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Up-regulation of pro-angiogenic factors and establishment of tolerance in malignant pleural effusions

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ABSTRACT

Introduction: Malignant pleural effusions (MPEs) are a significant source of cancer morbidity and mortality. Currently there is no cure for MPEs and treatments only palliate the symptoms. The purpose of this study was to determine if there are differences in markers of angiogenesis and immune phenotypes between adenocarcinoma-induced MPEs and benign pleural effusions (BPEs).
Methods: Pleural effusions were collected from patients with MPEs and BPEs. Cells were isolated from effusions and characterized using fluorescent cell sorting (FACS). Pleural effusions were evaluated by ELISA for VEGF-A. An angiogenesis protein array was completed to compare protein expression in malignant and non-malignant effusions.
Results: FACS analysis demonstrated lower accumulation of cytotoxic T-cells and significantly higher accumulation of monocytes, dendritic cells, mesothelial and tumor cells in MPEs compared to benign pleural effusions. MPEs were found to have 77-fold higher VEGF-A levels compared to BPEs. The angiogenesis protein array demonstrated elevated levels of pro-angiogenic factors VEGF-A, CXCL4 and MMP-8, and low levels of pro-inflammatory cytokines IL-8, MCP-1, and TGF-β1 in MPEs.
Conclusions: MPE is biased toward a Th2 dominant state. There is an increase in expression of VEGF-A and other pro-angiogenic factors in MPE. These data suggest there is a role for anti-angiogenesis therapy in patients with MPEs.

1. Introduction

Each year, 1.5 million people develop a pleural effusion in the United States [1]. Approximately 50% of patients with metastatic cancer will develop a malignant pleural effusion (MPE), with breast and lung cancers accounting for 75% of the cases [2]. MPEs are a significant source of cancer morbidity and mortality, and median survival of patients with metastatic cancer that acquire an MPE is 3 months [3]. Currently, there is no cure for MPEs and patients must deal with symptoms, such as dyspnea, orthopnea, chest pain and cough, which significantly affect their quality of life. Treatments, including pleural catheters, pleurodesis, and thoracentesis, provide some degree of symptomatic relief but do not address the underlying pathobiology. More research needs to be conducted to understand the cellular makeup and signaling that occurs in MPEs so to better treat and maybe even prevent lung effusions.

The immune system exists in numerous states in order to defend against infectious agents; the most well characterized being the Th1 and Th2 states [4]. Cell mediated immunity, or Th1 biased immunity, consists of cells having effector functions that are mediated through activation of $CD8^+$ T cells and inflammatory cytokines such as IL-2 and IFN-γ. Humoral mediated immunity, or Th2 biased immunity, is made up of cells that have a regulatory function that is mediated through $CD4^+$ T cells and tolerance inducing cytokines such as IL-4, IL-5 and IL-10. We and others have seen a shift toward immune tolerance and tumor promotion in the immune systems of patients with advanced cancer [5–9]. Whether this phenomenon occurs in pleural effusions has yet to be elucidated.

Angiogenesis is vital for cancer as it promotes vascular permeability, blood vessel development and tumor cell implantation. Vascular endothelial growth factor (VEGF), a potent growth factor for endothelial cells, plays a vital role in the promotion of angiogenesis and the formation of MPEs [10,11]. High levels of

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this pro-angiogenic factor have been found in MPEs of breast, lung and mesothelial origin [12–14]. Other pro-angiogenic factors are needed to promote malignancy, but their roles are less well understood.

Herein, we analyzed pleural effusions to phenotype immune cells, determine VEGF levels and explore other potential pro-angiogenic factors that differ between malignant and benign pleural effusions. The purpose of this study is to evaluate changes in immunity and angiogenic factors, which may give us additional insight into more effectively treating patients with malignant pleural effusions.

