Drug Discovery: Recent Progress and the Future

Regular Article

Docetaxel Upregulates HMGB1 Levels in Non-small Cell Lung Cancer

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Immune checkpoint inhibitors (ICIs) exert beneficial effects in non-small cell lung cancer (NSCLC) patients. However, ICIs are only advantageous for a limited population of NSCLC patients. Therefore to enhance their effects, combination therapies with ICIs have been developed. To identify preferable chemotherapy to combine with ICIs against lung cancer, we examined immunological effects of docetaxel compared with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI). We found no difference in peripheral lymphocyte counts and ratio of their subpopulations in lung cancer patients before and after both treatments. On the other hand, plasma levels of high-mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP) protein, showed significant increase after docetaxel treatment. Furthermore, we investigated effects of HMGB1 on tumor-infiltrating immune cells obtained from surgically resected tumor tissue from NSCLC patients. When the tumor infiltrating cells were stimulated with HMGB1, CD11c⁺ cells showed increased expression of activation markers. These findings imply that docetaxel could be involved in anti-tumor immunity *via* HMGB1. Therefore docetaxel might be a candidate for combination treatment with ICIs.

Key words docetaxel; high-mobility group box 1 (HMGB1); cancer immunotherapy; non-small cell lung cancer (NSCLC)

INTRODUCTION

Immune checkpoint inhibitors (ICIs) exert beneficial effects in non-small lung cancer (NSCLC) patients. In clinical trials conducted in patients with advanced, previously treated NSCLC, overall survival was longer with nivolumab, an anti-PD-1 ICI, than with docetaxel, a cytotoxic agent.^{1,2)} However, ICIs only exert beneficial effects in a limited population of NSCLC patients. Specifically, anti-PD-1 ICIs have shown limited efficacy in NSCLC patients with epidermal growth factor receptor (EGFR) mutation-positive tumors.^{3,4)}

There is a growing need for combination immunotherapies to improve the therapeutic efficacy of ICIs. One of the currently available combination immunotherapies is nivolumab plus ipilimumab for melanoma and renal cell carcinoma.^{5,6)} Other combination immunotherapies include pembrolizumab or atezolizumab plus platinum-based chemotherapy for NSCLC.^{7,8)} Although these combinations are more effective than monotherapy, adverse events are severe and frequent. Therefore it is eagerly desired to develop therapeutic agents that are safe and show synergistic effect for cancer immunotherapy.

Cytotoxic chemotherapeutics are able to induce immunostimulatory effects against cancer.⁹⁾ A key pathway in these effects is immunogenic cell death (ICD), which occurs as a

result of tumor cell endoplasmic reticulum stress and apoptosis induced by cytotoxic chemotherapeutics. During tumor cell death induced by cytotoxic chemotherapeutics, tumor cells release damage-associated molecular patterns (DAMPs) including high-mobility group box 1 (HMGB1). HMGB1 is an endogenous ligand that binds Toll-like receptor-4 (TLR-4) on dendritic cells (DCs), facilitating presentation of tumor-associated antigens to T cells.¹⁰⁾

In contrast to *in vitro* and *in vivo* animal studies, the rationale of ICD induced by cytotoxic chemotherapeutics with cancer patients remains to be evaluated. In this study we investigated chemotherapy-induced immunostimulatory effects in patients with NSCLC, focusing on docetaxel and EGFR-tyrosine kinase inhibitor (TKI), which are widely used in these individuals.

MATERIALS AND METHODS

Blood Sample Preparation Peripheral blood was obtained from 5 lung cancer patients treated with docetaxel administered every 3 weeks and 9 patients treated with EGFR-TKI (gefitinib, erlotinib, or afatinib) administered daily between May 2015 and August 2016. Peripheral blood was obtained from patients treated with docetaxel on days

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0, 14 (range, days 11–16), and 21 (range, days 21–36) and from those treated with EGFR-TKI on days 0, 14 (range, days 13–18), and 28 (range, days 28–52). Peripheral blood mononuclear cells (PBMC) were isolated by gradient density centrifugation using Lymphoprep (Axis Shield, U.K.). Isolated PBMCs and plasma were cryopreserved in liquid nitrogen.

