

Vascular Endothelial Growth Factor C–Induced Lymphangiogenesis Decreases Tumor Interstitial Fluid Pressure and Tumor Growth¹

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Abstract

Characteristically, most solid tumors exhibit an increased tumor interstitial fluid pressure (TIFP) that directly contributes to the lowered uptake of macromolecular therapeutics into the tumor interstitium. Abnormalities in the tumor-associated lymph vessels are a central brick in the development and prolonged sustaining of an increased TIFP. In the current study, vascular endothelial growth factor C (VEGF-C) was used to enhance tumor-associated lymphangiogenesis as a new mechanism to actively reduce the TIFP by increased lymphatic drainage of the tumor tissue. Human A431 epidermoid vulva carcinoma cells were inoculated in NMRI nu/nu mice to generate a xenograft mouse model. Seven days after tumor cell injection, VEGF-C was peritumorally injected to induce lymphangiogenesis. Tumor growth and TIFP was lowered significantly over time in VEGF-C–treated tumors in comparison to control or VEGF-A–treated animals. These data demonstrate for the first time that actively induced lymphangiogenesis can lower the TIFP in a xenograft tumor model and apparently reduce tumor growth. This model represents a novel approach to modulate biomechanical properties of the tumor interstitium enabling a lowering of TIFP *in vivo*.

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Introduction

Elevated tumor interstitial fluid pressure (TIFP) is a common observation in cancer patients and in experimental tumor models [1–4]. The increased pressure is driven by the abnormal blood and lymphatic vasculature in the tumor tissue that is hallmarked by the highly permeable and chaotic blood vessel network and non-functional lymphatic drainage [5–7]. The enhanced TIFP is the central biophysical protagonist that hampers the enrichment of macromolecular therapeutics within the tumor [8,9].

It has been shown that tumors seem to lack a functional lymphatic vascularization and establish high interstitial fluid pressure values, while in normal tissues excessive fluid is permanently drained by lymphatic vessels [10]. Respectively, it has been reported that in tumors that exhibit signs of lymphatic vessel architecture those were not able to drain tissue fluid. One reason for this phenomenon is the compression of the lymphatic vascular system in tumor tissue

by proliferating cancer cells [11]. Furthermore, impairment in the function of the lymphatic valves is under consideration to be another reason why intratumoral lymphatic vessels feature a non-functional state [12]. However, immunohistochemical staining against lymphatic markers often revealed a complete absence of lymph vessels in tumor tissue [7,13]. Nevertheless, no investigations were undertaken so far to

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investigate how the presence of functional lymphatic vessels in the tumor tissue influences the TIFP *in vivo*.

We have previously shown that TIFP induces mechanical stretch to tumor cells and contributes as a trigger factor to enhanced tumor cell proliferation [14,15]. Furthermore, we systemically lowered the TIFP and enabled an increased uptake of pharmaceutical active drugs into the tumor tissue [16]. In the latter experimental setup, we applied concentrated human serum albumin as an agent to decrease the TIFP through an enhanced colloid osmotic pressure [16].

In the current work, we are going to address the hypothesis that an increase in functional lymphatics can lower the TIFP through an increased draining of excessive tissue fluid. Subcutaneously, A431 vulva carcinoma-bearing NMRI nude mice were peritumorally treated with vascular endothelial growth factor C (VEGF-C) to enhance lymphangiogenesis in the tumor tissue. VEGF-C is a member of the VEGF family of vascular growth factors, which comprises VEGF-A, -B, -C, and -D and Orf virus VEGFs (or VEGF-E) [17,18]. VEGF-C has been identified to play a crucial role in the development of the lymphatic system [19,20]. In addition, the transgenic overexpression of VEGF-C induced lymphatic vessel enlargement in the skin, and a recombinant VEGF-C approach induced lymphangiogenesis in the chick chorioallantoic membrane [21,22].

In the experimental setup, TIFP and tumor growth were measured and compared with physiologic sodium chloride or VEGF-A–treated xenograft animals. Our findings indicate that the peritumorally applied VEGF-C is able to lower the TIFP and, in addition, delays tumor growth. Immunohistochemical staining of tumor tissue confirmed an increased lymphatic network after peritumoral VEGF-C application.