2. Patients and methods

2.1. Patients and sample acquisition

Pleural effusions were used from 19 patients who consented to use of their residual, drained effusions after diagnostic sampling was completed between 12/2009 and 12/2010. This study was approved by the Mayo Clinic IRB.

Pleural effusions were collected by thoracentesis or PleurX catheters and processed 4–6 h after acquisition to acquire cells and plasma. Pleural fluid was frozen down at −80 °C and cells were resuspended in cosmic calf serum + DMSO and stored in liquid nitrogen until analysis. In some cases biopsies were obtained at outside institutions and reviewed by a pathologist at our institution for confirmation. In other cases, primary lesions were biopsied and reviewed, or cells from pleural effusions underwent cytopathologic review at our institution. In addition to cytopathologic review, immunostains for MOC31, CK7, CK20, calretinin, mammoglobin, HER2, GCDFP-15, TTF-1, ER/PR and CDX2 were occasionally used. When cytology was obviously benign to an experienced pathologist, no further immunostains were utilized.

2.2. Assessment of cell phenotype by flow cytometry

Nineteen effusion cell samples were stained for T helper cells ($CD3^+/CD4^+$), cytotoxic T cells ($CD3^+/CD8^+$), NK cells ($CD16^+/CD56^+$), monocytes ($CD14^+/CD11c$), dendritic cells ($CD14^+/DR^+$), mesothelial cells and epithelial/tumor cells (EpCAM). All human monoclonal antibodies were purchased from BD Pharmingen (Franklin Lanes, NJ) and are as follows: anti-CD3 PC5, anti-CD4 PE, anti-CD8 FITC, anti-CD16 FITC, anti-CD56 PE, anti-CD14 FITC, anti-CD11c PC5, anti-DR PE, anti-CD3 PC7, anti-mesothelial PE, and anti-EpCAM FITC. Color and isotype controls were used for each different fluorescent combination. Cells were thawed and 200 μL aliquots were added to a round bottom 96 well plate. Five microliters of the desired antibody was added to each well and incubated for 30 min at 4 °C. Cells were washed twice in 1× PBS (Cellgro, Germany), 0.1% bovine serum albumin, and 0.05% sodium azide (Sigma–Aldrich, St. Louis, MO). Four color flow cytometry was run and analyzed on a Guava easyCyte 8HT flow cytometer (Millipore, Billebrica, MA). Percentages were calculated as totals of all cells in our sample population.

2.3. Determination of VEGF-A levels by ELISA

Nineteen pleural fluid samples were assayed in duplicate for concentrations of VEGF-A. Quantikine kits (R&D Systems, Minneapolis, MN) for VEGF-A were acquired and samples were analyzed as per manufacturer's instructions. A 96 well plate was incubated overnight at 4 °C with VEGF-A capture antibody. In the morning, wells were washed (0.05% Tween20 in PBS) and blocked for an hour at room temperature. After washing the wells, the samples and standards were added to the 96 well plate and incubated for 2 h at room temperature. Detection antibody was added and incubated for 2 h at 25 °C. Streptavidin–HRP solution was added to each well for 20 min and substrate solution was used to visualize the reaction. After 20 min in substrate solution, stop solution was added to the reaction and protein concentration was determined using a standard curve at 450 nm by nanospectroscopy.

