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Role of angiopoietins in mesothelioma progression

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ABSTRACT

Background and objective: Anti-angiogenic treatment has been recently shown to be clinically beneficial for mesothelioma patients. Angiopoietins-1 and -2 are key regulators of tumor angiogenesis. Ang-1 is mainly known to promote angiogenesis and vessel stability, while Ang-2 could serve as an antagonist of Ang-1 causing vessel regression and destabilization or enhance angiogenesis in a context-dependent manner. We hypothesized that Ang-1 would promote and Ang2 would halt experimental mesothelioma by affecting tumor angiogenesis.
Methods: To examine the effects of angiopoietins in mesothelioma angiogenesis and in vivo growth we constructed Ang-1 or Ang-2 overexpressing AE17 and AB1 mesothelioma cells and implanted them in the respective syngeneic animals. We also explored the clinical relevance of our observations using the human tumoral mRNAseq data available in the TCGA database.
Results and conclusions: Ang-1 promotes mesothelioma angiogenesis and growth while the effect of Ang-2 is context-dependent. Low Ang-1 levels in human mesotheliomas are associated with the epitheloid subtype. Tumors of high Ang-1, or concurrent high Ang-2 and VEGF expression present high PECAM-1 and CDH5 expression, markers of vascularity and vascular stability, respectively. Our results highlight the importance of angiopoietins in mesothelioma pathophysiology and pave the way for the clinical development of novel anti-angiogenic strategies.

1. Introduction

MalignantPleural Mesothelioma (MPM) is an aggressive tumor arising from the mesothelium of the pleural cavity and is characterized by poor prognosis. A burst of mesothelioma cases is expected to occur in the upcoming decades since asbestos (the main cause of mesothelioma) is still used in many populous countries [1]. Noteworthy, the developed countries (where asbestos use has been already banned) might be still under mesothelioma threat since modern fibrous material such as carbon nanotubes might also be involved in mesothelioma development [2,3]. No treatment has so far been substantially beneficial for patients with MPM [1]. Anti-angiogenic treatments have given promising results [4] implying a strong dependence of mesothelioma progression on tumor angiogenesis. A phase III clinical trial has already outlined the superiority of the combination of anti-angiogenic and standard chemotherapy over chemotherapy alone [5]. Angiopoietins-1 to -4 (Angs) are key regulators of tumor angiogenesis, but Ang-1 and -2 are the most extensively studied in the context of tumor biology [6]. They exert their actions mainly through binding to a tyrosine kinase receptor (Tie2) which is principally expressed by endothelial cells [7]. Ang-1 activates Tie-2 and promotes vessel maturation. Ang-2 causes vessel regression and destabilization or enhances angiogenesis in a context-dependent manner [8,9]. Little is known about the role of angiopoietins in mesothelioma progression. It has been shown that Ang-1 could enhance Tie-2-expressing mesothelioma cell proliferation and migration in vitro and that high Ang-1 serum levels are related to shorter patients' survival [10].

Based on the above, we aimed to investigate the role of angiopoietins (-1 and -2) in mesothelioma progression. We hypothesized that Ang-1 would promote and Ang2 would halt experimental mesothelioma

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by affecting tumor angiogenesis. Our hypothesis was tested using Ang-1 and Ang-2 overexpressing mesothelioma cells in two syngeneic models of pleural mesothelioma [11]. We subsequently attempted to explore the clinical relevance of our observations in the human tumor mRNAseq data that are freely available in the "The Cancer Genome Atlas" (TCGA) database.

2. Materials and methods

2.1. In vitro studies

2.1.1. Creation of Ang-1 and-2 overexpressing clones

Cells: We used AE17 and AB1 murine mesothelioma cells [11]. Both cell lines express Ang-1 and -2 as well as Tie-2. AB1 cells express more Ang-2 while AE17 cells express more Ang-1 than AB1 cells. AB1 cells and AB1 tumors present higher VEGF secretion levels [12]. Overexpression plasmids of murine Ang-1 and Ang-2 were constructed upon amplification of the complete coding sequences of mouse Ang-1 and Ang-2 genes (GenBank NM_009640 and NM_007426, respectively). Overexpression plasmids and respective vectors were stably transfected into mesothelioma cells. Overexpressing cell clones were maintained in the presence of blasticidin (10 μg/ml). Angiopoietin overexpression was confirmed by western blot and Real-time PCR as previously described [12]. Clones that overexpressed a 3–5 fold angiopoietin -1 or -2 were selected in order to better represent the magnitude witnessed among patients mesothelioma tumors (roughly estimated according to TCGA data). Potential effects of angiopoietin overexpression in cell cycle and viability was verified by DNA content analysis upon PI staining and MTS, respectively.

2.2. In vivo studies

Mice were purchased from Hellenic Pasteur Institute (Athens, Greece) and were housed at the Animal Model Research Unit of Evangelismos Hospital, (Athens, Greece) receiving food and water ad libitum. Experiments were approved (Decision No: 789/13-02-2014) by the Veterinary Administration Bureau, Prefecture of Athens, Greece under compliance to the national law and the EU Directives.