This work identifies the importance of a functional lymphatic system with regard to increased TIFP featured regularly in solid tumors. It can offer new perspectives for an approach to lower the TIFP through enhanced lymphangiogenesis and simultaneous targeting of tumor tissue with macromolecular therapeutics.

Materials and Methods

Drugs and Cells

The A431 epidermoid vulva carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum.

VEGF-A (mBA-165, #sc-4571; Santa Cruz Biotechnology, La Jolla, CA) and VEGF-C (#DA3519X; Acris Antibodies, Herford, Germany) were injected peritumorally. Anti-LYVE-1 antibody was obtained from Abcam (Cambridge, United Kingdom; #ab14917) to identify lymphatic endothelial cells. Immunofluorescence visualization was performed using anti-rabbit IgG–fluorescein isothiocyanate secondary antibody (#F0382; Sigma, Steinheim, Germany).

Tumor Models

Female NMRI nu/nu mice (5–6 weeks; 18–22 g; Janvier Elevage, Le Genest-Saint-Isle, France) were subcutaneously injected on both flanks with 5×10^6 A431 cells. All animal experiments were approved in accordance with the German animal welfare regulations (Regierungspräsidium Darmstadt, F79/47). The animals were kept under specific pathogen-free conditions. Sterilized food and tap water were given *ad libitum*. Mice were anesthetized using isoflurane gas anesthesia (Abbott, Wiesbaden, Germany) for TIFP and tumor growth measurements. For tumor excision, animals were anaesthetized using

ketamine/xylazine (100/10 mg/kg, intraperitoneally; Pharmacia, Erlangen, Germany and BayerVital, Leverkusen, Germany). All animals were placed on regulated heating pads to keep the body temperature at 37 to 37.5°C during the experiments. After tumor excision, anesthetized animals were sacrificed.

Tumor size was measured with calipers every 4 days, starting 7 days after subcutaneous tumor inoculation, and tumor volume was calculated as described previously [23,24]. Furthermore, peritumoral injection of VEGF-C (0.5 or 1.0 or 2.0 µg), VEGF-A (1.0 or 2.0 µg), or physiologic sodium chloride (0.9%) was performed 7 days after tumor cell injection.

TIFP Measurements

TIFP was evaluated as reported previously using the “wick-in-needle” technique [14,25,26]. TIFP measurements were done every 4 days using a 27-gauge–sized hollow needle inserted in the outermost tumor tissue layers. The needle was connected to a clinical pressure transducer APT 300 (HSE Harvard Apparatus, March-Hugstetten, Germany). The needle and the adjacent connecting tube were filled with a physiologic sodium chloride solution. Recording of TIFP started when tumors reached a volume of ≥ 500 mm³. Fluid communication between the needle and the tumor tissue was tested by compressing and decompressing the tubing, and data were accepted only if the recorded pressure returned rapidly to the value before tube compression.

Immunohistochemical Staining

Ki-67 immunohistochemical staining was performed using tumors fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sectioning was performed using a Leica RM2125 rotary microtome (Leica Microsystems, Wetzlar, Germany). Subsequently, tumor sections were dried for 15 minutes at 65°C, deparaffinized in xylene, rehydrated in series of graded alcohols, rinsed in distilled water followed by heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0; DakoCytomation, Hamburg, Germany). Then, tissue sections were cooled for 20 minutes, washed [3% H₂O₂, 0.1% Triton–phosphate-buffered saline (PBS), 2% glycine-PBS], and incubated for 10 minutes with protein blocking agent (Immunotech, Marseille, France). Consecutively, sections were incubated with anti-human Ki-67 (clone MIB-1, 1:50 in PBS; DakoCytomation) for 1 hour at room temperature. After three washes in PBS, sections were incubated with a biotinylated secondary antibody and streptavidin peroxidase reagent (Immunotech) for 45 minutes. Staining was performed using the AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Finally, sections were counterstained using Mayer's hematoxylin (AppliChem, Darmstadt, Germany).