2.4. Analysis of human angiogenic proteins and cytokines

Nine fluid samples were analyzed for 55 angiogenic proteins, cytokines and chemokines using the Human Angiogenesis Array kit (R&D Systems, Minneapolis, MN). Proteins included Activin A, ADAMTS-1, angiogenin, angiopoietin 1 and 2, angiostatin/plasminogen, amphiregulin, artemin, coagulation factor III, CXCL16, CD26, EGF, EG_VEGF, CD105, endostatin/collagen XVIII, endothelin-1, FGF acid and base, FGF-4, FGF-7, GDNF, GM-CSF, HB-EGF, HGF, IGFBP-1, 2 and 3, IL-1β, IL-8, TGF-β1, leptin, MCP-1, MIP-1α, MMP-8 and -9, NRG1-β1, pentraxin 3, PDGF, persephin, CXCL4, PLGF, prolactin, serpin B5, E1 and F1, TIMP-1 and 4, TSP-1 and 2, uPA, vasohibin, VEGF and VEGF-C. Instructions were followed as directed by the manufacturer. At room temperature, membranes were blocked then placed in effusion plasma. Membranes were then washed and placed in biotin-conjugated primary antibody. Following an overnight incubation at 4 °C, membranes were placed in streptavidin–HRP and dots were detected on X-ray film using ECL detection reagent (GE Healthcare, United Kingdom). Pixel density analysis (Western Vision Software, Salt Lake City, UT) was used to determine the state of angiogenesis in malignant and benign effusions. Membranes were analyzed using software from Western Vision software (Salt Lake City, UT) by correcting for background and averaging each sample's pixel density.

2.5. Data analysis

Medians and interquartile ranges were calculated for benign and malignant group analysis of ELISA, angiogenesis array proteins and cell phenotypes. Significance was determined using a two-sided Mann-Whitney *U* test for nonparametric samples at a confidence interval of 95%. Pixel densities were normalized for comparison across experiments and using Western Vision Software and presented as medians and interquartile ranges.

3. Results

3.1. Patients

There were 19 patients who consented to use of their pleural effusions during the study period. The samples were primarily from male (58%), elderly patients (median age 79 years, interquartile range 73–83; Table 1). Six patients were diagnosed with a benign effusion and 13 patients were diagnosed with a malignant effusion.

3.2. Malignant effusions show a more tolerant phenotype than benign effusions

There were noteworthy differences found in the frequencies of epithelial, mesothelial, dendritic cells, monocytes and cytotoxic ($CD8^+$) T cells between BPEs and MPEs (Fig. 1). Significantly higher levels of cytotoxic T cells were observed in benign effusions (49.31%, 34.12–79.16; median and interquartile range) when compared to malignant ones (20.69%, 11.84–32.75; $p = 0.0015$). Interestingly, there were no significant differences between levels of $CD4^+$ T cells in malignant (31.76%, 16.19–45.94) and benign effusions (24.85%, 11.97–82.76; $p = 0.957$). Natural killer cells were also not significantly different between MPEs (4.480%, 0.840–22.61) and benign effusions (14.10%, 9.05–19.18; $p = 0.1310$). In MPEs there was a significant increase in dendritic cells (15.20%, 9.870–24.17) and

