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Targeting of VEGF-dependent transendothelial migration of cancer cells by bevacizumab

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ABSTRACT

Cancer progression is often associated with the formation of malignant effusions. Vascular endothelial growth factor (VEGF) is a major regulator of vascular permeability and has been implicated as mediator of tumor progression.

We examined the production and secretion of VEGF₁₆₅ in various primary cancer cells derived from malignant effusions, and the role of exogenous VEGF₁₆₅ as a mediator of effusion formation. VEGF₁₆₅ was constantly secreted by all cultured tumor cells in an mTOR-dependent manner, as it was inhibited by the mTOR inhibitor rapamycin. Secreted VEGF₁₆₅ showed functional activity by inducing endothelial leakiness and tumor cell-transendothelial migration *in vitro*, effects which could be reverted by the anti-VEGF antibody bevacizumab.

Thus, mTOR inhibitors as well as bevacizumab should be considered as potential agents in cancer patients suffering from malignant effusions.

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1. Introduction

Cancer is one of the leading causes of morbidity and mortality in industrialized countries. Despite increasing efforts to understand the biology of the disease and to develop novel therapeutic strategies, the prognosis in most advanced neoplasms still remains poor (Cresanta, 1992; Parkin, 2001;

Gralow et al., 2008). During the last decade, our knowledge about clonal evolution of neoplastic cells, oncogene-dependent signaling, and the mechanisms that underly progression of the disease, has increased significantly (Hunter, 1997; Hahn et al., 1999; Carmeliet and Jain, 2000; Jones and Baylin, 2002). As a result, new treatment strategies and several new drugs have been developed, with the aim to

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prevent progression of advanced tumor lesions. These drugs act directly on tumor cells or/and may influence the tumor-associated microenvironment (Morin, 2000; Shawver et al., 2002; Daub et al., 2004; Schwartz and Shah, 2005; Gasparini et al., 2005).

A well established hypothesis is that (neo)angiogenesis plays an important role in tumor formation, tumor progression, and metastasis (Carmeliet and Jain, 2000; Kirsch et al., 2000; Bergers and Benjamin, 2003; Carmeliet, 2000; Dvorak, 2002; Ferrara, 2004). A key regulator of angiogenesis in normal and neoplastic tissues is VEGF (Kirsch et al., 2000; Bergers and Benjamin, 2003; Dvorak, 2002; Ferrara, 2004; Prager et al., 2004a, b, 2009). This cytokine activates endothelial cells within minutes (Prager et al., 2004a, b) and induces the formation of new blood vessels and thereby facilitates local tumor growth (Gasparini et al., 2005; Kirsch et al., 2000; Bergers and Benjamin, 2003; Dvorak, 2002; Ferrara, 2004). In addition, VEGF₁₆₅ is a well established mediator of vascular permeability, and therefore was originally termed vascular permeability factor (Dvorak et al., 1995). It has also been described that neoplastic cells in diverse tumors express VEGF₁₆₅ (Carmeliet and Jain, 2000; Gasparini et al., 2005; Kirsch et al., 2000; Bergers and Benjamin, 2003; Boockch et al., 1995; Mercurio et al., 2004; Weigand et al., 2005). In several instances, malignant cells also display VEGF receptors suggesting autocrine growth regulation (Boockch et al., 1995; Mercurio et al., 2004; Weigand et al., 2005; Giatromanolaki et al., 2006; Ochiuni et al., 2006; Timoshenko et al., 2007). All in all, VEGF₁₆₅ is considered an important factor contributing to systemic and/or local progression in various tumors. Therefore, several attempts have been made to develop therapeutic strategies targeting VEGF₁₆₅ or VEGF receptors (Ferrara, 2005; Herbst, 2006; Kerr, 2004; Yang et al., 2003; Hurwitz et al., 2004; Sandler et al., 2006; Miller et al., 2007; Tonra and Hicklin, 2007). Likewise, the VEGF₁₆₅-targeting antibody bevacizumab has been described to counteract progression of malignant lesions in patients with advanced colon cancer, renal cell carcinoma, non small cell lung cancer, and breast cancer (Kerr, 2004; Yang et al., 2003; Hurwitz et al., 2004; Sandler et al., 2006; Miller et al., 2007; Tonra and Hicklin, 2007).

In certain malignancies, disease progression and metastasis are often associated with the formation of malignant effusions. Effusion formation is considered to develop on the basis of a locally disturbed tumor-microenvironment, with 'leakiness' of local cell layers and transmigration of tumor cells (Yano et al., 1997, 2000; Hamed et al., 2004). In various studies, VEGF₁₆₅ has been implicated as a key mediator contributing to the formation of malignant effusions in solid tumors (Yano et al., 2000; Hamed et al., 2004). However, the exact mechanisms underlying effusion formation remain unknown.

We and others have recently shown that the anti-VEGF antibody bevacizumab profoundly counteracts effusion formation in patients with various solid tumors (Gerber and Ferrara, 2005; Pichelmayer et al., 2006; Numnum et al., 2006). The aims of the present study were to examine the production and secretion of VEGF₁₆₅ in neoplastic cells derived from malignant effusions, to explore the mechanisms of VEGF₁₆₅ production, the possible role of VEGF₁₆₅ as a regulator of effusion formation, and the effects of specific pharmacologic inhibitors.

2. Results

2.1. VEGF₁₆₅ is expressed in various tumor cells and cancer cell lines

As determined by immunocytochemistry, all tumor cell lines and all primary tumor cells analyzed were found to express the VEGF₁₆₅ protein (Fig. 1). As expected, the intensity of staining varied from donor to donor. Likewise, VEGF₁₆₅ was strongly expressed in neoplastic cells of 4 breast tumor patients, whereas in 2 patients with breast cancer, tumor cells expressed low amounts of VEGF₁₆₅. Fig. 1 shows examples of VEGF₁₆₅ staining obtained with primary tumor cells and cell lines. Preincubation of the anti-VEGF₁₆₅ antibody with recombinant VEGF₁₆₅ (absorption control) resulted in a negative stain (not shown). A summary of all staining results obtained with neoplastic cells is shown in Tables 1 and 2 for primary cells and Table 3 for tumor cell lines.

