Cell Surface Tetraspanin CD9 Mediates Chemoresistance in Small Cell Lung Cancer

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Abstract

Small cell lung cancer (SCLC) is an aggressive malignancy with extremely high mortality due to the appearance of widespread metastases early in its clinical course and rapid acquisition of chemoresistance after initial therapy. A theory of cell adhesion–mediated drug resistance is thought to be a principal mechanism in which extracellular matrix proteins provide a survival advantage against cytotoxic drug-induced apoptosis. We found that the tetraspanin family member CD9 was expressed preferentially in SCLC tumors and metastases from three of seven relapsed patients, whereas chemonaïve primary tumors from 16 patients were CD9 negative with only one exception. Additionally, CD9 was highly expressed on SCLC cell lines rendered resistant to cisplatin or etoposide, and was upregulated in parental chemosensitive cells within 48 hours after exposure to either of these compounds. CD9-expressing chemoresistant SCLC cells adhered more tightly to fibronectin via β1 integrin, but they were less motile than the respective chemosensitive parental lines. Notably, treatment of the chemoresistant cells with chemokine CXCL12 downregulated CD9 and transiently restored motility. Moreover, selective targeting of CD9 by treatment with specific monoclonal antibody ALB6 or a small interfering RNA triggered apoptosis in the chemoresistant cells. Taken together, our findings implicate CD9 in the cell adhesion–mediated drug resistance mechanism, highlighting CD9 as an attractive therapeutic target to improve therapeutic outcomes in SCLC. *Cancer Res; 70(20); 8025–35. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Approximately 15% of all histologic types consist of small cell lung cancer (SCLC) that ultimately bears poor outcome. The extreme aggressiveness of SCLC is due to its unique biological characteristic; namely it metastasizes microscopically to systemic organs even in the early stages and often recurs with a multidrug resistance (MDR) phenotype shortly after dramatic response to initial treatments, which is achieved in approximately 80% of cases (1, 2). It is noticeable for several molecular target drugs including gefitinib, erlotinib, cetuximab, and bevacizumab to have shown survival benefit in non-SCLC (3–7). However, no favorable therapeutic strategy has been established in recurrent SCLC to date. Development of novel drugs that overcome MDR and bring about significant survival benefit is urgently desired in SCLC.

Two well-characterized drug efflux pumps, P-glycoprotein and MDR-related protein, have been thought to be crucial for MDR of SCLC, but the importance of this mechanism is still controversial because ectopic overexpression of these pumps could induce partial resistance compared with the level achieved by long-term drug selection (8, 9). Clinical studies also revealed that expression of these pumps was not common and did not necessarily correlate with outcome in SCLC (10, 11). Therefore, these molecules cannot completely account for MDR of SCLC and substitutable mechanisms must be involved in the process.

SCLC cells exist in an extracellular matrix (ECM)–rich environment *in vivo,* and an interaction between the tumor and the host microenvironment is central for their survival and proliferation. Sethi and colleagues proposed the concept of cell adhesion–mediated drug resistance (CAM-DR) as a principal mechanism for SCLC cells to acquire the MDR phenotype. They showed that β1 integrin–mediated adhesion of SCLC cells to ECM protects them from chemotherapy-induced apoptosis through activation of several intracellular proteins (12, 13). Kraus and colleagues showed that chemoresistance of SCLC correlates with adhesion to ECM and constitutive activation of Akt and mitogen-activated protein kinase pathways (14). We also reported that adhesion

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of SCLC cells to fibronectin enhanced their viability and cytoskeletal function mainly by activating phosphatidylinositol-3-kinase and p125 focal adhesion kinase (15). Tsurutani and colleagues showed that laminin-mediated activation of the phosphatidylinositol-3-kinase/Akt pathway also enabled SCLC cells to escape from imatinib-induced apoptosis (16). Furthermore, Hodkinson and colleagues elucidated that interaction with ECM prevented SCLC cells from etoposide-induced G_2-M cell cycle arrest by overriding the upregulation of $p21^{Cip1/WAF1}$ and $p27^{Kip1}$, and the downregulation of cyclins E, A, and B (17). The importance of the CAM-DR mechanism has been also reported in various malignancies other than SCLC (18–22).

Tetraspanin is the generic term for the glycoprotein family containing four transmembrane domains. Members of this family form multimeric complexes with one another and other cell surface proteins including integrins, leukocyte antigens, and signaling molecules at specialized tetraspanin-enriched microdomains. As organizer, stabilizer, and facilitator of these molecular networks termed the "tetraspanin web," tetraspanins regulate cellular morphology, motility, fusion, and intracellular signals (23, 24). Among more than 30 members in mammals, CD9 was identified as a molecule that suppresses cellular motility and metastatic potential in a human lung adenocarcinoma cell line (25, 26). Clinicopathologic findings indicated that CD9 is a predictor for better prognosis in adenocarcinoma of the lung (27, 28). However, the biological relevance of CD9 in SCLC has not been well understood thus far. We reported that CD9 was absent in chemonaïve tumors at the primary site in SCLC patients as well as in the majority of SCLC cell lines, and that ectopic overexpression of CD9 suppressed their motility (29). This evidence prompted us to investigate whether CD9 was involved in the advance of the disease and the CAM-DR mechanism and whether CD9 could then be a therapeutic target to overcome MDR in SCLC.

Materials and Methods

Cell lines and cell culture

SCLC cell lines SBC-3 (30), SBC-3/CDDP (31), and SBC-3/ETP (32) were kindly provided by Dr. K. Kiura, Okayama University, Okayama, Japan. NCI-H69 (H69) was purchased from American Type Culture Collection. H69/CDDP (33) and H69/VP (34) were obtained from National Cancer Center, Tokyo, Japan. H69/CD9 and SBC-3/CD9, which stably overexpress ectopic CD9, were established as described previously (29). All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL).

Antibodies

Monoclonal antibodies (mAb) against CD9, clones 72F6 (Novocastra Laboratories), MM2/57 (Biosource International), and ALB6 (Immunotech), were used as indicated in each experiment. The mAbs for β1 integrin (4B4), β-actin (C4), and poly(ADP)-ribose polymerase (PARP; 46D11) were purchased from Beckman Coulter, MP Biomedicals, and Cell Signaling

Technology, respectively. Rabbit polyclonal antibodies against phosphorylated or regular extracellular signal–regulated kinase 1/2 (ERK1/2), c-*Jun* NH$_2$-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 mitogen-activated protein kinase, and Akt were available from Cell Signaling Technology. Normal mouse IgG$_1$ (Thermo Scientific) was used as a control in several experiments.