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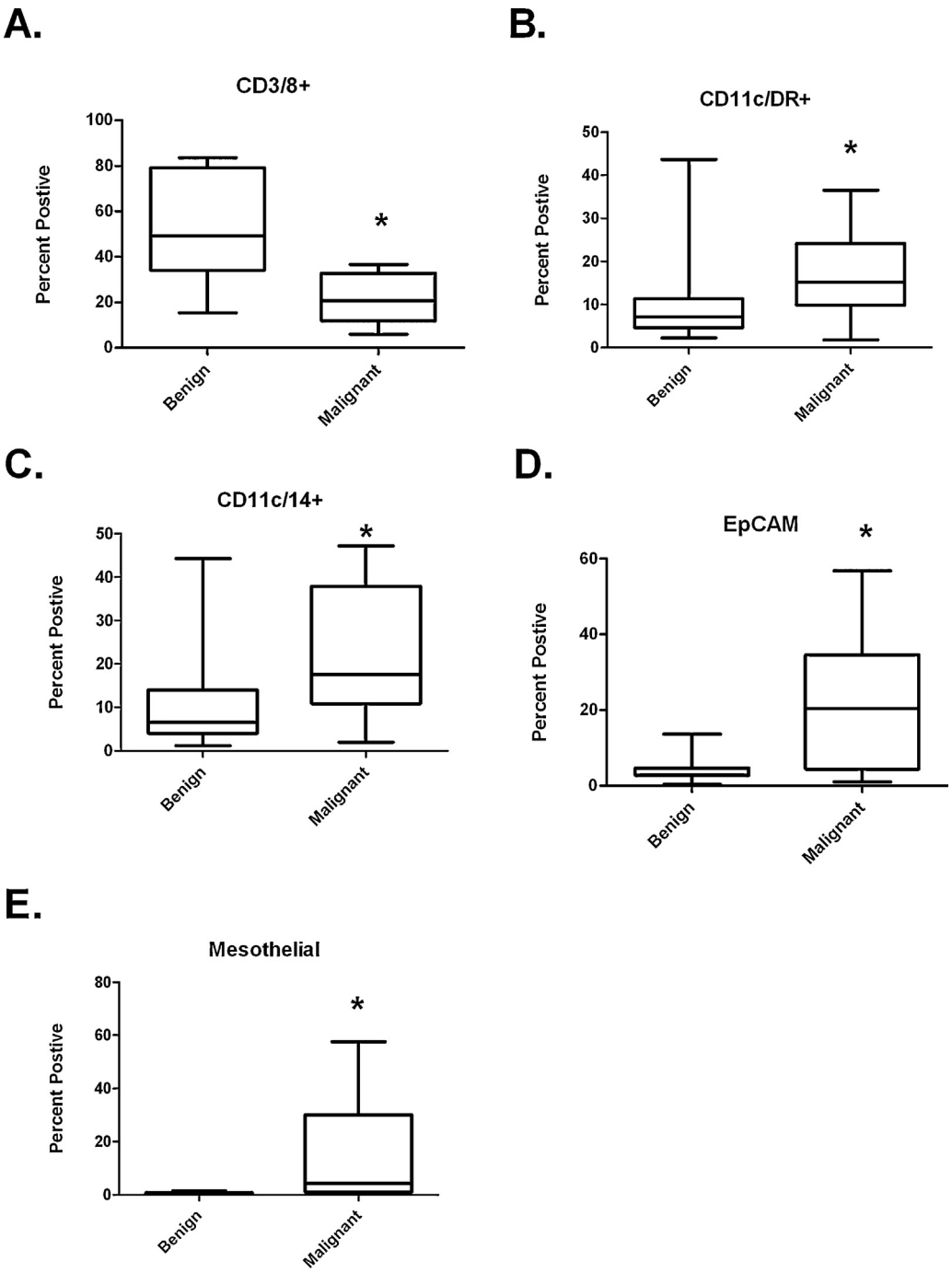

Fig. 1. MPEs contain an increased number of chronic inflammatory (Th2) cells compared to benign. Cell phenotypes determined by flow cytometry presented as the median percent and interquartile ranges ($n = 17$ MPE; $n = 9$ benign PE). (A) Cytotoxic T cells (CD3+ CD8+); $p = 0.0015$. (B) Dendritic cells (CD11c DR); $p = 0.0205$. (C) Monocytes (CD11c CD14); $p = 0.027$. (D) Epithelial/tumor (EpCAM); $p = 0.0082$. (E) Mesothelial cells; $p = 0.0015$.

monocytes (17.65%, 10.91–37.87) compared to levels of dendritic cells (7.130%, 4.595–11.37; $p = 0.0205$) and monocytes (6.620%, 4.020–14.08; $p = 0.0270$) found in BPEs (Fig. 1B and C). There was a major increase in EpCAM positive cells (20.39%, 4.430–34.56) in MPEs compared to benign (2.810%, 2.720–4.640; $p = 0.0082$; Fig. 1D). Our data further demonstrated MPEs are composed of a higher frequency of mesothelial cells (4.30%, 0.870–30.13) than are found in begin effusions (0.630%, 0.320–0.770; $p = 0.0015$; Fig. 1E). We also looked at a small sample of $CD4^+$ T cell subgroups, namely CD294 and TIM3. Although not significant, $CD4^+$ T cells in MPEs expressed higher levels of CD294 (8.894, 4.867–13.70) compared to benign effusions (1.609, 0.794–12.83; $p = 0.267$).

