Abstract. Non-small cell lung cancer (NSCLC) is one of the leading causes of death in all lung cancer patients due to its metastatic spread. Even though cisplatin treatment after surgical resection of the primary tumor has been established as a standard chemotherapy for residual disease including metastatic spread, NSCLC often acquires a resistance against chemotherapy, and metastatic disease is often observed. Amongst many potential mechanisms, epithelial-to-mesenchymal transition (EMT) has been considered as an important process in acquiring both metastatic spread and chemo-resistance of NSCLC. In this study, we identified MCL-1 as a critical molecule for chemo-resistance in A549 cells associated with TGF-β-induced EMT. Importantly, downregulation of MCL-1 by siRNA or inhibition of MCL-1 with pan-BCL2 inhibitor to inhibit MCL-1 was able to overcome the EMT-associated chemo-resistance in A549 cells. Collectively, MCL-1 can be a new therapeutic target for overcoming EMT-associated chemo-resistance in NSCLC patients in the context of post-operative chemotherapies.

Introduction

Non-small cell lung cancer (NSCLC) is one of the common causes of death in the world (1). Although cisplatin treatment after surgery is known as a standard and effective chemotherapy, metastatic spread in NSCLC patients often occurs (2-4). In addition, it is still controversial whether an adjuvant chemotherapy with cisplatin gives a significant survival advantage in stage I NSCLC patients compared with surgery alone (5-7). This may lead to the possibility that metastatic NSCLCs already acquire resistance to chemotherapy during tumor progression.

Cancer metastasis is one of the major causes of the high mortality rate in cancer patients and it consists of multiple biological steps, such as dissemination from primary tumor, intravasation, attachment to vessel of target tissue, extravasation, angiogenesis, and subsequent growth at the metastasis site (8). Some of these biological steps have been shown to be related to epithelial-to-mesenchymal transition (EMT) (9-12). Although EMT also has been shown to limit the sensitivities of cancer cells to chemotherapeutic drugs (13,14), the potential mechanism by which cancer cells acquire resistance to anticancer drugs associated with the EMT process is not well defined.

In this study, we demonstrated that EMT in human NSCLC cell line A549 induced by TGF-β treatment limits the sensitivities to various anticancer drugs, and further identified MCL-1 as a critical molecule of such EMT-associated chemo-resistance of A549 cells. Importantly, we showed that targeting MCL-1 by siRNA delivery or the pan-BCL2 inhibitor treatment could overcome the EMT-associated chemo-resistance in A549 cells.

Materials and methods

Reagents and plasmids. The reagents used were recombinant human TGF-β from Peprotech (London, UK), obatoclax from Selleck Chemicals (Houston, TX, USA), ABT-737 from AdooQ BioScience (Irvine, CA, USA), cisplatin, vinorelbine, gemcitabine and paclitaxel from Wako Pure Chemical Industries (Osaka, Japan). siRNAs against MCL-1 (L-004501-00, J-004501-16, and J-004501-17), Bcl2A1 (L-003306-00), and control (D-00181-02) siRNA were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The human MCL-1 cDNA was amplified from normal human cDNA and subcloned into pcDNA3.1-HA (from David E. Fisher, MGH, MA, USA).

Cell cultures. Human lung adenocarcinoma A549 cells were cultured in RPMI-1640 medium (Life Technologies Corp., Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 2 mM L-glutamine.
(Life Technologies Corp.), 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO₂ at 37°C. A549 cells stably expressing MCL-1 or vector control were established by transfecting pcDNA3.1-HA/MCL-1 or pcDNA3.1 under G418 (1 mg/ml). For siRNA transfection, each 25 nM of siRNAs was reverse-transfected using Lipofectamine™ RNAiMAX (Life Technologies Corp.) following the manufacturer's instructions, and the transfected cells were used for each experiment.

Cell viability assay. Cell viability was quantified using the cell proliferation reagent WST-1 (Dojindo, Japan) or CellTiter-Glo (Promega, Madison, WI, USA). A549 cells, siRNA-reverse transfected A549 cells, or stable MCL-1 expressing A549 cells were incubated for 24 h. The antitumor drugs were then added after pre-treatment with TGF-β for 48 h. After additional incubation for the indicated time, WST-1 solution or CellTiter-Glo reagent was added. Absorbance was measured at 450 nm using a Microplate reader for WST-1 assay and luminescence was measured using a GloMax Multi-detection system (Promega) for CellTiter-Glo assay. The cell viability was determined as percent viability compared with the vehicle control.

