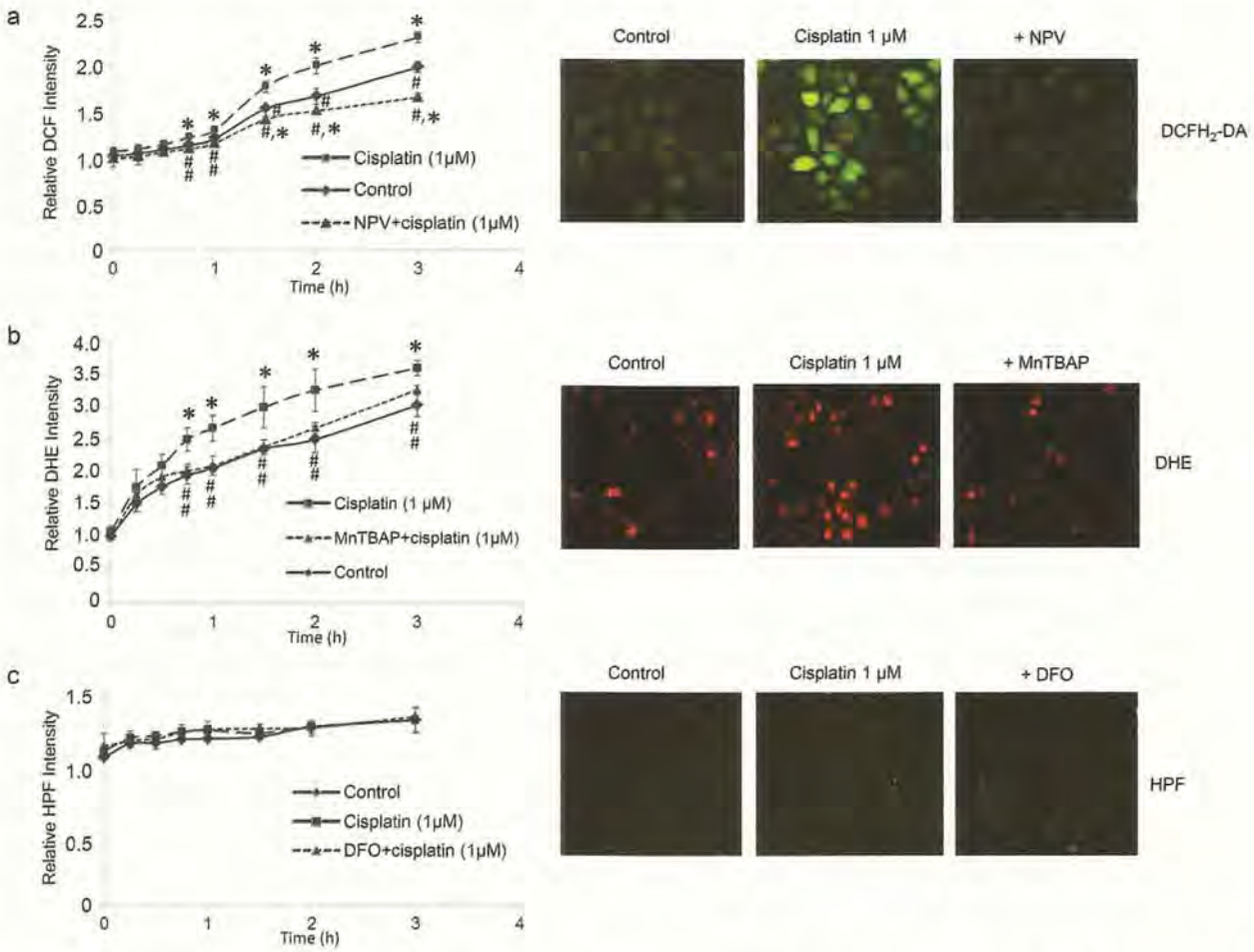

Figure 6. *Hydrogen peroxide and superoxide anion are key reactive oxygen species (ROS) induced by sub-toxic doses of cisplatin. a: H460 cells were left untreated or were pre-treated with 1 μM sodium pyruvate (NPV) for 30 min, prior to cisplatin treatment (1 μM); cellular ROS levels were determined by 2',7'-dichlorofluorescein diacetate (DCFH2-DA) probe. b: Cells were pre-treated with 50 μM Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) for 30 min, were then treated with cisplatin, and then the superoxide anion levels were detected by dihydroethidium (DHE) probe. c: Cells were incubated with 1 μM deferoxamine (DFO) for 30 min prior to cisplatin treatment, and hydroxyl radical levels were detected using a 3'-(p-hydroxyphenyl) fluorescein (HPF) probe. All ROS signals were determined by a fluorescence microplate reader and were visualized under fluorescence microscopy. Values are means±S.D. (n=3); *p<0.05 versus non-treated control; #p<0.05 versus cisplatin-treated cells.*

signals by fluorescence microscopy and by a fluorescence microplate reader. Cells were similarly treated with cisplatin in the presence of specific ROS probes, namely DHE for superoxide detection and HPF for hydroxyl radical detection, as well as specific ROS scavengers, namely sodium pyruvate (hydrogen peroxide scavenger), MnTBAP (superoxide anion inhibitor), and DFO (hydroxyl radical inhibitor). Figure 6a and b indicate that treatment with 1 μM cisplatin induced hydrogen peroxide and superoxide anion production, as indicated by the increased cellular fluorescence intensity detected by $DCFH_2$-DA and DHE, respectively. Although the DCFH2-DA probe was not specific for hydrogen peroxide detection, sodium pyruvate abolished the cisplatin-mediated ROS induction, suggesting that hydrogen peroxide was the main ROS induced by cisplatin. An induction of DHE intensity clearly indicated the presence of superoxide anion in the treated cells. Furthermore, MnTBAP treatment blocked the DHE intensity, confirming the induction of superoxide anion production in response to cisplatin in these cells (Figure 6b). Regarding hydroxyl radical, cisplatin treatment caused no induction in the HPF signal compared to the non-treated control. Moreover, addition of DFO did not alter the HFP signal compared to the cisplatin- and non-treated cells (Figure 6c), suggesting that hydroxyl radical was not significantly induced in this sub-toxic cisplatin-mediated induction of ROS.

Hydrogen peroxide induced by cisplatin regulates anoikis resistance in lung cancer cells. Having shown that the sub-toxic dose of cisplatin increases cellular superoxide anion and hydrogen peroxide and the subsequent ROS inductions were concomitant with the CAV1 up-regulation, we next tested the role of ROS on the cisplatin-mediated induction of CAV1. Cells were treated with cisplatin (1 μM) in the presence or absence of pan-ROS scavengers, GSH (1 mM) and NAC (1 mM) for 24 h, and CAV1 expression was determined by western blotting. Figure 7a shows that cisplatin treatment for 24 h caused a significant increase in CAV1 levels and this protein induction was abolished in cells pre-incubated with GSH and NAC. Furthermore, we identified which ROS were involved in this process. H460 cells were pre-treated with specific ROS antioxidants including DFO (1 mM), MnTBAP (50 μM), sodium pyruvate (1 mM) or catalase (5,000 U/ml) prior to cisplatin treatment (1 μM), and the CAV1 expression levels were analyzed by western blotting. Figure 7b shows that cisplatin treatment increased the cellular CAV-1 levels and the addition of hydrogen peroxide scavengers, sodium pyruvate and catalase, completely inhibited the cisplatin-induced CAV1 up-regulation. On the contrary, pre-treatment with DFO and MnTBAP had only a minimal effect on cisplatin-induced induction of CAV1.

Since our results suggest that hydrogen peroxide induced by cisplatin was able to regulate CAV1 expression in these cells, the effect of exogenous hydrogen peroxide treatment on the CAV1 levels was evaluated in order to confirm the above findings. H460 cells were treated with hydrogen peroxide (100 μM) alone and the CAV1 levels were analyzed by western blotting. Figure 7c shows that hydrogen peroxide significantly elevated CAV1 expression as compared to non-treated controls. These results indicate that hydrogen peroxide is a major positive regulator of CAV1 expression in response to cisplatin treatment.

Discussion

The acquisition of cisplatin resistance, frequently found in human lung cancer, has been considered to be an important but complex obstacle of effective chemotherapy (39-41). Although the exact mechanism by which lung cancer cells tolerate cytotoxic drugs remains elusive, higher aggressiveness of surviving cancer cells after cisplatin-based regimens, existing as the principal problem in cancer treatment, has continuously been reported. The present study demonstrated for the first time that sub-lethal concentrations of cisplatin render human lung carcinoma cells resistant to detachment-induced apoptosis. Furthermore, we found that cisplatin-generated ROS were responsible for CAV1 up-regulation and, subsequently, anoikis resistance.

It is widely known that cisplatin treatment causes an induction of several ROS, namely superoxide anion, hydrogen peroxide, and hydroxyl radical, and such a ROS increase causes cytotoxic effects on cells (42-44). However, less is known regarding the ROS generated by sub-toxic concentrations of cisplatin. We report herein, for the first time, that a low concentration of cisplatin is capable to increase production of specific ROS, namely superoxide anion and hydrogen peroxide (Figure 6). Our results further revealed the effect of hydrogen peroxide in rendering cells resistant to anoikis. Most metastatic cancer cells resist detachment-induced apoptosis (anoikis). Anoikis plays a principal role in inhibition of cancer cells spreading from the original site to other sites. Several studies suggested that CAV1 expression was correlated with aggressive behaviors of lung cancer cells, including multidrug-resistance (45-47) and anoikis resistance (20). The present study demonstrated that after treatment with sub-toxic concentrations of cisplatin, CAV1 was up-regulated in a dose-dependent manner (Figure 4) and such up-regulation of CAV1 had an inhibitory effect on cell anoikis (Figure 2). Furthermore, we revealed that CAV1 expression in cisplatin-treated H460 cells was dependent on the oxidative stress induced by cisplatin. Addition of antioxidant GSH and NAC was able to attenuate the ROS induction and, subsequently, the CAV1 up-regulation (Figure 5). Previous studies reported that cisplatin-mediated death was related to the induction of cellular hydrogen peroxide (42, 48) and hydroxyl radical (49) production. However, in this study, a low concentration of cisplatin up-regulated hydrogen peroxide but did not alter the hydroxyl radical levels (Figure 6a and c). Since induction of cellular ROS by cisplatin has been previously shown to be dose dependent (43), it is possible that the production of cellular hydroxyl radical may be attenuated on low-dose cisplatin treatment and is overwhelmed by cellular antioxidants.

Various effects of specific ROS have been shown in many studies. We thus identified the specific ROS involved in the mechanism of cisplatin-mediated CAV1 up-regulation and anoikis resistance. Specific ROS scavengers, as well as specific ROS probes were used and the obtained results indicated that superoxide anion and hydrogen peroxide are two key ROS present in cisplatin-treated cells. The increase of CAV1 in response to cisplatin exposure was mainly due to hydrogen peroxide but not superoxide anion (Figure 7b). Our results demonstrated that the treatment of cells with exogenous hydrogen peroxide promoted the up-regulation of CAV1 (Figure 7c), while the superoxide anion generator 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) had no effect on the cultures (data not shown). In accordance with a previous study reporting the crucial role of hydrogen peroxide on CAV1 expression and cell anoikis (20), the present study indicates that hydrogen peroxide induced by sub-toxic concentrations of cisplatin can help cancer cells resist detachment-induced apoptosis and may facilitate the metastatic ability of cancer cells.

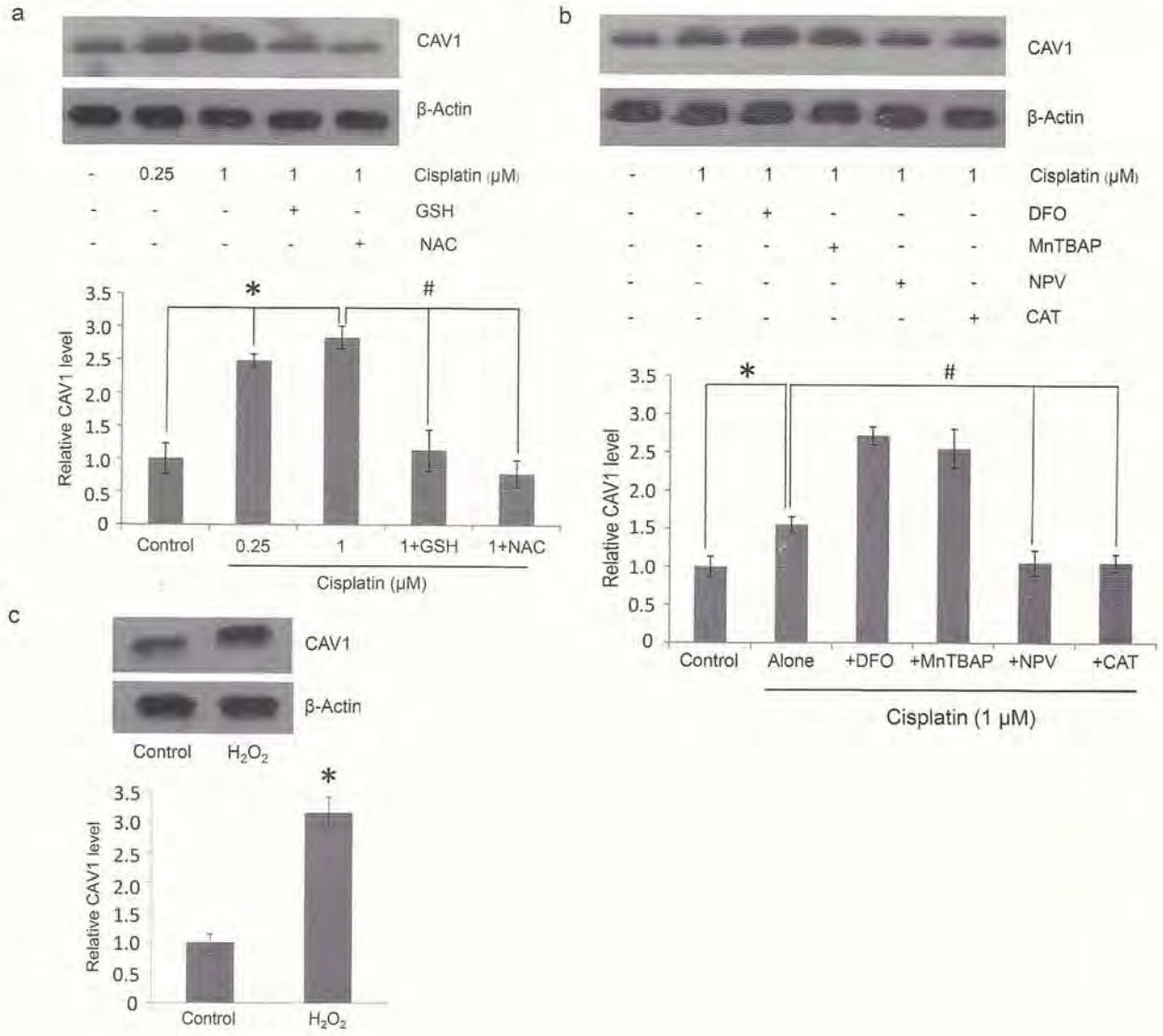

Figure 7. *Sub-toxic concentrations of cisplatin induce anoikis resistance in H460 cells through a hydrogen peroxide-dependent mechanism. a: H460 cells were left untreated or were pre-treated with 1 mM reduced glutathione (GSH) or 1 mM N-acetyl cysteine (NAC) for 30 min, and were then treated with 1 μM cisplatin for 24 h. The caveolin-1 (CAV1) level was evaluated by western blot analysis. b: Cells were left untreated or were pre-treated with 1 μM deferoxamine (DFO), 50 μM Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), 1 μM sodium pyruvate (NPV) or 5,000 U/ml catalase (CAT) for 30 min and the cells were then treated with cisplatin for 24 h before analyses of CAV1 by western blotting. (c) H460 cells were treated with hydrogen peroxide (100 μM) for 24 h and CAV1 levels were determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal sample loading. Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to non-treated cells. Values are means±S.D. (n=3); *p<0.05 versus non-treated control cells; #p<0.05 versus cisplatin-treated cells.*

In summary, we report a novel effect and an underlying mechanism of sub-toxic concentrations of cisplatin in regulating anoikis resistance in H460 human lung carcinoma cells. Exposure to cisplatin at the sub-toxic concentrations induced ROS generation (mainly superoxide and hydrogen peroxide). Hydrogen peroxide induced by such cisplatin exposure mediated CAV1 up-regulation and anoikis resistance in these cells. Since the ability to up-regulate cellular ROS production, especially of hydrogen peroxide, is found in a number of chemotherapeutic agents and other drugs, this finding might lead to further investigations which will facilitate a better understanding regarding cancer cell biology and benefit the design of more effective treatment strategies for chemotherapy.

Acknowledgements

This research was supported by Thailand Research Fund (to P. Chanvorachote) and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR11XXA-55). The Authors wish to thank Mr. Krich Rajprasit (Srinakharinwirot University) for proofreading.

References

1 Sleijfer DT, Meijer S and Mulder NH: Cisplatin: a review of clinical applications and renal toxicity. Pharm World Sci *7*: 237-244, 1985.

2 Loehrer PJ and Einhorn LH: Cisplatin. Ann Intern Med *100*: 704-713, 1984.

3 Ozols RF: Ovarian cancer, Part II: treatment. Curr Prob Cancer *16*: 63-126, 1992.

4 Wong E and Giandomenico CM: Current status of platinum-based antitumor drugs. Chem Rev *99*: 2451-2466, 1999.

5 Gerl A, Clemm C, Schmeller N, Hentrich M, Lamerz R and Wilmanns W: Late relapse of germ cell tumors after cisplatin-based chemotherapy. Ann Oncol *8*: 41-47, 1997.

6 Cognetti F, Ruggeri EM, Felici A, Gallucci M, Muto G, Pollera CF, Massidda B, Rubagotti A, Giannarelli D and Boccardo, F: Adjuvant chemotherapy with cisplatin and gemcitabine *versus* chemotherapy at relapse in patients with muscle-invasive bladder cancer submitted to radical cystectomy: an Italian, multicenter, randomized phase III trial. Ann Oncol *23*: 695-700, 2012

7 Parez RP: Clinical oncology update: cellular and molecular determinants of cisplatin Resistance. Eur J Cancer *34*: 1535-1542, 1998.

8 Kelland L: The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer *7*: 573-584, 2007.

9 Li HL, Xie SM, Zhang L, Cai CJ, Wang W, Huang J, Wang DY, Wen DP, Deng QH, Zhong NS and He JX: Establishment and characterization of a new drug surviving cell line Am1010, derived directly from muscle metastases of a human lung adenocarcinoma patient with multi-drug-resistance to cisplatin, taxol, and gefitinib. Acta Pharmacol Sin *31*: 601-608, 2010.

10 Oliver TG, Mercer KL, Sayles LC, Burke JR, Mendus D, Lovejoy KS, Cheng MH, Subramanian A, Mu D, Powers S, Crowley D, Bronson RT, Whittaker CA, Bhutkar A, Lippard SJ, Golub T, Thomale J, Jacks T and Sweet-Cordero EA: Chronic cisplatin treatment promotes enhanced damage repair and tumor progression in a mouse model of lung cancer. Genes Dev *24*: 837-852, 2010.

11 Baselga J, Trigo JM, Bourhis J, Tortochaux J, Cortés-Funes H, Hitt R, Gascón P, Amellal N, Harstrick A and Eckardt A: Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck . J Clin Oncol *24*: 5568-5577, 2005.

12 Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC and Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci USA *1*: 3070-3074, 1922.

13 Kelly SL, Basu A, Teicher BA, Hacker MP, Hamer DH and Lazo JS: Overexpression of metallothionein confers resistance to anticancer drugs. Science *241*: 1813-1815, 1998.

14 Hall MD, Okabe M, Shen DW, Liang XJ and Gottesman MM: The role of cellular accumulation in determining sensitivity to platinum-based chemotherapy. Annu Rev Pharmacol Toxicol *48*: 495-535, 2008.

15 Peklak-Scott C, Smitherman PK, Townsend AJ and Morrow CS: Role of glutathione S-transferase P1-1 in the cellular detoxification of cisplatin. Mol Cancer Ther *7*: 3247-3255, 2008.

16 Frisch SM and Francis H: Disruption of epithelial cell matrix interactions induces apoptosis. J Cell Biol *124*: 619-626, 1994.

17 Frisch SM and Screaton RA: Anoikis mechanisms. Curr Opin Cell Biol *13*: 555-562, 2001.

18 Grossmann J: Molecular mechanisms of detachment induced apoptosis-anoikis. Apoptosis *7*: 247-260, 2002.

19 Mehlen P and Puisieux A: Metastasis: a question of life or death. Nat Rev Cancer *6*: 449-458, 2006.

20 Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y and Chanvorachote P: Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin degradation. Am J Physiol Cell Physiol *300*: C235-245, 2011.

21 Terence MW and Michael PL: Caveolin-1 in oncogenic transformation, cancer, and metastasis. Am J Physiol Cell Physiol *288*: 494-506, 2005.

22 Di Vizio D, Sotgia F, Williams TM, Hassan GS, Capozza F, Frank PG, Pestell RG, Loda M, Freeman MR and Lisanti MP: Caveolin-1 is required for the upregulation of fatty acid synthase (FASN), a tumor promoter, during prostate cancer progression. Cancer Biol Ther *6*: 1263-1268, 2007.

23 Nasu Y, Timme TL and Yang G: Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. Nat Med *4*: 1062-1064, 1988.

24 Yang G, Truong LD, Timme TL, Ren C, Wheeler TM, Park SH, Nasu Y, Bangma CH, Kattan MW, Scardino PT and Thompson TC: Elevated expression of caveolin is associated with prostate and breast cancer. Clin Cancer Res *4*: 1873-1880, 1988.

25 Thompson TC: Metastasis-related genes in prostate cancer: the role of caveolin-1. Cancer Metastasis Rev *17*: 439-442, 1999.

26 Ho CC, Huang PH, Huang HY, Chen YH, Yang PC and Hsu SM: Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol *161*: 1647-1656, 2002.

27 Suzuoki M, Miyamoto M and Kato K: Impact of caveolin-1 expression on prognosis of pancreatic ductal adenocarcinoma. Br J Cancer *87*: 1140-1144, 2002.

28 Sies H: Oxidative stress: oxidants and antioxidants. Exp Physiol *82*: 291-295, 1997.

29 Polytarchou C, Hatziapostolou M and Papadimitriou E: Hydrogen peroxide stimulates proliferation and migration of human prostate cancer cells through activation of activator protein-1 and up-regulation of the heparin affin regulatory peptide gene. J Biol Chem *280*: 40428-40435, 2005.

30 Halliwell B: Biochemistry of oxidative stress. Biochem Soc Trans *35*: 1147-1150, 2007.

31 Klaunig JE, Kamendulis LM and Hocevar BA: Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol *38*: 96-109, 2010.

32 Brown NS and Bicknell R: Hypoxia and oxidative stress in breast cancer: oxidative stress its effects on the growth, metastatic potential and response to therapy of breast cancer. Breast Cancer Res *3*: 323-327, 2001.
33 Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, Nakada K, Honma Y and Hayashi J: ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science *320*: 661-664, 2008.
34 Nishikawa M, Hashida M and Takakura Y: Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. Adv Drug Deliv Rev *61*: 319-326, 2009.
35 Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H and Mural M: Role of reactive oxygen species in *cis*-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. Br J Cancer *76*: 206-210, 1997.
36 Schweyer S, Soruri A, Heintze A, Radzun HJ and Fayyazi A: The role of reactive oxygen species in cisplatin-induced apoptosis in human malignant testicular germ cell lines. Int J Oncol *25*: 1671-1676, 2004.
37 Seve P and Dumontet C: Chemoresistance in non-small cell lung cancer. Curr Med Chem Anticancer Agents *5*: 73-88, 2005.
38 Berndtsson M, Hagg M, Panaretakis T, Havelka AM, Shoshan MC and Linder S: Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. Int J Cancer *120*: 175-180, 2006.
39 Nishio K, Nakamura T, Koh Y, Suzuki T, Fukumoto H and Saijo N: Drug resistance in lung cancer. Curr Opin Oncol *11*: 109-115, 1999.
40 Hoffman PC, Mauer AM and Vokes EE: Lung cancer. Lancet *355*: 479-485, 2000.
41 Socinski MA: Cytotoxic chemotherapy in advanced nonsmall cell lung cancer: a review of standard treatment paradigms. Clin Cancer Res *10*: 4210s-4214s, 2004.
42 Wang L, Chanvorachote P, Toledo D, Stehlik C, Mercer RR, Castranova V and Rojanasakul Y: Peroxide is a key mediator of *BCL2* down-regulation and apoptosis induction by cisplatin in human lung cancer cells. Mol Pharmacol *73*: 119-127, 2007.
43 Chanvorachote P, Pongrakhananon V, Wannachaiyasit S, Luanpitpong S, Rojanasakul Y and Nimmannit U: Curcumin sensitized lung cancer cells to cisplatin-induced apoptosis through superoxide anion induction and Bcl-1 degradation. Cancer Invest *27*: 624-635, 2009.
44 Pongjit K and Chanvorachote P: Caveolin-1 sensitizes cisplatin-induced lung cancer cell apoptosis *via* superoxide anion-dependent mechanism. Mol Cell Biochem *358*: 365-373, 2011.
45 Lavie Y and Liscovitch M: A role for caveolae in multidrug resistance of cancer cells. Mol Biol Cell *8*: 207, 1997.
46 Lavie Y, Fiucci G and Liscovitch M: Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. J Biol Chem *273*: 32380-32383, 1998.
47 Shatz M and Liscovitch M: Caveolin-1 and cancer multidrug resistance: Coordinate regulation of pro-survival proteins? Leuk Res *28*: 907-908, 2004.
48 Choi BK, Choi CH, Oh HL and Kim YK: Role of ERK activation in cisplatin-induced apoptosis in A172 human glioma cells. Neurotoxicol *25*: 915-924, 2004.
49 Jiang M, Wei Q, Pabla N, Dong G, Wang CY, Yang T, Smith SB and Dong Z: Effects of hydroxyl radical scavenging on cisplatin-induced p53 activation, tubular cell apoptosis and nephrotoxicity. Biochem Pharmacol *73*: 1499-1510, 2007.

Received March 8, 2012
Revised April 3, 2012
Accepted April 4, 2012

ANTICANCER RESEARCH *32*: 483-490 (2012)

Caveolin-1 Attenuates Hydrogen Peroxide-induced Oxidative Damage to Lung Carcinoma Cells

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Abstract. *Background: Oxidative stress has been shown to play an important role in cancer progression. In lung cancer, increasing expression of caveolin-1 (Cav-1) has been found in both primary and metastatic carcinomas and may be critical in the regulation of the oxidative status of cancer cells. Materials and Methods: Using molecular and pharmacological manipulations, the role of Cav-1 in regulating cellular oxidative status in lung cancer cells was investigated. The level of Cav-1 was determined by western blot analysis and reactive oxygen species (ROS) were detected by specific fluorescence probes. Results: The treatment of lung cancer H460 cells with hydrogen peroxide (H_2O_2) significantly up-regulated ROS inside the cells and contributed to cell apoptosis. While cells stably transfected with Cav-1 overexpressing plasmids (H460/Cav-1) exhibited decreased ROS signal and attenuated cell death rate, shRNACav-1 transfected (H460/shCav-1) cells showed enhanced ROS signal and increased cell damage. The use of specific superoxide anion and the hydrogen peroxide detecting assays and hydroxyl radical inhibition assay indicated that the variable oxidative stress found in these cells was mainly due to the alteration of the cellular hydroxyl radical level. Conclusion: A novel role of Cav-1 protein is the suppression of cellular oxidative stress induced by H_2O_2.*

Abbreviations: CAT, catalase; Cav-1, caveolin-1; $DCFH_2$-DA, dichlorofluorescein diacetate; DFO, deferoxamine; DHE dihydroethidium; GSH, glutathione; H_2O_2, hydrogen peroxide; HPF, 3'-(p-hydroxyphenyl) fluorescein; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; PI, propidium iodide.

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Key Words: Caveolin-1, hydrogen peroxide, lung cancer, oxidative stress, reactive oxygen species, H460 cells.

Although reactive oxygen species (ROS) are widely accepted to be important mediators of normal cell processes, when in excess, they cause oxidative stress implicated in the damage of cellular components and subsequently cell death (1-3). Such cell and tissue damage has been shown to be associated with several pathological conditions (4-6). In cancer, ROS are considered as carcinogens since evidence has indicated their roles in facilitating carcinogenesis and tumor progression (5, 7-10), and they also affect cancer cell survival and behavior (11, 12). Chronic inflammation as found in the environments of several human carcinomas including lung cancer, is likely to be a major cause of increased ROS in such microenvironments (5, 13). Indeed, ROS, including superoxide anions, hydrogen peroxide (H_2O_2) and singlet oxygen, are released from inflammatory cells of the innate immune system, and cause oxidative damage to surrounding cancer cells (13, 14). Several studies have documented high ROS levels in the lung of lung cancer patients (15, 16).

In certain types of tumor including lung cancer, an up-regulation of caveolin-1 (Cav-1), a major protein component of caveolae, has been observed (17, 18) and the Cav-1 protein was shown to mediate anoikis resistance and other aggressiveness behaviors such as migration and invasion of cancer cells (19-21). Moreover, the expression of Cav-1 has been related to poor prognosis in lung (22) and prostate carcinomas (23, 24). Cav-1 has been shown to function as a scaffolding protein (25) and to regulate certain proteins such as Src-like kinases, endothelial nitric oxide, and H-Ras (26-30). Although the contributions of Cav-1 protein on cancer progression and metastasis have been intensively investigated and reported, information regarding the role of Cav-1 in controlling redox status and in oxidative stress-induced damage is still limited.

Among key ROS presenting in physiological and pathological conditions, H_2O_2 has gained the most attention since it is relatively stable in comparison to other principle ROS and can pass through biological membranes and spread in tissues (31, 32). In cancer microenvironments, an increase of ROS, especially H_2O_2, has been well recognized (16, 33).

Although the role of H_2O_2 in cancer cell viability either in promoting cell survival or inducing cell death is controversial, many researchers believe that H_2O_2 at basal level and low concentrations could favor cell proliferation and viability (2, 8), whereas at high doses, H_2O_2 could induce cell damage through the induction of intracellular oxidative stress (33, 34). According to this concept, cancer cells immerging in H_2O_2-rich microenvironments may possess mechanisms which allow them to survive.

Cav-1 protein could play a role in modulating the cellular oxidative condition. The present study investigated the role of Cav-1 protein in H_2O_2-induced cell death in human lung carcinoma H460 cells, using molecular and pharmacological manipulations.

Materials and Methods

Cells and reagents. Human non-small cell lung cancer H460 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin in a 5% CO_2 environment at 37°C. *N*-acetylcysteine (NAC), reduced glutathione (GSH), sodium pyruvate, H_2O_2, catalase (CAT), Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), propidium iodide (PI), Hoechst 33342, 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and deferoxamine (DFO) were obtained from Sigma Chemical Inc. (St. Louis, MO, USA); dihydroethidium (DHE) and 3'-(p-hydroxyphenyl) fluorescein (HPF) from Molecular Probes, Inc (Eugene, OR, USA); antibody for Cav-1 and peroxidase-conjugated secondary antibody from Abcam (Cambridge, MA, USA) and the transfecting agent Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA).

Plasmid and transfection. The Cav-1 overexpressed (H460/Cav-1) and Cav-1 knockdown (H460/shCav-1) cells were established by transfection of the H460 cells with Cav-1 plasmids obtained from the American Type Culture Collection and Cav-1 knockdown plasmid shRNACav-1 obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Briefly, 60% confluent cells were transfected with 15 μl of Lipofectamine 2000 reagent and 2 μg of Cav-1, shRNACav-1 or mock control plasmids. After 16 h, the medium was replaced with culture medium containing 10% fetal bovine serum. Approximately 3 days after the beginning of transfection, the single cell suspensions were plated onto 75 ml culture flasks and cultured for 60 days with an antibiotic selection. The expression of Cav-1 in the transfectants was quantified by western blot analysis.

ROS detection. Intracellular ROS were determined using the ROS-specific probe, $DCFH_2$-DA, superoxide anions were determined by DHE, and hydroxyl radicals were determined by HPF. The cells were incubated with 10 μM of $DCFH_2$-DA, HPF or DHE for 30 min at 4°C, after which they were washed and immediately analyzed for fluorescence intensity by fluorescence microplate reader (SpectraMax M5, Molecular Devices Corp., Sunnyvale, CA, USA) using a 480-nm excitation beam and a 530-nm band-pass filter for detecting DCF fluorescence, using a 490-nm excitation beam and a 515-nm band-pass filter for HPF or using a 488-nm excitation beam and a 610-nm band-pass filter for DHE, and visualized under a fluorescence microscope (Eclipse Ti-U, Nikon, Tokyo, Japan).

Cytotoxicity assay. To determine H_2O_2-mediated cytotoxicity, cell viability was determined by MTT assay. After the specified treatment, H460, H460/Cav-1 and H460/shCav-1 cells in 96-well plates were incubated with 500 μg/ml of MTT for 4 h at 37°C. The intensity of formazan product was measured at 570 nm using a microplate reader. All the analyses were performed in at least three independent replicate cultures. The absorbance ratio of treated to non-treated cells was calculated and presented as relative cell viability.

Apoptosis and necrosis assay. Apoptotic and necrotic cell death was determined by Hoechst 33342 and PI co-staining. After the specified treatments, the cells were incubated with 10 μM Hoechst and 5 μg/ml PI dye for 30 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

Western blotting. Cell lysates were obtained by incubating the cells in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 60 min on ice. The protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and equal protein samples (30 μg) were heated at 95°C for 5 min with Laemmli loading buffer. Then, the lysates were loaded on 10% SDS-polyacrylamide gel for electrophoresis. After separation, the proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20) and incubated with the anti-Cav-1 antibodies at 4°C for 10 h. The membranes were washed twice with TBST for 15 min and incubated with horseradish peroxidase–coupled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhanced chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad).

Statistical analysis. The mean data from at least three independent experiments were normalized to the results of the non-treated control. Statistical differences between the means were determined using an analysis of variance (ANOVA) and *post hoc* test at a significance level of $p<0.05$, and presented as the mean±S.D.

Results

Effect of hydrogen peroxide on oxidative stress and cell death. Subconfluent (90%) monolayers of H460 cells were treated with H_2O_2 (0-200 μM) for various times and ROS levels were analyzed using $DCFH_2$-DA as the probe. Figure 1a and b show that H_2O_2 exposure caused a dose-dependent increase in cellular DCF fluorescence intensity as early as 10 min after the start of treatment and a steady cellular ROS signal was observed after 70 min. While the control non-treated H460

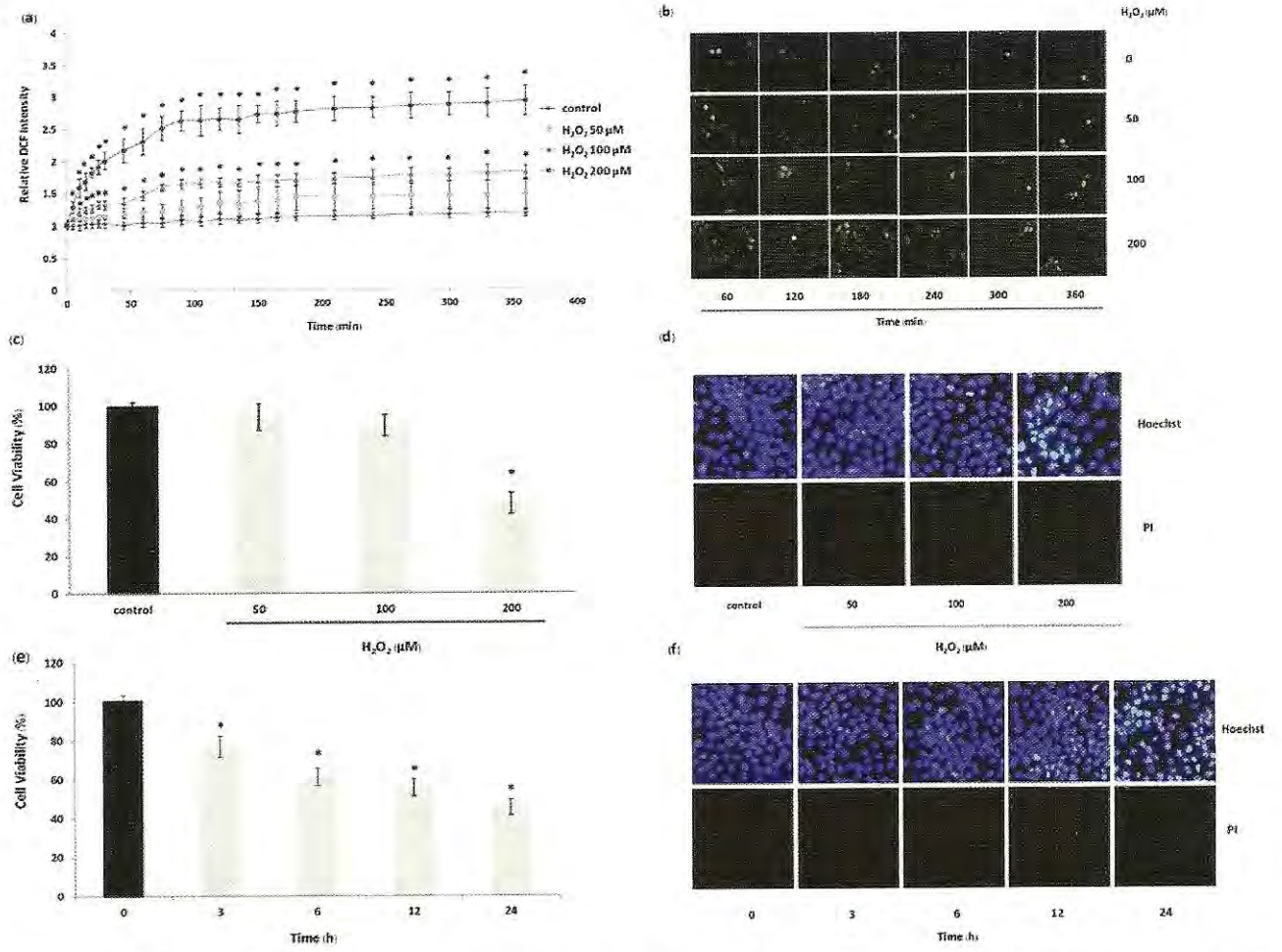

Figure 1. *Effect of H_2O_2 in lung carcinoma H460 cells. (a) Cellular ROS level after treatment with H_2O_2. (b) Cellular DCF signals visualized by fluorescence microscope. (c) Cell death determined by MTT assay after 24 h treatment and (d) Nuclear morphology of H_2O_2-treated and control cells stained with the Hoechst 33342 and PI dye. (e) Time-dependent effect of 200 μM H_2O_2 on cell death and (f) Hoechst and PI staining assays. Data points represent the mean±S.D. (n=3). *p<0.05 versus non-treated control.*

cells exhibited no significant change in cellular ROS content throughout the time of detection, treatment with 100-200 μM H_2O_2 caused significantly up-regulated intracellular ROS signals. Notably, the treatment with 200 μM H_2O_2 exhibited a dramatic increase in the ROS signal with approximately 2.75-fold induction in cellular ROS compared to a non-treated control. The MTT assay showed that this H_2O_2 dose caused a significant reduction of cell survival with decreasing cell viability as early as 3 h after treatment and the reduction further continued until 24 h with approximately 50% of the cells remaining viable (Figure 1c and e). An analysis of cell apoptosis by Hoechst 33342 nuclear staining assay further revealed that the decrease of cell viability was due to apoptosis, as indicated by the increasing number of cells with condensed nuclear fluorescence and nuclear fragmentation (Figure 1d and f), whereas PI-positive cells, indicating necrotic cells, were not detected under these conditions.