AE17 cells overexpressing Ang-1 (AE17Ang1over), Ang-2 (AE17Ang2over) and control cells (AE17vector) were intrapleurally injected (5×10^5 cells/mouse) to 8–10 week-old C57BL/6 mice (5–8 mice per group). Similarly, AB1 cells overexpressing Ang-1 (AB1Ang1over), Ang-2 (AB1Ang2over) and control cells (AB1vector) were intrapleurally injected (5×10^5 cells/mouse) to 8–10 week-old Balb/c mice (5–8 mice per group). In each case, animals were euthanized 14 days after pleural delivery of tumor cells. Pleural fluid, tumors, lungs and blood were collected and stored for subsequent analysis. Pleural fluids were retrieved and quantified; mesothelioma tumors were collected and weighed.

2.3. Immunohistochemistry and immunofluorescence

For immunohistochemistry, formalin-fixed, paraffin-embedded tumor tissue sections were stained with anti-PCNA antibody (Santa Cruz Biotechnology) for evaluation of tumor cell proliferation. Tumor cell apoptosis was assessed using the TUNEL assay as previously described [13]. For immunofluorescence, tumors were fixed, permeabilised (0.25% Triton-X) and subsequently stained for the presence of CD31 (BD Biosciences, Athens, Greece) and a-SMA (Sigma-Aldrich, Steinheim, Germany) for endothelial and pericyte cell staining, respectively. Vessel networks were examined under a confocal microscope (TCS SP5 confocal microscope (Leica Microsystems)). Images were subsequently analyzed for vessel density, vascular area and pericytes vessel coverage using ImageJ software (National Institutes of Health, Bethesda, MD).

2.4. Human mesothelioma studies

Data concerning Ang-1, Ang-2, VEGF, PECAM-1, CDH5 mRNA levels and overall survival, obtained from 87 patients with MPM, were retrieved from "The Cancer Genome Atlas" (TCGA) public access database (gdc.cancer.gov) [14]. Patients were divided to four groups according to their Ang-2 and VEGF tumoral mRNA levels. They were first divided according to their VEGF levels: Those whose VEGF RNA-Seq by Expectation-Maximization (RSEM) values were lower to 1314,83 (1st quartile) were characterized as 'low', and those with RSEM higher to 4095,37 (3rd quartile) were considered to be 'high'. These patients were subsequently subdivided to high or low Ang-2 if their Ang-2 RSEM levels were above or below the median, respectively.

2.5. Statistics

All values are presented as mean ± standard error of mean (SEM). Differences between groups were evaluated using the one-way ANOVA or Mann Whitney, as appropriate. Descriptive analysis was performed as well as correlation using the Pearson or Spearman correlation coefficient, as appropriate. N-Way tabulation was also performed using the data derived from TCGA public-access data base. Ang-1 or Ang-2 mRNA was considered as an independent variable, while overall survival was the dependent one. Age, sex, histological type and drug therapy were tested as confounding factors. P values < 0.05 were considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences v.13.0.0 (IMB, Armonk, NY).

3. Results

3.1. Characterization of angiopoietin overexpressing mesothelioma clones

As presented in Fig. 1, we have created AB1 and AE17 mesothelioma cell clones that stably overexpress Ang-1 or -2. We subsequently evaluated any changes in tumor cell proliferation rates upon angiopoietin overexpression by cell cycle analysis upon propidium iodide staining. No changes in cell cycle phases was witnessed (data not shown), suggesting that Ang overexpression does not affect mesothelioma cell proliferation in vitro.

3.2. Ang-1 overexpression promotes mesothelioma tumor growth. Ang-2 overexpression favors AB1 and abrogates AE17 tumor progression in vivo

In order to investigate whether angiopoietin overexpression affects mesothelioma progression in vivo we used two orthotopic and syngeneic murine models of mesothelioma developed by our team [11]. Ang-1 overexpression significantly promoted mesothelioma growth in both models (Fig. 2A). Interestingly, Ang-2 overexpressing clones behaved differently between the two models. In specific, Ang-2 overexpression abrogated AE17 mesothelioma growth, and favored AB1 tumors (Fig. 2A). Additionally, animals bearing AE17 Ang-2 overexpressing tumors also exhibited 90% lower pleural fluid volumes compared to control ones (Fig. 2B).

3.3. Tumor derived angiopoietins sculpt tumor vessel network by affecting vascularity and vessel stability

We then asked whether angiopoietins impact key structural features of mesothelioma vasculature. We thus investigated whether angiopoietin overexpression affects tumor vascularity (expressed by vessel density and the ratio of vascular area to total tumor area) and vascular stability (expressed by the percentage of vascular walls covered by pericytes). In AE17 mesotheliomas, Ang-1 overexpression conferred a significant increase in vessel density (Fig. 3A) and pericyte coverage (Fig. 3C), while Ang-2 overexpression exerted a strong anti-angiogenic effect, reducing vessel density, area and pericyte coverage (Fig. 3A–C).