Reagents

Nippon Kayaku Co. (Tokyo, Japan) supplied us with cisplatin (CDDP) and etoposide (VP-16). SN-38, the active form of irinotecan, and amrubicin were provided by Yakult Co. (Tokyo, Japan) and Dainippon Sumitomo Pharma Co. (Osaka, Japan), respectively. Recombinant human CXCL12 was purchased from R&D Systems.

Immunohistochemistry

Immunoperoxidase procedures were carried out as described elsewhere (29). After antigen retrieval, inactivation of endogenous peroxidase, and blockade of any nonspecific reaction, the sections were subjected to immunoreaction with primary anti-CD9 mAb 72F6 (diluted 1:400) overnight at 4°C and subsequently incubated with goat anti-mouse IgG biotinylated secondary antibody (diluted 1:300; Dako) for 10 minutes at room temperature and then labeled with streptavidin-peroxidase complex. They were developed and counterstained with Mayer's hematoxylin.

Flow cytometry

Cells (1×10^4) were incubated with 10 μg of primary mAb or normal mouse IgG$_1$ for 30 minutes at 4°C followed by labeling with FITC-conjugated goat anti-mouse IgG (Biosource). Stained cells were analyzed by FACScan (Becton Dickinson).

Reverse transcription-PCR

Total RNA was isolated from cells by the Isogen method (Nippon Gene), 1 μg of which was reverse-transcribed using the cDNA Synthesis Kit (Invitrogen). Generated cDNAs were amplified with the following forward and reverse primer sets: *CD9* (5′-TGCATCTGTATCCAGCGCCA-3′ and 5′-CTCAGG-GATGTAAGCTGACT-3′) and *β-actin* (5′-TGAACCCTAAGGC-CAACCGTG-3′ and 5′-GCTCA TAGCTCTTCTCCAGGG-3′). Using TaKaRa Ex Taq Hot Start Version (Takara Bio), the reaction mixtures were subjected to 30 amplification cycles with 30 seconds of denaturing at 94°C, 30 seconds of annealing at 60°C (CD9) and 61°C (β-actin), and a 60-second extension at 72°C. These primer pairs for *CD9* and *β-actin* amplified 799 bp and 397 bp fragments, respectively. Amplicons were electrophoresed on a 1% agarose gel and visualized with ethidium bromide.

Immunoblotting

Whole cell lysates were separated in a 10% to 20% gradient gel (Wako) by SDS-PAGE under nonreducing conditions, transferred to Immobilon-P membrane (Millipore) as described previously (35). Proteins were immunoblotted with proper primary antibodies (diluted 1:500–1,000) followed by

appropriate horseradish peroxidase–conjugated secondary antibodies (diluted 1:1,000, donkey anti-rabbit or sheep anti-mouse IgG; Amersham) for 1 hour at room temperature. Immunoreactive bands were visualized using a chemiluminescent technique with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Regulation of CD9 expression

Cells (1×10^6) were treated with CDDP or VP-16 at various concentrations ($\approx IC_{50}$ and $\approx 2 \times IC_{50}$ for respective parental clone) for 48 hours (Fig. 1D) or incubated in the presence (100 ng/mL) or absence of CXCL12 for 6 hours (Fig. 4B) in serum-containing (10% fetal bovine serum)
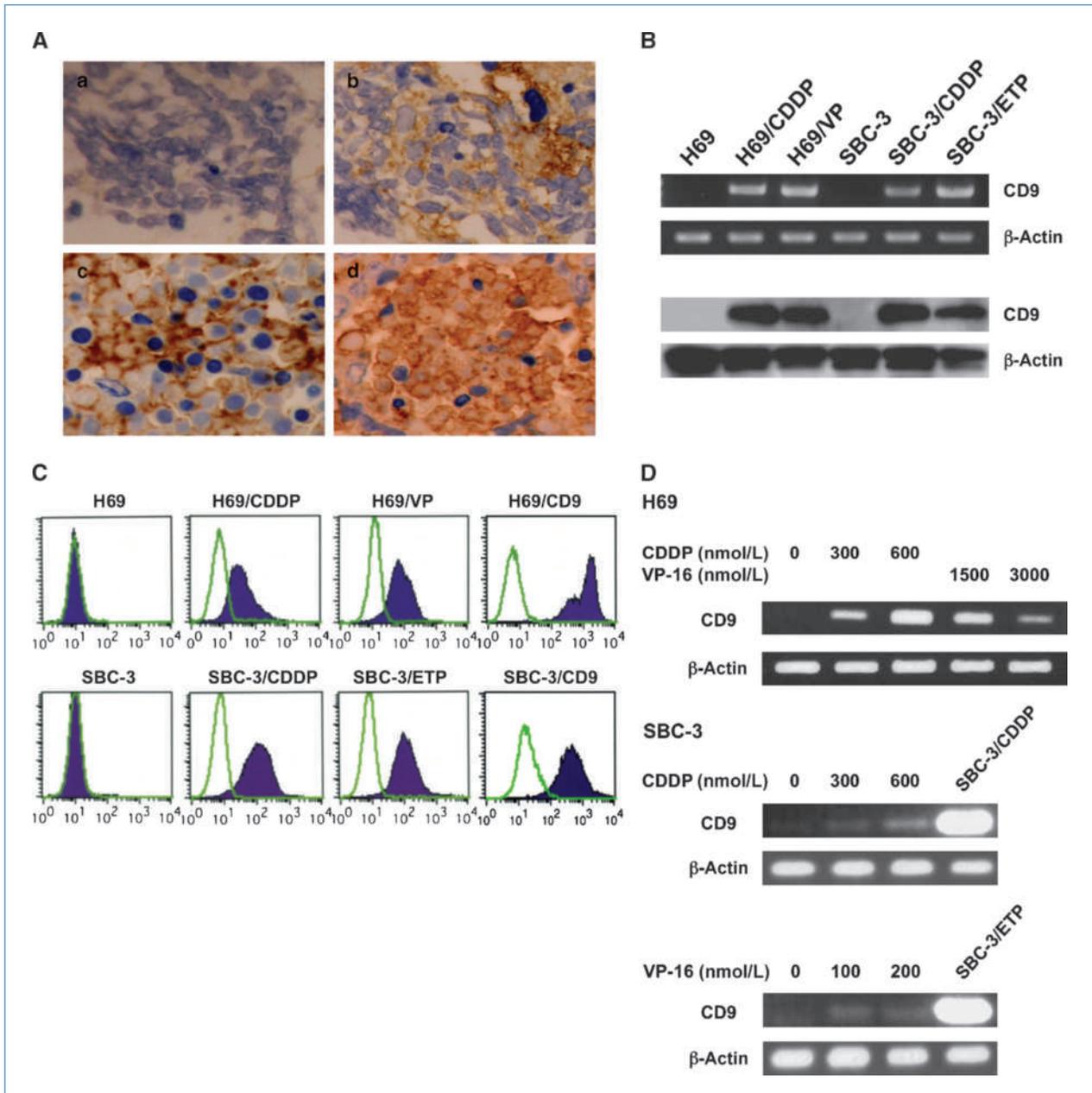
Figure 1. CD9 is expressed in recurred and metastasized tissues and upregulated by exposure to cytotoxic drugs in SCLC. A, tissues stained with mAb 72F6 from a representative SCLC patient. No immunoreactive CD9 is detectable in pretreated specimens from the primary site (a). In pathologically dissected tissues, CD9 is positive in the relapsed primary tumor (b), metastasized mediastinal lymph nodes (c), and the liver (d). B, CD9 is upregulated exclusively in chemoresistant cells at transcriptional (top) and protein (bottom) levels. C, FACS analysis confirms that CD9 is properly expressed on the cell surface of chemoresistant clones and CD9 transfectants. D, exposure of H69 and SBC-3 to various concentrations ($\approx IC_{50}$ and $\approx 2 \times IC_{50}$) of CDDP or VP-16 for 48 h induces transcriptional upregulation of *CD9*.

