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Cytotoxic chemotherapeutic agents and the EGFR-TKI osimertinib induce calreticulin exposure in non–small cell lung cancer

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ABSTRACT

Objectives: Synergistic anticancer efficacy of combination treatment with immune checkpoint inhibitors (ICIs) and platinum-based chemotherapy in patients with advanced non–small cell lung cancer (NSCLC) may be attributable in part to the phenomenon of immunogenic cell death (ICD), which is characterized by the release of damage-associated molecular patterns (DAMPs) from dying tumor cells. The ability of cytotoxic chemotherapeutic agents and molecularly targeted drugs such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) to induce DAMPs during the treatment of NSCLC has remained unclear, however.

Materials and methods: We investigated the ability of seven cytotoxic chemotherapeutic agents and the third-generation EGFR-TKI osimertinib to induce translocation of the DAMP calreticulin to the cell surface in multiple NSCLC cell lines. The plasma concentration of soluble CRT in advanced NSCLC patients treated with cytotoxic chemotherapy or osimertinib was measured.

Results: Antimetabolites and microtubule inhibitors induced expression of CRT at the cell surface (ecto-CRT) to a greater extent than did platinum agents in six NSCLC cell lines, exhibiting higher up-regulation of phosphorylation of eukaryotic initiation factor-2α (eIF2α). Ecto-CRT expression was positively correlated with apoptosis induction in NSCLC cells treated with these various chemotherapeutic agents. The drug-induced up-regulation of ecto-CRT in NSCLC cells was attenuated by the pan-caspase inhibitor Z-VAD-FMK. Osimertinib similarly increased ecto-CRT expression in association with apoptosis induction in five *EGFR*-mutated NSCLC cell lines. Furthermore, the plasma concentration of soluble CRT in 16 NSCLC patients treated with single-agent pemetrexed or docetaxel and in nine *EGFR*-mutated NSCLC patients treated with osimertinib was increased after treatment onset.

Conclusion: Our findings indicate that antimetabolites, microtubule inhibitors, and osimertinib are effective inducers both of CRT exposure in NSCLC cell lines and of soluble CRT release in patients with advanced NSCLC, suggesting that these agents might prove effective for promotion of antitumor immunity in combination immunotherapy.

1. Introduction

Lung cancer has a low survival rate and is increasing in incidence worldwide [1]. Immune checkpoint inhibitors (ICIs) have recently changed the treatment landscape for individuals with advanced solid tumors [2], with numerous clinical studies having demonstrated that the addition of ICIs—such as antibodies specific for programmed cell death–1 (PD-1) or for its ligand PD-L1—to platinum-based combination chemotherapy results in a significant extension of overall survival in patients with advanced non–small cell lung cancer (NSCLC) [3–7]. This improved antitumor efficacy of ICIs plus combination chemotherapy in the clinical setting has suggested that chemotherapeutic agents may

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increase the susceptibility of tumors to ICI treatment.

Many chemotherapeutic agents not only have direct cytotoxic effects but also are thought to modulate antitumor immunity, with one mechanism implicated in this modulation being the induction of immunogenic cell death (ICD) [8]. ICD is a form of regulated cell death that triggers an adaptive immune response through the release or exposure of damage-associated molecular patterns (DAMPs) including calreticulin (CRT), high-mobility group box 1 (HMGB1), and ATP [9]. Exposure of colon cancer cells to chemotherapeutic agents such as anthracyclines and doxorubicin was found to induce the translocation of intracellular CRT to the cell surface and thereby to elicit phagocytosis of the tumor cells by dendritic cells in vitro, whereas administration of recombinant CRT in vivo increased the antitumor effects of chemotherapeutic agents that do not induce ICD [10]. Whereas CRT normally resides in the lumen of the endoplasmic reticulum, CRT that has translocated to the plasma membrane (ecto-CRT) in association with the induction of apoptosis serves as an "eat me" signal for professional phagocytes and thereby promotes adaptive immunity [11]. Although the immunologic relevance of soluble CRT released from dying tumor cells in the clinical setting remains unclear, recent studies have shown that recombinant CRT stimulates monocytes-macrophages in vitro and promotes immunogenicity in vivo, with these effects depending in part on self-oligomerization of the protein [12,13]. These various observations suggest that both ecto-CRT and soluble CRT might function as DAMPs that contribute to the anticancer immune response, and they may provide a basis for the development of a new strategy for immunogenic chemotherapy.

We have now investigated the effects of a variety of anticancer agents that are currently administered for standard treatment of NSCLC—including cytotoxic chemotherapeutic drugs and an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI)—on the translocation of CRT to the cell surface in multiple NSCLC cell lines. In addition, we have measured the concentration of soluble CRT in plasma from individuals with advanced NSCLC during systemic anticancer treatment.

2. Materials and methods

2.1. Cell culture and reagents

Human NSCLC cell lines were obtained from American Type Culture Collection (A549, H1299, H1650, HCC827, HCC4006), from European Collection of Authenticated Cell Cultures (PC9 cells), or from RIKEN (EBC1 cells), or were kindly provided by Y. Maehara of Kyushu University (11−18 cells). All cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM, Gibco), each supplemented with 10 % fetal bovine serum and 1% penicillin-streptomycin (Gibco). Pemetrexed disodium (PEM) was obtained from LKT Laboratories; docetaxel (DTX), cisplatin (CDDP), and carboplatin (CBDCA) were from Tocris; gemcitabine (GEM) was from Tronto; and paclitaxel (PTX), vinorelbine ditartrate (VNR), dimethyl sulfoxide were from Wako, and Z-VAD-FMK was from Selleck.

