Decreased MUC1 Expression Induces E-Cadherin-mediated Cell Adhesion of Breast Cancer Cell Lines

Kelichi Kondo, Nobuoki Kohno, Akihito Yokoyama, and Kunio Hiwada
Second Department of Internal Medicine, Ehime University School of Medicine, Onsen-gun, Ehime 791-0295, Japan

INTRODUCTION

Cadherin-catenin complex is a Ca\(^{2+}\)-dependent and homophilic cell-cell adhesion molecule. In human carcinomas, most cells expressing E-cadherin have a tendency to be noninvasive (1). Dysfunction of the cadherin-catenin complex was reported to increase the invasiveness of cancer cells (2, 3). However, some gastric cancers and ductal carcinomas of the breast have invasive characteristics despite expressing E-cadherin (1, 4). In gastric cancer, the mutation of E-cadherin and catenins causes dysfunction of E-cadherin (5). In the case of breast cancer, however, the reasons for the invasiveness have not been clarified.

MUC1 is a nonsecretory mucin expressed on epithelial cells and is abundantly expressed on most cancer cells (6). A more than 10-fold increase of MUC1 expression in certain types of carcinoma, such as breast carcinoma, has been reported compared with the adjacent normal epithelial tissues (7-9).

We established two breast cancer cell lines, YMB-S (10) and ZR-75-1S, both of which expressed high levels of E-cadherin on their surface and proliferated in suspension culture without aggregation in a complete liquid medium. Both YMB-S and ZR-75-1S cells showed a high surface expression of MUC1.

NaB is one of the differentiation-inducing agents and has been reported to produce a wide variety of effects on cultured cells, including the arrest of cell growth, morphological changes of transformed cells, and the induction of proteins such as hemoglobin and peptide hormones (11). It has also been reported to induce differentiation of breast cancer cell lines (12). In addition, NaB was reported to be effective against acute leukemia (13). In this study, we examined the effect of NaB on both YMB-S and ZR-75-1S cells and found that NaB induced cell-cell and cell-surface adhesion by these cells. Because overexpression of MUC1 in its transfectants has been reported to interfere with cellular aggregation (14), we also investigated the influence of NaB on MUC1 expression by these cells.

MATERIALS AND METHODS

Cell Culture and Establishment of Cell Lines. Cells were cultured in a complete liquid medium (RPMI 1640 supplemented with 10% FCS, 4 mM HEPES, streptomycin (100 μg/ml), penicillin (100 units/ml), and 2 mM L-glutamine) in an incubator with a 5% CO\(_2\) atmosphere at 37°C. YMB-S cells were cloned from a human breast cancer cell line, YMB-1 (15). Most YMB-1 cells proliferated with adherence to the surface of a plastic tissue culture flask, resulting in an overgrowth of 5-30% of the cells growing in suspension. The cells in suspension were separated and placed into a new flask for further culture. After repeating passage once or twice a week for 6 months, YMB-S cells were finally established, proliferating in suspension without aggregation in a complete liquid medium (16). ZR-75-1S cells were cloned from a human breast cancer cell line, ZR-75-1, obtained from the American Type Culture Collection. Cloning was done in the same fashion as for YMB-S cells.

Induction of Cell Adhesion. To induce cell adhesion, cells in suspension were treated with indicated doses of NaB (Wako Pure Chemical, Osaka, Japan) or 4-hydroxybutyrate (Nippon Kayaku, Tokyo, Japan). To elucidate the mechanism of this adhesion, NaB-treated cells were cultured with mAb to human E-cadherin (HECD-1; Takara, Tokyo, Japan), human α-catenin, and β-catenin (HECD-1; Takara, Tokyo, Japan), human α-catenin (Telios Pharmaceuticals, San Diego, CA), human CD29 (β3 integrin; Southern Biotechnology Associates, Birmingham, AL), and β3 integrin (Bioline Diagnostic, Torino, Italy) 24 h after NaB treatment.

Cell Growth and Cell Cycle Analysis. Cells in suspension were collected by gentle pipetting, and then the cells adhering to the surface of flasks were harvested with 0.25% trypsin and 0.02% EDTA in DPBS (Life Technologies, Inc., Grand Island, NY). Cells were counted by the trypan blue dye exclusion method. The cell cycle distribution of cells was analyzed using a commercially available kit (Cycle TEST; Becton Dickinson, San Jose, CA) and a fluorescence-activated cell analyzer (EPICS-Profile; Coulter Immunology, Hialeah, FL) with Multi Cycle software (Coulter Immunology).

