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Lung Tumor-Associated Dendritic Cell-Derived Amphiregulin Increased Cancer Progression

Ya-Ling Hsu,* Ming-Shyan Huang,‡† Da-En Cheng,* Jen-Yu Hung,*†‡ Chih-Jen Yang,*†‡ Shah-Hwa Chou,‡ and Po-Lin Kuo*†‡

The interaction of cancer within a microenvironment is an important factor determining cancer development. This study analyzed the soluble factors secreted by tumor-associated dendritic cells (TADCs), which are responsible for increasing lung cancer growth, migration, invasion, and epithelial-to-mesenchymal transition. Addition of amphiregulin, present in large amounts in TADC-conditioned medium (CM), mimicked the inductive effect of TADC-CM on lung cancer progression, supported by the enhancement of cell proliferation, migration, and invasion as well as osteolytic bone metastases phenotypes. In contrast, neutralization of amphiregulin from TADC-CM decreased the advanced malignancy-inductive properties of TADC-CM. Significant upregulation of amphiregulin has been seen in tumor-infiltrating CD11c⁺ DCs in human lung cancer samples and patients' sera. The enhancement of amphiregulin in TADCs has also been noted in mice transplanted with lung cancer cells. Induction of lung cancer progression by TADC-derived amphiregulin is associated with increased STAT3 and AKT activation, which subsequently increases the expression of cyclin D, Twist, and Snail. Blocking AKT significantly decreases TADC-CM and amphiregulin-mediated migration by decreasing the upregulation of Snail, whereas inhibition of STAT3 reduced the modulation of TADC-derived amphiregulin on Twist and cyclin D expression, suggesting that cooperation of STAT3 and AKT plays a critical role in TADC-mediated cancer progression. Moreover, mice treated with anti-amphiregulin Abs showed decreased incidence of cancer development and increased survival rates. Our study suggests that inhibition of amphiregulin or amphiregulin-related signaling is an attractive therapeutic target in lung cancer patients.


Lung cancer, one of the leading causes of cancer mortality in the world, is associated with very poor prognoses even after surgical resection (1). Cancer microenvironments have been demonstrated to play an important role in modulating tumor progression and chemoresistance to anticancer drugs. Cancer microenvironments consist of fibroblasts, immune cells (lymphocytes, dendritic cells [DCs], and macrophages), extracellular matrix components, growth factors, and metabolites. A growing body of studies indicates that cancer development is also regulated by an orchestrated series of bidirecional interactions between nonneoplastic cells and cancer cells in the tumor microenvironment (2, 3). The lungs are notably complex in terms of the different types of DCs to be found. DC subsets of the lung can be divided into CD11c⁺ high conventional DCs and CD11c⁺ low plasmacytoid DCs (4). Different DC subsets may carry out different functions. We have suggested that lung cancer has the ability to promote immune tolerance and escape the host’s immune surveillance by inhibiting the differentiation, maturation, and activation of CD11c⁺ high DCs (5). A CD11c⁺ high myeloid DC, which is present in lung cancer, displays a “semimature” and immunosuppression phenotype (6). In contrast, a variety of immune cells, such as macrophages and myeloid-derived suppressor cells, have been reported to contribute to tumor metastasis (7). However, it is still not fully understood whether tumor-associated DCs (TADCs) are involved in lung cancer development or precisely which molecules are involved in the cross-talk of cancer and TADCs in the tumor microenvironment. Strategies that harness the immune system to react against tumors can be integrated with existing forms of therapy for optimal responses in treating this devastating disease.

Amphiregulin, an epidermal growth factor family growth factor, binds and activates the epidermal growth factor receptor (EGFR). Amphiregulin is produced as a transmembrane precursor that is released from the cell membrane by a disintegrin and metalloproteinases (ADAM17) (8). Elevated levels of amphiregulin have been characterized in various types of tumors, and amphiregulin is assumed to play a role in promoting cancer progression (9, 10). Amphiregulin also elevates angiogenic activity, promoting tumor growth and metastasis in pancreatic, colorectal, liver, and lung cancers (9–12). A recent study has indicated that cancer-derived ATP increased the expression of amphiregulin in DCs in a mouse lung cancer cell line (13). Patients with high levels of amphiregulin have poorer prognoses and higher resistance than patients with low levels of amphiregulin (14). After activation of EGFR, amphiregulin signaling promotes activation of the PI3K, thereby promoting the activation of AKT, protein kinase C, calcium...
mobilization MAPK, or STAT3 signaling cascades (12, 14–16). AKT and STAT3 are known to be critical mediators in the control of cancer growth and progression. Therefore, we hypothesized that amphiregulin may play a role in mediating the regulatory effects of lung cancer development via the DCs–lung cancer cell interaction.