Specific reactive oxygen species up-regulation in response to hydrogen peroxide treatment. The H460 cells were pretreated with pan- or specific-antioxidants, namely NAC, GSH, catalase, sodium pyruvate, DFO or MnTBAP for 1 h followed by H_2O_2 treatment (200 μM) for 24 h. The treatment with NAC and GSH, pan-antioxidants, significantly preserved viability of the H_2O_2-treated H460 cells (Figure 2a). The H_2O_2-scavengers catalase and sodium pyruvate and the hydroxyl radical inhibitor DFO also significantly prevented the cytotoxic effect of H_2O_2, whereas MnTBAP, a superoxide dismutase mimetic, had no protective effect. Confirmation studies using the $DCFH_2$-DA probe for ROS evaluation further showed a decrease of oxidative signal in all the antioxidant pre-treated cells excepted for the MnTBAP treated cells (Figure 2b).

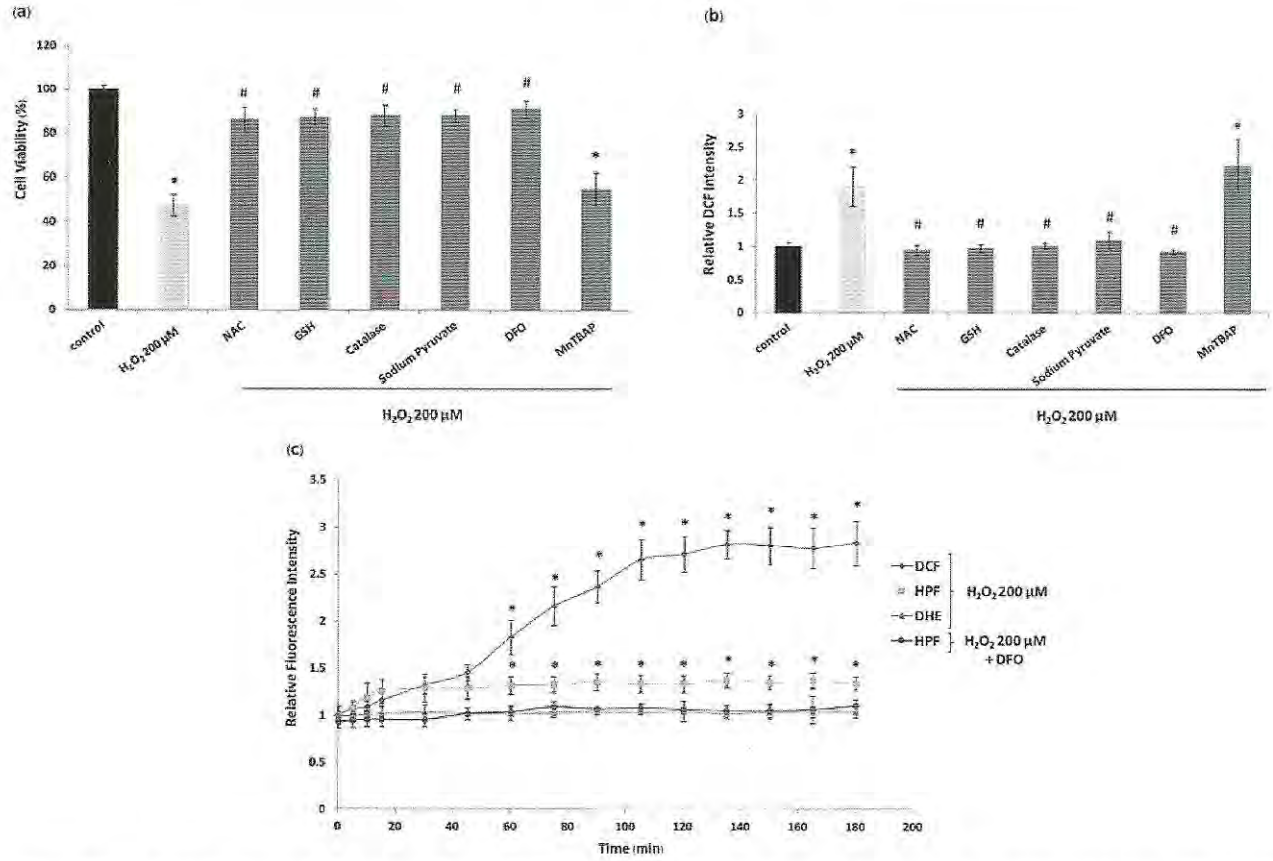

Figure 2. *Effects of antioxidants on H_2O_2-induced effects in H460 cells. H460 cells were left untreated or pre-treated with N-acetylcysteine (NAC 1 mM), glutathione (GSH 1 mM), catalase (5,000 units/ml), sodium pyruvate (1 mM), deferoxamine (DFO 1 mM) or Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP 50 μM) prior to 200 μM H_2O_2 treatment. (a) Cell viability after 24 h incubation. (b) Intracellular ROS levels 1 h post-treatment (optimal ROS signal) by $DCFH_2$-DA. (c) Cells treated with H_2O_2 (200 μM) in the presence or absence of DFO (1 mM) and analyzed for cellular ROS using $DCFH_2$-DA, superoxide anions using DHE, or hydroxyl radicals using HPF probes. Data points represent the mean±S.D. (n=3). *p<0.05 versus non-treated control; #p<0.05 versus 200 μM H_2O_2-treated cells.*

Importantly, administration of DFO prior to H_2O_2 could solely protect cell damage and completely prevented the induction of a DCF signal in response to H_2O_2. When the cells were treated with H_2O_2 in the presence of $DCFH_2$-DA for ROS detection, DHE for superoxide anion detection or HPF for hydroxyl radical detection, significantly increased in DCF and HPF signals occurred, but no effect on DHE signal was found (Figure 2a).

Effect of caveolin-1 on hydrogen peroxide-induced oxidative stress and cell death. Western blot analysis of Cav-1 expression in the stably transfected H460 cells showed a substantial increase in Cav-1 level in the H460/Cav-1 cells and significantly reduced Cav-1 level in the H460/shCav-1 cells (Figure 3a). When the cells were treated with 200 μM H_2O_2, a significantly higher ROS signal was found in the H460/shCav-1 cells in comparison to the parental H460 cells, while the H460/Cav-1 cells had a lower ROS signal compared to the H460 and H460/shCav-1 cells (Figure 3b and c). H460, H460/Cav-1 and H460/shCav-1 cells were also treated with H_2O_2 in the presence of the hydroxyl radical probe and as expected, the H460/shCav-1 cells exhibited the highest level of hydroxyl radical signal, whereas the H460/Cav-1 cells expressed the lowest level (Figure 3d).

To substantiate the role of Cav-1 in the regulation of oxidative stress-induced damage to these cells, H460, H460/Cav-1 and H460/shCav-1 cells were treated with 200 μM H_2O_2, and cell viability was verified by MTT assay after 24 h. The Cav-1 transfected cells exhibited a higher survival rate, while the shRNACav-1 transfected cells showed a lower percentage of cell survival over time as compared with the parental H460 cells (Figure 3e). At 24 h post-treatment, the H460/Cav-1 cells exhibited ~85% viability, whereas both the H460 and H460/shCav-1 cells showed a survival rate of <50%.

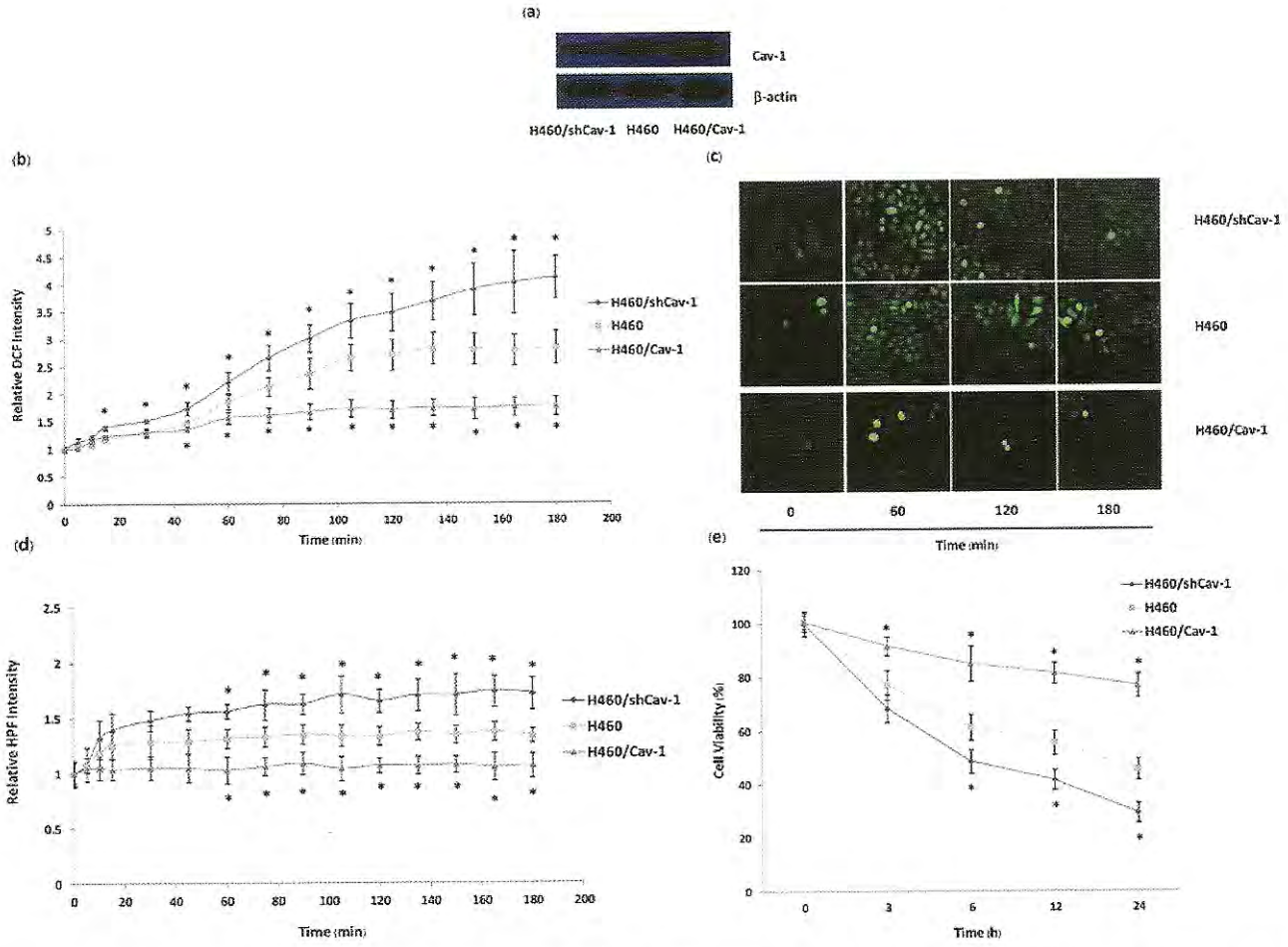

Figure 3. *Effect of caveolin-1 expression on oxidative stress and cell death. (a) Cav-1 protein expression by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. (b) H460, H460/Cav-1, and H460/shCav-1 cells treated with 200 μM H_2O_2 and analyzed for ROS signal by $DCFH_2$-DA over time and (c) cellular ROS signal detected by fluorescence microscope. (d) Cellular hydroxyl radical levels determined after 200 μM H_2O_2 treatment using HPF probe. (e) Cell survival determined using MTT assay. Data points represent the mean±S.D. (n=3). *p<0.05 versus control cells.*

Effect of deferoxamine and iron on H_2O_2-mediated oxidative stress and cell death. Figure 4 shows that treatment of the H460, H460/Cav-1 and H460/shCav-1 cells with 200 μM H_2O_2 for 24 h caused a significant decrease in cell survival in all the cells. The H460/shCav-1 cells exhibited the highest reduction in viability rate, whereas the H460/Cav-1 cells expressed the lowest rate of cell death. The addition of the hydroxyl radical inhibitor DFO significantly protected against cell death induced by H_2O_2 in all the cells. These observations were confirmed by the treatment of the cells with iron, which dramatically enhanced the cell death response to H_2O_2 in all the cells. Also, $DCFH_2$-DA and HPF probes were used for the detection of cellular ROS and hydroxyl radicals, respectively. Treatment of the H460, H460/Cav-1 and H460/shCav-1 cells with DFO successfully inhibited both ROS and hydroxyl radical inductions caused by H_2O_2 in all the cells (Figure 5a and c), whereas the treatment of these cells with iron caused a dramatic increase in both ROS (Figure 5b) and hydroxyl radical levels (Figure 5d).

Discussion

Cellular oxidative stress as well as cytotoxicity caused by H_2O_2 exposure were attenuated in Cav-1 overexpressing lung cancer cells while shRNA-mediated Cav-1 down-regulated cells were highly susceptible to H_2O_2-induced cell damage. It is well documented that H_2O_2 can be detoxified to water by cellular antioxidant enzymes including glutathione peroxidase and catalase (1, 35), however, in the presence of reduced transition metal ions such as iron or copper, H_2O_2 can be rapidly converted to highly reactive hydroxyl radicals

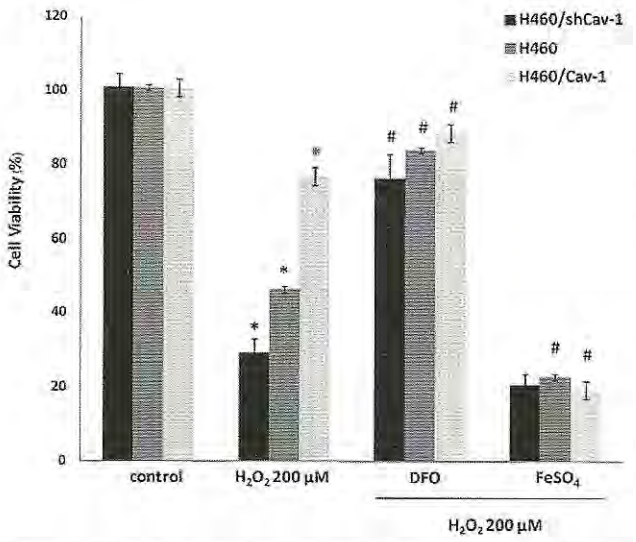

Figure 4. *Effect of hydroxyl radical modulators on viability of H_2O_2-treated cells. H460, H460/Cav-1, and H460/shCav-1 cells treated for 24 h with 200 μM H_2O_2 in the presence or absence of deferoxamine (DFO, 1 mM) or ferrous sulfate (50 μM). Cell viability was determined by MTT assay. Columns are mean±S.D. (n=3). *p<0.05 versus non-treated control; #p<0.05 versus respective H_2O_2-treated cells.*

through Fenton-like reactions (1, 36).

The H_2O_2 treatment of the cells in the present study resulted in an induction of cellular ROS, namely H_2O_2 and hydroxyl radicals. Increasing evidence has indicated different roles of specific ROS in regulation of cell behaviors (2, 8, 33, 37). Our previous experiments indicated that superoxide anions and H_2O_2 inhibited lung cancer cell migration and invasion whereas hydroxyl radicals had opposite effects (20). Also, endogenous H_2O_2, but not hydroxyl radicals could render cancer cells resistant to detachment-induced apoptosis (21). Previous experiments indicated that H_2O_2 generated in response to cisplatin treatment mediated renal cell necrosis, whereas hydroxyl radicals played a principle role in apoptosis induction (37). This finding was consistent with the present observation that blocking of hydroxyl radical formation by DFO pre-treatment dramatically inhibited H_2O_2-induced cell death in the H460 cells (Figure 4).

The cells stably transfected with Cav-1 overexpressing plasmids, showed a marked increase of Cav-1 level over the one of the parental H460 cells and the shRNA-transfected cells showed a dramatically reduced Cav-1 level (Figure 3a). Furthermore, Cav-1 functioned in attenuating the H_2O_2-derived hydroxyl radicals in the H460 cells and reduced H_2O_2-mediated death. In some studies, Cav-1 has been shown to sensitize cancer cells to apoptosis in response to death stimuli and a reduction of Cav-1 level has been shown to contribute to chemotherapeutic cisplatin and carboplatin resistance (38). Furthermore, we have recently reported that Cav-1 sensitizes cisplatin-induced cell death through superoxide anion induction (39). In contrast, the present study revealed that only minimal change in the cellular superoxide anion level occurred in response to H_2O_2 treatment (Figure 2c) and Cav-1 overexpression was able to protect against cell death. Although further investigations are necessary to clarify the mechanism(s) by which Cav-1 possess such opposite effects on the specific ROS mechanisms of cells, the present study has revealed for the first time that Cav-1 could attenuate the oxidative stress induced by H_2O_2.

In conclusion, Cav-1 modifies cellular oxidative stress induced by H_2O_2 treatment and renders non-small cell lung cancer cell resistant to apoptosis. Since sustained or elevated Cav-1 expression and H_2O_2 level could be concomitantly found in certain carcinomas, especially lung cancer, these findings may help providing better understanding of cancer biology.

Acknowledgements

This work was supported by Thailand Research Fund (Chanvorachote P.), research grant from the Faculty of Pharmacentical Sciences, and Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej (Suchaoin W.). The authors wish to thank Mr. Krich Rajprasit (Srinakharinwirot University) and Ms. Kanittha Pongjit.

References

1 Valko M, Izakovic M, Mazur M, Rhodes CJ and Telser J: Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem *266*: 37-56, 2004.

2 Burdon RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med *18*: 775-794, 1995.

3 Droge W: Free radicals in the physiological control of cell function. Physiol Rev *82*: 47-95, 2002.

4 Hunt CR, Sim JE, Sullivan SJ, Featherstone T, Golden W, Von Kapp-Herr C, Hock RA, Gomez RA, Parsian AJ and Spitz DR: Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. Cancer Res *58*: 3986-3992, 1998.

5 Klaunig JE, Kamendulis LM and Hocevar BA: Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol *38*: 96-109, 2010.

6 Madamanchi NR, Vendrov A and Runge MS: Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol *25*: 29-38, 2005.

7 Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS and Lambeth JD: Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Natl Acad Sci USA *99*: 715-720, 2002.

8 Polytarchou C, Hatziapostolou M and Papadimitriou E: Hydrogen peroxide stimulates proliferation and migration of human prostate cancer cells through activation of activator protein-1 and up-regulation of the heparin affin regulatory peptide gene. J Biol Chem *280*: 40428-40435, 2005.

9 Dreher D and Junod AF: Role of oxygen free radicals in cancer development. Eur J Cancer *32A*: 30-38, 1996.

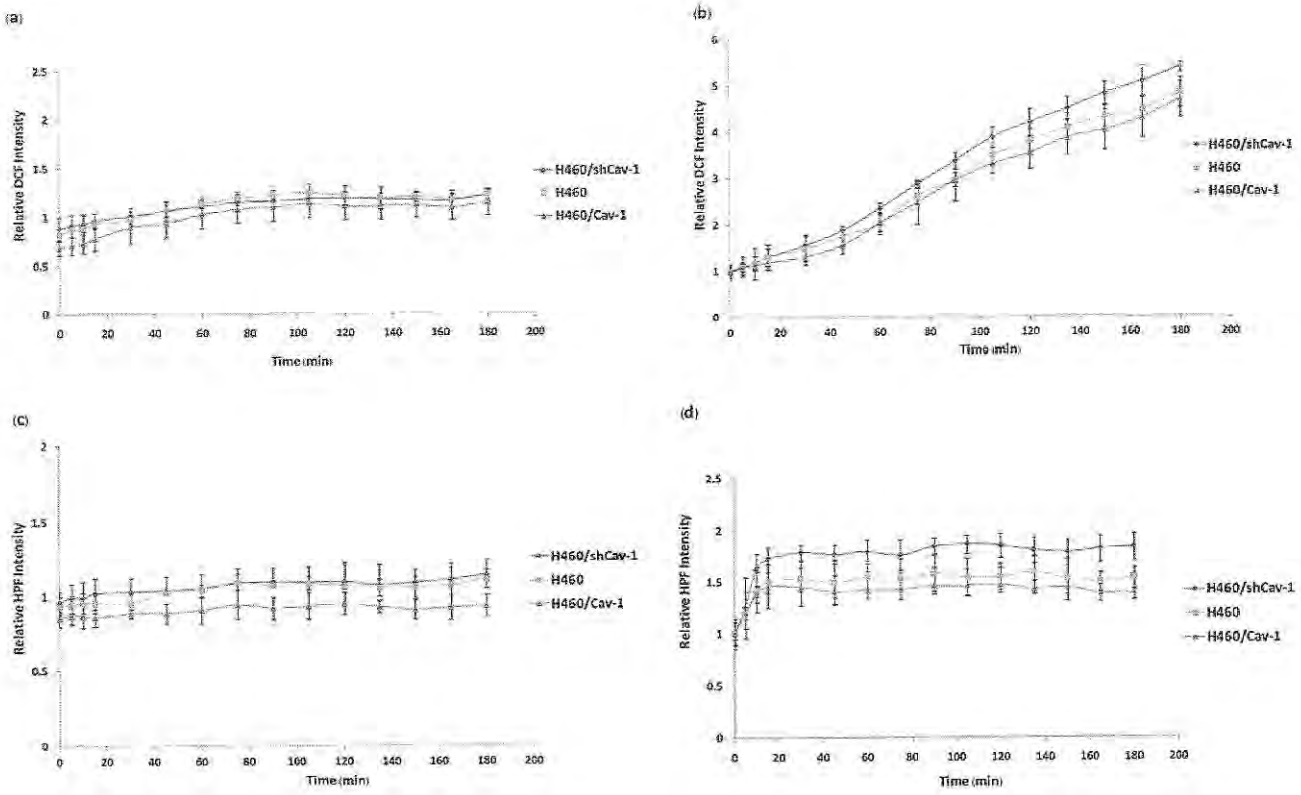

Figure 5. *Effect of hydroxyl radical modulators on cellular ROS and hydroxyl radical levels. H460, H460/Cav-1, and H460/shCav-1 cells were treated with 200 μM H_2O_2 in the presence of deferoxamine (DFO, 1 mM) (a and c) or ferrous sulfate (50 μM) (b and d) and cellular ROS and hydroxyl radical levels were detected using $DCFH_2$-DA (a and b) and HPF probes (c and d), respectively. Data are the mean±S.D. (n=3).*

10 Nakamura Y, Gindhart TD, Winterstein D, Tomita I, Seed JL and Colburn NH: Early superoxide dismutase-sensitive event promotes neoplastic transformation in mouse epidermal JB6 cells. Carcinogenesis *9*: 203-207, 1988.

11 del Bello B, Paolicchi A, Comporti M, Pompella A and Maellaro E: Hydrogen peroxide produced during gamma-glutamyl transpeptidase activity is involved in prevention of apoptosis and maintainance of proliferation in U937 cells. FASEB J *13*: 69-79, 1999.

12 Giannoni E, Fiaschi T, Ramponi G and Chiarugi P: Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. Oncogene *28*: 2074-2086, 2009.

13 Coussens LM and Werb Z: Inflammation and cancer. Nature *420*: 860-867, 2002.

14 Ames BN, Gold LS and Willett WC: The causes and prevention of cancer. Proc Natl Acad Sci USA *92*: 5258-5265, 1995.

15 Misthos P, Katsaragakis S, Milingos N, Kakaris S, Sepsas E, Athanassiadi K, Theodorou D and Skottis I: Postresectional pulmonary oxidative stress in lung cancer patients. The role of one-lung ventilation. Eur J Cardiothorac Surg *27*: 379-382, 2005.

16 Chung-man Ho J, Zheng S, Comhair SA, Farver C and Erzurum SC: Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res *61*: 8578-8585, 2001.

17 Sunaga N, Miyajima K, Suzuki M, Sato M, White MA, Ramirez RD, Shay JW, Gazdar AF and Minna JD: Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. Cancer Res *64*: 4277-4285, 2004.

18 Ho CC, Huang PH, Huang HY, Chen YH, Yang PC and Hsu SM: Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol *161*: 1647-1656, 2002.

19 Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang BH and Rojanasakul Y: Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. J Biol Chem *284*: 28476-28484, 2009.

20 Luanpitpong S, Talbott SJ, Rojanasakul Y, Nimmannit U, Pongrakhananon V, Wang L and Chanvorachote P: Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. J Biol Chem *285*: 38832-38840, 2010.

21 Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y and Chanvorachote P: Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. Am J Physiol Cell Physiol *300*: C235-C245, 2010.

22 Moon KC, Lee GK, Yoo SH, Jeon YK, Chung JH, Han J and Chung DH: Expression of caveolin-1 in pleomorphic carcinoma of the lung is correlated with a poor prognosis. Anticancer Res *25*: 4631-4637, 2005.

23 Karam JA, Lotan Y, Roehrborn CG, Ashfaq R, Karakiewicz PI and Shariat SF: Caveolin-1 overexpression is associated with aggressive prostate cancer recurrence. Prostate *67*: 614-622, 2007.
24 Tahir SA, Yang G, Ebara S, Timme TL, Satoh T, Li L, Goltsov A, Ittmann M, Morrisett JD and Thompson TC: Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. Cancer Res *61*: 3882-3885, 2001.
25 Sargiacomo M, Scherer PE, Tang Z, Kubler E, Song KS, Sanders MC and Lisanti MP: Oligomeric structure of caveolin: implications for caveolae membrane organization. Proc Natl Acad Sci USA *92*: 9407-9411, 1995.
26 Garcia-Cardena G, Oh P, Liu J, Schnitzer JE and Sessa WC: Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. Proc Natl Acad Sci USA *93*: 6448-6453, 1996.
27 Li S, Couet J and Lisanti MP: Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem *271*: 29182-29190, 1996.
28 Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I and Lisanti MP: Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J Biol Chem *270*: 15693-15701, 1995.
29 Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG and Michel T: Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. J Biol Chem *271*: 6518-6522, 1996.
30 Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M and Lisanti MP: Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem *271*: 9690-9697, 1996.
31 Bienert GP, Schjoerring JK and Jahn TP: Membrane transport of hydrogen peroxide. Biochim Biophys Acta Biomembr *1758*: 994-1003, 2006.
32 Ohno Y and Gallin JI: Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. J Biol Chem *260*: 8438-8446, 1985.
33 Miguel L-L: Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. Cancer Lett *252*: 1-8, 2007.
34 Mao Y, Song G, Cai Q, Liu M, Luo H, Shi M, Ouyang G and Bao S: Hydrogen peroxide-induced apoptosis in human gastric carcinoma MGC803 cells. Cell Biol Int *30*: 332-337, 2006.
35 Li S, Yan T, Yang JQ, Oberley TD and Oberley LW: The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res *60*: 3927-3939, 2000.
36 Lloyd RV, Hanna PM and Mason RP: The origin of the hydroxyl radical oxygen in the Fenton reaction. Free Radic Biol Med *22*: 885-888, 1997.
37 Baek SM, Kwon CH, Kim JH, Woo JS, Jung JS and Kim YK: Differential roles of hydrogen peroxide and hydroxyl radical in cisplatin-induced cell death in renal proximal tubular epithelial cells. J Lab Clin Med *142*: 178-186, 2003.
38 David JS: Mechanisms of resistance to cisplatin and carboplatin. Crit Rev Oncol Hematol *63*: 12-31, 2007.
39 Pongjit K and Chanvorachote P: Caveolin-1 sensitizes cisplatin-induced lung cancer cell apoptosis via superoxide anion-dependent mechanism. Mol Cell Biochem *358*: 365-373, 2011.

Received November 20, 2011
Revised December 21, 2011
Accepted December 23, 2011

Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells

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Am J Physiol Cell Physiol 302:C1284-C1292, 2012. First published 25 January 2012; doi:10.1152/ajpcell.00318.2011

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Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells

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[1]*Pharmaceutical Technology (International) Program;* [2]*Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; and* [3]*Department of Pharmaceutical Sciences, West Virginia University, Morgantown, West Virginia*

Submitted 26 August 2011; accepted in final form 21 January 2012

Chunhacha P, Pongrakhananon V, Rojanasakul Y, Chanvorachote P. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. *Am J Physiol Cell Physiol* 302: C1284–C1292, 2012. First published January 25, 2012; doi:10.1152/ajpcell.00318.2011.—Both caveolin-1 (Cav-1) and Mcl-1 have been implicated in the regulation of cancer cell anoikis, but their relationship and underlying mechanisms of regulation are not known. The present study demonstrated for the first time that Cav-1 regulates Mcl-1 through protein-protein interaction and inhibits its downregulation during cell anoikis in human lung cancer cells. Immunoprecipitation and immunocytochemistry studies showed that Cav-1 interacted with Mcl-1 and prevented it from degradation via the ubiquitin-proteasome pathway. Mcl-1 and Mcl-1-Cav-1 complex were highly elevated in Cav-1-overexpressing cells but were greatly reduced in Cav-1 knockdown cells. Consistent with this finding, we found that Mcl-1 ubiquitination was significantly attenuated by Cav-1 overexpression but increased by Cav-1 knockdown. Together, our results indicate a novel role of Cav-1 in anoikis regulation through Mcl-1 interaction and stabilization, which provides a new insight to the pathogenesis of metastatic lung cancer and its potential treatment.

lung cancer; anoikis resistance; metastasis; myeloid cell leukemia-1

RESISTANCE TO ANOIKIS, a form of apoptotic cell death induced by loss of cell anchorage to extracellular matrixes, has been accepted as a key determinant of cancer cell metastasis (1, 4). Recently, a number of proteins have been identified to facilitate anoikis resistance in various cancer types. Among these, caveolin-1 (Cav-1) has perhaps received the most attention since its expression has been linked to cancer progression and aggressiveness (27). Although some evidence has suggested a tumor suppressing role of Cav-1 (6, 11, 22), in lung cancer, Cav-1 potentiates cancer progression and aggressiveness. Cav-1 expression has been shown to relate to poor prognosis and reduced tumor-free periods in lung cancer patients (10). Moreover, Cav-1 was shown to facilitate metastasis and induce anoikis resistance in lung carcinoma cell lines (2, 23, 29). Not only does Cav-1 play a role in cell death and survival, it also plays a role in cell migration (17), invasion (26), and lipid transportation (21). Cav-1 was reported to exhibit scaffold function and to be essential in regulating several proteins such as endothelial nitric oxide synthase (eNOS), G protein subunit, and nonreceptor tyrosine kinases (14), supporting the wide range of activities of this protein in various cellular processes.[1]

The prosurvival member of Bcl-2 family protein named myeloid cell leukemia sequence 1 (Mcl-1) has recently been implicated as a key regulator of cell anoikis (24). In melanoma, the depletion of Mcl-1 renders mutant B-RAF melanoma cells sensitive to anoikis (1). Likewise, Mcl-1 degradation and Bim upregulation are a critical determinant of anoikis initiation in wild-type and c-Src-transformed NIH3T3 fibroblast cells. This protein is degraded through the ubiquitin-proteasomal pathway after cell detachment (28). Increasing evidence also indicates the role of Mcl-1 in progressive prostate cancer (31), supporting its clinical significance in cancer metastasis.

The objective of the present study was to investigate the possible relationship between Cav-1 and Mcl-1 and their regulation of anoikis in lung cancer cells. The hypothesis of this study is that Cav-1 mediates its effect on cancer cell anoikis through Mcl-1 regulation. Using gene overexpression and knockdown strategies, we demonstrate this relationship and elucidate the important role of Cav-1 in regulating Mcl-1 through protein interaction and stabilization, thus revealing the existence of a novel mechanism of anoikis regulation which could be important in cancer metastasis.

MATERIALS AND METHODS

Cells and reagents. Non-small-cell lung cancer (NSCLC)-H460 cells and melanoma G361 cells were obtained from American Type Culture Collection (Manassas, VA). H460 cells were cultured in RPMI 1640 medium, while G361 cells were cultured in DMEM medium. RPMI 1640 was supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. DMEM was supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. All cell cultures were incubated in a 5% CO_2 environment at 37°C. Lactacystin, MG 132, and dimethysulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO); propidium iodide (PI) and Hoechst 33342 were from Molecular Probes, (Eugene, OR); rabbit Cav-1 antibody, rabbit Mcl-1 antibody, mouse monoclonal ubiquitin antibody, mouse monoclonal Cav-1 antibody, and peroxidase-conjugated secondary antibody were from Abcam (Cambridge, MA); MitoTracker Red CMXRos, Alexa Fluor 350 goat anti-mouse IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L), and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Antibody for ubiquitin, protein G-agarose bead, and β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids and transfection. The Cav-1 expression plasmid [pEX_Cav-1-yellow fluorescent protein (YFP)] and control plasmid (pDS_XB-YFP) were obtained from American Type Culture Collection; the Mcl-1 expression plasmid 25375:pCDNA3.1-hMcl-1 was obtained from Addgene (Cambridge, MA); Cav-1 knockdown plasmid [Cav-1 short hairpin (sh)RNA plasmid] and control plasmid (control shRNA plasmid A) were obtained from Santa Cruz Biotechnology. Stable transfection of cells with Cav-1 expression plasmid or Cav-1 knockdown plasmid was performed by culturing H460 cells in a six-well plate until they reached ~60% confluence. Lipofectamine reagent (15 μl) and 2 μg of Cav-1, shRNA-Cav-1, or control plasmids were used to transfect the cells in the absence of serum. After 12 h, the medium was replaced with culture medium containing 5% FBS. Approximately 36 h after the beginning of transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24 to 28 days with G418

[1] This article is the topic of an Editorial Focus by Pamela G. Lloyd (16a).

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selection (600 μg/ml). The stable transfectants were pooled and the expression of Cav-1 protein in the transfectants was determined by Western blotting. The cells were cultured in antibiotic-free RPMI 1640 medium for at least two passages before being used in each experiment.

Anoikis assay. For anoikis evaluation, six-well tissue culture plates were coated with 200 μl (6 mg/ml in 95% ethanol) of poly 2-hydroxyethylmethacrylate (poly-HEMA; Sigma) and left for 10 h in a laminar flow hood. Cells in a single-cell suspension were seeded in poly-HEMA-coated plates at a density of 1×10^5 cells/ml and incubated for various times up to 24 h at 37°C. Cells were harvested, washed, and incubated with 20 μM 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) for 4 h at 37°C. Optical density was then determined using V-max photometer (Molecular Devices, Menlo Park, CA) at a 450-nm wavelength. Absorbance ratio of treated to nontreated cells was calculated and is presented as relative cell viability. For Hoechst 33342 and PI assays, cells were incubated with 10 μM Hoechst 33342 or 15 μM PI for 30 min at 37°C. Apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were scored under a fluorescence microscope (Olympus IX51 with DP70).

Western blot analysis. After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris·HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland) for 30 min on ice. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad, Hercules, CA). Equal amount of proteins of each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and were subsequently loaded on 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST [25 mM Tris·HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20] and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced with chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL) and quantified using analyst/PC densitometry software (Bio-Rad).

Immunoprecipitation. Cells were washed after treatment and lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14,000 *g* for 15 min at 4°C, the supernatants were collected and determined for protein content. Cell lysates were normalized, and equal amounts of protein per sample (60 μg) were incubated with anti-Cav-1 antibody conjugated to protein G plus-agarose beads (Santa Cruz) for 6 h at 4°C. The immune complexes were washed five times with ice-cold lysis buffer, resuspended in 2 × Laemmli sample buffer, and boiled at 95°C for 5 min. Immune complexes were separated by 10% SDS-PAGE and detected for Cav-1 and Mcl-1 complexes by Mcl-1 antibody. For detection of the ubiquitin-Mcl-1 complex, the anti-Mcl-1 antibody was incubated with the cell lysate in the immunoprecipitation step followed by Western blot analysis using anti-ubiquitin antibody.

Quantitative real-time RT-PCR. One microgram of TRIzol-extracted RNA was reverse-transcribed in a 100-μl reaction mixture containing 500 μM dNTP, 125 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), 40 units of RNase inhibitor, 2.5 μM oligo(dT), 1 × TaqMan reverse transcriptase buffer, and 5 mM $MgCl_2$ at 48°C for 40 min. The primers for *Mcl-1* (Hs03043899_m1*) and 18s rRNA (Hs99999901_s1) were obtained from Applied Biosystems. Amplification was performed at the following cycling conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A SYBR Green PCRMasterMix (Applied Biosystems) was used with 1 ng of cDNA and with 100–400 nM primers. A negative control without any cDNA template was run with every assay. All PCR reactions were performed by using ABI PRISM7900 Sequence Detection System (Applied Biosystems). Relative mRNA levels were determined by using the comparative C_T (threshold cycle) method (16), where the Mcl-1 target is normalized to the control and compared with a reference sample (assigned a relative value of 1) by the equation: $2^{-\Delta\Delta CT}$.

Immunofluorescence. Cells (0.5×10^6/well) were seeded in six-well plates for 24 h to allow the cell to completely adhere to the surface. Then, the cells were fixed in 3.7% formaldehyde for 10 min at room temperature and were then permeabilized and blocked in a solution containing 0.5% saponin, 1% FBS, and 1.5% goat serum for 30 min. After primary antibody incubation with both Cav-1 mouse monoclonal antibody (Abcam) at 1:100 dilution and Mcl-1 rabbit polyclonal antibody (Abcam) at 1:100 dilution for 1 h, cells were washed and incubated together with Alexa Fluor 350 goat anti-mouse IgG (H+L) conjugated secondary antibody (Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) conjugated secondary antibody (Invitrogen) for 30 min. Mitochondria were stained with MitoTracker Red CMXRos (Invitrogen). Cells were cytospun onto a glass slide and mounted using the anti-fade reagent Fluoromont-G (Southern Biotech, Birmingham, AL). Images were acquired by confocal laser scanning microscopy (Zeiss LSM 510).

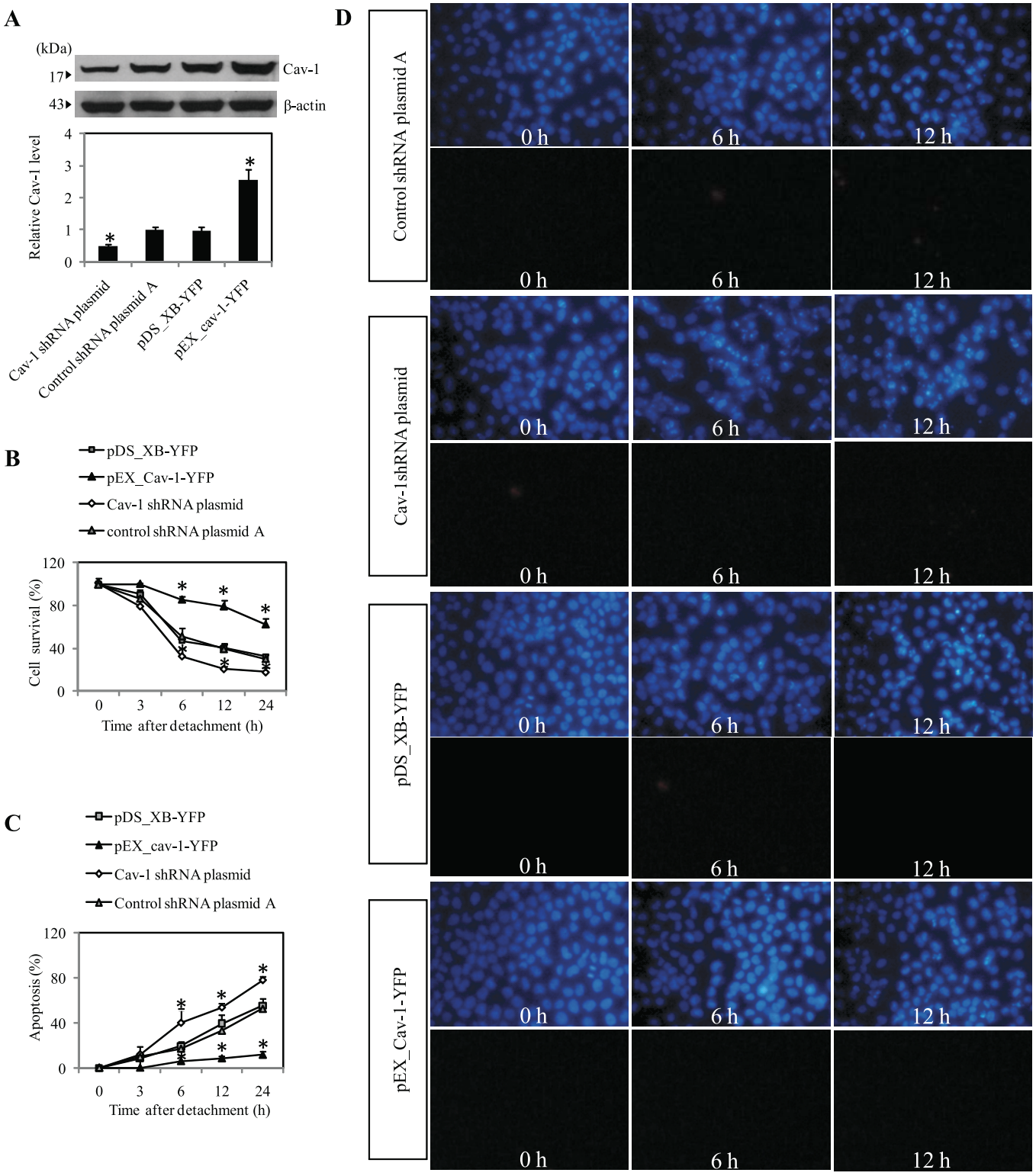
Statistical analysis. Mean data from independent experiments were normalized with control treatment groups. All of the experiments were repeated at least three times. A statistical analysis between treatments versus control was verified by Student's *t*-test. The strength of relationships, correlation coefficient (*r*), between each protein level after detachment was determined with SPSS software (version 16; SPSS, Chicago, IL). $P < 0.05$ was considered as statistically significant.