medium. Total RNAs were isolated and subjected to reverse transcription-PCR.

Drug sensitivity assays

Cells (5×10^3/well) were plated onto 96-well tissue culture–treated plates (Sumitomo Bakelite) and treated with serially diluted cytotoxic compounds in serum-containing medium for 120 hours. The relative number of viable cells was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (15, 36).

Adhesion assay

Cellular adhesive potential was evaluated as mentioned in our previous reports (15, 29, 36). Briefly, the wells of a 96-well plate (Linbro) were precoated with 10 μg/mL of human plasma fibronectin (Sigma-Aldrich) overnight at 4°C. After blocking nonspecific binding sites with RPMI 1640 containing 0.1% (w/v) bovine serum albumin, cells (5×10^4/well) were allowed to adhere with 1 μg/mL of 4B4 or control IgG$_1$ for 6 hours (H69 series) or 1 hour (SBC-3 series) at 37°C. The relative number of attached cells was determined by MTT assay.

Cell motility analysis by timelapse video microscopy

Cells (2×10^5) plated in fibronectin-coated 35-mm dishes (Corning) in serum-containing medium were untreated or treated with 100 ng/mL of CXCL12. After 6 hours of preincubation to urge cells to adhere, dishes were placed into a temperature- and humidity-controlled chamber (IX-IBM, Olympus). Cells were recorded for another 8 hours by timelapse video microscopy (TLVM) using an inverted microscope (IX70, Olympus) equipped with a charge-coupled device camera (Cool-Snap HQ cooled 12-bit; Roper Scientific). Digital video images were saved every 5 minutes and cellular movement was analyzed with MetaMorph software (Universal Imaging, Co.) and then plotted to show the trace of movement. The mean distance that a cell traversed for each 5-minute period was also calculated.

ALB6 treatment

Cells (1×10^4/well) were placed into 96-well plates (Sumitomo Bakelite) and then cultured either untreated or treated with 25 μg/mL of antibodies (ALB6 or IgG$_1$) in serum-containing medium. After 72 hours of treatment, cells were subjected to cell death detection assay to determine the biological toxicity of antibodies. The values of nucleosome enrichment that represent the increase of apoptosis were measured using Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics). In parallel, a 100 times larger scale (1×10^6 cells) treatment with antibodies or cytotoxic drugs was also carried out for immunoblotting.

Small interfering RNA treatment

Cells were transfected with either 40 nmol/L of small interfering RNA (siRNA) against human *CD9* (SHF27A-0631) or control cocktail RNAs (S30C-0126; B-Bridge International) using LipofectAMINE RNAiMAX (Life Technologies). After 48 hours of incubation, cells were replated in serum-containing medium (1×10^4/well of a 96-well plate for

MTT assay or 1×10^6/60-mm dish for immunoblotting and FACS) then cultured for another 72 hours.

Statistical analysis

All the studies for statistical evaluation were performed in triplicate in each experiment and repeated at least three times. Mean ± SD values were calculated and differences were evaluated by two-sided Welch's *t* test using Statcel-12 software (OMS). $P < 0.05$ values were considered statistically significant.

Results

CD9 is preferentially expressed in recurred and metastasized SCLC tissues

To address CD9 expression in recurred and metastasized SCLC tissues, immunohistochemistry was carried out in matched pre/posttreatment samples from seven patients who had undergone pathologic dissection. None of the pretreated primary tumors from these subjects showed immunoreactive CD9. Additional pretreatment samples from nine other patients were also negative for CD9 (with only one exception). However, relapsed primary tumor, mediastinal lymph nodes, and metastasized tumors in the liver were all positive for CD9 staining in two patients, tissues from one of whom are shown in Fig. 1A, and another patient also had CD9-positive tumors in the liver. These findings suggested that CD9 is involved in the disease progression and spread of SCLC.

CD9 is upregulated by exposure to cytotoxic drugs in SCLC cells

We next investigated CD9 expression in chemoresistant SCLC clones established by long-term exposure to cytotoxic agents. CD9 was upregulated at the transcriptional level (Fig. 1B) and abundantly expressed on the cell surface (Fig. 1C) in all resistant clones, but not in parental cells, even without chemotherapeutic drugs. To clarify if cytotoxic agents induce CD9 expression in chemosensitive SCLC cells, transcripts for CD9 were analyzed by reverse transcription-PCR after 48 hours of exposure to CDDP or VP-16 at approximately equal and double the concentrations of IC$_{50}$. CD9 was upregulated in both H69 and SBC-3 cells (Fig. 1D) but this effect was transient and abrogated by drug withdrawal (data not shown), in which the precise mechanism is unknown. These results suggested that chemotherapy upregulates CD9 in primarily sensitive SCLC cells rather than clonally selecting intrinsically resistant CD9-positive cells during treatment.

CD9 is involved in but not essential for acquisition of chemoresistance in SCLC cells

As SCLC often recurs with the MDR phenotype shortly after successful initial treatment, we carried out an MTT drug sensitivity assay. Proliferation rates were not significantly different among all clones including CD9 transfectants in both H69 and SBC-3 series (data not shown). H69/VP and SBC-3/ETP lost sensitivity to amrubicin compared with parental lines with IC$_{50}$ (nmol/L) values of 286 ± 65.1 (versus 42.3 ± 5.50 for H69, $P < 0.03$) and 310 ± 35.1 (versus 18.7 ± 12.6 for SBC-3, $P < 0.01$), respectively. SBC-3/CDDP showed

cross-resistance towards all of VP-16 (IC$_{50}$: 2,333 ± 416 versus 107 ± 11.6 for SBC-3, $P < 0.01$), amrubicin (IC$_{50}$: 70.7 ± 13.0 versus 18.7 ± 12.6 for SBC-3, $P < 0.01$), and SN-38 (IC$_{50}$: 23.3 ± 5.80 versus 1.13 ± 0.71 for SBC-3, $P < 0.03$). On the other hand, CD9 transfectants did not become resistant to any drug (Fig. 2). These data suggested that CD9 upregulation by chemotherapy might not be essential for the acquisition of drug resistance but could be a result of cellular adaptation.

