ANTICANCER RESEARCH *36*: 5883-5886 (2016)
doi:10.21873/anticanres.11174

Minodronic Acid in Combination with γδT Cells Induces Apoptosis of Non-small Cell Lung Carcinoma Cell Lines

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Abstract. *Background/Aim: Non-small cell lung carcinoma (NSCLC) is one of the leading causes of cancer-related death worldwide. Recent studies showed that nitrogen-containing bisphosphonates (N-BPs) directly and indirectly prevent proliferation, induce apoptosis, and inhibit metastasis of various types of cancer cell. In order to investigate the effect of combining minodronic acid (MDA) with γδ T-cells, NSCLC cells were treated with five concentrations of MDA. Materials and Methods: NSCLC cells were cultured with different concentrations of MDA alone or in combination with γδ T-cells for 24 h. Results: MDA with γδ T-cells had differential apoptotic effects on the NSCLC cell lines. The cells showed significant apoptotic effects in the presence of MDA in a dose-dependent manner. Conclusion: This study is the first to report an indirect effect of MDA against NSCLC. We report the induction of apoptosis by MDA in combination with γδ T-cells.*

Non-small cell lung carcinoma (NSCLC) is one of the leading causes of cancer death worldwide (1). Despite significant progress in therapy, the prognosis of patients with advanced NSCLC is still poor, with fewer than 5% long-term survivors at 5 years (2). Thus, newer agents must be developed to establish an effective therapeutic strategy against NSCLC.

Bisphosphonates (BPs) prevent bone loss and fractures, and are used for the treatment of osteoporosis, and for the prevention or treatment of multiple myeloma and skeletal metastases of solid tumors (3). Nitrogen-containing BPs (N-BPs), the so-called the second- and the third-generation BPs, are cytotoxic to osteoclasts, which is favorable for enhanced bone mineralization, and recent studies showed that N-BPs also have cytotoxic activities against tumors such as breast and prostate cancer (4, 5). These cytotoxic actions are attributable to a number of mechanisms, including apoptosis induction and anti-angiogenesis (6, 7). N-BPs have been shown to directly prevent proliferation, induce apoptosis, and inhibit metastasis of various types of cancer cell (4, 5, 8-11).

Furthermore, N-BPs inhibit farnesyl pyrophosphate synthetase, a critical enzyme in the mevalonate pathway, and increase the levels of the intermediate metabolic products of isopentenyl pyrophosphate (IPP), and induce cellular accumulation of IPP. IPP is a specific potent phosphoantigen for Vγ9Vδ2 T-cells, and activates γδ T-cells (12). Interestingly, γδ T-cells exhibit the capacity to kill a broad range of solid tumor and leukemia cells (13-15). It has been documented that activated human γδ T-cells produce large amounts of cytokines, including interferon-γ, consequently inducing antitumor immune responses (16, 17). The antitumor effects by γδ T-cells in tumors mainly involve granzyme/perforin release and death ligands, such as CD95L (FAS-ligand), tumor necrosis factor (TNF)-α release, and TNF-related apoptosis-inducing ligand (TRAIL) release via the caspase pathway in the γδ T-cells/target synapse (18, 19). Hence, N-BPs have indirect effects, that is to say γδ T-cell-mediated apoptotic effects, on various types of cancer cell through cellular accumulation of IPP (16, 17). N-BPs have been shown to directly and indirectly prevent proliferation, induce apoptosis, and inhibit metastasis of various types of cancer cell (6, 20).

Minodronic acid (MDA; YM529) is a third-generation N-BP, with antitumor effects on NSCLC cells that are poorly understood. Recently, it was reported that MDA directly induced apoptosis of NSCLC cells (21). To our knowledge, no report has demonstrated an indirect effect of MDA against NSCLC. Here, we report the induction of apoptosis in NSCLC cell lines by MDA in combination with γδ T-cells.

