Abstract. A human steroid hormone, ouabain, has been shown to play a role in several types of cancer cell behavior; however, its effects on cancer metastasis are largely unknown. Herein, we demonstrate that sub-toxic concentrations of ouabain facilitate cancer cell detachment from the extracellular matrix in human lung cancer cells. Ouabain at concentrations of 0-10 pM significantly enhanced cell detachment in dose- and time-dependent manners, while having minimal effect on cell viability. The detachment-inducing effect of ouabain was found to be mediated through focal-adhesion kinase and ATP-dependent tyrosine kinase pathways. Alpha-5 and beta-1 integrins were found to be down-regulated in response to ouabain treatment. Since detachment of cancer cells is a prerequisite process for metastasis to begin, these insights benefit our understanding over the molecular basis of cancer biology.

Despite advances in chemotherapy, as well as novel strategies to overcome lung cancer, this type of cancer remains a leading cause of death (1). The majority of deaths of patients with lung cancer are due to metastasis, a process by which cancer cells spread from their origin to other parts of the patient’s body (2-5). In order to elucidate molecular targets for the development of novel therapies, insights into lung cancer cell biology regarding metastasis are very important. Cancer metastasis consists of several key components (6, 7).

While other steps of metastasis such as anoikis, migration, invasion, and cancer cell adhesion to the endothelial surface have been intensively studied, the molecular basis of cancer cell detachment from the original tumor is largely unknown. This early dissemination of cancer cells from their extracellular matrix (ECM) was shown to be critical to successful metastasis and such a process occurs via the cascades of focal adhesion dissociation (8-11). Even though the mechanisms of cancer cell detachment are not yet defined, evidence indicates that focal adhesion kinase (FAK) and ATP-dependent tyrosine kinase (AKT) pathways play important roles in both cell detachment and cell motility (12, 13). Activation of FAK or AKT was shown to induce focal adhesion dissociation in many cell models (13, 14). Furthermore, integrins, proteins linking focal adhesion complex with components of the ECM were shown to be critical for proper cell adhesion. Among various types of integrins, alpha-5 and beta-1 integrins have garnered predominant attention in cancer research since their activity was shown to be important for adhesion in several types of cancers (15-19).

Ouabain, an endogenous substance that has been identified as a human hormone, has gained increasing attention in cancer research (20-23). Ouabain was reported found in plasma in concentrations ranging from 2-770 pM (24) and was shown to sensitize cancer cells to death induced by TNF-related apoptosis-inducing ligand (TRAIL) (25). Because cancer cells in the patient’s body are likely to be exposed to ouabain and there is no report indicating effects of ouabain in regulation of cancer cell detachment, the present study aimed to elucidate the possible regulatory role of the specific substance. The knowledge gained from this study may lead to a better understanding of cancer biology and may help in the search for new molecular targets for novel anticancer strategies.

Materials and Methods

Cells and reagents. Human lung adenocarcinoma H23 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin were obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD, USA) in 37°C with 5% CO2-
membranes (Bio-Rad, Hercules, CA, USA). The transferred proteins were transferred onto nitrocellulose using polyacrylamide gel electrophoresis (SDS-PAGE) to separate. After lysates were collected and assayed for protein content using the BCA (Bio-Rad, Hercules, CA, USA) for 40 min on ice, collected whole cell lysates and centrifuged in inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) twice with cold PBS and incubated with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 100 mM phenylmethyl sulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 4˚C for 15 min. The supernatants of the whole cell lysates were collected and incubated at 37˚C for 4 h. The optical density was then determined by a microplate reader (Anthos, Durham, NC, USA) at 570 nM, and the percentage of viable cells was calculated relative to control cells. 

Cell viability assay. Cell viability was evaluated using MTT assay. Briefly, H23 cells were seeded at a density of 10^4 cells/well in 96-well plate overnight. After that, they were treated with different concentrations of ouabain for 12 h. Cell viability was determined by incubation with MTT solution (5.0 mg/ml in phosphate-buffered saline (PBS)) and incubated at 37˚C for 4 h. The optical density was then determined by a microplate reader (Anthos, Durham, NC, USA) at 570 nM, and the percentage of viable cells was calculated relative to control cells.

