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Ubenimex Activates the E-Cadherin-mediated Adhesion of a Breast Cancer Cell Line YMB-S

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It has been reported that ubenimex, a biological response modifier, has a direct anti-tumor effect. To clarify the mechanism involved, we examined the effects of ubenimex on the growth and adhesive property of a breast cancer cell line YMB-S. The cells proliferate in a floating manner without aggregation in normal complete medium. Ubenimex induced cell-cell and cell-surface adhesion of the cells accompanied with growth suppression. E-Cadherin localized at cell-cell contact sites of adhered cells, and anti-E-cadherin antibody inhibited the adhesion. Both Western blot analysis and binding assay disclosed that there was no apparent difference between E-cadherin levels of the cells before and after the treatment with ubenimex. These results indicate that ubenimex inhibits the proliferation of YMB-S cells and augments cell-to-cell adhesion through the induction of E-cadherin-mediated adhesion resulting from the functional activation of pre-expressed but inefficient E-cadherin.

Key words: Ubenimex — Breast cancer cell line YMB-S — E-Cadherin — Induction of adhesion

Ubenimex is a dipeptide discovered in the culture medium of *Streptomyces olivoreticuli*[1] and is clinically used as a biological response modifier (BRM).[2,3] Ubenimex is known to inhibit competitively the activity of aminopeptidases B and N, which exist on the cell surfaces of mammalian cells,[4,5] and to inhibit the activity of leukotriene A_4 (LTA_4) hydrolase.[6] Through the inhibition of aminopeptidases on cell surfaces, ubenimex stimulates the release of various cytokines by immunocompetent cells and modulates the function of anti-tumor effectors such as macrophages, NK cells, and cytotoxic T cells.[7–9] In addition to these effects as a BRM, ubenimex has been reported to have direct anti-tumor effects on some cancer cells.[10,11] Further, Saiki *et al.* reported that ubenimex inhibited tumor cell invasion *in vitro*.[12] The mechanism of these anti-tumor effects, however, has not been elucidated. We therefore investigated the effects of ubenimex on the biological behavior of YMB-S cells, which proliferate in a floating manner without aggregation in liquid complete medium supplemented with 10% fetal calf serum (FCS).

MATERIALS AND METHODS

Agents Ubenimex was provided by Nippon Kayaku Co., Ltd. (Tokyo). Amastatin, which inhibits aminopeptidase N, was purchased from Protogen AG (Laufelfingen, Switzerland) and arphamenine B, which inhibits aminopeptidase B, was from Sigma Chemical Co. (St. Louis, MO).

Cell and cell culture YMB-S cells are a clone established from breast ductal cancer cells YMB-1.[13] Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, antibiotics and 2 mmol/liter of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (complete medium), and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cell number was counted by the trypan blue dye-exclusion method using a hemocytometer. The DNA synthesis of cells was determined by measuring ^{3}H-thymidine (^{3}H-TdR; ICN, Costa Mesa, CA) incorporation.

Cell cycle analysis To determine the cell cycle distribution of cells, the "Cycle Test" kit (Nippon Becton Dickinson, Tokyo) was used. The cell number was adjusted to 2.5×10^6 cells/ml in a sample buffer [citrate buffer, sucrose, and dimethyl sulfoxide (DMSO)] of the kit. The cell suspension (0.2 ml) and 1.8 ml of solution A (trypsin, spermine, and detergent) in the kit were mixed and incubated at room temperature for 10 min. To this mixture, 1.5 ml of solution B (trypsin inhibitor, RNase, and detergent) in the kit was added and the whole was mixed at room temperature for 10 min. Then 1.5 ml of solution C (propidium iodide, spermine, and detergent) in the kit was added to the suspension and the whole was mixed at room temperature for 10 min. The DNA content of each cell was measured using a fluorescence-activated cell analyzer EPICS-Profile (Coulter Immunology, Hialeah, FL). The data were analyzed using "Multi Cycle" software (Coulter Immunology).