2.2. MTS assay

Cell viability was evaluated with the MTS assay. Cells were seeded in 96-well flat-bottom plates (Greiner Bio-One) and incubated for 12 h before exposure to test agents and incubation for an additional 72 h. Cell Titer 96 Aqueous One Solution (Promega) was then added to each well, and absorbance at 490 nm was measured with a Multiskan FC instrument (Thermo Fisher Scientific). The median inhibitory concentration (IC_{50}) for each agent was extrapolated from survival curves with the use of Graphpad Prism version 7 or Microsoft Excel.

2.3. Detection of ecto-CRT

Cells were transferred to six-well plates and incubated for 12 h before exposure to test agents at their IC_{50} values and additional incubation for 72 h. CRT and annexin V at the cell surface were then detected by flow cytometry. The cells were thus collected, washed with Annexin V binding buffer (BioLegend), and incubated for 20 min on ice with rabbit antibodies to human CRT (Abcam) and fluorescein isothiocyanate–conjugated annexin V (BioLegend) in Annexin V Binding Buffer (BioLegend). They were then washed twice with FACS buffer containing 2 % FBS and 0.1 % sodium azide before incubation for 20 min on ice with Alexa Fluor 647–conjugated donkey antibodies to rabbit immunoglobulin G (Abcam) in Annexin V Binding Buffer. After an additional two washes with FACS buffer, the cells were subjected to flow cytometry with a FACSverse instrument (Becton Dickinson).

2.4. Patients and sample collection

Plasma was collected from 25 individuals with advanced NSCLC regardless of previous treatment history at four time points including before, on days 3 and 8 of, and at the end of the first cycle of chemotherapy with docetaxel or pemetrexed, or before, on days 3 and 8 of, and at ~1 month after the onset of osimertinib administration. Soluble CRT was measured with an enzyme-linked immunosorbent assay (ELISA) kit (#ELH-CALR-1, RayBiotech). All patients provided written informed content. Tumor diameter was measured at baseline and after the second cycle of treatment according to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1).

2.5. Immunoblot analysis

Cultured cells were lysed in RIPA buffer (Thermo Fisher), the lysates were fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated overnight at 4 °C with primary antibodies to phosphorylated eIF2α (#9721, diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA) and to eIF2α(#9722, diluted 1:1000; Cell Signaling Technology). Immune complexes were detected with horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (GE Healthcare UK, Amersham, UK), Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific), and the ChemiDoc XRS + system (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

All statistical analysis was performed with GraphPad Prism 7. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Cytotoxic chemotherapeutic agents induce cell surface exposure of CRT in NSCLC cell lines

We first performed the MTS assay to determine the IC_{50} of seven cytotoxic chemotherapeutic agents administered clinically for the treatment of NSCLC—CDDP, CBDCA, VNR, DTX, PTX, GEM, and PEM—in six human NSCLC cell lines (Table 1). We then examined the effect of each chemotherapeutic agent at its IC_{50} on the expression level of ecto-CRT in the same cell lines by flow cytometry. All seven chemotherapeutic agents induced a marked increase in the expression level of ecto-CRT in all six NSCLC cell lines (Fig. 1A), with these effects being significant for the two antimetabolites PEM and GEM and for the three microtubule inhibitors PTX, DTX, and VNR, but not for the two platinum agents CDDP and CBDCA (Fig. 1B). As eukaryotic initiation factor-2α (eIF2α)phosphorylation, as one of signs of ER (endoplasmic reticulum) stress, correlates with its downstream CRT exposure, we compared the

Table 1
IC_{50} values (μM) of cytotoxic chemotherapeutic agents for non–small cell lung cancer cell lines.

Drug classification	Drug	A549 (WT)	H1299 (WT)	EBC1 (WT)	PC9 (Ex19del)	H1650 (Ex19del)	11-18 (L858R)
Platinum agent	Cisplatin	25.2	10.3	9.79	14.9	17.2	20.2
	Carboplatin	22.8	9.65	18.5	14.6	16.8	20.3
Antimetabolite	Pemetrexed	17.3	3.78	1.03	0.456	5.86	2.92
	Gemcitabine	0.634	1.16	0.233	1.27	0.807	0.224
Microtubule inhibitor	Docetaxel	4.27	0.026	0.023	0.003	0.007	0.006
	Paclitaxel	0.075	0.063	0.009	0.008	0.030	0.006
	Vinorelbine	0.055	0.061	0.008	0.007	0.036	0.011

The *EGFR* status of each cell line is indicated in parentheses: WT, wild type; Ex19del, exon-19 deletion.