RESULTS

Caveolin-1 inhibits anoikis of H460 cells. We and others have previously reported the role of Cav-1 in anoikis regulation in various cell types (7, 23). To assure the role of this protein in anoikis regulation of the test cell system, we first characterized the effect of different ectopic Cav-1 expression levels on cell anoikis of H460 cells. Through stable gene transfection,

Fig. 1. Caveolin-1 (Cav-1) overexpression increases anoikis resistance in H460 cells. *A*: control, HCav-1 [expression plasmid (pEX)_Cav-1 plasmid transfectant H460], or short hairpin (sh)Cav-1-transfected H460 cells were constructed and grown in culture that was then analyzed for Cav-1 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells. *B*: subconfluent (90%) monolayers of control transfected, Cav-1-overexpressing cells, and Cav-1 knockdown cells were detached and suspended in poly-HEMA-coated plates for various times (0–24 h). At the indicated times after detachment, the cells were collected and their survival was determined by XTT assay. Viability of detached cells at *time 0* was considered as 100%. *C*: percentage of cell detachment-induced apoptosis was analyzed by Hoechst 33342 nuclear fluorescence. Data points represent means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells. *D*: detachment-induced apoptosis and necrosis in control transfected cells, Cav-1-overexpressing, and Cav-1 knockdown cells. Detached cells were suspended in poly-HEMA-coated plates for 0–12 h, and cell apoptosis and necrosis were determined by Hoechst 33342 and propidium iodide (PI) fluorescence measurements, respectively. pDS_XB-YFP, yellow fluorescent protein Cav-1 control plasmid.

we generated Cav-1-overexpressing (HCav-1) cells, shRNA knockdown (shCav-1) cells, and vector (pDS_XB-YFP and control shRNA plasmid A) control cells, as described in MATERIALS AND METHODS. These mutant clones were analyzed for Cav-1 expression by Western blotting (Fig. 1*A*). To study anoikis, Cav-1 overexpressed, Cav-1 knockdown, and vector control cells were detached and incubated in adhesion-resistant poly-HEMA-coated plates. Cell survival was then determined at various times by XTT assay. Analysis of cell viability showed that detachment of the cells caused a time-dependent



decrease in cell survival, with approximately 80%, 50%, and 30% of the overexpressed, vector control, and knockdown Cav-1 cells, respectively, remaining viable after 6 h (Fig. 1*B*). At 24 h postdetachment, HCav-1 cells exhibited ~60% viability, whereas both control and shCav-1 cells showed a survival rate of <40%. Control experiments, in which cells were allowed to attach in normal tissue culture plates, showed no significant change in cell viability over the 24-h test period (data not shown). Analysis of cell apoptosis by Hoechst 33342 assay showed that shCav-1 cells were most susceptible to apoptosis induced by cell detachment, whereas HCav-1 cells were least susceptible (Fig. 1*C*). This finding is consistent with the cell viability data showing the highest rate of survival of HCav-1 cells after detachment. Morphological analyses of apoptotic and necrotic cell death by Hoechst 33342 and PI assays showed that apoptosis was the primary mode of cell death induced by cell detachment in H460 cells (Fig. 1*D*).

Cell detachment induces Cav-1 and Mcl-1 downregulation. The role of Cav-1 and Mcl-1 in cancer cell anoikis is unclear. To provide evidence for the role of these proteins, we evaluated the expression profiles of Cav-1 and Mcl-1 after cell detachment in lung cancer H460 cells. The cells were detached, suspended in adhesion-resistant plates, and analyzed for Cav-1 and Mcl-1 protein expression by Western blotting. Figure 2, *A* and *B*, shows that after cell detachment, both Cav-1 and Mcl-1 expression gradually decreased over time concomitant with cell viability and death, suggesting their potential relationship and role in anoikis regulation. Like Cav-1, the role of Mcl-1 in anoikis regulation was studied using stable gene transfection. Figure 2*C* shows that stably transfected Mcl-1 (HMcl-1) cells expressed a high level of Mcl-1 protein as compared with vector-transfected control cells. The HMcl-1 cells also showed resistance to anoikis as indicated by their increased viability after cell detachment over control cells (Fig. 2*D*).

Mcl-1 downregulation during cell anoikis is regulated by Cav-1 interaction. Cav-1 has been shown to function as a scaffold protein regulating the stability and function of several proteins (14). The observations that both Cav-1 and Mcl-1 have a similar effect on anoikis and their expression is similarly downregulated during anoikis suggest the possible linkage and shared mechanism of anoikis regulation. Since Mcl-1 is recognized as a relatively short half-life protein (19) and its scaffolding interaction with Cav-1 has not been reported, we explored their possible interaction by generating a correlation plot between Cav-1 and Mcl-1 expression during cell anoikis (Fig. 3*A*). Not only did the reduction of these proteins correlate well with the induction of cell anoikis, but the plot also revealed a highly correlated profile of Cav-1 and Mcl-1 downregulation with the correlation coefficient of 0.98.

Next, we used immunoprecipitation techniques to determine the direct interaction between the two proteins. Cell lysates of HCav-1, shCav-1, and H460 cells were then prepared, immunoprecipitated using Cav-1 antibody, and analyzed for Cav-1-Mcl-1 complex by Western blotting using Mcl-1 antibody as a probe. The results showed that Cav-1-Mcl-1 complex formation was most pronounced in HCav-1 cells, which express the highest level of Cav-1, and least expressed in Cav-1 knockdown (shCav-1) cells (Fig. 3*B*). Moreover, consistent results were observed in the immunoprecipitation experiment using Mcl-1 antibody followed by Western blot analysis using Cav-1 antibody. These results suggest that Cav-1 plays a role in scaffolding Mcl-1 protein and that its interaction with Mcl-1 may play a role in regulating Mcl-1 level. To provide supporting evidence for the Cav-1 and Mcl-1 interaction, immunocytochemical studies were performed to evaluate the intracellular localization of the two proteins. Figure 3*C* shows immunofluorescent staining of Mcl-1 and Cav-1, which are strikingly similar and supportive of the protein colocalization.

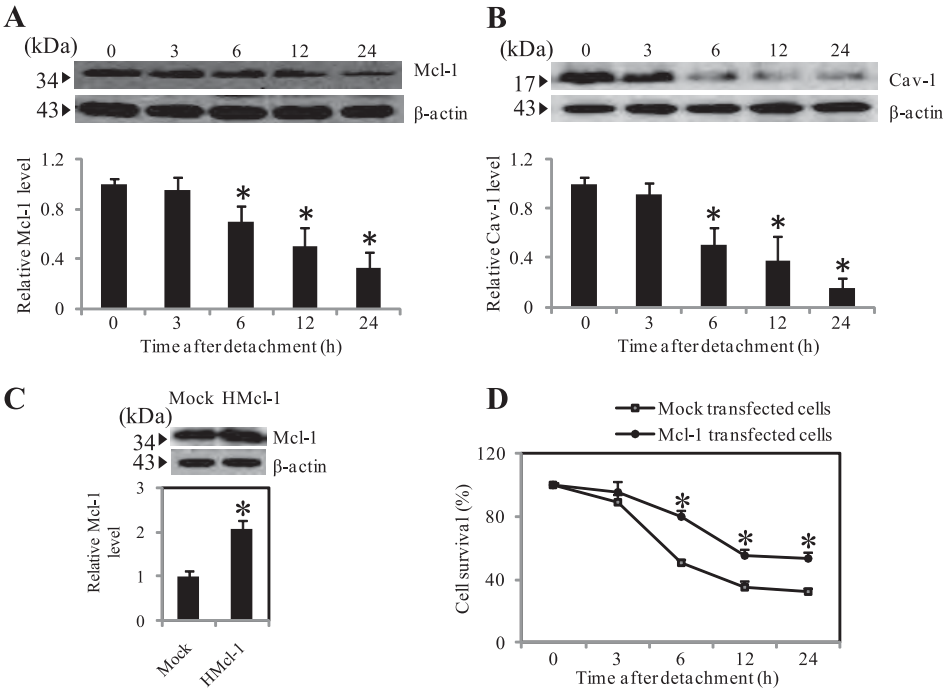


Fig. 2. Cav-1 and myeloid cell leukemia sequence 1 (Mcl-1) expression after cell detachment. *A* and *B*: H460 cells were detached and suspended in poly-HEMA-coated plates for various times (0–24 h). Blots were probed with antibodies specific to Mcl-1 and Cav-1 and were reprobed with β-actin antibody. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control at *time 0*. *C*: mock and HMcl-1 cells were grown in culture and analyzed for Mcl-1 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells. *D*: subconfluent (90%) monolayers of Mock and HMcl-1 cells were detached and suspended in poly-HEMA-coated plates for various times (0–24 h). At the indicated times, the cells were collected and their survival was determined by XTT assay. Viability of detached cells at *time 0* was considered as 100%. Data points represent means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells.

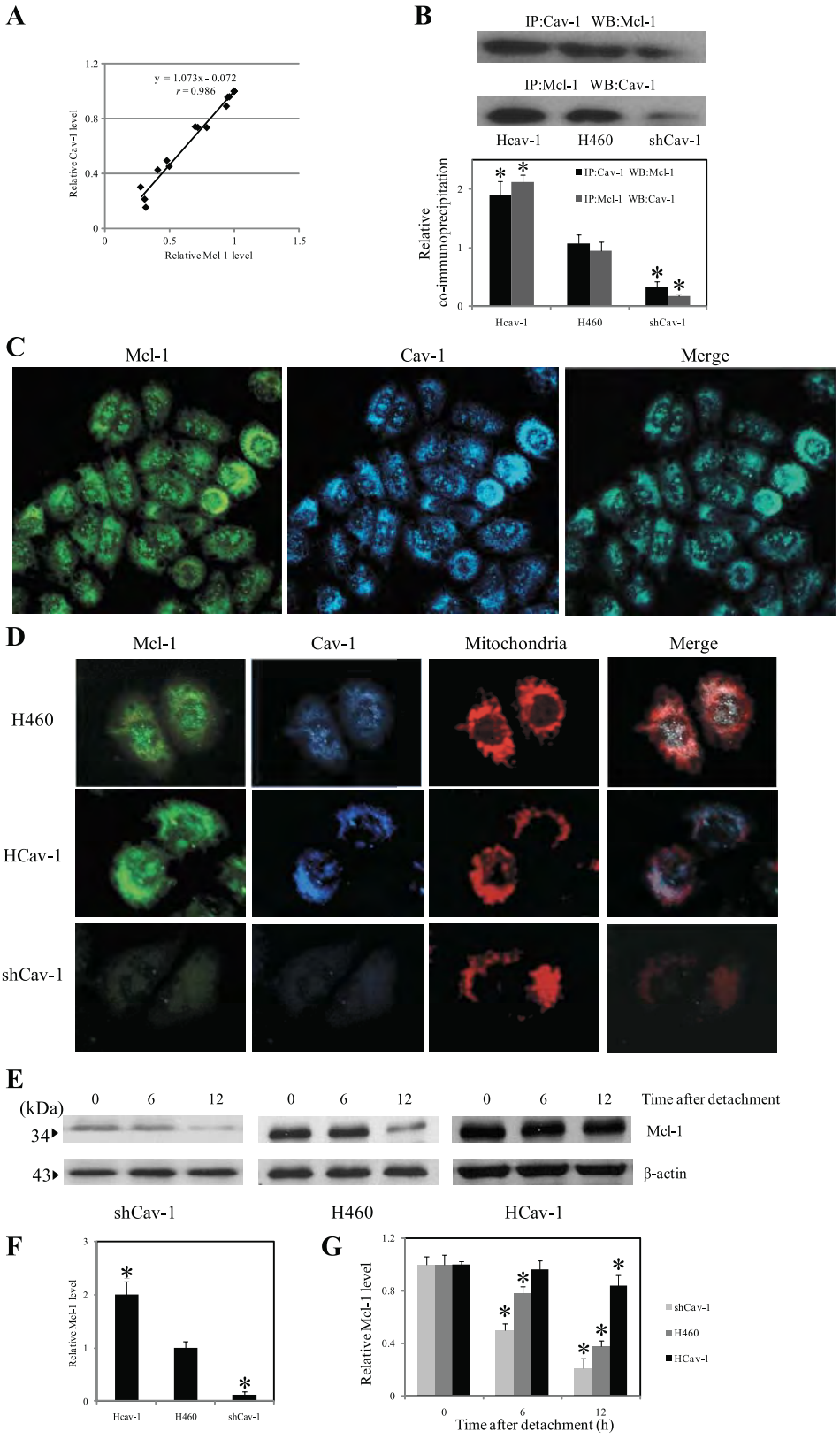


Fig. 3. Interaction and localization of Cav-1 and Mcl-1. *A*: correlation analysis of the expression of Cav-1 and Mcl-1 after detachment of H460 cells. *B*: immunoprecipitation (IP) experiments were performed using specific anti-Cav-1 antibody; immunoblots were probed with anti-Mcl-1 antibody and vice versa. Equal amounts of protein (25 μg) were loaded in each lane. WB, Western blotting. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells. *C*: H460 cells were analyzed for localization of Cav-1 and Mcl-1 by immunofluorescence microscopy. Immunofluorescence was performed using mouse anti-Cav-1 monoclonal antibody and rabbit anti-Mcl-1 polyclonal antibody, followed by appropriate secondary antibodies labeled with Alexa Fluor 350 and Alexa Fluor 488 to visualize Cav-1 and Mcl-1, respectively. Cells were also stained with MitoTracker Red CMXRos (300 nM) to aid visualization of mitochondria. *D*: differential expression of Cav-1 and Mcl-1 in HCav-1, shCav-1, and H460 cells. Cells were fixed and processed for immunofluorescence staining. *E*: dependence of Mcl-1 reduction after cell detachment on Cav-1 expression. HCav-1, shCav-1, and H460 cells were detached and suspended in poly-HEMA-coated plates for various times (0–12 h). Blots were probed with specific antibody to Mcl-1 and were reprobed with β-actin antibody to confirm equal loading of samples. *F*: relative Mcl-1 levels in attached cells. *G*: relative Mcl-1 levels in shCav-1, HCav-1, and H460 cells after detachment for 0, 6, and 12 h. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control at detachment time = 0 h.

Cav-1 stabilizes Mcl-1 in H460 cells. Having shown that Cav-1 interacts with Mcl-1, we further investigated whether such interaction is essential for Mcl-1 stability after cell detachment. Adhered HCav-1, shCav-1, and H460 cells were stained with antibodies for Mcl-1, Cav-1, and MitoTracker, and their fluorescent signals were observed by microscopy. While the MitoTracker signals are relatively constant in these cells, the intensities of Cav-1 and Mcl-1 signals in these cells vary greatly (Fig. 3*D*). Interestingly, cells that express a high level of Cav-1 (HCav-1) also exhibit a high level of Mcl-1, while those that express a low level of Cav-1 (shCav-1) also show a low level of Mcl-1, suggesting the stabilizing effect of Cav-1 on Mcl-1. To further study this effect, HCav-1, shCav-1, and H460 cells were detached and incubated in adhesion-resistant plates for 0–12 h. Western blot analysis of Mcl-1 was then performed at 0, 6, and 12 h postdetachment. Figure 3*E* shows that at various times of the detachment, Mcl-1 levels in these cells varied depending on the expression levels of Cav-1 in each cell type. These findings strengthen the above finding that Cav-1 interacts with Mcl-1 and stabilizes the protein under different attachment conditions.

Mcl-1 reduction after cell detachment is mediated through ubiquitin-proteasomal degradation. Although Mcl-1 has been reported to be degraded via the proteasomal pathway (28), we suspected both transcription and degradation to play a role in Mcl-1 downregulation during cell anoikis. To test this, we performed quantitative real-time RT-PCR and proteasome inhibition studies in detached H460 cells. Mcl-1 mRNA level was significantly reduced as early as 1 h (data not shown) and remained unchanged up to 24 h after detachment (Fig. 4*A*). This finding excluded the possibility that Cav-1 could stabilize Mcl-1 through a transcription-dependent mechanism. Therefore, we tested the involvement of ubiquitin-proteasomal system on Mcl-1 downregulation after cell detachment. Figure 4*B* shows that cell detachment caused a substantial reduction in Mcl-1 protein level and that treatment of the cells with specific proteasomal inhibitors, lactacystin and MG132, completely inhibited the Mcl-1 reduction. These results indicate that Mcl-1 downregulation after cell detachment is mediated mainly by the proteasome degradation pathway.

Cav-1 stabilizes Mcl-1 by attenuating Mcl-1 ubiquitination. Proteasomal degradation of a protein is triggered by protein ubiquitination. To test the potential involvement of ubiquitination in Mcl-1 stability and its regulation by Cav-1, Mcl-1 immunoprecipitation and ubiquitination studies were performed in various Cav-1 expressing cells. In normal H460 cells, the formation of ubiquitin-Mcl-1 complexes gradually increased as early as 1 h after cell detachment and peaked at ~6 h (Fig. 5*A*). The level of ubiquitin-Mcl-1 complex formation was minimal in Cav-1 overexpressing (HCav-1) cells and maximal in Cav-1 knockdown (shCav-1) cells as compared with normal H460 cells (Fig. 5*B*). These results indicate that Cav-1 attenuated the ubiquitination of Mcl-1 and stabilized the protein after cell detachment.

Cav-1 regulates Mcl-1 expression and anoikis in human melanoma G361 cells. An upregulation of Cav-1 and Mcl-1 has been found not only in non-small-cell lung cancer but also in other forms of cancer such as human melanoma (13, 20). To test whether Cav-1 might have a similar regulatory role on Mcl-1 and anoikis in other cancer cells, melanoma G361 cells were stably transfected with Cav-1, shCav-1, or control plasmids. After clonal selection, the cells were analyzed for Mcl-1, Cav-1, and anoikis. Figure 6*A* shows that the Cav-1 transfected cells (G361-Cav-1) expressed the highest level of Cav-1 protein, whereas the shCav-1 transfected cells (G361-shCav-1) exhibited the lowest level.

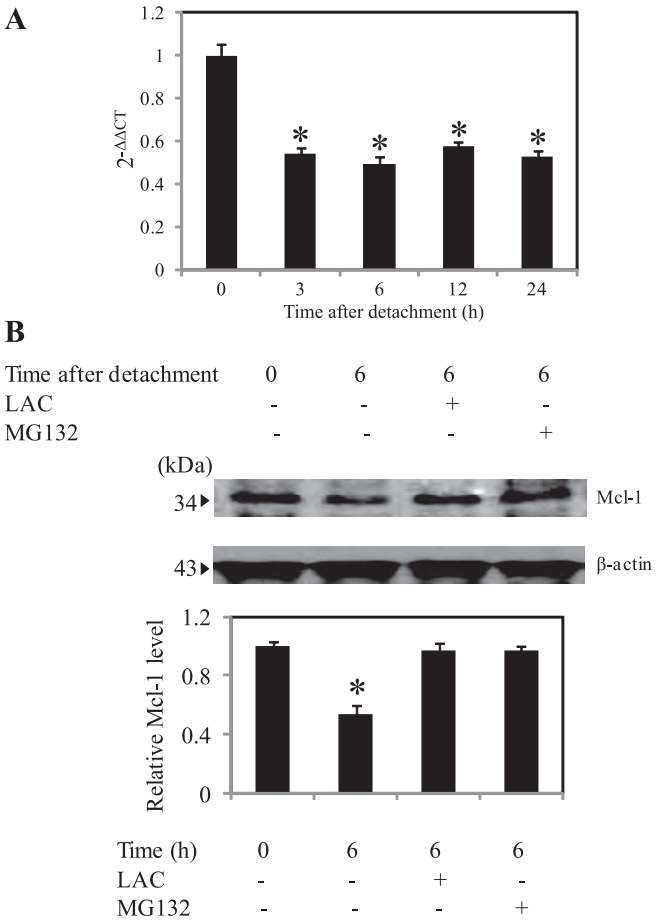


Fig. 4. Transcription and degradation of Mcl-1 after cell detachment. *A*: real-time PCR analysis of Mcl-1 mRNA expression after cell detachment. The relative mRNA expression was determined by using the comparative C_T method as described in MATERIALS AND METHODS. Columns are means ± SD ($n = 3$). *$P <$ 0.05 vs. control at detachment time = 0 h. *B*: relative Mcl-1 expression after detachment for 0–6 h in the presence or absence of lactacystin (LAC; 20 μM) or MG132 (10 μM) in H460 cells. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control at detachment time = 0 h.

To test the interaction between Cav-1 and Mcl-1, cells were harvested at *time 0* and at 6 h after detachment. Cell lysates were then prepared and analyzed for Mcl-1 expression by Western blotting. The results show that the levels of Mcl-1 in G361-Cav-1, G361-shCav-1, and G361 cells at 0 and 6 h postdetachment were highly dependent on the cellular level of Cav-1 in each cell (Fig. 6*B*). Cell anoikis studies also show that Cav-1 functioned as an anoikis inhibitor as evidenced by the inhibitory effect of Cav-1 overexpression and the promoting effect of Cav-1 knockdown on cell anoikis (Fig. 6*C*). The above results are consistent with the earlier findings in H460 cells and indicate the general role of Cav-1 in anoikis and Mcl-1 regulation.

DISCUSSION

When cells are detached from the extracellular matrix, the loss of anchorage-related signals results in an abrogation of

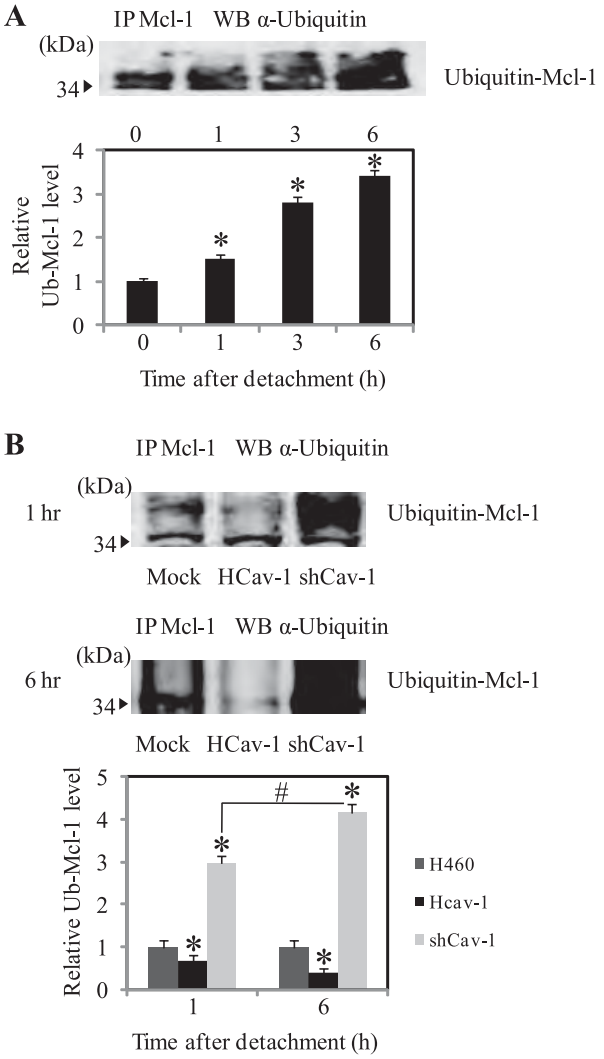


Fig. 5. Effect of Cav-1 expression on Mcl-1 ubiquitination. *A*: H460 cells were detached and suspended in poly-HEMA-coated plates for various times. Cell lysates were prepared and immunoprecipitated (IP) with anti-Mcl-1 antibody. The resulting immune complexes were analyzed for ubiquitin by Western blotting (WB) using anti-ubiquitin antibody. Maximum Mcl-1 ubiquitination was observed at 6 h after cell detachment. The immunoblot signals were quantified by densitometry. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control at detachment time = 0 h. *B*: HCav-1, shCav-1, and H460 cells were detached and suspended in poly-HEMA-coated plates for 1 and 6 h. Cell lysates were immunoprecipitated with anti-Mcl-1 antibody, and the resulting immune complexes were analyzed for ubiquitin (Ub) by Western blotting. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected at detachment time = 1 h; #$P < 0.05$ vs. the indicated control.

certain cellular processes such as cell survival and growth (12) and consequently initiates the process of anoikis (8). Since anoikis is an important cellular event controlling cancer metastasis, unraveling its underlying mechanisms is critical to the understanding of disease pathogenesis and its treatment. Among the many types of cancer, lung cancer has frequently been found to metastasize at the time of tumor detection. While the exact mechanisms of cancer metastasis have been extensively investigated, an upregulation of Mcl-1 (25) and Cav-1 (23) has been implicated in lung cancer aggressiveness and progression. Mcl-1 was found to overexpress in NSCLC cells and regulate their survival and sensitivity to diverse apoptotic stimuli (25). Apoptotic stimuli such as cell detachment induce Bim (activator of BH3-only protein) expression (3). Recently, Zhang et al. (30) have demonstrated that Mcl-1 can sequester Bim in NSCLC cells which supports the role of Mcl-1 in attenuating anoikis in this cancer cell type. Moreover, amplification or overexpression of Mcl-1 was shown to render cells resistant to detachment-induced apoptosis (24) and the decrease in Mcl-1 level is required in the initiation of cell anoikis (1, 28). Previously, we and others have shown that Cav-1 confers resistance to anoikis in cancer cells (7, 23). Furthermore, the expression of Cav-1 has been used as a biomarker for virulence of some cancers (5). The role of Cav-1 in cancer cell anoikis has been described in many ways such as the induction of survival pathways (15) and the reduction of Cav-1 and Mcl-1 (23, 28). We further demonstrated in this study that, during cell anoikis, Cav-1 and Mcl-1 reduction was tightly correlated. Cav-1 functioned as a scaffolding protein for Mcl-1 binding as demonstrated by immunoprecipitation studies (Fig. 3*B*). In addition, the Cav-1-Mcl-1 complex significantly increased in the Cav-1 overexpressing (HCav-1) cells, but decreased in the Cav-1 knockdown (shCav-1) cells. Immunocytochemistry studies further confirmed the colocalization of Cav-1 and Mcl-1 in the cells, which was largely associated with the mitochondria (Fig. 3*D*).

Since Mcl-1 is known to be a short half-life protein due to continuous proteasomal degradation (28), it is possible that its interaction with Cav-1 could affect its stability, which was first demonstrated in this study. Although a rapid decline in Mcl-1 mRNA level was observed at 1 h postdetachment, the mRNA level remained relatively constant during the next 24-h period (Fig. 4*A*). Because Mcl-1 protein level was significantly decreased at 6 h postdetachment and continued to decline during the 24-h period, this finding ruled out transcriptional regulation as responsible for the Mcl-1 downregulation. Moreover, the observation that proteasome inhibitors completely inhibited detachment-induced Mcl-1 downregulation (Fig. 4*B*) strongly supported protein degradation and stabilization of Mcl-1 by Cav-1 as a key control mechanism.

Proteasomal degradation of a protein is generally triggered by its ubiquitination (9). We tested and found that Mcl-1 is ubiquitinated during cell detachment and that this process is inhibited by Cav-1. The mechanism by which Cav-1 inhibits Mcl-1 ubiquitination is unclear but likely involves steric hindrance of the ubiquitination sites by Cav-1. The interaction between Cav-1 and Mcl-1 may also affect Mcl-1 phosphorylation which has been linked to its ubiquitination. For example, phosphorylation of Mcl-1 at Ser159 by glycogen synthase kinase-3 was observed during cell anoikis (28) and was found to promote Mcl-1 ubiquitination and subsequent degradation (18).

We extended our finding on the role of Cav-1 in Mcl-1 and anoikis regulation in lung carcinoma H460 cells to melanoma G361 cells due to their reported expression in human melanoma (13, 20). We found that Cav-1-Mcl-1 interaction could be detected in G361 cells and that such interaction is dependent on the cellular level of Cav-1 which determines cellular susceptibility to anoikis (Fig. 6). These results support the general role of Cav-1 as anoikis regulator through Mcl-1 interaction.

In conclusion, we report a novel finding on the role of Cav-1 in anoikis regulation of human lung carcinoma and

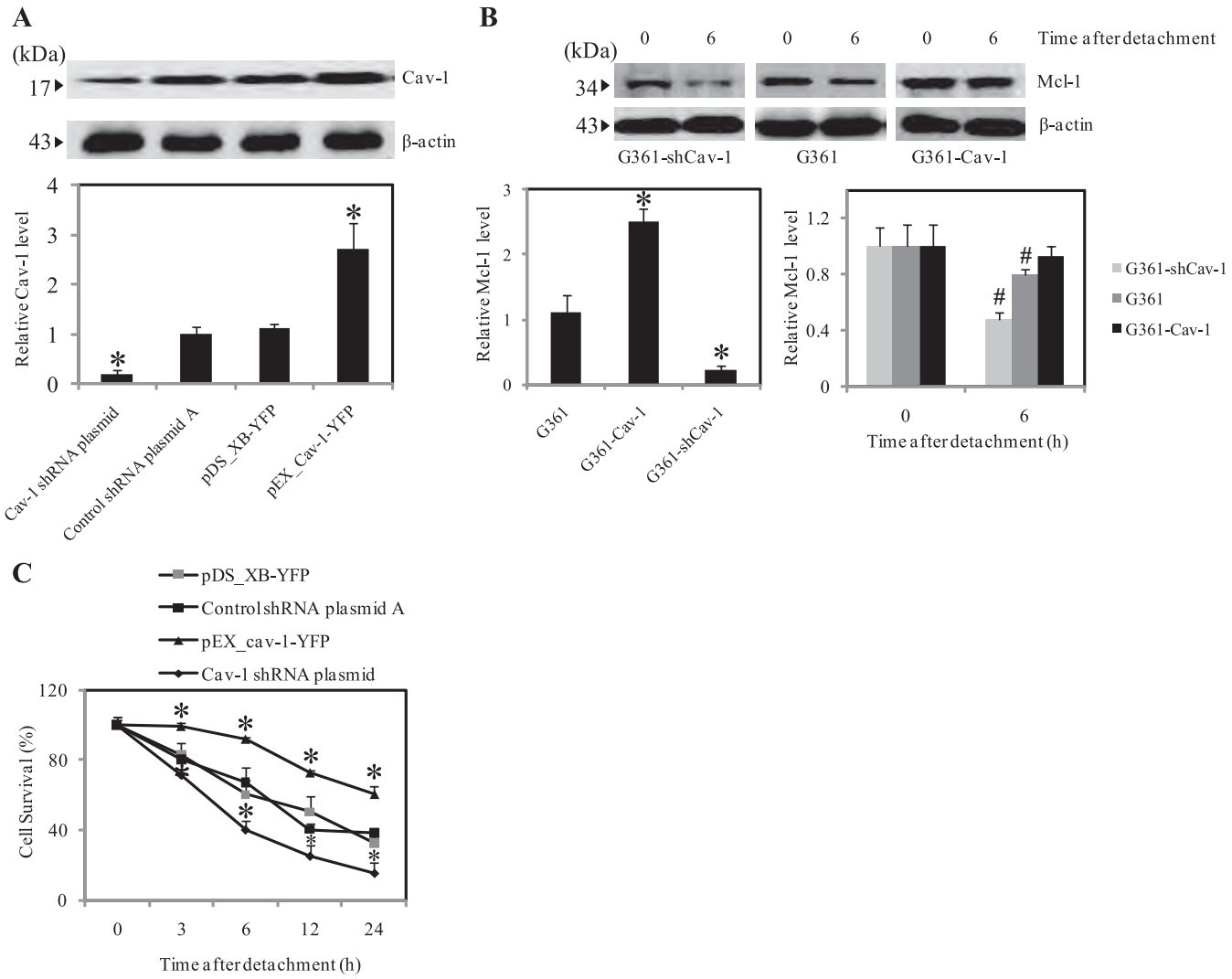


Fig. 6. Cav-1 regulates Mcl-1 expression and anoikis in melanoma G361 cells. *A*: control, G361-Cav-1, and G361-shCav-1 transfected cells were grown in and analyzed for Cav-1 expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the results. *B*: G361, G361-Cav-1 and G361-shCav-1 cells were detached and suspended in poly-HEMA-coated plates for various times (0–6 h). Blots were probed with specific antibody to Mcl-1 and were reprobed with β-actin antibody. Columns are means ± SD ($n = 3$). *$P < 0.05$ vs. control G361 cells. #$P < 0.05$ vs. control at detachment time = 0 h. *C*: subconfluent (90%) monolayers of mock, G361-Cav-1, and G361-shCav-1 cells were detached and suspended in poly-HEMA-coated plates for various times (0–24 h). At the indicated times, the cells were collected and determined for survival by XTT assay. Viability of detached cells at *time 0* was considered as 100%. Data represent means ± SD ($n = 3$). *$P < 0.05$ vs. control transfected cells.

melanoma cells. While the role of Cav-1 and Mcl-1 in anoikis regulation has been reported, their association and the underlying mechanisms of regulation are unclear. We found that Cav-1 interacts with Mcl-1 and stabilizes the protein by blocking its ubiquitination and subsequent degradation. Because an elevated expression of Cav-1 and Mcl-1 has been linked to the progression of cancer and metastasis, the findings of this study could be beneficial to the understanding of cancer etiology and metastasis mechanisms.

ACKNOWLEDGMENTS

The authors thank Mr. Krich Rajprasit for editing assistance.

GRANTS

This research was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Ratchadaphiseksomphot Endowment Fund (Chulalongkorn University), Thailand Research Fund (to P. Chanvorachote), and National Institutes of Health (R01-HL076340-04S1).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P. Chunhacha, V. Pongrakhananon, and P. Chanvorachote performed the experiments; P. Chunhacha and P. Chanvorachote analyzed the data; P. Chunhacha and P. Chanvorachote interpreted the results of the experiments; P. Chunhacha and P. Chanvorachote prepared the figures; P. Chunhacha and P. Chanvorachote drafted the manuscript; P. Chunhacha, V. Pongrakhananon, Y. Rojanasakul, and P. Chanvorachote approved the final version of the manuscript; Y. Rojanasakul and P. Chanvorachote edited and revised the manuscript; P. Chanvorachote, conception and design of research.

REFERENCES

1. **Boisvert AK, Longmate W, Abel EV, Aplin AE.** Mcl-1 is required for melanoma cell resistance to anoikis. *Mol Cancer Res* 7: 549–556, 2009.
2. **Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang BH, Rojanasakul Y.** Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. *J Biol Chem* 284: 28476–28484, 2009.
3. **Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ.** Bcl-2 and Bcl-xL sequester BH3 domain-only molecule preventing Bax and Bak-mediated mitochondrial apoptosis. *Mol Cell* 8: 705–711, 2001.
4. **Chiarugi P, Giannoni E.** Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol* 76: 1352–1364, 2008.
5. **Corn PG, Thompson TC.** Identification of a novel prostate cancer biomarker, caveolin-1: implications and potential clinical benefit. *Cancer Manag Res* 2: 111–122, 2010.
6. **Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, Kohtz DS, Lisanti MP.** Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. *FEBS Lett* 428: 205–211, 1998.
7. **Fiucci G, Ravid D, Reich R, Liscovitch M.** Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene* 21: 2365–2375, 2002.
8. **Frisch SM, Francis H.** Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124: 619–626, 1994.
9. **Glickman MH, Ciechanover A.** The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82: 373–428, 2002.
10. **Ho CC, Kuo SH, Huang PH, Huang HY, Yang CH, Yang PC.** Caveolin-1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy. *Lung Cancer* 59: 105–110, 2007.
11. **Hurlstone AF, Reid G, Reeves JR, Fraser J, Strathdee G, Rahilly M, Parkinson EK, Black DM.** Analysis of the CAVEOLIN-1 gene at human chromosome 7q31.1 in primary tumours and tumour-derived cell lines. *Oncogene* 18: 1881–1890, 1999.
12. **Hynes RO.** Cell adhesion: old and new questions. *Trends Cell Biol* 9: M33–M37, 1999.
13. **Keuling AM, Felton KE, Parker AA, Akbari M, Andrew SE, Tron VA.** RNA silencing of Mcl-1 enhances ABT-737-mediated apoptosis in melanoma: role for a caspase-8-dependent pathway. *PLoS One* 4: e6651, 2009.
14. **Lajoie P, Nabi IR.** Lipid rafts, caveolae, and their endocytosis. *Int Rev Cell Mol Biol* 282: 135–136, 2010.
15. **Li L, Ren CH, Tahir SA, Ren C, Thompson TC.** Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. *Mol Cell Biol* 23: 9389–9404, 2003.
16. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402–408, 2001.

16a. **Lloyd PG.** *Caveolin-1, antiapoptosis signaling, and anchorage-independent cell growth*. Focus on "Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells." *Am J Physiol Cell Physiol* (March 14, 2012). doi:10.1152/ajpcell.00075.2012.

