Induction of apoptosis by ubenimex (Bestatin®) in human non-small-cell lung cancer cell lines

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Summary - We studied the direct anti-tumor effects of ubenimex on five human lung cancer cell lines; ABC-1, RERF-LC-OK, RERF-LC-MS (adenocarcinoma) and SQ-5, EBC-1 (squamous cell carcinoma). Ubenimex dose-dependently inhibited the growth of these cancer cell lines except RERF-LC-MS. The results indicated that lung squamous cell carcinoma cell lines were more sensitive to ubenimex than lung adenocarcinoma cell lines. Coincidentally, histological observation by Hematoxylin eosine (HE) staining revealed that ubenimex induced nuclear condensation and apoptic body in the cancer cell lines. Immunohistochemical study showed ubenimex-treated cells expressed LeY antigen which is a useful phenotypic marker predictive of apoptosis. The induction of DNA fragmentation was also observed in the ubenimex treated cancer cell lines by ELISA. We conclude that ubenimex exhibits its direct anti-tumor effect against non-small-cell lung cancer cell lines, more effectively against squamous carcinoma cell lines, through the induction of apoptosis.

ubenimex (Bestatin®) / non-small-cell lung cancer / apoptosis

INTRODUCTION

Ubenimex is a low molecular weight agent (MW 308.38) isolated from culture supernatant of *Streptomyces olivoleticuli* by Umezawa et al in 1976 [1], and is of dipeptide nature. Ubenimex is thought to be a biological response modifier which shows anti-tumor effect through the augmentation of immune system in host. Ubenimex acted on macrophages, T cells and polyclonal bone marrow progenitor cells and augmented the activity of these cells.

Furthermore, the immunocompetent cells activated by ubenimex produced various cytokines which augment humoral and cellular immune response [2-7].

It has already been reported that ubenimex in combination with chemotherapy and radiotherapy decreases the size of tumors and prolongs survival in the patients with squamous cell lung carcinoma [8, 9]. But, there were no reports about the direct anti-cancer effect of ubenimex. Therefore, we examined the direct effect of ubenimex on the growth of non-small-cell lung cancer cell lines in vitro.

MATERIALS AND METHODS

Cell cultures

Human lung cancer cell lines ABC-1, RERF-LC-OK, RERF-LC-MS (adenocarcinoma) and SQ-5, EBC-1 (squamous cell carcinoma) were obtained from Japan Cancer Research Resources Bank and RIKEN Cell Bank. The cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified 5% CO2 atmosphere.

Chemicals

Ubenimex ((-)N-[(2S, 3R)-3-amino-2-hydroxy-4-phenylbutyryl]-L-Leucine), obtained from Nippon Kayaku Co, Ltd (Tokyo, Japan), was dissolved in saline. The tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St Louis, MO, USA). Anti-LeY-monoclonal-antibody (BM1/JIMRO) was purchased from Japan Immunoresearch Laboratory Co, Ltd (Takasaki City, Gunma, Japan).
Cell growth assay

Cell growth was measured by the colorimetric MTT assay according to the method by Twentyman [10] with some modifications. Briefly, after trypsinization, cells were washed with fresh medium and counted with a hemocytometer and suspended in RPMI1640 containing 10% FBS. Ten thousand cells in 200 μL of the culture medium were placed in one well of multiplates (Falcon Plastics). The number of cells was counted by the trypan blue dye exclusion test. Then, various concentrations of ubenimex (0.1, 1, 10, 100, 200 μg/mL) were added to each well in triplicate. After the plates were incubated in a CO2 incubator at 37 °C for adequate days, 20 μL MTT solution, dissolved in 5 mg/mL phosphate buffered saline (PBS) and filtered (Nalge Co, Rochester, NY, USA), were added to each well. Following an additional 4-hour incubation at 37 °C, supernatant was discarded and then 200 μL of dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, LTD, Osaka, Japan) were added to dissolve the MTT-formazan product. After mixing with a mechanical plate mixer (Iwaki Glass Co, CA, USA), optical density (OD) was measured with Kinetic microplate reader (Molecular Devices Co, CA, USA) using test and reference wavelengths of 490 and 650 nm. Experiments were tried three times and unpaired t-test was used for statistical examination.

Examination of morphological change

Ten thousand trypsinized cells aliquoted in 300 μL of RPMI1640 medium containing 10% FBS were placed on an 8 chamber glass slide (Lab-Tek R, Nunk Inc, USA) and cultured overnight. Then, ubenimex was added to wells at the concentration of 100 μg/mL. After the incubation of cells for 24 and 72 hours, the cells were histologically characterized by HE staining.