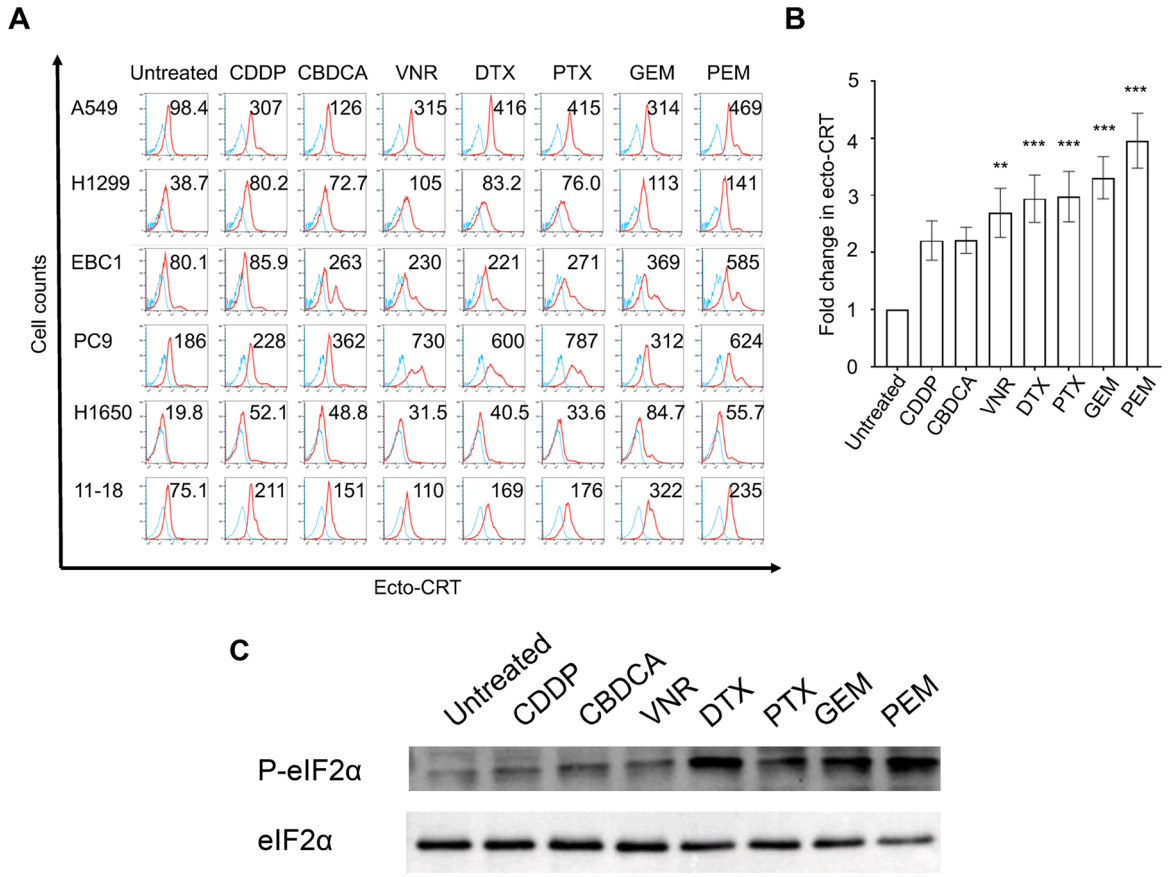

Fig. 1. Effects of cytotoxic chemotherapeutic agents on the expression level of ecto-CRT in human NSCLC cell lines. (A) A549, H1299, EBC1, PC9, H1650, and 11-18 cells were incubated with or without the indicated chemotherapeutic agents at their IC_{50} values for 72 h, after which the expression level of ecto-CRT was determined by flow cytometry. (B) Quantitation of the fold change in mean fluorescence intensity for ecto-CRT induced by each drug in the six cell lines for experiments similar to that shown in (A). (C) Effect of the chemotherapeutic agents on protein levels of eIF2αin NSCLC cells. EBC1 cells were incubated for 48 h in the absence or presence of the indicated cytotoxic drugs at their IC_{50}. The cells were then lysed and subjected to immunoblot analysis with antibodies to phosphorylated eIF2α (P-eIF2α) and to total eIF2α (loading control). Data are means ± SEM from 2 independent experiments with similar results. $^{**}p < 0.01$, $^{***}p < 0.001$ versus untreated cells (Dunn's multiple comparisons test).

eIF2α phosphorylation levels in NSCLC cells treated with these differential chemotherapeutic agents. Our results from immunoblot analysis showed that antimetabolites PEM and GEM as well as the microtubule inhibitors DTX and PTX induced a greater up-regulation of phosphorylation eIF2α in NSCLC cells compared with the platinum drugs CDDP and CBDCA (Fig. 1C).

3.2. Relation between ecto-CRT expression and apoptosis in NSCLC cells treated with cytotoxic chemotherapeutic agents

Given that chemotherapeutic agents have been shown to up-regulate ecto-CRT in cancer cells during the induction of apoptosis [10], we simultaneously assessed the effects of such agents both on ecto-CRT expression and on apoptosis, as detected by the appearance of annexin V at the cell surface. We detected a positive correlation between the proportions of cells positive for ecto-CRT and for surface expression of annexin V in the six NSCLC cell lines treated with each of the seven cytotoxic drugs, although the correlations for DTX and GEM did not achieve statistical significance (Fig. 2A). Given these positive correlations and the role of caspases in mediating the apoptotic response, we examined the effects of the pan-caspase inhibitor Z-VAD-FMK on the up-regulation of ecto-CRT expression by the seven chemotherapeutic agents in EBC1 cells. The increase in the expression level of ecto-CRT induced by CBDCA, VNR, PTX, GEM, or PEM was significantly inhibited in the presence of Z-VAD-FMK at 50 μM (Fig. 2B), indicating that caspase-dependent apoptosis accounts in part for the appearance of CRT