CD9 enhanced β1 integrin–mediated adhesion to fibronectin in SCLC cells

Integrins are essential cell surface receptors for ECM from which intercellular survival signals go downstream. Because almost all SCLC cell lines express β1 integrin subunit (37), and because fibronectin is one of the major ECM components and common substrates for integrins, adhesion assays were performed to evaluate adhesive potential of SCLC clones onto this ECM (Fig. 3). H69 cells usually grow as floating aggregates and a majority of them still kept floating even in the presence of fibronectin with only 8.15 ± 3.25% attached cells. In contrast, approximately half of both H69/CDDP (48.2 ± 2.39%, $P < 0.01$) and H69/VP (50.0 ± 4.69%, $P < 0.01$) adhered. Moreover, SBC-3 cells which usually grow in an anchorage-dependent fashion significantly increased adhesive faculty when they became resistant to CDDP and VP-16 with attachment ratios ranging from 55.0 ± 9.16% to 83.6 ± 3.26% ($P < 0.01$) and 86.6 ± 4.47% ($P < 0.01$), respectively. Treatment with function-blocking anti-β1 integrin mAb (4B4)

completely canceled this phenomenon in H69/CDDP (7.25 ± 3.23%, $P < 0.01$) and H69/VP (7.47 ± 4.19%, $P < 0.01$) to the level of H69 (8.64 ± 1.35%) and also markedly decreased the adhesive potential in all SBC-3/CDDP (28.7 ± 11.2%, $P < 0.01$), SBC-3/ETP (33.0 ± 14.0%, $P < 0.03$), and SBC-3 (12.4 ± 9.08%, $P < 0.01$). Ectopic overexpression of CD9 extremely enhanced adhesion in both H69/CD9 (85.8 ± 2.75%, $P < 0.01$) and SBC-3/CD9 (78.6 ± 5.08%, $P < 0.01$), which was abrogated by 4B4 (7.63 ± 0.70%, $P < 0.01$ and 25.4 ± 10.7%, $P < 0.01$, respectively). These results showed that CD9 is one of the key molecules in β1 integrin–mediated cell adhesion.

CD9 and CXCL12 participate in the CAM-DR mechanism regulating cell motility in SCLC

The tighter the cells attach to ECM, the less motile they become in general. We compared the cellular motility of SBC-3/CDDP, SBC-3/ETP, and SBC-3/CD9 with SBC-3 on fibronectin using TLVM. Movement distances (μm/5 min) of 10 randomly selected cells are shown in Fig. 4A. Not only SBC-3/CDDP (0.65 ± 0.28, $P < 0.005$) and SBC-3/ETP (0.98 ± 0.47, $P < 0.03$) but also SBC-3/CD9 (0.63 ± 0.41, $P < 0.005$) were significantly less motile than SBC-3 (2.12 ± 1.23). We previously reported that CXCR4, the unique receptor for a chemokine CXCL12, was commonly expressed in SCLC cell lines and CXCL12 enhanced their motility (36). As all the clones used here express similar amounts of CXCR4 (data not shown), we tested if CXCL12 could restore the motility of these resistant clones. Compared with the unstimulated

Figure 2. CD9 is involved but is not essential for acquired chemoresistance in SCLC cells. Cells of H69 series (top) and SBC-3 series (bottom) were treated with serially diluted drugs for 120 h. Relative number of viable cells was quantified by MTT assay. Columns, the mean IC$_{50}$ (nmol/L) values for parental (white), cisplatin-resistant (striped), and etoposide-resistant (black) clones or CD9 transfectants (gray) from three independent experiments; bars, SD. *, $P < 0.01$ and **, $P < 0.03$ versus respective parental clone AMR, amrubicin.
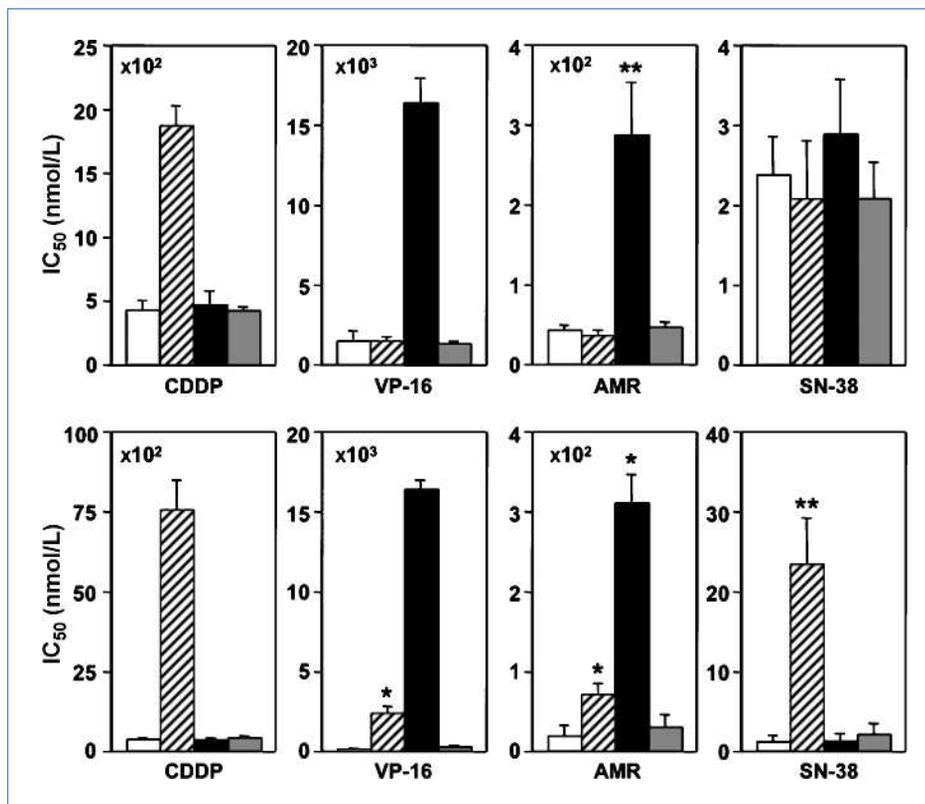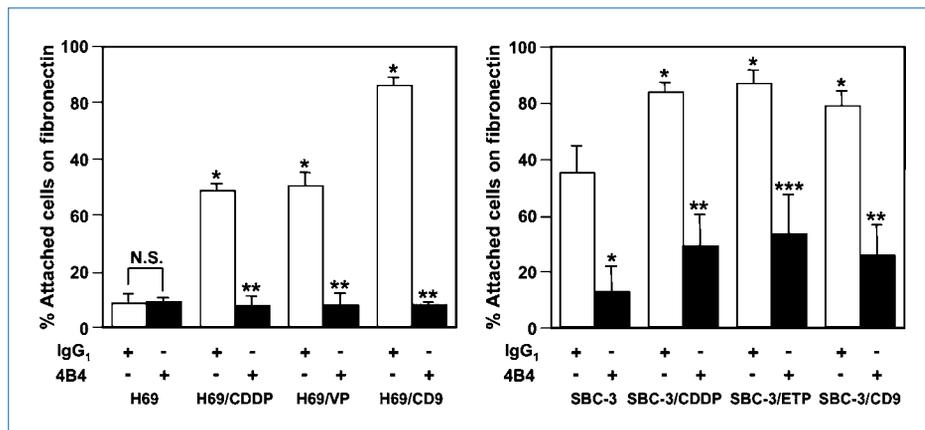
Figure 3. CD9 enhances β1 integrin–mediated adhesion to fibronectin in SCLC cells. Cells were allowed to adhere to the fibronectin-coated surface for 6 h (H69 series) or 1 h (SBC-3 series) in the presence of 1 μg/mL of control IgG$_1$ (white) or 4B4 (black). The relative number of attached cells was determined by MTT assay. Columns, mean relative ratios to unwashed well; bars, SD. *, $P < 0.01$ versus IgG$_1$-treated parental clone; **, $P < 0.01$ versus IgG$_1$-treated resistant clone; and ***, $P < 0.03$ versus IgG$_1$-treated SBC-3/ETP.

