Effect of malignant-associated pleural effusion on endothelial viability, motility and angiogenesis in lung cancer

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Abstract
Malignant pleural effusion (MPE) and paramalignant pleural effusion (PPE) remain debilitating complications in lung cancer patients with poor prognosis and limited treatment options. The role of vascular endothelial cells has not been explored in the pleural environment of lung cancer. By integrating MPE and PPE as malignant-associated pleural fluid (MAPF), the current study aimed to evaluate the effect of MAPF on cell proliferation, migration and angiogenesis of HUVEC. First, increased capillaries were identified in the subpleural layer of lung adenocarcinoma. Compatible with pathological observations, the ubiquitous elevation of HUVEC survival was identified in MAPF culture regardless of the underlying cancer type, the driver gene mutation, prior treatments and evidence of malignant cells in pleural fluid. Moreover, MAPF enhanced HUVEC motility with the formation of lamellipodia and filopodia and focal adhesion complex. Tube formation assay revealed angiogenic behavior with the observation of sheet-like structures. HUVEC cultured with MAPF resulted in a significant increase in MAPK phosphorylation. Accompanied with VEGFR2 upregulation in MAPF culture, there was increased expressions of p-STAT3, HIF-1α and Nf-kB. VEGF/VEGFR2 blockade regressed endothelial migration and angiogenesis but not cell proliferation. Our data indicate the angiogenic activities of MAPF on vascular endothelial cells that revealed increased pleural capillaries in lung cancer. Targeting the VEGF/VEGFR2 pathway might modulate the angiogenic propensity of MAPF in future clinical investigations.

KEYWORDS
angiogenesis, lung cancer, malignant pleural effusion, vascular endothelium, VEGFR2

Abbreviations: ZO-1, zonula occludens-1.
Changchien and Chen contributed equally to this research.

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1 | INTRODUCTION

Lung cancer is one of the leading causes of malignant pleural effusion (MPE). The diagnosis of MPE depends on the presence of malignant cells in pleural fluid cytology or pleural biopsy. In contrast, paramalignant pleural effusion (PPE) is described as an indirect effect of a tumor on the pleural space and is the absence of cancer cells in pleural examinations. Overall, more than 10% of lung cancer patients will develop a large amount of pleural fluid. Massive pleural effusion impairs patients’ daily activity because the volume of fluid affects vital capacity and causes symptoms such as dyspnea, coughing and chest pain. Unfortunately, current options for the management of pleural effusion are limited and mainly palliative, such as thoracentesis, pleural catheter placement and chemical pleurodesis. Since 2010, the presence of MPE has been reclassified as stage IV disease, characterized by frequent distal metastasis and poor prognosis. Characterization of the pleural microenvironment might identify druggable targets to alleviate massive pleural effusion in lung cancer patients.

Histologically, the pleural tissues consist of a monolayer of mesothelial cells and an underlying layer of loose connective tissues. Within the loose connective tissues, there are fibroblasts, adipocytes, and vascular and lymphatic endothelial cells. Previous studies of the pleural microenvironment have observed the translocation of lung carcinomas into the pleural space through pulmonary vessels. In addition, the seeding of pleural tumor cells is mediated by the angiogenic effects of pleural effusion. Host cells serve as partners that co-orchestrate the inflammatory response and vascular permeability in the pleural space. MPE-endothelial cell interactions and cancerous invasion of the pleura may trigger angiogenic effects and related signaling, although these processes are poorly investigated.

Within the tumor microenvironment, there are many aberrant blood vessels stimulated by cancer angiogenesis. Endothelial cells within tumors possess increased capacities for migration and angiogenesis. Compared with those of the tumor microenvironment, the characteristics of endothelial cells within the pleural space in lung cancer remain unclear. Accordingly, the current study aimed to evaluate the impact of the pleural microenvironment on vascular endothelial cells. The term malignancy-associated pleural fluid (MAPF) was adopted to encompass samples of MPE and PPE from lung cancer patients who underwent thoracocentesis. By applying HUVEC, our data provided insight into their proliferation, migration and angiogenesis in MAPF and investigated signaling changes that could serve as future therapeutic targets.

