FEBS Open Bio 3 (2013) 225–230

journal homepage: www.elsevier.com/locate/febsopenbio

Calretinin mediates apoptosis in small cell lung cancer cells expressing tetraspanin CD9☆

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ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form 5 April 2013
Accepted 26 April 2013

Keywords:
Small cell lung cancer
Proteomics
Tetraspanin
CD9
Calretinin
Apoptosis

ABSTRACT

A majority of small cell lung cancer (SCLC) cells lack a metastasis suppressor, tetraspanin CD9, and CD9 expression promotes their apoptosis. By a proteomics-based approach, we compared an SCLC cell line with its CD9 transfectant and found that a calcium-binding neuronal protein, calretinin, is upregulated in CD9-positive SCLC cells. Ectopic or anticancer drug-induced CD9 expression upregulated calretinin, whereas CD9 knockdown down-regulated calretinin in SCLC cells. When calretinin was knocked down, CD9-positive SCLC cells revealed increased Akt phosphorylation and decreased apoptosis. These results suggest that CD9 positively regulates the expression of calretinin that mediates proapoptotic effect in SCLC cells.

1. Introduction

Small cell lung cancer (SCLC) is highly malignant lung tumor that spreads early throughout the body. It is characterized by neuroendocrine features such as neuropeptide production and N-CAM expression [1]. At diagnosis in most cases, SCLC has already metastasized to regional lymph nodes and distant organs including brain, bone, liver, and adrenal gland, thus excluding the possibility of surgical resection. Currently, standard treatment against extended SCLC is chemotherapy including cisplatin and etoposide [2]. Despite its high sensitivity to these anticancer drugs, SCLC rapidly develops recurrent tumors locally and at the distant organs. Such malignant phenotype is at least partially caused by acquired resistance to apoptotic cell death [3]. Elucidation of its mechanisms is necessary to improve outcome of chemotherapy, but little has been clarified.

Tetraspanins are a family of membranous proteins that has characteristic structure spanning the membrane four times. Through association with other functional proteins including integrins, growth factor receptors, membrane proteases, and intracellular signaling molecules, tetraspanins organize multiprotein complexes at the tetraspanin-enriched microdomain (TEM) and regulate cell adhesion, migration, and survival [4,5]. Among 33 members in humans, CD9 and CD82 are known as a metastasis suppressor of solid tumors. Clinical and pathological findings suggest that decreased expressions of these tetraspanins are associated with progression of cancers of breast, pancreas, colon, and esophagus, and nonsmall cell lung cancer (NSCLC) and thus with poor prognosis [6,7].

We have shown that, among tetraspanins, CD9 is selectively absent in a majority of SCLC lines and SCLC tissues in contrast to NSCLC which frequently expresses CD9, and that ectopic expression of CD9 in SCLC cells suppresses integrin β1-dependent cell motility [8] and promotes apoptotic cell death through attenuation of PI3K/Akt signaling [9]. These results suggest that the absence of CD9 contributes to highly malignant phenotype of SCLC. We also found that CD9 expression is induced and cell motility is decreased when SCLC cells are exposed to cisplatin or etoposide [10]. In the present study, we compared an SCLC cell line with its CD9 transfectant by a proteomics-based approach and found that a calcium-binding neuronal protein,

Abbreviations: SCLC, small cell lung cancer; NSCLC, nonsmall cell lung cancer; PARP, poly(ADP-ribose)polymerase; PMF, peptide mass fingerprinting; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; RT-PCR, reverse transcription-PCR; KO, knockout

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2211-5463/$36.00
http://dx.doi.org/10.1016/j.fob.2013.04.005

calretinin, is upregulated in CD9-positive SCLC cells. We also show that calretinin mediates apoptotic cell death of SCLC.

