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# The Angiopoietin/Tie2 Axis Mediates Malignant Pleural Effusion Formation[1]

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## Abstract

*PURPOSE:* Angiopoietins and their receptor, Tie2, participate in angiogenesis, regulation of vascular permeability, and inflammation, all central to the pathogenesis of malignant pleural effusions (MPEs). In the present study, we aimed to examine the role of the angiopoietin/Tie2 axis in MPE pathogenesis. *EXPERIMENTAL DESIGN:* MPE was induced by intrapleural injection of murine adenocarcinoma cells in C57BL/6 mice. Animals were given twice-weekly intraperitoneal injections of 40 mg/kg MuTekdeltaFc or vehicle. MuTekdeltaFc is a soluble Tie2 (sTie2) receptor that binds murine angiopoietins thereby disrupting their interaction with Tie2 receptors expressed on tissues. Animals were killed on day 14. *RESULTS:* Angiopoietin/Tie2 axis blockade significantly reduced pleural fluid volume and pleural tumor foci. The mean ± SEM pleural fluid volumes were 617 ± 48 $\mu$l and 316 ± 62 $\mu$l for the control and treated groups, respectively ($P$ = .001), whereas the mean ± SEM tumor foci were 7.3 ± 1.0 and 3.0 ± 0.52 for the control and treated groups, respectively ($P$ = .001). In addition, tumor-associated cachexia, tumor angiogenesis, pleural vascular permeability, recruitment of inflammatory cells to the pleural cavity, and local elaboration of vascular endothelial growth factor and interleukin 6 were also downregulated, and tumor cell apoptosis was induced in animals treated with the inhibitor. *CONCLUSIONS:* Our results indicate that the angiopoietin/Tie2 axis is an important component of MPE pathogenesis. Further studies are required to determine whether therapeutic interventions targeting this pathway could be beneficial for patients with MPE.

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## Introduction

Malignant pleural effusion (MPE) is a common condition, affecting ~660 patients per million population each year and is associated with a very short survival and poor quality of life [1–3]. Current therapies used to prevent the reaccumulation of pleural fluid and relieve symptoms include pleurodesis (chemical-induced pleural fibrosis aiming at eliminating the pleural space), indwelling pleural catheters, and

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chemotherapy. All these are of variable efficacy and may cause serious adverse effects [1,4]. Nevertheless, very little is known about its pathogenesis. Unmasking the mechanisms of the disease would hopefully permit the development of more specific, effective, and safer treatment modalities.

Angiogenesis, pleural vascular hyperpermeability, and inflammation are considered central to the pathogenesis of MPE [3,5–8]. Angiopoietins 1 to 4 are important regulators of angiogenesis and exert their actions through binding a common tyrosine kinase receptor (Tie2) that is mainly expressed on the surface of endothelial cells [9]. An emerging body of evidence supports a central role for the angiopoietin/Tie2 pathway in tumor-associated angiogenesis [9,10] as well as in regulation of vascular permeability and inflammation [9,11–15], all central to the pathogenesis of MPE. We have previously reported that pleural expression of angiopoietin 2 is increased in patients with exudative pleural effusions, including MPE [16]. However, the role of angiopoietin/Tie2 signaling in the pathogenesis of MPE has not been examined. The aim of the present study was to determine the functional significance of the angiopoietin/Tie2 pathway in MPE formation using a recently developed model of MPE [6]. To interfere with angiopoietin/Tie2 axis signaling, we used MuTekdeltaFc, a sTie2 receptor that binds murine angiopoietins and prevents their interaction with Tie2 receptors expressed on tissues. We hypothesized that systemic administration of sTie2 would impair tumor angiogenesis and downregulate pleural inflammation and pleural vascular permeability, thereby inhibiting pleural tumor growth and pleural fluid accumulation that occur after intrapleural injection of cancer cells in mice.