state, movement of SBC-3/CDDP (1.95 ± 1.23, $P < 0.01$) and SBC-3/ETP (2.18 ± 1.01, $P < 0.005$) significantly increased to the level of SBC-3 in response to CXCL12. On the other hand, SBC-3 (3.35 ± 1.48, $P = 0.059$) and SBC-3/CD9 (0.83 ± 0.37, $P = 0.26$) did not respond to CXCL12, probably due to extremely low or high expression of CD9, respectively. Of interest, transcripts for CD9 were markedly reduced by CXCL12 within 6 hours in SBC-3/CDDP and SBC-3/ETP with movement recovery, but were marginal in SBC-3/CD9 (Fig. 4B). This effect did not last so long and CD9 transcriptional levels returned to baseline after 24 hours (data not shown). We also examined if CXCL12 treatment could affect the chemosensitivity of these cells. SBC-3/CDDP and SBC-3/ETP cells were not resensitized even in the presence of CXCL12, probably because its effect was transient (Fig. 4C).

These results could strongly support the CAM-DR theory that motility of cancer cells increases up to a peak then goes down again to the baseline, which forms a bell-shaped curve, along with continuously increasing cell-ECM adhesion and chemoresistance (38, 39). Upregulation of endogenous CD9 probably plays some important roles in CAM-DR mechanism and exogenous CXCL12 may transiently, but dynamically, regulate cellular motility by controlling CD9 expression in SCLC (Fig. 4D).

Selective inhibition of CD9 induced the apoptosis of chemoresistant SCLC cells

Because ALB6, a mAb against CD9, was reported to induce apoptosis of CD9-expressing malignant cells (40), we also examined if selective targeting of CD9 by ALB6 or siRNA could induce the apoptosis of chemoresistant SCLC cells. ALB6, but not control IgG$_1$, led to apoptotic cell death exclusively in CD9-expressing cells coupled with cleavage of PARP. When the extent of apoptosis was evaluated using an increase of nucleosome enrichment index by cell death detection assay, values for ALB6 and control IgG$_1$ were 0.50 ± 0.32 versus 0.02 ± 0.04 ($P < 0.05$) in H69/CDDP, 0.52 ± 0.16 versus 0.00 ± 0.05 ($P < 0.001$) in H69/VP and −0.09 ± 0.18 versus −0.02 ± 0.02 in H69 (N.S.), 0.79 ± 0.15 versus 0.04 ± 0.09 ($P < 0.005$) in SBC-3/CDDP, 0.71 ± 0.27 versus 0.02 ± 0.04 ($P < 0.03$) in SBC-3/ETP, and −0.02 ± 0.19 versus 0.03 ± 0.08 (N.S.) in SBC-3, respectively

(Fig. 5A). In terms of intracellular signaling, ALB6 induced the phosphorylation of JNK/SAPK and p38 but not Akt and ERK1/2 in SBC-3/CDDP cells within 10 minutes but the effect was transient and disappeared by 30 minutes, which was consistent with the previous report (ref. 40; Fig. 5B).

We next examined the effect of siRNA-induced CD9 depletion on survival of chemoresistant clones. Immunoblot and FACS analyses confirmed that CD9 was successfully ejected from the cell surface at least from day 2 to day 5 (Fig. 6A). Similar to ALB6, siRNA treatment also induced apoptosis exclusively in chemoresistant clones in parallel with cleavage of PARP. When evaluated by MTT assay, the percentage of viable cells treated with targeted versus scramble RNAs were 21.1 ± 9.3 versus 92.2 ± 6.7 ($P < 0.0001$) in H69/CDDP, 42.5 ± 1.9 versus 90.8 ± 4.3 ($P < 0.0001$) in H69/VP, and 91.5 ± 3.7 versus 92.7 ± 5.3 in H69 (not significant), 12.5 ± 3.2 versus 94.9 ± 3.0 ($P < 0.0001$) in SBC-3/CDDP, 15.4 ± 3.0 versus 94.7 ± 9.9 ($P < 0.0001$) in SBC-3/ETP, and 93.5 ± 6.8 versus 94.8 ± 5.6 (not significant) in SBC-3, respectively (Fig. 6B). These findings showed the possibility of using CD9 as a therapeutic target to overcome the drug resistance of SCLC.

Discussion

CD9 is a tetraspanin that is well characterized by its close association with β1 integrin and its ability to reinforce its diverse biological actions on cell adhesion, migration, motility, and survival via interaction with ECM (23, 24). We found that CD9 was preferentially expressed on SCLC cells at relapsed primary tumors and metastasized organs in patients who had received prior chemotherapy. Therefore, we hypothesized that CD9 upregulated by chemotherapy could participate in the progression and spread of SCLC.