2. Materials and methods

2.1. Cell lines

OS1, OS2-RA, and OS3-R5 were SCLC cell lines established in our laboratory, and their biological properties were previously characterized [8]. SCLC lines, OC10 and CADO LC6, a lung adenocarcinoma cell line, CADO LC9, and a mesothelioma cell line, OC-(MT)37, were provided by Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan) [11]. An SCLC line, SBC-3, and its chemoresistant subline, SBC-3/CDDP, were kindly provided by Dr. K. Kiura (Okayama University, Okayama, Japan) [10]. SCLC cell lines, NCI-H69, NCI-N231, and NCI-H209, a lung adenocarcinoma line, A549, and pleural mesothelioma lines, NCI-H226, NCI-H2452, NCI-H28, and MSTO-211H, were purchased from American Type Culture Collection (Rockville, MD). A lung squamous cell carcinoma line, HARA, was a kind gift from Dr. H. Iguchi (Kyusyu Cancer Center, Fukuoka, Japan). All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Antibodies and reagents

Mouse anti-CD9 mAb (MM2/57), anti-poly(ADP-ribose)polymerase (PARP) mAb (42/PARP), and anti-β-actin mAb (C4) were purchased from Biosource, BD Biosciences, and Santa Cruz Biotechnology, respectively. Mouse anti-CD9 mAb (72F6) was purchased from Novocastra. Goat anti-calretinin polyclonal Ab (AB1550) and rabbit anti-calretinin polyclonal Ab (DC8) were purchased from Chemicon International and Zymed Laboratories, respectively. Rabbit anti-cleaved PARP (Asp214) mAb (D64E10), anti-phospho-Akt (Ser473) mAb (D9E), and anti-Akt polyclonal Ab were purchased from Cell Signaling Technology. Cisplatin (CDDP) was provided by Nippon Kayaku Co. (Tokyo, Japan).

2.3. Flow cytometry

Cells (10^4) were incubated with 10 μg/ml primary mouse mAbs and labeled with FITC-conjugated goat anti-mouse immunoglobulin (Biosource International). Normal mouse IgG was used as a control. Stained cells were analyzed on a FACScan (Becton Dickinson).

2.4. cDNA and small interfering RNA (siRNA) transfection

Establishment of stable CD9-, NAG-2-, and mock-transfectants of OS3-R5 was previously described [8,9]. Cells were transfected with 40 nM cocktail siRNAs against human CD9 (No. SHF27A-0631; B-Bridge International) or human calretinin (No. SHF27A-0981; B-Bridge International), or negative control cocktail RNAs (No. S30C-0126; B-Bridge International) using LipofectAMINE 2000 Reagent (Invitrogen).

2.5. Two-dimensional electrophoresis (2-DE) and mass spectrometry analysis

Proteins were extracted from cells with the Complete Mammalian Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). For 2-DE, isoelectric focusing (IEF) was performed using the PROTEAN IEF cell (Bio-Rad laboratories) according to the manufacturer's instructions. Extracted proteins were reconstituted in a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributylphosphine (TBP), 0.0002% bromophenol blue (BPB), 0.2% Bio-lyte ampholyte 4–7) and applied to ReadyStrip™ IPG strips (11 cm, pH 4–7). IEF was run for 45,000 Vh. Two-dimensional electrophoresis was carried out in 10% Bis–Tris Criterion™ XT Precast gels. After staining with the Silver Stain MS Kit (Wako Pure Chemical Industries, Osaka, Japan), the gels were captured by transmission scanning and analyzed with Image Master 5.0 (Amersham Biosciences). Following analysis, selected protein spots were manually excised from the gels and digested with trypsin (Promega) according to published procedures [12]. All peptide mass fingerprinting (PMF) spectra were obtained by using an ultraflex TOF/TOF matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany).

2.6. Database search

PMF data were searched with Mascot software (Matrix Science, London, UK) against NCBInr or Swiss-Prot databases. Protein database searching was performed with following parameters: *Homo sapiens*, maximum of one missed, cleavage by trypsin, monoisotopic masse value, charge state of 1 +, allowing a mass tolerance of 100 ppm, and carbamidomethyl modification of cysteine. Protein scores of >64 indicate identity or extensive homology ($P < 0.05$) and were considered significant.