Immunocytochemistry, Western blotting and binding assay Localization of E-cadherin on ubenimex-treated cells was examined by an indirect immunoperoxidase

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method using anti-human E-cadherin monoclonal antibody (HECD-1; Takara, Tokyo) and Vectastain ABC kits (Vector Lab., Burlingame, CA), as described previously.[14] Immunofluorescent staining using HECD-1 and FITC-conjugated anti-mouse IgG (TAGO Inc., Burlingame, CA) was performed to examine the localization of E-cadherin on control cells.

Expression levels of E-cadherin on YMB-S cells were examined using Western blot analysis, as described elsewhere.[15, 16] As a positive control, PC-9 human lung carcinoma cells were used.[16] In brief, 1×10^6 cells were washed three times with Dulbecco's phosphate-buffered saline, pH 7.40 (DPBS). After removal of the supernatant, 500 μl of extraction buffer containing 1% Triton X-100, 5 mmol/liter of $CaCl_2$, 2 mmol/liter of phenylmethylsulfonyl fluoride (PMSF), and 2 mg/ml of leupeptin, were added to the pellet, and the cells were incubated on ice with pipetting for 15 min. They were then centrifuged at 15,000 rpm at 4°C for 30 min. An equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the supernatant, and the cell lysate was denatured at 100°C for 5 min. Each sample was loaded on 8% polyacrylamide gel at 30 μg-protein/lane. Proteins separated in the gel were then transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad Lab., Hercules, CA). The membrane was incubated successively with 5 μg/ml of HECD-1 antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG (Zymed Lab. Inc., San Francisco, CA) at room temperature for 1 h. Then 5 ml of enhanced chemiluminescence (ECL) detection reagent (Amersham International plc, Amersham, UK) was added to the membrane, which was incubated for 1 min, and exposed to an X-ray film at room temperature for 15 min.

Expression levels of E-cadherin on YMB-S cells were also determined by indirect radioimmunoassay, using HECD-1 antibody and ^{125}I-anti-mouse Ig, as described previously.[17] Whole living cells were used for this assay. As a negative control, spent culture medium of P3-NS1-Ag4/1 mouse myeloma cells was used. The specific binding was estimated as the difference of ^{125}I-bindings between HECD-1 and the negative control. Data were obtained in triplicate.

Cell invasion assay The invasion of YMB-S cells into Matrigel was determined by using Matrigel invasion chambers (Becton Dickinson, Bedford, MA), as described previously.[12] The Matrigel invasion chamber consists of a cell culture insert containing an 8 μm pore size membrane coated with solubilized basement membrane preparation. The cells were cultured in complete medium containing 2 mmol/liter or 0.2 mmol/liter of ubenimex for 3 days. Then 1×10^4 cells were re-suspended in 1 ml of fresh complete medium containing ubenimex at the same concentration as that of the preculture, seeded into the upper compartment of the chamber, and cultured for 24 h. The cell number in the lower chamber was counted.

Measurement of leukotriene B_4 LTB_4 in the cultured spent medium was measured by chromatography and radioimmunoassay. Briefly, 4 ml of cold methanol-acetic ether (2:1) was added to 1 ml of spent culture medium. The sample was centrifuged at 3000 rpm for 5 min, and 3 ml of 30% petroleum ether were added to the supernatant. To the lower compartment, 5 ml of 50 mmol/liter acetate buffer pH 4.0 was added. After clean-up on a reversed-phase column, Amprep (Amersham Int. plc), the extract was used as a sample. The concentration of LTB_4 in an appropriate fraction was assayed by the LTB_4-[^{3}H] assay system (Amersham Int. plc).

Statistical analysis The data are shown as mean±SD. The data were analyzed for intergroup significance by the Kruskal-Wallis test and two sample t test. The criterion of significance was taken as $P<0.01$.