Immunofluorescence

For immunofluorescence studies of lymphangiogenesis, tumors were embedded in TissueTek-Medium (Jung, Heidelberg, Germany) and immediately frozen. Frozen samples were cut in thin slices of 8 to 12 µm using a Leica Kryostat (Leica Microsystems) and placed on object slides. Slides were fixed using cold acetone (–20°C), incubated with anti-LYVE-1 antibody (1:200) for 2 hours at room temperature, and subsequently incubated with fluorescein isothiocyanate–coupled anti-rabbit antibody (1:100, 1 hour at room temperature). Afterward, slides were washed, mounted, and stored at 4°C in nontransparent boxes to protect fluorescent dyes from bleaching. Sections were examined using a BZ8000 fluorescence microscope (Keyence, Neu-Isenburg, Germany).

Quantification of Apoptotic Tumor Cells

The ApopTag Red *In Situ* Apoptosis Detection Kit (S7165; Chemicon, Temecula, CA) was used to assess cellular apoptosis in frozen sections of tumor tissue that employs terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) methodology. Frozen slides were fixed for 10 minutes in 1% paraformaldehyde in PBS, washed with PBS, incubated for 5 minutes in -20°C cold ethanol–acetic acid, and washed with PBS. After quenching of endogenous peroxidase activity, samples were incubated with a terminal deoxynucleotidyl transferase enzyme mix (60 minutes at 37°C in a humidified chamber). This step was followed by the incubation of the slides with anti-digoxigenin conjugate for 30 minutes in a light-protected humidified environment. Finally, slides were washed, counterstained, and embedded. We performed nuclear counterstaining using 4',6-diamidino-2-phenylindole dihydrochloride (AppliChem).

Results

Lowered Tumor Growth Rate in VEGF-C–Treated Xenograft Mice

Initially, we recorded the increase of tumor growth in VEGF-C–treated mice *versus* sodium chloride–treated control animals employing the A431 tumor xenograft model (NMRI nu/nu mice). Therefore, NMRI nude mice were subcutaneously inoculated into the left and right parts of the back with A431 tumor cells. We found that the tumor growth was significantly reduced in all animals treated with VEGF-C that was applied in a peritumoral region 1 week after the inoculation of the tumor cells. Already at 8 days after the VEGF-C application in A431 tumor cells, a significant decrease in tumor growth was visible in the 0.5 ($284 \pm 26 \text{ mm}^3$) and 1.0 μg ($258 \pm 26 \text{ mm}^3$) treated animals in comparison to the control group ($707 \pm 85 \text{ mm}^3$). At 16 days, all three VEGF-C–treated groups (0.5, 1.0, and 2.0 μg) showed a significant reduction in tumor volume (Figure 1A). While control tumors reached a volume of $2051 (\pm 259) \text{ mm}^3$, the VEGF-C–treated tumors exhibit tumor volumes of

$836 \pm 191 \text{ mm}^3$ (0.5 μg), $692 \pm 90 \text{ mm}^3$ (1.0 μg), and $1097 \pm 155 \text{ mm}^3$ (2.0 μg), respectively.

In addition, we tested if the measured effect is specific for VEGF-C or related to the family of VEGFs; we repeated the experiment with VEGF-A, an angiogenesis-related endothelial growth factor. In the new investigation, VEGF-A was applied peritumorally according to the previous VEGF-C experiments. The injections were performed using 1.0 or 2.0 μg of VEGF-A, respectively. In comparison to the previous results of VEGF-C–treated animals, the peritumoral application of VEGF-A significantly increased the tumor growth of the A431 tumors compared to control tissue (Figure 1B).