In this study, we discovered that TADCs secrete high amounts of amphiregulin and play a role in promoting cancer progression. Our data show that TADCs-derived amphiregulin increases A549 and H460 proliferation, migration, and epithelial-to-mesenchymal transition (EMT) by the paracrine effect. These findings also revealed that DCs infiltrating tumor tissues of mice and human patients express high levels of amphiregulin, suggesting a novel role of amphiregulin in the interaction of cancer and DCs. Furthermore, treatment of mice with anti-amphiregulin Abs decreased the incidence of lung tumors and increased survival rates. Investigating the mechanisms by which TADCs contributes to lung cancer progression may facilitate the discovery of a potential target for developing therapeutic strategies targeting immune cells in the tumor microenvironment.

Materials and Methods

Lung cancer cells and conditioned media

Human lung cancer cells A549 and H460 and mouse Lewis lung carcinoma (LLC) cells were obtained from the Bioresource Collection and Research Center (Hsinchu City, Taiwan). To obtain the A549 and H460-conditioned medium (CM), cells were seeded at 2 × 10^5 cells/100 mm dish and cultivated for 24 h. The medium was replaced, and the supernatants were harvested after 48 h of incubation.

Serum samples from lung cancer patients

Preoperative blood samples were obtained from 62 lung cancer patients and 19 healthy donors admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). Serum was separated by centrifugation and frozen at −80°C. Approval for these studies was obtained from the Institutional Review Board of Kaohsiung Medical University Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Measurement of secreted factors

Supernatants from TADC, monocyte-derived DCs (mdDCs), and A549 and H460 cells were collected. IL-6 was assessed by IL-6 ELISA kit. Amphiregulin and IL-8 levels were quantified using the DuoSet ELISA. Osteopontin, glycodelin, and matrix metalloproteinase (MMP) 9 levels were measured by the Bio-Plex assay (Bio-Rad, Hercules, CA). Gene expression of these cytokines and factors was measured by real-time PCR as described above.

Isolation of CD14^+ monocytes and differentiation of mdDCs

Monocytes were purified from PBMCs obtained from healthy consenting donors. Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, U.K.). CD14^+ monocytes were enriched using CD14+ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec). CD14+ monocytes were purified from PBMCs obtained from healthy consenting donors. Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, U.K.). CD14+ monocytes were enriched using CD14+ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec). Mouse bone marrow cells were harvested from the long bones of mice using wound-healing, QCM 24-well Cell Migration Assay and Invasion Analysis of cell proliferation, migration, and invasion

Analysis of cell proliferation, migration, and invasion

Cell proliferation was assessed by Premixed WST-1 Cell Proliferation Reagent (BD Clontech, Mountain View, CA), according to the manufacturer’s instructions. Cell migration and invasion assays were conducted using wound-healing, QCM 24-well Cell Migration Assay and Invasion System, as described previously (17). mdDC-CM and TADC-CM were added to the bottom wells for 24 h as a chemoattractant, and the fluorescence of the invading cells was read using a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm. The migration of A549 and H460 cells was also assessed by a Scratch wound-healing assay.

Quantitative real-time PCR

RNA isolation was performed using the Trizol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR mixture contained 200 nM of each primer, 10 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), and 5 μl cDNA and nuclease-free water, with a total volume of 20 μl. The PCR was carried out with a denaturation step at 95°C for 10 min and then for 40 cycles at 95°C for 15 s and at 60°C for 1 min. All PCRs were performed in triplicate and normalized to internal control GAPDH mRNA. Relative expression was presented using the 2^-ΔΔCT method.

Immunoblot

Cells were lysed on ice for 15 min by M-PEL lysis reagent (Pierce). Cell lysis was centrifuged at 14,000 × g for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by SDS-PAGE (5–12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% nonfat dry milk in TBS, the membrane was incubated with the desired primary Ab for 1–16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary Ab, and the immunoreactive proteins were detected using an ECL kit (Millipore), according to the manufacturer’s instructions.