RESULTS

Morphological change of YMB-S cells treated with ubenimex YMB-S cells cultured in complete medium proliferated in a floating manner without aggregation, as shown in Fig. 1A. The cells cultured in medium with

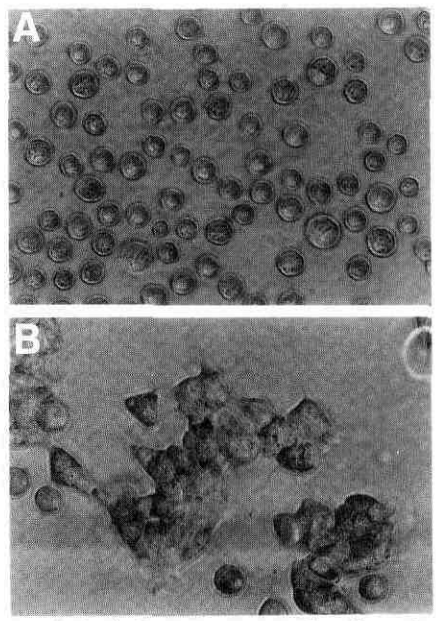

Fig. 1. Morphological appearance of cells cultured on a tissue culture dish. (A) YMB-S cells proliferated in a floating manner without aggregation. (B) YMB-S cells showed cell-cell and cell-surface adhesion when cultured in the presence of 2 mmol/liter of ubenimex for 6 days.

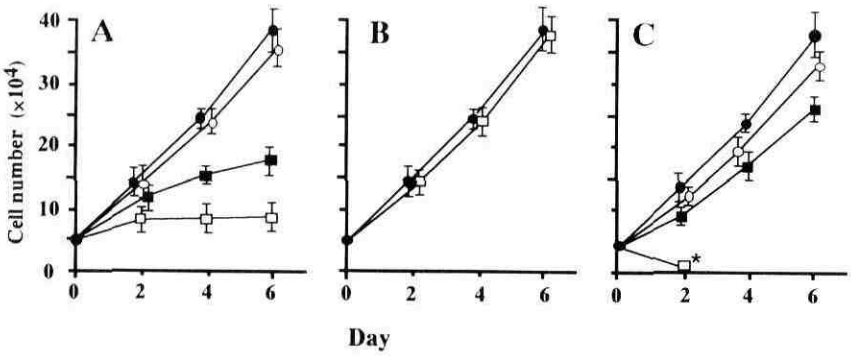

Fig. 2. Effects of aminopeptidase inhibitors on cell proliferation. Cells were cultured with ubenimex (A), amastatin (B), or arphamenine B (C). ●, control; □, 2 mmol/liter; ■, 0.2 mmol/liter; ○, 0.02 mmol/liter. ★ All cells were killed on day 2.

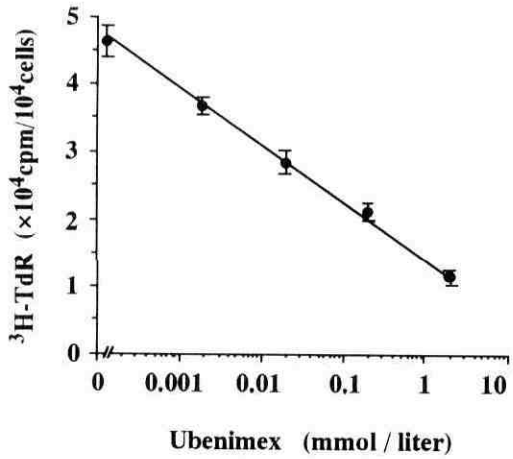

Fig. 3. Effect of ubenimex on ^{3}H-TdR incorporation of YMB-S cells. Cells (1×10^5/ml) were cultured with ubenimex for 3 days (quadruplicate).