2.7. Reverse transcription-PCR (RT-PCR)

One microgram of total RNA was reversely transcribed with a cDNA synthesis kit (Invitrogen) using random hexamers. The thermal cycling parameters were 30 cycles of 40 s at 94 °C, 40 s at 60 °C, and 90 s at 72 °C for CD9 and 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C for calretinin. We confirmed that these variables yielded amplification of template DNAs within a linear range. The sequences of upstream and downstream oligonucleotide primers for CD9 was previously described [8]. Upstream and downstream oligonucleotide primers used for calretinin were 5′-GGAAGCACTTTGACGCAGACG-3′ and 5′-CTCGCTGCAGAGCACAATCTC-3′, respectively.

2.8. Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer containing 1% Brij 99, 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Whole cell lysates or immunoprecipitates with anti-CD9 mAb (MM2/57) were separated by 10% SDS-PAGE under nonreducing conditions for CD9 or under reducing conditions for the other proteins. After transfer to Immobilon-P membranes (Millipore), immunoblotting was performed with primary Abs followed by peroxidase-conjugated secondary Abs. Immunoreactive bands were visualized with a chemiluminescent reagent (PerkinElmer).

2.9. Immunohistochemistry

A human SCLC tissue array was purchased from US Biomax Inc. It contained small cell carcinoma tissues from 30 individuals and normal tissues from three individuals. Each specimen was represented by two cores from different tissue spots. After antigen retrieval, inactivation of endogenous peroxidase, and blockade of non-specific reaction, the tissue microarray sections were stained with anti-CD9 mAb (72F6) or anti-calretinin Ab (DC8), followed by incubation with biotinylated goat anti-mouse and rabbit IgG Ab and streptavidin-conjugated peroxidase. These were counterstained with Mayer's hematoxylin [10]. Specimens were regarded as positive when staining was observed in more than 30% of tumor cells on average. The significance of association between CD9 staining and calretinin staining was evaluated by Fisher's exact test.