2 | MATERIAL AND METHODS

2.1 | Patient clinical information and procurement of malignancy-associated pleural fluid samples

Pleural fluid samples were collected from lung cancer patients receiving chest ultrasonography-guided thoracentesis, with informed consent for the use of the specimens in research. This study was
approved by the institutional review board (IRB) of the Tri-Service General Hospital (TSGH) Research Ethics Committee. Patient clinical information, including demographics, staging, treatment regimens, driver gene status and cytological examinations of malignant cells, was extracted from medical records. From each patient, a total of 5 mL of pleural fluid samples were collected and then centrifuged twice at 1000 g for 10 min. The supernatant fluid was filtered through a Millipore filter (0.22 µm) and stored at −80°C until use.

2.2 | Cell culture

The HUVEC cell line was purchased from the Bioresource Collection and Research Center (BCRC) and cultured in endothelial cell medium (ECM) (ScienCell Research Laboratories).

2.3 | Drugs and reagents

Sunitinib (Sun) and 2,3,3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were bought from Sigma-Aldrich. Bevacizumab was obtained from Roche.

2.4 | Migration and transwell assays

The migration ability of HUVEC was assayed by wound healing and transwell assays. The wound area was prepared by seeding HUVEC in a 3.5-cm culture dish to form a monolayer. After being scratched with a P200 pipette tip and photographed, the cells were cultured with 30% MAPF (V/V) for 6 hours. The wound area was analyzed with ImageJ. For the transwell migration assay, 2 × 10^4 HUVEC were seeded into the upper chamber of a Transwell plate (Corning Costar). After incubation at 37°C for 16 hours, the cells on the lower side of the insert were fixed with 10% formalin in PBS and stained with Coomassie Brilliant Blue G250 (Sigma). The migrated cells were examined in three randomly selected fields from each membrane in five independent experiments. The transwell invasion assay was prepared by seeding 4 × 10^4 HUVEC in the upper chamber of a Transwell plate. Before cell seeding, 0.5% Matrigel in a coating buffer solution (BD) was added to the upper chamber and then incubated for 16 hours at 37°C. The cells on the lower side of the insert were fixed and stained. The invaded cells were counted in three randomly selected fields from each membrane in five independent experiments.

2.5 | Tube formation assay

Matrigel (50 mL/well) was added to a prechilled 96-well plate and incubated for 1 hour at 37°C. HUVEC (1 × 10^4) were seeded into each well with 30% MAPF (V/V). After 6 hours of incubation, tube formation was imaged. Then, the cellular networks of angiogenesis were quantified and analyzed with ImageJ.

2.6 | Immunocytochemistry

After incubation with MAPF, HUVEC on coverslips were rinsed with PBS and then fixed with 10% formalin in PBS (pH 7.4). A blocking solution (5% milk in 0.1% Triton X-100) was used to block nonspecific binding sites. Then, a primary antibody against p-paxillin (BD Bioscience) in blocking buffer was incubated with the HUVEC at 4°C overnight. The next day, F-actin and FITC-conjugated goat anti–mouse IgG (both from Sigma-Aldrich) were applied for 1 hour. DAPI was used for counterstaining. Finally, the cells were mounted with mounting medium (Gel Mount Aqueous, Sigma) and recorded with a Nikon D1X digital camera (Carl Zeiss, Pberkochei).