Expression and Tyrosine Phosphorylation of the Cadherin-Catenin Complex. The expression and tyrosine phosphorylation of the cadherin-catenin complex on cells were examined using Western blotting and immunoprecipitation, as described previously (3). In brief, 5 × 10⁶ cells were washed with a buffer containing 1 mM sodium vanadate, 3 mM hydrogen peroxide, and DPBS. After removal of the supernatant, 1 ml of extraction buffer (1% Triton X-100, 1% NP40, 5 mM CaCl₂, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 3 mM hydrogen peroxide) was added to the pellet. The cells were incubated on ice for 15 min and then centrifuged at 14,000 rpm at 4°C for 20 min. The protein concentration of the supernatant was measured using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). For Western blot analysis, the sample was loaded onto an 8% SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane (Bio-Rad Laboratories), the membrane was incubated with a mouse anti-human E-cadherin antibody (HECD-1, mouse IgG1), a mouse anti-α-catenin mAb (mouse IgG1; Transduction Laboratories, Lexington, KY), or a mouse anti-β-catenin mAb (mouse IgG1; Transduction Laboratories), followed by a horseradish peroxidase-conjugated goat antimouse IgG1 antibody (Zymed Laboratories, Inc., San Francisco, CA). For the immunodetection of phosphoryrosine, cell extracts were incubated with 5 μg of anti-E-cadherin, anti-α-catenin, or anti-β-catenin antibody, followed by rabbit antimouse IgG1 antibody conjugated Sepharose 4B (Zymed Laboratories). After washing, the samples with SDS-PAGE sample buffer were denatured at 100°C for 5 min followed by centrifugation at 10,000 rpm at 4°C for 2 min. The supernatant was loaded onto an 8% SDS-PAGE gel.
polyacrylamide gel and transferred to a membrane in the same fashion as described for Western blotting. The membrane was incubated with 2 μg/ml mouse monoclonal anti-phosphotyrosine antibody [PY-20 (mouse IgG2b); Transduction Laboratories], followed by a horseradish peroxidase-conjugated rat antimouse IgG2b antibody (Zymed Laboratories). Then the membrane was reacted with enhanced chemiluminescence detection reagent (Amersham) for 1 min and exposed to X-ray film at room temperature for 15 min. To evaluate the level of expression of E-cadherin, α-catenin, β-catenin, and phosphotyrosine, densitometry was done with NIH Image 1.59 software (National Technical Information Service, Springfield, VA).

**Cytofluorometry.** Single cells harvested with 0.02% EDTA-DPBS were incubated with an antimilk mucin core antigen mAb [SM-3 (mouse IgG1); Cybus Biotechnology, Southampton, United Kingdom] or an antihuman milk fat globule mAb [anti-HMFG-1 (mouse IgG1); Ylem, Rome, Italy], followed by incubation with FITC-conjugated goat F(ab')2 antirat IgG + IgM (Biosource International, Camarillo, CA). Stained cells were analyzed with a FACSCalibur cytofluorometer (Becton Dickinson) and CELL Quest software (Becton Dickinson).

**Preparation of a MUC1 cDNA Probe.** A MUC1 cDNA probe corresponding to nucleotides 855-1141 of human pancreatic tumor mucin cDNA (17) was prepared from the total RNA of YMB-S cells by the reverse transcription-PCR method. The primers used for reverse transcription-PCR were previously described as P1 and P2 by Stern et al. (18). The resultant probes were subcloned into a pBluescript II KS(+) vector (Stratagene, La Jolla, CA), and the DNA sequence was determined with a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, WI). The probe was labeled with [α-32P]dCTP by the random hexamer priming method (Prime It II random primer labeling kit; Stratagene).