Tissue Sample Preparation Surgically resected NSCLC tumor tissues were obtained from 5 patients without any neoadjuvant therapy between February and April 2018. Fresh tumor tissues were minced in a 6-cm dish and digested to single cell suspension using a Tumor Dissociation Kit for humans (Miltenyi Biotec, Germany) and gentle MACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. The cell suspension was applied to a 70- μ m nylon cell strainer (BD Biosciences, U.S.A.) with lysis of red blood cells by BD Pharm lyse. Dead cells and debris were removed by centrifugation in isodensity Percoll solution (Pharmacia Biotech), followed by HMGB1 stimulation assay.

Cell Lines Human lung cancer cell lines A549, HCC827, NCI-H322, HCC4006, HCC2935, Calu-3, and NCI-H1975 were obtained from the American Type Culture Collection (ATCC). RERF-LC-AI and EBC-1 were obtained from RIKEN Bio Resource Center, Japan. PC-9 cells were obtained from Immuno-Biological Laboratories. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Sigma, U.S.A.) containing 10% fetal bovine serum (FBS; HyClone, Thermo Scientific, U.S.A.), penicillin (100 units/mL), and streptomycin (100 μ g/mL) (Nakalai Tesque, Japan).

Flow Cytometric Analysis Surface marker staining was performed after FcR block using Human TruStain FcX Fc receptor blocking solution (BioLegend, U.S.A.). Cells were incubated with Fixable viability dye (eBioscience, U.S.A.). Surface marker-stained cells were analyzed on BD LSRFortessa with FACSDiva software (BD Biosciences). The following antibodies were used for FACS staining: anti-CD25-PE (clone

BC96), anti-CD86-PE (IT22), anti-CD8a-fluorescein isothiocyanate (FITC) (RPA-T8), anti-CD4-BV711 (OKT4), anti-CD11b-BV711 (M1/70), anti-CD45-BV785 (H130), anti-CD8a-BV510 (RPA-T8), anti-CD11c-BV510 (3.9), anti-CD69-BV605 (FN50), and IgG1 isotype control (MOPC-21) purchased from Biolegend. Anti-CD4-PE-Cy7 (SK3), anti-PD1-PE-Cy7 (EH12.1), anti-CD134-PE-CF594 (ACT35), and IgG1 isotype control (A19-3) were purchased from BD Bioscience. Anti-ICOS-PerCP-eFluor710 (ISA-3) antibody was purchased from eBioscience. Anti-Foxp3-PE (PCH101) was purchased from Invitrogen, U.S.A.

HMGB1 Enzyme-Linked Immunosorbent Assay (ELISA) HMGB1 concentrations in plasma samples from NSCLC patients or cell culture supernatant were measured

Table 1. Patient Characteristics

	Docetaxel (n = 5)	EGFR-TKI (n = 9)
Sex		
Male/female	4/1	2/7
Histology		
Adenocarcinoma	5	9
EGFR mutation		
Ex21: L858R	0	0
Ex19 del	0	4
Ex18: G719S	0	4
T790M	1	1
WT	4	0
EGFR-TKI		
Gefitinib		5
Erlotinib		2
Afatinib		2
Smoking status		
Current/former/never	2/1/2	0/0/9

Fig. 1. Profile of Peripheral Lymphocytes in NSCLC Patients Treated with Docetaxel or EGFR-TKI

(a) The schedule of sampling for profiling is shown. (b) Ratio of CD4⁺ and CD8⁺ T cells was analyzed by flow cytometry. The data are shown as comparison *versus* baseline. Statistical analysis was done by paired *t*-test with a *p* value <0.05 indicating significant difference.

by HMGB1 ELISA Kit (LS Bio) according to manufacturer's instructions.

WST Assay of Lung Cancer Cell Lines Cancer cell lines were co-cultured with gefitinib (0.001–100 μ M) or docetaxel (0.001–100 μ M) for 24 h. Then, cultured cell survival rate was measured by Premix WST-1 Cell Proliferation Assay System (TaKaRa, Japan).