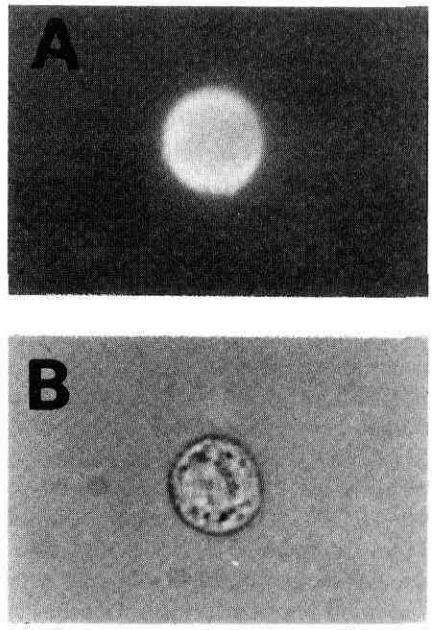

Fig. 5. Immunofluorescence staining of YMB-S cells untreated with ubenimex. (A) anti-E-cadherin antibody (HECD-1). (B) phase-contrast image.

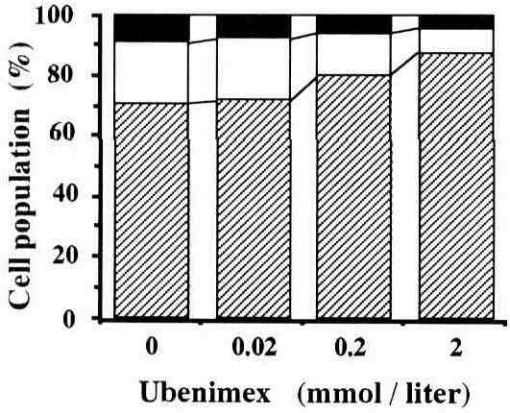

Fig. 4. Effect of ubenimex on the cell cycle of YMB-S cells. Cells were cultured with ubenimex for 3 days, and the cell cycle distribution of the cells was analyzed. ■, G2+M; □, S; ▨, G0/G1.

2 mmol/liter of ubenimex began to adhere on day 3, and most of the cells had completely adhered and spread on the culture plate by day 6, as shown in Fig. 1B. Amastatin and arphamenine B, each 0.02 to 2 mmol/liter, had no effect on the appearance.

Effects of ubenimex on the proliferation of YMB-S cells

Ubenimex inhibited the proliferation of YMB-S cells in a dose-dependent manner, as shown in Fig. 2A. From 0.02 to 2 mmol/liter of amastatin, however, had no effect (Fig. 2B). The viability levels of the cells cultured with 2 mmol/liter of ubenimex for 3 and 6 days were 92 ± 4 and $90\pm3\%$ (mean$\pm$SD), respectively. Arphamenine B, 0.02 and 0.2 mmol/liter, suppressed the cell growth (Fig.

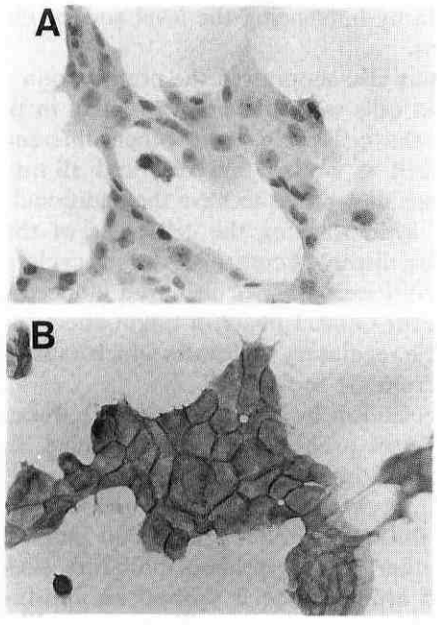

Fig. 6. Immunocytochemical staining of YMB-S cells treated with 2 mmol/liter of ubenimex for 6 days. (A) negative control (normal mouse IgG). (B) anti-E-cadherin antibody (HECD-1).