Western blot analysis. Whole cell lysates were prepared as described previously (15). The primary antibodies used were E-cadherin, N-cadherin, Snail, MCL-1, BCL2A1/Bfl-1, BCL-xL, PARP and caspase-3 (Cell Signaling Technology, Beverly, MA, USA), hemagglutinin (HA) (Roche, Indianapolis, IN, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All antibodies were used by 1:2000 dilution.

Real-time RT-PCR. Expression of MCL-1 and BCL2A1 mRNA was quantitatively determined by real-time PCR on an ABI PRISM 7300 Real Time PCR System (Life Technologies Corp.). Total RNAs were prepared using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Expression level of the targeted mRNAs was normalized to β-actin mRNA. The primers used were: 5'-TCG TAA GGA CAA AAC GGG AC-3' (sense) and 5'-CAT TCC TGA TGC CAC CTT CT-3' (antisense) for MCL-1 mRNA, 5'-CCC GGA TGT GGA TAC CTA TAA GGA GA-3' (sense) and 5'-GTC ATC CAG CCA GAT TTA GGT TCA-3' (antisense) for BCL2A1 mRNA, and 5'-GCA CAG AGC CTC GCC TT-3' (sense) and 5'-GTT GTC GAC GAC GAG CG-3' (antisense) for β-actin mRNA.

Apoptosis assay. Apoptotic cell number was determined using the MUSE Annexin V and Dead Cell kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, the stable cells were harvested after being treated with cisplatin and diluted with PBS containing 1% bovine serum albumin (BSA) as a dilution buffer to a concentration of 5x10⁶ cells/ml. Cell suspension (100 µl) was then added to 100 µl MUSE Annexin V and Dead Cell reagent (2x dilution), incubated for 20 min at room temperature, and analyzed using the MUSE Cell Analyzer. Total Annexin V-positive cells were determined as apoptotic cells.

Results

Acquired chemo-resistance in A549 cells associates with TGF-β-induced EMT. Although the relationship between epithelial-to-mesenchymal transition (EMT) and chemo-resistance has been implicated (14,16), the detailed molecular mechanism of such EMT-accompanying chemo-resistance has not been determined yet. Therefore, we first tested the various antitumor reagents, such as cisplatin, paclitaxel, gemcitabine, and vinorelbine, in A549 lung adenocarcinoma cell lines with or without inducing EMT by pre-treating with 5 ng/ml TGF-β (11,17). Consistent with our previous studies (17,18), A549 pre-treated with TGF-β showed EMT phenotype in both cell morphology and expression of protein markers such as E-cadherin reduction and N-cadherin induction (data not shown). In concert with EMT induction, A549 cells pre-treated with TGF-β showed significant resistance against all anticancer reagents tested (Fig. 1A and Table I), which was associated with the reduction of apoptosis marker expression (Fig. 1B). These findings indicate that A549 cells acquired a wide spectrum of chemo-resistance, possibly through apoptosis inhibition associated with EMT induced by TGF-β.
Critical role of MCL-1 in A549 chemo-resistance associated with TGF-β-induced EMT. We next investigated the expression levels of BCL2 family members in EMT-induced A549 cells. The members are related to chemo-resistance in various cancers by inhibiting apoptosis (15,19,20). Amongst many of BCL2 family members, the expression of MCL-1 and BCL2A1 were specifically increased associated with TGF-β-induced EMT in A549 cells in a time-dependent manner (Fig. 2A). We further confirmed the increased mRNA expression of MCL-1 and BCL2A1 after TGF-β treatments (Fig. 2B) consistent with their protein expression.

In order to further investigate whether either MCL-1 or BCL2A1 is required for EMT-associated chemo-resistance, we employed gene knock-down of MCL-1 or BCL2A1 by using siRNA pools in which four different siRNAs are contained. MCL-1 knock-down rescued the sensitivity to cisplatin treatment in EMT-induced A549 cells contrary to BCL2A1 knock-down which did not show any significant effect (Fig. 3A). Similar results were also confirmed in additional experiments using two siRNAs against MCL-1 with different target sequences (Fig. 3B). Furthermore, MCL-1 overexpression was able to significantly suppress the cisplatin-induced apoptosis in non-EMT-induced parental A549 cells (Fig. 3C). Collectively, these results strongly support the critical contribution of MCL-1 in acquiring EMT-associated chemo-resistance in A549 cells.