**Northern Blot Analysis.** Total RNA (10 μg) was denatured in formaldehyde and formamide at 65°C for 15 min, and electrophoresis was done on a 1% agarose gel containing formaldehyde (19). RNA was transferred to a nylon membrane (Hybond N+; Amersham, Buckinghamshire, United Kingdom) by capillary transfer with 20× SSC (3 m NaCl and 0.3 m sodium citrate). The membrane was fixed in 0.05 M NaOH for 5 min and baked at 80°C for 2 h. It was prehybridized in a buffer containing 50% formamide, 5× saline-sodium phosphate–EDTA (0.75 M NaCl, 50 mM sodium phosphate, pH 7.4), and 5 mM EDTA, 5× Denhardt’s solution, 0.5% SDS, and 0.2 mg/ml salmon sperm DNA at 42°C for 4 h. Hybridization was carried out with a MUC1 cDNA probe in the same buffer at 42°C for 24 h. At the end of hybridization, the filter was washed stringently in 0.1× saline-sodium phosphate–EDTA and 0.1% SDS at 55°C for 20 min. Autoradiography was performed with hyperfilm-MP (Amersham) using an intensifying screen at —80°C for 48 h. To evaluate the level of MUC1 mRNA expression, densitometry of transcripts was done with BAS 1000 (Fuji Film Corp., Kanagawa, Japan).

**Antisense MUC1 mRNA.** Antisense and control phosphorothioate oligonucleotides of MUC1 were purchased from Biognostik GmbH (Göttingen, Germany). The control was a randomized-sequence oligonucleotide of the same length as the antisense oligonucleotide. The antisense or control oligonucleotide (4 μM) was added to 2500 cells in 100 μl of complete medium. For the evaluation of MUC1 expression, immunocytochemistry was performed with anti-HMFG-1 and a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA).

All experiments were done at least in triplicate. Data are shown as the mean and SD or as the representative of identical results.

**RESULTS**

**NaB-induced, E-Cadherin-mediated Adherence of YMB-S and ZR-75-1S Cells.** YMB-S cells and ZR-75-1S cells proliferated in suspension cultures of complete medium without aggregation, as shown in Fig. 1, A and D. In the presence of 2 mM NaB, both types of cells showed cell-cell adhesion and adherence to the surface of plastic tissue culture flasks (Fig. 1, B and E). The adhesion started on...
Fig. 2. Characteristics of YMB-S cell proliferation in the presence of NaB. A, time course of cell numbers. NaB inhibited cell growth in a dose-dependent manner. In the presence of 0.5 mM NaB, the number of cells did not change for 6 days. In the presence of more than 0.5 mM NaB, cell numbers decreased. B, cell cycle analysis on day 4. NaB dose-dependently arrested cells in $G_0/G_1$ phase. Error bars, SD.

day 2 and was almost complete by day 4. An anti-E-cadherin antibody dose-dependently inhibited cell-cell and cell-surface adhesion, with concentrations from 0.1 to 10 µg/ml, as shown in Fig. 1, C and F. The anti-$\alpha_3$-, $\beta_1$, and $\alpha_3\beta_1$ integrin antibodies had no effect on NaB-treated cells.

**NaB-inhibited Proliferation of YMB-S and ZR-75-1S Cells.**
NaB inhibited the proliferation of YMB-S cells in a dose-dependent manner, as shown in Fig. 2A. Essentially the same results were obtained with ZR-75-1S cells (data not shown). Cell cycle analysis on day 4 showed that NaB dose-dependently arrested YMB-S cells in the $G_0/G_1$ phase (Fig. 2B).

**Effect of NaB on the Expression and Tyrosine Phosphorylation of E-Cadherin and Catenins.** NaB treatment did not change the expression of E-cadherin, $\alpha$-catenin, or $\beta$-catenin when compared with untreated cells (Fig. 3, A and C). Tyrosine phosphorylation of immunoprecipitated E-cadherin and coprecipitins is shown in Fig. 3, B and D (Lanes g and h). Phosphotyrosine was detected on 120-, 95-, and 83-kDa molecules, which might have been E-cadherin, $\beta$-catenin, and plakoglobin, respectively. The level of phosphorylation was markedly increased in NaB-treated cells compared with untreated cells. The immunoprecipitated $\alpha$-catenin and coprecipitins are shown in Fig. 3, B and D (Lanes i and j). Their tyrosine phosphorylation levels were similar to that of E-cadherin. The immunoprecipitated $\beta$-catenin and coprecipitins were differently phosphorylated (Fig. 3, B and D, Lanes k and l) from E-cadherin and $\alpha$-catenin. Only two molecules, E-cadherin and $\beta$-catenin, were detected. Plakoglobin was not coprecipitated with the anti-$\beta$-catenin antibody. In NaB-treated YMB-S cells, phosphorylation of the cadherin-catenin complex was approximately six times higher than that in untreated cells. In ZR-75-1S cells, NaB approximately doubled the level of phosphorylation.