Immunofluorescence

Noncancerous lung tissue specimens obtained from human lung cancer patients were embedded in OCT and frozen in liquid nitrogen. Sections (3–5 μm) were fixed with acetone at −20°C and then stained with amphiregulin Ab (Abcam) or costained by anti-CD11c Ab. After a wash with PBS containing 0.1% Tween 20, slides were incubated with Dylight 488- or Dylight 549-conjugated secondary Abs (Rockland, Gilbertsville, PA), with or without DAPI, for 1 h at room temperature. The data were analyzed with a confocal laser scanning microscope (Fluoview FV500, Olympus, Tokyo, Japan).

Osteoclast differentiation and activity assay

PBMCs were plated and incubated overnight at 37°C. Nonadherent cells were removed by washing with PBS, and the remaining adherent cells were grown in culture medium containing mdDC-CM, A549-TADC-CM, H460-TADC-CM, or amphiregulin (10 ng/ml) presenting in 200 ng/ml human M-CSF and 100 ng/ml human RANKL for 7 d. The medium was replaced every 3 d. Osteoclast formation was measured by quantifying cells positively stained by TRAP (Sigma-Aldrich, St. Louis, MO); osteoclasts were determined to be TRAP-positive staining multinuclear (more than three nuclei) cells by means of light microscopy. The TRAP-positive cells, and the number of nuclei per TRAP-positive cell in each well, were counted. Osteoclasts’ bone resorption activity was assessed by BD BioCoat Osteologic Bone Resorption assay (BD Biosciences, Bedford, MA), in the same culture conditions as described above.

Gene knockdown by small interfering RNA

Monocytes were transfected with 1 μmol/l nontarget or amphiregulin small interfering RNA (siRNAs) pool (Dharmacon) according to manufacturer delivery media (B-005000), according to the manufacturer’s instructions. Positive controls Accell GAPDH siRNA and scrambled Accell siRNA pool were used in the experiments. After 72 h transfection, the medium was changed to RPMI 1640 medium containing 10% FBS, 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 5 d. The medium was then replaced every 3 d. Osteoclast formation was measured by counting cells positively stained by TRAP (Sigma-Aldrich, St. Louis, MO); osteoclasts were determined to be TRAP-positive staining multinuclear (more than three nuclei) cells by means of light microscopy. Real-time PCR was performed with a denaturation step at 95°C for 10 min and then for 40 cycles at 95°C for 15 s and at 60°C for 1 min. All PCRs were performed in triplicate and normalized to internal control GAPDH mRNA. Relative expression was presented using the 2^-ΔΔCT method.

Animal models and isolation of CD11c^+ cells from lungs

LLC cells were transfected into C57BL/6 mice by tail vein injection. Lung tissue was collected 24 d after injection, then minced and incubated in RPMI 1640 medium with collagenase type 1 (400 U/ml) (Worthington Biochemicals) for 1 h at 37°C. The digested tissues were filtered through a 70-μm cell strainer and washed with RPMI 1640 medium. CD11c^+ DCs were isolated from the cell suspension by CD11c magnetic beads (Miltenyi Biotec). Mouse bone marrow cells were harvested from the long bones of mice using a procedure similar to that described above.
the limbs and then placed in RPMI 1640 containing murine GM-CSF (20 ng/ml) and murine IL-4 (20 ng/ml, R&D Systems), with or without LLC-CM, for 24 h. The expression of various mRNAs was assayed by real-time PCR. Survival rates were evaluated in mice injected with LLC cells. Iso-type control Ig (IgG) and amphiregulin (50 μg/mice) Ab were i.p. injected every 7 d three times after administration of LLC. Animals were sacrificed on day 24 after LLC cell transplantation and the number of tumor nodules recorded for the analysis of lung cancer incidence. For survival assay, all animals were closely monitored daily for signs of distress and were sacrificed when they appeared lethargic or to have increased breathing rates.

Statistical analysis
Data were expressed as means ± SD. Statistical comparisons of the results were made using ANOVA. Significant differences (p < 0.05) between the means of the two test groups were analyzed by Student t test. Survival studies were assessed using Kaplan–Meier survival curves and analyzed with the Mantel–Cox log-rank test.