17. **Luanpitpong S, Talbott SJ, Rojanasakul Y, Nimmannit U, Pongrakhananon V, Wang L, Chanvorachote P.** Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. *J Biol Chem* 285: 38832–38840, 2010.
18. **Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR.** Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 21: 749–760, 2006.
19. **Michels J, Johnson PW, Packham G.** Mcl-1. *Int J Biochem Cell Biol* 37: 267–271, 2005.
20. **Nakashima H, Hamamura K, Houjou T, Taguchi R, Yamamoto N, Mitsudo K, Tohnai I, Ueda M, Urano T, Furukawa K.** Overexpression of caveolin-1 in a human melanoma cell line results in dispersion of ganglioside GD3 from lipid rafts and alteration of leading edges, leading to attenuation of malignant properties. *Cancer Sci* 98: 512–520, 2007.
21. **Quest AF, Leyton L, Párraga M.** Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochem Cell Biol* 82: 129–144, 2004.
22. **Racine C, Belanger M, Hirabayashi H, Boucher M, Chakir J, Couet J.** Reduction of caveolin 1 gene expression in lung carcinoma cell lines. *Biochem Biophys Res Commun* 255: 580–586, 1999.
23. **Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y, Chanvorachote P.** Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. *Am J Physiol Cell Physiol* 300: C235–C245, 2011.
24. **Simpson CD, Anyiwe K, Schimmer AD.** Anoikis resistance and tumor metastasis. *Cancer Lett* 272: 177–185, 2008.
25. **Song L, Coppola D.** Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biol Ther* 4: 267–276, 2005.
26. **Tang Y, Zeng X, He F, Liao Y, Qian N, Toi M.** Caveolin-1 is related to invasion, survival, and poor prognosis in hepatocellular cancer. *Med Oncol*. In press.
27. **Thompson TC, Tahir SA, Li L, Watanabe M, Naruishi K, Yang G, Kadmon D, Logothetis CJ, Troncoso P, Ren C, Goltsov A, Park S.** The role of caveolin-1 in prostate cancer: clinical implications. *Prostate Cancer Prostatic Dis* 13: 6–11, 2010.
28. **Woods NT, Yamaguchi H, Lee FY, Bhalla KN, Wang HG.** Anoikis, initiated by Mcl-1 degradation and Bim induction, is deregulated during oncogenesis. *Cancer Res* 67: 10744–10752, 2007.
29. **Yeh D, Chen C, Sun MZ, Shao S, Hao L, Song Y, Gong L, Hu J, Wang Q.** Caveolin-1 is an important factor for the metastasis and proliferation of human small cell lung cancer NCI-H446 cell. *Anat Rec* 292: 1584–1592, 2009.
30. **Zhang H, Guttikonda S, Roberts L, Uziel T, Semizarov D, Elmore SW, Leverson JD, Lam LT.** Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene* 30: 1963–1968, 2011.
31. **Zhang S, Zhau HE, Osunkoya AO, Iqbal S, Yang X, Fan S, Chen Z, Wang R, Marshall FF, Chung LW, Wu D.** Vascular endothelial growth factor regulates myeloid cell leukemia-1 expression through neuropilin-1-dependent activation of c-MET signaling in human prostate cancer cells. *Mol Cancer* 9: 9, 2010.

Acquisition of Anoikis Resistance Up-regulates Caveolin-1 Expression in Human Non-small Cell Lung Cancer Cells

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Abstract. *Background: Anoikis is a key inhibitory step in the process of cancer cell metastasis. Knowledge regarding the adaptive response resulting in resistance to anoikis may benefit the development of new therapies. Materials and Methods: Anoikis-resistant cells were generated from anoikis-sensitive lung carcinoma cells and the underlying mechanism for this process was investigated. Results: Culturing H460 cells under suspended conditions caused spontaneous generation of anoikis-resistant H_AR1 and H_AR2 cells. We found that anoikis resistance in these cells caused caveolin-1 (CAV1) up-regulation. Using short hairpin RNA (shRNA), we confirmed that depletion of CAV1 rendered anoikis-resistant H_AR2 cells sensitive to anoikis. Furthermore, this study revealed that the acquisition of anoikis resistance induced CAV1 up-regulation through induction of CAV1 mRNA transcription. Conclusion: Our findings show CAV1 to be a key player in anoikis resistance and provide a novel mechanism regarding cancer cell adaptation, resulting in acquisition of anoikis resistance in lung cancer cells.*

Lung cancer is one of the most frequent causes of cancer-related death worldwide (1, 2), and the most common cause of death from this type of cancer is tightly associated with cancer metastasis. Metastasis is a multistep biological process and its regulation is extremely complicated (3). An early step of metastasis requires cancer cells to detach and migrate away from the primary tumor and to intravasate into the blood or the lymphatic circulation. This detachment in most cases leads to the cellular process of detachment-induced apoptosis called anoikis (4). Defects in anoikis lead to the survival of cells during their transportation through the circulatory system, by which they reach distant target organs, where they adhere, extravasate, and begin to form secondary tumors (5, 6).

Many phenotypic changes have been suspected to contribute to resistance of lung cancer cells to anoikis involving the up-regulation of oncogenes and the down-regulation of tumour suppressor genes (7-9). Caveolin-1 (CAV1), the protein that has been implicated in anoikis resistance, has gathered increased attention, since its regulatory role in several types of cellular behaviors has been consistently demonstrated (10-12). Indeed, as a 21-24 kDa structural protein, component of the plasma membrane microdomains termed caveolae, CAV1 has been shown to function in vesicular trafficking, signal transduction, and cancer progression (13-15). Although the up-regulation of this protein normally occurs in a variety of terminally differentiated cells including fibroblasts, adipocytes, smooth muscle cells, endothelial cells, and epithelial cells (16), CAV1's expression is greatly reduced in most oncogenically transformed cells and cancer cells (17-19). Interestingly, CAV1 was first explained to function as a tumor suppressor protein (20, 21). However, increasing evidence indicates its role as a tumor and metastasis promoter, as overexpression or re-expression of CAV1 was found in many advanced-stage and metastatic cancer cells. The up-regulation of CAV1 was shown to render Rat1A cells more resistant to apoptosis (22). Moreover, antisense-induced down-regulation of CAV1 sensitized human prostate cancer cells to apoptosis (23). Previous studies have shown that CAV1 acts as a negative regulator of anoikis (10, 11, 24, 25), and its elevated expression in lung carcinoma is closely associated with the increased metastatic capacity and the poor survival of patients (12).

Anoikis resistance can be driven in originally sensitive clones by altering culture cycles under adhered and non-adhered (suspended media) conditions (26). Since the underlying mechanism in this driving selection process for the acquisition of anoikis resistance in anoikis-sensitive cells remains poorly understood, the present study aimed at investigating the underlying mechanism for the acquisition of anoikis resistance of human lung cancer cells.

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Key Words: Anoikis resistance, cancer adaptation, caveolin-1, mRNA transcription, lung cancer, H460 cells.

Materials and Methods

Reagents. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT), cycloheximide and actinomycin D were obtained from Sigma Chemicals, Inc. (St. Louis, MO, USA). Propidium iodide (PI) and Hoechst 33342 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). PromoFectin transfecting agent was obtained from PromoKine (Heidelberg, Germany). A rabbit polyclonal anti-CAV1 antibody and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody were purchased from Abcam (Cambridge, MA, USA). The primary beta-actin antibody and the CAV1 shRNA plasmid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CAV1 (pEX_Cav-1-YFP) plasmid was obtained from the ATCC. The PcDNA3 plasmid was generously provided by Dr. Yon Rojanasakul (West Virginia University, Morgantown, WV, USA). All other biochemical reagents were from Sigma.

Cell culture. Non-small cell lung carcinoma H460 cells were obtained from the American Type Culture Collection (ATTC, Rockville, MD, USA) The cells were cultured and maintained in RPMI medium, supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA), 2 mmol/l L-glutamine (GIBCO) and 100 U/ml penicillin/streptomycin, at 37˚C in an atmosphere with 5% carbon dioxide. Anoikis-resistant cells were generated by sequential cycles of culturing under adherent and non-adherent conditions.

For non-adherent culture, tissue culture plates were coated with 1 ml of 10 mg/ml polyhydroxyethylmethacrylate polymer (polyHEMA) (Sigma-Aldrich, St. Louis, MO, USA) in 95% ethanol. After drying, plates were washed with phosphate-buffered saline (PBS) and dried overnight at room temperature. Cells were trypsinized and resuspended with RPMI medium, then added onto the polyHEMA-coated tissue culture plates. Cells were maintained in suspension culture for the indicated times of each experiment.

Selection for the anoikis-resistant cell line. Anoikis-resistant cells were generated by sequential cycles of culturing under adherent and non-adherent conditions. Briefly, H460 cells in monolayer were trypsinized and a single-cell suspension was seeded onto polyHEMA-coated tissue culture plates for 72 h. After incubation, the cells were washed and the transferred to tissue culture plates and left to recover. After the surviving clones were expanded, the cells were seeded again in polyHEMA-treated dishes as mentioned above, and the cycle was repeated for five more times. The resulting cells then were selected for single-cell colony, designated as H_AR1 and H_AR2. The two designated anoikis-resistant cells, H_AR1 and H_AR2 have different levels of resistance to anoikis. The cells were maintained in adhered condition and were passaged several times before being used in this study.

Generation of stable CAV1 overexpressing and CAV1 knock-down cells. CAV1 overexpressing H460 (H_Cav1-1 and H_Cav1-2) cells were generated by stable transfection of the cells with *CAV1* plasmid (pEX_Cav-1-YFP) from the ATTC. *CAV1* knock-down H460 (H_shCav1) cells were generated by stable transfection of the cells with *CAV1* shRNA plasmid (sc-29241-SH), purchased from Santa Cruz Biotechnology, Inc. Stable transfections were performed using the Promofectin® (Heidelberg, Germany) reagent, according to the manufacturer's instructions. Briefly, H460 cells were cultured on 24-well plates until they reached 50-60% confluence. One microgram of CAV1, *CAV1* shRNA or pcDNA3 plasmid and 2 μl of Promofectin solution were mixed and incubated at room temperature for 30 min, then added to the cells in the absence of serum. After 24 h, the medium was replaced with culture medium containing 10% FBS. For stable transfection of CAV1-overexpressing cells, H460 cells were maintained in medium containing 50 μg/ml of neomycin which gradually increased to 1000 μg/ml for one month. For *CAV1* knock-down cells, H460 cells were maintained in a medium containing 1 μg/ml puromycin for 2 weeks. After the selection periods, 200 μl of 100 cells/ml of each transfectant were seeded into each well of a 96-well cell culture plate and were cultured until a cell colony was clearly observed. A single colony from the wells was picked up and was further cultured for obtaining greater cell numbers. After the propagation period, all clones were identified by western blotting analysis and the clones that exhibited two different expression levels compared to the normal endogenous CAV1 level of H460 cells were designated as H_Cav1-1 and H_Cav1-2 cells. For the cells transfected with *CAV1* shRNA, the clones that stably expressed at least a 50% lower levels of *CAV1* compared to parental H460 cells were considered as H_shCav1 cells and one clone was picked for further investigation. *CAV1* overexpressing/*CAV1* knock-down cells were cultured in normal growth medium without neomycin or puromycin for two weeks before experiments were carried out.

Generation of CAV1 knock-down anoikis-resistant cells. CAV1 knock-down H_AR2 (H_AR2/shCav1) cells were generated by stable transfection of anoikis-resistant H_AR2 cells with *CAV1* shRNA plasmids (sc-29241-SH) from Santa Cruz Biotechnology, Inc. Stable transfection was performed using the Promofectin® (Heidelberg, Germany) reagent, according to the manufacturer's instructions. The cells were cultured for 14 days with puromycin selection (1 μg/ml). The pooled stable transfectant was identified by western blot analysis of CAV1 and was cultured in puromycin-free RPMI 1640 medium for two weeks before experiments were carried out.

Anoikis assay. The cells were trypsinized 1.0×10^4 cells were seeded onto polyHema-coated 96-well tissue culture plates. After incubating for 3, 6, 12 and 24 h, cell viability was determined by incubation with 0.3 mg/ml of XTT at 37˚C for 4 h. The intensity of the formazan product was determined at 450 nm using an ELISA plate reader. Relative cell viability was calculated by dividing the O.D. reading of the sample by that of the control at detachment time =0 h.

Apoptosis/anoikis and necrosis evaluation. After detachment for 12 h, 10 μg/ml of Hoechst 33342 and/or 5 μg/ml of propidium iodide (PI) were added to the cell culture and cells were visualized under a fluorescence microscope (Olympus I×51 with DP70). The number of apoptotic cells was calculated as percentage of cells in each sample. The use of different dyes for the determination mode of cell death was based on the dissimilar characteristics of necrotic and apoptotic cells. Cells that were undergoing apoptosis demonstrated cell shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation and apoptotic body formation, by which the cell membrane was not broken-up in early apoptosis; whereas necrotic cell death resulted in loss of membrane integrity, swelling and disruption of the cells. Hoechst 33342 is a permeable dye, which has the ability to stain the nucleic acids of apoptotic and necrotic cells, whereas PI is an impermeable dye staining necrotic cells that have lost their membrane integrity and which exhibit red PI staining throughout the nucleus. The apoptotic and necrotic cells can be, thus, distinguished under fluorescence microscopy.

Apoptosis was also assayed by cell cycle analysis. Cells were centrifuged, washed with phosphate buffer saline (PBS) and resuspended in 50 μg/ml PI with 0.1% Triton X-100 for 20 min at 4˚C. Cells were analyzed by flow cytometry and the sub G_0/G_1 fraction was used as a measure of the percentage of apoptotic cells.

Western blotting assay. Cells were incubated with lysis buffer containing 2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 100 mmol/l NaCl, 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA, and complete Mini cocktail protease inhibitor (Roche Diagnostic GmbH, Mannheim, Germany) for 30 min on ice. After insoluble debris were pelleted-down by centrifugation at 14,000 ×*g* for 15 min at 4˚C, the supernatant was collected and the protein content was determined using the Bradford method. Proteins (40 μg) were resolved on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred onto nitrocellulose membranes using standard procedures. The membranes were blocked in 5% skim milk in TBST (25 mmol/l Tris-HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween20) for 1 h, followed by incubation with CAV1 or beta-actin primary antibodies at 4˚C overnight. Membranes were washed three times with TBST for 10 min, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The immune complexes were detected by chemiluminescence and were quantified by image densitometry using the analyst/PC densitometer software (Bio-Rad, Richmond, CA, USA). Mean densitometry data from independent experiments were normalized the β-actin protein.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription-PCR was performed with the Access RT-PCR System (Promega, Madison, WI, USA), according to the manufacturer's instructions. The *CAV1* forward primer was 5'-CGTAGACTCGGAGGGAC ATC-3' and the reverse primer was 5'-TTTCGTCACAGTGAAGGTGG-3'. The forward primer for the internal control *GAPDH* was 5'-GCTGAGAACGGGAAGCTTGT-3' and the reverse primer was 5'-GCCAGGGGTGCTAAGCAG-3'. Reaction products were analyzed after 30 amplification cycles, each of which involved consecutive 1 min steps at 94, 55 and 72˚C. The PCR products were fractionated by electrophoresis on a 1.0% agarose gel, containing 0.5% ethidium bromide and were photographed.

Immunoflorescent staining. Cells were grown on collagen-coated glass coverslips, were fixed with 4% formaldehyde for 10 min, permeabilized using 0.1% Triton X-100 for 10 min, and blocked with 1% bovine serum albumin for 30 min. Cells were incubated with a antibody against CAV1 for 1 h at room temperature, were washed, and incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody. Digital images were acquired by a fluorescent microscope.

Statistical analysis. All the experiments were repeated at least three times. Statistical analysis was performed using one-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant.

Results

Acquisition of anoikis resistance by sequential cycles of culture under suspended and adherent conditions generates anoikis-resistant cells. In order to study the adaptive mechanism of lung cancer cells against anoikis, we established anoikis-resistant subline cells by subjecting lung carcinoma H460 cells to five sequential cycles of suspended and adherent cultures as described elsewhere (26). Two single-cells from anoikis-resistant subline cells were collected and cultured under adherent conditions, namely H_AR1 and H_AR2 cells. The levels of anoikis resistance of the cells were determined by analysis using the viability assay.

Detachment caused a time-dependent decrease in cell survival in both types of cells. At 12 h post-detachment, ~60% and ~80% of H_AR1 and H_AR2 cells remained viable respectively, while the parental control cells exhibited only ~40% survival (Figure 1). Adhered cells under normal culture conditions exhibited no significant change in cell viability during the tested period (data not shown). Hoechst 33342 and PI staining assays for cell apoptosis analysis, further showed that the decrease of cell survival after detachment was mainly due to apoptosis, as indicated by the increase of cells with intense nuclear fluorescence and chromatin condensation. To confirm this, cell cycle analysis of all cells was performed by PI DNA content assay and the apoptotic proportion of cells (sub-G_0/G_1 content) was determined by flow cytometry. Figure 1C indicates that the sub-G_0/G_1 content at 12 h after detachment of H_AR1 and H_AR2 cells was significantly lower than that of the control H460 cells. These results reveal the effects of cycles of suspended and adherent culture, in rendering lung cancer cells resistant to anoikis.

Regulatory roles of CAV1 on anoikis resistance. We generated cells exhibiting several levels of CAV1 expression by stable transfection with *CAV1* and *CAV1* shRNA plasmids. After selection, the derivative clones were evaluated for CAV1 expression by western blot analysis. Figure 2A shows that the *CAV1* plasmid-transfected cells exhibited ~1.4- and ~2.5-fold induction of CAV1 expression relative to that of the parental H460 cells; these cells were named H_Cav1-1 and H_Cav1-2, respectively. The expression of *CAV1* in shRNA transfectants was significantly down-regulated (~0.6-fold relative to that of control cells) and these cells were named H_shCav1. We confirmed the relative CAV1 level and determined the protein distribution by immunocytochemistry. Figure 2B indicates that CAV1 is localized in the plasma membrane and in the cytoplasm of all clones, similarly.

The clone cells were then evaluated for anoikis response. Figure 2C shows that CAV1-overexpressing cells, H_Cav1-1 and H_Cav1-2, exhibited a significant increase of cell survival after detachment, whereas H_shCav1 exhibited a higher degree of cell anoikis. Consistent results were also obtained from the Hoechst 33342 assay, indicating that the anoikis response was attenuated in the H_Cav1-1 and H_Cav1-2 cells, while the opposite effect was observed in the cells with CAV1 down-regulation. Cell cycle analysis exhibited a similar trend (Figure 2E), revealing that CAV1 functioned fowards inhibition of anoikis of the cells.

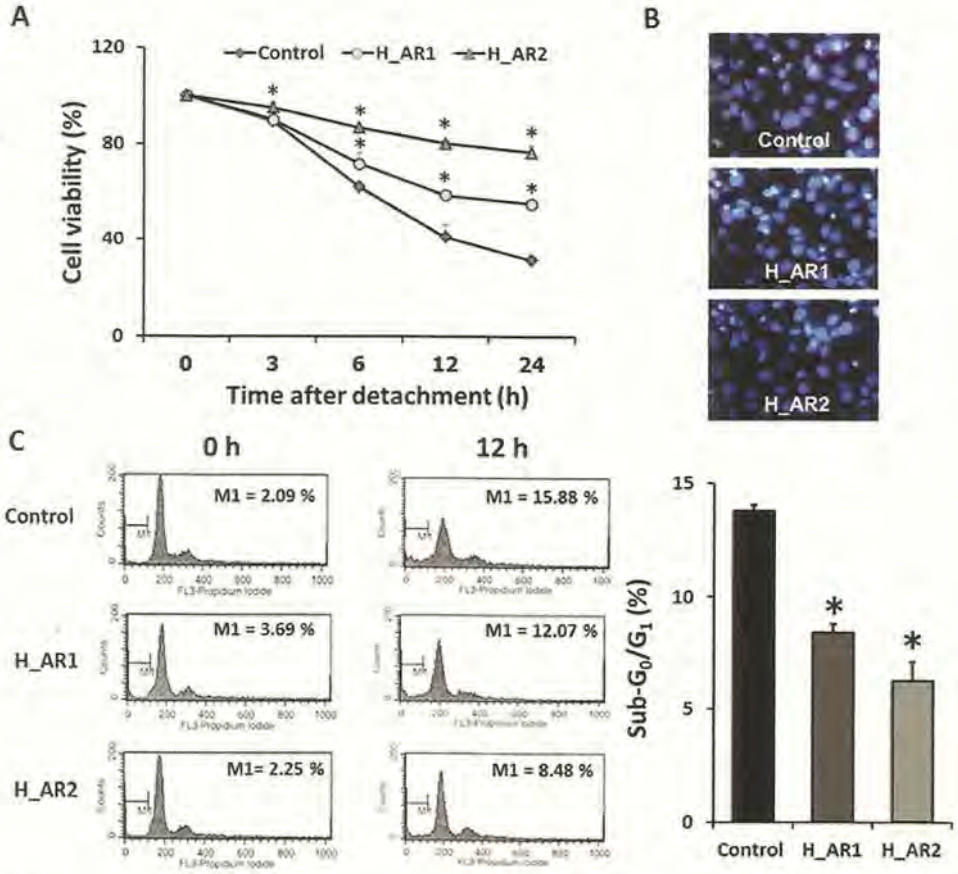

Figure 1. *Effects of acquisition of anoikis resistance on cell anoikis in lung cancer cells. H_AR1 and H_AR2, anoikis-resistant cells, were generated by sequential cycles of culture under suspended and adherent conditions. A: Cells were detached and suspended on polyhydroxyethylmethacrylate (poly-HEMA)-coated plates for the indicated times and cell viability was determined by the XTT assay. Values are means±SD (n=4); *p<0.05 versus the control H460 cells. B: Morphology of nuclei of detached cells stained with Hoechst 33342 and propidium iodide (PI) at 12 h after detachment was visualized under a fluorescent microscope. C: Cells were detached and suspended on poly-HEMA-coated plates for 12 h. Sub-G_0/G_1 cells were determined by flow cytometry. Values are means±SD (n=4); *p<0.05 versus the control H460 cells.*

CAV1 is up-regulated in anoikis-resistant lung carcinoma cells via a transcriptional-dependent mechanism. To further determine the association between CAV1 expression and anoikis resistance, we analyzed the CAV1 expression in anoikis-resistant subline cells with western blot analysis. Western blot analysis indicated that H_AR1 and H_AR2 cells exhibited ~1.4- and ~2.5-fold induction of CAV1 expression in relation to that of parental H460 cells (Figure 3). The levels in relation of CAV1 in the subline cells were confirmed with immunohistochemistry and the highest fluorescence signal was observed in H_AR2 cells. These results suggested that acquisition of anoikis resistance in these cells was likely due to the adaptation processes which resulted in the CAV1 up-regulation. As many studies showed that an increase of the pool of CAV1 in cells will subsequently increase the amount of phosphorylated CAV1

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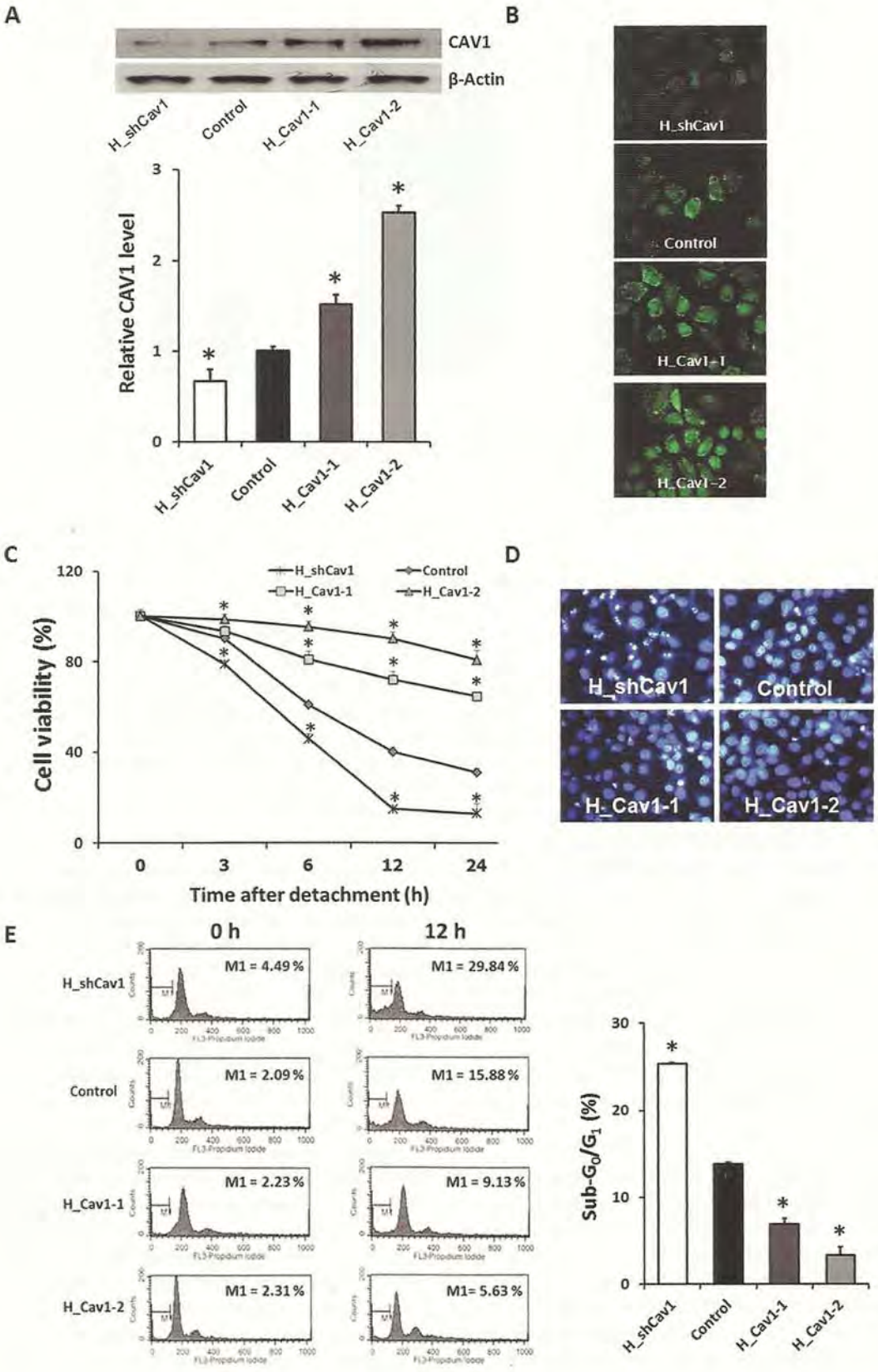
Figure 2. *Effects of caveolin-1 (CAV1) on anoikis resistance. A: H460 cells were stably transfected with CAV1-overexpressing or shRNA-CAV-1 plasmids to generate H_Cav1-1 and H_Cav1-2, and H460/shCav1 cells. The CAV1 protein expression was determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to those of the control transfected cells. B: Immunocytochemistry evaluation of CAV1 in the cells. C: Cells were detached and suspended on polyhydroxyethylmethacrylate (poly-HEMA)-coated plates for the indicated times and cell viability was determined by the XTT assay. Values are means ± SD (n=4); *p<0.05 versus the control transfected cells. D: The morphology of cell nuclei stained with Hoechst 33342 and propidium iodide (PI) was visualized under a fluorescent microscope at 12 h after cell detachment. E: Cells were detached and suspended on poly-HEMA coated plates for 12 h and the sub-G_0/G_1 proportion of cells was determined by flow cytometry. Values are means ± SD (n=4); *p<0.05 versus the control transfected cells.*


A
CAV1
β-Actin
H_shCav1
Control
H_Cav1-1
H_Cav1-2
Relative CAV1 level
B
H_shCav1
Control
H_Cav1-1
H_Cav1-2
C
H_shCav1
Control
H_Cav1-1
H_Cav1-2
Cell viability (%)
Time after detachment (h)
D
H_shCav1
Control
H_Cav1-1
H_Cav1-2
E
0 h
12 h
H_shCav1
M1 = 4.49 %
M1 = 29.84 %
Control
M1 = 2.09 %
M1 = 15.88 %
H_Cav1-1
M1 = 2.23 %
M1 = 9.13 %
H_Cav1-2
M1 = 2.31 %
M1= 5.63 %
Sub-G0/G1 (%)

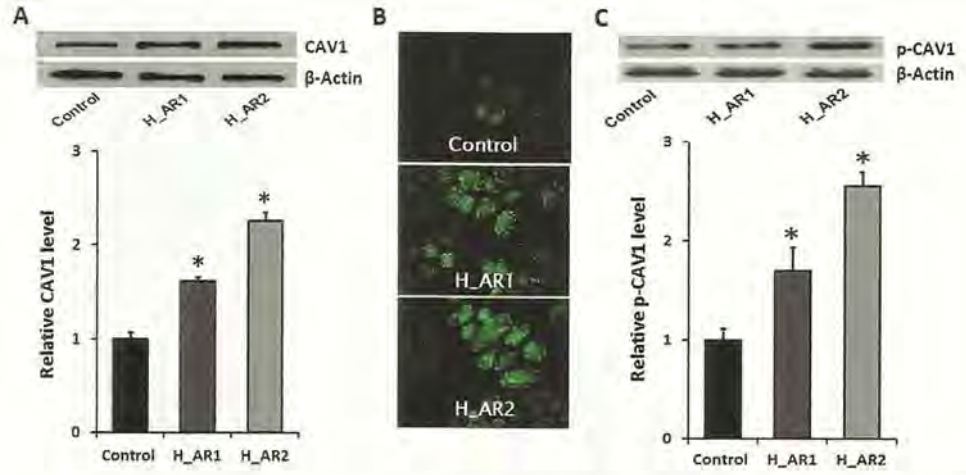


Figure 3. *Caveolin-1 (CAV1) is up-regulated in anoikis-resistant lung carcinoma cells. A: Anoikis-resistant sublines H_AR1 and H_AR2 cells were analyzed for CAV1 expression by western blotting. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to those of the control H460 cells. B: Immunocytochemistry of CAV1 in H_AR1 and H_AR2 cells. C: p-CAV1 of cells was analyzed by western blotting.*

resulting in more a aggressive behavior of cancer cells, we further evaluated p-CAV1 in these cells and found a significant increase of p-CAV1 in anoikis-resistant H_AR1 and H_AR2 (Figure 3C).

We next investigated the pathway involved in the up-regulation of CAV1 expression in H_AR1 and H_AR2 cells. Since the process of development of anoikis-resistant cells required the adaptation of the cells induced by anoikis stimuli, we hypothesized that the up-regulation of CAV1 in these cells was performed through mRNA induction. The level of *CAV1* mRNA in anoikis-resistant cells was evaluated by RT-PCR. Figure 4A shows that the mRNA levels of *CAV1* significantly increased in H_AR1 and H_AR2 cells in relation to that of parental control cells. We confirmed this finding by treating anoikis-resistant cells with the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide. Western blot analysis shows that treatment with actinomycin D and cycloheximide reduced the levels of the CAV1 protein in anoikis-resistant cells, suggesting that the up-regulation of CAV1 in acquisition of anoikis resistance was performed through a transcriptional mechanism.

CAV1 shRNA reduces CAV1 levels and reverses anoikis resistance. To provide supporting evidence that CAV1 was responsible for anoikis resistance of the H_AR2 cells, we tested whether the reduction of CAV1 in these cells sensitizes the cells to anoikis. Anoikis-resistant H_AR2 cells were transfected with shRNA-CAV1 plasmid and were named as H_AR2/shCav1 cells. Western blotting analysis for CAV1 was performed. Figure 5A indicates that the CAV1 levels in H_AR2/shCav1 cells were lower in comparison to these of H_AR2 cells. The H_AR2/shCav1 cells were then analyzed for anoikis resistance and the results indicate that H_AR2/shCav1 cells exhibited significantly lower cell viability after cell detachment compared to those of H_AR2 cells. These results suggest that the up-regulation of CAV1 during acquisition of anoikis resistance plays a key role in the suppression of anoikis in lung cancer cells.

Discussion

The ability of cancer cells to escape from anoikis is a pre-requisite for cell metastasis, through providing them with increased survival time while migrating to secondary sites. Thus, resistance to anoikis is considered as a crucial step during tumorigenesis. Acquisition of anoikis resistance in anoikis-sensitive cancer cells can be driven by alternating culture cycles under adherent and suspended conditions. Acquisition of anoikis resistance has been successfully developed in many cell such as osteosarcoma, colorectal, hepatoma, and lines oral tongue cancer cells (26-29). However, the underlying mechanism of acquisition of anoikis resistance in NSCLC is still unknown. In this study, we showed that when anoikis-susceptible lung carcinoma H460 cells were cultured under anoikis-inducing conditions *in vitro*, they spontaneously gave rise to anoikis-resistant cells.

The processes of removing exposure to an anoikis-inducing environment may act as a driving force towards cells with an anoikis-resistant phenotype, or forces adaptive anoikis resistance in cells (26). Although the mechanisms of acquisition of anoikis resistance are largely unknown, many

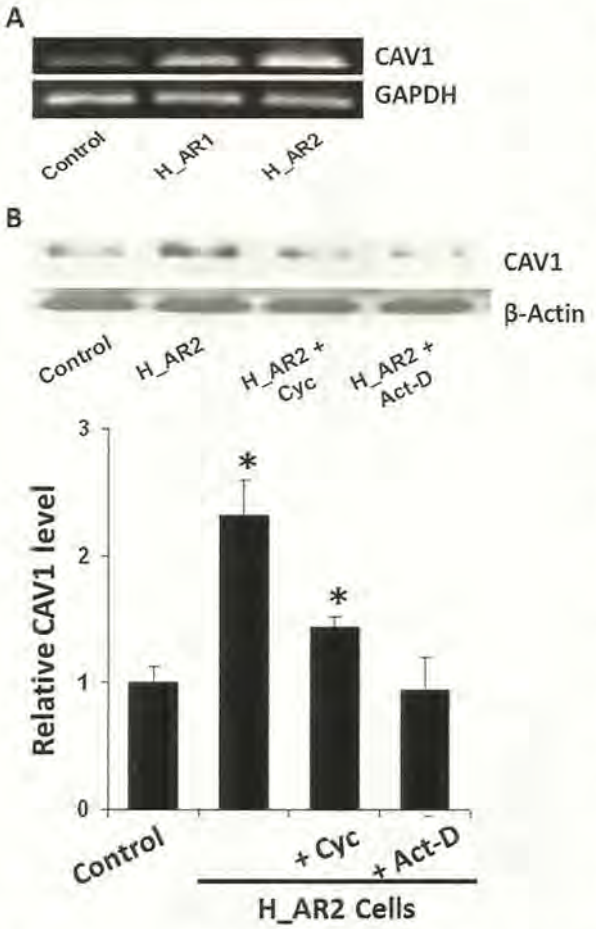


Figure 4. *Acquisition of anoikis resistance increases caveolin-1 (CAV1) expression through a transcriptional-dependent mechanism. A: CAV1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels of H_AR1 and H_AR2 were determined by RT-PCR. B: The cells were treated with the transcription inhibitor actinomycin D (Act D) and the translation inhibitor cycloheximide (Cyc) for 12 h and the CAV1 levels were determined by western blot analysis. Blots were reprobed with the β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to those of the control H460 cells.*

underlying mechanisms of cancer cells resisting detachment-induced apoptosis have been proposed, and the modification of oncogenes and of tumor suppressor genes, are by far the ones mentioned the most (7, 9, 30). Oncogenic versions of H-, K-, N-RAS, and RAS-related proteins teratocarcinoma oncogene (TC21) and R-RAS, have been shown to inhibit anoikis in RIE-1 rat intestinal epithelial cells (7). The study by McFall *et al*. showed that B-cell lymphoma 2 (Bcl-2) and cytokine response modifier A suppressed anoikis by attenuating the Jun-N-terminal kinase activation in canine kidney cells (7). Furthermore, the stabilization of EGFR has been shown to suppress anoikis in mammary epithelial cells (30). From epigenetic studies, alteration in the patterns of gene expression due to gene silencing and reprogramming, when exposed to an anoikis-inducing microenvironment, could be responsible, at least in part, for the acquisition of anoikis resistance (31-33). Moreover, the study by Kupferman *et al*. showed that the pattern of the S-100 protein (S100P), of kallikrein 6 (KLK6) and of catenin alpha-like 1 (CTNNAL1) expression was altered in anoikis-resistant cell lines compared to the anoikis-sensitive cells (34).

CAV1 protein has gathered increasing attention in cancer metastasis research and has been shown to play a crucial role in cancer progression and metastasis. Many studies have shown that CAV1 reduces anoikis in various cancer cell types including prostate cancer, small cell lung cancer, melanoma and hepatocellular carcinoma (35-38). Furthermore, the levels of CAV1 are high in metastatic and multidrug-resistant human cancer cell lines (39, 40). In breast cancer cells, CAV1 inhibited anoikis by blocking p53 activation (11). In NSCLC, our previous studies, and others showed that CAV1 increased cell resistance to anoikis (24, 25, 41). In addition, CAV1 was found to be highly expressed in brain metastases of NSCLC (42). Even though a role for CAV1 in anoikis resistance in NSCLC has been reported, whether CAV1 is responsible for exposure adaptation, resulting in acquisition of anoikis resistance is still unknown. Our study for the first time, showed that acquisition of anoikis resistance leads to up-regulation of CAV1 as shown by the higher levels of CAV1 in anoikis-resistant cell lines. We also showed that CAV1 is a key regulator in anoikis resistance since the levels of CAV1 correlated with an increase of anoikis resistance.

Our findings suggest that the CAV1 up-regulation was acquired during repeated response to anoikis stimuli. Furthermore, we provided information that such CAV1 up-regulation occurs through an increase of *CAV1* gene expression. Although the mechanism(s) that regulate CAV1 transcription in response to anoikis acquisition is still under investigation, some possible mechanisms have been proposed. Emergence of oncogenic *KRAS* mutations promoted by acquisition of anoikis resistance was shown to increase tumorigenicity *in vivo* in colorectal cancer cells (27). Interestingly, *KRAS*-mutated cell lines exhibited elevated expression of CAV1 (43), implying the possibility that the CAV1 up-regulation in the present study may be caused by a *KRAS* mutation.

In conclusion, we demonstrated for the first time that CAV1 is up-regulated by mRNA transcription in response to the acquisition of anoikis resistance. Understanding the underlying mechanisms in anoikis exposure adaptation resulting in acquisition of resistance to anoikis may therefore help delineate novel approaches for suppressing metastasis of lung cancer cells.

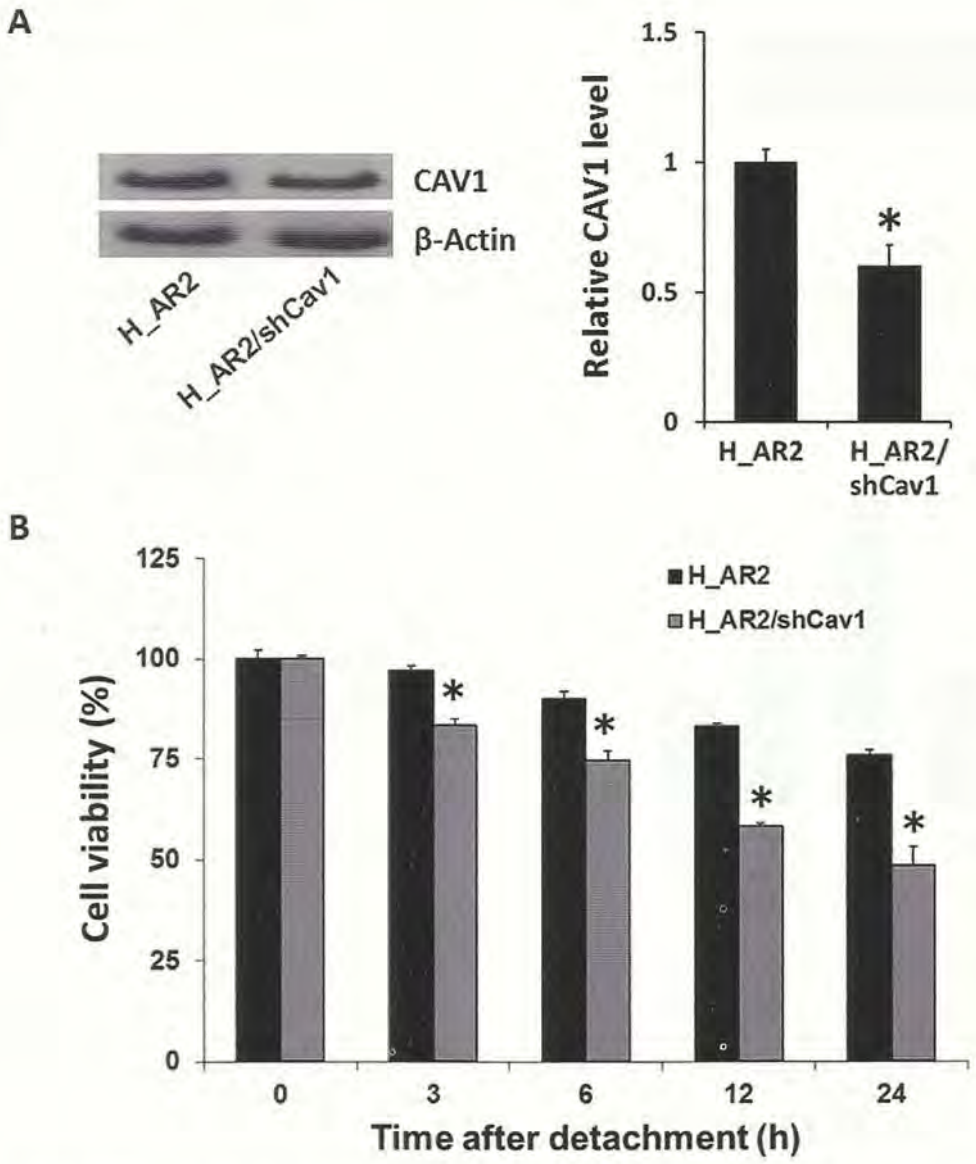


Figure 5. *The Caveolin-1 (CAV1) up-regulation is responsible for anoikis resistance in anoikis-resistant driven cells. A: Anoikis-resistant H_AR2 cells were transfected with shRNA CAV1 plasmids. The CAV1 levels of the cells were determined by western blotting. B: Cells were detached and suspended on poly-HEMA-coated plates for the indicated times and cell viability was determined by the XTT assay. Values are means±SD (n=4); *p<0.05 versus control transfected cells.*

Acknowledgements

The Authors would like to thank Mr. Krich Rajprasit. This research was supported by the Thailand Research Fund and Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University (P. Chanvorachote). The Young Lecturer Scheme granted by University Teknologi MARA (UiTM) to Hasseri Halim is gratefully acknowledged.