**Effect of Tyrosine Kinase Inhibitors.** There have been many reports that tyrosine phosphorylation of the cadherin-catenin complex suppresses the function of E-cadherin (20, 21), and dephosphorylation of $\beta$-catenin was reported to induce cadherin-mediated adhesion (22). Our results were contrary to these reports. If the adhesion of our cell lines required tyrosine phosphorylation, tyrosine kinase inhibitors might suppress it. Therefore, to test whether tyrosine phosphorylation was required for adhesion, tyrosine kinase inhibitors were added to YMB-S cells treated with NaB. As shown in Fig. 4, adherent cells and floating cells were mixed on day 2 of culture with NaB alone. Herbimycin A, a tyrosine kinase inhibitor, induced tight adhesion, and there were very few cells in the suspension. Genistein, another tyro-
sine kinase inhibitor, induced a weaker adhesion than herbimycin A but a tighter adhesion than that induced by NaB alone. There were no adherent cells in cultures with tyrosine kinase inhibitors alone.

**Effect of NaB on MUC1 Expression.** MUC1 expression by YMB-S cells was examined using SM-3 or anti-HMFG-1 antibody, which recognizes the MUC1 core protein. Expression was decreased on day 2 of culture with NaB but was unchanged between on day 0 and day 1, as shown in Fig. 5. On Northern blot analysis, the expression of MUC1 mRNA was decreased in both YMB-S cells (Fig. 6A) and ZR-75-1S cells (data not shown) after culture with NaB on days 1 and 2. The day 0:day 1:day 2 densitometric ratio was approximately 8:2:1.

**Effect of Ubenimex on MUC1 mRNA Expression.** YMB-S cells proliferated in suspension without aggregation, and adhesion was induced in the presence of 2 mM ubenimex, as described previously (16). The cells began to adhere on day 3. On Northern blotting, expression of MUC1 mRNA was decreased by ubenimex treatment of YMB-S cells on days 2 and 3 (Fig. 6B). The day 0:day 1:day 2:day 3 density ratio was approximately 10:10:8:3. Ubenimex induced the adhesion of these cells 24 h later than NaB. In addition, the adhesion induced by ubenimex was obviously weaker than that caused by NaB.

**Effect of Antisense MUC1 mRNA.** In the presence of 4 μM antisense oligonucleotide, YMB-S cells showed cell-cell adhesion on day 3 (Fig. 7B). The control oligonucleotide (4 μM) had no effect on YMB-S cells (Fig. 7C) as compared with control cultures on day 3 (Fig. 7A). Immunocytochemistry showed that MUC1 expression was decreased in cells treated with the antisense oligonucleotide, but not in those treated with the control oligonucleotide (Fig. 8).

**DISCUSSION**

We described two breast cancer cell lines that exhibited a complete loss of adhesive properties despite the expression of E-cadherin. These cells resumed adhesive properties by NaB treatment. The expression of the cadherin-catenin complex of these cells was unchanged by NaB treatment. An anti-E-cadherin antibody inhibited not only cell-cell but also cell-substrate adhesion. However, anti-α1 and anti-β1 integrin antibodies did not inhibit them. Therefore, the cadherin-catenin system might be most important in the induced adhesion. It is difficult to mention the mechanisms by which the inhibition of cadherin induced not only cell-cell but also cell-substrate adhesion, because cadherin has not been reported to be concerned with cell-substrate adhesion. Tyrosine phosphorylation of the cadherin-catenin complex was markedly increased in adherent cells. However, tyrosine kinase inhibitors such as herbimycin A and genistein induced tighter adhesion, so increased tyrosine phosphorylation might not be the cause of the adhesion. On the other hand, expression of MUC1, the overexpression of which in transfectants was reported to prevent cellular adhesion by masking the effect on adhesion molecules such as cadherins (14, 23), was decreased in NaB-treated cells. A MUC1 antisense oligonucleotide also induced adhesion in these cell lines. Therefore, we concluded that the function of E-cadherin expressed on
CELL ADHESION AND DECREASED MUC1 EXPRESSION

Fig. 5. Cytofluorometric measurement of MUC1 expression by YMB-S cells detected with anti-HMFG-1 antibody (A) or SM-3 (B). In the presence of 2 mM NaB, MUC1 expression was not different between day 0 and day 1, but it was decreased on day 2. The highest column represents control cells with the first antibody being nonimmune mouse IgG. X axis, fluorescence intensity; Y axis, cell counts.