Results
TADCs increased lung cancer cell proliferation, migration and EMT
To investigate the influence of TADCs on lung cancer progression, we assessed the effect of TADC-CM on lung cancer cell proliferation, migration, and invasion. As shown in Fig. 1A, A549-TADC-CM (20%) and H460-TADC-CM (20%) increased the proliferation of A549 and H460 cells after 72 h of treatment. In addition, both A549-TADC-CM and H460-TADC-CM increased the migration and invasive abilities of A549 and H460 cells (Fig. 1B, 1C). Furthermore, A549-TADC-CM and H460-TADC-CM also caused A549 and H460 cells to undergo EMT, including the downregulation of epithelial markers (E-cadherin, claudin-3, and ZO-1), and upregulation of fibroblast markers (N-cadherin, vimentin, and fibronectin) (Fig. 1D).

The effects of TADC on lung cancer migration and invasion were also confirmed by coculture system. As shown in Supplemental Fig. 1B and 1C, coculture of A549 or H460 cells with A549-TADCs or H460-TADCs enhances the migratory and invasive capability of A549 and H460 cells, respectively. These coculture system data coincide with that of TADC-CM treatment.

Amphiregulin plays an important role in TADCs-mediated cancer progression
To investigate which factor is responsible for TADCs-mediated lung cancer development, we used a microarray to assess the gene profile of A549-TADCs. The data showed that several soluble factors were upregulated in A549-TADCs when compared with mdDCs. Among these upregulated genes, levels of amphiregulin, a lung cancer-related growth factor, increased 11.73-fold in A549-TADCs (Fig. 2A). In addition, ADAM17, an enzyme responsible for amphiregulin release, was also increased in A549-TADCs. Quantitative real-time PCR (Q-PCR) analysis further revealed that A549-CM or H460-CM increased the expression of amphiregulin in A549-TADCs and H460-TADCs (Fig. 2A). Furthermore, protein levels of amphiregulin were enhanced in A549-TADCs and H460-TADCs, as determined by ELISA analysis (Supplemental Fig. 1D).

We also assessed the effect of amphiregulin protein on the proliferation, migration, invasion, and EMT of A549 and H460 cells. Amphiregulin increased the proliferation of A549 and H460 cells in a concentration-dependent manner (Supplemental Fig. 1E). Moreover, amphiregulin not only increased the migratory ability of A549 and H460 (Fig. 2B) but also enhanced their invasive ability and EMT (Fig. 2C, 2D).

To better understand the role of amphiregulin, we blocked the effect of amphiregulin on A549 and H460 proliferation and migration by using specific neutralization Ab and siRNA transfection. As shown in Fig. 3A, the effect of A549-TADC-CM and H460-TADC-CM on cell proliferation in A549 and H460 cells was neutralized by treatment with anti-amphiregulin Ab but not by isotype control. Similarly, amphiregulin neutralization Ab also decreased the stimulatory effects of A549-TADC-CM and H460-TADC-CM on lung cancer cell migration and invasion (Fig. 3B, 3C). The inhibitory effect of amphiregulin neutralization Ab on A549 migration and invasion was also confirmed by A549, H460, or mdDC coculture system (Supplemental Fig. 2A, 2B). Furthermore, inhibition of amphiregulin in monocytes by amphiregulin siRNA also reduced the effect of TADC-CM on proliferation, cell migration, and invasive ability of A549 and H460 cells (Fig. 4B–D). These data suggest that tumor-infiltrated DCs may increase lung cancer progression by amphiregulin production.

TADC-derived amphiregulin stimulated lung cancer to produce bone-metastasis factor (IL-6, IL-8 and parathyroid hormone-related protein) and osteoclastogenesis
Bone is a frequent target of lung cancer metastasis, and bone metastasis is mediated by complex interactions between tumor cells and resident stromal cells in the cancer microenvironment (18). We therefore assessed the potential role of TADC-derived amphiregulin in lung cancer metastasis. As shown in Fig. 5A and 5B, A549-TADC-CM and H460-TADC-CM increased A549 or H460 cells’ expression of two inflammatory cytokines, IL-6 and IL-8, which have been implicated in bone metastasis (19, 20). Similar to TADC-CM, amphiregulin also increases the expression of IL-6 and IL-8 in both A549 and H460 cells. PTHrP, a causative factor that contributes to osteolytic metastases, is also regulated in both A549-TADC-CM or amphiregulin-treated A549 and H460-TADC-CM or amphiregulin-treated H460 cells (Fig. 5C). Amphiregulin also increases the formation and activity of osteoclasts from PBMCs presenting in M-CSF and RANKL (Fig. 5D). In addition, knockdown of amphiregulin in monocytes by siRNA also decreases the upregulation of TADC-CMs on PTHrP expression in both A549 and H460 cells (Fig. 5E).