2.7 | Western blotting

HUVEC were rinsed once with PBS and lysed with buffer containing 0.15% Triton X-100, 10 mM EGTA, 2 mM MgCl₂, 60 mM PIPES, 25 mM HEPES, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM

### TABLE 1  Clinical characteristics of lung cancer patients

|                      | Sex, n (%) | Age, y, n (%) | ECOG performance status, n (%) | Smoking history, n (%) | Disease stage at diagnosis (AJCC 8th), n (%) | Histological classification, n (%) | Presence of PE, n (%) | Microscopic malignant cell in PE, n (%) | Number of prior regimens, n (%) |
|----------------------|------------|--------------|-------------------------------|------------------------|---------------------------------------------|-----------------------------------|---------------------|----------------------------------------|-------------------------------|
|                      | Female     | ≥65          | 0-2                           | Never                  | IV                                          | Adenocarcinoma                   | At diagnosis         | Positive                               | 0                             |
|                      | 10 (50%)   | 11 (55%)     | 13 (65%)                      | 14 (70%)               | 19 (95%)                                    | 18 (90%)                         | 8 (40%)             | 13 (65%)                               |                              |
|                      |            |              | ≥3                            |                        | 7 (35%)                                      | 1 (5%)                           | 12 (60%)            |                                        | 2 (10%)                       |
|                      |            |              |                               |                        |                                             | Small-cell carcinoma             |                     |                                        | 11 (55%)                      |
|                      |            |              |                               |                        |                                             |                                   |                     |                                        | ≥3                            |
|                      |            |              |                               |                        |                                             |                                   |                     |                                        |                               |
| Abbreviation: PE, pleural effusion.
β-glycerophosphate, 1 μg/mL leupeptin, 1 μg/mL pepstatin A and 1 μg/mL aprotinin (pH 6.9). Forty micrograms of each sample was loaded into the wells of a 10% SDS polyacrylamide gel. The protein samples were then transferred to a nitrocellulose membrane (Bio-Rad). The membranes were incubated with primary antibodies in TBST (150 mM sodium chloride, 50 mM Tris base and 0.1% Tween-20, pH 7.4) overnight at 4°C. The primary antibodies included rabbit antibodies specific for GAPDH, p65, focal adhesion kinase (FAK), myosin light chain (MLC), p-MLC, AKT and p-AKT (Cell Signaling Technology), and mouse antibodies specific for VEGFA (Santa Cruz), p-STAT3, p-FAK, p-paxillin, paxillin and zonula occludens-1 (ZO-1, BD Bioscience). Then, the membranes were incubated with primary antibodies in blocking solution overnight at 4°C. After washing, the strips were incubated with a 1:5000 or 1:10 000 dilution of HRP-conjugated anti-rabbit or anti-mouse IgG from Cell Signaling Technology. Next, the blots were incubated in the ECL substrate developing solution (Bio-Rad). The density of the bands was captured and quantified by densitometry using ImageJ. For immunoblotting analysis, the optical density of the test sample was expressed relative to the density of the internal control. Phosphorylated proteins were normalized to the total protein first.

2.8 | ELISA

The concentration of VEGFA in MAPF was measured using a commercially available ELISA kit (R&D Systems, 367) according to the guidelines of the manufacturer. The values obtained after ELISA were 368 corrected with a dilution factor and ultimately expressed in micrograms per milliliter.

2.9 | Statistical analysis

The collected data were expressed as the averages of at least triplicate samples and are presented as the mean ± SEM. P was analyzed
RESULTS

3.1 Characterization of increased pleural capillaries in lung adenocarcinoma

Assessment of H&E staining revealed the pattern of microvessels in pneumothorax and adenocarcinoma of the lung (Figure 1). Pleural tissues are presented in the pneumothorax section at 200× magnification. Some capillaries were observed beneath the layer of mesothelial cells. In contrast, the lung adenocarcinoma section revealed increased capillaries in the subpleural layer (Figure 1). Moreover, defoliation in the mesothelium and an increase in microvessels were observed at high power.

