Vascular endothelial growth factor enhances cancer cell adhesion to microvascular endothelium in vivo

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To investigate whether vascular endothelial growth factor (VEGF) enhances cancer cell adhesion to normal microvessels, we used in vivo video microscopy to measure adhesion rates of MDA-MB-435s human breast cancer cells and ErbB2-transformed mouse mammary carcinomas in the postcapillary venules of rat mesentery. An individual postcapillary venule in the mesentery was injected via a glass micropipette with cancer cells either in a perfusate of mammalian Ringer solution containing 1% bovine serum albumin as a control, or with the addition of 1 nM VEGF for test measurements. Cell adhesion was measured as either the number of adherent cells or the fluorescence intensity of adherent cells in a vessel segment for ~60 min. Our results showed that during both control and VEGF treatments, the number of adherent cells increased almost linearly with time over 60 min. The VEGF treatment increased the adhesion rates of human tumour cells and mouse carcinomas 1.9-fold and 1.8-fold, respectively, over those in control conditions. We also measured cancer cell adhesion after pretreatment of cells with an antibody blocking VEGF or an antibody blocking α6 integrin, and pretreatment of the microvessel with VEGF receptor (KDR/Flk-1) inhibitor, SU1498, or anti-integrin extracellular matrix ligand antibody, anti-laminin-5. All antibodies and inhibitor significantly reduced adhesion, with anti-VEGF and SU1498 reducing it the most. Our results indicate that VEGF enhances cancer cell adhesion to the normal microvessel wall, and further suggest that VEGF and its receptor, KDR/Flk-1, as well as integrins of tumour cells and their ligands at the endothelium, contribute to mammary cancer cell adhesion to vascular endothelium in vivo.

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The danger of cancer is organ failure caused by metastatic tumours that are derived from the primary tumour (Weiss et al. 1988; Steeg & Theodorescu, 2008). One critical step in tumour metastasis is adhesion of primary tumour cells to the endothelium forming the microvascular wall in distant organs (Steeg, 2006). Understanding this step may lead to new therapeutic concepts for tumour metastasis to target tumour cell arrest and adhesion in the microcirculation. In vitro static adhesion assays have been used to investigate tumour cell adhesion to endothelial cells (Lee et al. 2003; Earley & Plopper, 2006) and to extracellular matrix (ECM) proteins (Spinardi et al. 1995). Tumour cell adhesion has also been investigated using flow chambers (Giavazzi et al. 1993; Slattery et al. 2005; Chotard-Ghodsnia et al. 2007) or artificial blood vessels (Brenner et al. 1995) to address flow effects. Direct injection of tumour cells into the circulation has enabled the observation of tumour cell metastasis in target organs after killing the animals (Schluter et al. 2006), while intravital microscopy has been used to observe the interactions between circulating tumour cells and the microvasculature both in vivo and ex vivo (Koop et al. 1995; Al-Mehdi et al. 2000; Glinskii et al. 2003; Haier et al. 2003; Mook et al. 2003; Steinbauer et al. 2003). Regardless of these attempts, however, to date very little has been learned about the mechanisms governing tumour cell adhesion without loss of their physiological and dynamic microenvironment. This is largely due to the absence of an accurate in vivo model system.
Previous studies have found that breast cancer cells express vascular endothelial growth factor (VEGF) to a high degree (Brown et al. 1995; Lee et al. 2003), while the microvascular endothelium has abundant VEGF receptors, including VEGFR2 (KDR/Flk-1; Mukhopadhyay et al. 1998). This receptor has been implicated in normal and pathological vascular endothelial cell biology (Olsson et al. 2006); however, its role in tumour cell adhesion in general and in adhesion to normal microvessels in particular has not been examined in a well-controlled in vivo system. In addition, blood flow can enhance cell adhesion in certain conditions (Zhu et al. 2008). Microvasculature flow conditions, either in specific organs or in different physiological and pathological conditions, may alter tumour cell adhesion. Tzima et al. (2005) reported that the shear stress induced by the blood flow may activate VEGFR2 in a ligand-independent manner by promoting the activation of a mechanosensory complex, which functions upstream of integrin activation. Moreover, integrins, e.g. α6β4, α5β1, α6β1 and their ligands, e.g. laminin-5, -4, -2 and -1 of the ECM, have been suggested as key players for breast cancer cell adhesion (Spinardi et al. 1995; Giannelli et al. 2002; Guo & Giancotti, 2004; Guo et al. 2006).

Although VEGF has long been recognized as a vascular permeability-enhancing agent for normal endothelium both in vivo and in vitro (Collins et al. 1993; Bates & Curry 1996; Wu et al. 1996; Bates, 1997; Wang et al. 2001; Fu & Shen, 2004), at present, VEGF-induced microvessel hyperpermeability and its role in tumour metastasis remain poorly elucidated (Dvorak, 2002; Bates & Harper, 2003). Lee et al. (2003) used a transwell culture system with a human brain microvascular endothelial cell (HBMEC) monolayer as an in vitro model to investigate the effects of VEGF on adhesion and transendothelial migration of MDA-MB-231 breast cancer cells. They found that VEGF increased MDA-MB-231 adhesion and transmigration through increasing HBMEC monolayer permeability to inulin. Unfortunately, no well-controlled in vivo study of VEGF-mediated effects on tumour adhesion has been reported to date.