Tumor Growth Is Decreased due to Lowered Tumor Cell Proliferation

Herein, we investigated if the decelerated tumor growth is due to a reduced tumor cell proliferation or as a result of an increased tumor cell apoptosis. Tumors were excised 7 days after the peritumoral application of VEGF-C to assess cellular apoptosis and cellular proliferation by TUNEL staining or Ki-67 immunohistochemical staining, respectively. Figure 2, A to D, visualizes the results of TUNEL staining for tissue samples of VEGF-C–treated and control tumors. All slides show a similar pattern and number of apoptotic tumor cells. No significant changes are visible between the groups. In contrast, immunohistochemical staining against the cellular proliferation marker Ki-67 revealed a reduced staining in the VEGF-C–treated tumor tissue (Figure 2, F–H) compared to untreated control tumor tissue (Figure 2E). These findings show that the tumor growth rate in VEGF-C–treated tumors is lowered as a result of a decreased tumor cell proliferation, while tumor cell apoptosis is not affected by the VEGF-C application.

VEGF-C–induced Lymphangiogenesis in A431 Tumors

To clarify if the peritumoral application of VEGF-C enhanced lymphangiogenesis in tumor tissue, we excised tumors from all treatment groups at a size of approximately 1000 mm^3 and performed immunohistochemical staining with anti-LYVE-1. The antibody

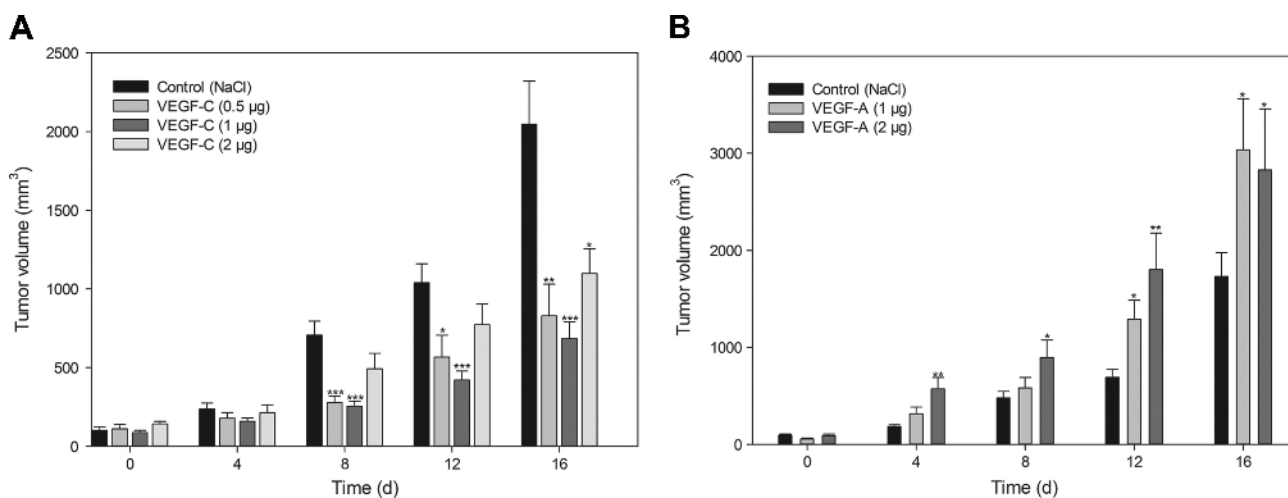


Figure 1. Measurement of tumor growth. (A) Peritumoral application of VEGF-C (0.5, 1.0, and 2.0 μg) at day 0. Tumor growth was measured every 4 days using a digital caliper. Data are shown as mean (\pm SEM) increase of TIFFP ($n = 10$). (B) Peritumoral application of VEGF-A (1.0 and 2.0 μg) at day 0. Tumor growth was measured every 4 days using a digital caliper. Data are shown as mean (\pm SEM) increase of TIFFP ($n = 10$). For both graphs, $*P = .05$, $**P = .01$, and $***P = .001$, treatment *versus* control.

