E-cadherin at the Cell-Cell Border in GnT-III Transfectants—Two clones with elevated GnT-III activity designated as B16-hm-III-1 and -2, and one clone with no detectable GnT-III activity designated as B16-hm-neo-1 were used in this investigation as positive transfectants and a control transfectant, respectively. Morphologically, the B16-hm cells and the control transfectant appeared fibroblastoid with loose cell-cell contacts, whereas positive transfectants were epithelioid and proliferated in compact organization (Fig. 2A).

E-cadherin expression was examined by indirect immunofluorescence (Fig. 2B). In B16-hm cells and a control transfectant, E-cadherin was weakly expressed at the cell-cell contacts. Positive transfectants, however, showed intense fluorescence with condensation at the cell-cell contacts, indicating elevated expression of E-cadherin at cell-cell contacts of positive transfectants.

Increased Glycosylated E-cadherin in GnT-III Transfectants—E-PHA lectin was used to analyze the alterations of carbohydrate structures of E-cadherin, since E-PHA has a high affinity for bisecting GlcNAc structure (16). Western blotting studies showed that the expression of E-cadherin was more increased in positive transfectants than in B16-hm cells and control transfectants (Fig. 3A, upper panel), which was consistent with the results of immunofluorescence microscopy. The signal for E-PHA binding to the positive cells was detected as the bands at 125 kDa, corresponding to immunoprecipitated E-cadherin, while these bands were almost undetectable in the B16-hm cells and a control transfectant (Fig. 3A, lower panel).

The relative E-PHA binding ratio (%) normalized by E-cadherin signal density was as follows: B16-hm, 0.9; B16-hm-neo-1, 1.5; B16-hm-III-1, 13.4; and B16-hm-III-2, 16.9; this indicated that the E-cadherin in the positive transfectants was glycosylated by GnT-III and few, if any, bisected oligosaccharides were attached to E-cadherin in B16-hm cells and a control transfectant. As compared with the B16-hm cells and the control transfectant, E-cadherin transcripts were not increased in positive transfectants, suggesting that the raised E-cadherin expression in GnT-III transfectants was not due to an increase of the transcriptional level (Fig. 3B).

Prolonged Turnover and Decreased Release of E-cadherin in GnT-III Transfectants—The turnover rate of E-cadherin was examined by chase studies. In B16-hm cells, immunoprecipi-
tated surface E-cadherin showed the most intense signal at 4 h and then declined to an undetectable level at 12 h (Fig. 4 A, lower panel). Immunoprecipitated surface E-cadherin from B16-hm-III-1 cells (upper panel) showed the most intensified signal at 8 h, and the signals of the immunoprecipitated E-cadherin were maintained at the detectable level during all the chase time periods. Released E-cadherin was detected as a band of 104 kDa in the supernatants from both B16-hm cells and positive transfectants, which was less in size than the surface E-cadherin (Fig. 4 B). In the supernatant of B16-hm cells, released E-cadherin was first detected at 2 h, gradually increased to show the most intensified signal at 8 h, and maintained to be at the detectable level during the incubation (upper panel). In B16-hm-III-1 cells, released E-cadherin was undetectable until 4 h during the incubation. The signals of released E-cadherin at 4 and 8 h were faint and much weaker than that detected in the supernatants of B16-hm cells and declined to the undetectable level at 12 and 24 h (lower panel). Turnover and release of E-cadherin of a control transfectant were similar to that of B16-hm cells, showing that transfection procedures did not affect the turnover of E-cadherin (data not shown). Collectively with these results, expression of GnT-III prolonged the turnover of E-cadherin and inhibited the release of E-cadherin from cell surface, which resulted in an increased level and accumulation of E-cadherin molecules at cell-cell contacts.

Increased Cell Aggregation in GnT-III Transfectants—To determine whether the increased E-cadherin expression was involved in the homotypic cell adhesion of B16-hm cells, cell aggregation was assayed using an antibody to E-cadherin that blocks E-cadherin-mediated adhesion (Fig. 5). In B16-hm cells and a control transfectant, more than 90% of cell aggregation was inhibited by 5 μg/ml ECCD-2. The inhibitory effect by ECCD-2 was, however, considerably lower in the positive transfectants, showing that the increased E-cadherin expression was associated with increased cell aggregation and that raised E-cadherin was biologically functional.

**DISCUSSION**

In many glycoproteins, the composition of oligosaccharides, especially N-linked oligosaccharides, exists in the structural components, which contribute to the folding, stability, and biological function of the molecules (17–19). Therefore, the activity and stability of the glycoprotein could be affected by subtle
Enhanced E-cadherin-mediated Adhesion by GnT-III

changes in oligosaccharide components. For example, an N-oligosaccharide on CD2 stabilizes the protein structure for the interaction with CD58 (19). Replication of the hepatitis B virus is suppressed by GnT-III gene transfer (20). Non-glycosylated growth hormone, which is secreted both from the apical and basolateral sides of Madin-Darby canine kidney cells, is secreted from the apical side when glycosylated (21). In this study, we examined the altered biological function of N-glycosylated E-cadherin by means of the introduction of the GnT-III gene (22) into mouse melanoma B16-hm cells. As the exogenous expression of GnT-III affects the processing of glycoproteins with complex-type oligosaccharides, the GnT-III gene transfection gives a global effect on many glycoproteins, including E-cadherin.