HMGB1 Secretion Assay Cancer cell lines were co-cultured with gefitinib (10 μ M) or docetaxel (10 μ M) for 24 h and the culture supernatant was collected for HMGB1 ELISA.

Ex Vivo HMGB1 Stimulation Assay Freshly isolated cells from tumor tissues were cultured with various concentrations of HMGB1 in PRMI medium 1640 (Nacalai Tesque) containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL) for 24 h. After the culture, expression of harvested cells was analyzed by flow cytometry. For positive control, lipopolysaccharide (LPS) (1 ng/mL) was used.

RT-qPCR Analysis Total RNAs were extracted from cells by RNAeasy Mini Kit (Qiagen). Total RNA 200 μ g was used for cDNA synthesis by High-Capacity RNA-to-cDNA Kit (Applied Biosystems, U.S.A.). The reaction mixture contained 200 ng of sample RNA, 5 pmol of each forward and reverse PCR primer, and the SYBR Green Realtime PCR Master Mix-Plus (TOYOBO, Japan) provided by the manufacturer in 50 μ L of the recommended buffer. Primer sequences for RT-qPCR are provided in Supplementary Table 1. Fluores-

cence was detected by Step One Real-Time PCR System (Applied Biosystems). Expression of *IFNG*, *IL1B*, *IL6*, *TNFA*, *IL8*, *MCPI*, and *CX3CL1* was measured. Expression data were normalized to the geometric mean of housekeeping gene β -actin.

Statistical Analysis An unpaired two-tailed Student's *t*-test was used to examine the significance of differences between samples, with a *p*-value <0.05 indicating significant difference. A paired *t*-test was performed to determine the significance of change within the group.

Study Approval This study was conducted according to the principles of the Declaration of Helsinki. The study protocol was approved by Osaka University Hospital Ethics Committee, and written informed consent was obtained from participants prior to their inclusion.

RESULTS

Effects of Peripheral T Cells by Chemotherapy To evaluate immunostimulatory effects of docetaxel or EGFR-TKI, we analyzed changes of expression profile on PBMCs during treatments. Patients' characteristics are shown in Table 1. CD4⁺ and CD8⁺ T cell counts in patients treated with docetaxel or EGFR-TKI were not changed significantly during treatments (Fig. 1). Subpopulations among CD4⁺ and CD8⁺ T cells, *e.g.* Foxp3, PD-1, OX40, and ICOS-positive cells, showed no significant change (Supplementary Fig. 1).

Immunostimulatory Effect by HMGB1 Elevation during Chemotherapy Next, in order to investigate the induction of ICD by treatment with docetaxel or EGFR-TKI, the changes of plasma HMGB1 concentrations during treatments was analyzed. Concentrations of plasma HMGB1 showed significant increases in all patients treated with docetaxel, whereas EGFR-TKI treatment had no effect on HMGB1 levels (Fig. 2). To confirm whether docetaxel could induce release of HMGB1 from lung cancer cells, HMGB1 secretion assay was performed with 10 kinds of lung cancer cell lines. Concentrations of docetaxel and EGFR-TKI used were confirmed to show similar levels of cytotoxicity in all cell lines (data not shown). Significantly higher levels of HMGB1 were observed in culture supernatant from 4 lung cancer cell lines originating from NSCLC treated with docetaxel than without treatment, whereas gefitinib had no effect on this parameter (Fig. 3).

Effects of HMGB1 on Tumor-Infiltrating Immune Cells of NSCLC Patients To determine immunostimula-

Fig. 2. Changes of Plasma HMGB1 Concentrations in NSCLC Patients during Chemotherapy

Plasma HMGB1 concentration of NSCLC patients was measured by ELISA before and after docetaxel (*n* = 5) or EGFR-TKI (*n* = 9) treatments. Statistical analysis was done by paired *t*-test with a *p* value <0.05 indicating significant difference.