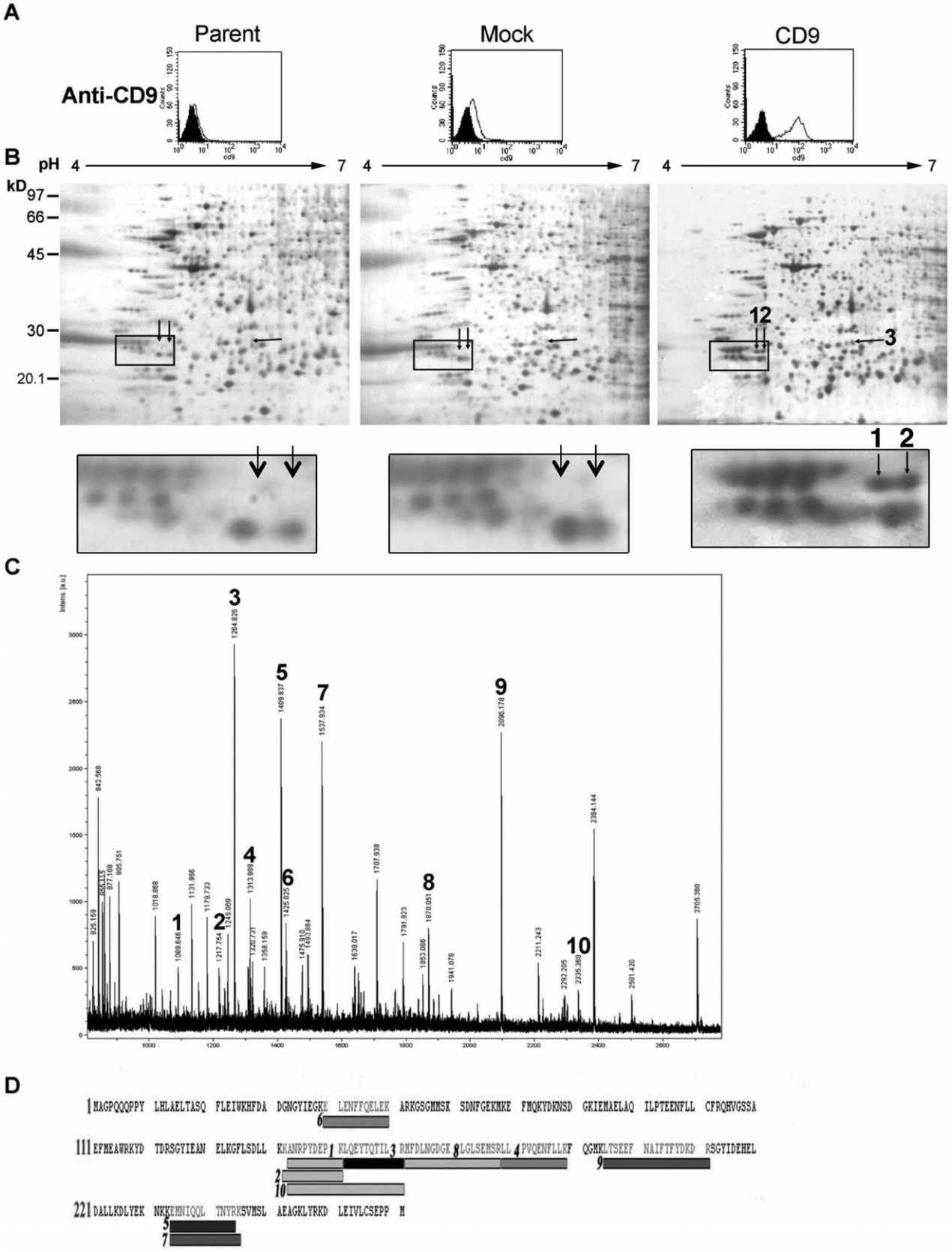

Fig. 1. Comparative proteomic analysis of the parent and CD9-overexpressing SCLC cells. (A) The parent, mock transfectant, and CD9 transfectant of OS3-R5 were stained with anti-CD9 mAb, labeled with FITC-conjugated goat anti-mouse immunoglobulin, and analyzed on a FACScan (*Open histograms*). *Closed histograms* indicate staining with control IgG. (B) Representative 2-DE maps of OS3-R5 and its transfectants. *Arrows* 1–3 indicate protein spots selectively identified in OS3-R5-CD9 by mass spectrometry. 1 and 2, calretinin; 3, PA28α. Images including the calretinin spots were enlarged in *lower columns*. (C) PMF spectra of spot 2 obtained by MALDI-TOF. Mass peaks, peptides of which were matched with human calretinin, are marked with numbers. (D) The matched peptides in panel (C) were indicated with bars, yielding 33% sequence coverage of calretinin.