An immunohistochemical study revealed high levels of immunoreactive ECM proteins in SCLC tissues. In tumor foci, collagen IV and fibronectin are deposited in adjacent host connective tissue; moreover, fibronectin and laminin are also visible in SCLC cells, suggesting that they might directly synthesize some components of the local ECM (12, 41). The production of fibronectin was also observed in all SCLC cell lines used in our experiments (data not shown). Thus, SCLC cells
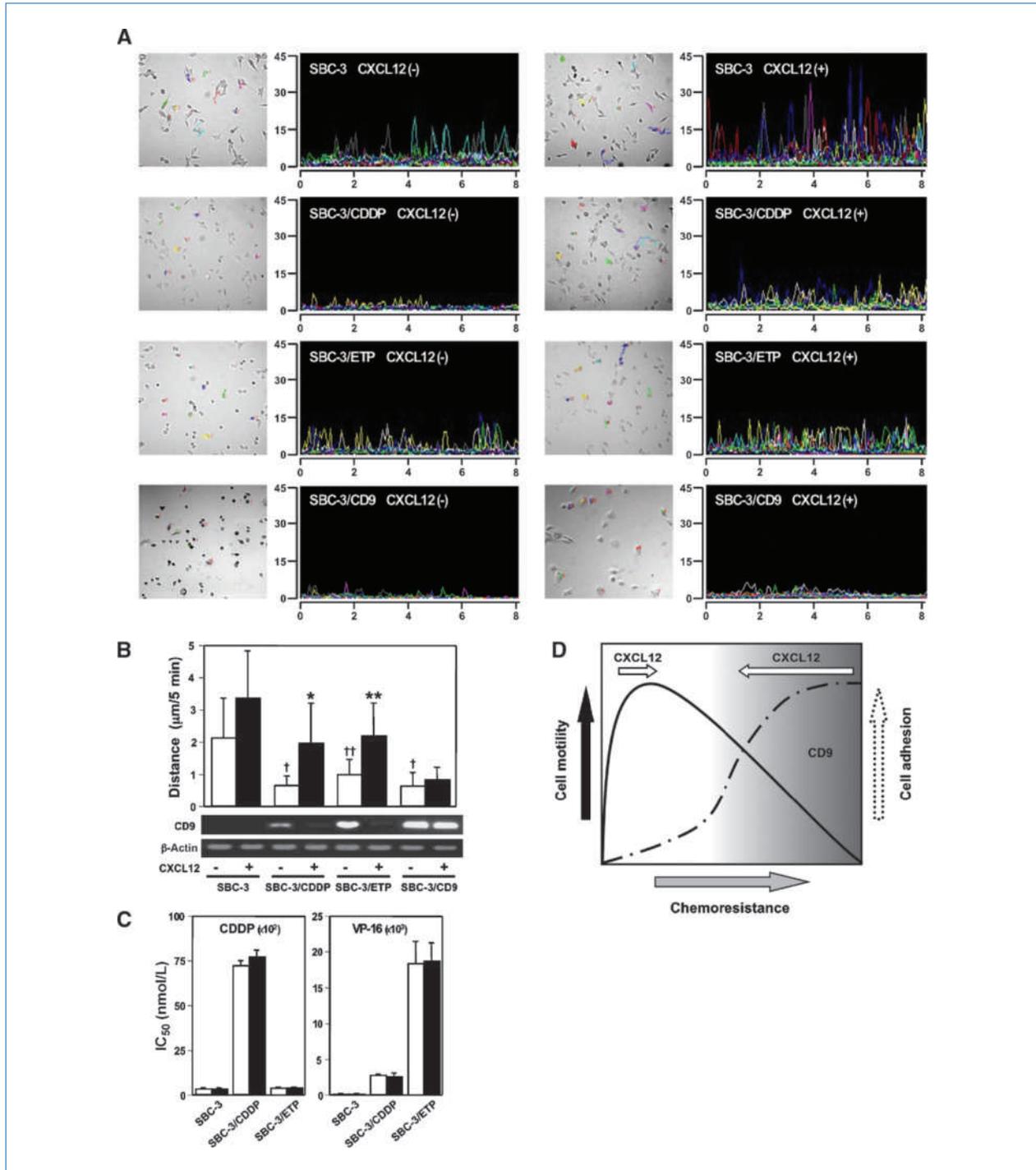
Figure 4. CD9 and CXCL12 participate in the CAM-DR mechanism regulating cell motility in SCLC. A, cells plated on fibronectin were untreated or treated with 100 ng/mL of CXCL12. After 6 h of preincubation, cells were recorded every 5 min for another 8 h by TLVM. Traces from 10 randomly selected cells are shown at ×80 magnification. Cellular movement is also presented in graphs with variables of time (h) as the X-axis and the distance that a cell traversed every 5 min (μm/5 min) as the Y-axis. B (top), columns, mean distances with (black) or without (white) CXCL12; bars, SD. *, $P < 0.01$ and **, $P < 0.005$ versus untreated resistant clone. †, $P < 0.005$ and ††, $P < 0.03$ versus untreated SBC-3; (bottom), reverse transcription-PCR analysis shows that CXCL12 downregulates CD9 in SBC-3–derived chemoresistant clones within 6 h. C, drug sensitivity in the presence (black) or absence (white) of CXCL12 was tested and shown as in Fig. 2. D, schema of CAM-DR mechanism with hypothetical involvement of CD9 