STAT3 and AKT activation are involved in the amphiregulin-mediated effect on lung cancer
Because TADC-derived amphiregulin increased cell proliferation, migration, and invasion, we assessed the effect of TADC-CMs and amphiregulin on several oncogenic signaling pathways, including AKT and STAT3 (15, 16). The results showed that exposure of A549 and H460 cells to A549-TADC-CM or H460-TADC-CM led to the phosphorylation of STAT3 and AKT. In contrast, the expression of STAT3 and AKT (unphosphorylated form) was unaltered by TADC-CM treatment in either A549 or H460 cells (Fig. 6A). Similar results were also found in amphiregulin-treated A549 and H460 cells (Fig. 7A). In addition, both TADC-CMs and amphiregulin increased the expression of Snail and Twist, two EMT-related transcriptional factors, and enhanced the levels of cyclin D in both A549 and H460 cells (Figs. 6A, 7A).

To investigate the functional impact of AKT and STAT3 activation on TADC-derived amphiregulin in lung cancer, we used chemical inhibitors Ly294002 and AG490 to specifically inhibit AKT and STAT3 signaling, respectively. STAT3 inhibitor AG490 decreased TADC-CM and amphiregulin-mediated cell proliferation, migration, and invasion (Figs. 6B–D, 7B–D). In contrast, AKT inhibitor Ly294002 markedly inhibited two types of TADC-CMs and amphiregulin-induced cell migration in both A549 and H460 cell lines, whereas it only slightly decreased TADC-CM and amphiregulin-driven cell proliferation. Furthermore, pretreatment of A549 and H460 cells with STAT3 inhibitor completely
abrogated expression of Twist and cyclin D but only partially decreased Snail caused by A549-TADC-CM, H460-TADC-CM, and amphiregulin (Figs. 6E, 7E). In contrast, AKT inhibitor completely inhibited Snail expression, but not Twist and cyclin D. Moreover, both STAT3 and AKT inhibitors reversed the inhibitory effect of TADC-CMs on E-cadherin in A549 and H460 cells. These data suggest that STAT3 and AKT play important roles in lung cancer progression (Figs. 6E, 7E).

To confirm the role of amphiregulin on TADC-mediated cancer progression in humans, we characterized the expression of amphiregulin in tumor-infiltrated CD11c⁺ DCs. Immunofluorescence staining of the marginal regions of a patient’s lung cancer reveals the presence of many CD11c⁺ DCs, which have infiltrated...
the area around the tumor. These areas express higher levels of amphiregulin in comparison with tissue sections of non-tumor regions (Fig. 8A). Furthermore, a marked statistical difference has also been found between lung cancer patients and healthy donor serum (Fig. 8B).

We also used animal experiments to determine whether lung cancer increased amphiregulin expression in DCs in vivo. We injected mouse lung cancer cell line LLC into mice and then allowed the cells to develop for 14 d. LLC-CM increased the production of amphiregulin in mice bone marrow cells presenting in IL-4 and GM-SCF (Supplemental Fig. 3A). These data coincide with human lung cancer data. Q-PCR and ELISA analysis have also shown that lung tumor-infiltrated CD11c+ DCs produce elevated levels of amphiregulin in mRNA and protein levels (Fig. 8C, Supplemental Fig. 3B).

Amphiregulin Ab decreased cancer metastasis in vivo
Finally, we assessed whether inhibiting amphiregulin production from tumor-infiltrated DCs could decrease lung cancer development. Treatment of mice by anti-amphiregulin mAb inhibited the growth of LLC in 25% (two of eight) of mice, in comparison with 85% (six of seven) of rat IgG-treated control mice (Fig. 8D). Furthermore, treatment by anti-amphiregulin mAb also improved long-term survival of mice (66.6%) with LLC tumors (up to 90 d), whereas all mice treated by control IgG died of cancer within 25 d (Fig. 8E).

Discussion
The microenvironment of a tumor is widely known to be an important factor regulating cancer development. Cells surrounding
between the two test groups, as analyzed by Student dependent experiments. The asterisk indicates a significant difference identified as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).

