Role of Activation Peptide of Procathepsin D in Proliferation and Invasion of Lung Cancer Cells

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Abstract. *Background: Procathepsin D (pCD) secreted by cancer cells, increases proliferation, metastasis and progression of breast cancer, however its role in lung cancer is still unclear. The purified pCD and its synthetic activation peptide (AP) have shown similar proliferative effect on various cancer cell lines. The aim of this study is to clarify the role of pCD and its AP in lung cancer by stable expression of pCD and pCD lacking its AP, in NCI-H23 lung cancer cells. Materials and Methods: The stable transfected clones were tested for cell proliferation, invasion and growth in nude mice. The effect of exogenous addition of purified pCD and its mutant proteins was also analyzed by proliferation assay. Results: The invasion and proliferation in vitro and tumor growth in vivo, demonstrated that the expression of pCD enhances the carcinogenic properties of NCI-H23 cells and that the AP is essential for these activities. Exogenous addition of purified proteins on various lung cancer cell lines showed that neither catalytic activity nor glycosylation are involved in the growth-promoting activity. Conclusion: This is the first report of pCD cDNA expression in lung cancer cells that enhances the growth and invasion of these cells both in vitro and in vivo.*

Lung cancer remains at epidemic proportions and continues to be the leading cause of cancer death in both men and women (1). Secreted proteases have an important role in cancer development, facilitating tumor invasion and growth (2). Some of these proteases are secreted more abundantly by cancer cells than by normal cells (3, 4).

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Procathepsin D (pCD) is the proform of lysosomal aspartic peptidase cathepsin D (CD), which is overexpressed and secreted by breast cancer cells with a documented role in tumor development (5). In normal cells, the mature CD participates in intracellular protein catabolism, hormone and antigen processing, as well as in the apoptotic pathway (6, 7). However, its preform pCD causes enhanced proliferation and neoplastic transformation (8, 9). Clinical studies using immunoassays have reported that pCD/CD is a marker of poor prognosis for breast cancer and is associated with high risk of metastasis (10-12).

CD is a lysosomal peptidase that is translated as preprocathepsin D in rough endoplasmic reticulum. It is translocated to golgi apparatus after removal of signal peptide, where its two N-linked oligosaccharide side chains are modified with mannose 6-phosphate moieties. The pCD tagged with mannose 6-phosphate is sorted in lysosomes, where it is activated at low pH into a single chain of proteolytically active form, CD, after removal of activation peptide (AP). The single chain is further cleaved into the two-chain form (13).

Elevated levels of pCD have been seen in many other types of malignancies, as reviewed by Leto *et al.* (8). Different studies have shown that pCD affects proliferation of cancer cells both *in vitro* and *in vivo* (14-17) and plays an essential role in tumor angiogenesis and apoptosis (18). Our laboratory studied the possible mechanism of pCD's action in breast cancer cell lines and localized the mitogenic activity to the AP region that interacts with an unknown cell surface receptor and exhibits growth factor-like activity on cancerous cells (19, 20). Further administration of anti-AP antibodies *in vivo* inhibited the growth of human breast tumors in athymic nude mice (16). Recently, it was shown that the AP-treatment of ZR-75-1 cells induced the expression of genes involved in signal transduction, cell cycle regulation, tumor invasion, and metastasis (21). Together, these studies establish the importance of pCD's role as a mitogen in breast cancer cells. However, further studies are necessary to establish the role of pCD in lung cancer.

In the present study, the mitogenic activity of pCD was tested both *in vitro* and *in vivo* in a lung cancer cell line. The full length of pCD and pCD without its AP were constructed and stably expressed as clones of the NCI-H23 cell line. The data clearly establish that the AP is responsible for the mitogenic activity of pCD.

Materials and Methods

Generation of constructs. The 1.1 Kb human pCD cDNA (a kind gift from Dr. Chirgwin) and the pCD(ΔAP) lacking the 44-amino acid coding sequence for the AP (22) were subcloned in the p3xFlag-CMV-14 vector (Sigma Chemical Co., St. Louis, MO, USA) at B*gl* II and X*ba* I sites. Authenticity of positive clones was established by DNA sequencing and restriction digestion with B*gl* II and X*ba* I enzymes, respectively.