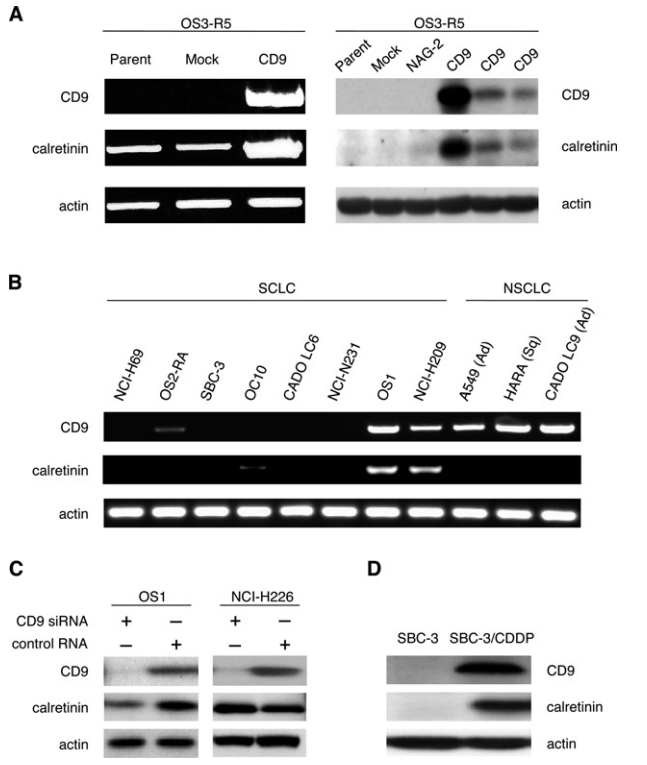

Fig. 2. Co-expression of CD9 and calretinin in SCLC cells. (A) Total RNA was extracted from the parent, mock transfectant, and CD9 transfectant of OS3-R5 and analyzed for expressions of CD9 and calretinin by RT-PCR. β-Actin amplification was used as the internal control (*left*). The parent, mock transfectant, NAG-2 transfectant, and CD9 transfectants of OS3-R5 were lysed with 1% Brij 99 lysis buffer. Cell lysates were analyzed for expressions of CD9 and calretinin by immunoblotting. Anti-β-actin blots were used as the internal control (*right*). (B) Total RNA was extracted from multiple SCLC and NSCLC cell lines and analyzed for expressions of CD9 and calretinin by RT-PCR. Ad, adenocarcinoma; Sq, squamous cell carcinoma. (C) An SCLC line OS1 (*left*) or a mesothelioma line NCI-H226 (*right*) was transfected with siRNAs against CD9 or control RNAs. Cell lysates were analyzed for expressions of CD9 and calretinin by immunoblotting. (D) Cell lysates of SCLC lines SBC-3 and SBC-3/CDDP were analyzed for expressions of CD9 and calretinin by immunoblotting.

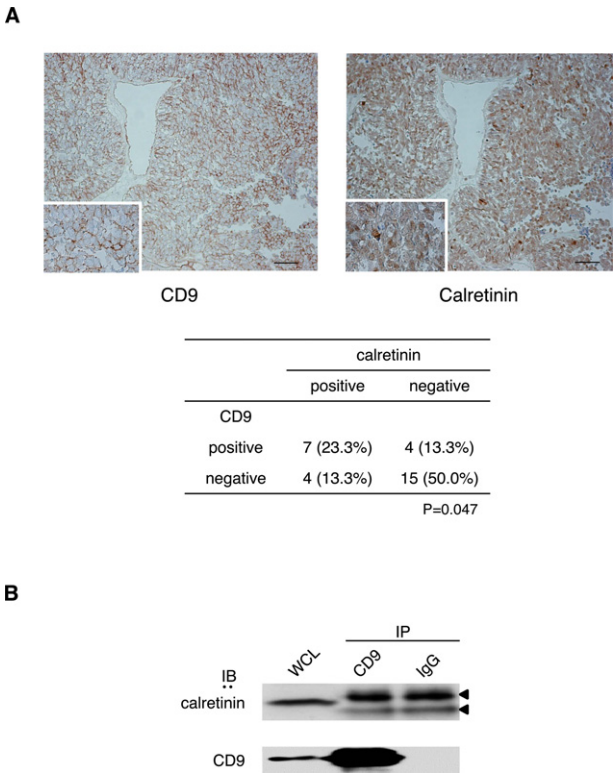

Fig. 3. CD9 is not physically associated with calretinin. (A) Immunohistochemical staining of CD9 and calretinin in a double-positive specimen from an SCLC tissue microarray. *Insets* show enlarged images of a part of sections. Bar, 50 μm. Significant association between CD9 and calretinin expressions in the tissue microarray was evaluated by Fisher's exact test (*table*). (B) CD9 and calretinin in whole cell lysate (WCL) and in immunoprecipitates (IP) with anti-CD9 mAb or control IgG of OS3-R5-CD9 were immunoblotted (IB). *Arrowheads* indicate nonspecific binding of secondary Abs.

2.10. Mice

The generation of CD9 knockout (KO) mice was described previously [13]. These mice were backcrossed more than six generations into the C57BL/6J background. The mice were bred in a barrier facility, and all animal procedures were performed in accordance with the Osaka University guidelines on animal care.

2.11. Apoptosis analysis

Cells were transfected with siRNA against calretinin or negative control RNAs. After 24 h, the cells were cultured in the absence or presence of CDDP in low-serum (0.1% FBS) RPMI 1640 for 48 h. Apoptotic cleavage of PARP [14] and decrease of Akt phosphorylation [9] were analyzed by immunoblotting. Viable cells were quantified with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Assays were performed in triplicate cultures and values are expressed as mean ± SD. Statistical differences were determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of proteins upregulated in OS3-R5-CD9 cells