## Materials and Methods

### In Vivo *Studies*

C57BL/6 mice (Hellenic Pasteur Institute, Athens, Greece) were inbred at the General Hospital "Evangelismos" (Athens, Greece). Experiments were approved by the Veterinary Administration Bureau, Prefecture of Athens, Greece. Intrapleural injections with mouse Lewis lung adenocarcinoma (LLC; ATCC, Manassas, VA) and sacrifice (14 days after LLC injection) were performed as described previously [6–8]. Starting 3 days after intrapleural injection of LLC cells, animals were given twice-weekly intraperitoneal injections of 40 mg/kg MuTekdeltaFc (Amgen, Inc., Thousand Oaks, CA), a murine sTie2, or vehicle (injection volume = 50 μl), for a total of four doses. Fourteen days after tumor cell injection, mice were killed by $CO_2$ for pleural fluid and tumor collection, as described previously [6–8].

### *Pleural Tumor Enumeration and Processing*

Pleural tumor nodules were enumerated by three independent and blinded readers under a dissecting microscope, and the average number was used for analyses. Pleural tumors were dissected avoiding adjacent normal tissue, snap-frozen in liquid nitrogen, and stored at −80°C. Tumor tissue was suspended in protein extraction buffer containing protease inhibitors (volume = 1 ml), and cytoplasmic protein extracts were collected after Dounce homogenization and centrifugation (16,000*g* for 5 minutes), as described previously [7,8]. All measurements in tumor cytoplasmic protein extracts were normalized for protein content.

### In Vivo *Vascular Permeability Assays*

To determine pleural vascular permeability in mice bearing MPEs, the animals received 200 μl of 4 mg/ml Evans' blue (total dose = 0.8 mg) i.v. on day 14 after LLC cell injection and were killed 1 hour later. Pleural fluid Evans' blue levels were determined by measuring absorbance at 630 nm in comparison to standards of known Evans' blue concentrations [6–8]. To evaluate the vascular permeability–enhancing properties of angiopoietins contained in pleural fluid, we used a modified Miles vascular permeability assay [7]. Malignant pleural effusion supernatants from untreated mice or PBS (volume = 50 μl) were injected at different spots of the shaved dorsal skin of C57B/6 mice. sTie2 (MuTekdeltaFc) was used to antagonize the effect of angiopoietins. Anti–vascular endothelial growth factor (VEGF)–neutralizing antibodies were used to abrogate the effect of VEGF, a factor with an established role in MPE-associated vascular hyperpermeability [5]. More specifically, five spots on each mouse received 1) PBS, 2) premixed MPE + PBS, 3) premixed MPE + 10 ng of sTie2, 4) premixed MPE + 400 ng of neutralizing anti-VEGF antibody (R&D systems, Abingdon, UK), or 5) premixed MPE + 10 ng of sTie2 + 400 ng of neutralizing anti-VEGF antibody. The mice received 200 μl of 4 mg/ml Evans' blue (total dose = 0.8 mg) i.v. immediately after dermal injections and were killed 30 minutes later. The skin was removed, the test sites were photographed, and the area of Evans' blue extravasation was determined using ImageJ freeware (Rasband 1997-2006, available at http://rsb.info.nih.gov/ij).

### *Cytology-Histology*

Nucleated pleural fluid cells count was assessed using a Neubauer hematocytometer. Cytocentrifugal specimens (cytospins) were prepared and stained with May-Gruenwald-Giemsa as described elsewhere [6,7]. Pleural tumor tissues were fixed in neutral-buffered formalin for 24 hours and 70% ethanol for 3 days, sectioned (thickness = 5 μm), and stained with hematoxylin and eosin or immunolabeled for proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA), terminal deoxynucleotidyl nick-end labeling (TUNEL; Roche, Penzberg, Germany), or factor VIII–related antigen (Invitrogen, San Francisco, CA), as described previously [7,8]. Quantification of immunoreactivity has been described elsewhere [17,18]. Factor VIII–related antigen labeling was quantified by the "hotspot" method [19]. Briefly, the whole tumor area was scanned at low power (A°, 40) to detect areas of enhanced angiogenesis (hotspots). Subsequently, five different, nonoverlapping, high-power fields (A°, 600) within hotspots from each tumor were assessed by two blinded readers for the number of individual cells or clusters of cells (counted as a unit) exhibiting immunoreactivity. Results were averaged for each tumor and then for each mouse.