MCL-1 inhibition by pan-BCL2 inhibitor treatment overcomes TGF-β-induced chemo-resistance. To test the clinical application of our findings, we examined the effects of pan-BCL2 inhibitors in EMT-associated chemo-resistance in A549 cells. As shown in Fig. 4A, obatoclax, which has a broad range of inhibition in BCL2 family members including MCL-1, re-sensitized EMT-induced A549 cells to cisplatin toxicity. Of note, the treatment with obatoclax (100 nM) showed only minor effect on TGF-β-treated A549 cell viability (Fig. 4A). In accordance with re-sensitizing to cisplatin toxicity, we also detected the cleavage of both caspase-3 and PARP in the combination of obatoclax with cisplatin in EMT-induced A549 cells by TGF-β (Fig. 4B). Collectively, these results implicate a clinical advantage for targeting MCL-1 in EMT-associated cisplatin-resistance in A549 cells.

**Discussion**

In this study, we demonstrated that human NSCLC A549 acquired chemo-resistance upon TGF-β-induced EMT and such EMT-associated chemo-resistance was mediated through MCL-1-dependent anti-apoptotic pathway. By treating with pan-BCL2 inhibitor, obatoclax, EMT-associated chemo-resistance in A549 cells can be reversed, therefore we propose that pharmacological inhibition of MCL-1 could be an attractive target to overcome EMT-associated chemo-resistance and further inhibit metastasis spread in NSCLC patients.

Although MCL-1 was a key molecule in EMT-associated chemo-resistance in this study (Fig. 3), other BCL2 family members are known to contribute for chemo-resistance in general. For example, enhanced BCL-2 expression is involved in nicotine- or matrilysin-induced cisplatin-resistance in lung cancer cells (21,22) and BCL2A1 confers resistance to BRAF inhibitors in melanoma (15). Considering other EMT inducers

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Table I. IC_{50} of various anticancer drugs in TGF-β-induced chemo-resistance.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (mean ± SD)</th>
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<tr>
<td>Cisplatin (µM)</td>
<td>7.6±0.8</td>
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<tr>
<td>Paclitaxel (nM)</td>
<td>11.1±1.1</td>
</tr>
<tr>
<td>Gencitabine (µM)</td>
<td>0.75±0.2</td>
</tr>
<tr>
<td>Vinorelbine (nM)</td>
<td>15.8±3.0</td>
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aInhibitory concentration at 50% (IC_{50}) was determined by WST-1 assay at 48 h. The values were calculated with data from at least three independent experiments. b\(p<0.05\) compared with A549 cells.
in tumor microenvironment, EGF or HGF, have been reported to induce both EMT and MCL-1 expression (20,23-26), MCL-1 induction might be a common mechanism for EMT-associated chemo-resistance. Although we do not show any direct connection between TGF-β and MCL-1, there are several reports that the EMT-related transcription factors, ZEB1 or Twist1, can regulate MCL-1 expression (27,28). In this context, we observed the induction of ZEB1 expression in A549 cells after TGF-β treatment (data not shown). Collectively, these observations suggest that ZEB1-mediated transcriptional control can be involved in EMT-associated chemo-resistance by regulating MCL-1 expression. Nevertheless, our current results implicate that mesenchymal-transitioned NSCLC could acquire the chemo-resistance through the induction of MCL-1. Consistent
with our findings, it is reported that EMT can be observed in the tumor specimens resected from NSCLC patients after chemo-radiotherapy (14) to acquire chemo-resistance (14,16); therefore, these lines of evidence support a clinical relevance of our presented findings. Importantly, we have demonstrated the importance of pharmacological targeting of MCL-1 to re-sensitize cisplatin treatment in A549 cells. In addition to its importance in EMT-associated chemo-resistance shown in this study, MCL-1 is involved in anoikis-resistance in NSCLCs, which can be critical for the survival of tumor cells during the metastatic process (29).

In conclusion, we newly identified MCL-1 as a key molecule for acquiring EMT-associated chemo-resistance in human NSCLC. Considering EMT-associated MCL-1 induction might play critical roles not only in chemo-resistance, but also metastatic spread and survival in distant tissue, pharmacological targeting of MCL-1 provides a new therapeutic opportunity in NSCLC particularly for combining with post-operative chemotherapies.

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