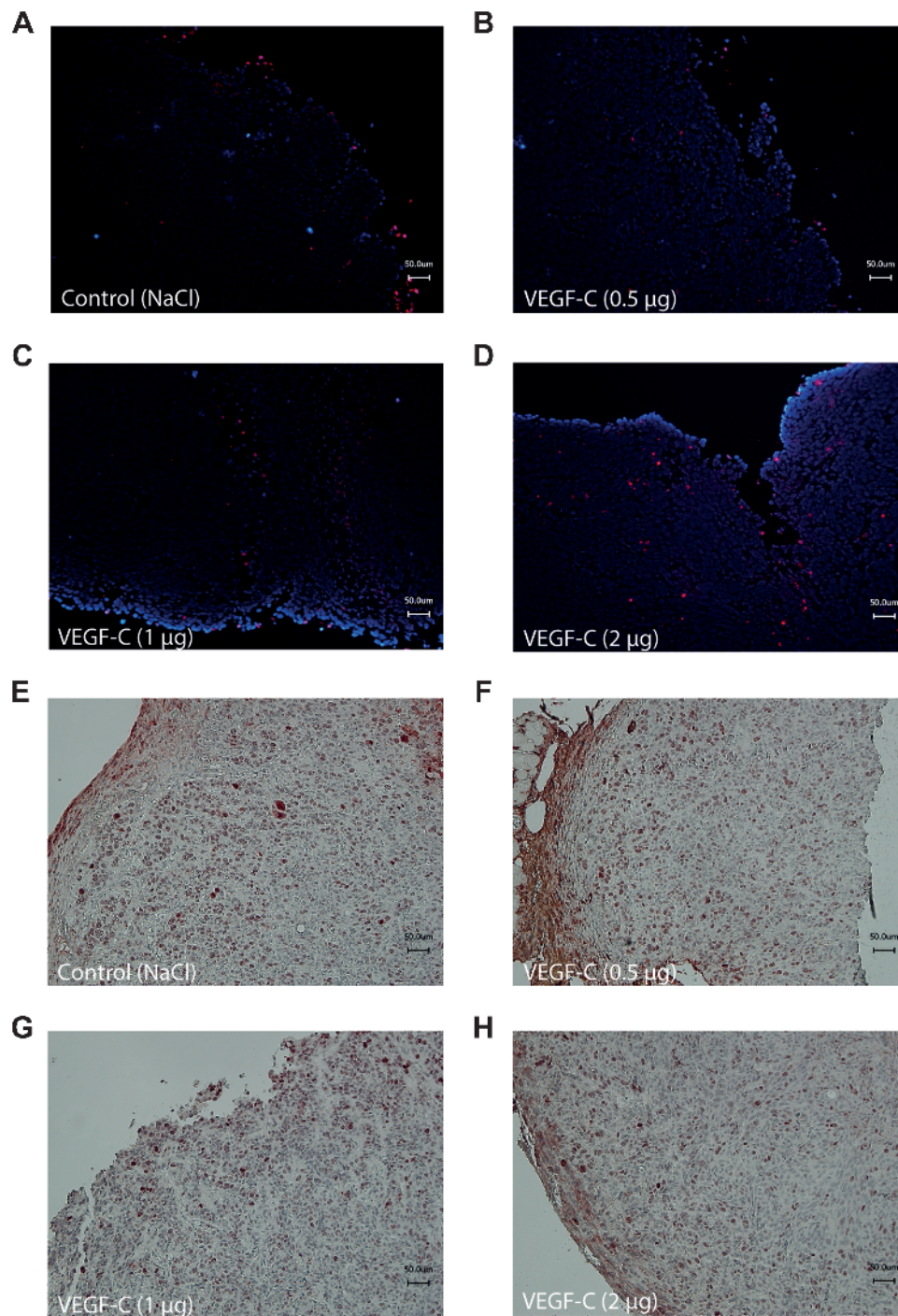


Figure 2. VEGF-C–treated tumors exhibit reduced tumor cell growth but no increase in tumor cell apoptosis. Cellular apoptosis was identified after TUNEL staining of frozen tumor tissue section of (A) control animals or (B) 0.5- μ g VEGF-C–, (C) 1.0- μ g VEGF-C–, or (D) 2.0- μ g VEGF-C–treated animals. Apoptotic cells are red labeled. Frozen sections were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride. Tumor cell proliferation was assessed by immunohistochemical staining of paraffin-embedded tumor tissue section of (E) control animals or (F) 0.5- μ g VEGF-C–, (G) 1.0- μ g VEGF-C–, or (H) 2.0- μ g VEGF-C–treated animals against the cellular proliferation marker Ki-67. Slides were counterstained using Mayer's hematoxylin.