Immunocytochemical staining

After cultured on an 8 chamber glass slide as described above, the cells were rinsed with PBS and fixed with 4% paraformaldehyde. They were then blocked with PBS and 10% horse serum, and incubated at the concentration of 20 μg/mL with anti-LeY monoclonal antibody (MoBiTec, Gottingen, Germany) overnight. After rinsing with PBS, they were incubated with biotinylated anti-mouse IgG at first and next with avidin-biotin-peroxidase complex (ABC kit, Vector). After rinsing in PBS, they were incubated in 0.1M Tris-HCl buffer containing 0.02% 3,3'-diaminobenzidine and 0.03% H2O2. Nuclear counterstaining was performed with Mayer’s hematoxylin solution.

Detection of DNA fragmentation

We used Cellular DNA Fragmentation ELISA Kit (Boehringer Mannheim GmbH, Germany) to check DNA fragmentation. Exponentially growing ABC-1 and SQ-5 cells (10 mL; 2 x 10^5 cells/mL) were incubated with 10 μM BrdU overnight at 37 °C, the cells were centrifuged at 250 g for 10 minutes and adjusted to 1 x 10^5 cells/mL in culture medium. 100 μL of cell suspension were mixed with 100 μL of either ubenimex solution or culture medium in a well of microtiter plate in triplicate. After incubation of the plate for 24 or 72 hours at 4 °C, the plate was centrifuged at 1,000 rpm for 10 minutes and 100 μL of supernatant were removed from each well. The cells in multiplate were lysed by adding an incubation buffer (100 μL/well) for 30 minutes at room temperature. The multiplate was centrifuged at 1,000 rpm for 10 minutes and 100 μL of supernatant were transferred directly to a well of a multiplate pre-coated with anti-DNA antibody. Then the samples were incubated for 90 minutes at room temperature. After washing, the samples were denatured and fixed by exonuclease III (10 U/mL in 66 mM Tris; 0.66 mM MgCl2; 1 mM 2-mercaptoethanol. pH 8.0; 100 μL/well) for 30 minutes at 37 °C. Then, peroxidase-conjugated anti-BrdU solution was added and incubated for an additional 90 minutes at room temperature. And then, adding substrate solution and incubating at room temperature in the dark on a plate shaker at 250 rpm for approximately 10-20 minutes until the color development was sufficient for photometric analysis, reaction mixture was stopped by adding 25 μL of stop solution (5.6% H2SO4) into each well and the absorbance at 450 nm (reference wavelength, 690 nm) against substrate solution as a blank.

Statistical analysis

Significant differences were determined by unpaired t-test.

RESULTS

Inhibition of cell growth by ubenimex

The inhibitory effects of ubenimex on the proliferation of lung cancer cell lines were investi-
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Gateway and the results are shown in Figures 1 and 2. Ubenimex inhibited the proliferation of SQ-5, EBC-1 squamous carcinoma cells and ABC-1, RERF-LC-OK adenocarcinoma cells in a dose-dependent manner, while no growth inhibition showed against RERF-LC-MS adenocarcinoma cells. Against SQ-5, EBC-1, ABC-1 and RERF-LC-OK cells, IC<sub>20</sub> values (drug concentration that induced 20% growth inhibition compared to untreated control) are 2.1, 2.7, 16.3, 46.7 µg/mL of ubenimex, and IC<sub>50</sub> are 72, 94, >200, >200 µg/mL of ubenimex, respectively (Table I). The results indicate that lung squamous cell carcinoma is significantly more sensitive to ubenimex than lung adenocarcinoma (P < 0.01).

Fig 1. Effects of ubenimex on proliferation of ABC-1, RERF-LC-OK, RERF-LC-MS adenocarcinoma. Cells (1 x 10^4/well) were incubated for seven days with various concentration of 0.1, 1, 10, 100, 200 µg/mL of ubenimex. The cell proliferation was determined by a colorimetric assay (MTT assay) on day 1, 3, 5, 7. Each optical density (OD) is shown in the mean of three times experiments of triplicate culture. *P < 0.05; **P < 0.01. Ubenimex (µg/mL): 0: control; +: 0.1; 0: 1; 0: 10; ◇: 100; □: 200.