Amphiregulin possesses mitogenic potential for cells and plays a role in the development of several organs and tissues, such as lungs, kidneys, mammary duct formation, nerve generation, bones, and prostate (22–25). Amphiregulin is upregulated in various cancers and associated with cancer progression, such as cancer cell growth, proliferation, migration or invasiveness after the activation of EGFR (9, 10, 25). In addition, amphiregulin is not only involved in cancer growth but also is responsible for resistance to conventional chemotherapeutic drugs, such as cisplatin, and to hormonal antitumor aromatase inhibitors and EGFR-tyrosine kinase inhibitors (12, 26, 27). Amphiregulin is suggested to be a biomarker for assessing tumors and in predicting patient prognosis and therapeutic strategies (14). However, our results have shown that the source of amphiregulin production is not only cancer cells but that it is also secreted from tumor-surrounding dendritic cells. TADCs produce high levels of amphiregulin, which increase lung cancer proliferation, migration, and invasion. Inhibition of amphiregulin by siRNA or neutralization Abs decreases TADCs’ effects on cancer progression. Furthermore, TADC-derived amphiregulin also stimulates cancer cells to produce IL-6, IL-8 and PTHrP, and TADC-CM, and amphiregulin increases osteoclast differentiation and bone resorption activity, which suggests TADC-derived amphiregulin is strongly associated with the development of osteolytic bone metastasis. Investigation of the paradoxical roles of DCs in lung cancer development and the malignancy of tumors is a very important insight into the tumor microenvironment.

It is of interest to note that amphiregulin-producing CD11c+ DCs were found to infiltrate the cancerous lung tissue of LLC-bearing mice. In addition, CD11c+ DCs located in human lung cancer tissues also produce high amounts of amphiregulin when compared with CD11c+ DCs in nontumorous regions. Amphiregulin levels in lung cancer patients’ serum are also higher than those of healthy donors. Furthermore, antagonism of amphiregulin by injection of specific Abs decreases the recurrence of LLC and increases the survival rates of LLC-bearing mice. These results from experimental cell studies, mouse models, and clinical patient sections strongly suggest that amphiregulin is an important effector molecule through which TADCs enhance the development of lung cancer.

STAT3 signaling has been demonstrated to participate in various cellular processes, including the inflammatory response, cell proliferation, differentiation, survival, and motility (28). STAT3 is activated by multiple receptor and nonreceptor tyrosine kinases in response to various cytokines, hormones, and growth factors, and abnormal activation of STAT3 signaling has been seen in multiple tumors (28, 29). STAT3 has been reported to be involved in epidermal growth factor-stimulated cancer proliferation and migration by increasing cyclin D and Twist expression, respectively (16, 30). Twist is the basic helix-loop-helix transcription factor essential for regulating EMT of cancer by decreasing E-cadherin expression and is upregulated in a number of human tumors, including lung cancer (31). In this study, we have shown that treatment of A549 and H460 with TADC-CM and amphiregulin resulted in increased STAT3 phosphorylation and Twist expression. Inhibition of STAT3 activity by specific inhibitor decreases

(figures and tables)

FIGURE 3. Neutralizing amphiregulin by its specific Ab decreased TADC-CM-mediated cancer progression. Amphiregulin Ab decreased the effect of TADC-CM on cell proliferation (A), migration (B), and invasion (C). A549 and H460 cells were treated with mdDC-CMs or TADC-CMs in the upper insert and were treated with or without amphiregulin Ab (2 μg/ml) or IgG (2 μg/ml), and TADC-CMs (20%) was added into the lower well to act as an attractive agent for 24 h. The migratory and invasive cells were quantified as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).
cyclin D and Twist expression, suggesting that STAT3 is an upstream regulator of cyclin D and Twist. Moreover, STAT3 inhibitor decreases the effects of TADC-CM and amphiregulin on bone metastasis factor PThrP production and cell migration in A549 and H460 cells, suggesting that the activation of STAT3 signaling plays a crucial role in the development of lung cancer growth and metastasis.