binds specific to lymphatic vessel endothelial hyaluronan receptor 1, which is expressed on lymphatic endothelial cells and the hepatic sinusoidal endothelium but not on vascular endothelial cells. Therefore, LYVE-1 staining can be used as a lymphatic endothelial marker [27]. As shown in Figure 3, all VEGF-C–treated tumors highlighted a strong LYVE-1 signal. In comparison to the control tissue that presents only a slight background staining (Figure 3A), tissues of VEGF-

C–treated tumors show distinct lymphangiogenesis and lymphatic vessels in the tumor tissue (Figure 3, B–D). Our results also indicated an increased level of lymphangiogenesis in tumors treated with 1.0 or 2.0 μ g of VEGF-C compared to tumors that received a peritumoral application of 0.5 μ g of VEGF-C. Tumor tissue sections of VEGF-A–treated animals featured no LYVE-1–positive staining (data not shown).

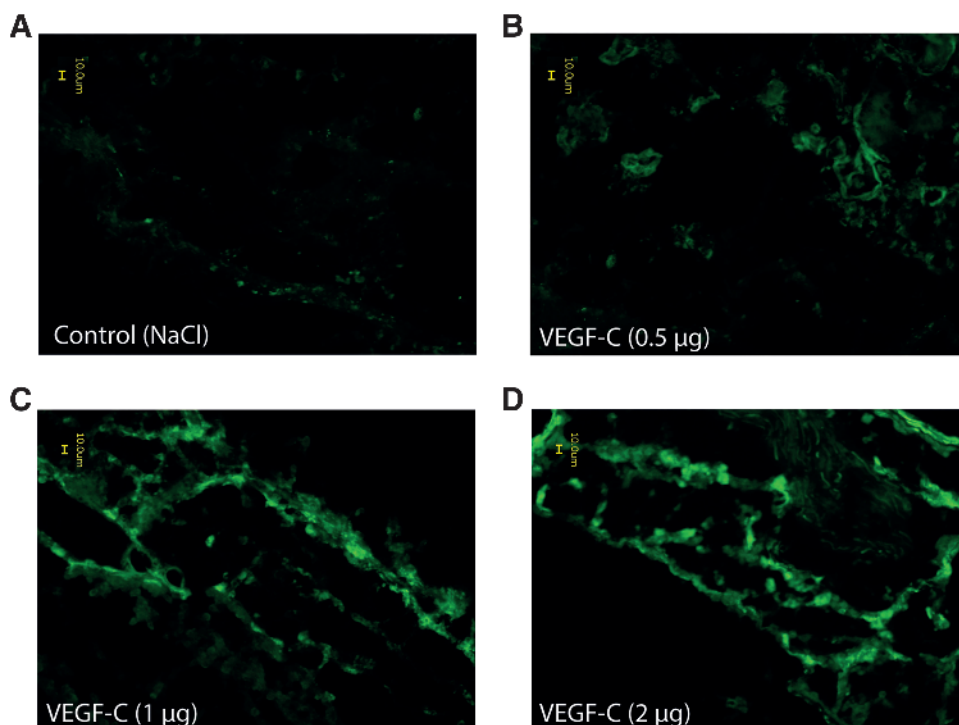


Figure 3. VEGF-C induced intratumoral lymphangiogenesis in A431 tumors. Immunohistochemical staining of frozen tissue sections of (A) control animals or (B) 0.5- μ g VEGF-C-, (C) 1.0- μ g VEGF-C-, or (D) 2.0- μ g VEGF-C–treated animals against the lymph-specific endothelial marker LYVE-1. Staining was performed on day 12 after the application of VEGF-C.