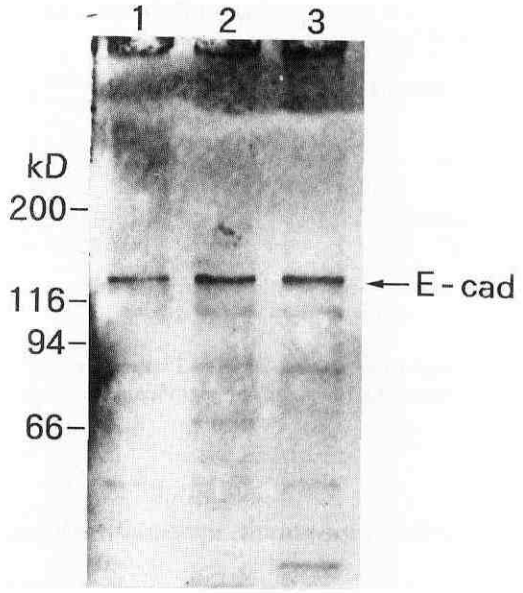

Fig. 7. Western blot analysis of E-cadherin. Positive control; PC9 cells, human lung carcinoma (lane 1). YMB-S cells cultured in complete medium (lane 2). YMB-S cells cultured with 2 mmol/liter of ubenimex for 6 days (lane 3).

2C), but all cells died when cultured with 2 mmol/liter of arphamenine B on day 2. Ubenimex suppressed ^{3}H-TdR incorporation of YMB-S cells dose-dependently, as shown in Fig. 3. In the examination of the effect of ubenimex on the cell cycle, it was found that the proportion of treated YMB-S cells in the G0/G1 phase was dose-dependently increased (Fig. 4).

Localization of E-cadherin and effects of anti-E-cadherin antibody Immunocytochemical studies showed that E-cadherin existed on the cell surface of YMB-S cells untreated with ubenimex (Fig. 5), and that E-cadherin of adhered cells was strongly expressed at cell-cell contact sites, as shown in Fig. 6. In both the Western blot analysis (Fig. 7) and the binding assay (Fig. 8), YMB-S cells had already expressed E-cadherin molecules even before the treatment with ubenimex. The expression levels of the cells treated with ubenimex were not changed. Since E-cadherin was expressed at the sites of cell-cell contact, we examined whether or not HECD-1 antibody inhibits the cell-cell adhesion. Ten μg/ml of the antibody was added to the culture medium with 2 mmol/liter of ubenimex on day 3. The cells were further cultured until day 9. The cells did not show cell-cell or cell-surface adhesion through the observation period.

Effect of ubenimex on the invasion of YMB-S cells into Matrigel membrane The number of cells treated with ubenimex which passed through the Matrigel membrane

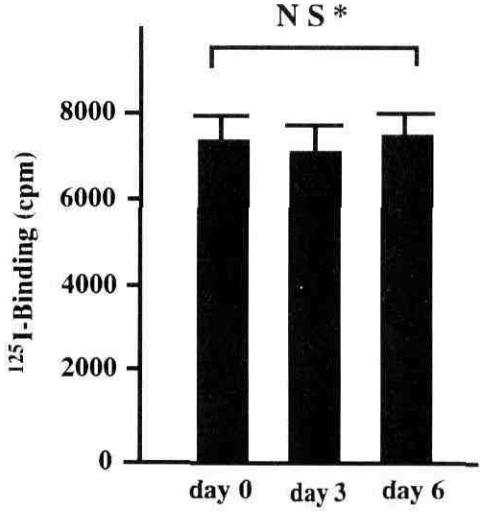

Fig. 8. Time course of E-cadherin expression levels on YMB-S cells cultured with 2 mmol/liter of ubenimex. Expression levels are indicated in terms of ^{125}I-binding. Columns and bars show the mean $\pm$SD experiments (triplicate). Differences among the three groups were analyzed by using the Kruskal-Wallis test. * NS; no significance ($P=0.384$).

decreased dose-dependently as shown in Table I. However, neither 2 mmol/liter amastatin nor 0.2 mmol/liter arphamenine B suppressed the number of cells which

Table I. Effects of Aminopeptidase Inhibitors on the Invasion of YMB-S Cell into Matrigel

Treatment (mmol/liter)		Invaded cells (mean±SD)
None		180±16
Ubenimex[a]	0.2	160±11
	2	48±12
Arphamenine B[b]	0.2	188±21
Amastatin[b]	2	186±18

a) Ubenimex dose-dependently suppressed the number of YMB-S cells that invaded the Matrigel ($P<0.01$, Kruskal-Wallis test).
b) Neither arphamenine B ($P=0.845$) nor amastatin ($P=0.774$) suppressed the invasion (no significance, t test).

passed through the membrane compared with untreated cells.