We previously established a CD9 (−) SCLC cell line OS3-R5 and its CD9 transfectant OS3-R5-CD9 and revealed that the CD9 transfectants were less motile on fibronectin [8] and revealed enhanced apoptosis in low-serum culture conditions [9]. To detect molecules that regulate these changes, cell lysates of the parent, mock transfectant, and CD9 transfectant of OS3-R5 (Fig. 1A) were subjected to 2-DE, and protein spots were visualized by silver stain of the gels. Spots selectively overexpressed in OS3-R5-CD9 were identified (Fig. 1B) and the corresponding proteins were analyzed by mass spectrometry. We repeated this experiment and found that two proteins, a calcium-binding protein, calretinin, and a proteasome activator subunit 1, PA28α, were reproducibly overexpressed in OS3-R5-CD9 cells (Table S1). Fig. 1C shows PMF spectra of spot 2 obtained by MALDI-TOF. Matched peptides were found to cover 33% of protein sequence of calretinin (Fig. 1D). Based on the fact that SCLC has neuronal features, we further analyzed calretinin, which is a protein distributed in the nervous system.

3.2. Co-expression of CD9 and calretinin in SCLC cells

RT-PCR revealed that the calretinin gene was minimally transcribed in the parent and mock-transfected OS3-R5 cells, and that ectopic expression of CD9 promoted its transcription (Fig. 2A, *left column*). To test the calretinin induction is specifically related to CD9, multiple CD9 transfectants and cells transfected with another tetraspanin NAG-2 were examined. As shown in Fig. 2A, *right column*,

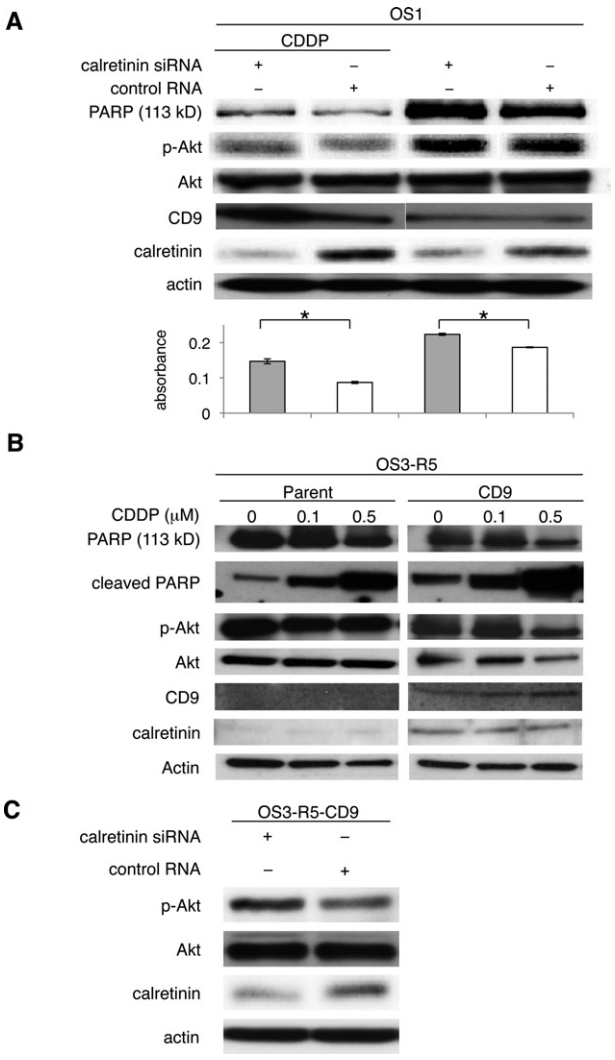

Fig. 4. Calretinin promotes apoptosis of CD9 (+) SCLC cells. (A) CD9 (+)/calretinin (+) OS1 cells were transfected with siRNAs against calretinin or control RNAs and cultured in low-serum conditions in the absence or presence of 5 μM CDDP for 48 h. Cell lysates were analyzed for expressions of PARP (113 kD), phosphorylated Akt (p-Akt), total Akt, CD9, and calretinin by immunoblotting. Anti-β-actin blots were used as the internal control (*upper*). Viable cells were quantified with a cell counting kit (*lower*). $^*P < 0.01$. (B) The parent and CD9 transfectant of OS3-R5 were cultured in low-serum conditions in the indicated concentrations of CDDP for 48 h. Cell lysates were analyzed for expressions of PARP (113 kD), cleaved PARP, phosphorylated Akt, total Akt, CD9, and calretinin by immunoblotting. (C) OS3-R5-CD9 cells were transfected with siRNAs against calretinin or control RNAs and cultured in low-serum conditions for 48 h. Cell lysates were analyzed for expressions of phosphorylated Akt, total Akt, and calretinin by immunoblotting.