References

1 Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin *55*: 74-108, 2005.

2 Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol *24*: 2137-2150, 2006.

3 Isaiah JF: The organ microenvironment and cancer metastasis. Differentiation *70*: 498-450, 2002.
4 Gilmore AP: Anoikis. Cell Death and Differ *2*: 1473-1477, 2005.
5 Hanahan D and Weiberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.
6 Craig DS, Kika A and Aaron DS: Anoikis resistance and tumor metastasis. Cancer Lett *272*: 177-185, 2008.
7 McFall A, Ulku A, Lambert QT, Kusa A, Rogers-Graham K and Der CJ: Oncogenic Ras blocks anoikis by activation of a novel effector pathway independent of phosphatidylinositol 3-kinase. Mol Cell Biol *21*: 5488-5499, 2001.
8 Rosen K, Rak J, Leung T, Dean NM, Kerbel RS and Filmus J: ROCKS: multifunctional kinase in cell behavior. J Cell Biol *149*: 447-456, 2000.
9 Frisch SM, Vuori K, Kelaita D and Sicks S: A role for Jun-N-terminal kinase in anoikis: suppression by bcl-2 and crmA. J Cell Biol *135*: 1377-1382, 1996.
10 Fiucci G, Ravid D, Reich R and Liscovitch M: Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene *21*: 2365-2375, 2002.
11 Ravid D, Maor S, Werner R and Liscovitch M: Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by up-regulation of insulin-like growth factor-1 receptors and signaling. Oncogene *21*: 1338-1347, 2005.
12 Ho CC, Huang PH, Huang HY, Chen YH, Yang PC and Hsu SM: Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol *161*: 1647-1656, 2002.
13 Galbiati F, Razani B and Lisanti MP: Emerging themes in lipid rafts and caveolae. Cell *106*: 403-411, 2001.
14 Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR and Anderson RG: Caveolin, a protein component of caveolae membrane coats. Cell *68(4)*: 673-682, 1992.
15 Scherer PE, Lisanti MP, Baldini G, Sargiacomo M, Mastick CC and Lodish HF: Induction of caveolin-1 during adipogenesis and association of GLUT4 with caveolin-rich vesicles. J Cell Biol *127*: 1233-1243, 1994.
16 Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Mark CB, Macaluso F, Russell RG, Li M, Pestell RG, Vizio DD, Hou HJ, Knietz B, Lagaud G, Christ GJ, Edelmann W and Lisanti MP: Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem *276*: 38121-38138, 2001.
17 Engelman JA, Zhang XL, Galbiati F and Lisanti MP: Chromosomal localization, genomic organization, and developmental expression of the murine caveolin gene family (Cav-1, -2, and -3) Cav1 and Cav2 genes map to a known tumor suppressor locus (6-A2/7q31). FEBS Lett *429*: 330-336, 1998.
18 Lee SW, Reimer CL, Oh P, Campbell DB and Schnitzer JE: Tumor cell growth inhibition by caveolin-1 re-expression in human breast cancer cells. Oncogene *16*: 1391-1397, 1998.
19 Koleske AJ, Baltimore D and Lisanti MP: Reduction of caveolin and caveolae in oncogenically transformed cells. Proc Natl Acad Sci USA *92*: 1381-1385, 1995.
20 Campbell L, Hollins AJ, Al-Eid A, Newman GR, von Ruhland C and Gumbleton M: Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolar epithelial primary cultures. Biochem Biophys Res Common *262*: 744-751, 1999.
21 Mikol DD, Hong HL, Cheng HL and Feldman EL: Caveolin-1 expression in Schwann cells. Glia *27*: 39-52, 1999.
22 Timme TL, Goltsov A, Tahir S, Li L, Wang J, Ren C, Johnston RN and Thompson TC: Caveolin-1 is regulated by c-myc and suppress c-myc-induced apoptosis. Oncogene *19*: 3256-3265, 2000.
23 Li L, Ren CH, Tahir SA, Ren C and Thompson TC: Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol *23*: 9389-9404, 2003.
24 Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y and Chanvorachote P: Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. Am J Physiol *300*: 235-245, 2011.
25 Chanvorachote P, Nimmanit U, Lu Y, Talbott S, Jiang B and Rojanasakul Y: Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. J Biol Chem *284*: 28476-28484, 2009.
26 Diaz-Montero CM and McIntyre BW: Acquisition of anoikis resistance in human osteosarcoma cells. Eur J Cancer *39*: 2395-2402, 2003.
27 Derouet M, Wu X, May L, Hoon Yoo B, Sasazuki T, Shirasawa S, Rak J and Kosen KV: Acquisition of anoikis resistance promotes the emergence of oncogenic K-ras mutations in colorectal cancer cells and stimulates their tumorigenicity *in vivo*. Neoplasia *9*: 536-545, 2007.
28 Zhang Z, Cao L, Li J, Liang X, Liu Y, Liu H, Du J, Qu Z, Cui M, Liu S, Gao L, Ma C, Zhang L, Han L and Sun W: Acquisition of anoikis resistance reveals a synoikis-like survival style in BEL7402 hepatoma cells. Cancer Lett *267*: 106-115, 2008.
29 Swan EA, Jasser SA, Holsinger FC, Doan D, Bucana C and Myers JN: Acquisition of anoikis resistance is a critical step in the progression of oral tongue cancer. Oral Oncol *39*: 648-655, 2003.
30 Schafer ZT: Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature *461*: 109-113, 2009.
31 Barcellos-Hoff MH: It takes a tissue to make a tumor: epigenetics, cancer and microenvironment. J Mammary Gland Biol Neoplasia *6*: 212-221, 2001.
32 Coomber BL, Yu JL, Fathers KE, Plumb C and Rak JW: Angiogenesis and the role of epigenetics in metastasis. Clin Exp Metastasis *20*: 215-227, 2003.
33 Belinsky SA: Epigenetics is alive and growing. Trends Mol Med *8*: 53-54, 2002.
34 Kupferman ME, Patel V, Sriuranpong V, Amornphimoltham P, Jasser SA, Mandal M, Zhou G, Wang J, Coombes K, Multani A, Pathak S, Gutkind JS and Myers JN: Molecular analysis of anoikis resistance in oral cavity squamous cell carcinoma. Oral Oncol *43*: 440-454, 2007.
35 Yeh D, Chen C, Sun M, Shao S, Hao L, Song Y, Gong L, Hu J and Wang Q: Caveolin-1 is an important factor for the metastasis and proliferation of human small cell lung cancer NCI-H446 cell. Anat Rec *292*: 1584-1592, 2009.
36 Karam JA, Lotan Y, Roehrborn CG Ashfaq R, Karakiewicz PI, and Shariat SF: Caveolin-1 overexpression is associated with aggressive prostate cancer recurrence. Prostate *67*: 614-622, 2007.

37 Felicetti F, Parolini I, Bottero L, Fechi K, Errico MC, Raggi C, Biffoni M, Spadaro F, Lisanti MP, Sargiacomo M and Care A: Caveolin-1 tumor-promoting role in human melanoma. Int J Cancer *125*: 1514-1522, 2009.

38 Zhang ZB, Cai L, Zheng SG, Xiong Y and Dong JH: Overexpression of Caveolin-1 in hepatocellular carcinoma with metastasis and worse prognosis: correlation with vascular endothelial growth factor, microvessel density and unpaired artery. Pathol Oncol Res *3*: 495-502, 2009.

39 Ho CC, Kuo SH, Huang PH, Huang HY, Yang CH and Yang PC: Caveolin-1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine based chemotherapy. Lung Cancer *59*: 105-110, 2008.

40 Lavie Y, Fiucci G and Liscovitch M: Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. J Biol Chem *273*: 32380-32383, 1998.

41 Sunaga N, Miyajima K, Suzuki M, Sato M, White MA, Ramirez RD, Shay JW, Gazdar AF and Minna JD: Different roles for caveolin-1 in the development of non-small cell lung cancer *versus* small cell lung cancer. Cancer Res *64*: 4277-4285, 2004.

42 Cassoni P, Daniele L, Maldi E, Righi L, Taraglione V, Novello S, Valante M, Scagliotti GV and Papotti M: Caveolin-1 expression in lung carcinoma varies according to tumor histotype and is acquired *de novo* in brain metastases. Histopathology *55*: 20-27, 2009.

43 Lee SH, Lee SJ, Chung JY, Jung YS, Choi SY, Hwang SH, Choi D, Ha NC and Park BJ: p53 secreted by K-Ras-Snail pathway is endocytosed by K-Ras-mutated cells; Implication of target-specific drug delivery and early diagnostic marker. Oncogene *28*: 2005-2014, 2009.

Received February 21, 2012
Revised April 16, 2012
Accepted April 18, 2012

Cell Biology International

Cell Biol. Int. (2012) 36, 1055–1066 (Printed in Great Britain)

Research Article

Long-term hydrogen peroxide exposure potentiates anoikis resistance and anchorage-independent growth in lung carcinoma cells

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Abstract

Hydrogen peroxide (H_2O_2) is upregulated in tumour microenvironments and may contribute to effects on metastatic cancer cells. This study demonstrates that treatment of lung carcinoma and melanoma cells with H_2O_2 for 14 days results in an induction of anoikis resistance and growth in an anchorage-independent condition. H_2O_2 exposure increased the Cav-1 (caveolin-1) level through an increase of Cav-1 mRNA with minimal effect on protein degradation. Upregulation of Cav-1 induced anoikis resistance and facilitated growth in a detached manner. The findings show a novel role of hydrogen peroxide in the regulation of metastatic potential of cancer cells.

Keywords: anoikis; caveolin-1; hydrogen peroxide; long-term; lung cancer

1. Introduction

Among the steps of metastasis, anoikis resistance and ability to grow in an anchorage-independent manner are associated with a high degree of metastasis and advanced stage of cancer (Hanahan and Weiberg, 2000; Shanmugathasan and Jothy, 2000; Mori et al., 2009). Cav-1 (caveolin-1) is the major protein found in caveolae that is associated with cancer progression (Glenney and Zokas 1989; Rothberg et al., 1992; Scherer et al., 1996; Galbiati et al., 2001; Fiucci et al., 2002; Ravid et al., 2005). Although Cav-1 is a tumour suppressor protein (Engelman et al., 1998) and its level is down-regulated in some cancers (Lee et al., 1998; Racine et al., 1999; Bender et al., 2000), increasing evidence supported the reverse role of Cav-1 as a cancer-potentiating protein in many cancers (Yang et al., 1998; Kato et al., 2002; Suzuoki et al., 2002). Moreover, we and others have provided supporting data indicating that Cav-1 expression is tightly related to anoikis resistance and facilitates metastasis of lung cancer (Ho et al., 2002; Moon et al., 2005; Cassani et al., 2009; Yeh et al., 2009). Regulation of the Cav-1 level is mainly through proteasome-mediated degradation (Chanvorachote et al., 2009), and several findings have indicated the tight correlation between the Cav-1 level during cell detachment and cancer cell resistance to anoikis (Hanahan and Weiberg, 2000; Ravid et al., 2005, 2006).

According to the widely accepted concept, the cancer microenvironment is critical in the facilitation of metastasis (Rofstad, 2000; Isaiah, 2002) and causes significant impacts on cancer cell behaviour, such as chemotherapeutic resistance, invasion, and migration (Rennebeck et al., 2005; Wu, 2006; Brabek et al., 2010). However, the information regarding effects of microenvironment-associated substances on Cav-1 regulation as well as the consequences on cancer cell anoikis are largely unknown. Elevation of H_2O_2 levels in the lungs of lung cancer patients compared to normal subjects has been reported (Zieba et al., 2000; Chung-Man et al., 2001; Chan et al., 2009), as has a high H_2O_2 production in many cancer cells including lung cancer (Szatrowski and Nathan; 1991; Burdon, 1995; Lim et al., 2005; Liou and Storz, 2010).

An inhibitory effect of endogenous H_2O_2 generated during cell detachment on anoikis resistance in lung cancer cells has been shown. Also, exogenous H_2O_2 obtained during cell detachment stabilized Cav-1 protein by inhibiting its degradation through the ubiquitin-proteasomal pathway and contributed to anoikis resistance (Rungtabnapa et al., 2011). However, there is a lack of knowledge regarding the effect of hydrogen peroxide on attached cancer cells, as well as longer periods of hydrogen peroxide exposure as found in tumour environments (Szatrowski and Nathan, 1991; Droge, 2002; Storz, 2005; Lopez-Lazaro, 2007; Chan et al., 2009), which has become the main focus of the present study. We have investigated the role of H_2O_2 in controlling lung carcinoma cell anoikis and anchorage-independent growth, and identified the underlying mechanism. A novel mechanism of anoikis regulation by H_2O_2 through Cav-1 regulation exists, which could be important in the understanding of anoikis resistance in metastatic cancers.

2. Materials and methods

2.1. Cell cultures and reagents

Human non-small cell lung cancer cells (NCI-H460) and human melanoma cells (G361) were obtained from American Type Culture Collection (Manassas, VA). H460 cells were cultured in

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Abbreviations: Cav-1, caveolin-1; $DCFH_2$-DA, dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; poly-HEMA, poly-2-hydroxyethylmethacrylate; ROS, reactive oxygen species; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

RPMI 1640 medium containing 5% FBS (fetal bovine serum), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin. G361 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin. For long-term exposure experiments, cells were cultured in medium containing H_2O_2 (0, 1, 2.5, 5 and 10 μM) or catalase (0, 1000, 2000 and 5000 units/ml) for 14 days. The culturing medium was replaced by medium containing freshly prepared H_2O_2 or catalase every 2 days. All cells were grown in humidified incubators containing an atmosphere of 5% CO_2 and 95% air at 37°C. Antibodies for Cav-1, β-actin, peroxidase-labelled secondary antibodies, FITC secondary antibodies and protein A-agarose were obtained from Santa Cruz Biotechnology. All other chemicals and reagents including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], Annexin V-FITC, hydrogen peroxide (H_2O_2), catalase (CAT) and Hoechst 33342 were obtained from Sigma.

2.2. Generation of stable Cav-1 overexpressed and Cav-1 knockdown cells

The Cav-1 expression plasmid pEX_Cav-1 was acquired from the American Type Culture Collection (Manassas, VA) and Cav-1 knockdown plasmid shRNA-Cav-1 was obtained from Santa Cruz Biotechnology. Stable transfections of Cav-1 expression plasmid or Cav-1 knockdown plasmid were generated by culturing H460 cells in a 6-well plate until they reached 60% confluence. 15 μl of Lipofectamine reagent and 2 μg of Cav-1, shRNA-Cav-1 or mock control plasmid were used to transfect the cells in the absence of serum. After 12 h incubation, medium was replaced with fresh medium and the cells were maintained for an additional 36 h before being plated on to 75-ml culture flasks. The cells were cultured with G418 selection (600 μg/ml) for 24–28 days. The pooled stable transfections were confirmed by Western blotting of Cav-1 and cultured in antibiotic-free medium for at least two passages before using in each experiment.

2.3. MTT assay

MTT assay was used to determine the cell viability of H_2O_2 and catalase in H460 cells. After specific treatments, cells were incubated with 0.5 mg/ml MTT for an additional 4 h and the intensity of the MTT product was measured at 550 nm using a microplate reader. The cell viability was calculated from optical density (OD) readings and represented as a percentage to the non-treated control value.

2.4. Anoikis assay

Tissue culture 96-well plates were coated with 200 μl (6 mg/ml in 95% ethanol) of poly-HEMA (poly-2-hydroxyethylmethacrylate; Sigma) and left to evaporate overnight in a laminar flow hood at room temperature for drying. Adherent cells in the culture plate were trypsinized into a single cell suspension in the medium and seeded in poly-HEMA-coated plates at 5×10^4 cells/ml. Suspended cells were incubated at 37°C for 6 h. Cell anoikis was determined by XTT assay. The cells were incubated with 20 μM XTT reagent for 4 h at 37°C. The intensity of formazan product was determined at 450 nm using a microplate reader. The cell viability was calculated from optical density (OD) readings and represented as a percentage to the non-treated control value. The mode of cell death was confirmed by incubating the cells with 10 μg/ml Hoechst 33342 and PI (propidium iodide) and visualized under a fluorescent microscope (Olympus IX51 with DP70).

2.5. Flow cytometry

Treated and untreated cells were collected and incubated for 30 min at room temperature in the presence of Annexin V-FITC and PI. Cells were scored as apoptosis and necrosis by flow cytometry using a 485 nm excitation beam and a 538 nm band-pass filter (FACSort, Becton Dickinson). The mean fluorescence intensity was quantified by CellQuest Software (Becton Dickinson).

2.6. ROS (reactive oxygen species) detection

Intracellular ROS were determined using $DCFH_2$-DA (dichlorofluorescein diacetate) as a specific ROS probe. After specific treatments, cells were incubated with 10 μM $DCFH_2$-DA for 30 min at 37°C, after which they were washed, trypsinized, resuspended in PBS, and immediately visualized for fluorescence intensity by a fluorescence microscope. For hydrogen peroxide determination, Amplex Red was added to the cells and further incubated for 2 h. Then fluorescence intensity was determined using a fluorescence microplate reader (Beckton Dickinson), using a 530-nm excitation beam and a 590-nm band-pass filter.

2.7. Soft agar colony formation assay

The anchorage-independent cell growth was determined by assaying colony formation in soft agar. Briefly, monolayer cells were prepared into a single-cell suspension by trypsinization and homogenization. Cells were suspended in culture medium and 0.33% low-melting-temperature agarose, and 2 ml containing 2×10^4 cells were seeded in a 35 mm dish over a 3 ml layer of solidified culture medium with 0.6% agarose. The medium was refreshed every 3 days. The colonies were attained by light microscope and photographed after 2 weeks of incubation at 37°C.

2.8. Western blotting

After specific treatments, total cell lysates were prepared by incubating the cells in lysis buffer containing 20 mM Tris/HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM PMSF and a commercial protease cocktail mixture (Roche Applied Science) for 30 min on ice. Protein concentrations of cell lysates were determined using the Bradford method (Bio-Rad). Equal amounts of proteins of each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and loaded on to SDS/PAGE. Resolved proteins were transferred on to

0.45 μm nitrocellulose membranes (Bio-Rad) and immunoblotted with appropriate antibodies. The immune complexes were detected by enhanced chemiluminescence substrate (Supersignal West Pico; Pierce) and quantified using analyst/PC densitometry software (Bio-Rad).

2.9. Immunofluorescent staining

Cells were grown on glass coverslips, fixed with 4% formaldehyde for 10 min, permeabilized using 0.1% Triton X-100 for 10 min, and blocked with 1% BSA for 30 min. Cells were incubated with a Cav-1 antibody for 1 h at room temperature, washed and incubated with FITC-conjugated anti-rabbit antibody. Digital Images were acquired by fluorescent microscope (Olympus IX51 with DP70).

2.10. Quantitative real-time PCR

Total RNA was extracted with Trizol (Invitrogen). One microgram of RNA was reverse-transcribed in a 100 μl reaction mixture containing 500 μM dNTP, 125 units of MultiScribe Reverse Transcriptase (Applied Biosystems), 40 units of RNase inhibitor, 2.5 μM oligo(dT), 1 × Taq-Man reverse transcriptase buffer and 5 mM $MgCl_2$ at 48°C for 40 min. The primers were designed using Primer Express software (Applied Biosystems): Cav-1 (#AI878826) forward 5′-CGA-GAAGCAAGTGTACGACGC-3′, and reverse 5′-ACCACGTCATCGTTGAGGTG-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward, 5′-GAAGGTGAAGGTCG-GAGTC-3′, and reverse 5′-GAAGATGGTGATGGGATTTC-3′. Amplification conditions included 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A SYBR Green PCR Master Mix (Applied Biosystems) was used with 1 ng of cDNA and with 100–400 nM primers. Gene expression levels were quantified using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative mRNA levels were determined by using comparative C_T (threshold cycle) method, where the Cav-1 target is normalized to the control and compared with a reference sample (assigned a relative value of 1) by the equation $2^{-\Delta\Delta CT}$.

2.11. Immunoprecipitation

Cells were lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14000 ***g*** for 15 min, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad). 60 μg of proteins were incubated with Cav-1 antibodies for 14 h at 4°C, followed by incubated with protein A-conjugated Sepharose. The immune complexes were washed with 20 volumes of lysis buffer, and boiled at 95°C for 5 min. The immune complexes were resolved in 10% SDS/PAGE and analysed by Western blotting as described above.

2.12. Statistical analysis

The data are presented as the mean ± S.D. from 3 or more independent experiments. Statistical analysis was performed by Student's *t* test at a significance level of $P<0.05$.

3. Results

3.1. H_2O_2 exposure induces anchorage-independent growth and anoikis resistance of H460 cells

Sub-toxic concentrations of H_2O_2 and catalase were determined by MTT assay. The cells were treated with H_2O_2 (0–10 μM) or catalase (H_2O_2 scavenger; 0–5000 units/ml) for 24 h in attached culturing conditions. H_2O_2 and catalase in the range of 0.5–10 μM and 1000–5000 units/ml respectively, caused neither toxic nor proliferative effects on H460 cells (Figure 1A). To test whether the treatments could be able to modulate intracellular ROS levels, we used the ROS-specific probe $DCFH_2$-DA for intracellular ROS determination, and detected the fluorescence signal under a fluorescence microscope. Treatment with H_2O_2 significantly increased DCF fluorescence intensity, whereas catalase dramatically decreased the signal (Figure 1B). Hydrogen peroxide was measured using an Amplex Red assay, which indicated the corresponding effects of hydrogen peroxide and catalase treatments. To investigate the role of long-term H_2O_2 exposure on anoikis and anchorage-independent growth, cells were cultured in medium containing H_2O_2 (0, 0.5, 1, 2.5, 5 and 10 μM) or catalase (0, 1000, 2000, 3000 and 5000 units/ml) for 14 days. Figure 2(A) shows that H_2O_2 increased anchorage-independent growth in a dose-dependent manner, whereas sub-toxic concentrations of catalase dramatically decreased anchorage-independent growth. Figure 2(B) shows that treatment with H_2O_2 caused a dose-dependent increase in colony diameter, and ~2- and 4.5-fold inductions in response to 1 and 10 μM H_2O_2 respectively. Interestingly, the cells treated with H_2O_2 had a significantly increased colony number in a dose-dependent manner (Figure 2C). The anoikis response of these H_2O_2-exposed cells versus control non-treated cells was investigated. Cells were similarly cultured in the presence of H_2O_2 and catalase before being suspended in poly-HEMA-coated plates for 6 h. Figure 2(D) shows that detachment of H460 cells reduced cell viability over time, with ~60% remaining viable at 6 h. Exposure to H_2O_2 rendered cells resistant to anoikis in a dose-dependent manner, with ~80 and 100% viability of cells in response to 2.5 and 10 μM of H_2O_2 respectively. Analysis of cell apoptosis and necrosis by Hoechst 33342 and PI assay showed that the decrease in cell survival after detachment was mainly due to anoikis, as indicated by the increase in number of cells with intense nuclear fluorescence and chromatin condensation stained with Hoechst 33342 (Figures 2E and 2F). An insignificant number of PI-positive nuclei were seen, confirmed with annexin V-FITC/PI cell death assay using flow cytometry. Thus H_2O_2 exposure induced anchorage-independent growth and anoikis resistance in H460 cells.

3.2. Cav-1 is the key regulator of anchorage-independent growth and anoikis resistance

Cav-1 is associated with anchorage-independent cell growth and anoikis resistance in cancers (Fiucci et al., 2002; Ravid et al., 2005; Yeh et al., 2009) We tested whether Cav-1 can regulate the

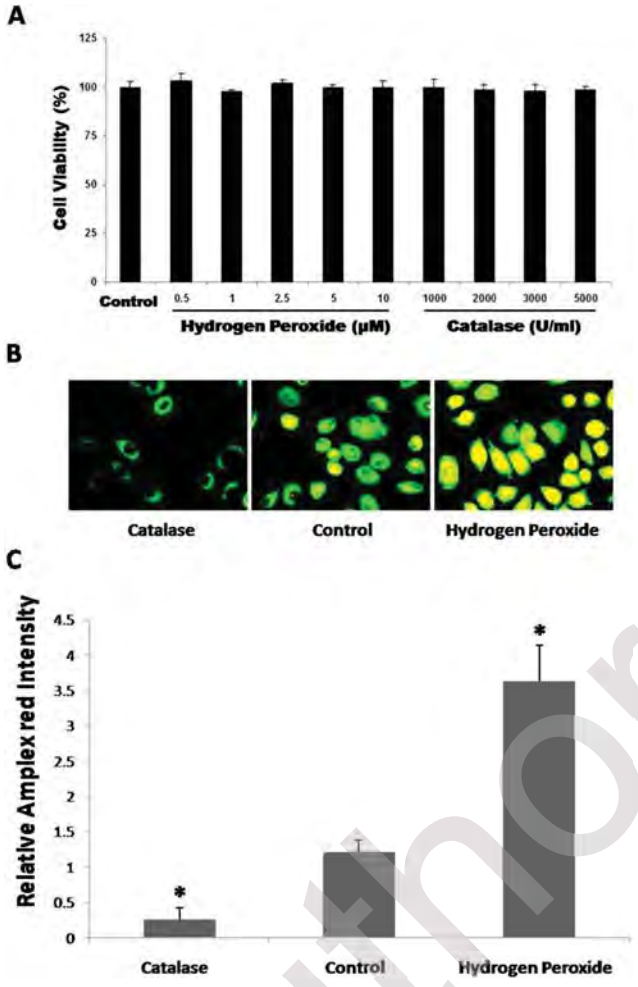

Figure 1 Effect of H_2O_2 and catalase treatments on viability and intracellular ROS level of H460 cells

(**A**) Cells were treated with H_2O_2 (0.5–10 μM) and catalase (1000–5000 units/ml) for 24 h and cell viability was determined by MTT assay. Values are mean ± S.D. (n=4). *P<0.05 versus control. (**B**) Cells were treated with H_2O_2 (2.5 μM) and catalase (2000 units/ml) for 2 h. ROS was determined by ROS-specific probe $DCFH_2$-DA and visualized under fluorescence microscope. (**C**) Hydrogen peroxide was determined by Amplex Red assay and quantified by fluorescence microplate reader. Values are mean ± S.D. (n=4). *P<0.05 versus control.

anchorage-independent growth and anoikis resistance in H460 cells. The H460 cells were stably transfected with Cav-1 or shRNA Cav-1 plasmid to increase or knockdown Cav-1 respectively. Western blot analysis of Cav-1 expression showed a substantial increase in Cav-1 protein level in Cav-1-transfected cells (H460/Cav-1), whereas a significant decrease in Cav-1 level was seen in shRNA Cav-1 transfected cells (H460/shCav1) (Figures 3D and 3E). These stable transfected cells were prepared in suspension and analysed for anchorage-independent growth by soft agar colony assay. H460/Cav-1 had a high capability to grow in the anchorage-independent manner with a ~2-fold induction of colony diameter and a 1-fold induction of colony number compared to the control vector cells, whereas H460/shCav-1 cells had a low ability to survive and grow (Figures 3A–3C). In addition, we investigated the role of Cav-1 in anoikis characteristics of H460 cells. H460/Control Vector, H460/Cav-1, H460/Control shRNA and H460/shCav-1 cells were detached for 0–24 h and cell viability was analysed by XTT assay. Figure 3(F) shows that H460/Cav-1 cells were significantly more resistant to anoikis compared to control cells, whereas H460/shCav1 cells were more sensitive to detachment-induced apoptosis. Cav-1 therefore plays a critical role in inhibition of detachment-induced cell death and facilitates growth in detached condition.

3.3. H_2O_2 exposure induces Cav-1 upregulation and consequently enhances cell growth and anoikis resistance of detached H460 cells

Having shown that Cav-1 caused H460 cell resistance to anoikis and encouraged growth in detached conditions, we hypothesized that H_2O_2 exposure could induce anchorage-independent cell growth and anoikis resistance by regulating the level of Cav-1 protein. Expression of Cav-1 was examined after cultivation with sub-toxic concentrations of H_2O_2 for 14 days; it was upregulated in response to H_2O_2 in a dose-dependent manner (Figure 4A). We determined whether such H_2O_2-induced Cav-1 upregulation could last long enough to render anchorage-independent growth of the cells. After 24 h, 72 h and 2 weeks, the cells in H_2O_2-free soft agar assays were extracted and analysed for Cav-1 expression by Western blotting. H_2O_2 significantly upregulated Cav-1 levels after 24 h of detachment (Figure 4B) and the increase was sustained in the cells cultivated in soft agar after 72 h and 2 weeks (Figure 4C–4D). Importantly, the higher dose of H_2O_2 exposure prior to detachment caused a higher Cav-1 upregulation at every time-point. These findings correlated with the above observation of the ability of cells to grow in anchorage-independent condition cells and develop anoikis resistance in a dose-dependent manner, supporting the conclusion that H_2O_2-mediated Cav-1 upregulation is responsible. To ensure the increase of the Cav-1 level in H_2O_2-treated cells, immunocytochemistry staining for Cav-1 in H460/10 μM-H_2O_2 cells (Figure 4E) shows that H460/10 μM-H_2O_2 cells have dramatically high Cav-1-related fluorescent intensity compared to H460 control cells.

3.4. H_2O_2 exposure enhances anoikis resistance and the growth under detached conditions in shRNA-transfected cells

To provide further support, we tested whether H_2O_2-exposure could render H460/shCav1 cell growth in detached conditions and resistant to anoikis. We stably transfected H460 cells with shRNA Cav-1 plasmid; these cells were either left untreated or treated with 10 μM H_2O_2 for 24 h and followed by soft agar assay for 72 h and 2 weeks. After 2 weeks, H460/shCav1 cells had a low ability to survive and grow in detached conditions, with a 0.5 reduction in colony diameter, a 0.3 reduction in colony number; and treatment with H_2O_2 could enhance such a capability as indicated in the increased size and appearance of the cell colony (Figures 5A–5C). After 72 h of soft agar culturing, the cells were extracted and the Cav-1 level determined by Western blot analysis. Figure 5(D) shows that H460/shCav1 cells expressed a significantly lower level of Cav-1 compared to the H460/Control shRNA cells, and treatment of H460/shCav1 cells with H_2O_2 resulted in a substantial

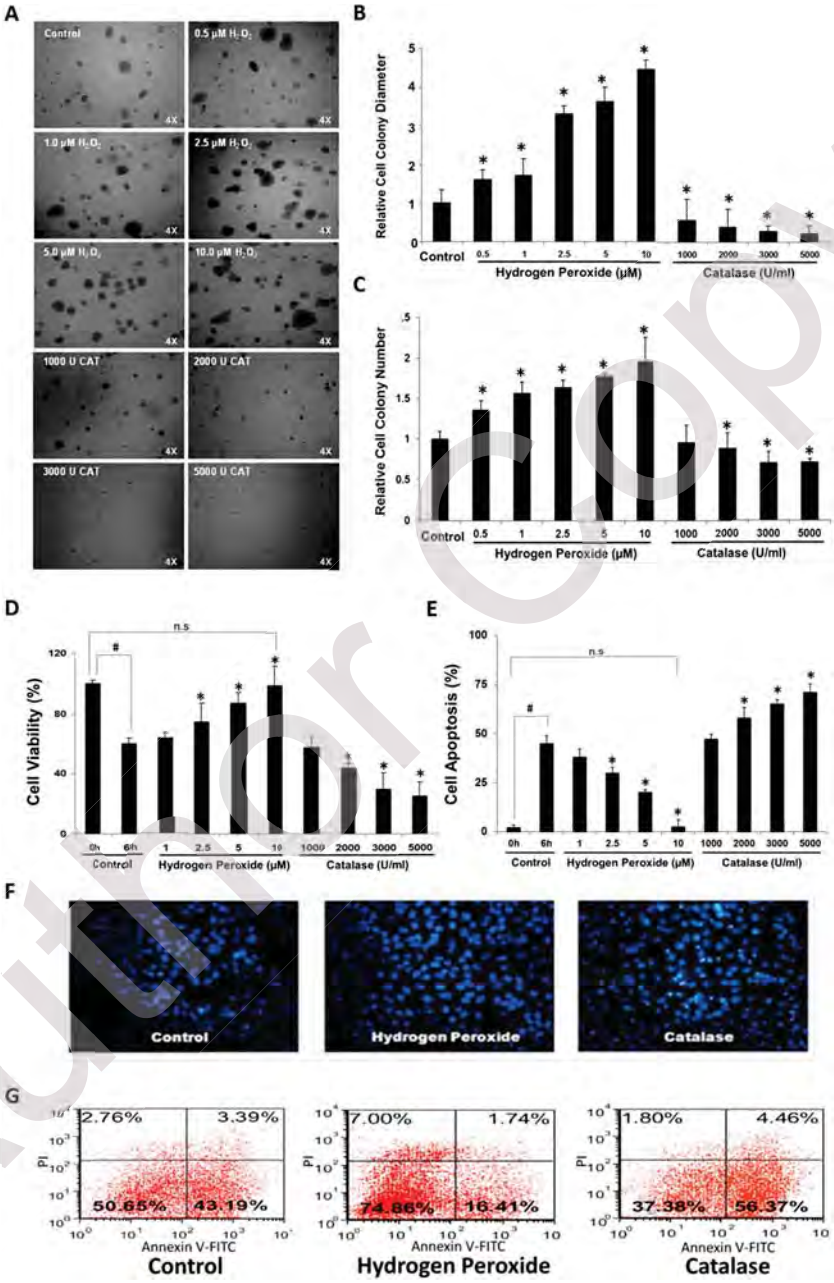

Figure 2 **H_2O_2 exposure induces anchorage-independent growth and anoikis resistance of H460 cells**
The cells were cultured in the presence of H_2O_2 (0–10 μM) or catalase (0–5000 units/ml) for 14 days. (**A**) Single cell suspensions were subjected to soft agar colony formation assay as described in Materials and methods. (**B**) Relative cell colony diameter and (**C**) relative cell colony number were determined by using image analyser. Values are mean ± S.D. ($n=3$). *$P<0.05$ versus control. (**D**) Cells were detached and suspended in poly-HEMA-coated plates for 6 h and cell viability was determined by XTT assay. Values are mean ± S.D. ($n=4$). $^{\#}P<0.05$ versus control at 0 h; *$P<0.05$ versus control at 6 h. (**E**) Cells were detached and suspended in poly-HEMA-coated plates for 6 h, anoikis cell nuclei stained with Hoechst 33342 and PI was visualized and quantified under fluorescence microscope. Values are mean ± S.D. ($n=4$). $^{\#}P<0.05$ versus control at 0 h; *$P<0.05$ versus control at 6 h. (**F**) H_2O_2 (2.5 μM) and catalase (2000 units/ml) exposed cells were detached for 6 h. Morphology of detached H460 cells anoikis nuclei stained with Hoechst 33342 and PI was visualized under fluorescence microscope. (**G**) Cell death was evaluated by annexin V-FITC/PI using flow cytometry.

increase in Cav-1 compared to H460/shCav1 cells. Furthermore, H460/shCav1 cells showed a significantly higher degree of anoikis compared to the control H460 cells, and the treatment of the H460/shCav1 cells with H_2O_2 significantly inhibited the reduction of cell viability after detachment as compared to H460/shCav1 cells (Figure 5E).

3.5. H_2O_2 exposure induces anchorage-independent growth and anoikis resistance of melanoma cells

The effects of H_2O_2 on human melanoma G361 cells was explored. Cells were exposed to H_2O_2 (0–10 μM) or catalase (0–5000 units/ml)

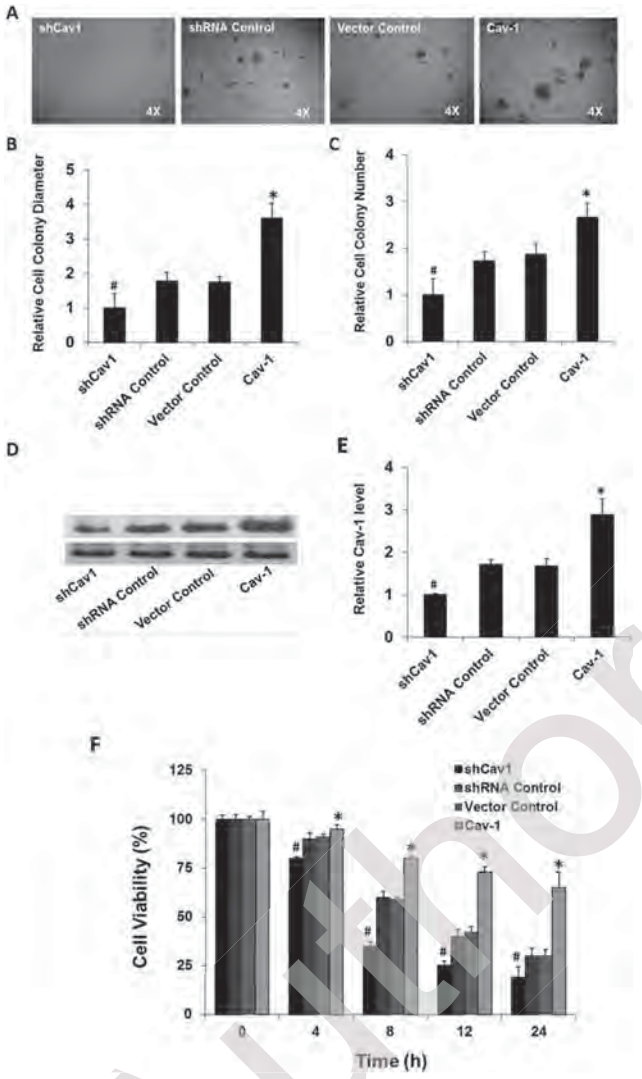

Figure 3 **Cav-1 is the key regulator of anchorage-independent growth and anoikis resistance.**

(**A**) H460 cells were stably transfected with Vector, Cav-1 overexpressing, Control shRNA or shRNA Cav-1 plasmids to generate H460/Vector, H460/Cav-1, H460/Control shRNA and H460/shCav1, respectively. The ability of anchorage-independent growth of transfected cells was evaluated by using soft agar colony formation assay. The relative cell colony diameter was determined by using an image analyser. Values are mean ± S.D. ($n=4$). $^{\#}P<0.05$ versus control shRNA; $^{*}P<0.05$ versus control vector. (**B**) Relative cell colony diameter was determined by using an image analyser. $^{\#}P<0.05$ versus control shRNA; $^{*}P<0.05$ versus control vector. (**C**) Relative cell colony number was determined by using an image analyser. $^{\#}P<0.05$ versus control shRNA; $^{*}P<0.05$ versus control vector. (**D**) Cav-1 protein expression was determined by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. (**E**) Immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the control cells. (**F**) Anoikis response of the transfected cells was determined at the indicated times after cells detachment by XTT assay. Values are mean ± S.D. ($n=4$). $^{\#}P<0.05$ versus control shRNA; $^{*}P<0.05$ versus control vector.

for 14 days. H_2O_2 enhanced anchorage-independent survival and growth of melanoma cells, whereas catalase treatment caused the opposite effect (Figures 6A–6C). H_2O_2 also significantly induced anoikis resistance in these cells, but treatment with catalase decreased cell survival after detachment (Figures 6D and 6E). Thus H_2O_2 exposure can give similar results in another type of cancer cell.