Fig 2. Effects of ubenimex on proliferation of SQ-5, EBC-1 squamous cell carcinoma. Cells (1 x 10^4/well) were incubated for seven days with various concentration of 0.1, 1, 10, 100, 200 µg/mL of ubenimex. The cell proliferation was determined by a colorimetric assay (MTT assay) on day 1, 3, 5, 7. *P < 0.05; **P < 0.01. Ubenimex (µg/mL): 0: control; +: 0.1; 0: 1; ◇: 10; 0: 100; □: 200.
Table I. Sensitivity of lung cancer cell lines against to ubenimex.

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<th>Squamous</th>
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<th>Adenocarcinoma</th>
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<tr>
<td></td>
<td>SQ-5</td>
<td>EBC-1</td>
<td>ABC-1</td>
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<tr>
<td>IC_{20}</td>
<td>2.1 ± 1.6</td>
<td>2.7 ± 0.3</td>
<td>16.3 ± 4.0*</td>
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<tr>
<td>IC_{50}</td>
<td>72.7 ± 17.0</td>
<td>94.7 ± 9.9</td>
<td>&gt; 200</td>
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IC_{20} and IC_{50} mean ± SD (μg/mL). IC_{20} or IC_{50} values are the concentration of ubenimex that induced 20% or 50% growth inhibition compared to untreated control. SD indicates standard deviation. * significant difference (P < 0.01) of IC_{20} between SQ-5 and ABC-1, and between EBC-1 and ABC-1; ** significant difference (P < 0.01) of IC_{20} between SQ-5 and RERF-LC-OK, and between EBC-1 and RERF-LC-OK; NE: indicates not effective; > 200 indicates more than 200 μg/mL of IC_{50} of ubenimex.

Morphological change induced by ubenimex

As shown in figures 3 and 4, ubenimex-treated ABC-1 and SQ-5 carcinoma cells showed typical apoptotic morphology by HE staining, judging from the nuclear condensation, cytoplasmic condensation and membrane blebbing. These morphological changes were observed at both 24 and 72 hours after treatment with ubenimex, and were more marked in SQ-5 squamous carcinoma.

Fig 3. Staining of ABC-1 by HE magnification × 500. ABC-1 cells were incubated without (A) or with (B) 100 μg/mL concentration of ubenimex for 72 hours. In contrast to control (A), ABC-1 cells treated with ubenimex showed more often shrinkage of cytoplasm, condensation of the nucleus (→).

Fig 4. Staining of SQ-5 by HE magnification × 500. SQ-5 cells were incubated without (A) or with (B) 100 μg/mL concentration of ubenimex for 72 hours. In contrast to control (A), SQ-5 cells treated with ubenimex showed more often shrinkage of cytoplasm, condensation of the nucleus (→) and apoptotic body (●).
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However, no morphological changes were observed in RERF-LC-MS that exhibited no growth inhibition by ubenimex treatment.

Detection of DNA fragmentation of cancer cells by ELISA

We next examined the amount of DNA fragmentation of ABC-1 adenocarcinoma and SQ-5 squamous cell carcinoma induced by ubenimex treatment using ELISA. As shown in fig 5, DNA fragmentation was observed in both ABC-1 and SQ-5 cells treated with ubenimex dose-dependently. A significant increase in DNA fragmentation was found in ABC-1 following treatment with 10 μg/mL (P < 0.01) and 100 μg/mL (P < 0.001) of ubenimex. In the case of SQ-5, ubenimex 10-100 μg/mL also caused a significant increase of DNA fragmentation (P < 0.001) after incubation for 24 hours. However, only a slight increase of DNA fragmentation was observed in SQ-5 following treatment for 24 hours with 1.0 μg/mL (P < 0.05). The amount of DNA fragmentation showed the same tendency between the 24 and 72 hour treatment groups.

LeY antigen expression on lung cancer cells treated by ubenimex

To confirm the effect of ubenimex on the induction of apoptosis, we examined the induction of LeY antigen expression immunohistologically on the cells treated with or without ubenimex. LeY expression appears to be an important predisposing marker for apoptosis [11]. As shown in figures 6 and 7, following treatment of ABC-1 and SQ-5 cells with ubenimex at a concentration of 100 μg/mL for 24 h or 72 h, they were positively stained by anti-LeY antibody MoAb BM1/IIMRO. The LeY antigen expression seem-
Fig 7. Immunological staining of SQ-5 with anti-LeY monoclonal antibody. A is control, B is the staining that SQ-5 cells were incubated with 100 pg/mL concentration of ubenimex for 72 hours. In contrast to control (A), SQ-5 cells treated with ubenimex showed LeY positive.