Cell culture and transfection. The human lung cancer cell line NCI-H23 was obtained from the ATCC (Manassas, VA, USA). The cell line was maintained in RPMI 1640 (Sigma) medium containing HEPES (Sigma) buffer supplemented with 10% heat-inactivated FCS (Hyclone Lab., Logan, UT, USA), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), in plastic disposable tissue culture flasks at 37˚C in a 5% CO_2/95% air incubator. The cell line was transfected at 70-80 % confluency with 0.8 μg of plasmid construct mixed with 2.5 μl of Lipofectamine 2000 reagent (Invitrogen Life Tech., Carlsbad, CA, USA) in Optimem-I (Invitrogen). After 5 h, the transfection medium was replaced with fresh medium and the cells were grown for an additional 24 h. For the generation of stable clones, the transfected cells were incubated in the presence of G418 (800 μg/ml) over a period of 2-3 weeks. Finally, G418-resistant cells were expanded in 96-well plate and the positive stable clones were checked for secretion of pCD by Western blot.

Western blot analysis. The control and the stably transfected NCI-H23 cells ($2x10^5$ cells/ml) were seeded in normal growth medium. After 24 h, the medium was replaced with RPMI containing 0.1% FCS and the cells were allowed to continue to grow for an additional 48 h. Conditioned media were collected, concentrated (10x) using a centricon (Pall Life Sciences, Ann Arbor, MI, USA) and subjected to protein estimation using the BCA protein Assay Kit (Pierce, Rockford, IL, USA). Ten micrograms of total protein samples were diluted 1:1 in Laemmli sample buffer, heated at 95˚C for 5 min and subjected to SDS-PAGE. Electrophoresis and immunoblotting were performed as described (23). The primary antibodies used were anti-AP (prepared in our lab) and anti-Flag M2 antibody (Sigma) and the anti-mouse IgG-alkaline phosphatase conjugate (Sigma) was used as secondary antibody.

To examine the effect of AP treatment on NF-κB2 at protein level, the NCI-H23 cells ($5x10^6$ cells) were treated with 20 nM AP for 4 h. The cell lysates containing an equal amount of total proteins were subjected to Western blot analysis using anti-NFkB2 antibody; the expression of β-actin was detected with anti-actin antibody, as loading controls.

Matrigel assay. A commercial kit manufactured by Chemicon International (Temecula, CA, USA) was used to evaluate the invasion across the Matrigel layer according to the manufacturer's instructions. Briefly, $3x10^5$ cells in serum free medium were added to each well and incubated for 48 h in a CO_2 incubator. Cells that migrated through the matrix and became attached to the other side of the insert were fixed, stained and quantitated by dissolving the stained cells in 10% acetic acid. An equal aliquot of stained cells was transferred to a 96-well plate for calorimetric reading at OD 560 nm.

In vitro cell proliferation assay. For growth experiments, cells were harvested and washed six times in Iscoves's modified Dulbecco's medium (Sigma) with HEPES buffer supplemented with glutamine (Sigma), antibiotics, 10 μg/ml of human transferrin (Sigma) and 0.1% FCS. Cells were seeded in 96-well tissue culture plates at a density of $5x10^4$ cells/ml (150 μl/well) in the presence or absence of purified proteins (10 ng/well) in triplicate. After four days in culture, the proliferation was evaluated using Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the instructions given by the manufacturer. The positive control included NCI-H23 cells in RPMI media with 10% FCS and the negative control comprised of NCI-H23 cells in RPMI media without serum.

MTT assay. Control and stably transfected NCI-H23 cells ($5x10^3$) were plated into each well (100 μl/well) of 96-well culture plates. At each specified time interval, MTT (Sigma) solution (10 μl from 5 mg/ml stock) was added to the wells and the plates were further incubated at 37 ˚C for 4 h in a humidified incubator with 5% CO_2. The medium was then aspirated to ease the formation of the formazan product that was then solubilized with the addition of 100 μl of acidic isopropanol. The optical density was measured at 570 nm with reference wavelength of 630 nm using a SLT ELISA reader (Tecan, Research Triangle Park, NC, USA). Similar experiments were repeated in triplicate.