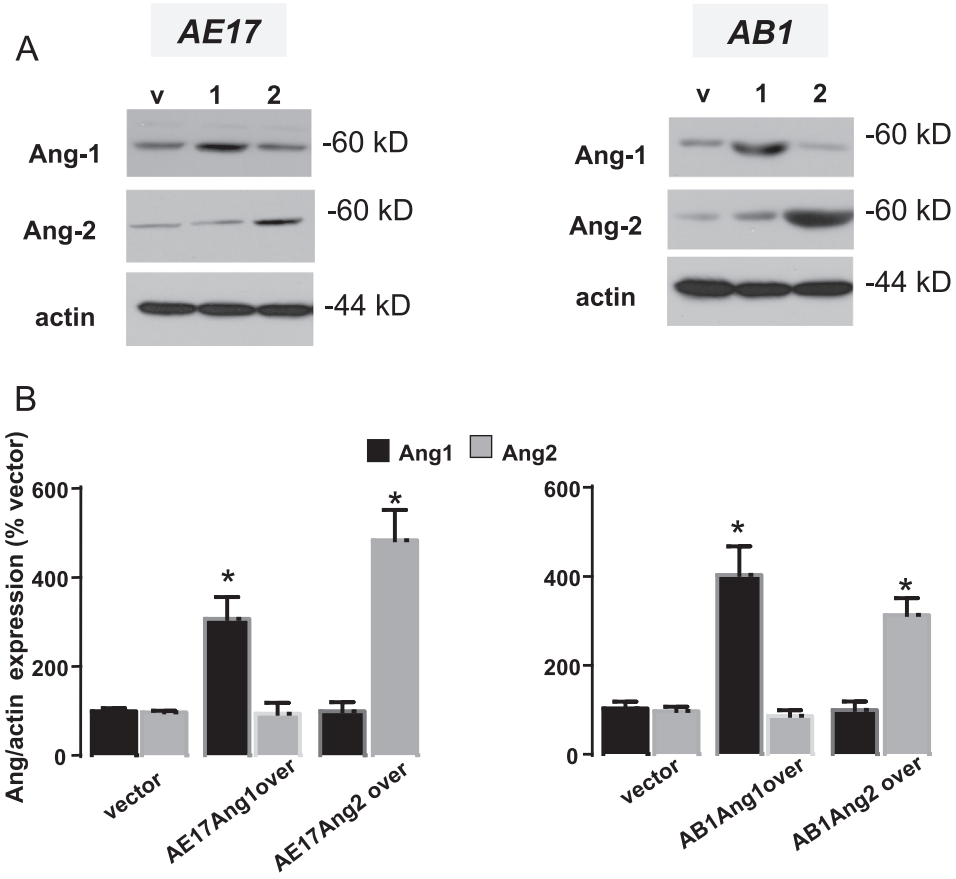


Fig. 1. Creation and characterization of Angiopoietin overexpressing mesothelioma clones. AE17 and AB1 mesothelioma cells were stably transfected with Ang-1 or Ang-2 overexpressing plasmids, or empty vector and overexpression was verified by western blot. Results were normalized to actin. (A) Representative blots. (B) Results of densitometric analysis. Data presented as mean ± SEM, n = 3, $^{*}p < 0.05$ compared to respective vector, $^{\#}p < 0.05$ compared to AB1 vector.

In AB1 mesotheliomas, although Ang-1 overexpression greatly expanded total vessel area (Fig. 3C), it had no effect on the density or the maturity of the new vessels (Fig. 3A and C). On the contrary, Ang-2 overexpression significantly enhanced both tumor vessel area and pericyte coverage (Fig. 3B, C and E lower panel) in AB1 mesotheliomas.

3.4. Angiopoietin overexpression affects in vivo mesothelioma cell proliferation and apoptosis

We subsequently investigated whether tumoral angiopoietin overexpression affects tumor cell proliferation and apoptosis rates. Ang-1 overexpressing AE17 tumors presented higher proliferation rates and lower tumor cell apoptosis compared to control ones (Fig. 4A and B). On the other hand, AE17 Ang-2 overexpressing tumors exhibited significantly higher tumor cell apoptosis (Fig. 4B). Both angiopoietin overexpression reduced tumor cell apoptosis in AB1 mesotheliomas (Fig. 4B), while Ang-2 overexpressing tumors showed enhanced proliferation rates (Fig. 4A).