3.2 Effect of malignancy-associated pleural fluid on HUVEC proliferation

To recapitulate the impact of the pleural microenvironment on endothelial cells, MAPF was adopted in the present study as conditioned medium in the HUVEC culture system. Through sonography-guided thoracentesis, MAPF was collected from 20 lung cancer patients, and their characteristics are summarized in Table 1. As shown in the MTT assay, the survival rate increased to approximately 50% in MAPF culture (Figure 2A). The trypan blue assay showed a two-fold increase in viable cells (Figure 2B). Immunofluorescence staining revealed more Ki67-positive cells in MAPF culture (Figure 2C). DAPI staining revealed few HUVEC undergoing mitosis, with a five-fold increase in the mitotic index. The above data demonstrated the ability of MAPF to promote HUVEC proliferation.

3.3 Stimulation of endothelial motility and angiogenesis by malignancy-associated pleural fluid

In addition to cell proliferation, endothelial cell migration during angiogenesis is critical during new vessel formation. In the scratch wound healing assay, supplementation with MAPF increased HUVEC directional migration (Figure 3A). Transwell migration assays revealed consistent results after incubation with MAPF (Figure 3B). The integration of focal adhesion and actin cytoskeleton rearrangement plays a fundamental role in cell movement. After phalloidin staining of F-actin, HUVEC incubated with MAPF were

with Student’s t-test and a P-value of less than 0.05 indicated statistical significance.
found to exhibit lamellipodium protrusion and filopodium formation (Figure 3C). In addition, the protrusion front of the cell presented increased staining of phosphorylated paxillin (Figure 3C). Focal adhesion complexes are recognized to contribute to endothelial motility during angiogenesis. Western blotting showed that MAPF culture significantly upregulated the expression of integrin β1 and β3 at 24 hours (Figure 4A). Increased phosphorylation of other focal adhesion components, including FAK and paxillin, was found (Figure 4B). Comparatively, the change of p-MLC2 was insignificant after MAPF incubation (Figure 4C). The above data suggested the ability of MAPF to stimulate HUVEC migration and activate focal adhesion signaling.

Next, a tube formation assay was used to examine the ability of HUVEC to form capillary networks under MAPF treatment (Figure 5A). Instead of inducing tubular structures, supplementation with MAPF induced HUVEC to form sheet-like structures.
Malignancy-associated pleural fluid (MAPF)-induced changes in the angiogenic behavior of HUVEC. HUVEC were incubated with MAPF or control medium. A, Micrographs show HUVEC tube formation after culture with MAPF for 6 and 12 h (N = 10). B, Image analysis of tube length, branch points and tube width at 6 and 12 h. C, Morphological change of HUVEC cultured with MAPF for 8 h (N = 8). (**P < 0.01; ****P < 0.0001 compared to the control group.)

This tendency reflected the statistical significance of tube width in the MAPF-treated group (Figure 5B). Moreover, a capillary-like structure (CLS) was identified after MAPF culture for 8 hours, in which a single cell expanded, while cells around its periphery elongated and arranged into circles (Figure 5C). In conclusion, HUVEC treated with MAPF possessed a distinct angiogenic capacity.

3.4 | Upregulation of the endothelial VEGFR2/VEGFA signaling network by malignancy-associated pleural fluid

The dominance of VEGFR2/VEGFA signaling in endothelial cells during angiogenesis has been well established. Compared with culture medium, MAPF revealed elevated VEGFA concentration up to several hundred-fold (Figure 6A). Following MAPF culture for 24 hours, HUVEC showed an upward trend of VEGFR2 expression but not VEGFR1 level (Figure 6B). In contrast, there was insignificant change in the ZO-1 level (Figure S1A). Next, intracellular signaling relevant to proliferation was examined due to increased cell viability in MAPF culture. There was increased phosphorylation of p38, ERK and JNK, but not Akt, in HUVEC (Figure 6C, Figure S1B). Third, transcriptional factors related to the VEGFA/VEGFR2 network were investigated. The differences in the protein expression of p-STAT3, p-p65 and HIF-1α reached statistical significance after MAPF culture for 24 hours (Figure 6D). Along with increased p-p65, there was increased expression of nitric oxide synthase 2 in HUVEC (Figure S1C).

