Phospholipase D signaling pathway is involved in lung cancer-derived IL-8 increased osteoclastogenesis

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Bone is a frequent target of lung cancer metastasis, which is associated with significant morbidity and a dismal prognosis. This study analyzed the soluble factors secreted by lung cancer cells, which are responsible for increasing osteoclast differentiation. Addition of recombinant human interleukin-8 (rhIL-8), present in large amounts in A549-conditioned medium (CM) and NCI-H460-CM, mimicked the inductive effect of A549-CM and NCI-H460-CM on osteoclastogenesis. In contrast, depletion of interleukin-8 (IL-8) from A549-CM and NCI-H460-CM decreased the osteoclastogenesis-inductive properties of A549-CM and NCI-H460-CM. Induction of osteoclast differentiation by lung cancer-derived CM and rhlIL-8 was associated with increased phospholipase D (PLD) activation, and the activations of protein kinase C (PKC) α/βII, extracellular signal-regulated kinase (ERK) 1/2 and AKT/the mammalian target of rapamycin (mTOR). Blocking PLD by a specific inhibitor significantly decreased osteoclast formation by inhibiting PKCs activation and subsequently attenuating the phosphorylation of ERK1/2. PLD inhibitor also completely decreased AKT and mTOR phosphorylation, whereas phosphatidylinositol-3-kinase (PI3K) inhibitor only partially decreased mTOR phosphorylation, suggesting that mTOR activation by PLD is through both PKC/ATK-dependent and PI3K/ATK-independent manner. In addition, blocking AKT and ERK1/2 by a specific inhibitor also suppressed lung cancer-derived CM and rhIL-8-induced osteoclast differentiation. Moreover, treatment of peripheral blood mononuclear cells with sera from invasive lung cancer patients increased the formation of osteoclasts. Our study suggests that IL-8 or IL-8-mediated PLD/ PKC/ERK1/2 or PLD/ATK signaling is an attractive therapeutic target for osteolytic bone metastases in lung cancer patients.

Introduction

The skeleton is a frequent target of lung cancer metastasis. Approximately 30–40% of patients with advanced lung cancer will develop bone metastasis, resulting in a significant impact on both morbidity and survival (1,2). Within the skeleton, lung cancer metastasis causes osteolytic lesions characterized by increased osteoclast activity (1). Osteoclasts have long been recognized as the cells that resorb bone in normal bone remodeling and in pathological conditions when bone resorption is increased (3). Cancer cells secrete numerous factors, which stimulate osteoclast-mediated bone resorption, and the consequent release of some factors from the skeletal matrix, which were proved to affect tumor cells, promoting cancer cells spreading and bone destruction (3). Recently, various factors that contribute to cancer cell migration to bone, such as stromal-derived factor-1, monocyte chemotactic protein 1 and receptor activator of nuclear factor-κB ligand (RANKL), and factors that enhance tumor cell growth within bone, such as transforming growth factor-β and parathyroid hormone-related protein, have been identified (4–6). However, it is still not fully clear if there are other factors involved in lung cancer-mediated bone destruction, nor the precise molecules involved in the formation of osteolytic lesions.

Interleukin-8 (IL-8 or CXCL8), a proinflammatory CXC chemokine, is associated with the promotion of neutrophil chemotaxis and degranulation (7). Elevated levels of IL-8 and/or its receptors have been characterized in cancer cells, endothelial cells, infiltrating neutrophils and tumor-associated macrophages, suggesting that IL-8 may function as a significant regulatory factor within the tumor microenvironment (8,9). After activation of heterotrimeric small G proteins, IL-8 signaling promotes activation of the phosphatidylinositol-3-kinase (PI3K), phospholipase C and phospholipase D (PLD), promoting activation of AKT, PKC, calcium mobilization or mitogen-activated protein kinase signaling cascades (7,10). Increased AKT expression and activity have been detected in several types of cancer, and its role in regulating cell survival, proliferation, angiogenesis and metastasis has been confirmed (11). AKT has been identified as being involved in the regulation of osteoclast survival and differentiation, whereas deficiency of AKT in osteoclasts results in impairment of bone resorption (12,13). However, the precise mechanism of AKT-mediated osteoclast differentiation still remains unknown.