### *Biochemical and Cytokine Determinations*

Pleural fluid and cell supernatant protein content was determined using the Bio-Rad protein assay (Hercules, CA). Mouse VEGF, tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) were determined using ELISA (Peprotech EC, London, UK, for VEGF; R&D Systems for TNF-α and IL-6). The minimum detectable levels were 31.3, 5.1, and 1.6 pg/ml for VEGF, TNF-α, and IL-6, respectively.

### In Vitro *Cell Viability Assay*

Lewis lung adenocarcinoma cells were plated at equal densities in 96-well culture dishes and treatment with sTie2 (10, 30, and 100 μg/ml) was started when cells were ~30% confluent. Cell viability was assessed after 4, 24, and 48 hours by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium inner salt (MTS) reduction assay (Promega, Madison, WI).

### *Statistical Analysis*

Data are presented as mean ± SE. To assess the difference in the means between different groups, unpaired and paired Student's *t*-tests and one-way analysis of variance with least squared difference *post hoc* tests were used, as appropriate. All *P* values are 2-tailed; $P < .05$ were considered significant. Statistical analyses were done using SPSS v.13.0.0 (SPSS, Inc., Chicago, IL).

## Results

### *sTie2 Inhibits Intrapleural Tumor Dissemination, Pleural Fluid Accumulation, and Tumor-Associated Cachexia*

We initially examined the effects of angiopoietin/Tie2 signaling blockade on macroscopic parameters of MPE induced by intrapleural injection of adenocarcinoma cells. The primary end points were the volume of pleural fluid and the number of pleural tumors. The sTie2-treated animals had significantly less pleural fluid (Figure 1*A*) and fewer pleural tumor implantations (Figure 1*B*) compared with the controls. In particular, the mean ± SEM pleural fluid volume was 617 ± 48 µl for the control and 316 ± 62 µl for the treated group ($P = .001$), whereas tumor foci were 7.3 ± 1 and 3.0 ± 0.52 for the control and treated groups, respectively ($P = .001$). In addition, whereas at the beginning of the experiment, body weight did not differ significantly between groups ($P = .24$), on the day of sacrifice, the animals in the treatment group lost 0.23 ± 0.54 g, which was significantly less ($P = .03$) than the animals in the control group, 1.95 ± 0.54 g (Figure 1*C*). These data clearly indicate that the angiopoietin signal transduction pathway promotes MPE formation by lung adenocarcinoma cells.

### *sTie2 Limits Tumor Angiogenesis and Pleural Vascular Hyperpermeability*

We next studied the effect of sTie2 on tumor angiogenesis and pleural vascular hyperpermeability, both central to the excessive pleural fluid production and the resultant formation of a MPE [5–7]. Tumor angiogenesis, assessed by tumor tissue immunostaining using an anti–factor VIII–related antigen antibody (fVIIIra), was found to be significantly suppressed. The mean ± SEM numbers of fVIIIra-positive clusters per high-power field were 2.9 ± 0.27 for the animals treated with the inhibitor compared with 11.7 ± 1.19 ($P < .001$) in those treated with the control solution (Figure 2*A*). For quantification of pleural vascular hyperpermeability, control and sTie2-treated mice received 0.8 mg of Evans' blue (an albumin-binding dye) i.v. 1 hour before sacrifice [7]. The total mean ± SEM pleural fluid level of Evan's blue, a marker of pleural vascular plasma leakage, was 28 ± 3 µg in treated animals, which was significantly lower than that in the control animals, 69 ± 10 µg ($P = .003$). This indicates that sTie2 limits pleural vascular permeability associated with the growth of the tumor cells in the pleural cavity (Figure 2*B*).