3.3. *Angiogenic factors, including VEGF-A, are increased in malignant pleural effusions*

There was a significant increase in the levels of VEGF-A in malignant effusions (1804 pg/mL, 422.6–3411) when compared to benign effusions (5.185 pg/mL, 2.644–46.46; $p < 0.0001$; Fig. 2A). Results of the protein array showed a trend in the up-regulation of pro-angiogenic factors and down-regulation of pro-inflammatory cytokines in MPEs compared to benign effusions (Table 2). However, this method is qualitative unlike ELISA, thus cannot accurately measure protein concentration. We found that in MPEs, levels of VEGF-A (100.2, 69–149.7), CXCL4 (65.03, 63.8–113.5; Fig. 2B)

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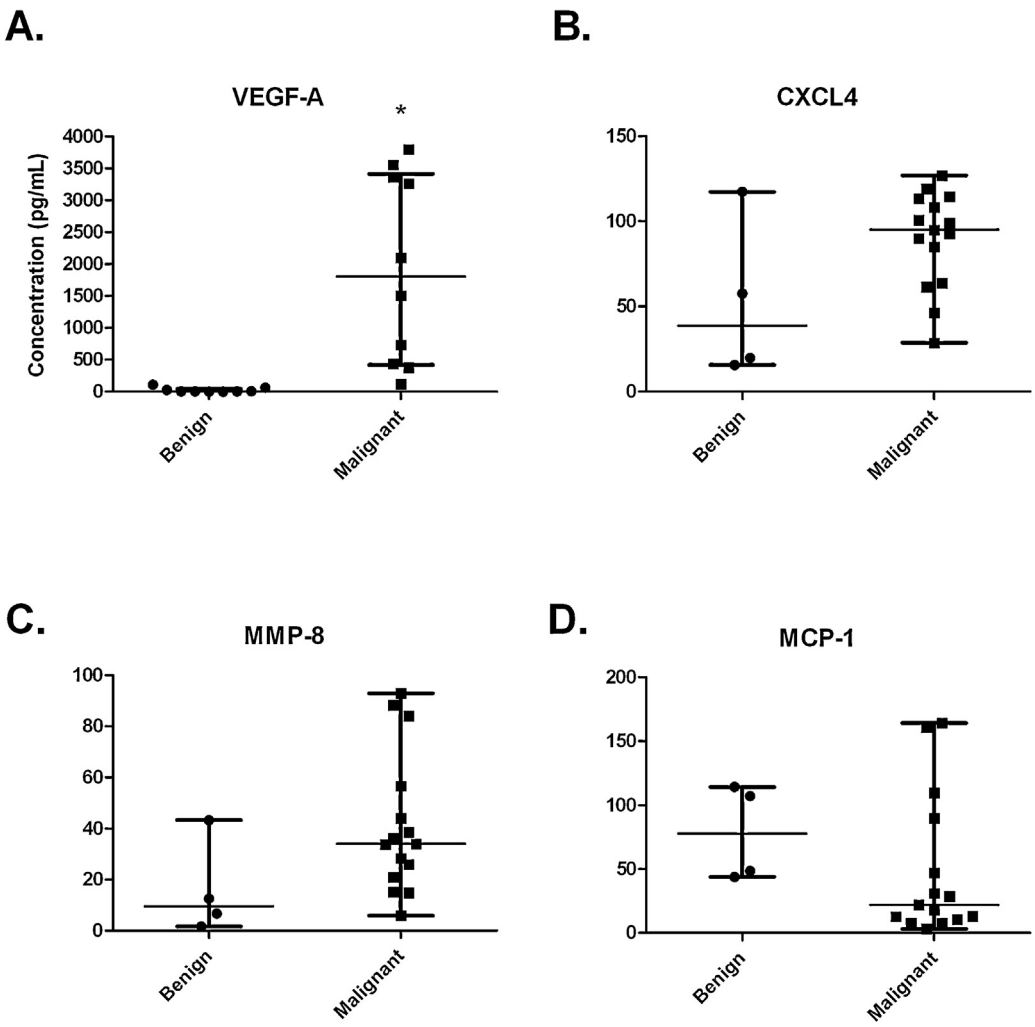