PLD has been implicated in intracellular signal transduction, endocytosis, exocytosis, cytoskeletal reorganization, cell migration, mitosis, differentiation and survival (10,14–16). PLD has to be activated by IL-8 (10), and in turn catalyzes the hydrolysis of the phosphodiester bond of phosphatidlycholine to generate the lipid second messenger phosphatidic acid (PA) and choline (14). PA, widely considered to be the intracellular lipid mediator of many biological functions, has many downstream targets, including nicotinamide adenine dinucleotide phosphate oxidase, the mammalian target of rapamycin (mTOR), PI3K and PKC (17,18). To date, 10 isotypes of PKC family enzymes for mammals have been identified, which are subgrouped into three categories depending on their structural homology and dependency on Ca2+ and cofactors. Classical PKCs (cPKCs; PKC-α, β and γ) require Ca2+ and diacylglycerol and phospholipids; novel PKCs (nPKCs; PKC-δ, ε, η and θ) are Ca2+ independent but diacylglycerol and phospholipid dependent; and atypical PKCs (aPKCs; PKC-ζ, η, ι and μ) are insensitive to Ca2+ and diacylglycerol (19). cPKCs are known to be critical mediators in the control of osteoclastic survival and formation through the activation of mitogen-activated protein kinase (20,21).

Therefore, we hypothesized that PLD and cPKC may play a role in osteoclastogenesis mediated by lung cancer. Although the regulation of bone metastasis has been a focus of intensive investigation, relatively little is known about the molecular mechanisms that control the process. Understanding molecular
mechanisms contributing to bone metastatic is essential for developing biomarkers of cancer progression, as well as for the development of more effective therapies. In this study, we examined the effect of osteoclastogenesis on human peripheral blood mononuclear cells (PBMCs) by the conditioned medium (CM), which was derived from human lung cancer cell lines A549 and NCI-H460. We also demonstrated the molecular mechanism involved in lung cancer-mediated osteoclastogenesis.

Material and methods

Cell culture and CM

Normal lung fibroblast IMR-90 cells and human lung cancer cell A549 and NCI-H460 were obtained from the American Type Culture Collection (Rockville, MD). To obtain the CM, cells were seeded at 2 × 10⁴ cells per 100 mm dish and cultivated until 60% confluence. The medium was replaced and the supernatants were harvested at 48 h of incubation. IL-8 depletion from A549-CM and NCI-H460-CM was performed by using anti-IL-8 antibodies (2 µg/ml) and Sepharose A/G beads following regular immunoprecipitation techniques. Cytokine depletion was confirmed by IL-8 ELISA assay kit.

Expansion of PBMCs

Human peripheral blood, obtained from healthy adult volunteers, was collected in syringes containing 1000 U/ml of preservative-free heparin. PBMCs were isolated by density centrifugation using Ficoll–Hypaque and were resuspended in RPMI supplemented with 10% heat-inactivated fetal bovine serum. PBMCs were then plated and incubated overnight at 37°C. Non-adherent cells were removed by washing with phosphate-buffered saline, and the remaining adherent cells were grown in culture medium containing vehicle or 200 ng/ml human macrophage colony-stimulating factor (M-CSF) and 100 ng/ml human RANKL for 7 days. The medium was replaced every 3 days (22). Blood samples were obtained from 25 stage IV lung cancer patients admitted to Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital. Serum was separated from blood and frozen at −70°C. Approval for these studies was obtained from the Institutional Review Board of the Kaohsiung Medical University Choung-Ho Memorial Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Osteoclast differentiation assay

Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase (TRAP) (Sigma–Aldrich, St Louis, MO). Briefly, cells were fixed for 30 s and then stained with naphthol AS-BI-phosphate and a tartrate solution for 1 h at 37°C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells by means of light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted (22).