To evaluate the contribution of angiopoietins in the induction of MPE-associated vascular permeability, we performed a modified Mile's assay injecting pleural fluid supernatants from control animals pretreated with PBS, sTie2 or/and anti-VEGF neutralizing antibody into the skin of mice previously administered Evan's blue i.v. and measured the area of the dermal dye extravasation. As shown in Figure 2, *C* and *D*, both sTie2 and anti-VEGF significantly ($P < .001$) reduced MPE-induced skin vascular permeability. Interestingly, the inhibition of MPE-induced vascular permeability conferred by combination of sTie2 and anti-VEGF was significantly greater ($P < .05$) than that caused by anti-VEGF alone. In accord with our hypothesis, these results show that the angiopoietin/Tie2 axis enhances new vessel formation and vascular leakiness associated with MPE pathogenesis.

### *sTie2 Suppresses Pleural Inflammation Associated with MPE Formation*

In addition to the effects on plasma leakage in the pleural cavity, angiopoietin/Tie2 axis blockade resulted in significant down-regulation of malignancy-associated pleural inflammation. Treatment with the inhibitor resulted in a significant reduction of pleural fluid inflammatory cells ($P = .003$), including mononuclear cells ($P = .038$), lymphocytes ($P = .002$), and neutrophils ($P = .001$; Figure 3*A*). We further examined whether sTie2 treatment affects local elaboration of the proinflammatory and angiogenic mediators, VEGF, IL-6, and TNF-α, because all of the above have been previously shown to promote MPE through induction of angiogenesis and enhancement of pleural vascular permeability [5–8,20]. The pleural fluid levels of VEGF and IL-6 were significantly decreased in the treated group (Figure 3, *B–D*). In contrast the tumor levels of VEGF, TNF-α, or IL-6 did not differ significantly between the treated and control groups (Figure 3, *B–D*). These data suggest that angiopoietin signaling promotes proinflammatory and angiogenic mediator expression in MPE.

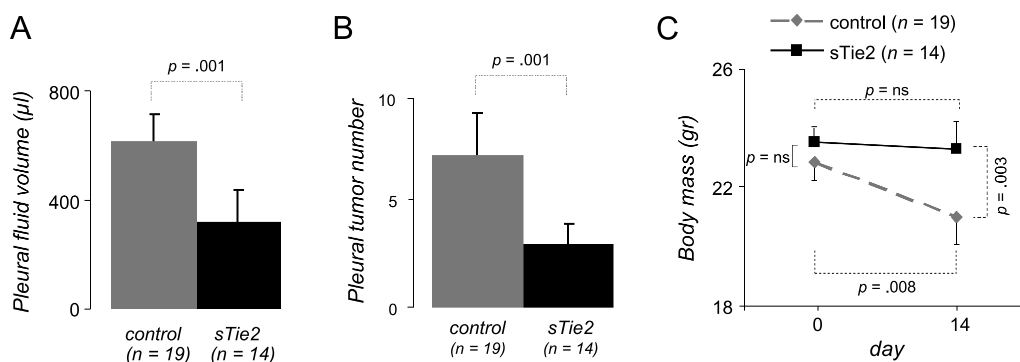


**Figure 1.** sTie2 treatment inhibits pleural fluid accumulation, intrapleural tumor dissemination, and tumor-associated cachexia. sTie2-treated animals had significantly less pleural fluid (A) and pleural tumor implantations (B). In addition, treatment with the inhibitor resulted in reduced weight loss compared to controls (C). *Columns*, *points* indicate mean; *bars*, SEM; *P*, probability value; *n*, sample size; ns, not significant.