Fig. 2. Differences in the levels of pro- and anti-angiogenic factors found in MPEs and benign effusions. (A) ELISA of median VEGF-A levels (IQR) expressed in pg/mL of MPEs ($n = 10$) and benign effusions ($n = 9$) ($p < 0.0001$). (B) Median pixel density and IQR of CXCL4 (not significant). (C) Median pixel density and IQR of MMP-8 (not significant). (D) Median pixel density and IQR of MCP-1 (not significant).

and MMP-8 (34.08, 20.99–56.64; Fig. 2C) were non-significantly higher compared to benign effusions (80.32, $p = 0.2501$; 38.73, $p = 0.471$; 9.635, $p = 0.0801$, respectively). On the other hand, important pro-inflammatory promoting genes such as MCP-1 (77.95, 45.04–112.6; Fig. 2D), IL-8 (12.31, 6.10–92.7), and TGF-β1 (41.57, 24.16–63.67) were increased compared to MPEs (22.16, $p = 0.1211$; 7.59, $p = 0.3953$; 36.22, $p = 0.5157$, respectively). Anti-angiogenic genes were found at similar levels in both benign and MPEs. MPEs expressed slightly higher levels of prolactin (86.82, 64.87–93.39) and endostatin (83.48, 57.28–97.2) compared to benign (54.74, $p = 0.2113$; 67.41, $p = 0.6527$, respectively).

Table 1
Clinical characteristic of study patients and their diagnosis.

Sample ID	Age	Gender	Side	Diagnosis
MB 1	91	Male	Right	Chronic inflammation
MB 2	84	Male	Left	Acute inflammation
MB 3	60	Female	Right	Primary peritoneal ACA
MB 11	74	Male	Right	Squamous lung
MB 12	83	Male	Right	Lung ACA
MB 13	74	Male	Right	Squamous lung
MB 14	80	Female	Right	Ovarian
MB 15	69	Female	Right	Lung ACA
MB 16	68	Female	Right	Lung ACA
MB 17	72	Male	Right	Lung ACA
MB 18	78	Male	Left	Lung ACA
MB 19	87	Male	Right	Melanoma
MB 20	83	Male	Right	Colon ACA
MB 21	82	Female	Right	Reactive mesothelium
MB 22	75	Male	Right	Epithelial mesothelium
MB 23	63	Female	Left	Squamous lung
MB 24	79	Female	Left	Breast
MB 25	82	Female	Right	Reactive mesothelium
MB 26	85	Male	Right	Inflammation

ACA = adenocarcinoma.

4. Discussion

MPEs are enriched for immunosuppressed/Th2 biased cells such as monocytes and dendritic cells, and have low levels of $CD8^+$ T cells. We found increased levels of both epithelial (EpCAM) and mesothelial cells in MPEs and also detected significant elevations of VEGF-A in MPEs compared to BPEs, similar to what others have reported [10,12,15]. Additionally, we show that the increase in VEGF-A in MPEs is accompanied by increases in numerous other pro-angiogenic factors.