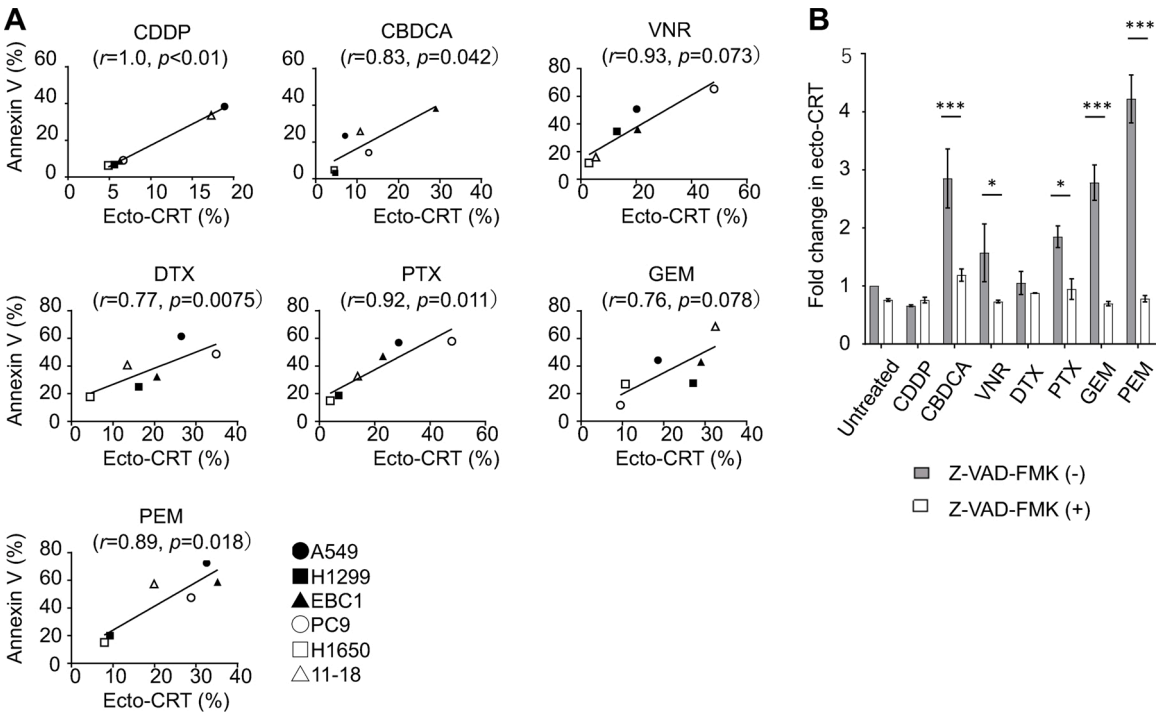

Fig. 2. Relation between ecto-CRT expression and apoptosis induced by cytotoxic chemotherapeutic agents in NSCLC cell lines. (A) Positive correlation between the proportion of cells positive for ecto-CRT or for surface annexin V as detected by flow cytometry in NSCLC cell lines treated with the indicated cytotoxic agents at their IC_{50} values for 72 h. Each dot represents representative data for each cell line (A549, H1299, EBC1, PC9, H1650, or 11-18). Spearman's correlation coefficient (*r*) and *p* values are indicated (Spearman test). (B) EBC1 cells were incubated for 72 h in the absence or presence of the indicated cytotoxic drugs at their IC_{50} values as well as in the presence of Z-VAD-FMK (50 μM) or dimethyl sulfoxide vehicle, after which the fold change in the expression level of ecto-CRT relative to control cells was measured by flow cytometry. Data are means ± SEM from 2 independent experiments with similar results. $*p < 0.05$, $***p < 0.001$ (Sidak's multiple-comparison test).

at the cell surface.

3.3. Osimertinib up-regulates ecto-CRT expression in EGFR-mutated NSCLC cell lines

Osimertinib is a third-generation EGFR-TKI that is administered as a standard first-line treatment for *EGFR* mutation–positive NSCLC [14]. Given that the effects of EGFR-TKIs on ecto-CRT expression in *EGFR*--mutated NSCLC cell lines had not previously been described, we examined the effects of osimertinib on ecto-CRT expression in PC9, H1650, HCC827, and HCC4006 cells, all of which harbor an exon-19 deletion of *EGFR*; in 11-18 cells, which are positive for the L858R point mutation of *EGFR*. Treatment with osimertinib at its respective IC_{50} values (Table 2, Supplementary Fig. 1) increased the percentage of ecto-CRT–positive cells in all five of these *EGFR*-mutated NSCLC cell lines (Fig. 3A). The average fold change in ecto-CRT expression induced by osimertinib in these five cell lines was statistically significant (Fig. 3B). There was also a significant positive correlation between the proportions of cells positive for ecto-CRT and for annexin V in these five cell lines (Fig. 3C). The antitumor effect of osimertinib on both ecto-CRT and annexin V expression in PC9 cells was significantly reduced in the presence of Z-VAD-FMK (Fig. 3D and E).