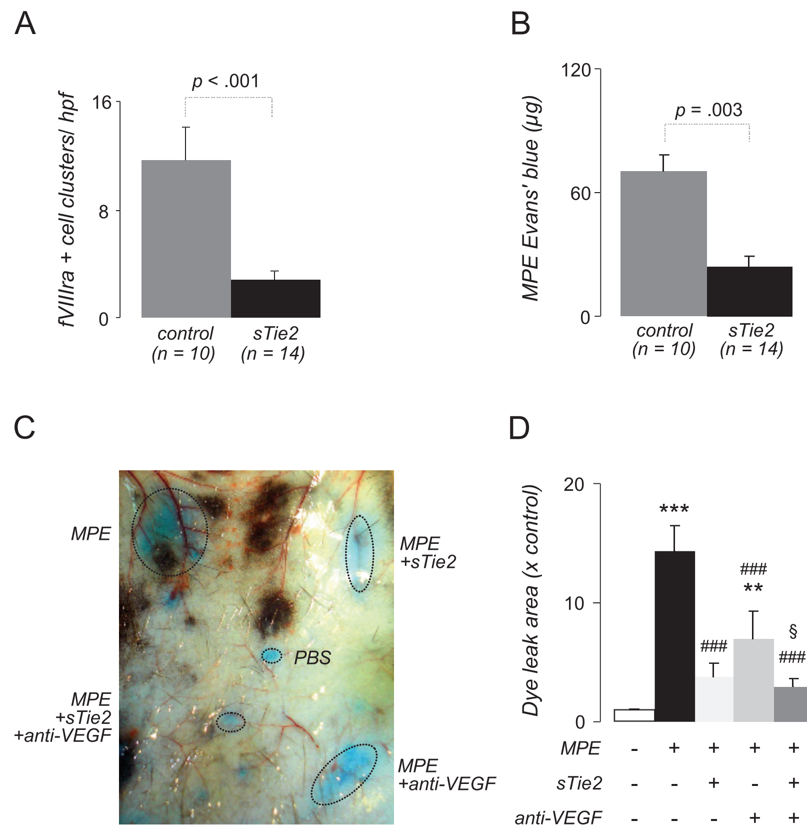


**Figure 2.** sTie2 treatment suppresses pleural tumor angiogenesis and vascular hyperpermeability in the pleura and the skin. (A) sTie2-treated animals were characterized by reduced pleural tumor angiogenesis as assessed by immune-labeling of fVIIIra. (B) Determination of pleural fluid levels of albumin-binding Evans' blue after i.v. injection of the dye demonstrated that sTie2-treated mice were characterized by markedly reduced vascular leakiness of albumin compared with the controls. (C) Seven C57B/6 mice received intradermal injections of PBS (negative control) or cell-free MPE fluid with or without sTie2 and/or anti-VEGF neutralizing antibody at different spots of the shaved dorsal skin. Inverted dermis of representative mouse with test sites in dashed circles. (D) Summary of data obtained from experiment described under (C). sTie2 reduced the vascular leakage induced by MPE fluid and had an additive effect on the reduction of permeability caused by VEGF neutralization ($^{**}P < .01$ and $^{***}P < .001$ compared with PBS; $^{###}P < .001$ compared with MPE; $^{\S}P < .05$ compared with MPE + anti-VEGF). *Columns* indicate mean; *bars*, SEM; *P*, probability values; *n*, sample size.

### *sTie2 Induces Tumor Cell Apoptosis* In Vivo

Tumor cell proliferation *in vivo* assessed by PCNA immunolabeling of pleural tumor tissue was not impaired by sTie2 treatment (Figure 4*A*). In contrast, *in vivo* tumor cell apoptosis assessed by TUNEL was significantly increased by sTie2 treatment (Figure 4*B*). The mean ± SEM TUNEL-positive cells per high-power field were 19 ± 2.8 in sTie2-treated animals and 7.8 ± 1 in the control animals ($P < .001$). *In vitro*, treatment of LLC cells with sTie2 for 24 and 48 hours have no effect on tumor cell viability assessed by MTS assay (data not shown).

## Discussion

In this study, we assessed whether angiopoietin/Tie2 signaling participates in the pathogenesis of MPE. We postulated that the systemic administration of a compound that prevents the interaction of the angiopoietins with their receptor would reduce MPE formation in mice. This proved to be the case: inhibition of the angiopoietin/Tie2 axis suppressed pleural fluid accumulation, impaired tumor dissemination in the pleural cavity, and halted tumor-induced cachexia. These effects were linked to the attenuation of pleural inflammation, reduction of VEGF and IL-6 local release, partial inhibition of malignancy-associated tumor angiogenesis, and pleural vascular hyperpermeability as well as induction of tumor cell apoptosis.