Fig. 6. Northern blot analysis of MUC1 mRNA expression by YMB-S cells. The cDNA probe of MUC1 hybridized to two transcripts of approximately 4.5 and 6.5 kb. A, in the presence of 2 mM NaB, the expression of MUC1 mRNA was obviously decreased on days 1 and 2 compared with day 0. B, in the presence of 2 mM ubenimex, the expression of MUC1 mRNA started to decrease on day 2 and was obviously decreased on day 3 compared with day 0 or day 1.

these cells might be suppressed by the masking effect of MUC1 and then restored by suppressing MUC1 expression.

NaB has a variety of morphological and biological effects on cultured cells (11). Charollais et al. (24) reported that NaB inhibited G1 phase progression in the cell cycle. Russo et al. (25) showed that NaB might play a role in regulating CKII phosphorylation. Our results indicated that NaB reduced the expression of MUC1 mRNA, and that this change might have caused adhesion. It is not known at present whether NaB acts on a very specific target in cells or on several cell components, although 24 years have passed since NaB was first reported to be a strong inhibitor of cell growth (26).

MUC1 is a transmembrane mucin expressed on most cancer cells (6), and our breast cancer cell lines also expressed MUC1. It projects at least 200–500 nm above the plasma membrane, whereas most proteins on the cell surface remain inside the boundaries of the glycocaryx, which is approximately 10 nm thick (7). MUC1 is released from the cell just above the plasma membrane within several hours (T1/2 = 24 h) after synthesis (7). From the results of Northern blot analysis, the expression of MUC1 mRNA was decreased by 24 h of treatment with NaB, but cytofluorometry showed that the expression of MUC1 mucin core protein decreased on day 2. Induction of adhesion started on day 2 and progressed gradually day by day. It seems unlikely that E-cadherin-mediated adhesion suppressed the expression of MUC1, because the expression of MUC1 mRNA was decreased on the day after NaB treatment, when most cells did not adhere.

MUC1 antisense oligonucleotide suppressed the expression of MUC1 and induced cell adhesion. We previously reported that ubenimex induced E-cadherin-mediated adhesion of YMB-S cells without changing the level of E-cadherin expression between untreated and ubenimex-treated cells (16). However, the mechanism of this adhesion was not clarified. In this study, we showed that ubenimex reduced the expression of MUC1 mRNA 24 h before the beginning of adhesion. NaB induced adhesion of breast cancer cell lines and reduced the expression of MUC1, as ubenimex did, but NaB induced an earlier and stronger adhesion than ubenimex. We cannot be sure that the mechanism of adhesion induced by NaB is the same as that of ubenimex. However, in our cell lines, these

Fig. 7. Effect of a MUC1 antisense oligonucleotide on YMB-S cells. A, control culture on day 3. B, supplementation with 4 μM MUC1 antisense oligonucleotide for 3 days. C, culture with the control oligonucleotide for 3 days. Bar, 50 μm.
agents induced morphological changes similar to those induced by the MUC1 antisense oligonucleotide. MUC1-specific targeting immunotherapy for breast cancer has recently been investigated (27, 28). Our results support these approaches and suggest that the regulation of MUC1 might contribute to cancer therapy.

REFERENCES

12. Abe, M., and Kufe, D. W. Effect of sodium butyrate on human breast carcinoma (MCF-7) cellular proliferation, morphology, and CEA production. Breast Cancer Res. Treat., 4: 269–274, 1984 of 4 µM MUC1 antisense oligonucleotide (A) and 4 µM control oligonucleotide (B) on day 3. Anti-HMFG-1 antibody was used as the first antibody and stained with VECTASTAIN Elite ABC kit. MUC1 expression was decreased in antisense-treated cells on day 3. Counterstaining was performed with methyl green for 1 min. C, negative control; the first antibody was nonimmune mouse IgG. D, positive control; control culture on day 3. Bar, 50 µm.

Fig. 8. Immunocytochemistry of MUC1 expression by YMB-S cells in the presence of 4 µM MUC1 antisense oligonucleotide (A) and 4 µM control oligonucleotide (B) on day 3. Anti-HMFG-1 antibody was used as the first antibody and stained with VECTASTAIN Elite ABC kit. MUC1 expression was decreased in antisense-treated cells on day 3. Counterstaining was performed with methyl green for 1 min. C, negative control; the first antibody was nonimmune mouse IgG. D, positive control; control culture on day 3. Bar, 50 µm.

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