Consistent with the phenotype of increased endothelial angiogenesis, our protein analysis revealed upregulation of VEGFR2, activation of intracellular MAPK signaling, and the involvement of the transcription factors p-STAT3, p-p65 and HIF-1α.

3.5 | Attenuation of malignancy-associated pleural fluid-induced endothelial migration and angiogenesis by blockade of VEGF signaling

Due to the finding that angiogenic factors were increased by MAPF incubation, the efficacy of VEGF signaling blockade was
evaluated in terms of endothelial proliferation, motility and angiogenesis. Sunitinib and bevacizumab were applied as VEGFR2 inhibitor and anti-VEGF antibody, respectively. After cotreatment with 1 μM sunitinib (marketed as Sutent) for 24 hours, the MTT assay revealed a negligible impact on MAPF-induced endothelial proliferation (Figure 7A). However, the abrogation of HUVEC motility was observed in the transwell migration assay when the cells were coincubated with MAPF and sunitinib (Figure 7B). In the tube formation assay, sunitinib effectively suppressed the increased tube width induced by MAPF (Figure 7C). Although statistically insignificant, the cotreatment with 0.01 mg/mL bevacizumab (marketed as Avastin) revealed a downward trend in MAPF-induced endothelial proliferation from the MTT assay (Figure 8A). In the transwell assay, bevacizumab significantly suppressed endothelial motility in MAPF incubation (Figure 8B). In the tube formation assay, increased tube width in the MAPF-treated group was attenuated by bevacizumab treatment (Figure 8C). The above results revealed the potential of targeting VEGF pathway to treat MAPF-induced endothelial migration and angiogenesis but not proliferation.

4 | DISCUSSION

The present study provided the first pathological description of capillary formation in the subpleural layer of lung adenocarcinoma. To simulate the observation of increased pleural microvessels at the cellular level, we cultured HUVEC with pleural fluid from lung cancer patients. In a total of 20 lung cancer patients receiving thoracocentesis, the pleural fluids exhibited a universal ability to promote HUVEC proliferation. First, this phenomenon was present not only in lung adenocarcinoma but also in squamous cell carcinoma and small-cell lung cancer. Second, neither patients harboring gene mutations nor...
patients receiving multiple-line treatments showed differences in the bioactivity of MAPF. Third, regardless of the presence of malignant cells in the pleural fluid, lung cancer-related pleural fluids induced similar HUVEC viability. Thus, the analysis of patient characteristics revealed the consistent ability of MAPF to increase the proliferation of endothelial cells, suggesting the potential to induce angiogenesis in the subpleural layer of lung cancer.