Malignant pleural effusion develops mostly as a result of increased pleural fluid production, which, in turn, is secondary to mutually dependent biological phenomena including an ongoing angiogenic process and the associated enhanced vascular permeability and pleural inflammation [3,5–8,20]. The present study demonstrates that angiopoietin/Tie2 axis blockage impairs new vessel formation in the tumor, attenuates pleural vascular leakage and decreases recruitment of inflammatory cells. More interestingly, a Miles assay in the mouse skin disclosed that the propermeability effects mediated by angiopoietin/Tie2 signaling are independent and complementary to that exerted by VEGF. The above observations are in concert with the established role of the axis in the regulation of angiogenesis, vascular permeability, and inflammation [9,10,12–15]. In this regard, angiopoietins, along with VEGF, are central to the regulation of tumor angiogenesis [10]. It is classically considered that angiopoietin 2 overexpression in the tumor microenvironment destabilizes the existing vasculature to allow microvessel formation from the surrounding host vessels, whereas angiopoietin 1 acts as a maturation and stabilization signal [9]. Strict temporal regulation of angiopoietin expression is required for tumor angiogenesis to be accomplished because sustained angiopoietin 2 expression disrupts vessel formation and inhibits tumor growth [21]. However, the exact role of individual angiopoietin family members

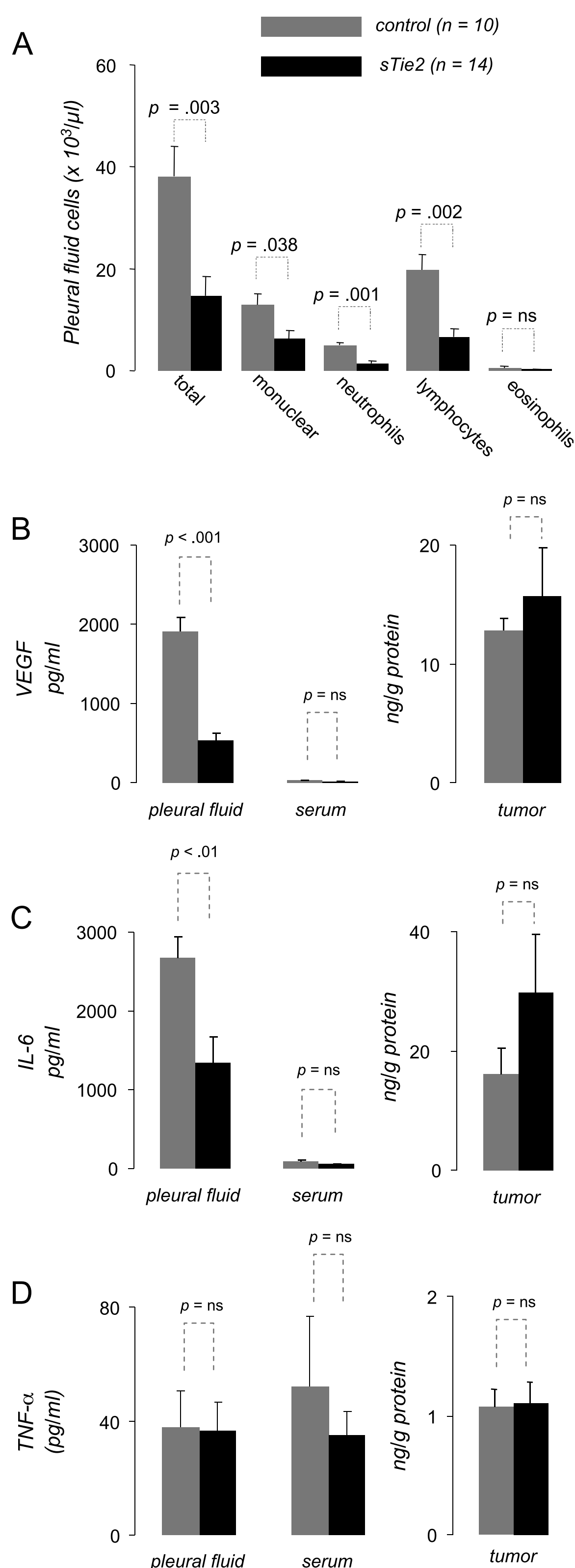


**Figure 3.** sTie2 downregulated the expression of proangiogenic and proinflammatory mediators in the tumor microenvironment. (A) Nucleated and differential cell counts from control and sTie2-treated mice. (B) Vascular endothelial growth factor levels in MPE and serum from mice treated as in Figure 1 ($n$ = 8 per data point). (C) Interleukin 6 levels in MPE and serum from mice treated as in Figure 1 ($n$ = 8 per data point). *Columns* indicate mean; *bars*, SEM; *P*, probability values; ns, not significant.