Our group has previously shown that systemic immunity is biased toward a Th2 tolerant state in metastatic melanoma, with tumor derived VEGF being a key driver of this process [5]. Similarly, suppressive changes, such as a decrease in $CD8^+$ T cells, occur in the sentinel lymph node which precedes melanoma metastasis into the tissue [6]. In MPEs we observe a very similar phenomenon. Th2 mediated immune cells, such as dendritic cells and monocytes,

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Table 2
Proteins important for the promotion or suppression of angiogenesis and inflammation in malignant and benign effusions. Data are shown as the median pixel density with interquartile ranges ($n = 5$ MPE; $n = 4$ benign).

Angiogenesis factor	Median pixel density (IQR)		*p*-value
	Benign effusions	Malignant effusions	
Pro-inflammatory			
CXCL16	61.75 (58.16–117.4)	89.65 (48.48–100.9)	0.8026
MCP-1	77.95 (45.04–112.6)	22.16 (10.56–89.85)	0.1211
Pentraxin 3	56.74 (22.57–113.5)	86.51 (68.72–101.5)	0.2113
uPA	12.21 (0.942–51.92)	21.04 (4.545–28.68)	0.5823
IL-8	12.31 (6.10–92.7)	7.59 (5.085–11.54)	0.3953
TGF-β1	41.57 (24.16–63.67)	36.22 (21.45–44.63)	0.5157
Pro-angiogenic			
Angiogenin	110.3 (104.1–148.5)	139.6 (98.93–162.8)	0.5157
Angiopoietin-2	8.663 (3.55–24.88)	11.65 (6.045–16.22)	0.6527
CD26	57.79 (57.79–71.69)	76.08 (40.07–91.90)	0.3953
CD105	57.17 (32.83–102.7)	70.63 (42.96–78.96)	0.7263
CXCL4	38.73 (16.70–102.4)	95.03 (63.8–113.5)	0.1471
FGF-7	13.56 (4.191–38.01)	28.07 (16.05–39.10)	0.2937
HGF	48.23 (33.01–91.28)	50.48 (21.60–78.25)	0.8026
IGFBP-1	94.05 (89.75–148)	121.1 (97.46–138.3)	0.5823
IGFBP-2	51.64 (10.28–114.5)	65.71 (45.46–98.78)	0.7263
IGFBP-3	93.37 (80.90–152.8)	120.4 (86.33–137.3)	0.6527
Leptin	110.1 (94.83–124.4)	120 (101.2–157.1)	0.3953
MMP-8	9.635 (2.928–35.59)	34.08 (20.99–56.64)	0.0801
MMP-9	63.35 (19.01–150.2)	93.52 (61.56–101.5)	0.5157
PLGF	59.94 (25.42–77.58)	58.54 (36.71–94.74)	0.7263
Serpin E1	63.49 (15.50–134.7)	81.96 (63.57–119.8)	0.6527
TSP-1	9.415 (4.206–65.79)	15.43 (11.40–23.09)	0.4533
VEGF	80.32 (41.97–118.9)	100.2 (69–149.7)	0.2501
Anti-angiogenic			
Endostatin	67.41 (46.64–109.5)	83.48 (57.28–97.20)	0.6527
Prolactin	54.74 (43.07–102.5)	86.82 (64.87–93.39)	0.2113
TIMP-1	30.13 (3.129–114.3)	81.64 (47.48–92.49)	0.3953
TIMP-4	55 (32.12–132.7)	85.65 (55.77–113.1)	0.6527

Factors not present in our sample: Activin A, ADAMTS-1, angiopoietin-1, angiostatin/plasminogen, amphiregulin, artemin, coagulation factor III, EGF, endothelian-1, FGF-4 GDNF, GM-CSF, HB-EGF, IL-1β, MIP-1α, NRG1-β1, PDGF-AA, PDGF-AB, persephin, serpin B5, serpin F1, TSP-2 and VEGF-C.