3.5. Tumoral Ang-1 mRNA level is associated with the more aggressive mesothelioma phenotype and correlates with markers of increased angiogenic density and stability. Tumors having high Ang-2 and VEGF levels display increased angiogenesis and stability

Having shown that angiopoietins significantly affect progression of murine mesotheliomas by modulating tumor vasculature, we subsequently asked whether these observations could be scaled up to mesothelioma patients. We thus retrieved the freely available results of RNAseq analyses of mesothelioma patients' tumors from the TCGA databank. We first, examined whether Ang-1 or -2 levels in tumors are associated with patients' survival. Multivariate statistical analysis regarding 87 patients with mesothelioma, revealed that neither Ang-1, nor Ang-2 expression is an independent predictor of survival. Among the four factors tested (age, sex, drug therapy and histological type), in line with previous observations [15], epithelioid type was found to be an independent predictor of longer survival (Fig. 5A). Nevertheless, a strong association between low Ang-1 mRNA levels and epithelioid type was observed (Fig. 5A).

We subsequently examined whether angiopoietin tumor expression is associated with PECAM-1/CD31 expression (marker of tumor vascularity) and expression of CDH5 gene which codes for VE-cadherin (marker of vessel stability). There was a significant correlation between Ang-1 and PECAM-1 expression as well as Ang-1 and CDH5 expression in mesotheliomas (Fig. 5B and C). No significant association was found among Ang-2 and the aforementioned genes (data not shown). Since Ang-2 overexpressing tumors behaved differently in the two models and given the fact that AE17 and AB1 produce different amount of VEGF [12] we evaluated Ang-2 in relation with VEGF levels of mesotheliomas. Patients with high Ang-2 and high VEGF levels had higher PECAM-1 levels compared to those with low Ang-2 and high VEGF, or those with high Ang-2 and low VEGF (Fig. 5D). Additionally, patients with high Ang-2 and high VEGF had significantly higher CDH5 levels compared to those with high Ang-2 and low VEGF (Fig. 5D).

4. Discussion

The present study aimed to examine the role of angiopoietins in mesothelioma progression in vivo. We therefore constructed Ang-1 and Ang-2 overexpressing mesothelioma cells and used them in the respective syngeneic murine models. We demonstrated that: A. tumor derived Ang-1 significantly enhanced mesothelioma progression, while Ang-2 differentially affected mesotheliomas. B. Ang-1 enhanced tumor angiogenesis and vessel pericyte recruitment in AE17 mesotheliomas and expanded vessel area of AB1 tumors. C. Ang-2 reduced the vascular