2.2. Detection of VEGF mRNA in primary neoplastic cells and tumor cell lines

To demonstrate expression of VEGF mRNA, tumor cell lines and primary tumor cells were subjected to RT-PCR analysis. In these experiments, VEGF mRNA was detectable in all primary tumor cell samples (Table 2) and in all tumor cell lines examined (Table 3). These data suggest that neoplastic cells produce VEGF₁₆₅ in a constitutive manner. By contrast, not all cell lines were found to express KDR (Table 3). As assessed by RT-PCR, substantial amounts of KDR transcripts were detectable in MDA-MB-231 cells which is consistent with previous data (Timoshenko et al., 2007), and less abundant amounts of KDR mRNA were found in A427 cells, HCT-8 and EGI-1 cells, whereas in the other cell lines, KDR mRNA was not detected (Table 3). In MDA-MB-231 cells, we also confirmed expression of surface KDR on neoplastic cells using flow cytometry (Table 3). Unexpectedly, however, surface KDR was not detectable in A427 cells and EGI-1 cells. Another interesting observation was that KDR mRNA was also found in 3 of 3 primary tumor cell populations examined, i.e. in one breast carcinoma, one gastric cancer, and one pancreatic carcinoma (patients #14, #15, and #16 as described in Table 2).

2.3. Production and secretion of the VEGF₁₆₅ protein in tumor cell lines

To further demonstrate production of VEGF₁₆₅ in tumor cells, lysates and supernatants from tumor cells were prepared before VEGF₁₆₅ expression was quantified by ELISA. In all cell lines tested, we found measurable amounts of VEGF₁₆₅ in cell lysates as well as in cell-free supernatants (Table 2). In time course experiments, VEGF₁₆₅ was found to accumulate in supernatants of primary tumor cells and in supernatants of all cell lines tested, suggesting constant production and secretion of the cytokine (Supplemental Figure 1). Levels of VEGF₁₆₅ in tumor cell lysates tested did not change significantly during the culture period (not shown), which is consistent with permanent secretion of expressed VEGF₁₆₅.

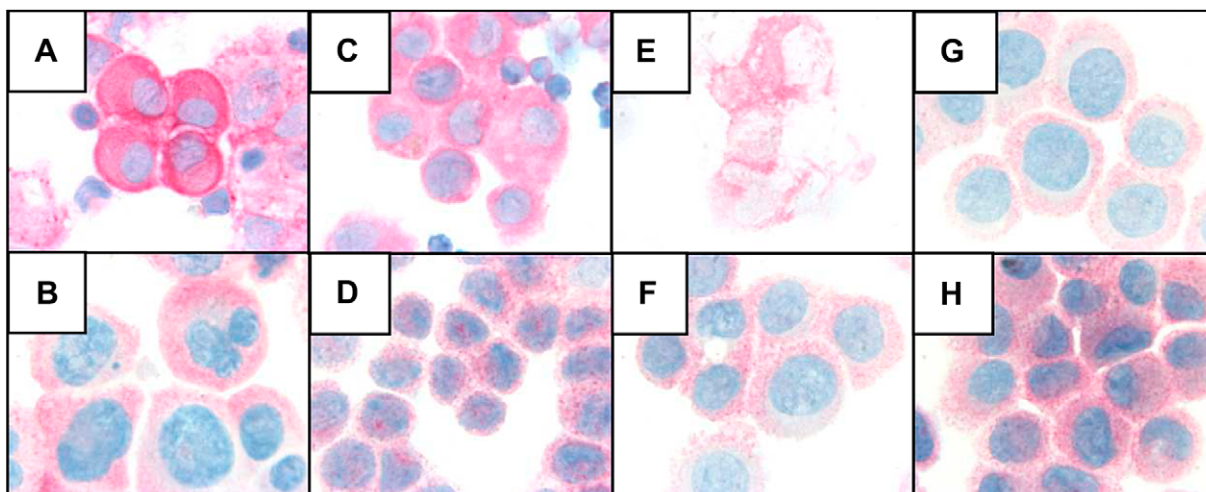


Figure 1 – Immunocytochemical detection of VEGF in primary tumor cells and cell lines. Tumor cells were spun on cytospin slides and examined for expression of VEGF₁₆₅ by immunocytochemistry using an anti-VEGF antibody as described in Section 2. VEGF₁₆₅ was detected in primary breast carcinoma cells in patient #10 in Table 1(A), in the breast carcinoma cell line MDA-MB-231 (B), in primary gastric carcinoma cells in patient #11 (C), as well as in the gastric carcinoma cell line MKN-45 (D). Tumor cells either derived from pancreatic carcinoma of patient #8 (E), from the pancreatic carcinoma cell line BxPC-3 (F), from the colon carcinoma cell line HCT-8 (G), or from the lung carcinoma cell line A427 (H) were spun on cytospin slides and were examined for VEGF₁₆₅ expression using a monoclonal anti-VEGF₁₆₅ antibody. VEGF₁₆₅ was detected in all tumor cells examined. Figures (magnification $\times 400$ each) were prepared using an Olympus DP11 camera connected to an Olympus BX50F4 microscope equipped with $100\times/1.35$ UPlan-Apo objective lens (Olympus, Hamburg, Germany). Images were prepared using Adobe Photoshop CS2 software version 9.0 (Adobe Systems, San Jose, CA) and processed with PowerPoint software (Microsoft, Redmond, WA).

2.4. Effects of rapamycin on VEGF₁₆₅ protein expression in tumor cells

Recent data suggest that the mammalian target of rapamycin (mTOR) is involved in the regulation of expression of VEGF₁₆₅ in neoplastic (hematopoietic) cells (Mayerhofer et al., 2002, 2005). Therefore, we were interested whether rapamycin also affects expression of VEGF₁₆₅ in tumor cells, which were derived from malignant effusions. To address this question, we first confirmed the effect of rapamycin on phosphorylation of S6 kinase1, a downstream signalling effector of mTORC1 pathway (Supplemental Figure 2). Next, we incubated primary tumor cells and tumor cell lines with two different doses of rapamycin (20 or 100 nM) for a time period of up to 6 days (cell lines) or of up to 10 days (primary tumor cells). In these experiments, rapamycin substantially decreased VEGF₁₆₅ expression in supernatants of primary tumor cells as well as in various tumor cell lines (Fig. 2). Thus, rapamycin had an inhibitory effect on VEGF₁₆₅ production and secretion already at 20 nM (Fig. 2). We observed that rapamycin affected VEGF mRNA levels indicating that rapamycin inhibited VEGF synthesis (Supplemental Figure 4).

2.5. Evaluation of apoptosis in tumor cell lines and primary neoplastic cells after incubation with rapamycin

We could previously show that rapamycin not only blocks VEGF₁₆₅ secretion but also affects growth and survival of neoplastic cells in myeloid leukemias (Mayerhofer et al., 2005). In the present study, we were therefore interested whether rapamycin would induce apoptosis in solid tumor cells and in corresponding tumor cell lines. To address this question, we

incubated a number of tumor cell lines (MDA-MB-231, BxPC-3, A-427, HCT-8, EGI-1) with various concentrations of rapamycin. However, over the dose-range tested (2–200 nM), rapamycin did not induce apoptosis, i.e. did not promote the numbers of apoptotic cells compared to control medium (not shown), nor significantly affected cell proliferation (Supplemental Figure 3). From these data we conclude that in these set of experiments rapamycin led to a direct reduction of VEGF expression and did not affect viability of tumor cells examined.