make up a larger percent of cells in effusions compared to cells in benign effusions, and cytotoxic T cells are significantly lower in MPEs. We also saw a significant increase in the level of mesothelial cells in malignant effusion compared to benign. Although a pleural effusion due to tuberculous has shown a significant decrease in the population of mesothelial cells, malignancies may need these cells to support continued proliferation and invasion [16]. Using EpCAM as a marker for neoplastic cells, we detected a higher level of EpCAM positive cells in malignant effusions as expected. Interestingly, EpCAM has been associated with the development of at Th2 response through the secretion of IL-4, promoting tumor immune evasion [17]. Clinically, cancer progression has been shown to occur more rapidly in patients having an immune system that is biased toward a Th2 response compared to those having a cell mediated response [18]. In a mouse model, it has been shown that tumor specific T cells drive inflammation and a Th1 response which is protective in both myeloma and B-cell lymphoma [19]. In humans, the combination of a vaccine containing antigens specific to melanoma with chemotherapy increased $CD8^+$ T cell response against the tumor [20]. These findings further support our hypothesis that tumors induce a tolerant Th2 biased state within their host in order to support further tumorigenesis.

Our data indicate that malignant pleural effusions exist in a chronic inflammatory and pro-angiogenic state. Thus, treatment with anti-angiogenic therapy should be considered for the palliative management of malignant effusions. Animal studies have shown the use of bevacizumab, an anti-VEGF monoclonal antibody, interferes with acute inflammation reducing the volume of fluid accumulation in the lungs [21]. Bevacizumab following chemotherapy in the treatment of non-small cell lung cancer (NSCLC) resulted in a progression free survival of 312 days without recurrence of a pleural effusion [22]. A phase II of bevacizumab plus chemotherapy for first line treatment in non-squamous NSCLC resulted in an overall survival of 14.7 months and a progression free survival of 6 months [23]. Currently, bevacizumab plus carboplatin and paclitaxel is FDA approved for use in unresectable, local, recurrent, and metastatic NSCLC having shown to improve median overall survival in this disease from 10.3 to 12.3 months [24].

The fact that VEGF levels are significantly higher in MPE suggests that targeted administration of anti-VEGF therapy could be beneficial. For example, a group in France used a Port-A-Cath system for delivering interferon-γ intrapleurally to 29 patients with malignant mesothelioma and found it to be well tolerated for long-term therapy delivery and a 64% reduction in the infection rate compared to open chest tubes [25]. More recently, PleurX catheters have been widely accepted in the management of effusions due to their ease of use by trained family members allowing significant palliation of symptomatic effusions. Sixty-four patients with benign and malignant effusions who underwent PleurX catheter placement reported a high satisfaction rate, few infections and none had recurrence of the effusion at their 3 month follow-up [26]. Others believe pleurodesis with talc to be a better option for patients because it resolves more than 90% of MPE cases and is less expensive [27]. Nevertheless, these catheters could provide an excellent path for delivering therapies, such as anti-angiogenic therapy, directly to the site of the effusion.

Limitations of this study include a small sample size of pleural effusions from patients with either a benign or malignant diagnosis. Due to the difficulty in obtaining samples we generalized all effusions into malignant or benign groups and analyzed them together. This likely caused the large differences seen within each category. Analysis demonstrated large sample variances, likely due to the

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fact that many clinical diagnoses were grouped together into two broad categories. Also, specimens were frozen 4–6 h after they were collected which might not be optimal for detecting more unstable proteins and cytokines in the fluid. Lastly, due to the difficulty of obtaining tissue biopsies and exhaustion of cell pellets made from pleural effusions, we were unable to confirm expression of significant protein markers by IHC.

5. Conclusion

In conclusion, we have shown that cells in malignant effusions are biased toward a Th2 tolerant, and pro-angiogenic phenotype. We also demonstrated up-regulation of many additional pro-angiogenic proteins and a decrease in pro-inflammatory factors in MPEs compared to benign effusions. These data support a role for immunotherapeutics and targeted anti-angiogenic therapy in the treatment and management of MPEs.

Conflict of interest statement

None declared.

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