Bone resorption assay

PBMCs were plated into a calcium phosphate apatite-coated 16-well plate (BD BioCoat Osteologic Bone Resorption assay (BD Biosciences, Bedford, MA)) in the same culture conditions as described above. After a 7 day culture, each well was washed with saline and one solution of 5% sodium hypochlorite was left in the well for 5 min to detach the cells. The number of pits in each well was counted under a microscope.

Immunoblot/kinase assay

PBMCs were seeded at 2 × 10⁴ cells per 100 mm dish and cultivated until 60% confluence. The medium was replaced and the supernatants were harvested at 48 h of incubation. IL-8 depletion from A549-CM and NCI-H460-CM was performed by using anti-IL-8 antibodies (2 µg/ml) and Sepharose A/G beads following regular immunoprecipitation techniques. Cytokine depletion was confirmed by IL-8 ELISA assay kit.

Results

Soluble factors produced by lung cancer cells stimulate osteoclast formation and function

Previous studies have demonstrated that spontaneous osteoclastogenesis is present in patients affected by different solid tumors with osteolytic metastases (23). We first assessed the effect of normal lung fibroblast IMR-90-CM and lung cancer cell lines A549-CM and NCI-H460-CM in osteoclastogenesis. As shown in Figure 1A, exposure of PBMCs with A549-CM and NCI-H460-CM increased osteoclastogenesis of PBMCs present in RANKL and M-CSF (Figure 1A). In contrast, IMR-90-CM did not increase osteoclastogenesis of PBMCs present in RANKL and M-CSF (control group) (Figure 1A). To confirm whether these osteoclasts derived from CM-treated PBMCs were functional and capable of bone resorption, PBMCs were cultured on artificial bone discs. As shown in Figure 1B, PBMCs treated with A549-CM and NCI-H460-CM caused more bone resorption areas compared with IMR-90-CM-treated PBMCs. Concurrently, NFATc1, TRAF6, OSCAR and Cathepsin K, markers of differentiated osteoclasts, were also upregulated, when compared with IMR 90-CM treatment alone. These data suggest that A549-CM and NCI-H460-CM increased differentiation of osteoclastic precursors (Figure 1C).

IL-8 is necessary for the induction of osteoclastogenesis by lung cancer cell

IL-8 has been reported to play an important role in osteoclast formation (8). Therefore, we assessed the expression of IL-8 in A549-CM and NCI-H460-CM and IMR-90-CM. As shown in Figure 2A, significantly high levels of IL-8 were found in the A549-CM and NCI-H460-CM, whereas IL-8 level was relatively low in IMR-90-CM. Next, we assessed the effect of rhIL-8 on the formation of osteoclasts. Addition of rhIL-8 to PBMCs increased osteoclastogenesis from PBMCs present in RANKL and M-CSF (Figure 2B). Moreover, rhIL-8 also increased the capacity of bone resorption present in RANKL and M-CSF (Figure 2C).

Successful depletion of IL-8 from A549-CM and NCI-H460-CM was confirmed by IL-8 enzyme-linked immunosorbent assay kit, as shown in supplementary Figure S1 (available at Carcinogenesis Online). IL-8-depleted CMs were added into PBMCs. Depletion of IL-8 effectively reversed the induction of osteoclastogenesis caused by A549-CM and NCI-H460-CM (Figure 2D). These data clearly show that IL-8 in A549-CM and NCI-H460-CM mediates the changes in the formation of osteoclasts.