RNA extraction and reverse transcriptase-PCR. NCI-H23 cells ($5x10^7$) were incubated in serum-free medium containing 20 nM AP for 4 h at 37˚C. Total RNA was extracted from control and AP-treated NCI-H23 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as specified by the manufacturer. RNA quality and quantity were determined by ultraviolet spectrophotometry and formaldehyde agarose gel electrophoresis. Total RNA (200 ng) from both samples was reverse transcribed and PCR amplified using SuperScript™ One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers of NF-κB2, CDC-42 and β-actin were used and particular RT-PCR conditions were used, as described earlier (21). RT-PCR products were then separated on 1.0% agarose gel, visualized under UV light and photographed.

Gross pathology and histopathology. Four-to-six week old athymic nude mice (Jackson lab, Bar Harbor, ME, USA) were injected with 0.1 ml of a cell suspension containing NCI-H23 cells ($4x10^6$) stably transfected with pCD or with pCD (ΔAP). Eight weeks after cell injection, the mice were euthanized by inhalation of CO_2. Euthanasia was confirmed by pneumothorax. A complete necropsy was performed on all mice and the heart, lungs, kidney, liver, spleen and parts of the intestinal tract were removed and fixed in 10% buffered formalin. Fixed tissues were trimmed and paraffin-embedded for processing. The blocks were cut into 4 μm sections and stained with hematoxylin and eosin for histopathology examination.

Statistical analysis. The Student's unpaired *t*-test was used to calculate statistical differences between data sets. Significant difference was taken at $p<0.05$. Additionally, proliferation data

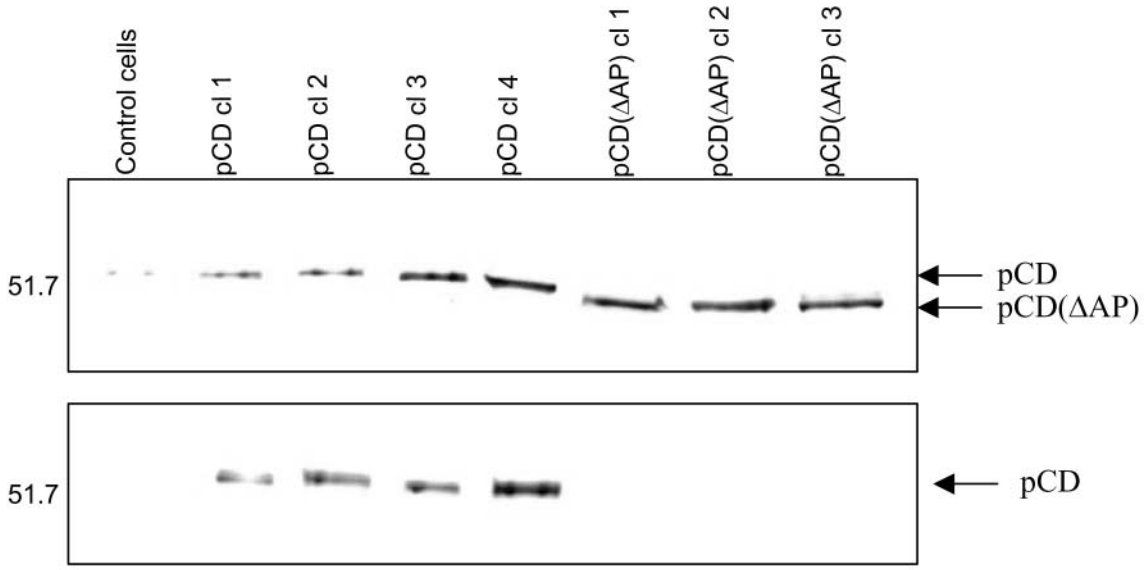

Figure 1. *Analysis of expression and secretion of pCD and pCD(ΔAP) from NCI-H23 stably transfected cells. Conditioned media from control and transfected cells were collected over 2 days. The conditioned media was then analyzed by immunoblotting using anti-Flag (upper panel) and anti-AP (lower panel) antibodies.*

were assessed by one-way analysis of variance (ANOVA), using SigmaSTAT software with Holm-Sidak method for multiple comparison ($p \leq 0.05$).