Nuclear staining assay. Apoptotic and necrotic cell death were determined by Hoechst 33342 and PI co-staining. After treatment with non-toxic concentrations of ouabain, cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml of 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).

Cell-ECM adhesion assay. Cell-ECM adhesion assay was performed as follows. Briefly, matrigel was thawed at 4˚C for 12 h, diluted to 0.2 μg/ml, spread evenly on the surface of 96-well plates, and incubated at 37˚C for 12 h. Cells were treated with ouabain at sub-toxic concentrations (0-10 pM) for 0-6 h and were dissociated from matrigel surfaces by trypsinization. After trypsinization, matrigel was thawed at 4˚C for 12 h, diluted to 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37˚C, and the concentration range of 0-50 pM of ouabain was selected for the present study. H23 cells were cultured in presence and absence of ouabain (0-50 pM) for 12 h, and cell viability was determined by the MTT viability assay. Figure 1A shows that when cells were treated with ouabain, at concentrations ranging from 0-10 pM, neither cytotoxicity nor proliferative effects were observed. A significant decline in cell viability was detected in cells treated with 50 pM ouabain, with approximately 70% of the cells remaining viable. Accordingly, the Hoechst33342 staining assay indicated that at 0-10 pM, ouabain did not cause apoptosis (Figure 1B and C).

Effect of ouabain on the viability of human lung cancer H23 cells. Firstly, we characterized the effects of ouabain on the survival of human lung cancer H23 cells. Because previous studies have reported the concentrations of ouabain and ouabain-like substances in human plasma to range from 2-770 pM, the concentration range of 0-50 pM of ouabain was selected for the present study. H23 cells were cultured in presence and absence of ouabain (0-50 pM) for 12 h, and cell viability was determined by the MTT viability assay.

Results

Effect of ouabain on the viability of human lung cancer H23 cells. To elucidate the effect of ouabain on lung cancer cell detachment, we performed cell-ECM adhesion and detachment assays. For cell-ECM adhesion assay, cells were incubated with ouabain at sub-toxic concentrations (0-10 pM) for 0-6 h and were subjected to the cell-ECM adhesion assay. After trypsinization, cells were allowed to adhere to the matrigel surface for 30 min and adhered cells were determined using the MTT assay and cell counting. Figure 2 shows that treatment with ouabain significantly reduced the number of adhered cells in dose- (Figure 2A-C) and time-dependent manners (Figure 2D-F) compared to the non-treated H23 control cells. Treatment with ouabain at 5 and 10 pM reduced the adhesion of cells to approximately 65% and 50%, respectively (Figure 2A and B).

Furthermore, we elucidated the effect of ouabain on cancer cell detachment using cell detachment assay. Cells were sparsely seeded in order to avoid cell-cell contact. After 12 h incubation, the cells were incubated with 0-10 pM ouabain for 6 h and were dissociated from matrigel surfaces by incubating with 0.1% trypsin at 37˚C for 5-10 min. The
percentage of remaining cells to that of total cells was assessed by the MTT assay and cells scored under an inverted microscope. Figure 3 shows that treatment with ouabain significantly enhanced the detachment of cells in dose-dependent manner. Taken together, these results suggest that ouabain at physiological concentration weakened cancer cell adhesion to ECM surface and facilitate detachment.

Ouabain induces FAK and AKT activation and reduction of alpha-5 and beta-1 integrin. Having demonstrated the potentiating effect of ouabain on lung cancer cell detachment, we next examined the underlying mechanism, focusing on the expression level and activation of the proteins known to play roles in cancer cell-ECM adhesion. Cells were treated with ouabain for 6 h and expression of FAK, activated FAK (phosphorylation at Tyr 397), AKT, activated AKT (phosphorylation at Ser 473), alpha-5 integrin, and beta-1 integrin were evaluated by western blot analysis. Figure 4 indicates that treatment with ouabain significantly increased the levels of phosphorylated FAK (Tyr 397) and phosphorylated AKT (Ser 473), whereas ouabain exposure had no significant effect on the levels of total FAK and total AKT. For dose-dependent assessment, cells were treated with non-toxic concentrations of ouabain for 6 h and expression levels of these proteins were evaluated. Figure 4A and B indicate that treatment with ouabain resulted in the activation...
of FAK and AKT in a dose-dependent fashion. The time-dependent effect of ouabain on FAK and AKT activation were also evaluated. Cells were treated with 10 pM ouabain for 0-6 h, and the level of proteins was detected by western blotting. Figure 4C and D show that an increase of ouabain exposure caused an increase in activation of FAK and AKT. These results indicate the involvement of the FAK and AKT pathways in the ouabain-facilitated detachment of these cells.