2.6. Recombinant human VEGF₁₆₅ as well as tumor-derived VEGF₁₆₅ promote leakiness and tumor-transmigration in endothelial cell monolayers

VEGF₁₆₅ is known to mediate vascular permeability as an initial step in angiogenesis. In the present study, we examined the effects of tumor-derived VEGF₁₆₅ (from supernatants either of tumor cell lines or primary tumor cells, which were isolated from malignant effusions) on endothelial leakiness and tumor cell transmigration using a transwell chamber assay. By this set of experiments we aimed to assess a potential functional role of tumor-derived VEGF₁₆₅ in malignant effusion formation. Both, recombinant as well as tumor-derived VEGF₁₆₅ were found to increase vascular permeability determined by an increase in phenol red diffusion through the endothelial monolayer. Bevacizumab was found to inhibit enhanced permeability mediated by recombinant VEGF₁₆₅ or tumor cell supernatants containing VEGF₁₆₅ (Fig. 3A). From these data we conclude that VEGF₁₆₅ is the tumor cell-derived factor, which drives trans-endothelial diffusion.

Table 1 – Patients' characteristics.

Patient no.	Age years	Gender f/m	Diagnosis	Investigated tumor site	Previous therapy
01	68	f	Breast adenocarcinoma	Pleural effusion	Letrozole, Fluvestrant, FEC 3×, Docetaxel 5×, Gemcitabine 5×
02	63	f	Breast adenocarcinoma	Pleural effusion	Late relapse after 10 years
03	68	f	Breast adenocarcinoma	Ascites	FEC 6×, CMF 6×, Tamoxifen, Anastrozole, Novantron
04	40	f	Breast adenocarcinoma	Pleural effusion	CMF 6×, Trastuzumab 25×, Paclitaxel/Carboplatin 3×, Temozolomid, Fluvestrant, Goserelin, Paclitaxel 1×, Lapatinib
05	83	m	Parotis adenocarcinoma	Pleural effusion	Gemcitabine 3×
06	70	m	Oesophageal adenocarcinoma	Ascites	Cisplatin/5-FU 3×, FLEP 2×
07	72	f	Ovarial cystadenocarcinoma	Ascites	Carboplatin/Paclitaxel 6×, Carboplatin/Docetaxel 6×, Doxorubicin liposomal 9×, Topotecan 6×, Gemcitabine/Paclitaxel 9×, Cisplatin/Vinorelbine 2×
08	82	f	Pancreatic adenocarcinoma	Ascites	Gemcitabine 5×
09	70	m	Pancreatic adenocarcinoma	Ascites	No previous therapy
10	47	f	Breast adenocarcinoma	Pleural effusion	Tamoxifen, Epirubicin/Paclitaxel 1×, CMF/Goserelin 14×, Xeloda low dose continuous, Fluvestrant/Goserelin 17×, Vinorelbine/Goserelin 1×, Anastrozole/Goserelin, Doxorubicin liposomal 3×, Aromasin, Docetaxel 3×
11	68	f	Gastric signet-ring cell tumor	Ascites	DCF 6x
12	56	m	Lung adenocarcinoma	Pleural effusion	Gemcitabine/Cisplatin 4x
13	59	m	Lung adenocarcinoma	Ascites	Gemcitabine/Carboplatin 4x, Gemcitabine 1x
14	56	f	Breast adenocarcinoma	Pleural effusion	Doxorubicin/Cyclophosphamid 3x, Anastrozole, Capecitabin
15	71	m	Gastric carinoma	Pleural effusion	No information available
16	72	m	Pancreatic adenocarcinoma	Ascites	Gemcitabine 6x, Erlotinib/Capecitabin, Oxaliplatin/Erlotinib/Capecitabin 6x, Gemcitabine/Erlotinib/Capecitabin 3x, Irinotecan/Raltitrexed 3x, Mitomycin C 1x.

No patient had received previous bevacizumab (Avastin®).

f, female; m, male; ca, carcinoma; 5-FU, Flourouracil; FLEP, Cisplatin - 5-Flourouracil-Leucovorin-Etoposid; FEC, Flourouracil-Epirubicin-Cyclophosphamid; CMF, Cyclophosphamid-Methotrexat-Flourouracil; DCF, Docetaxel-Cisplatin-Flourouracil; AC, Epirubicin-Cyclophosphamid.

Next, we were interested whether an increase in endothelial leakiness upon VEGF₁₆₅ stimulation is accompanied with increased transmigration of tumor cells. In these experiments, a biotin-labelled tumor cell line derived from a malignant effusion (MDA-MB-231) was placed in the upper chambers on top of the endothelial monolayer, before VEGF₁₆₅ (in the presence or absence of 400 ng/ml bevacizumab) was placed in the lower chambers. We found that recombinant human VEGF₁₆₅ as well as tumor-derived VEGF₁₆₅ promoted transmigration of MDA-MB-231 cells through endothelial monolayers (Fig. 3B). Bevacizumab was found to block tumor-derived VEGF₁₆₅-induced cell migration as well as recombinant human VEGF₁₆₅-induced cell migration of MDA-MB-231 cells. From these data, we conclude that VEGF₁₆₅ was the responsible factor inducing migration. Finally, we were able to show that VEGF₁₆₅ promotes trans-endothelial migration of primary tumor cells in our double chamber assay (Fig. 4). As expected, bevacizumab was found to inhibit VEGF₁₆₅-induced transmigration of primary tumor

cells in a similiar manner as bevacizumab blocked transmigration of MDA-MB-231 cells (Fig. 4).

3. Discussion

Tumor progression and metastasis are often accompanied by the formation of malignant effusions, which is associated with very short survival and poor quality of life. Such effusions are often resistant to conventional antineoplastic therapy and thus represent a major challenge in oncology. VEGF₁₆₅ is a well established mediator of vascular permeability (Dvorak, 2002; Dvorak et al., 1995) and thus a potential trigger of malignant effusion formation (Yano et al., 2000; Hamed et al., 2004). This assumption is supported by the observation that the neutralizing anti-VEGF antibody bevacizumab is an effective agent in patients with advanced cancer presenting with malignant effusions (Pichelmayer et al., 2006; Numnum et al., 2006). In the present study, we were able to show that

Table 2 – Detection of VEGF₁₆₅ in neoplastic cells derived from malignant effusions.