Reduced Tumor Interstitial Fluid Pressure in VEGF-C–Treated Xenograft Mice

To determine if the VEGF-C–induced lymphatic network influences the TIFP, we used the “wick-in-needle” technique to measure TIFP intratumorally. We started the measurement at day 8 and with a minimum tumor volume of approximately 500 mm³. The TIFP measurement was performed every 4 days. A significant reduction in TIFP is visible for all VEGF-C–treated animals at day 16 in comparison to the sodium chloride–treated control mice (Figure 4). Animals that were previously treated with 1.0 μ g of VEGF-C exhibit the highest reduction in TIFP. At day 16, TIFP values of 5.5 (\pm 0.5) mm Hg for 1.0 μ g of VEGF-C *versus* 11.7 (\pm 0.6) mm Hg for the control animals were measured. Furthermore, TIFP values were 8.9 (\pm 1.1) mm Hg for 0.5 μ g of VEGF-C and 7.5 (\pm 0.5) mm Hg for 2.0 μ g of VEGF-C, respectively. Both values were also significantly lower compared to the control tumors.

Discussion

Elevated TIFP is known to hamper the uptake of macromolecular molecules due to a reduced convectionally driven transendothelial fluid transport [28]. Particularly, the transport and uptake of therapeutic drugs, e.g., monoclonal antibodies, is reduced, contributing to a lowered retention time of chemotherapeutics within tumor tissue [3,29,30]. The lack of a functional lymphatic drainage has been proposed as a key factor in the development of increased values of TIFP in human and experimental tumors [10].

Here, we pursued a new approach to lower the TIFP, highlighting the importance of the lymphatic vasculature in the development and homeostasis of high TIFP values. Initially, VEGF-C was administered

peritumoral of vulva carcinoma–derived epithelial A431 tumors that were grown subcutaneously in immunosuppressed NMRI nu/nu mice. As reported previously, A431 tumors exhibit increased TIFP values of up to 15 mm Hg and show a strong correlation between TIFP and tumor volume [14,16]. Respectively, it has been shown by others that TIFP increases with the tumor volume in a number of different tumor species [1,31–33]. In our investigations, we examined

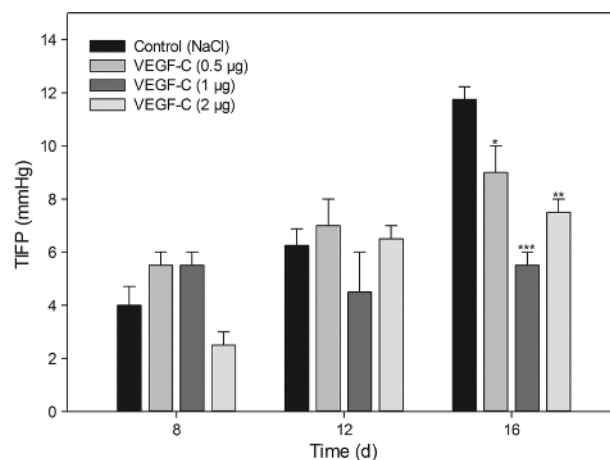


Figure 4. TIFP decrease in VEGF-C–treated animals. TIFP was measured for the first time at day 8 starting at a tumor volume of approximately 500 mm³. TIFP measurements were performed every 4 days consecutively. Data are shown as mean (\pm SEM) increase of TIFP ($n = 4$ per group). * $P = .05$, ** $P = .01$, and *** $P = .001$, treatment *versus* control.