Deduced from the amino acid sequence, mouse E-cadherin has four putative N-glycosylation sites (12). Lectin blot analysis revealed that immunoprecipitated E-cadherin from both B16-hm cells and a control transfectant had undetectable bisecting GlcNAc residues. In contrast, E-cadherin from the positive transfectant did bind E-PHA, indicating bisecting GlcNAc residues. In contrast, E-cadherin from the positive transfectants, as judged by cell aggregation assay. Thus, this indicates that cell-cell aggregation was quantitatively enhanced by the increased expression of E-cadherin, although there also remains a possibility that the glycosylation may give the qualitative effect on the adhesive function of E-cadherin. We conclude that the suppression of metastasis in B16-hm cells expressing GnT-III (9) is at least partly due to the increased level of glycosylated E-cadherin.

To examine further whether the increased E-cadherin at the cell-cell border of positive transfectants was biologically functional as a homotypic adhesion molecule, we performed cell aggregation assay and evaluated adhesive function of glycosylated E-cadherin. Compared with B16-hm cells and control transfectants, cell-cell binding was more enhanced in positive transfectants, as judged by cell aggregation assay. Thus, this indicates that cell-cell aggregation was quantitatively enhanced by the increased expression of E-cadherin, although there also remains a possibility that the glycosylation may give the qualitative effect on the adhesive function of E-cadherin. We conclude that the suppression of metastasis in B16-hm cells expressing GnT-III (9) is at least partly due to the increased level of glycosylated E-cadherin.

Acknowledgment—We thank Dr. M. Takeichi (Kyoto University, Kyoto, Japan) for kindly providing mouse E-cadherin cDNA and valuable suggestions.

REFERENCES

1. Yousefi, S., Higgins, E., Daling, Z., Pollex-Kruger, A., Hindsgaul, O., and Dennis, J. W. (1991) J. Biol. Chem. 266, 1772–1782
2. Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L., and Kerbel, R. S. (1987) Science 236, 582–585
3. Saitho, O., Wang, W.-C., Lotan, R., and Fukuda, M. (1992) J. Biol. Chem. 267, 5700–5711
4. Fernandez, B., Sagnan, U., Auger, M., Demetriou, M., and Dennis, J. W. (1995) J. Cell Biol. 130, 383–392
5. Schachter, H. (1986) Biochem. Cell Biol. 64, 163–181
6. Fuji, S., Nishiyama, A., Nishikawa, A., Miura, R., and Taniguchi, N. (1990) J. Biol. Chem. 265, 6009–6018
7. Gu, J., Nishikawa, A., Tsuroka, N., Ohno, M., Yamaguchi, N., Kangawa, K., and Taniguchi, N. (1993) J. Biochem. (Tokyo) 113, 614–619

Enhanced E-cadherin-mediated Adhesion by GnT-III

Fig. 4. Turnover of surface E-cadherin and release of E-cadherin from B16-hm cells and GnT-III transfectants, as determined by pulse-chase study. B16-hm-III-1 cells (upper panel) and B16-hm (lower panel) were 35S-radiolabeled for 15 min. At 2, 4, 8, 12, and 24 h after pulse-labeling, E-cadherin was immunoprecipitated from cell lysates (A) or supernatants (B) using ECCD-2 simultaneously. After the separation of immunoprecipitated E-cadherins by 10% SDS-PAGE, the gel was dried and autoradiographed to x-ray film for 2 days. The results were reproducible in three independent experiments.
Enhanced E-cadherin-mediated Adhesion by GnT-III

9. Yoshimura, M., Nishikawa, A., Ihara, Y., Taniguchi, S., and Taniguchi, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8754–8758
10. Hart, I. R., and Saini, A. (1992) Lancet 339, 1453–1457
11. Takeichi, M. (1993) Curr. Opin. Cell Biol. 5, 806–811
12. Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. (1987) Nature 329, 341–343
13. Oda, T., Kanai, Y., Oyama, T., Yoshiura, K., Shimoyama, Y., Birchmeier, W., Sugimura, T., and Hirohashi, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1858–1862
14. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
15. Jiang, W. G., Hiscox, S., Hallett, M. B., Horrobin, D. F., Mansel, R. E., and Puntis, M. C. (1995) Cancer Res. 55, 5043–5048
16. Yamashita, K., Hitoi, K., and Kobata, A. (1987) J. Biol. Chem. 258, 14753–14755
17. Dwek, R. A. (1995) Science 269, 1234–1235
18. Taniguchi, N., and Ihara, Y. (1995) Glycocon. J. 12, 733–738
19. Wyss, D. F., Choi, J. S., Li, J., Knoppers, M. H., Willis, K. J., Arulanandam, A. R. N., Smolyar, A., Reinherz, E. L., and Wagner, G. (1995) Science 269, 1273–1278
20. Miyoshi, E., Ihara, Y., Hayashi, N., Fusamoto, H., Kamada, T., and Taniguchi, N. (1995) J. Biol. Chem. 270, 28311–28315
21. Scheiffele, P., Peranen, J., and Simons, K. (1995) Nature 378, 96–98
22. Nishikawa, A., Ihara, Y., Hatakeyama, M., Kangawa, K., and Taniguchi, N. (1992) J. Biol. Chem. 267, 18199–18204