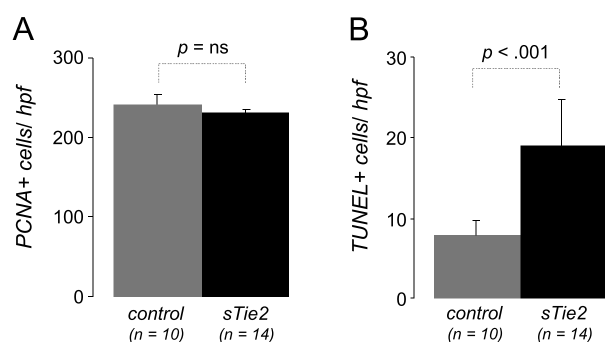


**Figure 4.** sTie2 treatment enhances tumor cell apoptosis *in vivo*. Pleural tumor tissue from control and sTie2 treated as evaluated for PCNA (A) and TUNEL (B) immunoreactivity. sTie2 treatment did not affect tumor cell proliferation *in vivo* but significantly increased tumor cell apoptosis *Columns* indicate mean; *bars*, SEM; *P*, probability values; *n*, sample size.

is still controversial [22]. In addition, angiopoietin 2 increases vascular permeability of the existing vasculature [15], an effect that, along with the leakage from newly formed, yet immature microvessels, contributes to fluid exudation in tumor and surrounding tissues, including the pleural cavity. We believe that the robust antiangiogenic [23,24] and antipermeability [15] properties of sTie2 explain for the most part the MPE-inhibiting effect of the compound in our system. Interestingly, enhanced tumor cell apoptosis was observed in treated animals albeit *in vitro* cell viability was not affected by sTie2, suggesting that the inhibitor blocks a survival-promoting event that exclusively occurs *in vivo*. Hence, most likely tumor cell apoptosis is secondary to tissue hypoxia, which results from impairment of tumor angiogenesis. One could thus propose that MPE-limiting effects of sTie2 are secondary to the reduction of pleural tumor burden. However, the observation that pretreatment of pleural fluid from control mice with sTie2 reduced MPE-induced vascular permeability, and given the central role of vascular leakage in MPE pathobiology, it is reasonable to assume that the compound exerts a direct inhibitory effect in pleural fluid accumulation. From a clinical perspective, we believe that the ideal treatment of patients with MPE should target both pleural fluid production and the tumor *per se*.

Along with their role in angiogenesis, angiopoietins have been recently implicated in the regulation of inflammation, an increasingly recognized factor in the pathogenesis of MPE [6–8]. Although angiopoietin 1 was initially reported as an anti-inflammatory factor [25,26], it was subsequently shown that it may augment adhesion onto endothelial cells and chemotaxis of Tie2-expressing neutrophils [11,12]. Similarly, angiopoietin 2 was shown to promote neutrophil recruitment at sites of acute inflammation [11–13] and to induce local inflammation and plasma leakage when injected in mouse paw [15]. More interestingly, Tie2 is also expressed by and mediates the angiopoietin 2–induced recruitment of a subset of blood monocytes with potent angiogenic properties at the tumor microenvironment [14,27–29]. We recently were able to study MPE-associated pleural inflammation using a novel model of the disease in immunocompetent mice that permits the development of a host antitumor immune response [6–8]. Using this model, we demonstrate in the present study that the influx of inflammatory cells in the pleural cavity and the pleural fluid concentrations of VEGF and IL-6 were remarkably decreased in sTie2-treated animals with MPE. Interestingly, the inhibitor did not impair VEGF and IL-6 expression by the tumor. It is thus reasonable to speculate that

a significant portion of VEGF and IL-6, both important for MPE formation [5,20], comes from the inflammatory cells invading the pleural space. From the data presented above, it follows that sTie2 treatment might affect tumor angiogenesis and pleural vascular permeability not only directly, that is, by intervening on the Tie2 signaling in endothelial cells, but also indirectly, through a potent inhibitory effect on MPE-associated inflammatory response.