Patient no	Diagnosis	VEGF mRNA (RT-PCR)	VEGF protein by immunocytochemistry	VEGF protein in cell culture supernatants (ELISA)
01	Breast adenocarcinoma	+	+	+
02	Breast adenocarcinoma	+	+/-	+
03	Breast adenocarcinoma	+	+/-	+
04	Breast adenocarcinoma	+	+	+
05	Parotis adenocarcinoma	+	+	+
06	Osophageal adenocarcinoma	n.t.	+	+
07	Ovarial cystadenocarcinoma	+	n.t.	+
08	Pancreatic adenocarcinoma	-	+	+
09	Pancreatic adenocarcinoma	+	+	+
10	Breast adenocarcinoma	n.t.	+	+
11	Gastric signet-ring cell ca	+	+	+
12	Lung adenocarcinoma	n.t.	+	n.t.
13	Lung adenocarcinoma	n.t.	+	n.t.
14	Breast adenocarcinoma	+/-	+	n.t.
15	Gastric carcinoma	+	+	n.t.
16	Pancreatic carcinoma	+	+	n.t.

VEGF₁₆₅, vascular endothelial growth factor; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; ca, carcinoma; n.t., not tested. No patient had received previous bevacizumab (Avastin[®]) therapy.

in various solid tumors, neoplastic cells produce and secrete VEGF₁₆₅ in a constitutive manner, and that tumor-derived VEGF₁₆₅ induces endothelial leakiness as well as tumor cell transmigration through endothelial monolayers. Our results also show that VEGF₁₆₅-mediated tumor cell migration can be blocked by bevacizumab, confirming the role of VEGF₁₆₅ as a tumor-derived mediator contributing to the development of malignant effusions.

It is commonly accepted that neoplastic cells in a variety of different solid tumors can express VEGF₁₆₅ (Carmeliet and Jain, 2000; Boockook et al., 1995; Mercurio et al., 2004; Weigand et al., 2005; Giatromanolaki et al., 2006). In this study, we were able to show that VEGF₁₆₅ is commonly expressed by tumor cells derived from malignant effusions at mRNA- as well as protein level. We have examined tumor cells derived from malignant effusions of breast cancer, pancreatic carcinoma, ovarian cancer, esophageal carcinoma, lung cancer, colon cancer, and gastric cancer patients. In each case, isolated tumor cells as well as tumor cell lines expressed VEGF mRNA and

the VEGF₁₆₅ protein in a constitutive manner. Moreover, we were able to show that isolated primary tumor cells constantly secrete VEGF₁₆₅, resulting in a time-dependent accumulation of this cytokine in cell-free culture supernatants. All in all, these data strongly suggest that tumor cells derived from malignant effusions produce and secrete VEGF₁₆₅ in a constitutive manner.

We and others have recently shown that the production of VEGF₁₆₅ in myeloid leukemias is regulated by the mammalian target of rapamycin, mTOR, and that rapamycin not only counteracts VEGF₁₆₅ expression but also the growth and survival of leukemic cells (Mayerhofer et al., 2002, 2005). More recent data suggest that mTOR and VEGF₁₆₅ may also play a role in the pathogenesis of renal cell carcinomas and other tumor types (Cho et al., 2007; Mellado and Gascon, 2006). In the present study, we were able to show that rapamycin inhibits expression and secretion of VEGF₁₆₅ in all tumor cell types examined. In particular, exposure of cultured tumor cells to rapamycin resulted in a dose-dependent decrease in VEGF₁₆₅ levels

Table 3 – Detection of VEGF₁₆₅ in tumor cell lines.

Cell line	Tumor type	VEGF mRNA (RT-PCR)	VEGF protein (ICC) (pg/10 ⁵ cells) ^a	VEGF in lysates	VEGF in sups (ng/ml) ^a	KDR mRNA (RT-PCR)	KDR mRNA (light-cycler)	KDR protein (FACS)
MDA-MB-231	breast cancer	+	+	55.0 ± 7.3	19.9 ± 3.6	+	572.9 ± 31.7	+
EGI-1	cholangiocellular (bile duct) carcinoma	+	+	45.1 ± 13.7	14.5 ± 2.6	+/-	1.0 ± 0	-
BxPC-3	pancreatic carcinoma	+	+	154.1 ± 62.7	19.5 ± 3.8	-	0.6 ± 0.4	-
A-427	lung carcinoma	+	+	203.0 ± 57.3	52.9 ± 2.1	+/-	1.6 ± 0.8	-
HCT-8	colon carcinoma	+	+	43.3 ± 3.6	62.4 ± 10.6	+/-	0.5 ± 0.1	-
MKN-45	gastric carcinoma	+	+	n.t.	n.t.	-	1.2 ± 0.2	-

VEGF, vascular endothelial growth factor; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction. n.t., not tested; +, clearly detectable; +/- weakly expressed; -, not detectable. Light cycler data are given as fold increase mRNA levels compared to EGI-1 (set as 1.0).

^a The VEGF₁₆₅ protein was measured in cell lysates and in cell-free supernatants of cultured cell lines on day 6 by ELISA.