and CXCL12 is shown. Motility (solid line and arrow) of cancer cells increases up to a peak then goes down again to the baseline level in company with continuous increase in chemoresistance (gray arrow) and adhesive potential (dashed line and arrow). Upregulation of endogenous CD9 is probably involved in CAM-DR mechanism (gradient gray zone) and exogenous CXCL12 may dynamically regulate cellular motility controlling CD9 expression (open arrows).
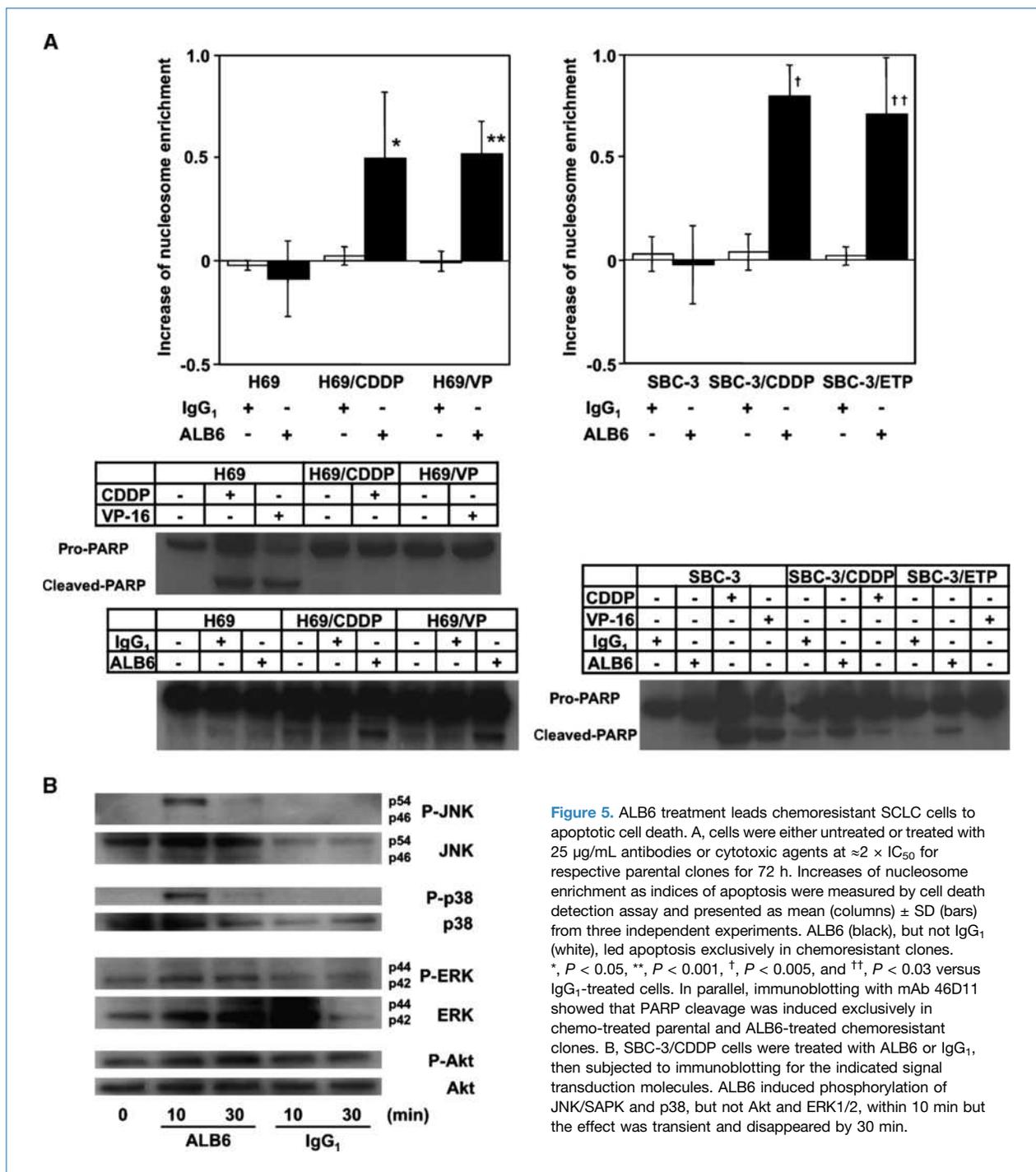
Figure 5. ALB6 treatment leads chemoresistant SCLC cells to apoptotic cell death. A, cells were either untreated or treated with 25 μg/mL antibodies or cytotoxic agents at ≈2 × IC$_{50}$ for respective parental clones for 72 h. Increases of nucleosome enrichment as indices of apoptosis were measured by cell death detection assay and presented as mean (columns) ± SD (bars) from three independent experiments. ALB6 (black), but not IgG$_1$ (white), led apoptosis exclusively in chemoresistant clones. *, $P < 0.05$, **, $P < 0.001$, †, $P < 0.005$, and ††, $P < 0.03$ versus IgG$_1$-treated cells. In parallel, immunoblotting with mAb 46D11 showed that PARP cleavage was induced exclusively in chemo-treated parental and ALB6-treated chemoresistant clones. B, SBC-3/CDDP cells were treated with ALB6 or IgG$_1$, then subjected to immunoblotting for the indicated signal transduction molecules. ALB6 induced phosphorylation of JNK/SAPK and p38, but not Akt and ERK1/2, within 10 min but the effect was transient and disappeared by 30 min.