Whereas the present study demonstrates a potent antiangiogenic, antipermeability, and anti-inflammatory effect of sTie2 in mice with MPE, it is limited by the fact that it does not reveal the exact molecular events influenced by the compound. Because LLC cells are known to express both angiopoietins 1 (although weakly) and 2, this difficulty is inherent to the nonselective nature of sTie2 that binds all members of the angiopoietin family [30]. More intriguingly, different angiopoietins interact with the naturally occurring receptor to mediate complementary and often opposing effects [9,10]. Accordingly, whereas angiopoietins 1, 3, and 4 elicit Tie2 phosphorylation (activation), angiopoietin 2 may act both as a Tie2 agonist and as an antagonist depending on the cellular context [10,31–36]. The agent used in the present study prevents both angiopoietins 1 and 2 binding to their receptor and abolishes Tie2 phosphorylation on endothelial cells [9,37]. The last action can partly explain our findings because Tie2 activation is a crucial proangiogenic molecular event [9,10]. We furthermore speculate that the angiopoietin 2–neutralizing properties of sTie2 may also be important for MPE suppression and that angiopoietin 2 is part of the complex mechanism that promotes MPE for the following reasons: 1) Excess amounts of angiopoietin 2 but not angiopoietin 1 are found in the pleural fluid of patients with MPE [16]. 2) Locally upregulated angiopoietin 2 is required to initiate tumor-associated angiogenesis [10]. 3) The attenuation of pleural vascular permeability in animals treated with the compound and the reduction of the permeability potential of MPE fluid conferred by sTie2 (skin Miles assay) should be most likely attributed to angiopoietin 2 blockade because angiopoietin 2, but not other members of the angiopoietin family, has permeability-promoting properties [15]. In contrast, both angiopoietins 1 and 4 have been shown to inhibit vascular leakage [25,37,38]. 4) sTie2 suppressed experimental ocular angiogenesis, a process that, similar with the formation of MPE, is characterized by angiopoietin 2 overexpression [23]. However, we would like to emphasize that, whatever the exact mechanisms, our observations illustrate the importance of the angiopoietin/Tie2 axis in MPE pathogenesis and suggest that blockade of the pathway effectively suppresses pleural fluid accumulation and intrapleural tumor dissemination.

Although this study is the first to report on the effect of angiopoietin/Tie2 axis blockade in MPE progression, similar interventions have been previously shown to downregulate angiogenesis and halt tumor growth in other models of malignant disease [39–42], as well as benign conditions characterized by neovascularization [23,24]. However, in most of the above studies, the inhibitor was administered with intratumoral injections [39,40], it was systemically overexpressed through the use of intravenously injected adenoviruses [24,42], or it was produced by stably transfected tumor cells [41]. Systemic administration of the inhibitor has been only used to suppress retinal neovascularization in mice [23]. Hence, our study is the first to demonstrate that intermittent systemic administration of sTie2 is effective against tumor-associated angiogenesis and can halt cancer growth. Because our treatment protocol is more clinically relevant than the above-mentioned inhibition techniques, we suggest that our findings pave the road for clinical studies using angiopoietin/Tie2 signaling blockade to treat MPE.

In conclusion, the findings reported here indicate that angiopoietin/Tie2 axis participates in MPE formation, promoting tumor angiogenesis, tumor-associated pleural inflammation, local release of VEGF and IL-6, and enhanced pleural vascular permeability. Our results warrant further research to explore the role of individual angiopoietins and probably to define the clinical efficacy and safety of targeting angiopoietin/Tie2 signaling to treat patients with MPE.

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