Fig. 3. HMGB1 Concentration in Culture Medium of Lung Cancer Cell Lines

Lung cancer cell lines were cultured with gefitinib (10 μ M) or docetaxel (10 μ M) for 24 h. The supernatant of the duplicated culture medium served for measuring HMGB1 concentration by ELISA. Statistical analysis was done by Student's *t*-test. ***p* < 0.001; **p* < 0.05.

Fig. 4. Change of Cytokine and Chemokine mRNA Expression in Tumor-Infiltrating Cells of NSCLC Patients by HMGB1 Treatments

The mRNA expression levels of cytokines and chemokines in tumor-infiltrating cells of NSCLC patients ($n=4$) were analyzed after 12-h treatment with HMGB1. Expression levels were measured by RT-qPCR. The values by LPS stimulation are shown as positive control. Data are shown as comparison with unstimulated control samples. Statistical analysis was done by paired *t*-test. ** $p < 0.05$; * $p < 0.1$.

Fig. 5. Expression of CD86 on CD11c⁺ Cells Stimulated by HMGB1

Tumor-infiltrating cells of NSCLC patients ($n=5$) with various concentrations of HMGB1 for 24h were tested. The data show CD86 positive cells percentage and mean fluorescence intensity (MFI) of CD86 in the subpopulation. Expression was measured by flow cytometry. The values by LPS stimulation are shown as positive control. Data are shown as comparison with unstimulated control samples. Statistical analysis was done by paired *t*-test. * $p < 0.05$.

tory effects of HMGB1 on NSCLC patients, we analyzed tumor-infiltrating cells from surgically resected tumor tissues of NSCLC patients in *ex vivo* HMGB1 stimulation assay. Tumor-infiltrating cells from NSCLC patients were cultured with HMGB1 for 12h. After the culture, cells were collected and the increase of cytokines and chemokines of the collected cells were analyzed by RT-qPCR. RT-qPCR analysis showed that mRNA levels of all cytokines (*IFNG*, *TNFA*, *IL1B*, and *IL6*) and chemokines (*MCP1*, *CX3CL1*, and *IL8*) were increased by HMGB1 stimulation (Fig. 4).

Next, we focused on activation of each tumor-infiltrating immune cell induced by HMGB1. Tumor-infiltrating cells from NSCLC patients were cultured with HMGB1 for 24h. After the culture, the flow cytometric analysis of the collected cells revealed that the expression of CD86, a co-stimulatory molecule on macrophages and DCs, was increased on CD11c⁺ cells, including macrophages and DCs (Fig. 5). These results suggest that HMGB1, induced by docetaxel treatment for NSCLC patients, increased co-stimulation by macrophages and DCs to T cells in tumor tissues.

DISCUSSION

In the present study, based on analyses of clinical specimens from NSCLC patients, we demonstrate that docetaxel treatment increased plasma HMGB1 levels and that HMGB1 increased CD86 co-stimulatory molecules on macrophages and DCs.

One of the concerns for immunological effects of docetaxel treatment is drug-induced lymphopenia. Changes in the proportion of T cell subsets in peripheral blood during platinum-based combination chemotherapy have been reported in several cancers, including thoracic malignancies,¹¹⁾ ovarian cancers,¹²⁾ pancreatic cancers,¹³⁾ and head and neck squamous cell carcinomas.¹⁴⁾ On the other hand, our analysis suggests that numbers of CD4⁺ and CD8⁺ T cells during docetaxel monotherapy are not changed significantly. Therefore it is estimated that effects on T cells during chemotherapy vary due to regimens of cytotoxic chemotherapeutics.

The immunostimulatory effects of ICD are mainly mediated by DAMPs, including HMGB1. In our analysis, the effects of docetaxel on release of HMGB1 from cancer cells vary due to each cell line, consistent with previous reports.^{15,16)} In contrast, we found that plasma HMGB1 concentrations were elevated in all patients during docetaxel treatment. Taken together with non-lymphopenia and HMGB1 release by docetaxel treatment, docetaxel might be a preferable chemotherapy to combine with ICIs against lung cancer.

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Conflict of Interest Department of Clinical Research in Tumor Immunology is a collaborating laboratory of Osaka University and Shionogi Co., Ltd.

Supplementary Materials The online version of this article contains supplementary materials.

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