exist in an ECM-rich environment and ECM proteins produced by adjacent stroma and tumor cells probably offer them a suitable microenvironment to support their proliferation and survival.

This evidence prompted us to study if CD9 is involved in the CAM-DR mechanism and how SCLC cells use CD9 to intensify this mechanism to circumvent chemotherapy-induced apoptosis. According to our findings, CD9 expression would consistently increase in parallel with augmentation of chemoresistance from the initial escape from chemotherapy-mediated apoptosis to the ultimately resistant stage (Fig. 1). Although CD9 might not be essential for the acquisition of drug resistance but a result of cellular adaptation to protect cells from chemotherapy-driven stress (Fig. 2), chemoresistant
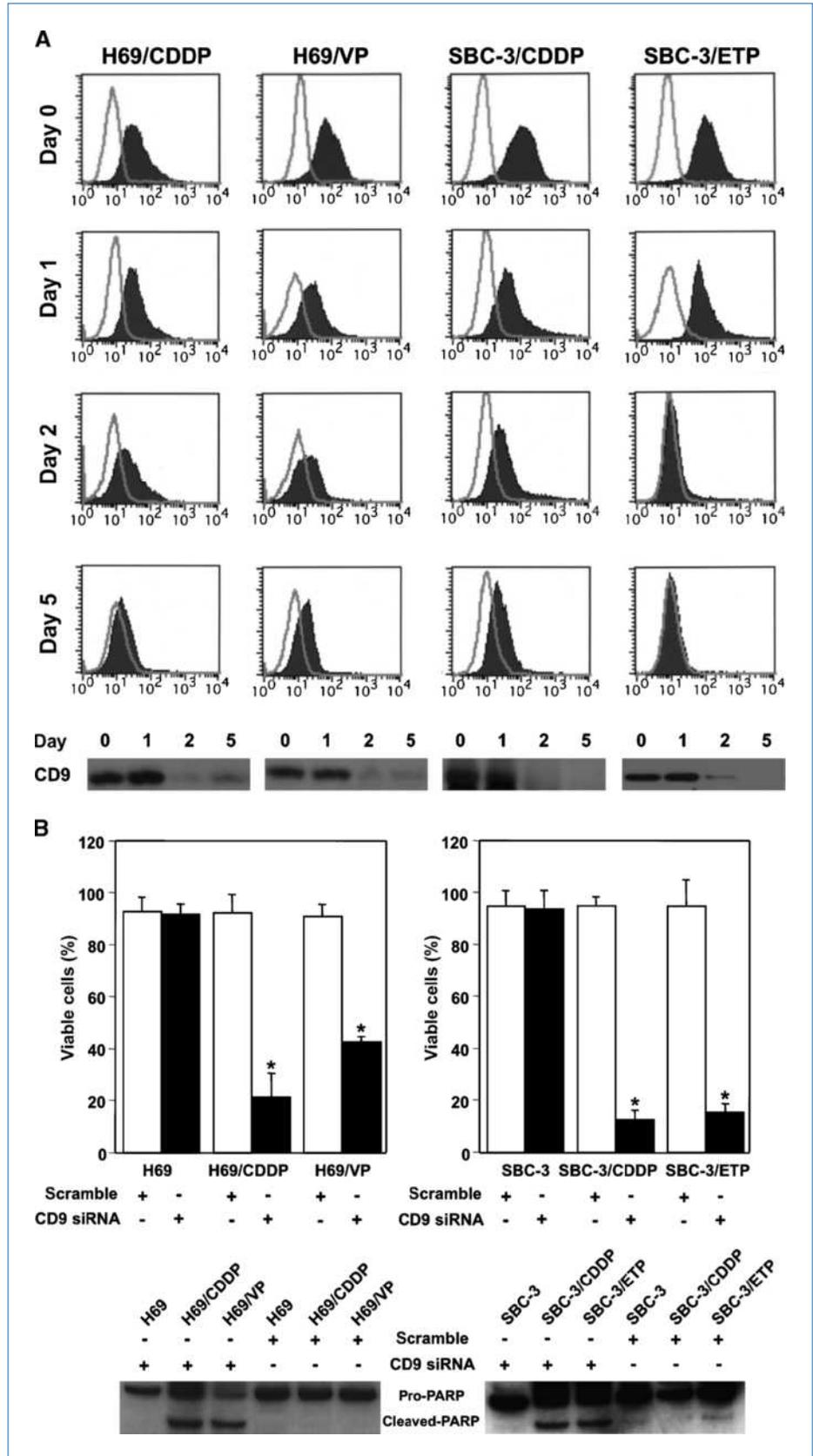
Figure 6. *CD9* siRNA leads chemoresistant SCLC cells to apoptotic cell death. A, successful knockdown of CD9 from day 2 to day 5 was confirmed by immunoblot and FACS analyses. B, relative values of viable cell numbers were quantified by MTT assay on day 5 and shown as mean (columns) ± SD (bars) from three independent experiments. *CD9* siRNA (black) but not scramble RNA (white) induced apoptosis accompanied with PARP cleavage exclusively in chemoresistant clones. *, $P < 0.0001$ versus scramble-transfected cells.

clones adhered to fibronectin via $\beta 1$ integrin significantly more tightly than the respective parental clones in which CD9 was strongly concerned (Fig. 3). Therefore, chemoresistant SCLC cells probably strengthen survival signals from ECM in cooperation with upregulated CD9 in their local environment.

Based on the CAM-DR theory, stubborn adhesion to ECM would make cancer cells less mobile but offer them integrin-mediated survival advantage in the face of chemotherapy (38, 39), and in this setting, upregulated CD9 might play a role in strengthening this mechanism. Chemoresistant clones and CD9 transfectants actually became less mobile than their respective parental cells (Fig. 4A and B). Conversely, loose adhesion to ECM would give cancer cells adequate mobility at the sacrifice of increased susceptibility to chemotherapy-induced apoptosis. It is necessary for chemoresistant SCLC cells to restore motility to metastasize to new distant sites during the "treatment holiday." We have previously reported that CXCL12 secreted from stroma in such organs as bone marrow and lymph nodes enhanced the motility of SCLC cells and contributed to their site-specific metastasis to these organs (36). The motility of chemoresistant clones transiently increased to the level of parental cells in response to CXCL12 coupled with downregulation of CD9 (Fig. 4A and B). To form de novo metastases without affecting their chemosensitivity, it is reasonable and advantageous for chemoresistant cells that biological activity of CXCL12 on their motility and CD9 expression does not last so long (Fig. 4C). Thus, CD9 and CXCL12 might dynamically regulate cellular motility to provide cancer cells with suitable conditions spatially as well as temporally in the CAM-DR mechanism (Fig. 4D).

Selective targeting of CD9 by ALB6 and siRNA both led CD9-expressing SCLC cells to apoptotic cell death, suggesting that CD9 is a regulator of cell survival (Figs. 5 and 6). Murayama and colleagues reported that CD9 was commonly expressed in cancer cells originating from the digestive system, including stomach, colon and pancreas, and that ALB6 induced the apoptosis of these CD9-expressing cells coupled with transient activation of JNK/SAPK and p38 (40), which was consistent with our results in SCLC (Fig. 5B). ALB6 treatment of mice bearing CD9-expressing human gastric cancer cells has been recently shown to successfully inhibit tumor progression via not only antiproliferative and proapoptotic effects on malignant cells but also antiangiogenic effects on tumor vessels reducing microvessel density (42). Inhibitory effects of anti-CD9 mAb on in vivo tumorigenicity as well as in vitro proliferation of human colon carcinoma cells has also been reported (43). Regulation of tumor angiogenesis by ALB6 in vivo is likely and

acceptable because another anti-CD9 mAb (ALMA.1) inhibited human vascular endothelial cell migration toward ECM proteins at wound lesion and impaired its repair (44). Because targeting tumor vessels by bevacizumab provides advantages over traditional antitumor approaches (7), the anti-CD9 antibody is an attractive tool targeting both tumor cells and vessels in one molecule.

Furthermore, antibody-dependent cellular cytotoxicity might have participated in antitumor activity by anti-CD9 antibodies in vivo (42, 43), although the authors did not refer to the matter. IgG_1 subclass antibodies such as trastuzumab, rituximab, and cetuximab are well known to preferentially induce this immune reaction because they have longer half-lives and could bind to all subtypes of $Fc\gamma R$ on effector cells (45–48). In this context, IgG_1 antibody directed against CD9 such as ALB6 is expected not only as an apoptosis inducer for cancer cells and inhibitor of angiogenesis but also as a potent antibody-dependent cellular cytotoxicity mediator. Thus, targeting CD9 is still the more attractive option in antitumor strategy.

Collectively, our present study clarified the molecular significance of CD9 in CAM-DR mechanism in SCLC. Moreover, we showed the possibility of using this molecule as a novel therapeutic target to overcome drug resistance especially in the refractory stage. Finally, the implication of targeting CD9 for therapeutic strategies in SCLC is promising to bring about better prognosis of this devastating malignancy and is valuable for future clinical applications.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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