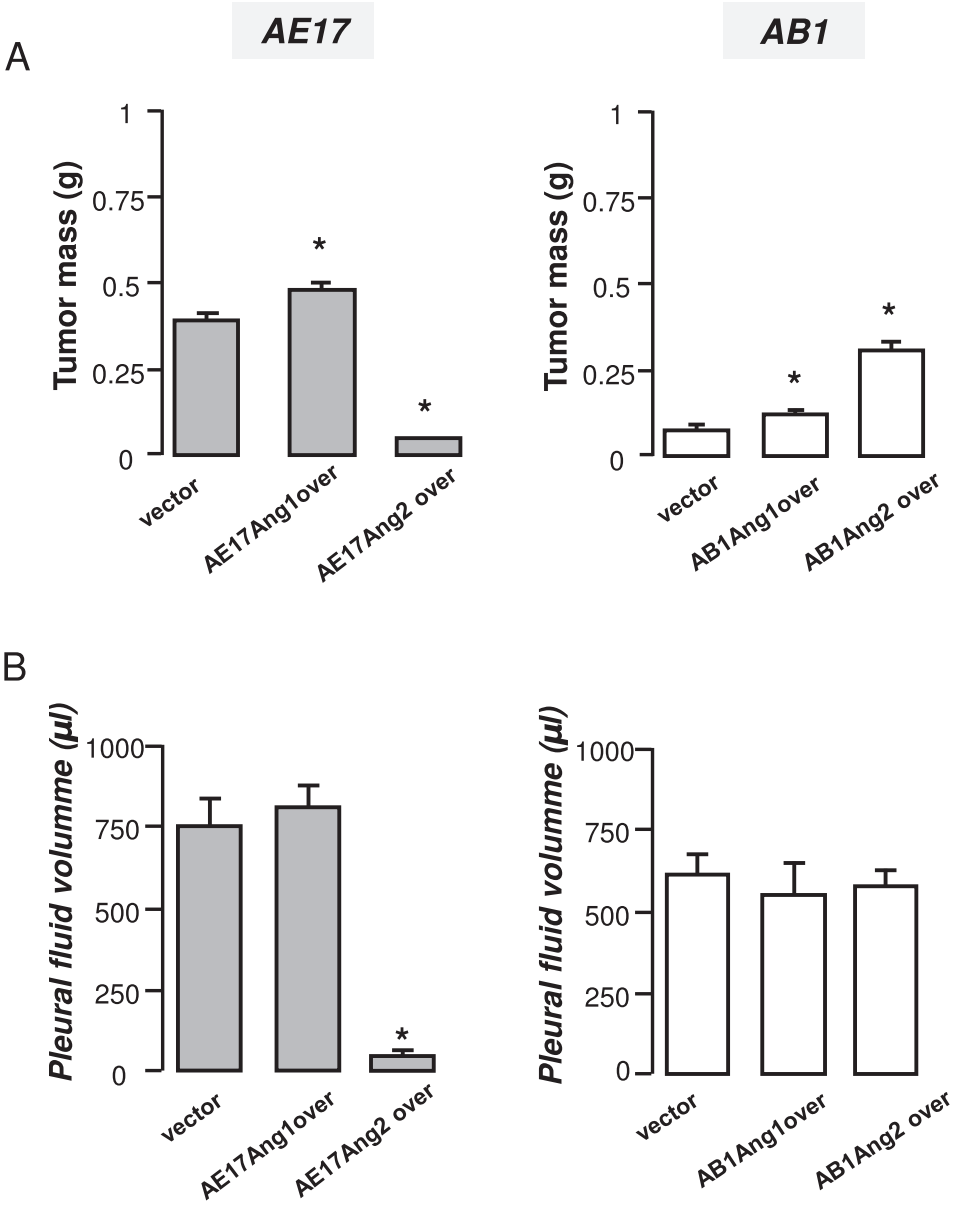


Fig. 2. Ang-1 overexpression elevates mesothelioma tumor growth. Ang-2 overexpression abrogates AE17 and favors AB1 tumor progression in vivo. 5x105 AE17vector, AE17 Ang1over, AE17 Ang2over cells were intrapleurally injected into syngeneic C57Bl/6 mice. Similarly, AB1 vector and angiopoietin overexpressing cells were implanted into Balb/c mice. Fourteen days later mice were sacrificed and mesothelioma tumors were excised and weighed (A) and pleural fluid was retrieved and quantified (B). Experiments were repeated twice. Data presented as mean ± SEM, n = 11–13, $^{*}p < 0.05$ compared to respective vector.

density of AE17 tumors but expanded vessel area of AB1 tumor vessels. D. Ang-1 overexpression promoted cell proliferation of AE17 mesotheliomas and reduces apoptosis in both models, while Ang-2 overexpression enhanced apoptosis in AE17 tumors and promoted proliferation of AB1 ones, in vivo. Analysis of the mRNA seq data of mesothelioma patients available in TCGA database revealed that low Ang-1 levels in tumors are associated with the epithelioid subtype. Finally, tumors with high Ang-1, or concurrent high Ang-2 and VEGF expression present high PECAM-1 and CDH5 expression.

This is the first study to demonstrate the impact of angiopoietins in mesothelioma progression in vivo. Our observations suggest that angiopoietins-1 and -2 are more than a 'yin and yang' case in mesothelioma progression. Ang-1 was found to favor mesothelioma growth in both models. Tumor promoting effects of Ang-1 have been also demonstrated in several preclinical models such as prostate cancer [16] and glioma [17]. In the present study, since angiopoietin overexpression was not found to impact tumor cell cycle, the effect on tumor growth and tumor cell apoptosis/proliferation should be mainly attributed to its effect on angiogenesis. Ang-1 overexpression fortified tumors with a more efficient network that provided the tumor cells with all nutrients and oxygen needed for their growth [6]. Although Ang-1 promoted tumor growth in both models, tumor vessel networks were differentially affected. In AE17 tumors, overexpression of Ang-1 enhanced vessel density and stabilized vessels promoting pericyte recruitment. The latter were also more tightly associated with the endothelial cells, compared to control tumors. On the other hand, in AB1 tumors, Ang-1 overexpression enlarged vessel area but had no effect on vessel density or pericyte recruitment. These divergent effects of Ang-1 can most likely be attributed to the different background VEGF concentration between the two models used in the present study [12]. High VEGF, which characterizes the AB1/Balb-c but not the AE17/C57bl-6 model, can partially reverse the vessel-stabilizing effect of Ang-1 [18] and at the same time act synergistically with Ang-1 to expand vessel area as observed in a rat's fat pad model of angiogenesis [19]. It should