3.4. Soluble CRT levels in plasma of NSCLC patients treated with cytotoxic agents or osimertinib

Given that the opportunities to obtain tumor tissue for quantitative assessment of ecto-CRT expression in advanced NSCLC patients receiving systemic anticancer treatment are limited in the clinical setting, we next examined whether it is possible to detect changes in the concentration of soluble CRT in plasma from such patients with an ELISA. We studied NSCLC patients receiving DTX or PEM single-agent regimens, given that both of these cytotoxic agents were found to have a relatively large effect on ecto-CRT expression in NSCLC cell lines (Fig. 1). Plasma was collected from 16 such patients at four different time points including before (pre), on days 3 and 8 of, and at the end of the first cycle of chemotherapy. We found that the maximum level of soluble CRT in plasma after the onset of chemotherapy was significantly greater than the baseline level (Fig. 4A). Finally, we performed a similar analysis for nine patients with advanced NSCLC positive for *EGFR* activating mutations who were treated with osimertinib. Again, the maximum level of soluble CRT after the onset of osimertinib treatment was significantly higher than the baseline level (Fig. 4B).

4. Discussion

The identification of molecules that reflect the extent of ICD and are

Table 2
IC_{50} values (nM) of osimertinib for *EGFR*-mutated non–small cell lung cancer cell lines.

Drug classification	Drug	PC9 (Ex19del)	H1650 (Ex19del)	11-18 (L858R)	HCC827 (Ex19del)	HCC4006 (Ex19del)
Tyrosine kinase inhibitor	Osimertinib	48.6	1790	781	10.2	43.1

The *EGFR* status of each cell line is indicated in parentheses: Ex19del, exon-19 deletion.