Results

Stable expression of pCD increases proliferation of NCI-H23 cells. Various lung cancer cell lines NCI-H727, NCI-H23, DMS-53 and SW900, for active pCD secretion, were previously examined (23). As NCI-H23 cells had the lowest secretion among all the cell lines tested, this cell line was used to determine the effect of experimentally overexpressed pCD. The pCD and pCD(ΔAP) were cloned downstream of the CMV promoter in the p3xFlag expression vector and were stably transfected into NCI-H23 cells. The positive clones were analyzed for the secretion of expressed pCD and pCD(ΔAP) by Western blot (Figure 1) using anti-Flag antibody (upper panel) and anti-AP antibody (lower panel). Additional studies were conducted with clone 4 of pCD and clone 2 of pCD(ΔAP), as these showed highest expression level of proteins. The NCI-H23 stable clones expressing and secreting pCD and pCD(ΔAP) were then analyzed for cell growth. The cell proliferation data in Figure 2, indicate that the original inoculated population of NCI-H23 pCD increased to 1.6-fold, as compared to NCI-H23 control cells, over a period of 7 days. The lack of any enhancement in proliferation by NCI-H23 pCD(ΔAP) suggested that AP region is essential for the growth activity of pCD.

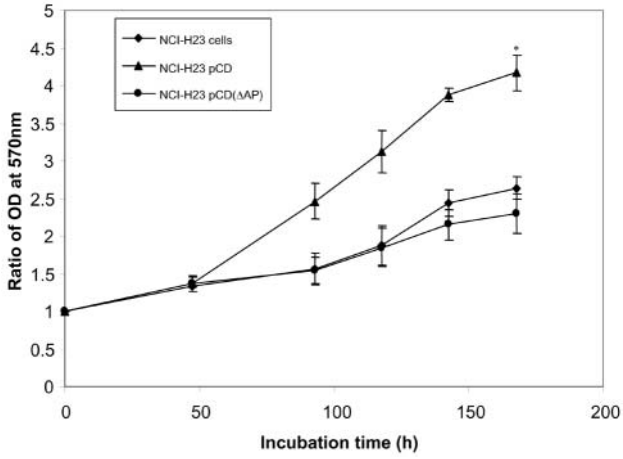

Figure 2. *Effect of pCD and pCD(ΔAP) expression on cell proliferation of NCI-H23 cells. Control and stably transfected cells were incubated for 7 days in the presence of 0.1% FCS and proliferation was monitored over the specified time period by MTT assay. All values of OD are relative to the OD at 0 h. *p<0.05 versus control cells (t-test).*

Effect of purified pCD and its mutant forms on proliferation of different lung cancer cell lines. The pCD and its mutants were affinity purified from the supernatant of the breast cancer cell line stably transfected with these mutant constructs (Ohri *et al.*, submitted manuscript). These mutants include: i) the pCD lacking the AP region designated as pCD(ΔAP), ii) nonglycosylated pCD designated as pCD(70+199m) and iii)