DISCUSSION

In this paper, we studied the direct anti-tumor effects of ubenimex on non-small cell lung carcinoma cell lines in vitro. This is the first report that ubenimex has direct anti-tumor activity against lung carcinoma cell lines through the induction of apoptosis.

Ubenimex dose-dependently inhibited the proliferation of ABC-1, RERF-LC-OK adenocarcinoma cell lines and SQ-5, EBC-1 squamous cells. As these cultured cell lines did not contain immune cells such as macrophages or T cells, these results suggest that ubenimex has direct anti-tumor effect on lung carcinoma cell lines.

After treatment of lung carcinoma cell lines with ubenimex, shrinkage of cytoplasm, nuclear condensation and membrane blebbing of cells were observed histologically. These histological findings are compatible with apoptosis reported by Willie et al [12, 13]. Judging from these findings, it was suggested that ubenimex exerts a direct anti-cancer effect through the induction of apoptosis in cancer cells. We performed two additional experiments to confirm whether ubenimex induced apoptosis or not.

We examined the DNA fragmentation of the cells treated with ubenimex by using ELISA method. DNA fragmentation could be observed at 24 hours after the treatment of the cells with ubenimex. The amount of DNA fragmentation at 72 hours after the treatment was the same as that at 24 hours. These results revealed that ubenimex induced apoptosis within 24 hours after the treatment.

We examined the expression of LeY antigen using immunostaining method to confirm the induction of apoptosis of ubenimex-treated cells (figs 6 and 7). The difucosylated type 2 chain determinant (Fuc α 1 → 2 Gal β 1 → 4 [Fuc α 1 → 3 GlcNAc β 1 → R]) was originally detected among a number of oligosaccharides released from ovarian cyst mucin blood group [14], and termed LeY [15]. This LeY antigen was confirmed as a tumor-associated antigen from the monoclonal antibody approach [16-18]. Recently, Hiraishi et al [11] demonstrated that the LeY antigen detected by MoAb BM1/JIMRO immunohistologically was useful for phenotypic marker predictive of apoptosis. Ubenimex induced LeY antigen on the surface of ABC-1 and SQ-5 cells.

Clinical efficacy data have been reported in 21 Phase II, Phase II/III or Phase III clinical trials. Recently, Fukuoka et al [8] reported that a multicenter cooperative controlled study of ubenimex against squamous cell lung cancer was performed and it was confirmed that ubenimex when used in combination with chemotherapy and radiotherapy, not only enhances the tumor-reducing effect but also prolongs the survival time. However, their pilot study conducted prior to the controlled study showed that ubenimex did not enhance anti-tumor effect on lung adenocarcinoma. Difference between squamous cell carcinoma and
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Adenocarcinoma in sensitivity to ubenimex is not clear but regulation of the growth signals by aminopeptidases may be involved in the mechanism. Ubenimex is a potent aminopeptidase inhibitor and shows the competitive inhibition dose dependently. The direct anti-tumor activity and induction of apoptosis by ubenimex treatment might occur by aminopeptidase inhibition.

However, the single oral dose of 30 mg of ubenimex (1 capsule of Bestatin®) produced an average peak level of 2.2 μg/mL at 1 hour [19], suggesting that such administration of ubenimex would not attain serum concentration sufficient for a favorable therapeutic effect and higher doses of ubenimex would probably be required for the induction of apoptosis of carcinoma cells. Tsunogake et al reported that ubenimex inhibited growth of leukemic cells dose- and time-dependently [20]. If cancer cells were treated with ubenimex for longer time periods, ubenimex might be more effective. Further study, therefore, of the effects of ubenimex administered at higher doses or for frequent longer time periods are required to evaluate its clinical potential as a direct tumoricidal drug.

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REFERENCES

5 Schorlemmer HU, Bosset K, Sedlacek HH. Ability of the immunomodulating dipeptide Bestatin® to activate cytoxic mononuclear phagocytes. Cancer Res 1983;43:4148
10 Twentymain PR, Lascombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 1987;56:279
18 Lloyd KO, Larsson G, Stromberg N, Thulin J, Karlsson KA. Mouse monoclonal antibody F-3 recognizes the difucosyl Type 2 blood group structure. Immunogenetics 1983; 17:537