3.6. H_2O_2 exposure induces Cav-1 upregulation through gene expression without altered Cav-1 degradation pathway

Regarding the mechanism involved in the upregulation of Cav-1 level, our previous study showed that H_2O_2 increased Cav-1 in detached cells by inhibiting its degradation (Rungtabnapa et al., 2011). Immunoprecipitation assay was used to determine the involvement of the protein degradation pathway. Figure 7(A) shows the same level of Cav-1 ubiquitin complex in control and H_2O_2-exposed cells, suggesting that ubiquitin-mediated protein degradation was not involved in upregulation by H_2O_2 exposure in attached conditions. Quantitative real-time PCR was used to determine the involvement of protein synthesis pathway. Figure 7(B) shows H_2O_2 exposure significantly increased Cav-1 mRNA in H_2O_2-exposed cells. We confirmed this finding by treating H_2O_2-exposed cells with actinomycin D and cycloheximide. Figure 7(C) shows that treatment with these drugs decreased Cav-1 in H_2O_2-exposed cells. Since Cav-1 mRNA expression is increased after H_2O_2 exposure, and this is reversed by actinomycin D and cycloheximide, it suggests that the upregulation occurs through new gene expression.

4. Discussion

Along with ROS in cancer development (Ishikawa et al., 2008; Kumuar et al., 2008), the presence of ROS in tumour microenvironments is associated with the accentuation of aggressiveness in many cancers (Storz, 2005; Droge, 2002). Importantly aggressiveness of cancer cells includes anoikis resistance, which allows the cells to survive until reaching distant sites and growth in anchorage-independent conditions, thereby promoting metastasis, leading to a poor prognosis in, e.g. lung cancer (Moore et al., 1998; Hanahan and Weiberg, 2000; Wei et al., 2001; Laurent et al., 2005; Craig et al., 2008). Three species of ROS frequently found in either normal or cancerous tissue are superoxide anion ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical (·OH) (Kirkinezos and Moraes, 2001; Klaunig et al., 2010). These ROS are of intracellular origin from several sources, including the electron transport chain of mitochondria, NAD(P)H oxidoreductase complex, xanthine oxidase, and cyclooxygenases (Lander, 1997; Pourahmad, 2002). Mitochondrial-derived ROS seems to be the predominant source of cellular ROS associated with oxidative stress induced by many stimuli (Ku et al., 1993; Barber and Harris, 1994). Although $O_2^{\bullet-}$ is the first ROS to be produced by the mitochondrial chain reaction, H_2O_2, a product of the detoxifying mechanism of $O_2^{\bullet-}$, has a more pronounced role on normal and tumour cell proliferation (Laurent et al., 2005). H_2O_2 possesses relatively low reactivity that allows this kind of ROS to diffuse and function in distant areas compared to the others (Droge, 2002; Ku et al., 1993; Barber and Harris, 1994; Cadenas and Davies, 2000).

The mechanisms by which H_2O_2 enhances cancer progression and metastasis include the fact that it increases NF-κB (nuclear factor κB) activity and facilitates hepatic cancer cell metastasis (Kobayashi et al., 2008). H_2O_2 also activates AP1 (activator protein

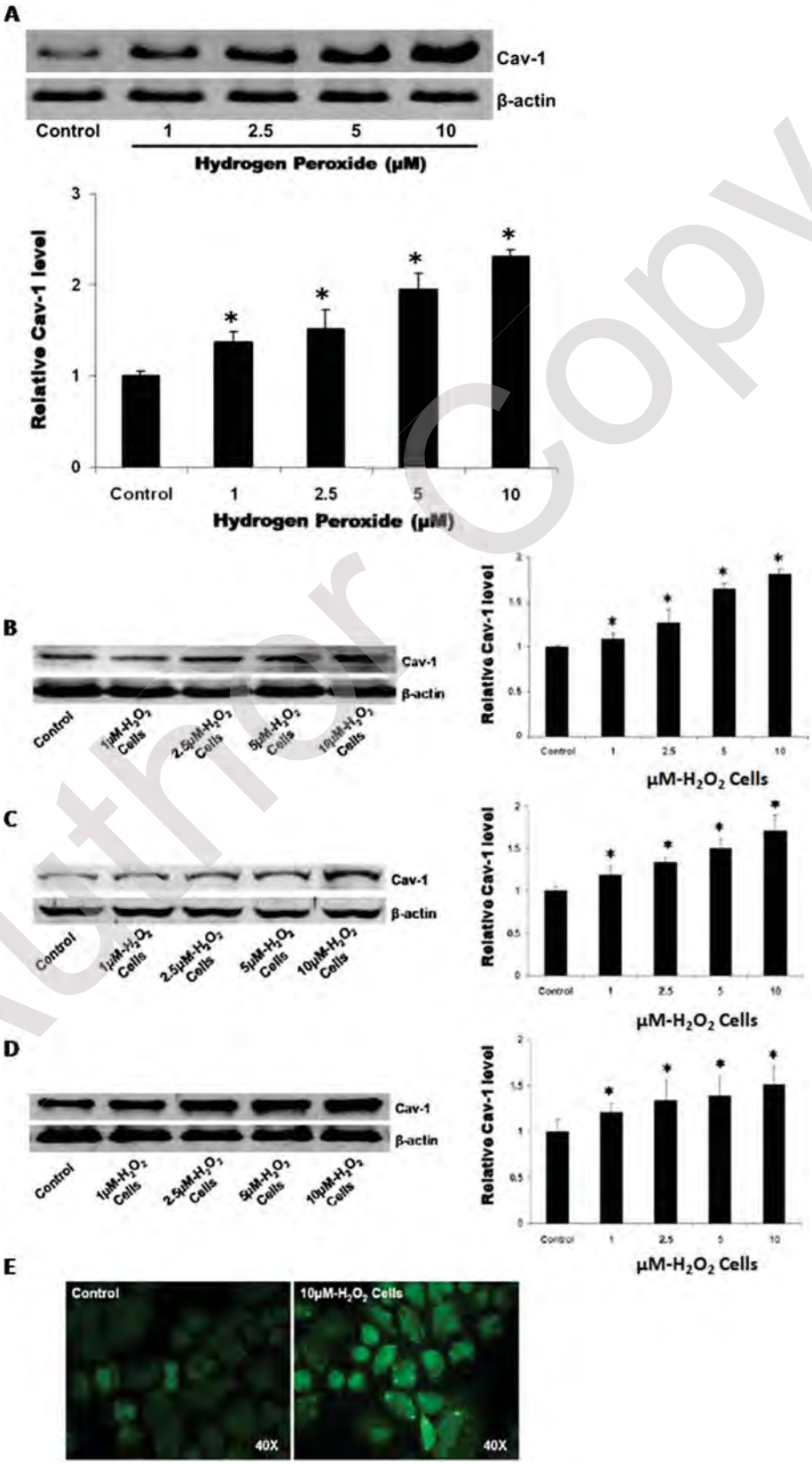

Figure 4 **H_2O_2 exposure induces Cav-1 upregulation**
(**A**) After cultivation of the cells with indicated concentrations of H_2O_2 for 14 days, Cav-1 protein expression was determined by Western blotting. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the control cells. H_2O_2-exposed cells were subjected to soft agar for 24 h, 72 h and 2 weeks. The cells were collected after culturing in soft agar for (**B**) 24 h, (**C**) 72 h and (**D**) 2 weeks, and the Cav-1 level was determined by Western blot analysis. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the control cells. (**E**) Immunocytochemistry staining for Cav-1 of H460/10 μM-H_2O_2 and non-treated control cells after 2 weeks in soft agar culture.

1), upregulates HARP (heparin affine regulatory peptide) gene, enhances proliferation and migration of prostate cancer cells (Polytarchou et al., 2005), reduces catalase expression via hypermethylation of CpG island II on the catalase promoter, and contributes to a higher stage of hepatocellular carcinoma (Min et al., 2010). It also affects angiopoietin-mediated activation of signalling pathways of Akt and MAPK (mitogen-activated protein kinase) phosphorylation, and increased angiogenesis of endothelial cells (Kim et al., 2006).

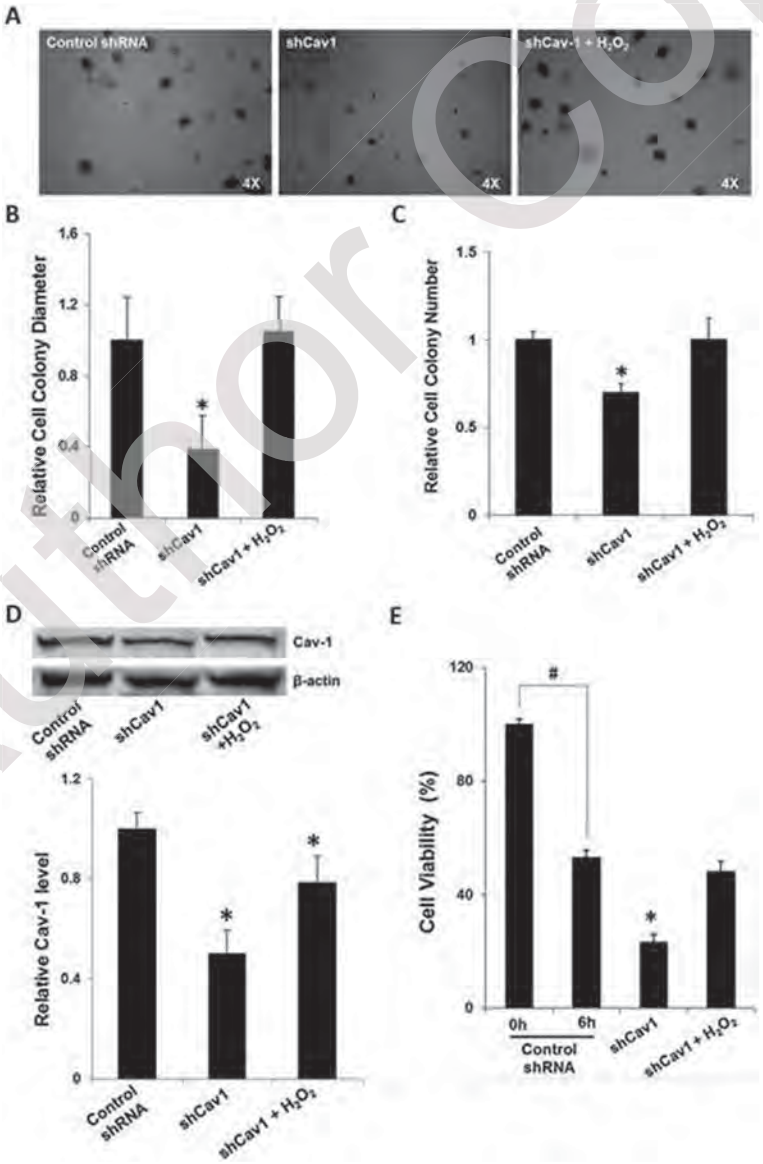

Figure 5 **H_2O_2 exposure enhances anoikis resistance and the growth under detached conditions in shRNA transfected cells**
H460/Control shRNA or H460/shCav1cells were left untreated or treated with 10 μM H_2O_2 for 24 h. (**A**) Cells were subjected to H_2O_2 free soft agar assays for 2 weeks and visualized under microscope. (**B**) The relative cell colony diameter and (**C**) relative colony number were determined by using an image analyser. Values are mean $\pm$ S.D. (n=3). *P<0.05 versus control shRNA. (**D**) After 72 h in soft agar culturing, cells were extracted from soft agar and Cav-1 protein expression was determined. (**E**) Cells were subjected to anoikis assay for 6 h and cell viability was determined by XTT assay. Values are mean $\pm$ S.D. (n=4). $^{\#}P$<0.05 versus control at 0 h; *P<0.05 versus control shRNA at 6 h.

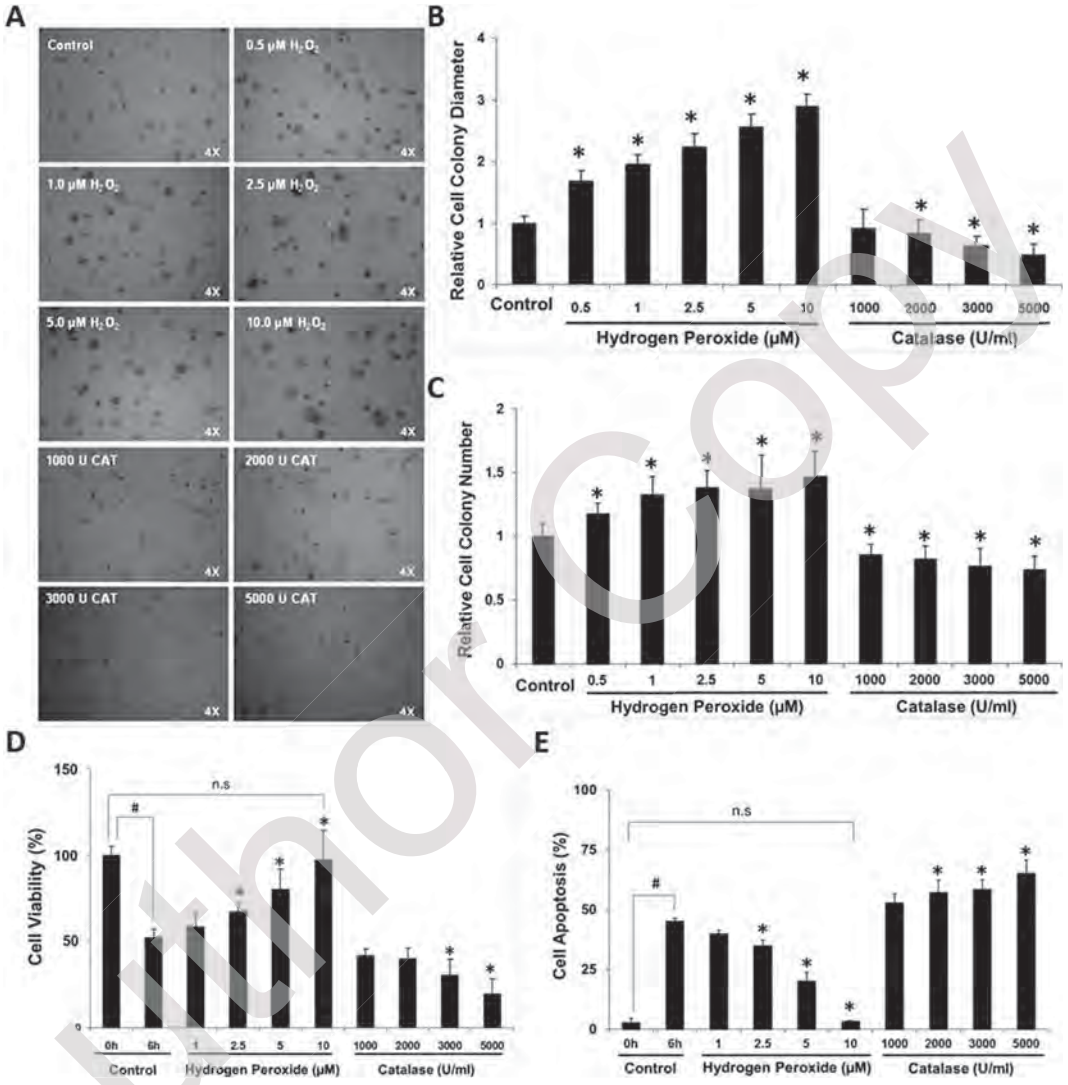

Figure 6 **H_2O_2 exposure induces anchorage-independent growth and anoikis resistance of melanoma cells**
Cells were exposed to H_2O_2 or catalase for 14 days. (**A**) Cells were detached and single cell suspensions were subjected to soft agar colony formation assay, and (**B**) relative cell colony diameter, and (**C**) relative cell colony number were determined by an image analyser. Values are mean ± S.D. (n=3). *P<0.05 versus control. (**D**) Cells were detached and suspended in poly-HEMA plates for 6 h and cell viability was determined by XTT assay. Values are mean ± S.D. (n=4). #P<0.05 versus control at 0 h; *P<0.05 versus control at 6 h. (**E**) Cells were detached and suspended in poly-HEMA-coated plates for 6 h, anoikis cell nuclei stained with Hoechst 33342 and PI was visualized and quantified under fluorescence microscope. Values are mean ± S.D. (n=4). #P<0.05 versus control at 0 h; *P<0.05 versus control at 6 h.

We have demonstrated that the exposure of lung carcinoma and melanoma cells to H_2O_2 prior to cell detachment dramatically increases their ability to resist detachment-induced apoptosis and encourage independent growth of the cells. Western blot analysis showed that long-term exposure of the cells to H_2O_2 significantly upregulates Cav-1 protein, which lasted as long as 2 weeks in soft agar assay. Regarding Cav-1 ubiquitin complex and Cav-1 mRNA level determinations, H_2O_2 exposure of the attached cells significantly increased Cav-1 expression without affecting its degradation. Both microenvironment-derived and cell-generated H_2O_2 are rapidly detoxified by 2 cellular enzymes, catalase and glutathione peroxidase. Thus, the half-life of H_2O_2 is considerably short in living systems (Pryor, 1986; Volk et al., 1997), even though roles of H_2O_2 in controlling cellular behaviour and functioning have been extensively reported. Our findings correlated with previous findings and further reveal the underlying mechanism how H_2O_2 treatment alters the expression of Cav-1 protein and facilitates cancer aggressiveness over relatively long periods. We also confirmed the regulatory role of Cav-1 in anoikis resistance and induced cell growth in detached conditions by transfecting the cells with Cav-1 overexpressing and shRNA Cav-1 plasmids. Both plasmids were able to change the level of cellular Cav-1 protein, and Cav-1 overepression dramatically rendered H460 cells anoikis resistant, whereas a decrease in Cav-1 mediated by shRNA transfection increased anoikis. To ensure that H_2O_2-mediated anoikis resistance and enhanced cancer cell growth in detached conditions were through Cav-1 modulation, shRNA transfected cells treated with increased Cav-1 level and cancer cell aggressiveness

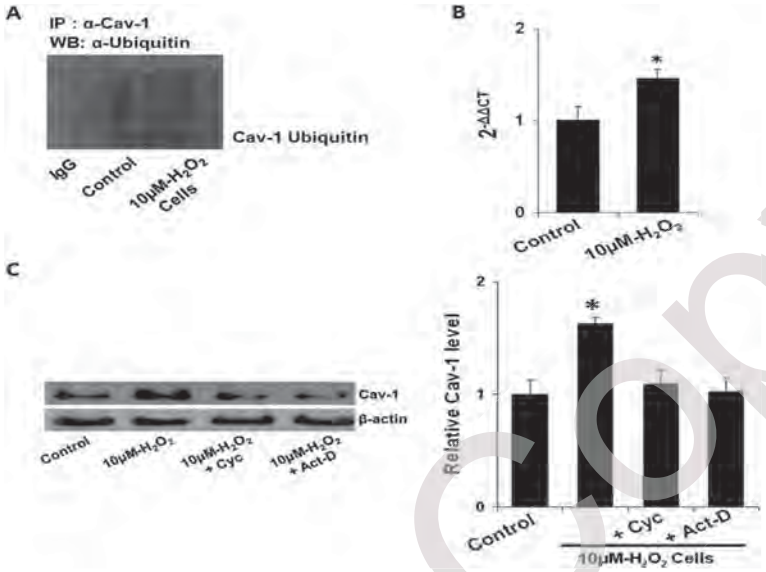

Figure 7 **H_2O_2 exposure induces Cav-1 upregulation through gene expression without altering Cav-1 degradation pathway**
H_2O_2-exposed cells were subjected to soft agar assay and the cells were collected after 2 weeks. (**A**) Cell lysates were prepared and immunoprecipated with anti-Cav-1 antibody. IgG without cell lysate was used as a non-specific antibody control. The resulting immune complexes were analysed for ubiquitin by Western blot analysis. (**B**) Relative Cav-1 mRNA expression was determined by quantitative real-time PCR using the comparative C_T method as described in the Materials and methods. (**C**) Cells were treated with Actinomycin D (Act D) and cycloheximide (Cyc) for 12 h before being collected from soft agar. Cav-1 level was determined by Western blot analysis. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the control cells.

(Figure 6). Thus an increase in Cav-1 level caused by H_2O_2 exposure is responsible for increased ability of H460 cells to resist anoikis and growth in a detached manner. Many studies have shown that Cav-1 increased cell survival and growth in detached conditions through an Akt-dependent pathway (Li et al., 2003; Ravid et al., 2005; Li et al., 2009), suggesting that Cav-1 may regulate anchorage-dependent growth via an Akt-dependent pathway.

In conclusion, H_2O_2 plays an important role in potentiating cancer cell aggressivenesses, anoikis resistance and growth in anchorage-independent conditions in human lung carcinoma H460 and melanoma G361 cells. Exposure to sub-toxic doses of hydrogen peroxide significantly upregulates Cav-1, and the increase is responsible for anoikis resistance and anchorage-independent growth of cancer cells. These findings suggest a linkage between elevated H_2O_2 in a cancer environment and an increase in tumour aggressiveness that are important in understanding tumour progression and metastasis.

Author contribution

Hasseri Halim carried out the experimental work, data analysis and drafted the manuscript. Pithi Chanvorachote conducted and design experiments, carried out the experimental work, research summary, and manuscript preparation and revision. Both authors read and approved the final manuscript.

Acknowledgements

We thank Mr Krich Rajprasit for editing assistance before submission.

Funding

This work was supported by the Thailand Research Fund and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

References

Barber DA, Harris SR. Oxygen free radicals and antioxidants: a review. Am Pharm 1994;34:26–35.

Bender FC, Reymond MA, Bron C, Quest AFG. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res 2000;60:5870–8.

Brabek J, Mierke CT, Rosel D, Vesely P, Fabry B. The role of the tissue microenvironment in the regulation of cancer cell motility and invasion. J Cell Commun Signal 2010;8:22–30.

Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radical Biol Med 1995;18:775–94.

Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biol Med 2000;29:222–30.

Cassani P, Daniele L, Maldi E, Righi L, Taraglione V, Novello S et al. Caveolin-1 expression in lung carcinoma varies according to tumor histotype and is acquired de novo in brain metastasis. Histopathology 2009;55:20–7.

Chan HP, Tran V, Lewis C, Thomas PS. Elevated levels of oxidative stress markers in exhaled breath condensate. J Thorac Oncol 2009;4:172–8.

Chanvorachote P, Nimmanit U, Lu Y, Talbott S, Jiang B, Rojanasakul Y. Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteosomal degration of caveolin-1. J Biol Chem 2009;284:28476–84.

Chung-Man Ho J, Zheng S, Comhair SA, Farrer C, Erzurm SC. Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res 2001;61:8578–85.

Craig DS, Kika A, Aaron DS. Anoikis resistance and tumor metastasis. Cancer Lett 2008;272:177–85.
Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.
Engelman JA, Zhang XL, Galbiati F, Lisanti MP. Chromosomal localization, genomic organization, and developmental expression of the murine caveolin gene family (Cav-1, -2, and -3) Cav-1 and Cav-2 genes map to a known tumor suppressor locus (6-A2/7q31). FEBS Lett 1998;429:330–6.
Fiucci G, Ravid D, Reich R, Liscovitch M. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene 2002;21:2365–75.
Galbiati F, Volonte D, Liu J, Capozza F, Frank PG, Zhu L et al. Caveolin-1 expression negatively regulates cell cycle progression by inducing g_0/g_1 arrest via a $p53/p21^{waf1/cip1}$-dependent mechanism. Mol Biol Cell 2001;12:2229–44.
Glenney JR, Zokas L. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. J Cell Biol 1989;108:2401–8.
Hanahan D, Weiberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
Ho CC, Huang PH, Huang HY, Chen YH, Yang PC, Hsu SM. Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol 2002;161:1647–56.
Isaiah JF. The organ microenvironment and cancer metastasis. Differentiation 2002;70:498–505.
Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H et al. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science 2008;320:661–4.
Kato K, Hida Y, Miyamoto M, Hashida H, Shinohara T, Itoh T et al. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer 2002;94:929–33.
Kim YM, Kim KE, Koh GY, Ho YS, Lee KJ. Hydrogen peroxide produced by angiopoietin-1 mediates angiogenesis. Cancer Res 2006;66:6167–74.
Kirkinezos IG, Moraes CT. Reactive oxygen species and mitochondrial diseases. Semin Cell Dev Biol 2001;12:449–57.
Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol 2010;38:96–109.
Kobayashi Y, Nishikawa M, Hyoudou K, Yamashita F, Hashida M. Hydrogen peroxide-mediated nuclear factor κB activation in both liver and tumor cells during initial stages of hepatic metastasis. Cancer Sci 2008;99:1542–52.
Ku HH, Brunk UT, Sohal RS. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. Free Radical Biol Med 1993;15:621–7.
Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. Cancer Res 2008;68:1777–85.
Lander HM. An essential role for free radicals and derived species in signal transduction. FASEB J 1997;11:118–24.
Laurent A, Nicco C, Chereau C, Goulvestre C, Alexandre J, Alves A et al. Controlling tumor growth by modulating endogenous production of reactive oxygen species. Cancer Res 2005;65:948–956.
Lee SW, Reimer CL, Oh P, Campbell DB, Schnitzer JE. Tumor cell growth inhibition by caveolin-1 re-expression in human breast cancer cells. Oncogene 1998;16:1391–7.
Li L, Rec C, Yang G, Goltsor AA, Tabata K, Thompson TC. Caveolin-1 promotes autoregulatory, Akt-mediated induction of cancer-promoting growth factors in prostate cancer cells. Mol Cancer Res 2009;7:1781–91.
Li L, Ren CH, Tahir SA, Ren C, Thompson TC. Caveolin-1 maintains activated AKT in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol 2003;23:9389–404.
Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L et al. Increased Nox1 and hydrogen peroxide in prostate cancer. Prostate 2005;62:200–7.
Liou GY, Storz P. Reactive oxygen species in cancer. Free Radical Res 2010;44:479–96.
Lopez-Lazaro M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. Cancer Lett 2007;252:1–8.
Min JY, Lim S, Jung G. Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the catalase promoter. FEBS Lett 2010;584:2427–32.
Moon KC, Lee GK, Yoo SH, Jeon YK, Chung JH, Han J, Chung DH. Expression of caveolin-1 in pleomorphic carcinoma of the lung is correlated with a poor prognosis. Anticancer Res 2005;25:4631–7.
Moore SM, Rintoul RC, Walker TR, Chilvers ER, Haslett C, Sethi T. The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and $p71^{s6k}$-dependent pathway. Cancer Res 1998;58:5239–47.
Mori S, Chang JT, Andrechek ER, Matsumura N, Baba T, Yao G et al. Anchorage-independent cell growth signature identifies tumors with metastatic potential. Oncogene 2009;28:2796–805.
Polytarchou C, Hatziapostolou M, Papadimitriou E. Hydrogen peroxide stimulates proliferation and migration of human prostate cancer cells through activation of activator protein-1 and up-regulation of the heparin affin regulatory peptide gene. J Biol Chem 2005;280:40428–35.
Pourahmad J. Identification of intracellular sources responsible for endogenous reactive oxygen species formation. Iran J Pharm Res 2002;1:21–9.
Pryor WA. Oxy-radicals and related species. Their formation, lifetimes, and reactions. Annu Rev Physiol 1986;48:657-67.
Racine C, Belanger M, Hirabayashi H, Boucher M, Chakir J, Couet J. Reduction of caveolin 1 gene expression in lung carcinoma cell lines. Biochem Biophys Res Commun 1999;255:580–6.
Ravid D, Maor S, Werner H, Liscovitch M. Caveolin-1 inhibits anoikis and promotes survival signaling in cancer cells. Adv Enzyme Regul 2006;46:163–75.
Ravid D, Maor S, Werner H, Liscovitch M. Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by upregulation of insulin-like growth factor-1 receptors and signaling. Oncogene 2005;24:1338–47.
Rennebeck G, Martelli M, Kyprianou N. Anoikis and survival connections in the tumor microenvironment is there a role in prostate cancer metastasis? Cancer Res 2005;65:11230–5.
Rofstad EK. Microenvironment-induced cancer metastasis. Int J Radiat Biol 2000;76:589–605.
Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. Cell 1992;68:673–82.
Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y, Chanvorachote P. Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. Am J Physiol 2011;300:235–45.
Scherer PE, Okamoto T, Chun M, Nishimoto I, Iodish HF, Lisanti MP. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. Proc Natl Acad Sci USA 1996;93:131–5.
Shanmugathasan M, Jothy S. Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. Pathol Int 2000;50:273–9.
Storz P. Reactive oxygen species in tumor progression. Front Biosci 2005;10:1881–96.
Suzuoki M, Miyamoto M, Kato K, Hiraoka K, Oshikiri T, Nakakubo Y et al. Impact of caveolin-1 expression on prognosis of pancreatic ductal adenocarcinoma. Br J Cancer 2002;87:1140–4.
Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991;51:794–8.
Volk T, Hensel M, Kox WJ. Transient Ca^{2+} changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. Mol Cell Biochem 1997;171:11–21.
Wei L, Yang Y, Yu Q. Tyrosine kinase-dependent, phosphatidylinositol 3′-kinase, and mitogen-activated protein kinase-independent signaling pathways prevent lung adenocarcinoma cells from anoikis. Cancer Res 2001;61:2439–44.
Wu WS. The signaling mechanism of ROS in tumor progression. Cancer Metastasis Rev 2006;25:695–705.
Yang G, Truong LD, Timme TL, Ren C, Wheeler TM, Park SH et al. Elevated expression of caveolin is associated with prostate and breast cancer. Clin Cancer Res 1998;4:1873–80.

Yeh D, Chen C, Sun M, Shao S, Hao L, Song Y et al. Caveolin-1 is an important factor for the metastasis and proliferation of human small cell lung cancer NCI-H446 cell. Anat Rec 2009;292:1584–92.

Zieba M, Suwalski M, Kwiatkowska S, Piasecka G, Gizelewska-Rzymowska I, Stolarek R, Nawak D. Comparison of hydrogen peroxide generation and the content of lipid peroxidation products in lung cancer tissue and pulmonary parenchyma. Resp Med 2000;94:800–5.

Received 3 March 2012/28 June 2012; accepted 30 August 2012

Published as an Immediate Publication 30 August 2012, doi 10.1042/CBI20120111

J Nat Med
DOI 10.1007/s11418-012-0719-y

ORIGINAL PAPER

Imperatorin sensitizes anoikis and inhibits anchorage-independent growth of lung cancer cells

Kanuengnit Choochuay · Preedakorn Chunhacha · Varisa Pongrakhananon · Rataya Luechapudiporn · Pithi Chanvorachote

Received: 6 August 2012 / Accepted: 10 October 2012

Abstract The anoikis-sensitization activity of imperatorin, an active furanocoumarin component of *Angelica dahurica* root, is reported herein for the first time. The present study demonstrated that the imperatorin treatment at sub-toxic concentrations enhanced human lung cancer H23 cell apoptosis after detachment. A Western blot analysis showed that imperatorin significantly enhanced the p53 protein level, which subsequently down-regulated Mcl-1 protein and up-regulated Bax, while it had a minimal effect on Bcl-2 expression. In addition, an anchorage-independent growth assay was performed to support the anti-metastasis potential of imperatorin. Consistent with anoikis assay, imperatorin exhibited a strong inhibitory effect on the anchorage-independent growth of the cells. Further, this study demonstrated that imperatorin sensitizes anoikis in other lung cancer cells, namely, H292 and A549. Because anoikis was shown to be a critical hindrance in preventing cancer cell metastasis, the knowledge regarding such an activity and an underlying mechanism may lead to the development of this compound for a cancer therapy.

Keywords Imperatorin · Anoikis · Anchorage-independent growth · Lung cancer cells

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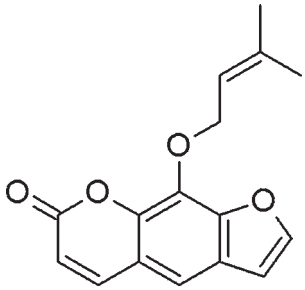
Introduction

Imperatorin (IM), a major active furanocoumarin enriched in a root of *Angelica dahurica*, has been reported to possess a variety of pharmacological actions against cancers, including the oncogene suppression, the inhibition of cancer cell proliferation, and the induction of cancer cell apoptosis both in vitro and in vivo (Fig. 1) [1–4]. However, until now, a role of imperatorin in the inhibition of cancer cell metastasis as well as cancer cell anoikis is largely unknown.

Metastasis is the major cause of cancer-related death in many human cancers, including lung cancer [5, 6]. In particular, lung cancer patients are frequently found to have metastatic tumors at the time of diagnosis, which may be the cause of a high rate of death in this type of cancer [7, 8]. Cancer metastasis is a complex process, consisting of cancer cell detachment, migration, extravasation, and adhesion to target sites. As an important barrier for cancer metastasis, apoptosis mediated by cell detachment, termed "anoikis", has garnered increasing attention in the cancer research domain. The anoikis process of the cells initiates after the cells lose contact from neighboring cells or from their extracellular matrix (ECM), and leads to the activation of p53 and the subsequent alteration of pro- and anti-apoptotic protein in the Bcl-2 family [9–13]. The balance between pro-apoptotic Bcl-2 proteins such as Bim, Bmf, Bax, and Bid and anti-apoptotic proteins such as Bcl-2 and Bcl-XL are found to be disturbed in response to cell detachment signals [14–20]. The mitochondrial membrane is then disrupted, followed by the release of cytochrome *c* and the activation of caspases [21, 22]. Recently, the role of anti-apoptosis Mcl-1 protein in the inhibition of anoikis has been intensively pronounced. A high level of endogenous or the overexpression of Mcl-1 was shown to be closely related to an anoikis response in many cancers [23–26]. So far, it has been well

Published online: 30 October 2012

Fig. 1 Structure of imperatorin



accepted that the resistance to anoikis is a hallmark of successful metastatic cancers. Also, an ability of cancer cells to grow in anchorage-independent conditions was shown to be an important characteristic of cancer aggressiveness and to be related to metastasis potential in different types of human malignancies, including lung cancer [27, 28].

As an ongoing research for anti-cancer drug development, the present study aimed to investigate the effects of imperatorin on cancer cell anoikis and anchorage-independent growth, and to explore a possible underlying mechanism.

Materials and methods

Cells and reagents

NCI-H23, NCI-H460, A549, and HK-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cancer cells were cultured in RPMI 1640 medium, while the HK-2 cells were cultured in DMEM medium. All mediums were supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5 % CO_2 environment at 37 °C. Imperatorin, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), Hoechst 33342, and propidium iodide were obtained from Sigma. Antibodies for Mcl-1, Bcl-2, Bax, P53, β-actin, and peroxidase-labeled secondary antibodies were obtained from Abcam Inc. (Cambridge, MA).

Cytotoxicity assay

Cell viability was determined by the XTT assay. After specific treatments, cells were incubated with 20 μM of XTT for 4 h at 37 °C. The optical density was then determined by a V-max photometer (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 450 nm.

Anoikis assay

To prevent cell adhesion, tissue culture six-well plates were coated with 200 μl (6 mg/ml in 95 % ethanol) of poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma) and left to evaporate overnight in a laminar flow hood at room temperature. Adherent H23, H292, and A549 cells in culture plates were trypsinized into a single-cell suspension in RPMI medium and then seeded in poly-HEMA-coated plates at a density of 1×10^5 cells/ml. Suspended cells were incubated at 37 °C for various times up to 24 h. For the cell survival assay, cells were incubated with XTT for 4 h at 37 °C. The optical density was then determined by a V-max photometer (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 450 nm.

Annexin V detection and sub-G0/G1 fraction analysis by flow cytometry

Cell anoikis were evaluated by Annexin V-FITC staining assay. Cells were collected, re-suspended, and incubated with Annexin V-FITC for 30 min at 37 °C. Apoptotic cells were scored by flow cytometry using a 485-nm excitation beam and a 538-nm band-pass filter (FACSort, Becton Dickinson, Rutherford, NJ). The mean fluorescence intensity was quantified by CellQuest software (Becton Dickinson). For sub-G0/G1 analysis, cells following detachment for 24 h in the presence or absence of imperatorin were harvested, re-suspended, and incubated with propidium iodide (PI) buffer for 15 min at 37 °C and determined for cell cycle profile by flow cytometry.

Nuclear staining assay

Apoptotic and necrosis cell death was determined by Hoechst 33342 and PI co-staining. After specific treatments, cells were incubated with 10 μM of Hoechst and 5 μg/ml of PI for 30 min at 37 °C. The apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

Colony formation assay

Anchorage-independent growth was determined by the colony formation assay in soft agar, as described by Koleske et al. [29], with minor modifications. Briefly, H23 cells from six-well plate monolayer cultures were prepared into a single-cell suspension by the treatment with a mixture of 350 μl trypsin and 1.5 mM EDTA. Cells were suspended in RPMI containing 10 % FBS and 0.33 % low melting temperature agarose, then 2 ml containing 5×10^3 cells were plated in a six-well plate over a 2-ml layer of solidified RPMI–10 % FBS–0.6 % agarose. The cells were fed every 3 days by adding 200 μl of RPMI–10 % FBS. Colonies survival was determined by incubation with resazurin 1:10 for 1 h and the fluorescence intensity of resazurin product (resorufin) was measured at 530 nm

(excitation wavelength) and 590 nm (emission wavelength) and photographed at ×10 magnification after 2 weeks.

Western blot analysis

After specific treatments, cells were incubated in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 % Triton X-100, 150 mM sodium chloride, 10 % glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Applied Science) at 4 °C for 20 min. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad). Proteins (40 μg) were resolved under denaturing conditions by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h in 5 % nonfat dry milk in TBST (25 mM Tris–HCl, pH 7.4, 125 mM sodium chloride, 0.05 % Tween 20) and incubated with appropriate primary antibodies at 4 °C for 10 h. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were then detected by an enhanced chemiluminescence detection system (Amersham Biosciences) and quantified using Analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The mean densitometry data from independent experiments were normalized to the results in cells in the control. The values are presented as mean ± standard deviation (SD) from three or more independent experiments and were analyzed by analysis of variance (ANOVA) and a post hoc test at a significance level of $p < 0.05$.

Results

Cytotoxic effect of imperatorin on lung cancer H23 and normal renal HK-2 cells

To explore the anoikis-sensitization activity of imperatorin, sub-toxic concentrations of such a compound were first characterized. Human lung cancer H23 cells were incubated with various concentrations of imperatorin (0–10 μg/ml) for 24 h and cell viability was determined by the XTT assay. Figure 2a shows that a significant cytotoxic effect of imperatorin was found at the concentration of 10 μg/ml, with approximately 85 % of the cells remaining viable, whereas the concentrations less than 5 μg/ml had no significant effect on H23 cell viability. In addition, the nuclear morphology study supported the above findings that no apoptotic and necrotic cell death was detected in response to 0–5 μg/ml imperatorin (Fig. 2b, c).