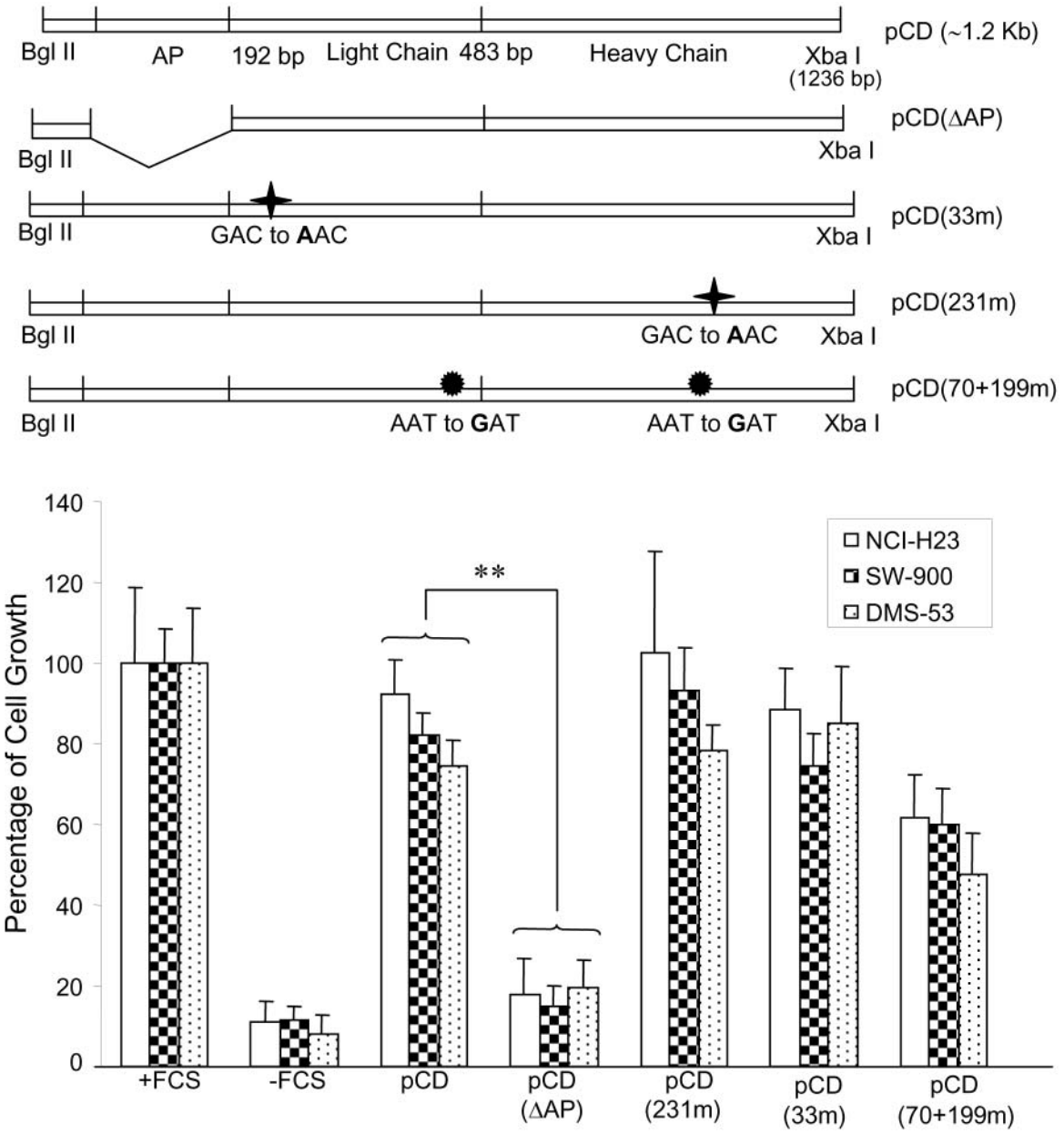

Figure 3. *Effect of exogenous addition of purified pCD and its mutant proteins [pCD(ΔAP), pCD(231m), pCD(33m), pCD(70+199m)] on the proliferation of lung cancer cell lines. A) Schematic representation of cDNA constructs of pCD and its mutants used in this study. ✦Refers to the catalytic site mutation (GAC to AAC) at 33 and 231 amino acid position, respectively, and ✹ refers to the glycosylation site mutation (AAT to GAT) at 70 and 199 amino acid position, respectively. B) Equal concentrations of pCD and its mutants were used to analyze the effect on proliferation of DMS-53, NCI-H23 and SW900 in serum-free conditions. The growth in FCS-containing medium was taken as 100% and without FCS was taken as negative control. Data represent the percentage of cell growth and are the mean±SD of three independent experiments performed in triplicates. Analysis of data was performed by ANOVA with overall significance at p=0.05. **p≤0.002.*

two mutants aborting the proteolytic activity by mutation in the active site aspartates, designated as pCD(33m) and pCD(231m), respectively. The schematic representation of the cDNA constructs used for these proteins is depicted in Figure 3A. The proliferation profile of three lung cancer cell lines NCI-H23, DMS-53 and SW900 (Figure 3B) was assessed with respect to the affinity purified fractions containing pCD, pCD(ΔAP), pCD(33m), pCD(231m) and pCD(70+199m) proteins using Biotrac Elisa assay where the incorporation of BrdU in actively dividing cells is measured. Purified pCD