the tumor growth in regard to the treatment with different concentrations of VEGF-C. The tumor growth rate was significantly reduced in VEGF-C–treated animals compared with sodium chloride– or VEGF-A–treated mice. Immunohistochemical stainings confirmed that the reduced growth rate of VEGF-C–treated tumors was due to a decreased tumor cell proliferation rate and not apoptosis induced. Hence, we investigated the lymphatic architecture in A431 tumors and found an enhanced lymphatic vascularization in VEGF-C–treated tumors. In addition, a lowered TIFP was measured in mice after being treated peritumorally with VEGF-C. This reduction in TIFP can be explained by the enhanced lymphatic drainage of the tumor interstitium after the VEGF-C application. Furthermore, the investigation of reduced tumor growth is in concert with our previous findings that lowered TIFP reduces tumor growth through mechanical relaxation of the tumor cortex [14,15]. However, studies performed in osteosarcoma cells identified increased VEGF-C levels in tumor cells grown under pressure compared to benign osteoblast values that expressed low VEGF-C levels under the same high pressure cell culture environment [34]. These findings indicate that tumor cells are more sensitive to pressure gradients in interstitial fluid than normal cells. Nevertheless, those findings are preliminary as they have been performed in cell culture and not in tumor models. The current *in vivo* data do not corroborate an increased lymphatic network in tumors with high TIFP levels in comparison to tumors with low TIFP.

Previous attempts to increase the functional lymphatic vasculature in tumor tissue through overexpression of VEGF-C in tumor cells were capable to induce the formation of functional lymphatics [12]. Nevertheless, those lymphatics were immature and unable to prevent lateral flow into side lymphatics [12]. In our attempt, we induced lymphangiogenesis by applying VEGF-C directly by subcutaneous peritumoral injection. This induced and established intratumoral lymphatic vascularization of the tumor tissue. The significant reduction of TIFP after the administration of VEGF-C can be used as a marker for a functional draining of excessive interstitial fluid. Yet, we cannot affirm that the functionality of the newly developed intratumoral lymphatics equates the drainage capacity of lymphatics outside of the tumor tissue.

In addition, peritumoral application of VEGF-A enhanced tumor growth but did not exhibit a significant effect on the growth of new intratumoral lymphatics in our experimental setup. On a first glance, this finding is in contrary to a range of publications showing that VEGF-A can induce hemangiogenesis and lymphangiogenesis. A closer look reveals that tissue inflammation might play a crucial role in the ability of VEGF-A to induce lymphangiogenesis [35,36]. It is supposed that VEGF-A mediates lymphangiogenesis by binding to the VEGF receptor 1 and recruiting of macrophages, which, in turn, can release additional lymphangiogenic markers [35]. Furthermore, VEGF-A was identified to promote lymphangiogenesis in VEGF-A–overexpressing transgenic animals [37]. However, VEGF-A–transfected H293 could not induce lymphangiogenesis due to lower expression levels of VEGF-A in comparison with transgenic approaches [38]. We assume that a single peritumoral injection as used in our experiment did not provide a VEGF-A level for the induction of lymphangiogenesis.

It has been proven that an increased lymphangiogenesis has a high therapeutic potential in the treatment of lymphedema [39]. Here, we show that using the TIFP-lowering effect of lymphangiogenesis can propose a new approach to increase the uptake of macromolecular therapeutics into tumor tissue. However, it has to be kept in mind that the hypothesized usage of a pro-lymphangiogenic treatment of tumors

with the aim of reducing the TIFP has a critical bottleneck that needs to be precluded prior. The active recruitment of functional lymphatic vessels into the tumor can increase the risk of lymphatic tumor cell metastasis. It is known that functional lymphatics in the margin of the tumor surface are sufficient for lymphatic-driven metastasis and intratumoral lymphatic vessels are not necessary for the tumor cells to metastasize [13]. Furthermore, enhanced lymphatic metastasis has been shown previously in VEGF-C–overexpressing tumors indicating that increased VEGF-C levels potentiate the risk of lymphatic metastases significantly [13,40]. During the time course of our experiments, we could not identify an increased risk of metastases in the VEGF-C–treated xenograft animals. Further investigations will have to take place including sentinel lymph node excisions to investigate the risk of increased metastasis due to improved lymphatic drainage of the tumor tissue.

In conclusion, our data show for the first time that the peritumoral application of VEGF-C induces lymphangiogenesis and lowers TIFP in the herein used animal model. Moreover, it was shown that besides lowering of TIFP, VEGF-C–treated tumors also exhibited a delayed growth rate. Thus, the present work displays new insights into the importance of the lymphatic vasculature pertaining to the development and maintenance of high TIFP in solid tumors.

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