The consensus is that cancer-related neovascularization encompasses endothelial proliferation and migration.\textsuperscript{17} To determine the effect of MAPF on endothelial motility, wound healing and transwell assays were applied, and both assays showed an increased migration rate. Moreover, phalloidin staining revealed morphological changes, with lamellipodia and filopodia. The formation of lamellipodia and filopodia at the leading edge could be recognized as progressive actin remodeling that is correlated with sprouting angiogenesis in vivo.\textsuperscript{18-20} Upregulation of integrins and focal adhesion signaling were found in HUVEC cultured with MAPF, which indicated not only cytoskeletal rearrangement but also an active cellular response to angiogenic stimulation.\textsuperscript{21,22} According to previous studies on VEGF-mediated angiogenesis, our results of abundant VEGF levels in MAPF could induce phosphorylation of FAK and paxillin with lamellipodum and filopodium formation in endothelial cells.\textsuperscript{23-25} To evaluate the potency of MAPF in angiogenesis, a tube formation assay was performed and showed significant increased tube width. As more cells were plated, HUVEC demonstrated a tendency to clump and form monolayers rather than capillary networks.\textsuperscript{26,27} This phenomenon of sheet-like structures in the tube formation assay could, thus, be attributed to the increased viability of cells after MAPF culture. Furthermore, Ahmad et al reported CLS formation in cultured HUVEC on days 2-3 after reaching cell confluency.\textsuperscript{28} In contrast, the presence of CLS was identified in MAPF culture for 8 hours, which implied the acceleration of angiogenesis. In summary, cell-free MAPF had a significant impact on endothelial cell proliferation, migration and angiogenesis.
VEGFA is an abundant effector molecule that is secreted from tumors, mesothelial cells and inflammatory cells and contributes to MPE formation as a potent permeability factor. VEGF is one of the major receptors for VEGFA on endothelial cells and might be more important than VEGFR1 in VEGF-induced cell proliferation and angiogenesis. Increases in VEGF and VEGFR expression were observed in HUVEC cultured with MAPF, while the changes in VEGFR remained nonsignificant. As a result, we postulated that VEGFR2 signaling is important for MAPF-mediated HUVEC proliferation, migration and angiogenesis. Along with VEGFR2 activation, there were several transcription factors involved in the pathogenesis of MPE, such as p-STAT3, NF-κB and HIF-1α. Our results revealed increased protein expression of p-STAT3, NF-κB and HIF-1α in HUVEC cultured with MAPF. The coordination of hypoxia and inflammation signaling is frequently observed in the tumor microenvironment; therefore, we proposed that MAPF culture might synergistically activate the HIF-1α and NF-κB pathways to increase HUVEC proliferation and angiogenesis. The induction of HIF-1α had been observed through a hypoxia-independent mechanism by STAT3 and NF-κB in cancer cells. Therefore, our results of increased HIF-1α protein level could be associated with the upregulation of p-p65 and p-STAT3 in MAPF-treated HUVEC. Upon activation, VEGFR2 undergoes autophosphorylation and switches on several intracellular cascades, including MAPK and Akt. MAPF culture activated endothelial VEGFR2, HIF-1α and NF-κB signaling, with cell proliferation and angiogenesis.
Administration of drugs targeting VEGF has the potential to improve the efficacy of current management for MPE. Based on our results showing the abundant VEGFA in MAPF and elevated VEGFR2 expression in HUVEC, the effects of bevacizumab and sunitinib on cell proliferation, migration and angiogenesis were, therefore, evaluated. Reduced cell migration and less tube width indicated the effect of both VEGFR2/VEGF blockade on MAPF-induced HUVEC motility and angiogenesis. These findings elaborated the vital role of VEGF pathway in MAPF-associated angiogenesis. Neither sunitinib nor bevacizumab elicit a significant impact on MAPF-induced endothelial proliferation. However, other mechanisms involving endothelial cell proliferation in MAPF may exist. Although further studies are required, our results exposed the rationale of targeting the VEGF pathway to attenuate MAPF-mediated endothelial angiogenesis.

There are some limitations and future directions that are relevant to the interpretation of these MAPF results. First, the bioactivity of MAPF was only weakly correlated with individual treatment response. The effect of MAPF on endothelial viability relied on the collection of pleural fluid from the same patient at different time points. Second, animal models of MPE are required to build on the cellular findings of HUVEC cultured with MAPF and establish the contribution of endothelial cells to fluid accumulation. Third, future studies are required to identify other bioactive factors that mediate MAPF-induced endothelial cell proliferation.

Consistent with the finding of increased numbers of pleural microvessels in clinical specimens, MAPF from lung cancer patients demonstrated a potent ability to drive vascular endothelial cell proliferation, migration and angiogenesis (Figure 9). Targeting effectively abolished MAPF-induced HUVEC migration and angiogenesis but not cell proliferation, which might require the blockade of multiple signaling pathways.

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DISCLOSURE
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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