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Key Words: Non-small cell lung carcinoma cells, γδ T-cells, bisphosphonate, apoptosis.

Furthermore, we investigated the indirect effect of MDA against NSCLC.

Materials and Methods

Reagents. MDA was provided by Astellas Pharma (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human T-cell receptor (TCR) γδ (11F2, IgG1) and phycoerythrin (PE)-conjugated CD3 [Muromomab-CD3 (OKT3), IgG2a] were purchased from BD Bioscience (San Jose, CA, USA) and Affymetrix (Santa Clara, CA, USA), respectively.

Preparation of human γδ T-cells. Informed consent was obtained for the collection of peripheral blood from a healthy volunteer, and peripheral blood mononuclear cells (PBMCs) were prepared by density-gradient centrifugation (Lymphoprep; Axis-Shield PoCAS, Oslo, Norway). For the preparation of γδ T-cells, human PBMCs were placed in a T25 culture flask (Corning, NY, USA) containing AIM-V medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% autologous plasma, 1 μM MDA (Astellas Parma Inc.) and 200 IU/ml recombinant human (rh) IL2 (Nipro Co., Osaka, Japan). The cultures were maintained and expanded in AIM-V medium supplemented with 200 IU rhIL2 for 14 days.

Cell lines. Human NSCLC cell lines, RERF-LC-KJ (JCRB0137) and LK-2 (JCRB0829) cells were purchased from JCRB Cell Bank (Osaka, Japan). Cell lines were maintained in RPMI-1640 (Life Technologies) with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 μg/ml penicillin and streptomycin (Life Technologies) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell treatment. Subconfluent NSCLC cells were cultured in 24-well plates seeded at 1.0×10^5 cells per well in 400 μl of medium supplemented with 10% FBS and incubated for 24 h with different concentrations of MDA alone or in combination with γδ T-cells at a 1:1 the effector to target cell (E:T) ratio. To investigate the effect of MDA with and without γδ T-cells, the cells were treated with five concentrations (0, 1, 5, 10, or 50 μM) of MDA.

Flow cytometry. Samples of treated cells were stained with appropriate antibody FITC-conjugated mouse anti-human TCR γδ and PE-conjugated CD3 and analyzed using a BD FACS Calibur flow cytometer. The data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

Determination of apoptosis. Apoptotic cell death was analyzed to examine the antitumor effects of MDA with γδ T-cells on the NSCLC cell lines.

After co-culture, the cells were harvested by trypsinization and washed twice with cold PBS (0.15 mol/l, pH 7.2). The cells were centrifuged at 840 × *g* for 5 min, then the supernatant was discarded and the pellet was resuspended in 1× binding buffer at a density of 1.0×10^5-1.0×10^6 cells per ml. Next, 100 μl of the suspension was transferred to a 5 ml culture tube and incubated with 5 μl of FITC-conjugated annexin-V and 5 μl of propidium iodide (PI) for 15 min at room temperature in the dark. Then 400 μl of 1× binding buffer was added to each sample tube, and the samples were analyzed by FACS using CellQuest software (BD Biosciences) to distinguish and quantitatively determine the percentage of apoptotic cells. We considered annexin V-FITC-positive/PI-negative cells as being apoptotic. Approximately 5,000 cells were analyzed for each treatment. Experiments were repeated independently at least three times.

Statistical analysis. Results are presented as the mean±standard error (SE). The statistical significance of differences was determined using a *t*-test or one-way analysis of variance (ANOVA) followed by Scheffe's test. Single and double asterisks indicated *p*-values of <0.05 and <0.01, respectively. A probability of <0.05 was considered statistically significant.

Results

Phenotypic analysis of γδ T-cells expanded in culture. The expression of cell-surface antigens on γδ T-cells expanded in culture was analyzed by flow cytometry. CD3 PE-positive and TCR γδ FITC-positive cells from human PBMCs were expanded from 2.03, 1,57, and 1.68% to 96.21, 95.57, and 96.37%, respectively, after culturing for 14 days with MDA (Figure 1).