We also assessed the osteoclastogenesis of PBMC treated with 15% serum from 25 stage IV lung cancer patients. As shown in supplementary Figure S2A (available at Carcinogenesis Online), TRAP-positive multinuclear cells were drastically increased in the presence of patients’ sera. Our results convincingly prove that patients with invasive lung cancer have soluble factors released into their sera,
which effectively increases osteoclast differentiation (P value = 1.55 x 10^{-10}). To further elucidate the nature of the soluble factors present in the sera of these lung cancer patients, we examined the levels of IL-8 in healthy donors and these patients. The average IL-8 serum level of these patients and healthy donors was 109.5 ± 104.3 pg/ml (range: 29.6–352.5 pg/ml; compared with healthy donor, P value is 0.00063) (supplementary Figure S2B is available at Carcinogenesis Online). The level of IL-8 in healthy donors ranges from very low (range: 0.22–0.94 pg/ml) to undetectable.

Lung cancer cell derived-CM and rhIL-8 activate AKT and ERK1/2 signaling

Because ERK1/2 and AKT signaling have been shown to be involved in osteoclastic differentiation (12,21), we investigated the role of ERK1/2 and AKT in lung cancer cell derived-CM and rhIL-8-mediated osteoclastogenesis. As shown in Figure 3A, treatment of PBMCs with A549-CM, NCI-H460-CM and rhIL-8 (20 ng/ml) increased the phosphorylation of AKT protein without affecting the protein level of total AKT. Exposure of PBMCs to A549-CM, NCI-H460-CM and rhIL-8 resulted in increased phosphorylated (activated) form of mTOR, a downstream target of PI3K/AKT. Similar responses were observed for the phosphorylated forms of another AKT downstream target, glycogen synthase kinase (GSK)-3β (Figure 3A). In addition, A549-CM, NCI-H460-CM and rhIL-8 also increased the phosphorylation of ERK1/2 in PBMCs, without affecting the expression of ERK1/2 protein (Figure 3A).

The activation of AKT and ERK1/2 was further confirmed by assessing phosphorylation of their substrates: GSK-3α/β for AKT and Elk-1 for ERK1/2. As shown in Figure 3B, phosphorylation of GSK-3α/β and Elk-1 were both increased after PBMCs exposed to A549-CM, NCI-H460-CM and rhIL-8. We also confirmed the role of IL-8 on the activation of AKT, mTOR and ERK1/2 by using IL-8 depleted A549-CM and NCI-H460-CM. As shown in Figure 3C, depletion of IL-8 effectively decreased the phosphorylation of AKT, mTOR and ERK1/2 in A549-CM, NCI-H460-CM-treated PBMCs (Figure 3C).

Lung cancer cell derived-CM and rhIL-8 activate PLD- and PKCα/βII-signaling pathway during osteoclastogenesis

PLD catalyzes the conversion of phosphatidylcholine to PA and is activated by IL-8 signaling in neutrophils (24). We therefore assessed the status of PLD cascade signaling after PBMCs treated with lung cancer cell derived-CM and rhIL-8. As shown in Figure 4A, treatment of PBMCs with A549-CM, NCI-H460-CM and rhIL-8 resulted in an increase of PLD activity in PBMCs.

Next, we investigated whether PKCα/βII, a downstream of PLD, is involved in lung cancer cell derived-CM and rhIL-8-mediated osteoclastogenesis. As shown in Figure 4B, exposure of PBMCs to A549-CM, NCI-H460-CM and rhIL-8 increased phosphorylation of PKCα/βII. In addition, PKC activity was also increased by A549-CM, NCI-H460-CM and rhIL-8 in PBMCs (Figure 4C).

We also confirmed the role of IL-8 on the activation of PLD and PKCα/βII by using IL-8 depleted A549-CM and NCI-H460-CM. Depletion of IL-8 effectively decreased the activation of PLD and PKCα/βII in A549-CM, NCI-H460-CM-treated PBMCs (Figure 4D and E).

The role of PLD in lung cancer cell derived-CM and rhIL-8-mediated PKCα/βII, AKT/mTOR and ERK1/2 activation

To further clarify if activation of PLD is the upstream signal for the activation of PKCα/βII, AKT and ERK1/2, specific PLD inhibitor, OSCAR is assessed by immunoblot assay. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test (P < 0.05). All experiments were performed independently at least three times.
0.3% (vol/vol) butan-1-ol was used. As shown in Figure 5A, pretreatment of PBMCs with butan-1-ol attenuated the phosphorylation of PKCα/βII, ERK1/2, AKT and mTOR in A549-CM, NCI-H460-CM and rhIL-8-treated PBMCs. This data suggests that PLD is the upstream molecule of PKCα/βII, AKT and ERK1/2.