Accordingly, the objective of this study was to investigate both in vivo breast cancer cell adhesion to normal microvascular endothelium and the effect of VEGF on adhesion in an individual microvessel in well-controlled permeability and flow conditions. Quantitative microscope photometry was used to quantify microvessel permeability to solutes of differing sizes. Adhesion rates of the human malignant breast cancer cell MDA-MB-435s and oncogenic receptor tyrosine kinase ErbB2-transformed mouse mammary carcinoma in a single postcapillary venule of rat mesentery in vivo were measured by intravital video microscopy. Our method includes at least two advantages over previous studies in this area. First, we investigate breast tumour cell adhesion in intact microvessels, rather than in a traditional cultured cell monolayer model, which lacks a true physiological microenvironment. Second, our work is performed on an individually perfused microvessel, in which experimental conditions can be well defined. This approach overcomes the uncertainties associated with whole-animal studies.

**Methods**

**Animal preparation**

All in vivo experiments reported in this paper were performed on female Sprague–Dawley rats (250–300 g, age 3–4 months), supplied by Simonson Laboratory (Gilroy, CA, USA) and Hilltop Laboratory Animals (Scottsdale, PA, USA). All procedures were approved by the Animal Care and Use Committees at both the University of Nevada Las Vegas and at the City College of the City University of New York. The methods used to prepare rat mesenteries, perfuse solutions and micropipettes for microperfusion experiments have been described in detail elsewhere (Fu & Shen, 2004). A brief outline of the methods is given below, with emphasis on the special features of the present experiments. At the end of experiments, the animals were killed with excess anesthetic (100 mg kg$^{-1}$ pentobarbitone intravenous injection). The thorax was opened to ensure death.

Rats were first anaesthetized with pentobarbitone sodium given subcutaneously. The initial dosage was 65 mg kg$^{-1}$, and an additional dose of 3 mg was administered as needed. After a rat was anaesthetized, a mid-line surgical incision (2–3 cm) was made in the abdominal wall. The rat was then transferred to a tray and kept warm on a heating pad. Its body temperature was kept at ~37°C by adjusting the heating pad temperature and monitored regularly by a thermometer. The mesentery was gently taken out from the abdominal cavity and spread on a glass coverslip, which formed the base of the observation platform as previously described (Fu et al. 2005). The gut was gently pinned out against a silicon elastomer barrier to maintain the spread of the mesentery. The upper surface of the mesentery was continuously superfused by a dripper with mammalian Ringer solution at 35–37°C, which was regulated by a controlled water bath and monitored continuously using a thermometer probe. The microvessels chosen for the study were straight, non-branching postcapillary venules, with diameters of 40–50 μm. All vessels had brisk blood flow immediately before cannulation and had no marginating white cells.

**Cell culture**

Human breast ductal carcinoma (MDA-MB-435s) cells were purchased from ATCC (Manassas, VA, USA) and cultured in 75 cm$^2$ plastic tissue culture flasks (Corning Inc., Corning, NY, USA) in Leibovitz’s L-15 medium
with 2 mM l-glutamine (ATCC), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA) and 100 U ml\(^{-1}\) penicillin (Sigma). Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide and subcultured every third day. Cells were cultured at 10\(^6\) cells ml\(^{-1}\) and grown to confluence (90%), and routinely passaged using trypsin/EDTA (ATCC) at a ratio of 1:4 (Earley & Plopper, 2006).

The ErbB2-transformed mouse mammary carcinoma wild-type (WT) cells were cultured in Sloan-Kettering Cancer Center (Guo et al. 2006). Briefly, WT cells were grown in 75 cm\(^2\) tissue culture flasks (Corning) with Dulbecco’s modified Eagle’s medium containing F12, non-essential amino acids, 10% fetal bovine serum (Sigma), hydrocortisone (1 \(\mu\)g ml\(^{-1}\)), cholera toxin (10 \(\mu\)g ml\(^{-1}\)) and insulin (10 \(\mu\)g ml\(^{-1}\)) in a humidified environment of 95% air and 5% CO\(_2\) at 37°C. On the day of experiments, cells were collected by brief trypsinization, then counted and suspended in phosphate-buffered saline (PBS, Sigma). To remove any remaining cell clumps, the cell suspension was filtered through 70 and 40 \(\mu\)m nylon meshes, and adjusted to contain \(\sim4\) million cells ml\(^{-1}\) for the final perfusate.