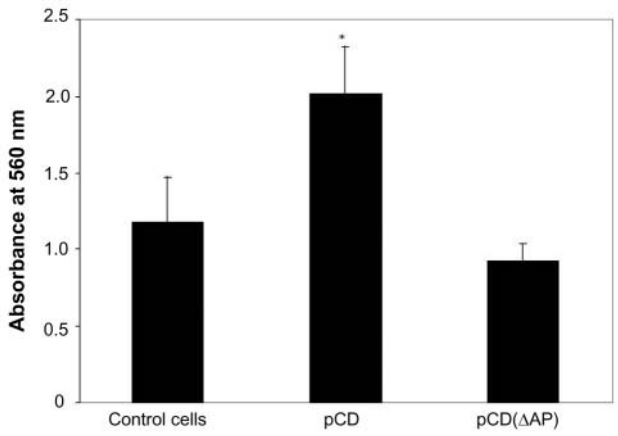

Figure 4. *Invasive potential of control and stably transfected NCI-H23 cells were evaluated using a commercial kit from Chemicon International. Columns represent the mean of three independent invasion experiments; bars, SD. *p<0.05 versus control (Student's t-test).*

along with pCD(33m) and pCD(231m) elicited an enhanced proliferation in the three cell lines tested, as opposed to pCD lacking the AP. Increased proliferation was also observed in glycomutant, pCD(70+199m), however the effect was found to be 15-20% lower compared to pCD.

Presence of AP imparts invasiveness to NCI-H23 cells and increases tumor growth in nude mice. The effect of expression of pCD and pCD(ΔAP) on invasive potential of NCI-H23 cells was checked by the *in vitro* invasive model-Matrigel. A significant increase in invasiveness was observed with NCI-H23 pCD cells when compared to control NCI-H23 cells (Figure 4). The NCI-H23 cells harboring the pCD(ΔAP) showed no enhancement in invasion.

In order to examine whether the AP region of pCD was indeed responsible for mitogenicity, as determined by our *in vitro* studies, stable clones of NCI-H23 pCD, NCI-H23 pCD(ΔAP) and control NCI-H23 cells were injected *i.v.* into nude mice consisting of 6 animals/group that were analyzed after a period of 8 weeks. As evidenced from the histology slides of the lung tissue (Figure 5), NCI-H23 pCD showed nests of tumor cells with moderate inflammation (numerous lymphocytes and neutrophils are seen around lung cancer cells) and minimal tissue necrosis, whereas NCI-H23 pCD(ΔAP) had few isolated tumor cells with minimal inflammation and no tissue necrosis. This data support our hypothesis that the AP region of pCD is indeed responsible for mitogenic activity both *in vitro* and *in vivo*.

Enhanced expression of NF-κB2 in response to AP treatment. To elucidate the direct effect of AP on NCI-H23 cells at the cellular level, NCI-H23 cells were treated with AP and RT-PCR was performed to test the up-regulation of two important genes, NF-κB2 and Cdc42. Distinct up-regulation of NF-κB2 and Cdc42 expression was observed (Figure 6A). The up-regulation of NF-κB2 expression in NCI-H23 cells treated with AP was further checked at the protein level. Western blot analysis (Figure 6B) of the control and AP-treated NCI-H23 cell lysates indeed revealed an increase in NF-κB2 levels (top panel). β-Actin served as a control for equal loading of cell lysates (bottom panel).

Discussion

Fontanini *et al.* (24) first detected CD in non-small cell lung cancer using an immunohistochemical technique. Later studies by Wang and Zhao (25) showed that the expression of CD was higher in stage III-IV patients, as compared to stage I-II patients. Nevertheless, no significant relationship could be established between the level of CD expression and the enzyme activity in tumor supernatant (26, 27). Previous studies have shown that pCD and its synthetic AP had similar proliferating effect on different lung cancer cell lines (23). This is the first study where the stable transfected clones of pCD and pCD(ΔAP) were constructed in NCI-H23 to check the direct effect of pCD and its AP on experimental metastasis *in vivo*.

The full length pCD and pCD (ΔAP) were successfully expressed and secreted by NCI-H23 cells, as confirmed by Western blotting. The NCI-H23 pCD cells showed a significant enhancement in cell proliferation, when compared to the NCI-H23 pCD(ΔAP) cells. An increase in proliferation by transfection of pCD in NCI-H23 is in line with the earlier observed effect, where rat cancer cell line transfected with human pCD cDNA showed enhanced proliferation and increased metastasis (17).