Similar to PLD inhibition, inhibition of PKCα/βII by bisindolylmaleimide I (Bis) decreased A549-CM, NCI-H460-CM and rhIL-8-induced ERK1/2 activation, suggesting that PKCα/βII is the potential upstream activator of ERK1/2 signaling (Figure 5B). In contrast, bisindolylmaleimide I (Bis) did not affect the phosphorylation of AKT and mTOR in A549-CM, NCI-H460-CM and rhIL-8-treated PBMCs (Figure 5B), suggesting that the activation of AKT is through a PKCα/βII-independent manner.

Previous studies reported that PLD increases mTOR by PI3K/AKT-dependent and PI3K/AKT-independent pathways (17,25). As shown in Figure 5C, pretreatment of PBMCs with PI3K inhibitor LY294002 completely decreased the activation of AKT but only partially decreased the mTOR phosphorylation caused by A549-CM, NCI-H460-CM and rhIL-8. In contrast, PLD inhibitor completely inhibited mTOR activation (Figure 5A). These data suggest that the activation of mTOR is through direct PLD and indirect PLD–PI3K/AKT signaling.
The role of PLD, AKT and ERK1/2 in lung cancer cell derived-CM and rhIL-8-mediated osteoclastogenesis

We further investigated the involvement of PLD, AKT and ERK1/2 in lung cancer cell derived-CM and rhIL-8-induced osteoclastogenesis. We examined the effects of the inhibitors of PLD, AKT and ERK1/2 on the formation of TRAP-positive multinuclear cells. As shown in Figure 6A, pretreatment of PBMCs with PLD inhibitor blocked A549-CM, NCI-H460-CM and rhIL-8-induced osteoclastogenesis. AKT inhibitor (10-((4'-((N-diethylamino)butyl)-2-chlorophenoxazinyl)) and mitogen-activated protein kinase inhibitor (PD98059) could also decrease A549-CM, NCI-H460-CM and rhIL-8-induced osteoclastogenesis (Figure 6B and C).

Discussion

Bone is a common site of distant metastasis in lung cancer (1). Current studies show that lung cancer cells could secrete IL-8 (8,27,28). Bendre et al. (8) further demonstrated that IL-8 from lung cancer cell line could increase osteoclast formation. And serum from advanced lung cancer patients contains high levels of IL-8 (6,29). Recently, Cai et al. (6) reported that levels of monocyte chemotactic protein 1 and IL-8 are higher in CM of lung cancer cell lines and in serum of lung cancer patients with bone metastasis (29). But the mechanism about osteoclastogenesis mediated by IL-8 was not detailed. From our results, the induction effects of A549-CM and NCI-H460-CM on osteoclastogenesis could be reproduced by exposure of PBMCs with rhIL-8 in the culture medium. And depleting IL-8 from A549-CM and NCI-H460-CM could reverse this induction effect. Serum IL-8 level of advanced lung cancer patients was higher than healthy adults, and the osteoclastogenesis of PBMCs treated with these sera was increased. Thus, we clearly demonstrated the role of IL-8 in osteoclastogenesis in advanced lung cancer patients.