calretinin was almost absent in the parent, mock transfectant, and NAG-2 transfectant in immunoblotting, whereas calretinin was obviously present in three independent CD9 transfectants and its level was parallel to that of CD9. We further examined if CD9 and calretinin are co-expressed in a panel of lung tumor cell line (Fig. 2B). Consistent with our previous report that most SCLC cells lack CD9 [8], five of eight SCLC lines revealed no transcription and one cell line (OS2-RA) showed only marginal transcription of *CD9* gene. The other two lines (OS1 and NCI-H209) clearly expressed CD9, and these CD9 (+) lines also expressed calretinin. Meanwhile, all three NSCLC lines were CD9 (+), but none of them expressed calretinin (Fig. 2B).

CD9 was next deleted by knockdown with siRNA in the SCLC line OS1, which expresses endogenous CD9. As shown in Fig. 2C, *left column*, the knockdown of CD9 suppressed the level of calretinin. In an additional experiment, we have studied CD9 and calretinin expressions in multiple pleural mesothelioma cell lines and found that only NCI-H226 expresses both CD9 and calretinin in immunoblotting (Fig. S1). CD9 was knocked down in this mesothelioma line, but unlike OS1, the calretinin level was not affected (Fig. 2C, *right column*). Our recent report showed that endogenous CD9 is induced when the CD9 (−) SCLC line SBC-3 is exposed to an anticancer drug, cisplatin [10]. As shown in Fig. 2D, calretinin was co-induced with endogenous CD9 in the cisplatin-exposed SBC-3 cells.

We further investigated if CD9 is generally required for the expression of calretinin using tissues from wild-type and CD9 KO mice. Calretinin was expressed in liver and brain lysates of wild-type mice, and its levels were not affected by the loss of CD9, as evidenced by abundant expression of calretinin in the lysates from CD9 KO mice (Fig. S2). Together, regulation of calretinin expression by CD9 seemed to be specific to human SCLC cells.

3.3. *CD9 is not physically associated with calretinin*

To investigate co-expressions of CD9 and calretinin *in vivo*, a tissue microarray of SCLC was analyzed by immunohistochemistry. Among 30 patients, 11 were CD9 (+), 11 were calretinin (+), and 7 were double-positive, and association between CD9 expression and calretinin expression was weakly significant (Fig. 3A, *table*). Higher positive rate of CD9 (11/30) in SCLC tissues compared with that in SCLC cell lines [8] might be because some biopsy specimens were obtained from relapsed or metastatic lesions, which were more frequently CD9 (+) than pretreated primary tumors [10]. Representative staining of CD9 and calretinin in a double-positive specimen indicated that expression patterns of these proteins were different (Fig. 3A). CD9 was stained at the cell periphery, whereas calretinin showed nuclear and cytoplasmic staining (Fig. 3A, *insets*). Tetraspanins including CD9 are characterized by their propensity to form multiprotein complexes at the plasma membrane. To examine if calretinin is present in large protein complexes including CD9, co-precipitated proteins with CD9 was immunoblotted with anti-calretinin Ab. As shown in Fig. 3B, calretinin did not co-precipitate with CD9 in OS3-R5-CD9 cell lysate even using non-stringent detergent, Brij 99. Thus, calretinin was not present in the protein complex including CD9.