Effects of MDA and γδ T-cells on apoptosis of NSCLC cell lines. As shown in Figure 2, treatment of MDA with γδ T-cells led to differential apoptotic effects on the NSCLC cell lines. Co-culture of MDA-treated RERF-LK-KJ cells with purified γδ T-cells led to dose-dependent cancer cell death, which was statistically significant at MDA concentrations of 10 and 50 μM. In addition, MDA concentrations of ≥10 μM induced significant apoptosis of LK-2 cells cultured with purified γδ T-cells compared with cells co-cultured without γδ T-cells. NSCLC cells treated with MDA alone had no statistically significant effect at any concentration in these settings.

Discussion

To our knowledge, this is the first report to describe the ability of MDA combined with γδ T-cells to induce apoptosis of NSCLC cells. We demonstrated an indirect effect of MDA against NSCLC cells, in contrast with the findings of Koshimune *et al*., who reported that MDA directly induces apoptosis of NSCLC cells (21). In this study, the apoptotic effect observed in NSCLC cell lines co-cultured with MDA and γδ T-cells was significantly higher than that of the controls. However, in this study, MDA alone did not significantly induce apoptosis of NSCLC cells. Koshimune *et al*. reported that treatment with MDA for 72 h directly induced apoptosis of NSCLC cells. We suspect that we could have demonstrated direct antiproliferative effects of MDA on NSCLC cells if we had extended the treatment period beyond 24 h because several *in vitro* studies (4, 5, 8, 14, 22, 23) have shown that other N-BPs (zoledronic acid and pamidronate) exert direct cytostatic and apoptotic effects on a variety of human tumor cell lines (myeloma, breast, prostate, pancreas,

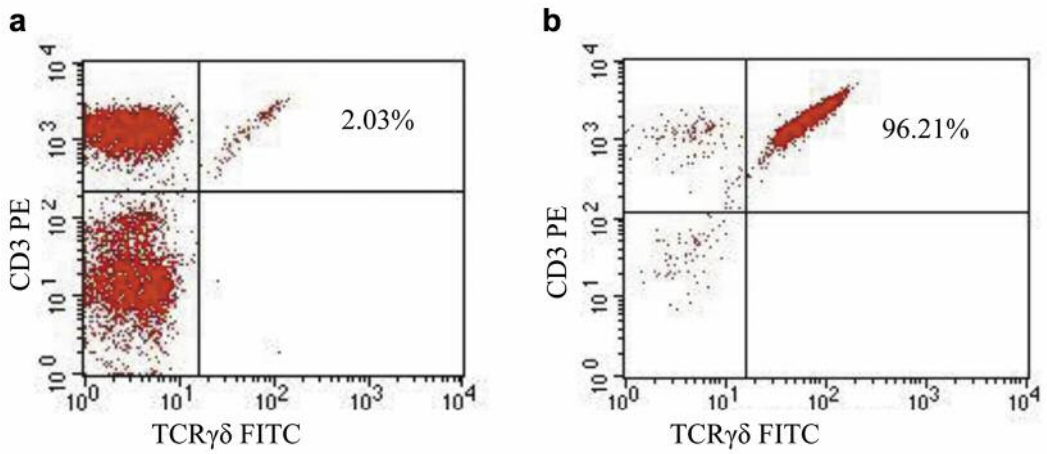

Figure 1. *Minodronic acid (MDA) induced γδ T-cell expansion from peripheral blood mononuclear cells. γδ T-Cell proliferation was analyzed by flow cytometry after 14 days in culture. The expanded cellular population was a T-cell receptor (TCR) γδ-positive cell population that was usually over 95% pure. a: γδ T-Cell proliferation was 2.03% before expansion. b: γδ T-Cell proliferation was 96.21% after 14 days in culture. CD3 PE: Phycoerythrin-conjugated CD3 ; FITC: Fluorescein isothiocyanate.*