AKT is a downstream effector of PI3K and has been shown to be a critical mediator of cell proliferation, survival and differentiation in a variety of cell types. Sugatani et al. (30) have reported that AKT plays a critical role in osteoclast differentiation, whereas loss of AKT protein inhibited osteoclast differentiation. In addition, mTOR, a downstream molecule of AKT, has also been reported to be involved in the regulation of osteoclast survival (30). Administration of rapamycin, an inhibitor of mTOR, has been found to decrease the number of TRAP-positive multinucleated osteoclasts in the chondro-osseous junction in rats (31). In our study, AKT signaling of PBMCs treated with lung cancer-derived-CM or rhIL-8 was dramatically increased compared with PBMCs treated with M-CSF/RANKL alone. Such inductive effects of lung cancer-derived-CM or rhIL-8 on AKT are further confirmed by the increase of phosphorylation of its

Fig. 3. The effect of lung cancer-derived-CM and rhIL-8 on AKT and ERK1/2 signaling. (A) The activation of AKT and ERK1/2 signaling. Adherent PBMCs were cultured in the presence of RANKL (100 ng/ml) and M-CSF (200 ng/ml), with or without A549-CM, NCI-H460-CM (20%) and rhIL-8 (20 ng/ml) for the indicated times. The amount of proteins was assessed by immunoblot assay. (B) The activity of AKT and ERK1/2. The activity of AKT and ERK1/2 was determined by in vitro AKT and ERK1/2 kinase assay kit. (C) Depletion of IL-8 from A549-CM and NCI-H460-CM decreased the activation of AKT and ERK1/2. All experiments were performed independently at least three times.
downstream target, GSK-3. In addition, exposure to lung cancer-derived-CM or rhIL-8 also activated mTOR pathway, another downstream signaling pathway of PI3K/AKT. This was demonstrated as phosphorylation of mTOR and its downstream target p70S6K were both increased after treatment with lung cancer-derived-CM or rhIL-8. Furthermore, selective inhibition of AKT of lung cancer-derived-CM and rhIL-8 by chemical inhibitor decreased the effects on osteoclastogenesis, suggesting that PI3K/AKT pathway plays a crucial role in lung cancer cell derived-CM and rhIL-8-mediated osteoclastogenesis.

Recent studies have shown that PKC signaling pathways regulate various types of cell differentiation, including osteoclasts (20,32,33). Lee et al. reported that PKCβII, as well as PKCβIII, were increased during osteoclast formation in mouse bone marrow cell cultures in the presence of M-CSF and RANKL. And inhibition of PKCβII by antisense oligonucleotide resulted in a reduction of RANKL-induced