AKT, a downstream effector of PI3K has been shown to be a critical mediator of cell proliferation, survival, and metastasis in a variety of cell types. AKT has been reported to act as an important factor in amphiregulin-mediated cell survival and migration (32, 33). In our study, we found that treating A549 and H460 cells with TADC-CM or amphiregulin dramatically increases the activation of AKT. The inductive effect of TADC-
FIGURE 5. TADC-derived amphiregulin increased bone metastasis-related factors in lung cancer. A, TADCd and amphiregulin increased the production of IL-6 (A), IL-8 (B), and PTHrP (C) in A549 and H460 cells. A549 and H460 cells were treated with A549-TADC-CM (20%) or H460-TADC-CM (20%) and amphiregulin (10 ng/ml) for 24 h, and then, the supernatants were collected and assessed by ELISA kits. A549 and H460 cells were treated with A549-TADC-CM (20%) or H460-TADC-CM (20%) and amphiregulin (10 ng/ml) for 24 h, and then, the supernatants were collected and assessed by ELISA kits. D, Amphiregulin increased osteoclast differentiation and activity. PBMCs were treated with amphiregulin (10 ng/ml) presenting in RANKL (100 ng/ml) and M-CSF (200 ng/ml) for 7 d. Osteoclast cells were stained for TRAP activity, and bone resorption activity was determined by the Osteologic MultiTest Slide. E, Inhibition of amphiregulin decreased the enhancement of TADCs on PTHrP expression. A549 and H460 cells were treated with various amphiregulin siRNA or scrambled siRNA-transfected A549 or H460-associated DC-CMs (20%) for 24 h, and then, the cells’ supernatants were collected and assessed by PTHrP ELISA kits. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference with control, as analyzed by Student t test (*p < 0.05).
FIGURE 6. TADC-CM promote cancer progression by STAT3 and AKT activation. A, TADC-CM increased STAT3 and AKT activation and enhanced Snail and Twist expression. Cells were treated with TADC-CM (20%) for the indicated times, and the expression of various proteins was assessed by immunoblot assay. STAT3 and AKT inhibitors decreased TADC-mediated cell proliferation (B), migration (C), invasion (D), and EMT (E). Cells were pretreated with or without AKT (5 μM) or STAT3 (1 μM) inhibitor and then cultured with TADC-CM (20%) for the indicated times (72 h for cell proliferation, 24 h for E-cadherin, and 6 h for Snail, Twist, and cyclin D). Cell proliferation was assessed by WST-1 assay. The invasiveness and migration ability of A549 and H460 cells were quantified by QCM 24-well Cell Migration and Invasion assay. Cells were seeded in the upper inserts were treated with or without AKT and STAT3 inhibitors, with amphiregulin acting as a chemoattractant for cancer migration and invasion. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).
CM or amphiregulin on AKT is responsible for the increase of Snail expression, a repressor of epithelial type marker E-cadherin. Furthermore, selective inhibition of AKT by chemical inhibitor also decreases the effects of TADC-CM or amphiregulin on cell migration and invasion, suggesting that phenotypic transition of lung cancer by TADC involves amphiregulin-mediated

FIGURE 7. Amphiregulin promotes cancer progression by STAT3 and AKT activation. A, Amphiregulin increased STAT3 and AKT activation and enhanced Snail and Twist expression. Cells were treated with amphiregulin (10 ng/ml) for the indicated times, and the expression of various proteins was assessed by immunoblot assay. STAT3 and AKT inhibitors decreased amphiregulin-mediated cell proliferation (B), migration (C), invasion (D), and EMT (E). Cells were pretreated with or without AKT or STAT3 inhibitor and then cultured with amphiregulin (10 ng/ml) for the indicated times (72 h for cell proliferation, 24 h for E-cadherin, and 6 h for Snail, Twist, and cyclin D). Cell proliferation, invasiveness, and migration ability of A549 and H460 cells were quantified by as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).
FIGURE 8. Elevated amounts of amphiregulin on CD11c⁺ DCs in the lung cancer-bearing patient and mice. A. Amphiregulin⁺CD11c⁺ DCs infiltrated cancer sections. Nontumor and tumor regions (n = 15) were cut and stained and then samples were analyzed by confocal microscopy (original magnification ×40). Arrows indicate the CD11c⁺/amphiregulin⁺ cells. B. High levels of amphiregulin were found in the serum of lung cancer patients. Amphiregulin levels of lung cancer patients (n = 62) and healthy donors' sera (n = 19) were assessed by ELISA. C. Elevated levels of amphiregulin were found in CD11c⁺ DCs isolated from the lungs of lung-cancer bearing mice. Culture media and LLC were injected into mice via the tail vein (control group, n = 10; LLC group, n = 8). After 24 d, nontumorous and tumorous regions of the lungs were harvested. CD11c⁺ DCs were isolated from fresh lung or tumor tissue, and amphiregulin levels were assessed by Q-PCR. Neutralization of amphiregulin decreased the occurrence of lung cancer (D) and improved survival rates (E) in mice. LLCs were injected into mice via the tail vein. Mice were dosed every 7 d with i.p. injections IgG (n = 7) or amphiregulin Abs (50 µg/ml) (n = 8). Arrows indicate the time of administration of amphiregulin Ab. The asterisk indicates a significant difference with the control, as analyzed by ANOVA with Student t test post hoc (*p < 0.05).
activation of AKT and subsequent AKT-activated Snail gene expression. Taken together, our findings suggest that a new mechanism of TADCs promotes lung cancer progression, in which TADC-derived amphiregulin causes phenotypic transition and reinforces cancer progression. Amphiregulin enhances the cooperation of STAT3 and AKT signaling and subsequently increases the expression of Twist and Snail, resulting in the promotion of lung cancer development. Neutralization of amphiregulin by amphiregulin Ab significantly decreases the incidence of cancer development in mice. Our findings provide important insights into the biology of tumors with vicious interactions of DCs and cancer as well as establish new rationales for using anti-amphiregulin strategies to target aggressive invasive lung cancer.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure Legends