As alpha-5 and beta-1 integrins have been implicated in cancer cell adhesion to the ECM surface (19), we tested whether detachment of H23 cells in the present study was associated with the decrease of these integrins. Interestingly,
ouabain treatment significantly decreased the cellular level of alpha-5 and beta-1 integrins in dose- and time-dependent manners (Figure 5). Because cellular integrins were previously shown to be tightly regulated via degradation through the lysosomal pathway (34, 35), we further tested whether treatment with ouabain could facilitate this degradation of integrins. Cells were treated with ouabain in the presence or absence of concanamycin A (CMA, a specific inhibitor that blocks lysosomal degradation of proteins) for 0-6 h and level of integrins was determined. Western blot analysis revealed that treatment with ouabain down-regulated alpha-5 and beta-1 integrins in a time-dependent manner and the addition of CMA significantly inhibited such effects of ouabain (Figure 5C and D). These results indicate that ouabain facilitates cancer cell detachment by mediating alpha-5 and beta-1 integrin degradation through a lysosomal mechanism. Taken together, we see that it is likely that this endogenous substance at specific biological concentrations could enhance cancer cell detachment via FAK and AKT activation, as well as facilitating of integrin degradation.

**Discussion**

Ouabain, a cardiac glycoside member isolated from plants, was found to be endogenously produced in humans and classified as a human hormone (20, 26). It has lately garnered increasing attention in cancer research. Here, we demonstrate for the first time that ouabain enhances lung cancer cell detachment.
time that ouabain at physiological concentrations (0-10 pM) facilitates lung cancer cell detachment. Even though detachment of cancer cells from their original tumor is accepted as an important initial process of cancer metastasis, there are only few studies to elucidate the cellular mechanisms of cell detachment.

Interestingly, ouabain and its related compounds are found in human plasma at different concentrations depending on the status of individuals as well as methods of assessment. In most cases, plasma ouabain concentrations are reported to be between 2-770 pM (24). The present study aimed to investigate the effect of this endogenous substance at its physiological levels, we therefore selected concentrations of 0-50 pM; however, ouabain at concentrations of more than 50 pM caused significant cytotoxicity towards human lung cancer cells. This result is consistent with the previous experiments indicating that ouabain possesses activity against many types of cancers (21, 22, 25, 27). Besides its direct cytotoxicity, we previously found that ouabain at non-toxic concentrations sensitized TRAIL-mediated death by down-regulating anti-apoptotic myeloid cell leukemia sequence-1 (MCL-1) protein in H292 and H460 cells (25).

Cell-ECM adhesion plays an important part for survival of the cancer cells by activating the survival signal through integrin-AKT-dependent mechanisms (28). Although the
detachment of cells will disrupt such survival and trigger detachment-mediated apoptosis (anoikis), certain cancer cells have the ability to resist anoikis and further spread (29, 30). For cell detachment, integrin binding to extracellular compartments must be disrupted and previous studies showed that such dissociation of integrin resulted from the activation of FAK and AKT pathways (10, 11, 31). In certain studies, FAK action was shown to involve downstream AKT (32). In addition, activation of AKT was found to regulate internalization and recycling of integrin (33). The internalization of integrins, under certain conditions, leads to degradation of cellular integrins via the lysosomal pathway (34, 35). Consistent with this finding, our results indicate that ouabain-mediated cell detachment involves FAK and AKT activation, and down-regulation of cellular integrins. Furthermore, using specific inhibitors of lysosomal degradation pathway, we found that ouabain reduced cellular integrins through the lysosomal pathway.

In summary, the present study demonstrated the possible role of ouabain at its physiological levels on the detachment of lung cancer cells. Ouabain exposure activated the FAK-AKT signaling pathway and reduced the level of integrins through lysosomal degradation. The knowledge gained from the present study may benefit our understanding on cancer biology and cancer metastasis.

Conflicts of Interest
The Authors declare that there are no conflicts of interest regarding this research.

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