Enhanced proliferation with addition of exogenous pCD and its synthetic AP was observed in prostate (28), breast (15) and lung (23) cancer cell lines. In the present investigation, the molecular features of pCD contributing to its growth-promoting activity were evaluated. Various mutant forms of pCD, *i.e.,* catalytically inactive mutants, nonglycosylated mutant and pCD lacking its AP were purified and assessed on different lung cancer cell lines by proliferation assay. The pCD and its catalytic mutant showed enhanced growth, while pCD(ΔAP) had no growth-promoting effect in any of the tested cell lines. The effect of nonglycosylated mutant of pCD on proliferation was 15-20% less, as compared to wild type pCD. This is in concurrence with the earlier observation that removal of sugar moiety from pCD slightly lowered the mitogenic activity, in both proliferation and activation experiments (19).

A positive correlation has been shown between the proportion of pCD secreted and the invasive potential in different cancer cell lines (23, 29). The effect of stable expression of pCD and pCD(ΔAP) on the invasive ability of

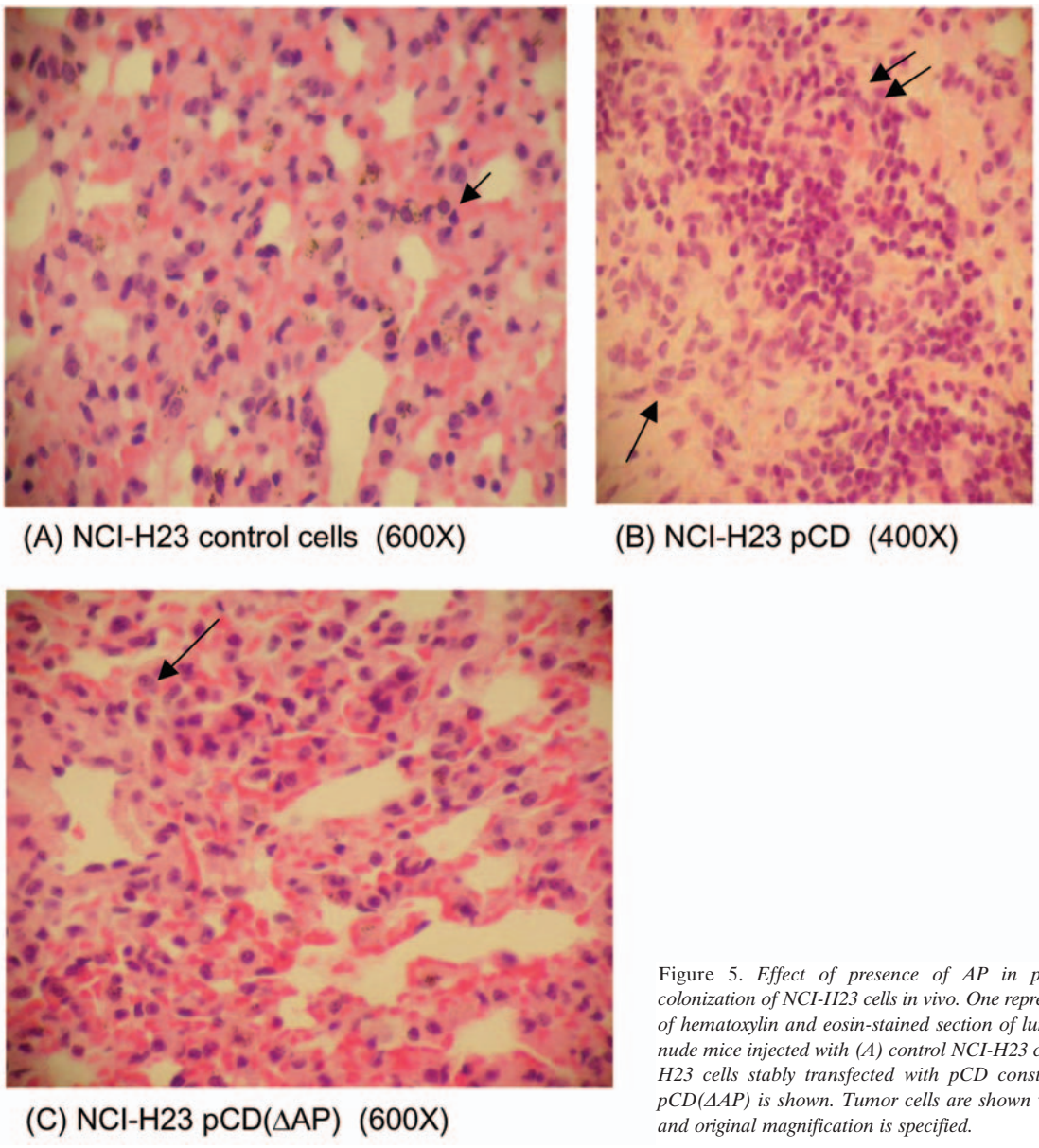

Figure 5. *Effect of presence of AP in pCD on lung colonization of NCI-H23 cells in vivo. One representative slide of hematoxylin and eosin-stained section of lung tissue from nude mice injected with (A) control NCI-H23 cells, (B) NCI-H23 cells stably transfected with pCD construct and (C) pCD(ΔAP) is shown. Tumor cells are shown with an arrow and original magnification is specified.*

NCI-H23 cells was examined. The NCI-H23 pCD cells showed a significant enhanced invasion compared to the pCD(ΔAP)-expressing cells, suggesting that AP is essential for enhancing the invasive potential of this cell line. An increase in lung colonization of cancer cells was also observed in nude mice injected with NCI-H23 cells harboring pCD, while no increase was observed in cells transfected with pCD(ΔAP), further elucidating the importance of AP in metastasis.