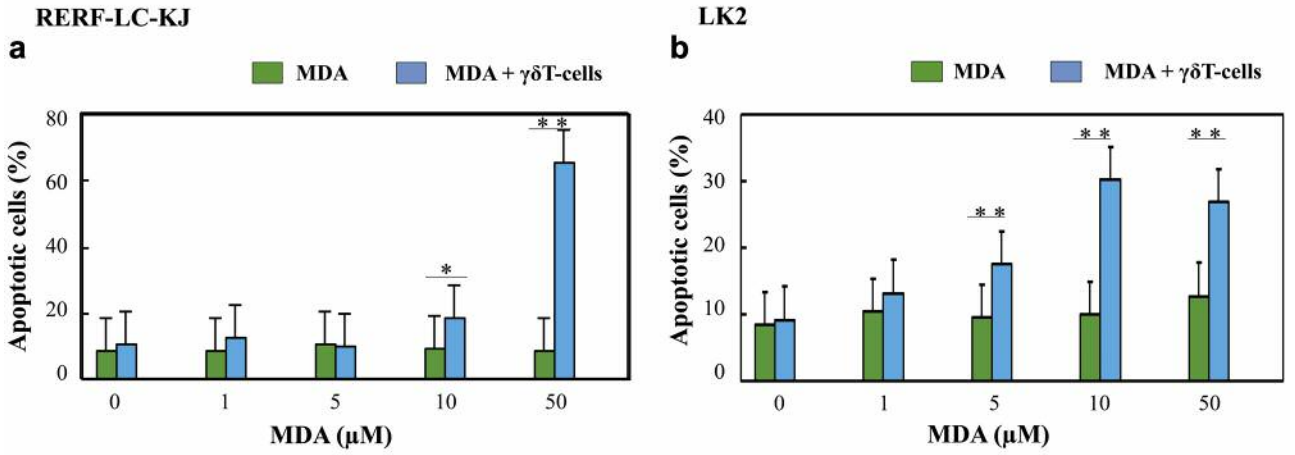

Figure 2. *Minodronic acid (MDA) sensitized non-small cell lung carcinoma (NSCLC) cell lines to γδ T-cell-mediated apoptosis. RERF-LC-KJ (a) and LK-2 (b) cells were untreated or co-cultured with γδ T-cells at a 1:1 ratio for 24 h. Cells from the co-cultures were measured by fluorometry. Each bar represents the mean±SE of values obtained in at least three independent experiments. p-Values were determined using a t-test to compare MDA-treated and MDA plus γδ T-cell treated cell lines. *p<0.05, **p<0.01.*

and glioblastoma) in a concentration- and time-dependent manner. The concentrations of BP used to produce these cytostatic and apoptotic effects *in vitro* were in the range of 5-2,000 μM. Although this appears rather high in comparison with the peak plasma levels usually achieved by intravenous BP infusion, it lies within the concentration range thought to occur locally in bone at an active resorption site (6,24). Hence, we carried out this study in the MDA range of 1-50 μM based on the actual clinical concentration of MDA of around 1 μM.

Interestingly, when BPs are combined with other common antineoplastic drugs, marked synergy occurs. Thus, the antiproliferative and apoptotic effects of BPs on breast cancer cells *in vitro* are enhanced with low concentrations of paclitaxel (25), and with tamoxifen (26). Thus, we expect that a similar synergistic effect also occurs on NSCLC cells.

Inducing apoptosis using MDA in combination with γδ T-cells has apoptotic effect on NSCLC cell lines *in vitro*. Further investigations are needed to show the synergistic effect on NSCLC cell lines *in vivo*. Ultimately, large-scale clinical trials will be required to investigate whether the promising antitumor potential of BPs can be exploited to reduce morbidity or increase survival in patients with NSCLCs.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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Received April 3, 2016
Revised April 30, 2016
Accepted May 8, 2016