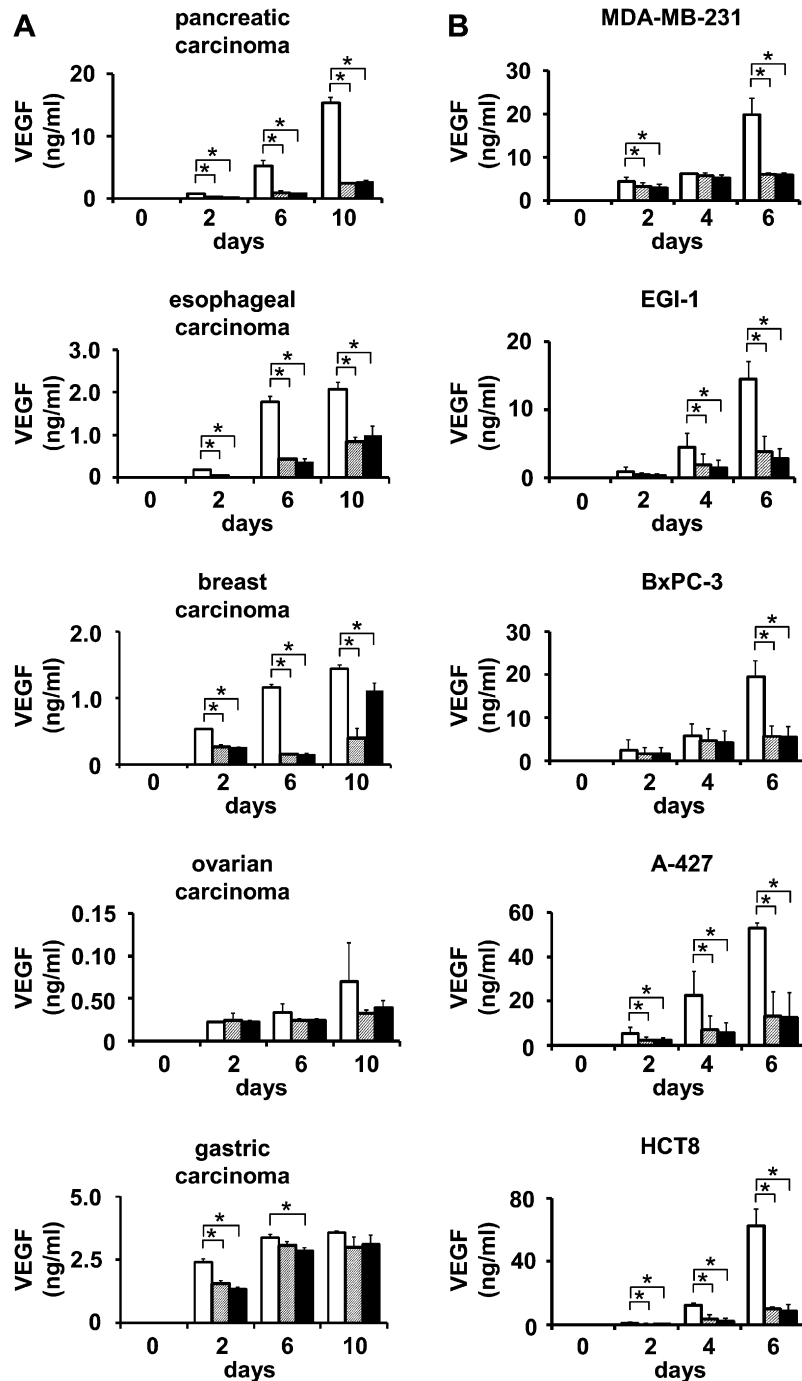


Figure 2 – Effects of the mTOR inhibitor rapamycin on VEGF₁₆₅ secretion in tumor cells. Primary tumor cells (A) and tumor cell lines (B) were cultured at 37 °C for 10 days (primary cells) or 6 days (cell lines) in the absence (open bars) or presence of rapamycin (20 nM, grey bars; 100 nM, black bars). At indicated time points, cell-free supernatants were collected and VEGF₁₆₅ concentrations were determined by ELISA. Results are given as ng/ml (VEGF₁₆₅) and represent the mean ± S.D. of triplicate cultures of primary tumor cells from each respective donor (A). Primary cells were derived from pancreatic carcinoma (patient #8 in Table 1), oesophageal carcinoma (patient #6), breast carcinoma (#10), ovarian carcinoma (#7), and gastric carcinoma (#11). VEGF₁₆₅ concentrations in supernatants of tumor cell lines (B) are given as mean ± S.D. of three independent experiments, whereby the following cell lines were examined: MDA-MB-231, EGI-1, BxPC-3, A-427, HCT8. As shown, rapamycin blocked secretion of VEGF₁₆₅ in most primary tumor cells as well as in all cell lines examined. Asterisk indicates a *p*-value < 0.05.