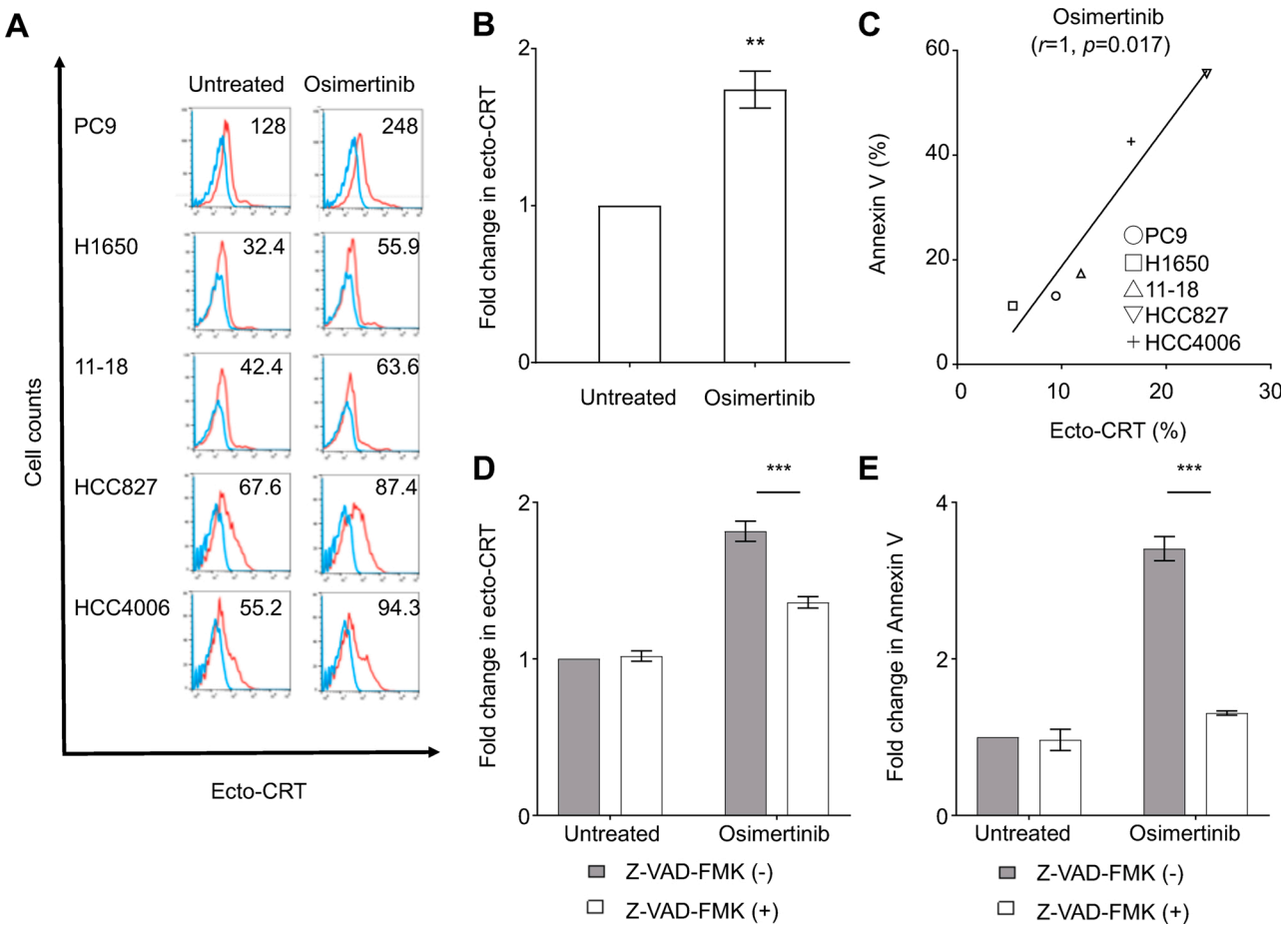

Fig. 3. Effects of osimertinib on ecto-CRT expression and apoptosis in human NSCLC cell lines positive for activating *EGFR* mutations. (A) Flow cytometric analysis of ecto-CRT expression in the indicated cell lines incubated in the absence or presence of osimertinib at its respective IC_{50} values for 72 h. (B) Fold change in mean fluorescence intensity for ecto-CRT induced by osimertinib in the five *EGFR*-mutated NSCLC cell lines for experiments similar to that shown in (A). Data are means ± SEM from 2 independent experiments with similar results. $^{**}p < 0.01$ (Wilcoxon matched-pairs signed-rank test). (C) Positive correlation between the proportion of cells positive for ecto-CRT or for surface annexin V as determined by flow cytometry in *EGFR*-mutated NSCLC cell lines treated with osimertinib at its respective IC_{50} values for 72 h. Each dot represents representative data for each cell line (PC9, H1650, 11-18, HCC827, or HCC4006). Spearman's correlation coefficient (r) and p values are indicated. (D, E) PC9 cells were incubated for 72 h in the absence or presence of osimertinib at its IC_{50} as well as in the presence of Z-VAD-FMK (50 μM) or dimethyl sulfoxide vehicle, after which the fold change in the expression level of ecto-CRT (D) or Annexin V (E) relative to control cells was measured by flow cytometry. Data are means ± SEM from 3 independent experiments with similar results. $^{***}p < 0.001$ (Sidak's multiple-comparison test).

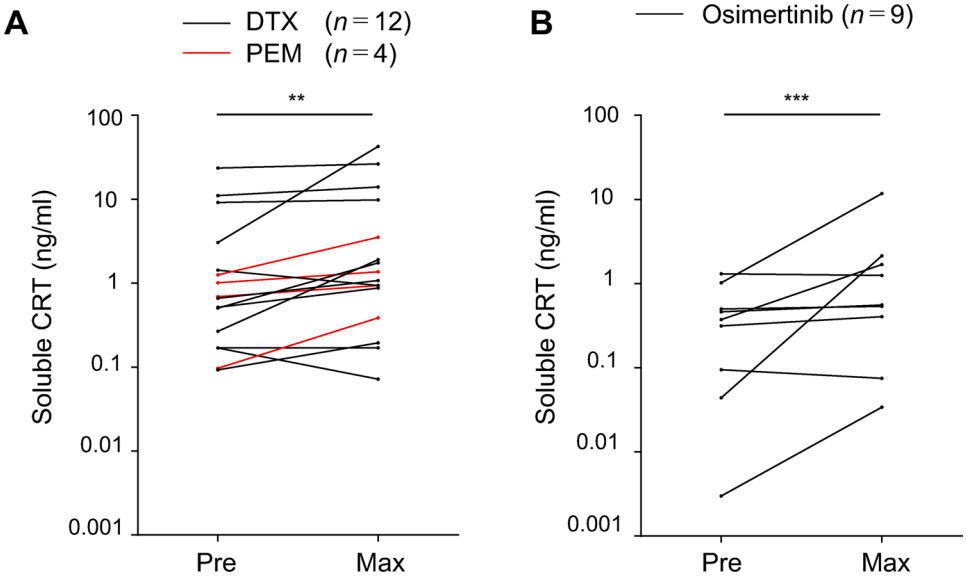

Fig. 4. Concentrations of soluble CRT in plasma of NSCLC patients before and after the onset of treatment with cytotoxic agents or osimertinib. (A) The plasma concentration of soluble CRT was determined before (pre), on days 3 and 8 of, and after the end of the first cycle of chemotherapy with DTX ($n = 12$) or PEM ($n = 4$) in patients with advanced NSCLC. The maximum (max) value for soluble CRT observed after the onset of treatment was then compared with the corresponding baseline value. $^{**}P < 0.01$ (Wilcoxon matched-pairs signed-rank test). (B) The plasma concentration of soluble CRT was determined before (pre), on days 3 and 8 of, and about 1 month after the onset of osimertinib therapy in patients with *EGFR*-mutated advanced NSCLC ($n = 9$). The maximum (max) value for soluble CRT observed after the onset of treatment was then compared with the corresponding baseline value. $^{***}P < 0.001$ (Wilcoxon matched-pairs signed-rank test).

able to trigger an anticancer immune response is likely to inform the design of more effective combination regimens involving immunotherapy. The exposure of CRT at the cell surface has been found to contribute to the immunogenicity of cancer cell death and to be associated selectively with ICD versus non-ICD forms of cell death [9,10,15,16]. Certain chemotherapeutic agents such as cyclophosphamide, doxorubicin, oxaliplatin, and mitoxantrone have been shown to trigger DAMP induction and to stimulate an antitumor immune response [17–21]. Exposure of cancer cells to such cytotoxic agents induces translocation of the chaperone protein CRT to the cell surface, where it promotes phagocytosis of tumor cells by antigen-presenting cells and thereby elicits a robust antitumor immune response [22]. However, the effects of many cytotoxic chemotherapeutic agents—including CDDP, CBDCA, PEM, GEM, DTX, PTX and VNR—used in the standard treatment of NSCLC on DAMPs such as ecto-CRT in lung cancer cells have remained unclear. We have now shown that the antimetabolites PEM and GEM as well as the microtubule inhibitors DTX, PTX, and VNR induced a more pronounced up-regulation of ecto-CRT in NSCLC cell lines compared with the platinum drugs CDDP and CBDCA. Given that endoplasmic reticulum stress was shown to trigger the exposure of CRT at the cell surface in human osteosarcoma cell lines [23], our results suggest that antimetabolites and microtubule inhibitors may have a greater capacity to induce such stress in NSCLC cell lines compared with platinum agents.