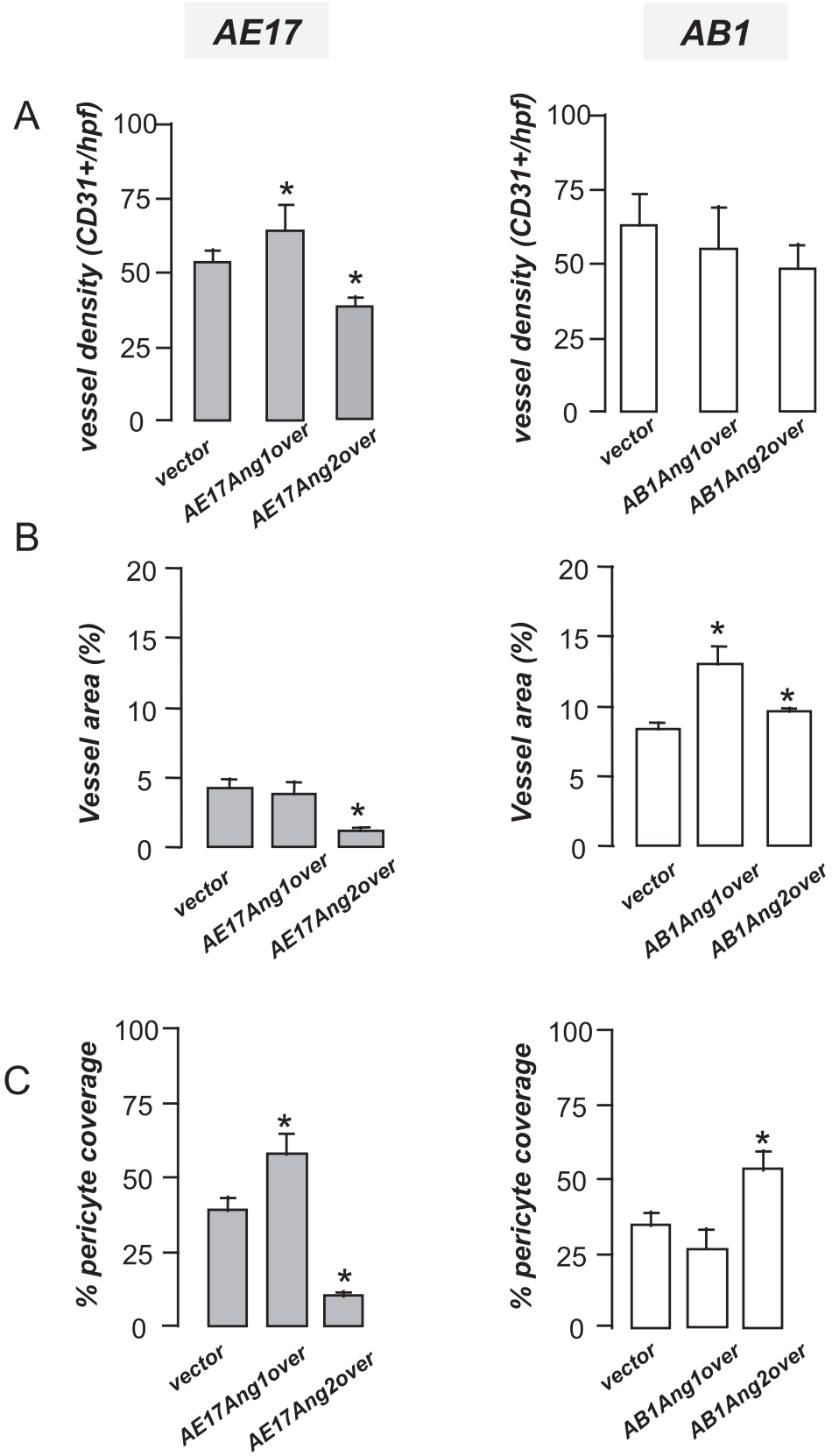


Fig. 3. Tumor derived Angiopoietins sculpt tumor vessel network by affecting vessel density, area and stability. Tumors of all groups were immunofluorescently stained for CD31 and aSMA expression and analyzed for proportional vessel density (A), vessel area (B) and pericyte coverage (C).Data presented as mean ± SEM, n = 5–8, $^{*}p < 0.05$ compared to respective vector.