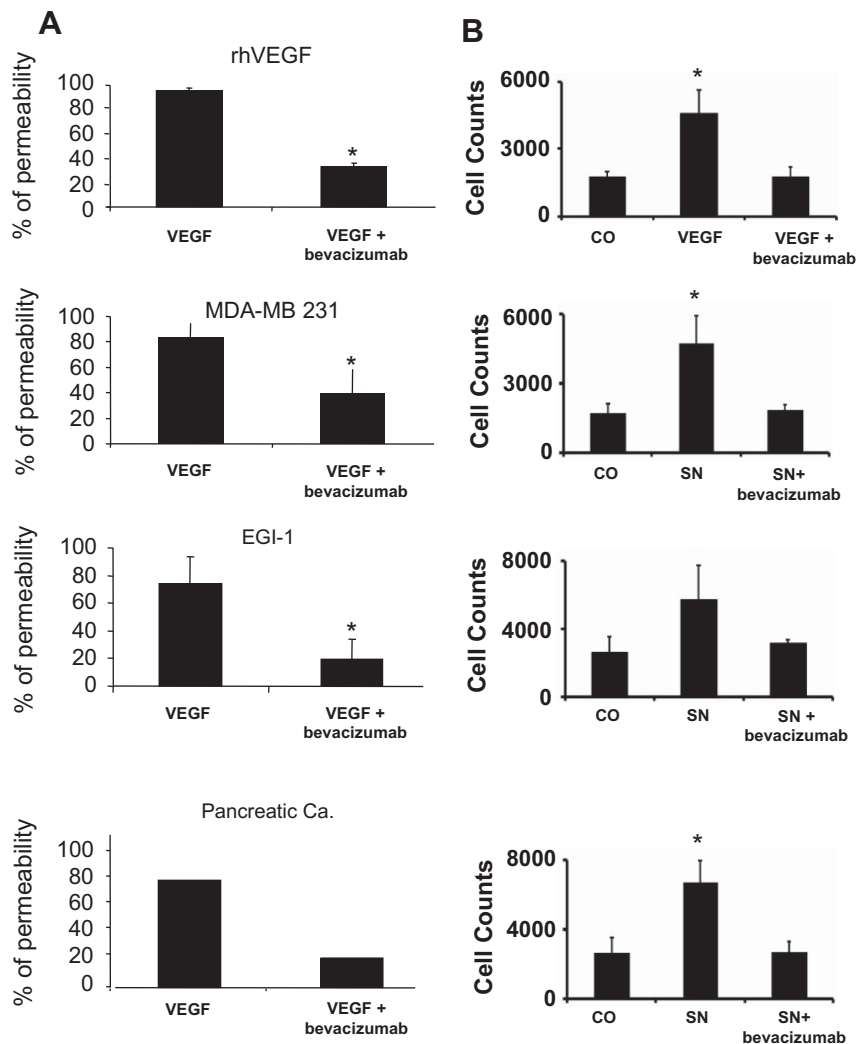


Figure 3 – (A) Tumor-derived VEGF₁₆₅ affects permeability of endothelial cell monolayers. HUVECs were seeded on a gelatine coated 3.0 μm pore size polyester membrane and were grown to confluency under serum depleted conditions. Addition of either recombinant human VEGF₁₆₅ (upper panel), or VEGF₁₆₅ secreted by tumor cell lines (middle upper panel: MDA-MB-231; middle lower panel: EGI-1) as well as VEGF₁₆₅ derived from primary tumor cells (lower panel: pancreatic cancer, patient #8) induced an increase in permeability as measured by phenol red diffusion through endothelial cell monolayers (60 min). % permeability reflects percentage of phenol red diffusion under conditions indicated compared to cell free membranes (100%). Error bars: SEM, $n = 3$; $*p < 0.05$. **(B) Effects of tumor-derived VEGF₁₆₅ on trans-endothelial monolayer migration by tumor cells.** A two-chamber assay was employed (for details see Section 2) to determine functional activity of tumor cell-derived VEGF₁₆₅ (from cell lines and primary tumor cells adapted to 5 ng/ml VEGF₁₆₅) compared to recombinant VEGF₁₆₅ (50 ng/ml) in the presence or absence of bevacizumab (400 ng/ml) on tumor cell transmigration through an endothelial cell monolayer. As control (CO), medium without VEGF₁₆₅ was applied. Biotinylated MDA-MB-231 cells were placed in upper chambers and were allowed to migrate upon recombinant human VEGF₁₆₅ (upper panel), upon VEGF₁₆₅-containing supernatants (SN) of EGI-1 cells (middle upper panel), SN of primary breast cancer cells (patient #1 in Table 1) (middle lower panel), or SN of primary pancreatic carcinoma cells (patient #8) (lower panel) stimulation. After transmigration through endothelial monolayers (37 °C for 24 h), cells were collected in lower chambers and counted under an inverted fluorescence microscopy. Results show the number of migrated cells and represent the mean \pm S.D. of three independent experiments (A, B). $*p < 0.05$ compared to CO.

measured in culture supernatants. Interestingly and importantly, in our set of experiments rapamycin did not affect *in vitro* growth or survival of cancer cells at dose-ranges (2–100 nM) and time-ranges (up to 10 days) examined. Whether higher concentrations of rapamycin blocks growth of cancer cells was not investigated and remains so far unknown. However, the pharmacologic levels of the drug that can be reached *in vivo* without major toxicity supposedly range between about 2

and 30 ng/ml (Jimeno et al., 2008). These concentrations apparently can lead to suppression of VEGF₁₆₅ expression, but not to growth inhibition. From these data, one could speculate that rapamycin in patients affects VEGF₁₆₅ expression in tumor cells and thus VEGF₁₆₅-induced progression, but would not directly affect proliferation of malignant cells.

From angiogenesis studies it is known that VEGF₁₆₅ is a key mediator of vascular permeability and thus was

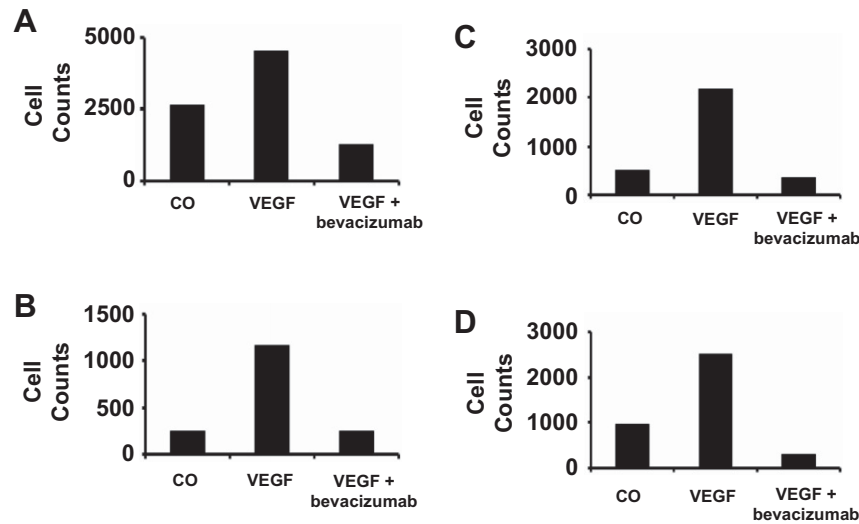


Figure 4 – (A–D) Effects of recombinant VEGF₁₆₅ on trans-endothelial migration of primary tumor cells. Biotinylated primary tumor cells from two patients with lung carcinoma (A: #12 and B: #13 in Table 1), one patient with gastric carcinoma (C: #14), and one with breast carcinoma (D: #15) were placed into the upper chambers. Control medium (CO) or recombinant VEGF₁₆₅ (50 ng/ml) with or without bevacizumab (400 ng/ml) were placed into the lower chambers as indicated. Tumor cells were allowed to transmigrate through endothelial monolayers at 37 °C for 24 h. Thereafter, migrated cells were recovered and counted by microscopy. Results show the number of migrated cells under each condition (each one experiment per donor).

furthermore suspected to be a potential trigger of malignant effusion formation in cancer (Yano et al., 2000; Hamed et al., 2004). We were therefore interested to study direct consequences of tumor-derived VEGF₁₆₅ on endothelial cell permeability and tumor cell transmigration *in vitro*. Results of our study show that recombinant VEGF₁₆₅ as well as tumor cell-derived VEGF₁₆₅ (supernatants) induce endothelial leakiness as well as transmigration of KDR-bearing tumor cells through endothelial monolayers. As expected, the effects of recombinant VEGF₁₆₅ and of tumor-derived VEGF₁₆₅ on tumor cell transmigration were effectively inhibited by the neutralizing anti-VEGF antibody bevacizumab. These observations are consistent with the clinical observation that bevacizumab counteracts the formation of malignant effusions in cancer patients (Pichelmayer et al., 2006; Numnum et al., 2006). From these data we conclude that VEGF₁₆₅ is an important mediator and potential therapeutic target of malignant effusion formation.

Malignant effusions are usually detected in advanced tumors and represent a particular medical problem. In fact, malignant effusions are often resistant to conventional cytotoxic treatment. In addition, malignant effusions supposedly represent an active site of further tumor development and origin of further metastasis. In line with this assumption, it has been described that cancer-initiating cells (cancer stem cells) are detectable in malignant effusions (Al-Hajj et al., 2003). However, the factors and conditions that regulate cancer stem cell migration into effusions remain at present unknown. From our data and from recently published data (Al-Hajj et al., 2003) one could speculate that VEGF₁₆₅ also attracts cancer stem cells and that these cells migrate into malignant effusions. Whether indeed cancers stem cells express VEGF₁₆₅ receptors (KDR) and whether these cells can also transmigrate through an endothelial monolayer is currently under investigation.

It has become increasingly evident that VEGF₁₆₅ as well as VEGF receptors represent major targets of cells forming the tumor microenvironment, and that drugs directed against VEGF₁₆₅ or its receptors may thus also influence tumor formation by disrupting interactions between tumor cells and the surrounding nutritive microenvironment (Guba et al., 2002). Together, all these data suggest that therapeutic intervention with VEGF₁₆₅ inhibitors and/or mTOR inhibitors in patients with advanced cancer, especially when associated with the formation of malignant effusions, may be a novel and promising therapeutic approach. One attractive possibility may be to combine mTOR inhibitors and bevacizumab to block both VEGF₁₆₅ secretion and VEGF₁₆₅ activity in local sites of tumor/metastasis and/or effusion formation.