We also found that the extent of ecto-CRT expression in NSCLC cell lines treated with cytotoxic drugs was positively correlated with apoptosis induction as reflected by appearance of annexin V at the cell surface. This finding suggests the possibility that monitoring of apoptotic markers in tumor samples or plasma of cancer patients may reflect the expression of ecto-CRT and induction of ICD during anticancer treatment. Consistent with previous preclinical findings that the pan-caspase inhibitor Z-VAD-FMK attenuated the exposure of CRT induced by doxorubicin [10] or mitoxantrone [24] in mouse colon cancer cells, we found that ecto-CRT expression induced by cytotoxic drugs in human NSCLC cells was also inhibited by Z-VAD-FMK. These results thus implicate apoptotic cell death in the up-regulation of ecto-CRT associated with ICD in NSCLC cells.

The concentration of soluble CRT in serum was found to be higher for lung cancer patients than for healthy individuals [25]. It was also found to be higher for the patients after chemotherapy than for the patients without chemotherapy [25]. In the clinical setting, quantitative assessment of ecto-CRT expression in tumor specimens of patients with advanced NSCLC is problematic because of limited accessibility for rebiopsy, especially after tumor debulking followed by systemic anticancer therapy. As an alternative means to detect changes in CRT exposure in tumor cells, we here performed serial measurements of soluble CRT in plasma of advanced NSCLC patients during single-agent treatment with PEM or DTX as a representative antimetabolite and microtubule inhibitor, respectively. We found that such treatment was associated with a significant increase in the soluble CRT concentration during the first treatment cycle. Although the relation between the expression level of ecto-CRT in tumor tissue and the concentration of soluble CRT in plasma during anticancer treatment remains unclear, our results suggest the possibility that soluble CRT in plasma reflects exposure of ecto-CRT in tumor cells and tumor cell destruction. Further studies will be necessary to distinguish this possibility from others.

We have also for the first time shown that osimertinib increased the expression level of ecto-CRT in *EGFR*-mutated NSCLC cell lines and that this effect correlated with the extent of apoptotic cell death. Moreover, osimertinib treatment was associated with an increase in the plasma concentration of soluble CRT in patients with advanced NSCLC positive for *EGFR* mutations. These findings thus suggest that osimertinib-induced tumor cell death may result in the exposure and release of CRT and thereby promote antitumor immunity in such patients. However, our correlation analysis could not detect positive correlation between tumor shrinkage (Supplementary Fig. 2), although the number of patients in current study might be small and insufficient making it harder to find statistical significance. Combination therapy with osimertinib and durvalumab, an antibody to PD-L1, was recently found to be unfeasible in patients with *EGFR*-mutated lung cancer as a result of an increased incidence of interstitial lung disease [26]. Our results now suggest the possibility that this adverse event may have been due to an excessive or aberrant immune response to self-antigens in lung tissue triggered by osimertinib-induced up-regulation of ecto-CRT or other DAMPs in tumor cells.

Cytotoxic chemotherapy can promote anticancer immunity either in a direct manner by reducing the frequency of peripheral blood regulatory T cells [27] or indirectly by subverting the immunosuppressive tumor microenvironment and increasing tumor susceptibility to attack by immune cells [28]. The establishment of ICD-relevant biomarkers such as DAMPs may therefore guide the implementation of personalized combination immunotherapy to restore or improve the anticancer immune response.

In conclusion, we have here shown that a wide range of cytotoxic chemotherapeutic agents as well as the third-generation EGFR-TKI osimertinib induce ICD associated with CRT exposure in NSCLC cell lines in vitro. In addition, treatment with such agents was associated with an increase in the plasma concentration of soluble CRT in patients with advanced NSCLC. Our findings thus suggest that these anticancer modalities might be suitable partners for ICI therapy as promoters of antitumor immunity.

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CRediT authorship contribution statement

Rie Furukawa: Methodology, Formal analysis, Investigation, Writing - original draft. **Hiroyuki Inoue:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Resources. **Yasuto Yoneshima:** Writing - review & editing, Visualization, Resources. **Hirono Tsutsumi:** Investigation. **Eiji Iwama:** Writing - review & editing, Visualization, Resources. **Yuki Ikematsu:** Visualization. **Nobuhisa Ando:** Visualization. **Yoshimasa Shiraishi:** Resources. **Keiichi Ota:** Visualization, Resources. **Kentaro Tanaka:** Writing - review & editing, Visualization, Resources. **Yoichi Nakanishi:** Supervision. **Isamu Okamoto:** Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2021.03.018.

References

[1] P.M. de Groot, C.C. Wu, B.W. Carter, R.F. Munden, The epidemiology of lung cancer, Transl. Lung Cancer Res. 7 (3) (2018) 220–233.
[2] C. Gridelli, F. Casaluce, Frontline immunotherapy for NSCLC: alone or not alone? Nat. Rev. Clin. Oncol. 15 (10) (2018) 593–594.