**Fig. 4.** The effect of lung cancer-derived-CM and rhIL-8 on the activation of PLD and PKC. (A) The activity of PLD. (B) The phosphorylation of PKCα/βII. (C) The activity of PKC. Depletion of IL-8 from A549-CM and NCI-H460-CM decreased the activation of PLD (D) and PKCα/βII (E). Adherent PBMCs were cultured in the presence of RANKL (100 ng/ml) and M-CSF (200 ng/ml), with or without A549-CM and NCI-H460-CM (20%), as well as rhIL-8 (20 ng/ml), for the indicated times. PLD and PKC activity was assessed by PLD and PKC activity kits, respectively. The amount of total and phosphorylated PKC was determined by immunoblot assay. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test ($P < 0.05$). All experiments were performed independently at least three times.
Fig. 5. The role of PLD on PKCs, AKT, mTOR and ERK1/2 activation. (A) The effect of PLD inhibitor on the activation of PKC, AKT, mTOR and ERK1/2. (B) The effect of PKC inhibitor on the phosphorylation of AKT, mTOR and ERK1/2. (C) The effect of PI3K inhibitor on the phosphorylation of AKT and mTOR. PBMCs were pretreated with various inhibitors [0.3% (vol/vol) butan-1-ol for PLD, bisindolylmaleimide I (Bis) for PKC, LY294002 (LY) for PI3K] for 1 h and then cultured in the presence of RANKL (100 ng/ml) and M-CSF (200 ng/ml), with or without A549-CM and NCI-H460-CM (20%), as well as rhIL-8 (20 ng/ml), for 30 min. The amount of total and phosphorylated proteins was determined by immunoblot assay. All experiments were performed independently at least three times. The intensity of the bands by the SigmaGel software.
Fig. 6. The role of PLD, AKT and ERK1/2 on lung cancer-mediated osteoclastogenesis. (A) The effect of PLD inhibitor on osteoclastogenesis. (B) The effect of AKT inhibitor on osteoclastogenesis. (C) The effect of ERK1/2 inhibitor on osteoclastogenesis. PBMCs were pretreated with various inhibitors [0.3% (vol/vol) butan-1-ol for PLD, 10-4-(N-diethylamino)butyl]-2-chlorophenoxyazine (AKT inhibitor) for AKT, PD98059 (20 μM) for ERK1/2] for 1 h and then cultured in the presence of RANKL (100 ng/ml) and M-CSF (200 ng/ml), with or without A549-CM and NCI-H460-CM (20%), as well as rhIL-8 (20 ng/ml) for indicated times. Cells were stained for TRAP activity kit. The asterisk indicates a significant difference between the control and test groups, as analyzed by Dunnett’s test (P < 0.05). All experiments were performed independently at least three times.
osteoclastogenesis (20). ERK1/2, a member of mitogen-activated protein kinase, is an important downstream molecule of PKCζ/II in RANKL-mediated osteoclastogenesis (19). Engagement of alphaVbeta3 integrin triggered ERK1/2 phosphorylation by a PKCζ-dependent pathway, resulting in increasing osteoclast bone resorption (20). In this study, treatment of PBMCs with A549-CM, NCI-H460-CM and rhIL-8 resulted in an increase of phosphorylated PKCζ/II and phosphorylated ERK1/2. Inhibition of PKC activity decreased ERK1/2 activation, suggesting that PKCζ/II is the upstream molecule of ERK1/2 in mediating osteoclastogenesis. Moreover, both PKC inhibitor and MEK1/2 inhibitor decrease osteoclastic differentiation in A549-CM, NCI-H460-CM and rhIL-8-treated PBMCs, suggesting that PKCζ/II/ERK1/2 pathway plays a crucial role in lung cancer-derived osteoclastogenesis.

IL-8 activates PLD and subsequently converts phosphatidylcholine to PA, stimulating polymorphonuclear leukocyte function and migration (14,15,34). PA has been shown to directly activate PKC, protein-tyrosine phosphatase, phosphoinositide-4 kinase and sphingosine kinase (35,36). Recent studies have shown that PLD signaling pathways regulate various types of cell differentiation, including myoblasts, granulocytes, keratinocytes and neurons (16,37,38). PKC-activating 12-O-tetradecanoylphorbol 13-acetate increases keratinocyte differentiation by activating PLD and ERK1/2 (39). From our study, treatment of PBMCs with A549-CM, NCI-H460-CM and rhIL-8 resulted in increased PLD activity and subsequently triggered the activation of PKCζ/II. And inhibition of PLD effectively blocked PKCζ/II activation. These results suggest that PLD acts as the upstream activator of PKCζ/II/ERK1/2 signaling in response to lung cancer-derived IL-8. Furthermore, PLD plays a crucial role in lung cancer-induced osteoclast differentiation.

PLD can be involved in AKT and mTOR activation at different levels in different mechanisms, including either direct interacting with PI3K or inhibiting the expression of tensin homologue deleted on chromosome 10 (PTEN) (36,40). In contrast, Chen et al. (41) have shown that PA directly mediates stimulation of mTOR in a PI3K/AKT independent manner. In our study, treatment of PBMCs with A549-CM, NCI-H460-CM and rhIL-8 resulted in increased mTOR activity and subsequently triggered the activation of PKCζ/II. And inhibition of PLD effectively blocked PKCζ/II activation. Taken together, our findings indicate that CM from lung cancer cell lines stimulate osteoclast differentiation. IL-8 in the CM cooperates to result in such enhanced effect on osteoclast differentiation. PLD/AKT and PLD/PKC/ERK1/2 were both found to be responsible for induction of osteoclastogenesis. In light of this finding, inhibition of IL-8 signaling is an attractive therapeutic target for osteolytic bone metastasis in advanced lung cancer patients.

Supplementary material
Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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