To gain further insight, RT-PCR of NCI-H23 control cells and NCI-H23 cells treated with AP was conducted. Our results indicate the up-regulation of NF-κB2 and CDC-42, as previously observed with ZR-75-1 and MCF-7 breast cancer cells (21). The NF-κB2 up-regulation is considered to be of prime importance, since members of this family are important regulators of cell cycle progression, cell survival, cell adhesion/angiogenesis, invasion, and inflammatory responses (30, 31). The up-regulation of NF-κB2 at protein level was also observed by AP treatment. Further studies are necessary to understand the association between NF-κB2 up-regulation and mitogenic activity of pCD.

In conclusion, the data presented clearly indicate that the AP is responsible for mediating the mitogenic activity of pCD in a lung cancer cell line. The *in vitro* results of cell proliferation, as

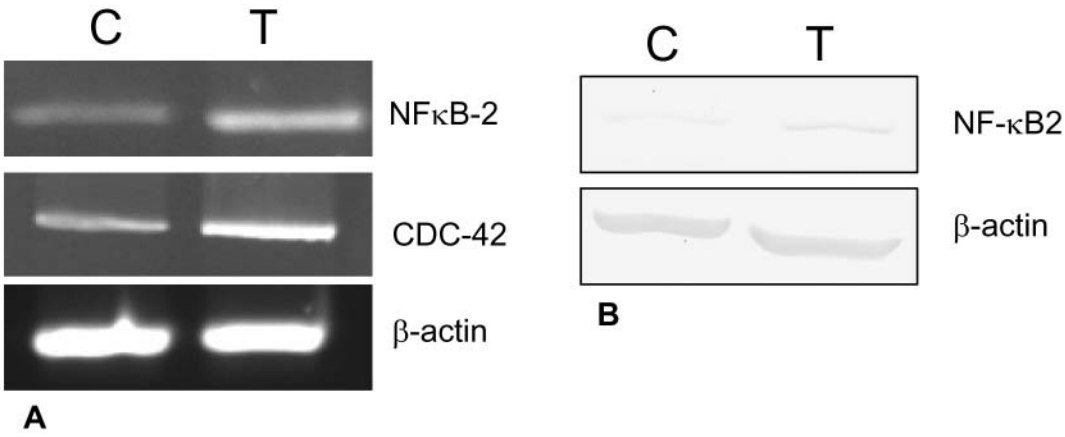

Figure 6. *(A) Differential expression of genes in response to AP treatment. RNA from control (C) and AP-treated NCI-H23 cells (T) was subjected to RT-PCR with gene-specific primers. Expression of β-actin served as an internal control. (B) Western blot analysis of total proteins from control (C) and AP-treated NCI-H23 cells (T) using NF-κB2 antibody; β-actin corresponds to equal loading of proteins.*

well as these of invasion, were confirmed by the *in vivo* studies in nude mice. Therefore, targeting the AP or cell-surface receptor recognizing this region represents an important approach for the suppression of growth in lung cancer.

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