All in all, our data suggest that VEGF₁₆₅ is a key regulator of malignant effusion formation in cancer patients. The effects of tumor-derived VEGF₁₆₅ can be blocked by the neutralizing anti-VEGF antibody bevacizumab, and production and secretion of VEGF₁₆₅ in tumor cells can be targeted by mTOR inhibitors. These observations may have implications for the pathogenesis of solid tumors and for the development of new therapeutic strategies.

Malignant effusions are common in advanced carcinomas and associated with short survival. Currently available therapies aim at preventing progression and relieving symptoms by puncture, local chemotherapy, or/and diuretics. However, in most cases, the effect of such therapy is short-lived and may cause adverse side-effects, whereas no specific therapy is available which is best explained by the fact that little is known about the pathogenesis of malignant effusion formation. In this article, mTOR-dependent VEGF secretion by tumor cells is characterized as a potential key-trigger of effusion formation in cancer patients, which may have

clinical implications, as agents targeting mTOR or VEGF-production in cancer cells are available.

4. Experimental procedures

4.1. Reagents

Rapamycin was purchased by Calbiochem (San Diego, CA), RPMI 1640 medium, penicillin/streptomycin, and fetal calf serum (FCS) from PAA Laboratories (Pasching, Austria), bovine serum albumin (BSA), gelatine, and phenol red from Sigma Aldrich (St. Louis, MO), recombinant human VEGF₁₆₅ from Promocell (Heidelberg, Germany), a polyclonal rabbit anti-VEGF antibody from SantaCruz (San Diego, CA), the anti-VEGF antibody bevacizumab (Avastin®) from Roche Austria (Vienna, Austria), goat anti-rabbit IgG and alkaline phosphatase complex from Biocare Medical (Concord, CA), Neofuchsin from Histofine (Nichirei, Japan), M199 medium and Dulbecco's phosphate-buffered saline (DPBS) from GIBCO Invitrogen (Auckland, New Zealand), Mayers Haemalaun from Merck (Darmstadt, Germany), a Quantikine human VEGF₁₆₅ Enzyme-linked Immunosorbent Assay (ELISA) from R&D Systems (Minneapolis, MN), RNeasy Mini Kit from QIAGEN (Hilden, Germany), First Strand cDNA Synthesis kit from Roche Diagnostics (Mannheim, Germany), HotStarTaq Master Mix (QIAGEN), and RT-PCR primers (VEGF₁₆₅, KDR, β -actin) from VBC Biotech (Vienna, Austria) or Eurofins MWG Operon (Ebersberg, Germany).

4.2. Cell lines

Cell lines used in this study were the breast cancer cell line MDA-MB-231 established from a malignant pleural effusion (Cailleau et al., 1978), the pancreatic carcinoma cell line BxPC-3, lung cancer cell line A-427, colon carcinoma cell line HCT-8, the bile duct carcinoma cell line EGI-1, and the gastric carcinoma cell line MKN-45. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics. Cell lines were passaged weekly using trypsin/EDTA (1:250-solution; PAA Laboratories).

4.3. Patients' characteristics

Primary tumor cells were obtained from malignant effusions of cancer patients in whom a diagnostic or therapeutic paracentesis was performed. A total number of 16 cancer patients were examined, namely 6 with breast cancer, 3 with pancreatic carcinoma, 1 with ovarian cancer, 1 with parotid carcinoma, 1 with esophageal carcinoma, 2 with lung cancer, and 2 suffering from gastric cancer. The patients' characteristics are shown in Table 1. Puncture material was collected and used for *in vitro* experiments after informed consent was given by patients.

4.4. Isolation and culture of primary neoplastic cells

Primary tumor cells were obtained from malignant effusions (8 pleural effusions and 8 ascites) by centrifugation in 250 ml tubes (Corning Inc, Corning, NY) at 2500 rounds per minute

(rpm) for 10 min. After centrifugation, cells were washed and recovered in RPMI 1640 medium containing 10% FCS. The presence and percentage of tumor cells were determined by Giemsa staining on cytopsin slides. Cell viability was examined by trypan blue exclusion test.

4.5. Culture of tumor cells with rapamycin and evaluation of apoptosis

Cell lines and primary tumor cells were incubated with rapamycin at various concentrations (2–200 nM) at 37 °C and 5% CO₂ for up to 10 days. Rapamycin was added every 48 h. Cell viability was determined by trypan blue exclusion test. The percentage of apoptotic cells was determined on Wright-Giemsa-stained cytopsin slides by microscopy. Apoptosis was defined according to conventional cytologic criteria (cell shrinkage, condensation of chromatin structure) as reported (Van and Den, 2002). MTT assays (Invitrogen, USA) were performed according to manufactory's protocol. 3H-thymidine incorporation assays were performed according standard operating procedures (1 μ curie [3H]thymidine per 10,000 cells seeded).

4.6. Immunocytochemistry

Immunocytochemistry was performed on cytopsin preparations of primary neoplastic cells and cell lines. VEGF₁₆₅ expression was analyzed using a polyclonal rabbit anti-VEGF₁₆₅ antibody (work dilution 1:30) and a biotinylated second-step goat anti-rabbit IgG antibody. Cytopsin slides were incubated with the primary antibody for 60 min at room temperature (RT), washed, and then incubated with the second step antibody for 30 min at RT. As chromogen, streptavidin-alkaline-phosphate complex was used. Antibody-reactivity was made visible using Neofuchsin. Cells were then counterstained with Mayer's hemalaun. The antibody reactivity was controlled by omitting the first step (anti-VEGF) antibody. In absorption control experiments, the anti-VEGF antibody was preincubated with recombinant VEGF₁₆₅ before applied.

4.7. Analysis of VEGF levels by ELISA

In typical experiments, cell lines (1×10^4 cells/ml) and primary tumor cells (1×10^5 cells/ml) were incubated with various concentrations of rapamycin (2–200 nM) in RPMI 1640 medium containing 10% FCS in 24 well plates (Corning & Costar, Corning, NY) at 37 °C for up to 6 days (cell lines) or up to 10 days (primary tumor cells). Rapamycin was replaced every 48 h. Cell lines were analyzed for VEGF₁₆₅ levels on days 0, 2, 4, and 6. Primary tumor cells were analyzed on days 0, 2, 6, and 10. VEGF₁₆₅ levels were determined in cell lysates and cell-free supernatants (after centrifugation) by ELISA following the manufacturer's instructions (R&D Systems). The detection limit of VEGF₁₆₅ by ELISA was 5 pg per ml.