3.4. *Calretinin promotes apoptosis of CD9 (+) SCLC cells*

Our previous reports have shown that ectopic expression of CD9 increases apoptosis by attenuation of postadhesive phosphorylation of Akt [9] and that anticancer drugs induce endogenous CD9 in SCLC cell lines [10]. To examine the involvement of calretinin in apoptosis of SCLC cells, calretinin was knocked down with siRNA in CD9 (+)/calretinin (+) OS1 cells, and expression of PARP was examined as an indicator of apoptotic cell death [14]. Exposure of OS1 cells to 5 μM cisplatin increased endogenous CD9 and calretinin and enhanced apoptosis as evidenced by decrease of 113-kD PARP and decreased phosphorylation of Akt (Fig. 4A, control RNA). Knockdown of calretinin prevented the OS1 apoptosis regardless of the exposure to cisplatin (Fig. 4A, calretinin siRNA). It appeared that the calretinin knockdown slightly upregulated CD9; this might reflect an unknown feedback mechanism. Proapoptotic role of calretinin was also studied in OS3-R5 cells. The exposure to CDDP for only 48 h did not induce endogenous CD9 and calretinin in this cell line and, when compared with the parent cells, OS3-R5-CD9 cells expressing calretinin revealed higher sensitivity to CDDP, as evidenced by enhanced PARP cleavage and decreased Akt phosphorylation (Fig. 4B). After the exposure to 1 μM CDDP for 48 h, viable cells of OS3-R5 and OS3-R5-CD9 were 56.1 ± 1.8% and 20.1 ± 2.7%, respectively ($P < 0.01$). As shown in Fig. 4C, the knockdown of calretinin in OS3-R5-CD9 increased phosphorylation of Akt. These results suggest that calretinin may be a downstream mediator of apoptosis in CD9 (+) SCLC cells.

4. Discussion

Our previous studies have proposed that the absence of tetraspanin CD9 contributes to highly malignant phenotype of SCLC and that CD9 may be a pivotal regulator of SCLC cell survival [8–10]. In the present study using a proteomics-based approach, we identified calretinin as a possible mediator of CD9-induced apoptosis in SCLC. Calretinin was present in CD9 (+) SCLC cell lines but not in CD9 (−) SCLC lines and CD9 (+) NSCLC lines. Ectopic or CDDP-induced expression of CD9 upregulated calretinin in SCLC lines. Knockdown of CD9 conversely down-regulated calretinin in an SCLC line but not in a mesothelima cell line. Furthermore, knockdown of calretinin increased Akt phosphorylation and decreased apoptosis in CD9 (+)/calretinin (+) SCLC cell lines. Although statistical significance in the association of CD9 and calretinin expressions was weak ($P = 0.047$) in SCLC tissues, we speculate that this might be due to elimination of apoptotic CD9 (+)/calretinin (+) tumor cells *in vivo*.

Calretinin is a member of the calcium-binding protein EF-hand family first identified in the retina. Calcium-binding proteins including calretinin are expressed in neuronal subpopulations of the nervous system. Calretinin is involved in cellular functions including intracellular calcium buffering, messenger targeting, and the modulation of neuronal excitability. Modulation of calcium signaling by calretinin is important for timing and plasticity of synaptic events in neuronal networks. Some studies have suggested neuroprotective role of calretinin against calcium-induced cytotoxicity, whereas others reported opposite effects [15,16]. Of note, a recent report using colorectal cancer cells indicated that calretinin is induced following treatment with oxaliplatin or 5-FU and positively regulates apoptotic signals via as yet unknown mechanisms [14]. In line with this report, the present study suggested that calretinin mediates proapoptotic signaling in SCLC cells and for the first time showed that CD9 positively regulates the expression of calretinin. It has been established that tetraspanins including CD9 work as organizer of multiprotein complexes at the membrane [4,5]. Although calretinin has been reported to concentrate beneath the plasma membrane during maturation in neurons [17], it did not co-precipitate with CD9 in OS3-R5-CD9 cells, suggesting the presence of other mediators linking CD9 to enhanced expression of calretinin.

In conclusion, by proteomics-based approach, we have proposed a novel proapoptotic pathway that links the metastatic suppressor CD9 to the neuronal calcium-binding protein, calretinin, in SCLC. Induction of CD9/calretinin may at least partially account for its high sensitivity to chemotherapy and might provide clues to new therapeutic approach to suppress early growth, metastasis, and recurrence of SCLC.

Acknowledgments

We thank Y. Habe for secretarial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.04.005.

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