[3] S.M. Gadgeel, M.C. Garassino, E. Esteban, et al., KEYNOTE-189: updated OS and progression after the next line of therapy (PFS2) with pembrolizumab (pembro) plus chemo with pemetrexed and platinum vs placebo plus chemo for metastatic nonsquamous NSCLC, J. Clin. Oncol. 37 (15_suppl) (2019), 9013–9013.
[4] L. Gandhi, M.C. Garassino, Pembrolizumab plus chemotherapy in lung cancer, N. Engl. J. Med. 379 (11) (2018) e18.
[5] M.A. Socinski, R.M. Jotte, F. Cappuzzo, et al., Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC, N. Engl. J. Med. 378 (24) (2018) 2288–2301.
[6] H. West, M. McCleod, M. Hussein, et al., Atezolizumab in combination with carboplatin plus nab-paclitaxel chemotherapy compared with chemotherapy alone as first-line treatment for metastatic non-squamous non-small-cell lung cancer (IMpower130): a multicentre, randomised, open-label, phase 3 trial, Lancet Oncol. 20 (7) (2019) 924–937.
[7] P. Martinez, S. Peters, T. Stammers, J.C. Soria, Immunotherapy for the first-line treatment of patients with metastatic non-small cell lung cancer, Clin. Cancer Res. 25 (9) (2019) 2691–2698.
[8] L. Galluzzi, A. Buque, O. Kepp, L. Zitvogel, G. Kroemer, Immunological effects of conventional chemotherapy and targeted anticancer agents, Cancer Cell 28 (6) (2015) 690–714.
[9] L. Galluzzi, I. Vitale, S.A. Aaronson, et al., Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018, Cell Death Differ. 25 (3) (2018) 486–541.
[10] M. Obeid, A. Tesniere, F. Ghiringhelli, et al., Calreticulin exposure dictates the immunogenicity of cancer cell death, Nat. Med. 13 (1) (2007) 54–61.
[11] S.J. Gardai, K.A. McPhillips, S.C. Frasch, et al., Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte, Cell 123 (2) (2005) 321–334.
[12] S.H. Huang, L.X. Zhao, C. Hong, et al., Self-oligomerization is essential for enhanced immunological activities of soluble recombinant calreticulin, PLoS One 8 (6) (2013), e64951.
[13] C. Hong, X. Qiu, Y. Li, et al., Functional analysis of recombinant calreticulin fragment 39-272: implications for immunobiological activities of calreticulin in health and disease, J. Immunol. 185 (8) (2010) 4561–4569.
[14] J.C. Soria, S.S. Ramalingam, Osimertinib in EGFR mutation-positive advanced NSCLC, N. Engl. J. Med. 378 (13) (2018) 1262–1263.
[15] M. Obeid, A. Tesniere, T. Panaretakis, et al., Ecto-calreticulin in immunogenic chemotherapy, Immunol. Rev. 220 (2007) 22–34.
[16] C. Clarke, M.J. Smyth, Calreticulin exposure increases cancer immunogenicity, Nat. Biotechnol. 25 (2) (2007) 192–193.
[17] E. Vacchelli, L. Galluzzi, W.H. Fridman, et al., Trial watch: chemotherapy with immunogenic cell death inducers, Oncoimmunology 1 (2) (2012) 179–188.
[18] I. Martins, A. Tesniere, O. Kepp, et al., Chemotherapy induces ATP release from tumor cells, Cell Cycle 8 (22) (2009) 3723–3728.
[19] O. Kepp, L. Galluzzi, I. Martins, et al., Molecular determinants of immunogenic cell death elicited by anticancer chemotherapy, Cancer Metastasis Rev. 30 (1) (2011) 61–69.
[20] L. Zitvogel, O. Kepp, L. Senovilla, L. Menger, N. Chaput, G. Kroemer, Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway, Clin. Cancer Res. 16 (12) (2010) 3100–3104.
[21] L. Zitvogel, O. Kepp, G. Kroemer, Immune parameters affecting the efficacy of chemotherapeutic regimens, Nat. Rev. Clin. Oncol. 8 (3) (2011) 151–160.
[22] L. Galluzzi, A. Buqué, O. Kepp, L. Zitvogel, G. Kroemer, Immunogenic cell death in cancer and infectious disease, Nat. Rev. Immunol. 17 (2) (2017) 97–111.
[23] L. Bezu, A. Sauvat, J. Humeau, et al., eIF2alpha phosphorylation is pathognomonic for immunogenic cell death, Cell Death Differ. 25 (8) (2018) 1375–1393.
[24] T. Panaretakis, O. Kepp, U. Brockmeier, et al., Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death, EMBO J. 28 (5) (2009) 578–590.
[25] R. Liu, J. Gong, J. Chen, et al., Calreticulin as a potential diagnostic biomarker for lung cancer, Cancer Immunol. Immunother. 61 (6) (2012) 855–864.
[26] G.R. Oxnard, J.C. Yang, H. Yu, et al., TATTON: a multi-arm, phase Ib trial of osimertinib combined with selumetinib, savolitinib, or durvalumab in EGFR-mutant lung cancer, Ann. Oncol. 31 (4) (2020) 507–516.
[27] Y. Hijikata, T. Okazaki, Y. Tanaka, et al., A phase I clinical trial of RNF43 peptide-related immune cell therapy combined with low-dose cyclophosphamide in patients with advanced solid tumors, PLoS One 13 (1) (2018), e0187878.
[28] C. Pfirschke, C. Engblom, S. Rickelt, et al., Immunogenic chemotherapy sensitizes tumors to checkpoint blockade therapy, Immunity 44 (2) (2016) 343–354.