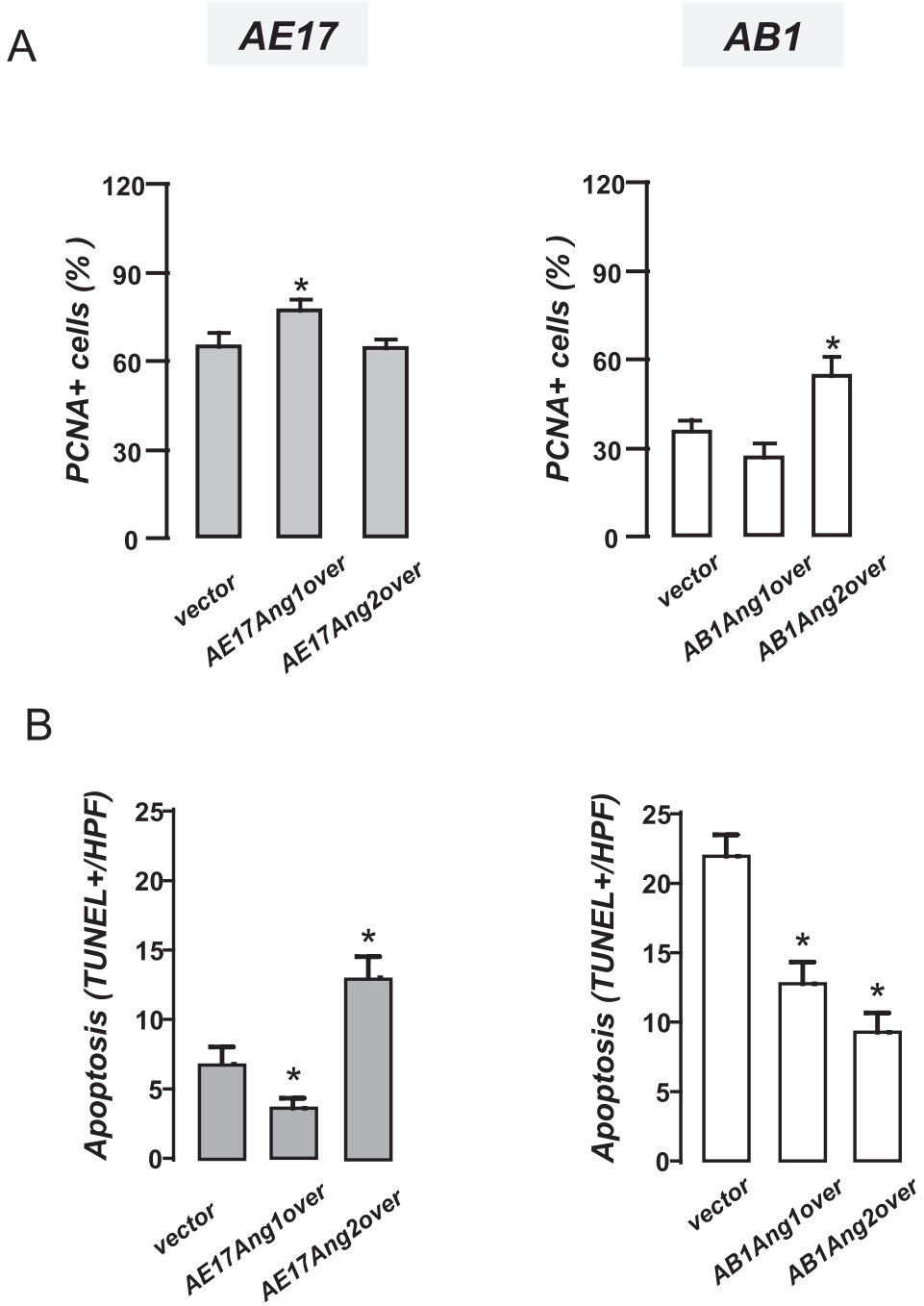


Fig. 4. Angiopoietin overexpression affects tumor cell proliferation and apoptosis of mesotheliomas. Tumor tissue sections from all groups' animals were analyzed for apoptosis by TUNEL assay (A) while tumor cell proliferation rate was evaluated by PCNA staining (B). Data presented as mean ± SEM, n = 6–9, $^{*}p < 0.05$ compared to vector. HPF: High Power Field.

be however noted, that the effect of Ang-1 in the AE17/C57bl-6 model was more consistent with the observation in human mesotheliomas: using TCGA data we found a significant correlation between tumor Ang-1 levels and the levels of both PECAM-1, a marker of tumor vascularity [20] and CDH5, a marker of tumor vessel stability [21].

In contrast to Ang-1, Ang-2 differentially affected mesothelioma growth in the two models. In specific, it halted AE17 mesothelioma growth and angiogenesis and favored those of AB1 ones. This discrepancy could be also attributed to the high background levels of VEGF in AB1 tumors [12]. In relation to this, while Ang-2 causes vessel regression in a low VEGF environment [22,23] it can also stimulate angiogenesis when VEGF is abundant [24,25]. Observations on human mesothelioma data corroborate our mouse findings on VEGF-Ang-2 functional inter-relationship. Indeed, among tumors with similar VEGF levels, those with higher Ang-2 presented higher PECAM-1 levels (marker of tumor vascularity) and tumors with high VEGF and high Ang2 exhibited the highest levels of CDH5, a marker of tumor vessel stability. Summing up, both angiopoietins can functionally affect mesothelioma growth through the regulation of tumor angiogenesis. Ang-1 exerts a supportive role, while Ang-2 has a dual effect which is probably context-dependent.

From a clinical point of view, our findings clearly display the importance of angiopoietins in the pathogenesis of mesothelioma and support the notion that anti-angiopoietin agents could be useful for

A

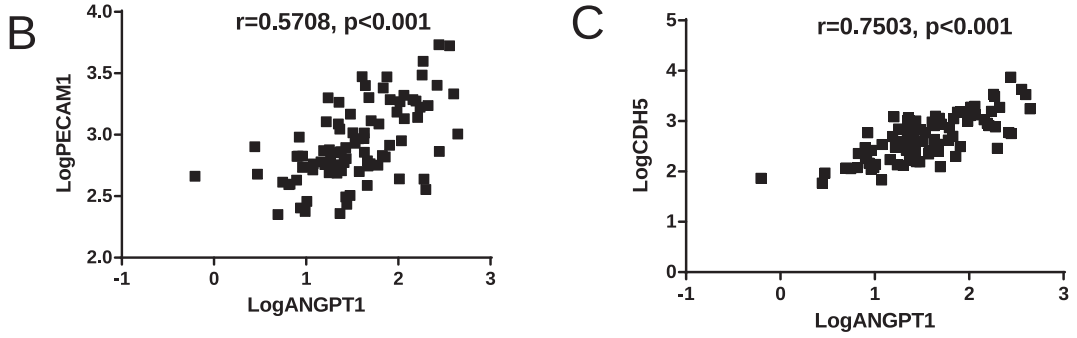
Table 1: Conditional table for DUMMYALIVE =0:

Count		TYPEEPTHELOID 0	1	Total
ANGPT1_284	[0, 100)	5	17	22
	[100, 200)	6	0	6
	[200, 300)	0	1	1
	[300, 400)	0	0	0
	[400, 500)	0	0	0
	Total	11	18	29

Measures of Association	Value
Phi Coefficient	0.658873
Cramer's V	0.658873
Contingency Coefficient	0.550187

Table Statistics	df	Value	Prob
Pearson X2	2	12.58930	0.0018
Likelihood Ratio G2	2	14.91384	0.0006

WARNING: Expected value is less than 5 in 66.67% of cells (4 of 6).



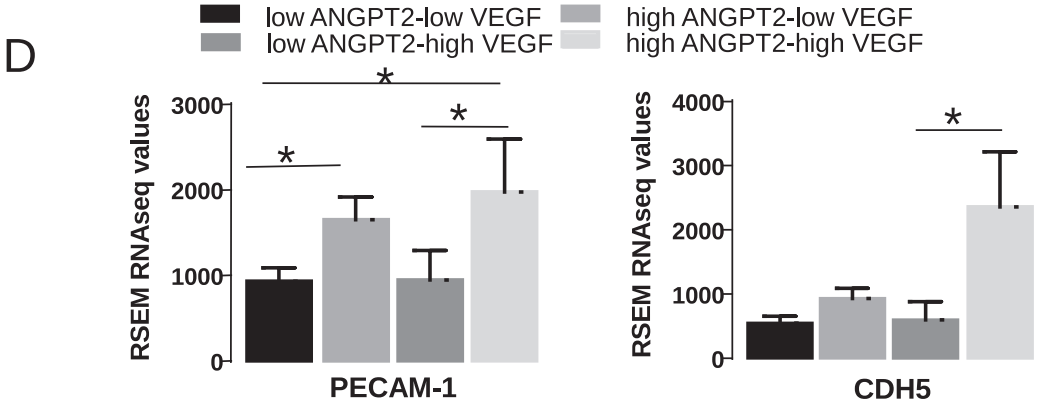


Fig. 5. Ang-1 mRNA levels in tumors of mesothelioma patients is associated with the more aggressive phenotype. High tumoral Ang-1 levels correlate with increased angiogenic density and stability. Patients with high tumoral Ang-2 and VEGF levels also have increased angiogenesis and stability Ang-1, Ang-2, VEGF, PECAM-1 and CDH5 RSEM mRNA values and overall survival, of 87 mesothelioma patients were retrieved from TCGA. (A) N-Way tabulation was performed on Ang-1, Ang-2 and clinical data. Ang-1 or Ang-2 mRNA was considered as an independent variable, while overall survival was the dependent one. Age, sex, histological type and drug therapy were tested as confounding factors. P values < 0.05 were considered significant. (B, C) Patients' tumoral Ang-1 RSEM values were correlated to PECAM-1 and CDH5. N = 87, r = Pearson's correlation. (D) Patients were divided to four groups according to their Ang-2 and VEGF tumoral mRNA levels and differences in PECAM-1 and CDH5 RSEM mRNA values were evaluated among groups. Data presented as mean ± SEM, n = 7–14, $^{*}p < 0.05$.

mesothelioma treatment, a strategy that has already been proven to be beneficial to other malignancies [26–28]. Most importantly, our observations imply that the effects of anti-angiopoietin treatment might be dependent on the tumor profile of angiogenic mediators. More specifically, while targeting Ang-1 could halt mesothelioma growth, anti-Ang-2 agents might either suppress or promote mesotheliomas, dependent on their VEGF content. Interestingly, in a recent preclinical study of our group, a dual angiopoietin inhibitor suppressed AB1 (VEGF overexpressing) tumors [12] in which both Ang-1 and Ang-2 were here found to be tumor promoting. On the other hand, it did not affect low-

VEGF AE17 tumors, in which most likely its suppressive effect caused by Ang-1 neutralization was counterbalanced by a tumor-promoting effect caused by Ang-2 blockade.

In conclusion, preclinical evaluation of the role of angiopoietins in mesothelioma progression demonstrated that Ang-1 promotes mesothelioma angiogenesis and growth while the effect of Ang-2 is context-dependent. This work highlights the central role of angiopoietins in mesothelioma pathophysiology and paves the way for the clinical development of novel anti-angiogenic strategies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.08.006.

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