4.8. Reverse transcription PCR (RT-PCR)

RT-PCR analysis was performed on neoplastic cells (cell lines and primary tumor cells) essentially as described (Vales et al., 2007). In brief, total RNA was isolated using the RNeasy Mini

Kit according to the manufacturers' instructions (QIAGEN). The following primer pairs were used: human VEGF₁₆₅ forward: 5' ATG AAC TTT CTG CTG TCT TGG G 3', VEGF₁₆₅ reverse: 5' CCG CCT CGG CTT GTC ACA TCT GC 3'; human KDR forward: 5' GTG TAA CCC GGA GTG ACC AAG GAT 3', KDR reverse: 5' GAT GTG ATG CGG GGG AGG AA 3', and as control human β -actin forward: 5' ATG GAT GAT GAT ATC GCC GCG 3', β -actin reverse: 5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC 3'. RT-PCR reactions were performed using First Strand cDNA Synthesis kit and HotStarTaq Master Mix Kit. PCR conditions were as follows: initial activation step at 95 °C for 15 min, following denaturation at 94 °C for 40 s, annealing at 58 °C for 1 min, polymerization at 72 °C for 1 min (35 cycles), and a terminal extension step at 72 °C for 10 min. PCR products were analyzed using 1% agarose gels.

4.9. Flow cytometric analysis of expression of KDR on cancer cells

In select experiments, primary tumor cells and cell lines were incubated with a PE-labelled monoclonal antibody against KDR (R&D Systems) or with a PE-labelled control monoclonal antibody (R&D Systems). Then, cells were washed and analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA, USA). For determining effects of rapamycin on p6S protein, cells (after incubation in control medium, in 0.01% DMSO or with rapamycin 100 nM, for 2 h) were fixed in formaldehyde (2%) and permeabilized with ice-cold methanol at –20 °C at least for 10 min (Supplemental Figure 2). Then cells were washed with PBS plus 0.1% bovine serum albumin and were incubated with a mouse anti-S6(pS235/pS236) antibody conjugated with Alexa Flour 647 (BD Biosciences) or with an isotype-matched control antibody (BD Biosciences). After washing cells were analyzed on a FACSCalibur (BD Biosciences).

4.10. Relative quantitative reverse transcriptase-polymerase chain reaction (Q-PCR)

For isolation of RNA Trizol reagent (Invitrogen) was used, as described previously (Pfaffl, 2001). cDNA was generated from 900 ng of total RNA with MuLV reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems). Primers were designed with PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA) using the reference mRNA sequences of respective genes from the GeneBank. Primers for Porphobilinogen-deaminase (PBGD) and β 2-microglobulin were used as described by us before (Prager et al., 2009). Q-PCR was performed by LightCycler technology using the Fast Start DNA Master Plus SYBR Green I kit for amplification and detection (Roche Diagnostics). The reactions were performed using 3 μ L DNA Master Mix Plus (containing 25 mM MgCl₂); 10.1 μ L H₂O; 0.4 μ L of each primer (10 μ M) and cDNA corresponding to 2.5 ng of total RNA used previously for reverse transcription. Relative quantification of any investigated gene was calculated by normalization to a housekeeping gene using the mathematical model by Pfaffl (2001), and presented as fold variation over the unstimulated control.

4.11. Tumor cell-endothelial cell transmigration assay

Tumor cell transmigration through intact endothelium was examined using monolayers of cultured human umbilical vein endothelial cells (HUVEC, Technoclone, Vienna, Austria) (4×10^4 cells per well) and a two-chamber assay essentially as reported (Trojanovsky et al., 2001). The integrity of HUVEC monolayers was confirmed by DAPI-stain (Vector Laboratories, Burlingame, CA). Transwell chambers were prepared using gelatine-precoated 8.0 μ m pore sized membranes (Nunc, Roskilde, Denmark). HUVEC were grown on membranes in M199 medium with 10% FCS, heparin, antibiotics, and endothelial cell growth supplement (Technoclone, Vienna, Austria) for 48 hours. Prior to use in transmigration assays, membranes were washed three times in M199 medium with 3% FCS. Before applied to upper chambers, primary tumor cells (lung cancer, $n = 2$; breast cancer, $n = 1$; gastric cancer, $n = 1$) and the VEGF receptor-2/KDR-positiv tumor cell line MDA-MB-231 were biotinylated with NHS-S-S biotin (Pierce, Rockford, IL) according to the manufacturer's protocol. After labeling, tumor cells (each 2×10^4 per well) were suspended in medium with 3% FCS, and placed into upper chambers. Lower chambers were filled with medium (plus 3% FCS) containing recombinant VEGF₁₆₅ (50 ng/ml) with or without bevacizumab (400 ng/ml). In case of MDA-MB-231-transmigration, lower chambers were also filled with supernatants of primary tumor cells or of tumor cell lines. Each supernatant was adjusted to 5 ng/ml 21 VEGF₁₆₅ by diluting in M199 medium plus 3% FCS. After 24 hours, migration membranes together with transmigrated cells were fixed in Carnoy (methanol and glacial acetic acid; 3:1 dilution), before placed upside down onto coverslips. Thereafter, cells were stained by FITC Streptavidin according to the instructions of the manufacturer (BD Biosciences Pharmingen, Erembodegem, Belgium), and then were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Cell numbers were determined by microscopy under an inverted fluorescence microscope (Axioplan-2, Zeiss, Jena, Germany)

4.12. Endothelial cell permeability assay

To confirm that tumor secreted VEGF₁₆₅ induces leakiness in HUVEC monolayers, a two-chamber permeability assay was performed as described (Jovov et al., 1991). In brief, HUVEC (2×10^4 per well) were grown to confluency on transwell polyester membranes (3.0 μ m pore size, Costar, High Wycombe, United Kingdom) precoated with 1% gelatine in M199 medium (without phenol red) supplemented with 5% FCS, heparin (EBEWE Pharma, Unterbach, Austria), and antibiotics. Before used, HUVEC were kept in M199 medium and 1% BSA for 4 h. Phenol red (200 ng/ml) was placed into lower chambers together with control medium (M199 plus 1% BSA) or control medium containing (i) recombinant VEGF₁₆₅ (50 ng/ml), (ii) tumor cell supernatants (VEGF₁₆₅ concentration adjusted to 7.5 ng/ml), (iii) tumor cell supernatants or recombinant VEGF₁₆₅ plus bevacizumab (400 ng/ml), or (iv) bevacizumab (400 ng/ml) without VEGF₁₆₅. After various time periods (30, 60, and 90 min), phenol red concentrations in upper chambers were measured at 479 nm in a spectrophotometer (NanoDrop ND-1000, PEQLAB Biotechnologie, Erlangen, Germany).

Transwell membranes without HUVEC were used to measure spontaneous diffusion.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.molonc.2010.01.002](https://doi.org/10.1016/j.molonc.2010.01.002).

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