Anti-cancer drugs are frequently found to cause toxic effects on the normal cells and such cytotoxic effects may become an important hindrance of successful chemotherapy. We also provided the supportive data regarding the cytotoxic effect of imperatorin on normal renal cells. Human renal HK-2 cells were treated with various concentrations of imperatorin and cell viability was determined

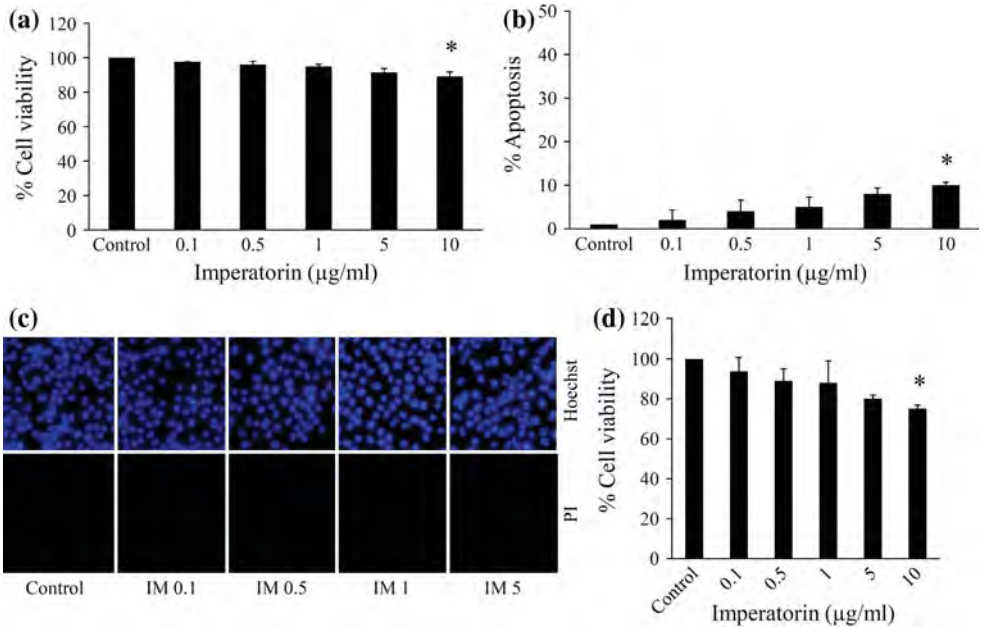


Fig. 2 Effect of imperatorin on cytotoxicity in lung cancer H23 cells. Cells were treated with various concentrations of imperatorin (0–10 μg/ml) for 24 h. **a** Cell viability was determined by the XTT assay. **b**, **c** Mode of cell death was determined by Hoechst 33342/PI co-staining assay. The apoptotic cells were visualized under a fluorescence microscope. **d** Cytotoxic effect of imperatorin on normal renal HK-2 cells. Cell viability was determined by the XTT assay. Values are means of independent triplicate samples ± SD ($n = 5$). *$p < 0.05$ versus non-treated control

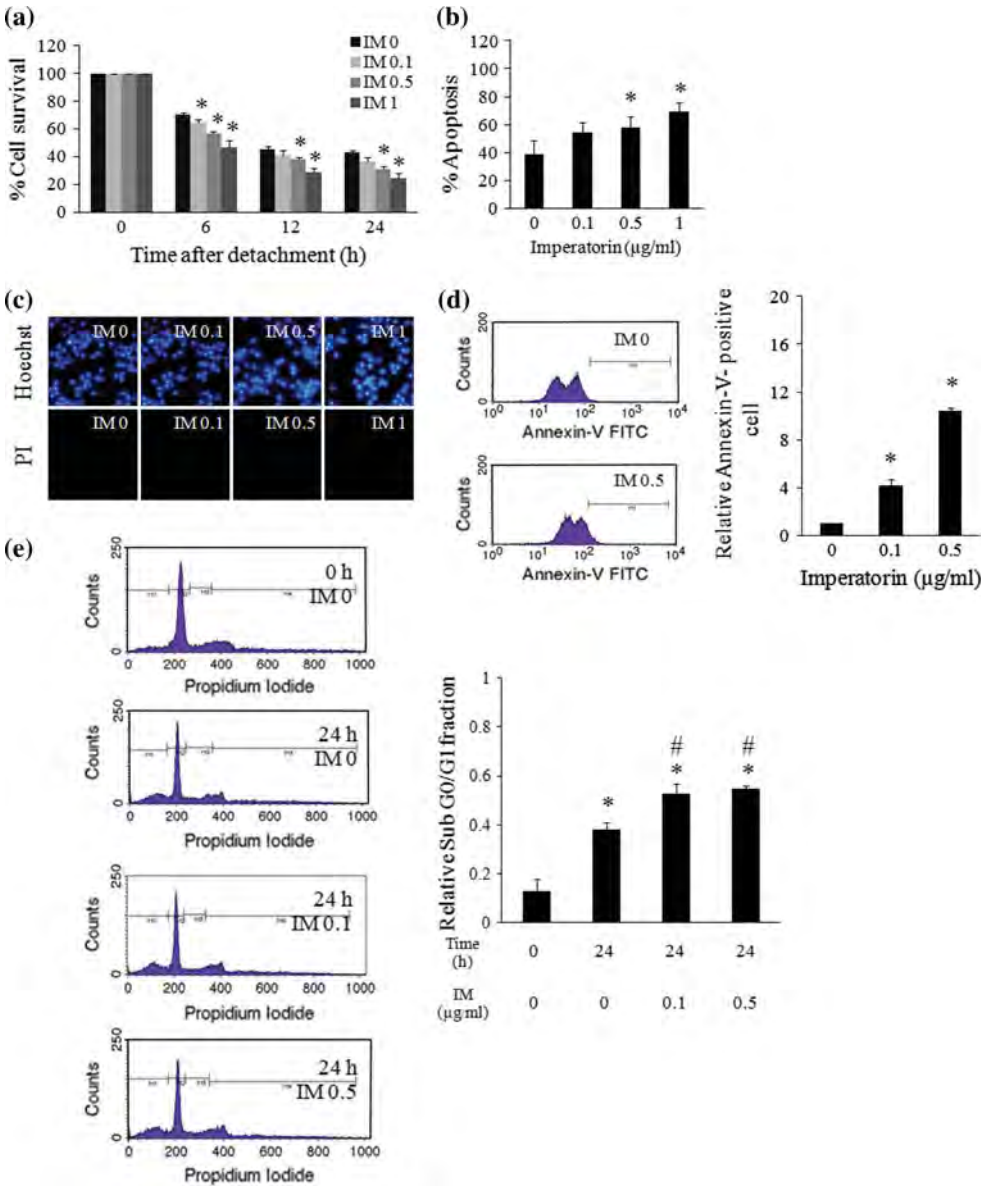


Fig. 3 Imperatorin sensitizes detachment-induced cell death in H23 cells. **a** Cells were detached and treated with various concentrations of imperatorin (0–1 μg/ml). Cell survival at the indicated times was determined by the XTT assay. Values are means of independent triplicate samples ± SD. $^{*}p < 0.05$ versus control at time 0 h. **b** Percentage of apoptotic detection by scoring DNA condensed and/or fragmented nuclei by Hoechst 33342 at 12 h after detachment. Values are means of independent triplicate samples ± SD. $^{*}p < 0.05$ versus non-treated control. **c** Morphology of cell nuclei after 12 h detachment was visualized under a fluorescence microscope after Hoechst 33342/PI co-staining. **d** Apoptosis was evaluated by Annexin V-FITC at 12 h after detachment. **e** Sub-G0/G1 fraction determined by flow cytometry. At the indicated time, cells were harvested, re-suspended and incubated with PI buffer for 15 min at 37 °C and determined for cell cycle profile by flow cytometry. $^{*}p < 0.05$ versus attached cell control and $^{\#}p < 0.05$ versus detached cell control

as previously described. Interestingly, the sub-toxic concentrations of imperatorin obtained from lung cancer H23 testing were found to be non-toxic to HK-2 cells (Fig. 2d). Even though further investigations were needed, these data supported the safety of the use of this compound for an anti-cancer aspect.

Imperatorin sensitizes lung carcinoma H23 cells to detachment-induced cell death

An anoikis-sensitization effect of imperatorin was then evaluated using non-toxic concentrations. Cells were detached and cultured in the attachment-resistant poly-HEMA-coated plates in the presence or absence of sub-toxic concentrations of imperatorin (0–1 μg/ml) and the cell survival was determined at the indicated times by the XTT assay. Figure 3a shows that, in the absence of imperatorin, cell detachment induced a time-dependent decrease in cell viability and the decrease was first detectable at 6 h after cell detachment, with approximately 70 % of the cells remain survival. Importantly, the addition of the detached cells with imperatorin caused a significant reduction of viable cells after detachment in a dose-dependent manner. Imperatorin at the concentration of 1 μg/ml could reduce the viability of the cells to approximately 50 and 30 % at times of 6 and 24 h, respectively. Further, the mode of cell death in response to imperatorin was investigated by Hoechst 33342 and PI staining assay. Figure 3b and c show that the addition of imperatorin to the detached cells significantly enhanced anoikis response, indicated by the increase of cells containing condensed DNA. The percentage of apoptosis cells at 12 h after cell

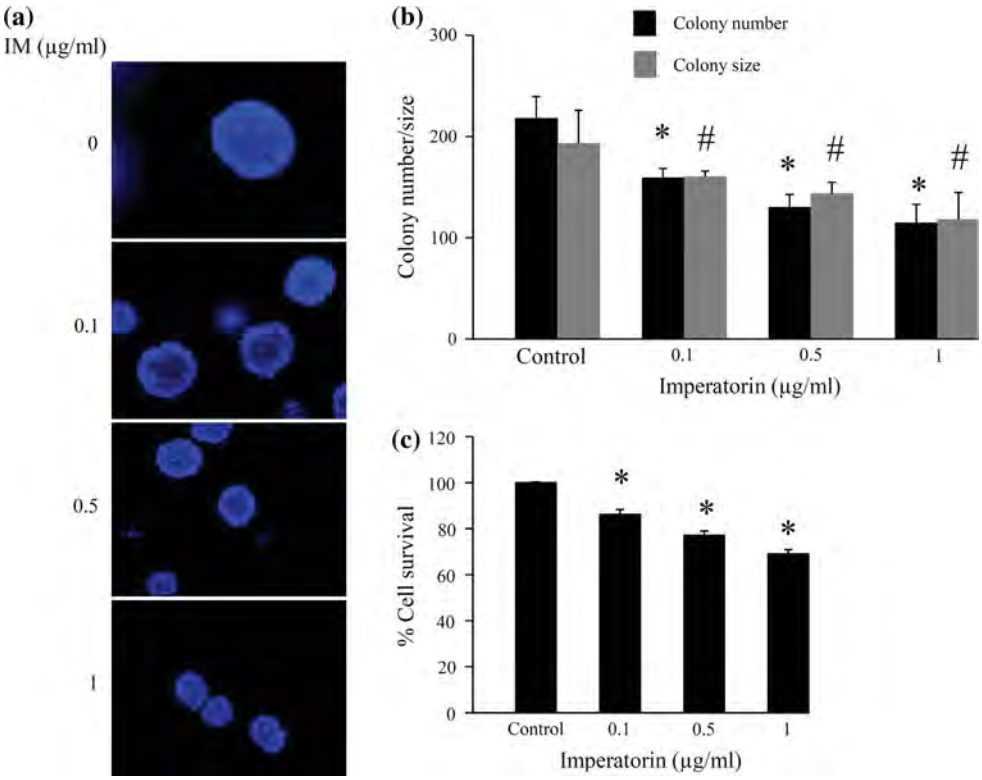


Fig. 4 Effect of imperatorin on anchorage-independent growth of H23 cells. Cells were subjected to soft agar colony formation assay, as described in "Materials and methods". **a** Colonies were stained with Hoechst 33342 and observed by a fluorescence microscope. **b** Colony number and colony size were determined by using an image analyzer. **c** Cell survival was determined with a resazurin-based assay. Values are means of independent triplicate samples ± SD. $^{*}p < 0.05$ versus non-treated control (colony number) and $^{\#}p < 0.05$ versus non-treated control (colony size)

detachment was found to be 54, 58, and 69 % in response to imperatorin at concentrations of 0.1, 0.5, and 1 μg/ml, respectively. It is interesting to note that there was no necrotic cell death detected in the present study. Annexin V staining assay was performed in order to determine the apoptosis response of the cells (Fig. 3d). Consistent with previous findings, annexin V-stained cells were found to be increased in response to imperatorin. In addition, cell cycle analysis showed that imperatorin treatment significantly increased the sub-G0/G1 fraction in a dose-dependent manner (Fig. 3e). These results indicated that the apoptosis induced by cell detachment or anoikis is the primary mode of cell death in our experiments.

Imperatorin inhibits anchorage-independent growth of H23 cells

Having shown the effect of imperatorin in sensitizing anoikis of lung cancer cells, we next tested whether such a compound could influence the growth of cancer cells in the anchorage-independent condition. As a well-accepted characteristic of metastatic cancer cells, the anchorage-independent growth or the cell growth in the detached condition was shown to be a potentiating factor presenting in highly aggressive cancers [27, 28]. H23 cells were subjected to soft agar assay in the presence or absence of sub-toxic concentrations of imperatorin and cultured for 2 weeks. Cell colony number as well as colony size were determined by microscopy. Figures 4a and b show that, in the absence of imperatorin, H23 cells were able to survive, grow under anchorage-independent conditions, and form large cell colonies. In contrast, the addition of imperatorin resulted in the reduction in both colony number and colony size. In order to quantify cell survival in the anchorage-independent assay, a resazurin-based cell viability assay was performed. Figure 4c shows that the results are consistent with the above-mentioned observation that viable cells in colony formation assays decreased in response to the imperatorin treatment in a dose-dependent manner. Together, our results indicated that imperatorin was able to inhibit cancer cell growth in the anchorage-independent condition.

Imperatorin-induces Mcl-1 down-regulation

To further clarify the mechanism by which imperatorin sensitizes detachment-induced apoptosis, we evaluated the level of proteins associating in an anoikis process, namely, p53, Mcl-1, Bcl-2, and Bax. Cells were detached and treated with 0–1 μg/ml imperatorin and the cells were subjected to Western blotting, as described in "Materials and methods". Figure 5a and b show that imperatorin significantly enhanced an increase of p53 level in a dose-dependent manner in comparison to that of the non-treated control. Further, imperatorin was found to decrease the anti-apoptotic Mcl-1 level in this lung cancer cells,

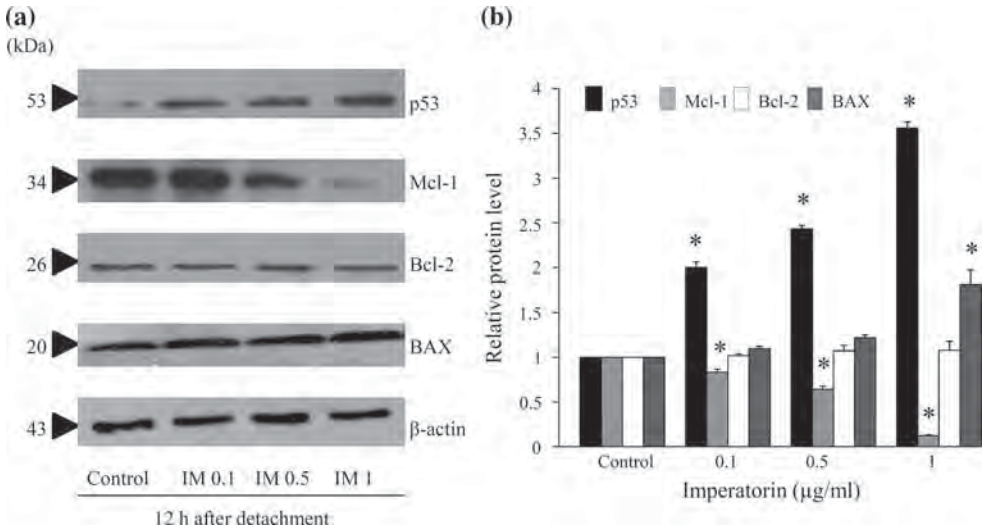


Fig. 5 Effect of imperatorin on proteins associated with the anoikis process. **a** Cells were detached and treated with various concentrations of imperatorin (0–1 μg/ml) for 12 h and the expression of p53, Mcl-1, Bcl-2, and Bax proteins was determined by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. **b** The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the results. Values are means on triplicate samples ± SD. $*p < 0.05$ versus non-treated control

whereas it exhibited only a minimal effect on Bcl-2 protein (Fig. 5a). The pro-apoptotic Bax was found to be significantly up-regulated in response to 1 μg/ml imperatorin. As Mcl-1 was shown to be a key regulator for anoikis resistance and p53 function was found to be attenuated in many cancer cells, these findings have highlighted the possible use of imperatorin for cancer therapy.

Anoikis-sensitization effect of imperatorin on other lung carcinoma cells

We further investigated the effect of imperatorin on anoikis in other lung cancer cells. Human lung cancer H292 and A549 cells were treated with various concentrations of imperatorin for 24 h. The cell viability assay indicated that imperatorin at a concentration of less than 10 μg/ml had neither cytotoxic nor proliferative effects on attached H292 and A549 cells (data not shown). To test the effect of imperatorin on the anoikis response of these cells, cells were incubated with 0–1 μg/ml imperatorin in the detached condition and cell viability was determined after 24 h by the XTT assay. Figure 6a and b show that cell detachment induced a gradual decrease in cell survival and imperatorin significantly sensitized both H292 and A549 cells to anoikis in a dose-dependent manner. After 24 h post-detachment, H292 cells exhibited approximately 60, 50, and 40 % viability in response to 0.1, 0.5, and 1 μg/ml of imperatorin treatments, respectively (Fig. 6a). Besides, the imperatorin treatment decreased the viability of A549 cells to 50, 40, and 35 % at concentrations of 0.1, 0.5, and 1 μg/ml, respectively (Fig. 6b). The morphology of apoptotic nuclei were observed in the imperatorin-treated cells (Fig. 6c–e), while PI-positive necrotic cells were not detected. These data supported the sensitizing effect of imperatorin on lung cancer cell anoikis.

Discussion

Among various human cancers, lung cancer is accepted as the leading cause of cancer mortality worldwide, and most deaths are associated with cancer metastasis [1, 2]. To metastasize, a malignant cell must detach from its primary tumor, invade the nearby circulatory or lymphatic system, and establish itself in a new site. An anoikis, a detachment-induced apoptosis, has been shown to play a critical role in the induction of most cancer cell death during travel in the blood or lymphatic circulations. Indeed, the resistance to anoikis is a prerequisite capability of metastatic cancer cells [24, 30–32]. Anoikis has primarily been described as an intrinsic apoptotic pathway mainly through a mitochondrial death pathway and was shown to be tightly related with the disturbance in the balance of proteins in the Bcl-2 family [33]. Previous studies have suggested that anti-apoptotic Bcl-2 and Mcl-1 proteins play an important role in mediating anoikis resistance, as well as the aggressive behaviors of lung cancer cells [34–37].

Increasing attention is paid to natural substances as a source for novel anti-cancer drugs. Imperatorin is a furanocoumarin isolated from the root of *Angelica dahurica*, which was previously reported to have several pharmacological effects [1–4]. The present study has further

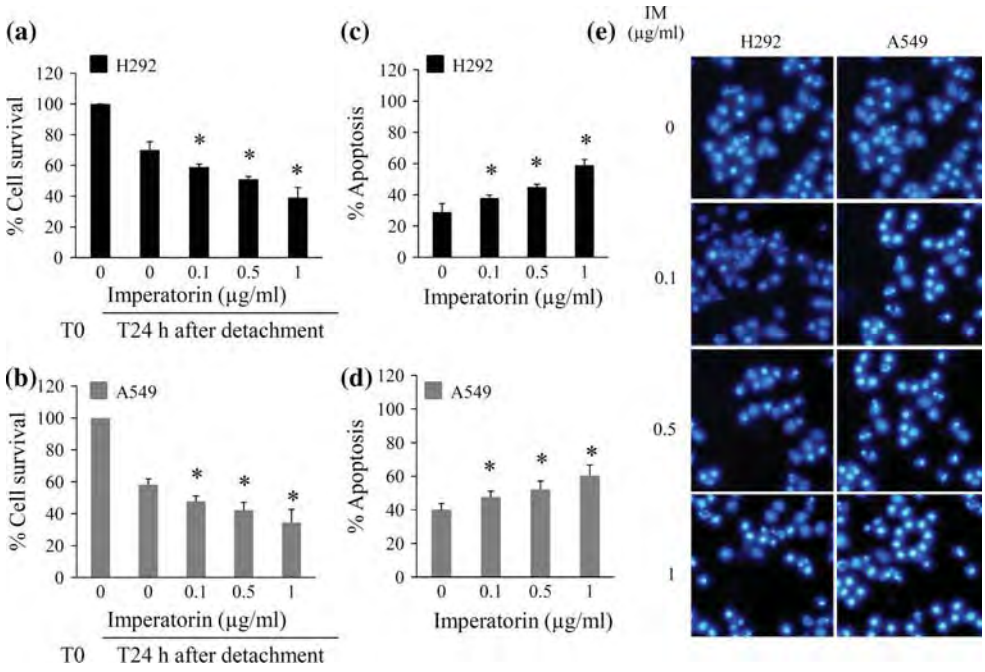


Fig. 6 Anoikis-sensitization effect of imperatorin on H292 and A549 lung cancer cells. **a** H292 and **b** A549 cells were detached and incubated with various concentrations of imperatorin (0–1 μg/ml) for 24 h, and cell survival was determined by the XTT assay. **c** H292 and **d** A549 cells were subjected to Hoechst 33342/PI co-staining assay. **e** The nuclear morphology of apoptotic cells was visualized under a fluorescence microscope. Values are means of independent triplicate samples ± SD. $*p < 0.05$ versus non-treated control at 24 h after cell detachment

provided evidence supporting the potential of this natural compound to be used for anti-metastasis aspects. We reported herein for the first time that imperatorin at sub-toxic concentrations could sensitize lung cancer cell anoikis and inhibit the growth of cancer cells in the detached condition, as an increase in Mcl-1 protein in certain cancer cells was reported to be a mechanism by which such cells resist anoikis [24, 34]. Moreover, the Mcl-1 protein was shown to be a key regulator for anoikis in many cell systems [23, 24, 34]. Imperatorin, which was reported herein to down-regulate Mcl-1, may be able to be developed for anti-metastasis approaches. Furthermore, we demonstrated that imperatorin at the concentrations used for anoikis sensitization was not toxic to normal renal HK-2 cells.

The loss of integrin engagement was shown to initiate the p53-dependent mitochondrial apoptosis pathway [38, 39]. Certain cancer cells develop mechanisms to attenuate p53 activation and, thus, resulted in anoikis resistance [40–45]. We found that the treatment with imperatorin significantly enhanced p53 activation in H23 lung cancer cells and influenced the cell anoikis response. Subsequently, the up-regulation of Bax concomitant with the down-regulation of Mcl-1 protein was detected in the present study and was shown to be a mechanism by which imperatorin sensitized H23 cell anoikis. However, in this study, the sub-toxic concentrations of imperatorin caused no effect on the Bcl-2 expression.

In conclusion, we have demonstrated that imperatorin at sub-toxic concentrations sensitized human lung carcinoma H23 cell anoikis and inhibited the growth of the cells in anchorage-independent conditions. Our results also revealed the roles of imperatorin treatment on p53 and proteins in the Bcl-1 family which could be important in understanding the mechanism of action of imperatorin and benefit the development of this compound. This new finding suggests the possible anti-metastasis role of imperatorin, which may be beneficial for cancer therapy.

Acknowledgments This research was supported by the Thailand Research Fund (to P. Chanvorachote) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). The authors wish to thank Mr. Krich Rajprasit (Srinakharinwirot University) for proofreading the manuscript.

References

1. Kostova I (2005) Synthetic and natural coumarins as cytotoxic agents. Curr Med Chem Anticancer Agents 5:29–46
2. Baek NI, Ahn EM, Kim HY, Park YD (2000) Furanocoumarins from the root of *Angelica dahurica*. Arch Pharm Res 23:467–470
3. Pae HO, Oh H, Yun YG, Oh GS, Jang SI, Hwang KM, Kwon TO, Lee HS, Chung HT (2002) Imperatorin, a furanocoumarin from *Angelica dahurica* (Umbelliferae), induces cytochrome c-dependent apoptosis in human promyelocytic leukaemia, HL-60 cells. Pharmacol Toxicol 91:40–48
4. Luo KW, Sun JG, Chan JYW, Yang L, Wu SH, Fung KP, Liu FY (2011) Anticancer effects of imperatorin isolated from *Angelica dahurica*: induction of apoptosis in HepG2 cells through both death-receptor- and mitochondria-mediated pathways. Chemotherapy 57:449–459
5. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008) Cancer statistics, 2008. CA Cancer J Clin 58:71–96
6. Herbst RS, Heymach JV, Lippman SM (2008) Lung cancer. N Engl J Med 359:1367–1380
7. Sun S, Schiller JH, Gazdar AF (2007) Lung cancer in never smokers—a different disease. Nat Rev Cancer 7:778–790

8. Mountzios G, Fouret P, Soria JC (2008) Mechanisms of disease: signal transduction in lung carcinogenesis—a comparison of smokers and never-smokers. Nat Clin Pract Oncol 5:610–618
9. Frisch SM, Screaton RA (2001) Anoikis mechanisms. Curr Opin Cell Biol 13:555–562
10. Christofori G (2003) Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J 22:2318–2323
11. Frisch SM (1999) Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. Curr Biol 9:1047–1049
12. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70
13. Martin SS, Vuori K (2004) Regulation of Bcl-2 proteins during anoikis and amorphosis. Biochim Biophys Acta 1692:145–157
14. Galante JM, Mortenson MM, Bowles TL, Virudachalam S, Bold RJ (2009) Erk/Bcl-2 pathway in the resistance of pancreatic cancer to anoikis. J Surg Res 152:18–25
15. Coultas L, Strasser A (2003) The role of the Bcl-2 protein family in cancer. Semin Cancer Biol 13:115–123
16. Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS (2003) Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol 5:733–740
17. Schmelzle T, Mailleux AA, Overholtzer M, Carroll JS, Solimini NL, Lightcap ES, Veiby OP, Brugge JS (2007) Functional role and oncogene-regulated expression of the BH3-only factor Bmf in mammary epithelial anoikis and morphogenesis. Proc Natl Acad Sci USA 104:3787–3792
18. Valentijn AJ, Metcalfe AD, Kott J, Streuli CH, Gilmore AP (2003) Spatial and temporal changes in Bax subcellular localization during anoikis. J Cell Biol 162:599–612
19. Valentijn AJ, Gilmore AP (2004) Translocation of full-length Bid to mitochondria during anoikis. J Biol Chem 279:32848–32857
20. Frankel A, Rosen K, Filmus J, Kerbel RS (2001) Induction of anoikis and suppression of human ovarian tumor growth in vivo by down-regulation of Bcl-XL. Cancer Res 61:4837–4841
21. Scorrano L, Korsmeyer SJ (2003) Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304:437–444
22. Grossmann J, Walther K, Artinger M, Kiessling S, Schölmerich J (2001) Apoptotic signaling during initiation of detachment-induced apoptosis (“anoikis”) of primary human intestinal epithelial cells. Cell Growth Differ 12:147–155
23. Boisvert-Adamo K, Longmate W, Abel EV, Aplin AE (2009) Mcl-1 is required for melanoma cell resistance to anoikis. Mol Cancer Res 7:549–556
24. Kim YN, Koo KH, Sung JY, Yun UJ, Kim H (2012) Anoikis resistance: an essential prerequisite for tumor metastasis. Int J Cell Biol 2012:306879
25. Li Z, Zhao J, Du Y, Park HR, Sun Sy, Bernal-Mizrachi L, Aitken A, Khuri FR, Fu H (2008) Down-regulation of 14-3-3ζ suppresses anchorage-independent growth of lung cancer cells through anoikis activation. Proc Natl Acad Sci USA 105:162–167
26. Woods NT, Yamaguchi H, Lee FY, Bhalla KN, Wang HG (2007) Anoikis, initiated by Mcl-1 degradation and bim induction, is deregulated during oncogenesis. Cancer Res 67:10744–10752
27. Ramachandra M, Atencio I, Rahman A, Vaillancourt M, Zou A, Avanzini J, Wills K, Bookstein R, Shabram P (2002) Restoration of transforming growth factor β signaling by functional expression of smad4 induces anoikis. Cancer Res 62:6045–6051
28. Zhu Z, Sanchez-Sweatman O, Huang X, Wiltrout R, Khokha R, Zhao Q, Gorelik E (2000) Anoikis and metastatic potential of cloudman S91 melanoma cells. Cancer Res 61:1707–1716
29. Koleske AJ, Baltimore D, Lisanti MP (1995) Reduction of caveolin and caveolae in oncogenically transformed cells. Proc Natl Acad Sci USA 92:1381–1385
30. Cao L, Han L, Zhang Z, Li J, Qu Z, Du J, Liang X, Liu Y, Liu H, Shi Y, Liu S, Gao L, Sun W (2009) Involvement of anoikis-resistance in the metastasis of hepatoma cells. Exp Cell Res 315:1148–1156
31. Kodama K, Ishii G, Miyamoto S, Goya M, Zhang SC, Sangai T, Yoshikawa T, Hasebe T, Hitomi Y, Izumi K, Ochiai A (2005) Laminin 5 expression protects against anoikis at aerogenous spread and lepidic growth of human lung adenocarcinoma. Int J Cancer 116:876–884
32. Wei L, Yang Y, Zhang X, Yu Q (2002) Anchorage-independent phosphorylation of p130(Cas) protects lung adenocarcinoma cells from anoikis. J Cell Biochem 87:439–449
33. Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87:99–163
34. Zuo J, Ishikawa T, Boutros S, Xiao Z, Humtsoe JO, Kramer RH (2010) Bcl-2 overexpression induces a partial epithelial to mesenchymal transition and promotes squamous carcinoma cell invasion and metastasis. Mol Cancer Res 8:170–182
35. Yang TM, Barbone D, Fennell DA, Broaddus VC (2009) Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. Am J Respir Cell Mol Biol 41:14–23
36. Song L, Coppola D, Livingston S, Cress WD, Haura EB (2005) Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol Ther 4:267–276
37. Chunhacha P, Pongrakhananon V, Rojanasakul Y, Chanvorachote P (2012) Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. Am J Physiol Cell Physiol 302:C1284–C1292
38. Schuler M, Green DR (2001) Mechanisms of p53-dependent apoptosis. Biochem Soc Trans 29:684–688
39. Benchimol S (2001) p53-dependent pathways of apoptosis. Cell Death Differ 8:1049–1051
40. Ravid D, Maor S, Werner H, Liscovitch M (2005) Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by upregulation of insulin-like growth factor-I receptors and signaling. Oncogene 24:1338–1347
41. Song W, Wang J, Yang Y, Jing N, Zhang X, Chen L, Wu J (2012) Rewiring drug-activated p53-regulatory network from suppressing to promoting tumorigenesis. J Mol Cell Biol 4:197–206
42. Guadamillas MC, Cerezo A, Del Pozo MA (2011) Overcoming anoikis—pathways to anchorage-independent growth in cancer. J Cell Sci 124:3189–3197
43. Derouet M, Wu X, May L, Yoo BH, Sasazuki T, Shirasawa S, Rak J, Rosen KV (2007) Acquisition of anoikis resistance promotes the emergence of oncogenic K-*ras* mutations in colorectal cancer cells and stimulates their tumorigenicity in vivo. Neoplasia 9:536–545
44. Grossmann J (2002) Molecular mechanisms of “detachment-induced apoptosis—Anoikis”. Apoptosis 7:247–260
45. Zhang Y, Lu H, Dazin P, Kapila Y (2004) Squamous cell carcinoma cell aggregates escape suspension-induced, p53-mediated anoikis: fibronectin and integrin alphav mediate survival signals through focal adhesion kinase. J Biol Chem 279:48342–48349

Artonin E Mediates MCL1 Down-regulation and Sensitizes Lung Cancer Cells to Anoikis

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Abstract. *Background: Anoikis, or detachment-induced apoptosis, is recognized as a key inhibitory process of cancer metastasis. Since lung cancer cells possess an ability to resist anoikis, resulting in a high rate of metastasis and death, the present study aimed to investigate the possible anoikis-sensitizing effect of artonin E (AE). Materials and Methods: AE was extracted from bark of Artocarpus gomezianus. Anoikis sensitization of AE was investigated in H460, A549 and H292 human lung cancer cells. The level of anoikis-related proteins was determined by western blot analysis and viable cells were measured by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method. Results: AE was shown to enhance anoikis of H460 cells in a dose-dependent manner. We investigated the underlying mechanisms of AE on anoikis sensitization and found that AE sensitized the cells by down-regulating the anti-apoptotic myeloid leukemia cell sequence-1 (MCL1) protein but had no significant effect on other proteins of the B-cell lymphoma-2 (BCL2) family, including BCL2 and BCL2-associated X protein (BAX). Anoikis sensitization of AE was consistently observed in A549 and H292 lung cancer cells. Conclusion: The present study demonstrates a novel activity of AE on lung cancer cell anoikis for the first time which might lead to the development of a new strategy for lung cancer therapy.*

Lung cancer is often found with metastatic tumors at the time of diagnosis (1, 2). As in other types of cancer, once metastasis occurs, the prognosis of such disease drops dramatically. Therefore strategies that effectively inhibit cancer cell spreading are of interest and benefit the development of new cancer therapies. Among multiple steps of cancer cell metastasis, the process of anoikis, or cell detachment-induced apoptosis, has been recognized as the most crucial cellular mechanism that prevents solid cancer from successfully spreading (3-7). In lung cancer, innate and acquired anoikis resistance was frequently found and has been linked to a high degree of tumor metastasis and an advanced stage of this disease (8-10). Many mechanisms of anoikis resistance have been proposed; however, most are regarded as an increase of anti-apoptotic or a decrease of pro-apoptotic proteins of the B-cell lymphoma-2 (BCL2) family (7, 11-14). An up-regulation of BCL2 and myeloid leukemia cell sequence-1 (MCL1) proteins was shown to render anoikis resistance in several cancer types (15-18). Since the oligomirization of pro-apoptotic BCL2-associated X protein (BAX) is an essential step generating pores on mitochondria, facilitating the release of cytochrome *c* (19, 20), it has been found that upon detachment, BAX is rapidly translocated to the mitochondrial membrane prior to triggering cell anoikis (21-23). Conversely, the depletion of BAX has been shown to cause anoikis resistance (5). Recently, the negative regulatory role of caveolin-1 (CAV1) protein on lung cancer cell anoikis has been reported (16).

Artonin E (AE) is a 3-prenylflavone compound extracted from the bark of *Artocarpus gomezianus* Wall. ex Tréc. (Moraceae) (Figure 1). AE is also found in other species of genus *Artocarpus* such as *A. scortechinii*, *A. rotunda*, *A. rigida* and *A. altili* (24). Among 3-prenylflavones isolated from the genus *Artocarpus*, AE was well-established as exhibiting potential pharmacological properties including arachidonate 5-lipoxigenase inhibition (25), antimicrobial (26), antimalarial (27), antituberculosis (27) and cytotoxicity (26, 27). Since there is no evidence indicating an effect of AE in the regulation of cancer cell anoikis, the present study aimed to investigate the effects of AE on modulating lung carcinoma H460 cell anoikis.

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Key Words: Artonin E, MCL1, anoikis, lung cancer cells, metastasis, H460, A549, H292, HK2 cells.

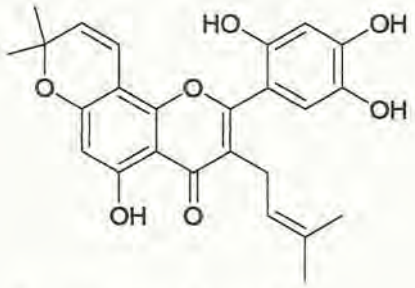

Figure 1. *Chemical structure of artonin E.*

Materials and Methods

Cell culture and reagents. H460, A549 and H292 non-small cell lung carcinoma cells and human kidney-2 (HK2) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). H460 and H292 cells were cultured in Roswell Park Memorial Institute medium (RPMI) medium and human kidney 2 (HK2), respectively and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin. Propidium iodide (PI), and Hoechst 33342 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Rabbit anti-MCL1 antibody was purchased from Cell Signaling (Danvers, MA, USA).

Anoikis assay. Six-well tissue culture plates were coated with 6 mg/ml poly-(2-hydroxyethyl-methacrylate (poly-HEMA) (Sigma–Aldrich, St. Louis, MO, USA) in 95% ethanol and incubated at 37°C overnight. Cells in the culture plate were trypsinized into a single cell suspension and then seeded in poly-HEMA-coated plates at a density of 5×10^4 cells/ml. After incubation, 20 μM of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent (Sigma–Aldrich, St. Louis, MO, USA) was added to the cells for 4 h at 37°C. The intensity of the formazan product was determined at 450 nm using a microplate reader. Cell viability was calculated from the optical density (OD) readings and is represented as a percentage to that of the non-treated control value. The mode of cell death was confirmed by incubating the cells with 10 μg/ml of Hoechst 33342 and visualization under a fluorescence microscope (Olympus IX51 with DP70) (Olympus, Japan).

Annexin-V detection by flow cytometry. Cell anoikis was confirmed by annexin V-fluorescein isothiocyanate (FITC) staining assay and flow cytometry. Cells were collected, re-suspended, and incubated with annexin V-FITC for 30 min at 37°C. Cells were scored by flow cytometry using a 485 nm excitation beam and a 538 band-pass filter (FACsort, Becton Dickinson, Rutherford, NJ, USA). The mean fluorescence intensity was quantified by the CellQuest software (Becton Dickinson).

Hoechst 33342 and PI staining. Apoptotic and necrotic cell death was determined by Hoechst 33342 and PI co-staining H460 and HK2 cells were incubated with different concentrations of AE (0-100 μg/ml) for 24 h in attachment condition and H460, H292 and A549 cells were also detached and suspended for 6 and 12 h in the presence or absence of AE at 0-5 μg/ml. Then, cells were incubated with 10 μM of the Hoechst and 5 μg/ml PI for 30 min at 37°C. Apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70) (Olympus, Japan).

Western blot analysis. H460 cells were incubated with different concentrations of AE (0-5 μg/ml) for 12 h in detachment conditions. Cells were then incubated with lysis buffer containing 2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 100 mmol/l NaCl, 10 mmol/l Tris–HCl (pH 7.5), 1 mmol/l EDTA, and Complete Mini cocktail protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min on ice. After insoluble debris was pelleted by centrifuging at 14,000 ×*g* for 15 min at 4°C, the supernatant was collected and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (40 μg) were resolved on 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto nitrocellulose membranes (Bio-Rad) using standard procedures. The membranes were blocked in 5% skimmed milk in TBST (25 mmol/l Tris–HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h, followed by incubation with rabbit anti-MCL1 antibody, (Cell Signaling, Danvers, MA, USA), rabbit anti-CAV1 antibody, rabbit anti-BCL2 antibody (Abcam, Cambridge, MA, USA), rabbit anti-BAX HRP and rabbit anti-p53 HRP conjugated antibody (Santa Cruz biotechnology, Inc. Santa Cruz, USA) at 4°C overnight. Membranes were washed three times with TBST for 10 min, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 2 h at room temperature. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified by imaging densitometry using the analyst/PC densitometry software (Bio-Rad). Mean densitometric data from independent experiments were normalized to β-actin protein.