Effect of ubenimex on LTB_4 release from YMB-S cells

The LTB_4 levels in the cultured spent medium of YMB-S cells, treated with and without 2 mmol/liter of ubenimex for 6 days, were 118±14 and 41±7 pg/ml, respectively. The level was significantly increased by culture with ubenimex ($P<0.001$, t test).

DISCUSSION

Our results indicate that ubenimex induced cell-cell adhesion of a breast cancer cell line YMB-S by activating pre-expressed but "lazy" E-cadherin molecules on the cell surface. Since loosely adhering cells have higher motilities compared with tightly adhering ones, the former are more easily able to invade and metastasize.[18] E-Cadherin is a Ca^{2+}-dependent and homophilic adhesive molecule.[19] Cancer cells which do not express E-cadherin on the cell surface are relatively more invasive than those expressing the molecules.[18, 20, 21] E-Cadherin is accepted to be one of the invasion suppressor molecules.[18, 22] Recent studies have proven that catenins, which are E-cadherin-associated proteins, have an important role in the regulation of E-cadherin-mediated adhesion.[22, 23] Even in the presence of E-cadherin on the cell surface, lack of α-catenin caused dysfunction of E-cadherin-mediated adhesion.[23] The YMB-S cells proliferate in a floating manner without aggregation in complete medium, although the cells express E-cadherin on the cell surface. This is because the E-cadherins do not work in normal culture conditions. We found that ubenimex brought about the resumption of activity of such non-functioning E-cadherin. The mechanism involved is not clear. There is, however, a possibility that ubenimex might activate E-cadherin by influencing the level and/or the function of catenins.

Ubenimex also suppressed the proliferation of YMB-S cells. Most cells were induced to remain in the G0/G1 phase of the cell cycle. Like other differentiation inducers, such as sodium butyrate and all-*trans*-retinoic acid,[24] ubenimex seems to have the additional biological action of synchronizing the cell cycle of the cells. In cancer cells, disturbed control of the cell cycle allows continuous proliferation. Since ubenimex induced the cells to remain in the G0/G1 phase, it might affect the function of cell cycle regulatory molecules which cause the cells to progress from G1 to S phase.

The mechanism by which ubenimex induces adhesion and suppresses proliferation was examined. In previous studies which described the direct anti-tumor effects of ubenimex, it was reasoned that the effect might be achieved through an ability to inhibit aminopeptidases, because other aminopeptidase inhibitors such as amastatin and arphamenine B showed an anti-tumor effect similar to that of ubenimex.[7, 12] In contrast, amastatin showed no effect on YMB-S cells. Arphamenine B suppressed the proliferation, but neither induced adhesion nor suppressed the invasion of the cells into the Matrigel membrane. These results indicate that the inhibition of aminopeptidase N and B activities was not enough to suppress the invasion and the proliferation, or to induce adhesion. It has been reported that ubenimex is an inhibitor of LTA_4 hydrolase, which is a rate-limiting enzyme for LTB_4 biosynthesis.[6] Since the amount of LTB_4 in the spent medium of YMB-S cells cultured in the presence of ubenimex was increased compared with that in the spent medium cultured without ubenimex, the LTA_4 hydrolase activity of the cells might not be inhibited. Thus, the mechanism of the direct anti-tumor effect of ubenimex remains unknown.

Ubenimex is clinically used as a BRM for patients with acute myelogenous leukemia in Japan[2, 25, 26] because it has been proven that the drug has a potent action to stimulate the immune system through the inhibition of aminopeptidases. The stimulatory action should also operate in patients with breast cancer. Ubenimex may be a potential therapeutic drug for the disease, because the drug has both direct tumor-suppressing and immunocyte-stimulating effects.

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