**Figure 1.** TADCs increased migratory and invasive ability in A549 and H460 cells in a co-culture system. (A) Flow chart of production of A549-TADC, H460-TADC, A549-TADC-CM and H460-TADC-CM. TADCs increased migratory (B) and invasive (C) ability in A549 and H460 cells in a co-culture system. Two lung cancer cells (2×10^4) were seeded into the upper insert and TADCs (5×10^5) was cultured in the bottom plate. The two cell lines were co-cultured for 24 h. The invasiveness and migration ability of A549 and H460 cells were quantified by QCM™ 24-well Cell Migration and Invasion assay. (D) The expression of amphiregulin in A549-TADCs and H460-TADCs. The supernatants of mdDCs, A549-TADCs and H460-TADCs were collected and the levels of amphiregulin protein determined by ELISA kit. (E) Amphiregulin increased the cell proliferation in A549 and H460 cells. A549 and H460 cells were treated with various concentrations of amphiregulin for 72 h. Cell proliferation was assessed by WST-1 assay. Data are representative of three independent experiments. The asterisk indicates a significant difference with the control, as analyzed by ANOVA with Student's t test post hoc (*, P < 0.05).

**Figure 2.** Blockade of amphiregulin by antibody decreased TADC-mediated cell migration (A) and invasion (B) in a co-culture system. Two lung cancer cells (2×10^4) were seeded into the upper insert presenting in IgG or amphiregulin antibodies (2
μg/ml), and TADC \((5 \times 10^5)\) was cultured in the bottom plate. The two cells were co-cultured for 24 h. The invasiveness and migration ability of A549 and H460 cells were quantified by QCM™ 24-well Cell Migration and Invasion assay. Data are representative of three independent experiments. The asterisk indicates a significant difference between two test groups, as analyzed by ANOVA with Student's \(t\) test post hoc (*, \(P < 0.05\)).

**Figure 3.** Elevated amounts of amphiregulin on CD11c\(^+\) DCs in mice. (A) LLC-CM increased the expression of amphiregulin in mice bone marrow cells. Mouse bone marrow cells were treated with RPMI 1640, LLC-CM (20%) and mouse amphiregulin (10 ng/ml) presenting mouse GM-CSF (20 ng/ml) and mouse IL-4 (20 ng/ml) for 1 day. Culture media were collected and the levels of amphiregulin were assessed by ELISA kit. (B) Elevated levels of amphiregulin found in CD11c\(^+\) DCs isolated from the lungs of lung-cancer bearing mice. Culture media (control group) and LLC were injected into mice via the tail vein. After 24 days, nontumorous and tumorous regions of the lungs were harvested. CD11c\(^+\) DCs were isolated from fresh lung or tumor tissue, and the culture medium collected after 24 h incubation. Amphiregulin levels in the culture medium were assessed by ELISA. The asterisk indicates a significant difference with the control, as analyzed by ANOVA with Student's \(t\) test post hoc (*, \(P < 0.05\)).
sFigure 1
Figure 2

(A) Cell migration (OD at 520 nm)

(B) Cell invasion (OD at 520 nm)
Figure 3