Statistical analysis. Mean data from at least three independent experiments were normalized to values for the non-treated controls, analyzed by one-way ANOVA at a significance level of $p<0.05$, and are presented as the mean±S.D.

Results

Effect of AE on H460 lung cancer cell viability. In order to study the effect of AE on lung cancer cell anoikis, we first characterized the cytotoxic effect of AE on H460 lung cancer cells. Cells were incubated with different concentrations of AE (0-100 μg/ml) for 24 h, and cell viability was followingly analyzed. Figure 2A shows that AE at low doses (0-5 μg/ml) caused neither toxic nor proliferative effects on these lung cancer cells. Notably, cytotoxic effect of AE was observed at concentrations higher than 10 μg/ml, with approximately 80% of the cells remaining viable (Figure 2A). Concurrent with these findings, Hoechst 33342/ PI staining assay revealed that apoptotic and necrotic cells were not observed in response to AE at the concentrations of 0-5 μg/ml (Figure 2B and C).

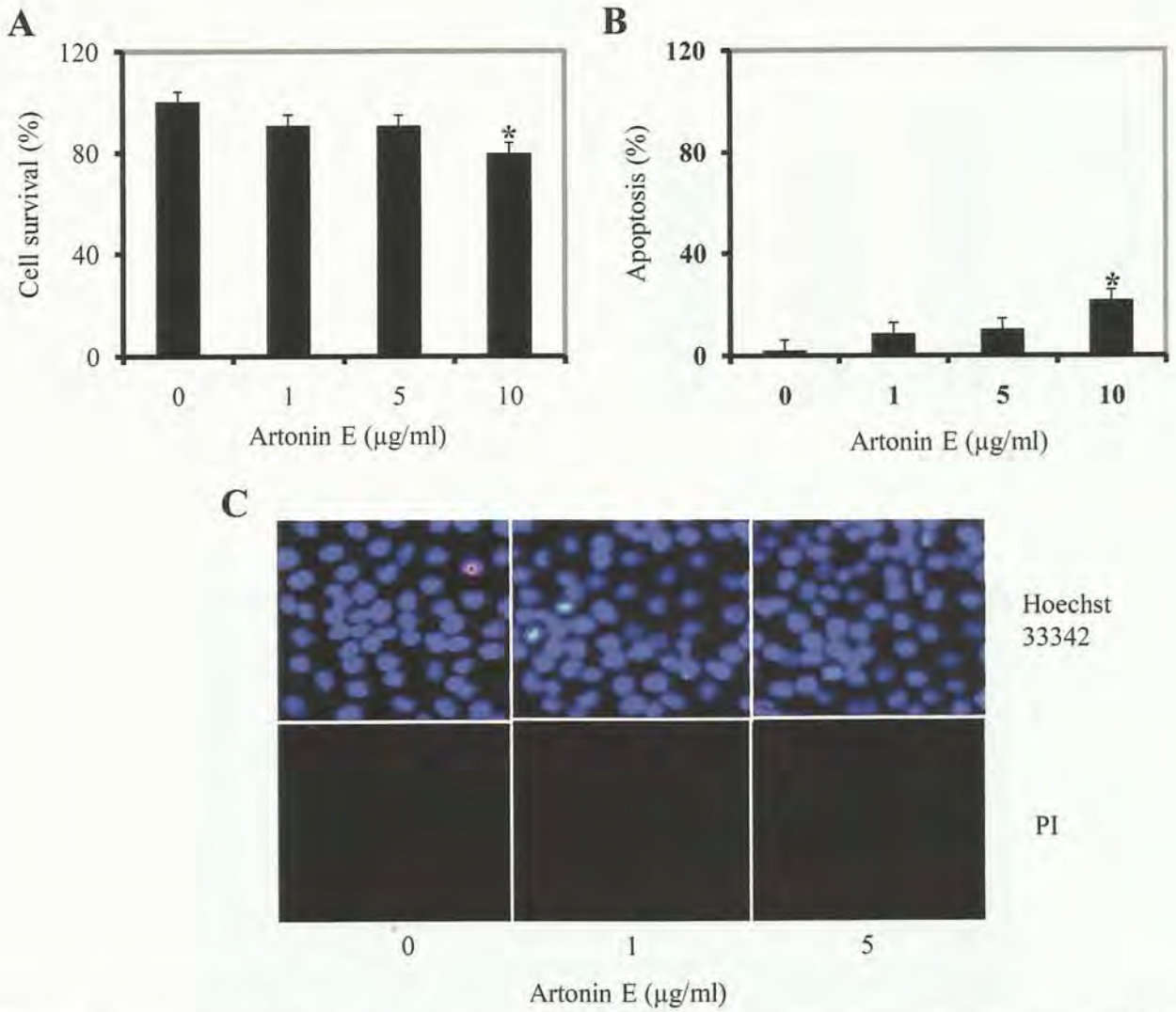

Figure 2. *Effect of artonin E on H460 lung carcinoma cell viability. Cells were treated with different concentrations of AE (0-10 μg/ml) for 24 h. A: Cell viability was determined by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. B: Percentage of cell apoptosis was obtained from Hoechst 33342/ propidium iodide (PI) assays. C: Nuclear morphology of Hoechst33342/PI-stained cells was captured under fluorescence microscopy.*

Effects of AE on cell viability of HK2 normal human renal cells. Since a major concern for anticancer drug use is the cytotoxicity on normal cells, we tested whether AE at the mentioned concentrations caused significant toxicity to HK2 human renal cells. The renal cells were treated with AE at the concentrations of 0-5 μg/ml and cell viability, apoptosis, and necrosis were evaluated after 24 h. Figure 3A demonstrates that viability of AE-treated HK2 cells was not significantly altered in comparison to that of the non-treated controls. We also found a very limited number of apoptotic and necrotic cells in response to 1-5 μg/ml of AE. These results suggest that AE at the concentrations of 1-5 μg/ml exhibited no cytotoxic effects towards neoplastic nor towards normal cells.

AE sensitizes H460 lung carcinoma cells to detachment-induced cell death. Having shown that concentrations of AE of 0-5 μg/ml were non-cytotoxic to H460 lung cancer as well as HK2 normal renal cells, we further evaluated an anoikis-sensitizing effect of AE. H460 cells were detached and suspended in the presence or absence of AE at subtoxic concentrations and cell viability, apoptosis, and necrosis were analyzed at various times (0-24 h). Figure 4A shows that after detachment, H460 cells exhibited a gradual

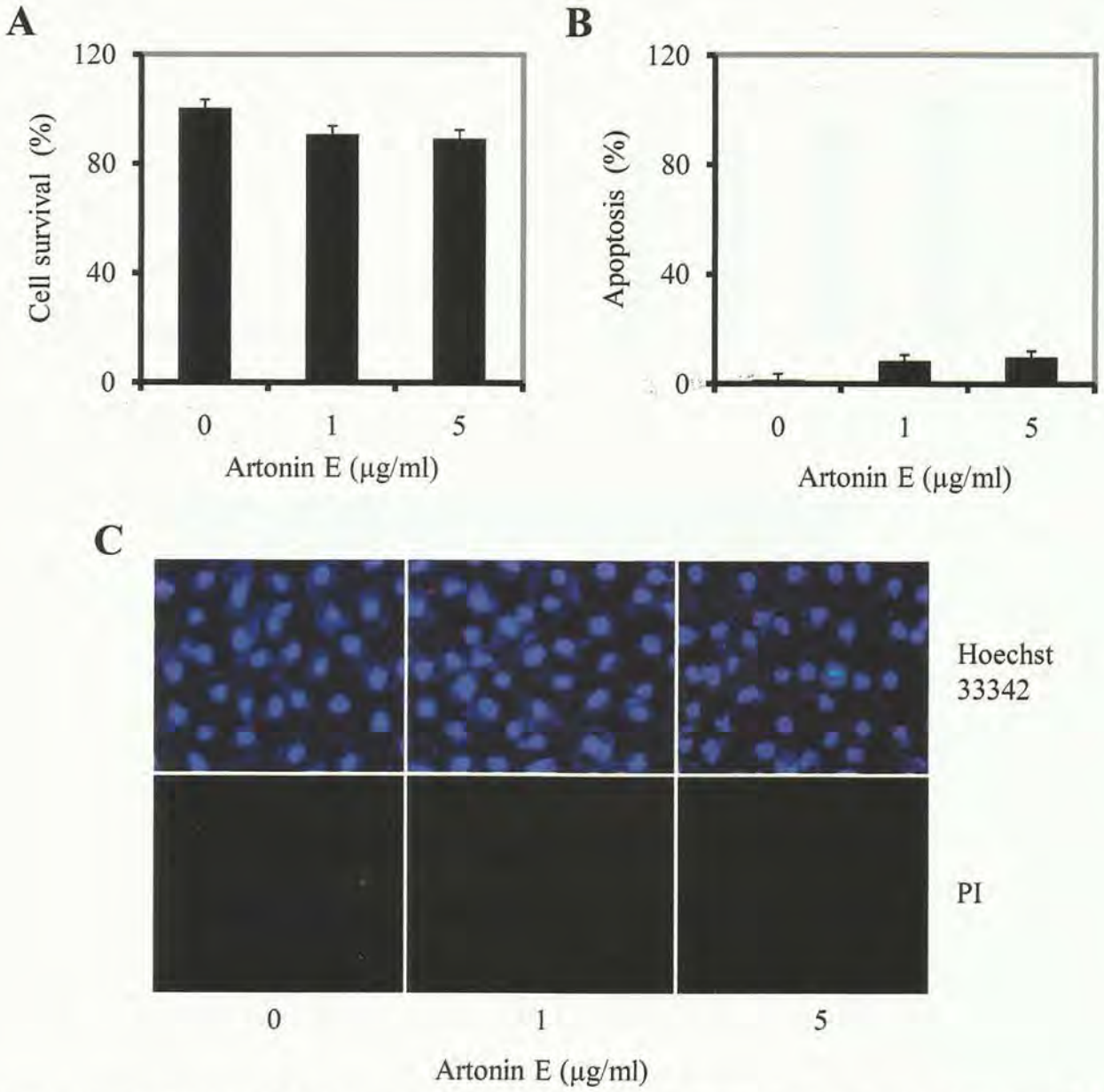

Figure 3. *Cytotoxic effect of artonin E in HK2 normal kidney cells. Cells were incubated in the presence or absence of AE (0-5 μg/ml) for 24 h. A: Cell viability was determined by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. B: Percentage of cell apoptosis. C: Nuclear morphology of cells stained with Hoechst33342 and propidium iodide (PI) was visualized under fluorescence microscopy.*

decrease in cell viability over time. Importantly, treatment with AE significantly sensitized these cells to anoikis in a concentration-dependent manner. A significant decrease in cell viability, in comparison to that of non-treated controls, was observed as early as 6 h after cell detachment, with approximately 50% and 20% of cells remaining viable in response to 1 and 5 μg/ml of AE, respectively. In control cells, approximately 85% cell viability was observed at 6 h after detachment and a dramatic decrease in cell viability was observed, firstly, at 24 h. To investigate the mechanisms of cell death, detached cells were incubated with Hoechst 33342 and PI fluorescent dyes and visualized and quantified under fluorescence microscopy. Figure 4B and C show a remarkable increase of the intensity of nuclear fluorescence

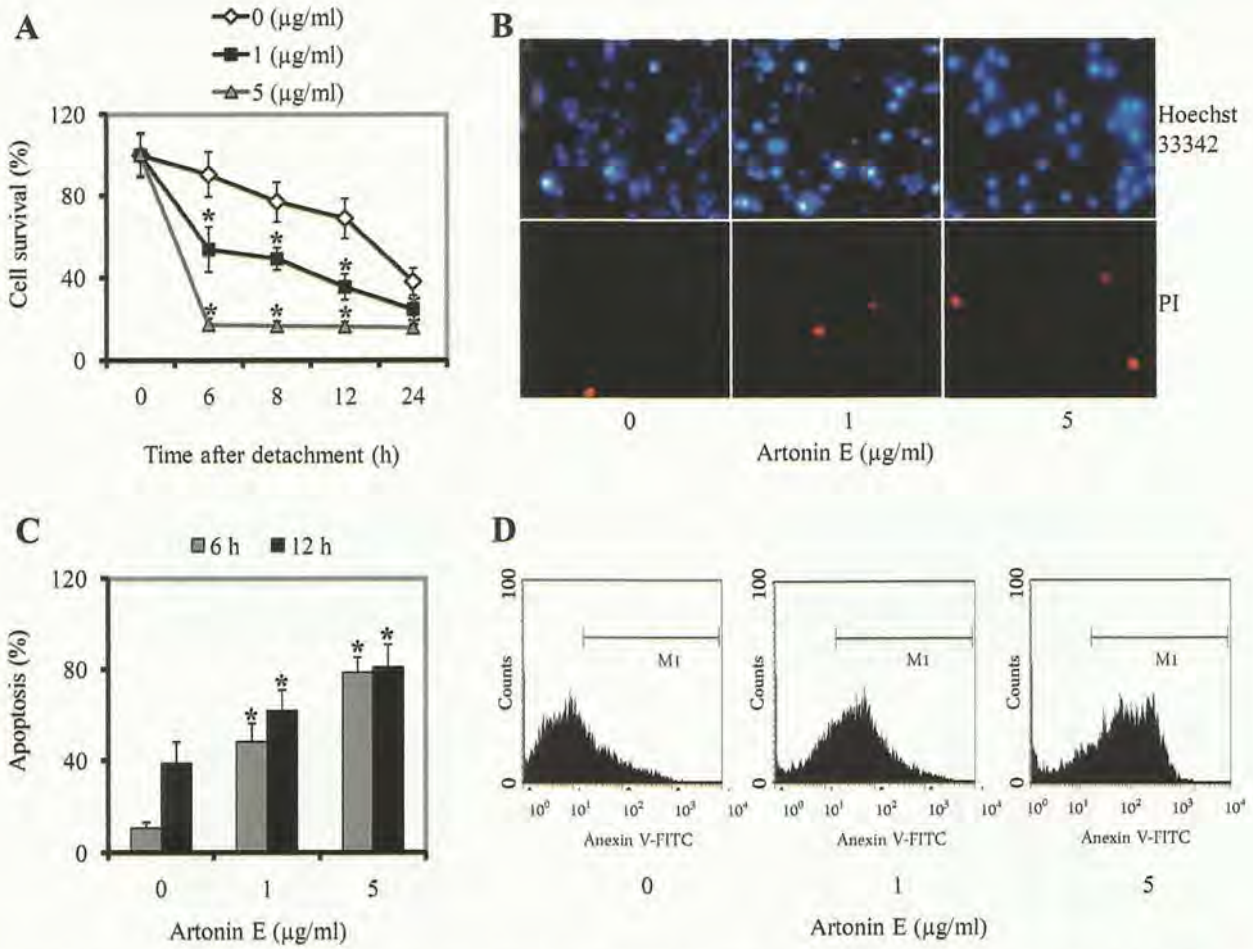

Figure 4. *Artonin E sensitizes H460 cells to detachment-induced apoptosis. A: Cells were treated with different concentrations of AE (0-5 μg/ml) and cell viability was determined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay at the indicated times. Values are means±S.D. of triplicate experiments. *p<0.05 versus non-treated control. B: Apoptosis and necrosis were detected by Hoechst 33342 and propidium iodide (PI) staining assay. C: Nuclear morphology of cells in response to AE treatment by staining with Hoechst 33342 and PI. D: Apoptosis was evaluated by annexin V-fluorescein isothiocyanate (FITC) and flow cytometry.*

and chromatin condensation of apoptotic cells. Approximately 70% and 80% apoptosis was recorded in response to 1 and 5 μg/ml AE treatment for 12 h, respectively, whereas necrotic cell death was minimal. Annexin-V staining assay was also performed to confirm the apoptotic response of the cells. Consistent with the above findings, annexin-V-stained cells detected by flow cytometry were found to increase in a dose-dependent manner in response to AE treatment (Figure 4D). These results indicated a potential role of AE in anoikis sensitization in human lung cancer cells.

MCL1 down-regulation is responsible for anoikis sensitization by AE. The mitochondrial apoptotic pathway has been shown to be involved in the process of cell anoikis (5-7). Among anti-apoptotic members of BCL2 family proteins, MCL1 has garnered most attention since it has been shown to mediate anoikis resistance in many cancer cell types (18, 28, 29). The role of MCL1 on anoikis regulation was confirmed in the present study by stable plasmid transfection. Cells were transfected with MCL1-overexpressing, MCL1 knock-down, or control plasmids. After selection periods, transfectant cells were evaluated for their MCL1 levels by western blot analysis. Figure 5A shows that the highest MCL1 expression was detected in MCL1-overexpressing H460 (HMCL1) cells, while the lowest MCL1 expression was observed in short hairpin ribonucleic acid (shRNA) MCL1-transfected H460 (shMCL1) cells. Notably, control transfectants exhibited MCL1 levels comparable to those of the parental cells. These transfectants were evaluated for anoikis at different times. The

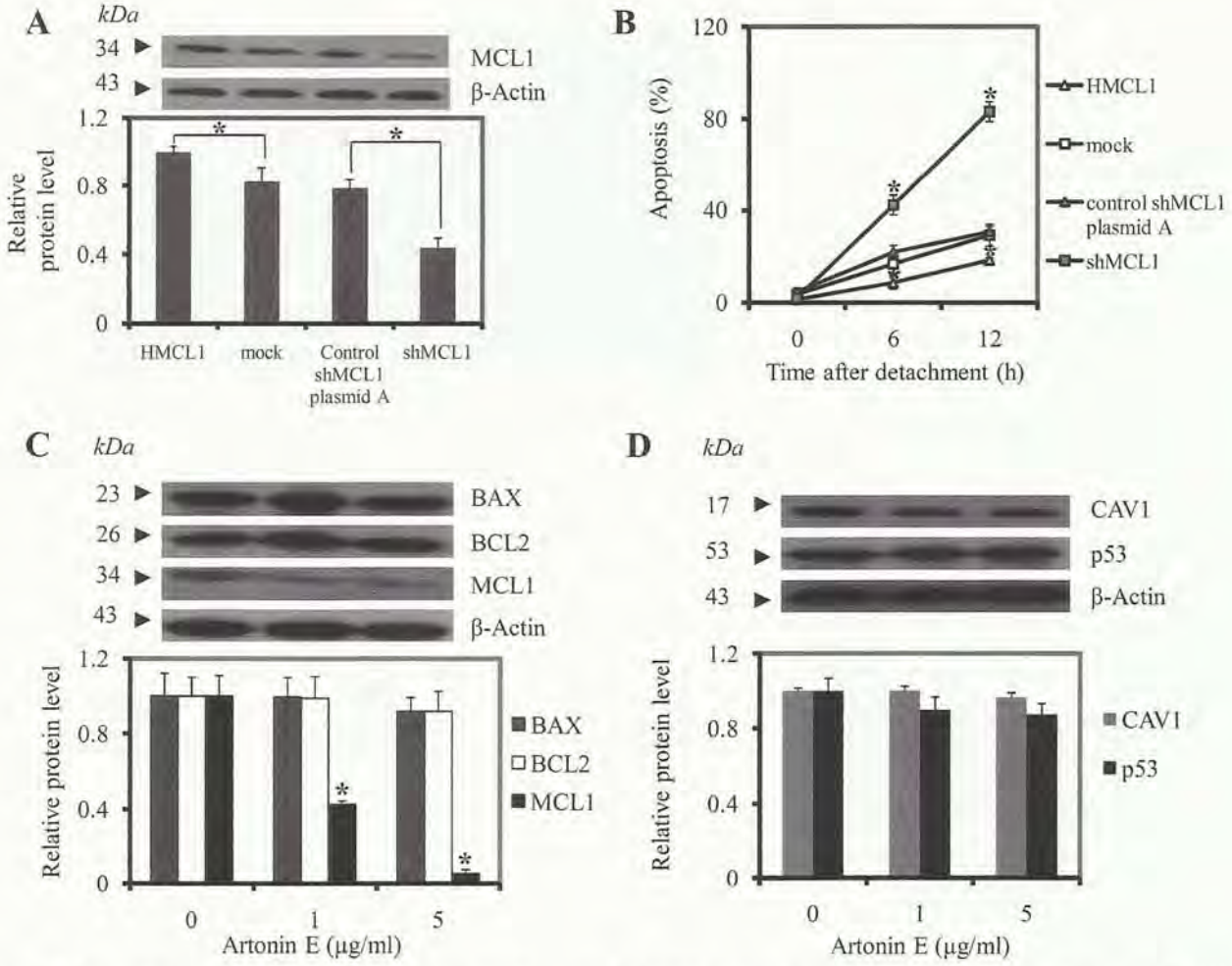

Figure 5. *Artonin E sensitizes anoikis via myeloid leukemia cell sequence-1 (MCL1) down-regulation. A: MCL1-mediated anoikis resistance in H460 cells. High expression of MCL1 protein (HMCL1), mock, control plasmid A and short hairpin MCL1 (shMCL1) cells were cultured in poly-2-hydroxyethyl methacrylate (HEMA)-coated plates and MCL1 levels were determined by western blotting at 12 h. B: Detached MCL1-overexpresing, -knock-down, and control cells were detached at 6, 12 h and viability of the cells was determined with the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. *p<0.05 versus mock-transfected control. Cells were detached and incubated with 1 and 5 μg/ml of AE for 12 h. C: B-cell lymphoma-2 (BCL2), BCL2-associated X protein (BAX) and myeloid leukemia cell sequence 1 (MCL1) expressions were determined by western blotting. D: p53 and caveolin-1 (CAV1) expressions were determined by western blot analysis. Values are means±S.D. (n=3). *p<0.05 versus non-treated control. Blots quantified related to β-actin, used to confirm equal loading of the samples.*

results showed that cell viability after detachment declined in a time-dependent manner in all cells. Corresponding to MCL1 levels in these cells, HMCL1 cells exhibited characteristics indicating the most anoikis resistance, with fewer than 10% of apoptotic cells being detected at 12 h after detachment. On the other hand, shMCL1 cells expressing the lowest level of MCL1 protein were shown to be very sensitive to detachment-induced apoptosis (Figure 5B). These results suggested that MCL1 plays an important role in anoikis regulation.

In order to clarify the mechanisms of AE in sensitizing cells to anoikis, the effect of AE on apoptosis-regulating proteins, namely p53, MCL1, BCL2, and BAX was evaluated. Detached H460 cells were treated with 1 and 5 μg/ml of AE or left untreated for 12 h and the expression of these proteins was evaluated by western blot analysis. Figure 5C and D show that while p53, BCL2 and BAX protein expressions were barely altered, the MCL1 expression significantly decreased in response to AE treatment (Figure 5C). Since the CAV1 protein has been shown in many

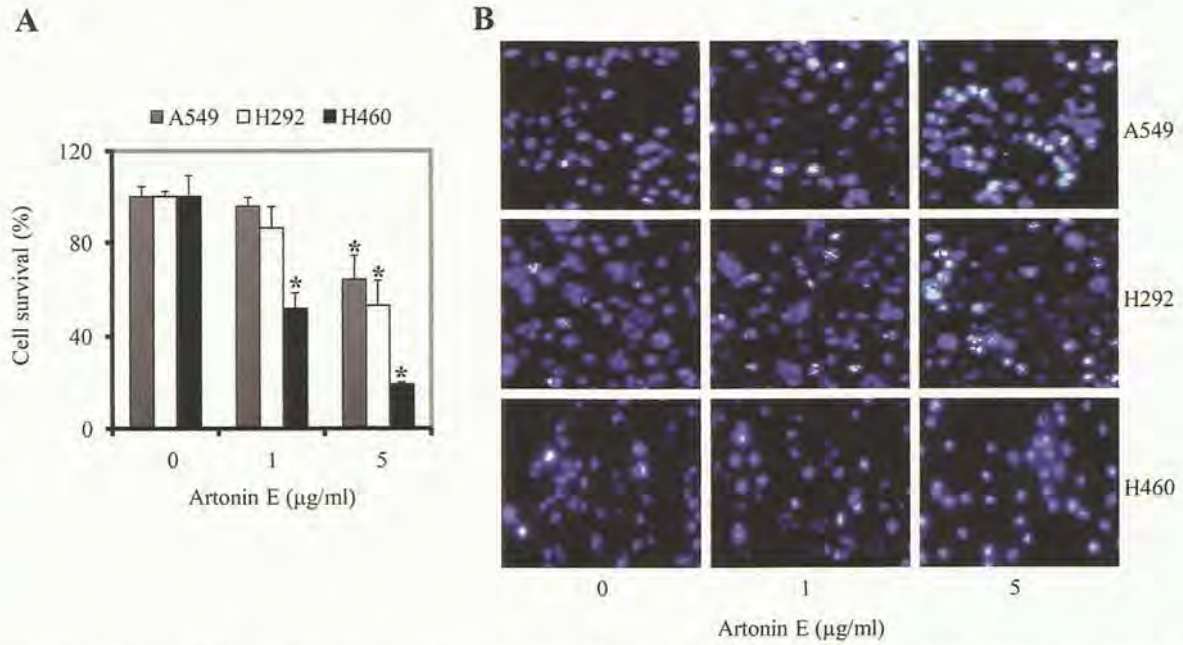

Figure 6. *Effect of artonin E (AE) of A549 and H292 lung cancer cells. A: A549, H292 and H460 cells were detached and cultured in poly-2-hydroxyethyl methacrylate (HEMA)-coated plates in the presence or absence of AE for 12 h and cell viability was determined as described in Materials and Methods. Values are means±S.D. (n=3). *p<0.05 versus non-treated control. B: Apoptotic and necrotic cells were detected by the Hoechst 33342 and propidium iodide (PI) staining assay. Representative photographs are shown from three independent experiments.*

studies to play an important role in anoikis resistance (16, 30-32), we also tested the effect of AE on CAV1 expression during anoikis. The results indicated that although CAV1 protein was found to be down-regulated after cell detachment in these cells (data not shown), AE treatment did not cause further reduction of expression of the protein (Figure 5D). These findings suggested that AE may, at least in part, have sensitized H460 cell anoikis *via* MCL1 down-regulation.

Anoikis-sensitizing effect of AE in A549 and H292 cells. Having demonstrated anoikis-enhancing activity of AE in H460 human lung cancer cells, we further confirmed such an effect in other lung cancer cell models. Anoikis after detachment of A549 and H292 human lung cancer cells in the presence or absence of AE was similarly evaluated, as mentioned above. Figure 6A shows that AE significantly reduced cell viability after detachment of both A549 and H292 cells. Even though A549 and H292 cells exhibited less susceptibility to AE sensitization, both cells exhibited reduced viability in response to 5 μg/ml of AE, with approximately 70% of A549 and 50% of H292 cells remaining viable in comparison to 10% of H460 cells under the same conditions. Apoptotic cells were also remarkably increased in these cells in response to AE treatment, correlating with earlier results (Figure 6B). These results indicated the general role of AE in anoikis sensitization of lung cancer cells.

Discussion

Anoikis resistance enables cancer cells to spread and establish their secondary tumors (5-7). In lung cancer, metastasis is considered as the most important cause of death (33-35) and such a concept has lead to the development of novel anti-metastasis agents and strategies (36-40). Considering plants as an important source of pharmacologically active compounds, AE, a compound isolated from the bark of *Artocarpus gomezianus* was shown to possess several activities such as arachidonate 5-lipoxigenase inhibition, anti-microbial, anti-malarial and anti-tuberculosis activity, and cytotoxicity (24-27). AE was also demonstrated to have a cytotoxic effect against p-388 leukemia cells (26). However, there is no evidence indicating the effect of this compound on cancer metastasis. We have reported, to our knowledge for the first time, that AE at concentrations with minimal toxic effects on normal renal cells exhibited a significant anoikis-sensitizing activity against lung cancer cells. We found that AE, at 5 μg/ml, significantly sensitized H460, A549, and H292 lung cancer cells to detachment-induced apoptosis and provided evidence of MCL1 being involved in the underlying mechanism.

Anoikis is a form of apoptosis initiated by loss of or inappropriate contact with the extracellular matrix or surrounding cells (5-7). Cancer cells either acquire anoikis resistance or possess an innate-resistant ability which enables them to survive after detachment, travel in blood or lymphatic circulations, and establish themselves at distant locations (7, 41, 42). Mechanisms responsible for anoikis resistance in cancer have been intensively investigated and the key proteins, which are MCL1 and CAV1 have garnered dominant attention. Overexpression of CAV1, a major protein component in caveolae, has been shown to mediate anoikis resistance in lung cancer cells (31, 32). Evidence was further provided that expression of such a protein is related to poor prognosis in lung cancer (43, 44). Likewise, MCL1 was shown to mediate anoikis resistance in melanoma (18), as well as lung cancer cells (29). As MCL1 is classified as an antiapoptotic protein, it functions in interacting and neutralizing pro-apoptotic functions of pro-apoptotic proteins, preventing the release of cytochrome *c* from mitochondria (45, 46). Herein, we found that AE reduced the cellular levels of MCL1 in detached cells. The results in this study showed that MCL1 down-regulation mediated by shRNA also resulted in increasing sensitivity of the cells to anoikis (Figure 5B). These results, together with our finding that other proteins, such as BCL2, BAX, CAV1, and p53, were not affected by the addition of AE indicates that AE may sensitize cells to anoikis by reducing cellular MCL1 expression. Since MCL1 was shown to be an important therapeutic target for the treatment of many types of cancer (47-49), compounds targeting MCL1 protein, such as AE, could be of greater interest for development and use in cancer therapy.

In summary, the present study has provided information regarding the role of AE in the regulation of anoikis in lung cancer cells. Detailed molecular analysis of AE on anoikis-resistant cells provides insights into the mechanisms of this compound which may be useful for the development of novel therapeutic strategies for prevention of cancer dissemination.

Acknowledgements

This work was supported by a Thailand research fund, a research grant from the 90th Aniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University. Thanks are also due to the Research Instrument Center of Faculty of Pharmaceutical Sciences, Chulalongkorn University, for providing research facilities. The Authors would like to thank Mr. Krich Rajprasit, a proofreader.

References

1 Bae HM, Lee SH, Kim TM, Kim DW, Yang SC, Wu HG, Kim YW and Heo DS: Prognostic factors for non-small cell lugn cancer with bone metastasis at the time of diagnosis. Lung cancer *77(3)*: 572-577, 2012.

2 Cheran SK, Herndon JE 2nd and Patz EF Jr.: Comparison of whole-body FDG PET to bone scan for detection of bone metastases in patients with a new diagnosis of lung cancer. Lung Cancer *44*: 317-325, 2004.

3 Zhong X and Rescorla FJ: Cell surface adhesion molecules and adhesion-initiated signaling: understanding of anoikis resistance mechanisms and therapeutic opportunities. Cell Signal *24*: 393-401, 2012.

4 Chiarugi P and Giannoni E: Anoikis: A necessary death program for anchorage-dependent cells. Biochem Pharmacol *76*: 1352-1364, 2008.

5 Gilmore AP: Anoikis. Cell Death Differ 12: 1473-1477, 2005.

6 Kim YN, Koo KH, Sung JY, Yun UJ and Kim H: Anoikis resistance: An essential prerequisite for tumor metastasis. Int J Cell Biol 2012: 2012.

7 Simpson CD, Anyiwe K and Schimmer AD: Anoikis resistance and tumor metastasis. Cancer Lett *272*: 177-185, 2008.

8 Li Z, Zhao J, Du Y, Park HR, Sun SY, Bernal-Mizrachi L, Aitken A, Khuri FR and Fu H: Down-regulation of 14-3-3zeta suppresses anchorage independent growth of lung cancer cells through anoikis activation. Proc Natl Acad Sci USA *105*: 162-167, 2008.

9 Kodama K, Ishii G, Miyamoto S, Goya M, Zhang SC, Sangai T, Yoshikawa T, Hasebe T, Hitomi Y, Izumi K and Ochiai A: Laminin 5 expression protects against anoikis at aerogenous spread and lepidic growth of human lung adenocarcinoma. Int J Cancer *116*: 876-884, 2005.

10 Wei L, Yang Y, Zhang X and Yu Q: Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. Oncogene *23*: 9052-9061, 2004.

11 Frisch SM and Francis H: Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol *124*: 619-626, 1994.

12 Rosen K, Rak J, Leung T, Dean NM, Kerbel RS and Filmus J: Activated Ras prevents downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. J Cell Biol *149*: 447-456, 2000.

13 Rosen K, Rak J, Jin J, Kerbel RS, Newman MJ and Filmus J: Down-regulation of the proapoptotic protein Bak is required for the Ras-induced transformation of Intestinal epithelial cells. Curr Biol *8*: 1331-1334, 1998.

14 Coll ML, Rosen K, Ladeda V and Filmus J: Increased Bcl-xL expression mediates v-Src induced resistance to anoikis in intestinal epithelial cells. Oncogene *21*: 2908-2913, 2002.

15 Galante JM, Mortenson MM, Bowles TL, Virudachalam S and Bold RJ: ERK/BCL2 pathway in the resistance of pancreatic cancer to anoikis. J Surg Res *152*: 18-25, 2009.

16 Pongrakhananon V, Nimmannit U, Luanpitpong S, Rojanasakul Y and Chanvorachote P: Curcumin sensitizes non-small cell lung cancer cell anoikis through reactive oxygen species-mediated BCL2 down-regulation. Apoptosis *15*: 574-585, 2010.

17 Bondar VM and McConkey DJ: Anoikis is regulated by BCL2-independent pathways in human prostate carcinoma cells. Prostate *51*: 42-49, 2002.

18 Boisvert-Adamo K, Longmate W, Abel EV and Aplin AE: MCL1 is required for melanoma cell resistance to anoikis. Mol Cancer Res *7*: 549-556, 2009.

19 Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D and Reed JC: Bax directly induces release of cytochrome *c* from isolated mitochondria. Proc Natl Acad Sci USA *95*: 4997-5002, 1998.

20 Antonsson B, Montessuit S, Lauper S, Eskes R and Martinou JC: Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome *c* release from mitochondria. Biochem J *2*: 271-278, 2000.
21 Valentijn AJ, Metcalfe AD, Kott J, Streuli CH and Gilmore AP: Spatial and temporal changes in Bax subcellular localization during anoikis. J Cell Biol *162*: 599-612, 2003.
22 Valentijn AJ and Gilmore AP: Translocation of full-length Bid to mitochondria during anoikis. J Biol Chem *279*: 32848-32857, 2004.
23 Owens TW, Valentijn AJ, Upton JP, Keeble J, Zhang L, Lindsay J, Zouq NK and Gilmore AP: Apoptosis commitment and activation of mitochondrial BAX during anoikis is regulated by p38MAPK. Cell Death Differ *16*: 1551-1562, 2009.
24 Jagtap UB and Bapat VA: *Artocarpus*: A review of its traditional uses, phytochemistry and pharmacology. J Ethnopharmacol *129*: 142-166, 2010.
25 Barron D and Ibrahim RK: Isoprenylated flavonoids a survey. Phytochemistry *43*: 921-982, 1996.
26 Suhartati T, Yandri and Hadi S: The Bioactivity Test of Artonin E from the Bark of *Artocarpus* rigida Blume. Eur J Sci Res *23*: 330-337, 2008.
27 Boonphong S, Baramee A, Kittakoop P and Puangsombat P: Antitubercular and antiplasmodial prenylated flavones from the roots of *Artocarpus* altilis. Chiang Mai J Sci *34*: 339-344, 2007.
28 Woods NT, Yamaguchi H, Lee FY, Bhalla KN and Wang HG: Anoikis, initiated by MCL1 degradation and Bim induction, is deregulated during oncogenesis. Cancer Res *67*: 10744-10752, 2007.
29 Chunhacha P, Pongrakhananon V, Rojanasakul Y and Chanvorachote P: Caveolin-1 regulates MCL1 stability and anoikis in lung carcinoma cells. Am J Physiol Cell Physiol *302*: 1284-1292, 2012.
30 Fiucci G, Ravid D, Reich R and Liscovitch M: Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene *21*: 2365-2375, 2002.
31 Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y and Chanvorachote P: Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. Am J Physiol Cell Physiol *300*: 235-245, 2011.
32 Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang BH and Rojanasakul Y: Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. J Biol Chem *284*: 28476-28484, 2009.
33 Martini N, Flehinger BJ, Zaman MB and Beattie EJ Jr.: Results of resection in non-oat cell carcinoma of the lung with mediastinal lymph node metastases. Ann Surg *198*: 386-397, 1983.
34 Wakelee H and Belani CP: Optimizing first-line treatment options for patients with advanced NSCLC. Oncologist *10*: 1-10, 2005.
35 Gompelmann D, Eberhardt R and Herth FJ: Advanced malignant lung disease: What the specialist can offer. Respiration *82*: 111-123, 2011.
36 Kataoka M, Schumacher G, Cristiano RJ, Atkinson EN, Roth JA and Mukhopadhyay T: An agent that increases tumor suppressor transgene product coupled with systemic transgene delivery inhibits growth of metastatic lung cancer in vivo. Cancer Res *58*: 4761-4765, 1998.
37 Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, Iizasa T, Nakayama T, Taniguchi M and Fujisawa T: A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res *11*: 1910-1917, 2005.
38 Pallis AG, Serfass L, Dziadziusko R, van Meerbeeck JP, Fennell D, Lacombe D, Welch J and Gridelli C: Targeted therapies in the treatment of advanced/metastatic NSCLC. Eur J Cancer *45*: 2473-2487, 2009.
39 Silvestri GA and Rivera MP: Targeted therapy for the treatment of advanced non-small cell lung cancer: A review of the epidermal growth factor receptor antagonists. Chest *128*: 3975-3984, 2005.
40 Indovina P, Marcelli E, Pentimalli F, Tanganelli P, Tarro G and Giordano A: Mass spectrometry-based proteomics: The road to lung cancer biomarker discovery. Mass Spectrom Rev. 2012.
41 Berezovskaya O, Schimmer AD, Glinskii AB, Pinilla C, Hoffman RM, Reed JC and Glinsky GV: Increased expression of apoptosis inhibitor protein XIAP contributes to anoikis resistance of circulating human prostate cancer metastasis precursor cells. Cancer Res *65*: 2378-2386, 2005.
42 Douma S, Van Laar T, Zevenhoven J, Meuwissen R, Van Garderen E and Peeper DS: Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. Nature *430*: 1034-1039, 2004.
43 Yoo SH, Park YS, Kim HR, Sung SW, Kim JH, Shim YS, Lee SD, Choi YL, Kim MK and Chung DH: Expression of caveolin-1 is associated with poor prognosis of patients with squamous cell carcinoma of the lung. Lung Cancer *42*: 195-202, 2003.
44 Ho CC, Kuo SH, Huang PH, Huang HY, Yang CH and Yang PC: Caveolin-1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy. Lung Cancer *59*: 105-110, 2008.
45 Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM and Huang DC: Proapoptotic Bak is sequestered by MCL1 and Bcl-xL, but not BCL2, until displaced by BH3-only proteins. Genes Dev *19*: 1294-1305, 2005.
46 Clohessy JG, Zhuang J, de Boer J, Gil-Gómez G and Brady HJ: MCL1 interacts with truncated Bid and inhibits its induction of cytochrome *c* release and its role in receptor-mediated apoptosis. J Biol Chem *281*: 5750-5759, 2006.
47 Gores GJ and Kaufmann SH: Selectively targeting MCL1 for the treatment of acute myelogenous leukemia and solid tumors. Genes Dev *26*: 305-311, 2012.
48 Akgul C: MCL1 is a potential therapeutic target in multiple types of cancer. Cell Mol Life Sci *66*: 1326-1336, 2009.
49 Warr MR and Shore GC: Unique biology of MCL1: Therapeutic opportunities in cancer. Curr Mol Med *8*: 138-147, 2008.

Received September 30, 2012
Revised October 30, 2012
Accepted October 31, 2012