The cell survival rate was \(\sim95\%\) before perfusion, and \(\sim90\%\) after \(\sim1.5\) h perfusion at driving pressures of 2–15 cmH\(_2\)O in perfusing micropipettes.

Fluorescent tagging of MDA-MB-435s cells

When the cells reached confluence, medium was removed. Cells were washed in PBS and incubated with CellTracker Green (Invitrogen, Eugene, OR, USA) at 0.5 \(\mu\)M for 40 min. After staining, the probe solution was replaced with fresh, prewarmed medium. The cells were then incubated for another 30 min at 37°C and collected by brief trypsinization, blocked with medium, and washed extensively twice with PBS. The immediate toxicity of this procedure was minimal, leaving 95–100% of the cells alive as determined by Trypan Blue (Sigma) exclusion. Finally, the cells were stored in 1% BSA (bovine serum albumin, Sigma) mammalian Ringer solution at 5 \(\times\) 10\(^6\) to 10 \(\times\) 10\(^6\) cells ml\(^{-1}\), kept in darkness at room temperature, and were ready for immediate use.

Solutions and reagents

**Mammalian Ringer solution.** Mammalian Ringer solution was used for all dissections, perfusates and superfusate (Fu & Shen, 2004). The solution composition was (mM): 132 NaCl, 4.6 KCl, 1.2 MgSO\(_4\), 2.0 CaCl\(_2\), 5.0 NaHCO\(_3\), 5.5 glucose and 20 Hepes. Its pH was balanced to 7.4 by adjusting the ratio of Hepes acid to base. In addition, the perfusate into the microvessel lumen contained BSA at 10 mg ml\(^{-1}\) (1% BSA–Ringer solution). A 1 \(\mu\)M solution of VEGF (human recombinant VEGF\(_{165}\), Peprotech, Rocky Hill, NJ, USA) was made in mammalian Ringer solution containing 1% BSA. Anti-\(\alpha\)6 and anti-laminin-5 antibodies and a control antibody, W632, were obtained from Sanquin Reagents (Amsterdam, The Netherlands), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma, respectively. Anti-human VEGF monoclonal antibody was purchased from Leinco Technologies, Inc. (St Louis, MO, USA), and the inhibitor to VEGFR2 (KDR/Flik-1), SU-1498, from Alomone Labs Ltd (Jerusalem, Israel). The concentration of antibodies was 20 \(\mu\)g ml\(^{-1}\), and the concentration of SU-1498 was 50 \(\mu\)M in 1% BSA–Ringer solution. All of the solutions described above were made at the start of the experiment and were discarded at the end of the day.

**Sodium fluorescein.** Sodium fluorescein (F6377, Sigma; molecular weight 376) was dissolved at 0.1 mg ml\(^{-1}\) in the Ringer solution containing 10 mg ml\(^{-1}\) BSA.

**Fluorescein isothiocyanate (FITC)-labelled \(\alpha\)-lactalbumin and BSA.** \(\alpha\)-Lactalbumin (L6010, Sigma; molecular weight 14 176) and BSA (A4378, Sigma; molecular weight 66 000) were labelled with FITC (F7250, Sigma, molecular weight 389.4) as detailed by Adamson et al. (1988). The FITC-labelled \(\alpha\)-lactalbumin and BSA were stored frozen and used within 2 weeks of preparation. On the day of use, unlabelled BSA was added to aliquots of the labelled protein. The final FITC–\(\alpha\)-lactalbumin and FITC–BSA concentrations used in the experiment were 1 mg ml\(^{-1}\) in the Ringer solution (Fu & Shen, 2004).

Perfusion of a single mesenteric microvessel

A single venular microvessel (40–50 \(\mu\)m diameter) was cannulated with a glass micropipette (~30 \(\mu\)m tip diameter, World Precision Instruments Inc., Sarasota, FL, USA) and perfused with the BSA–Ringer solution with either MDA-MB-435s or ErbB2-transformed mouse mammary carcinoma (WT) cells. An initial pressure of 10–20 cmH\(_2\)O, depending on the downstream resistance controlled by a water manometer, was applied through the pipette to the microvessel lumen. The initial pressure was set to balance the downstream blood pressure. Then the pressure was increased to a perfusion pressure of 12–30 cmH\(_2\)O. The difference between the initial pressure and the perfusion pressure was denoted as the driving pressure. The perfusion flow velocity was determined by the driving pressure and was calculated from the movement of a marker tumour cell. The relationship between driving pressure and perfusion velocity is demonstrated in Fig. 1 for three vessels. We tested two perfusion velocities in our study, a reduced velocity of \(~150\ \mu\text{m}\ \text{s}^{-1}\) when the driving pressure was controlled at \(~2\ \text{cmH}_2\text{O}\), and \(~1000\ \mu\text{m}\ \text{s}^{-1}\), which was a mean blood flow velocity in this type of microvessel when the driving pressure was \(~10\ \text{cmH}_2\text{O}\).
Cell adhesion and solute permeability measurement

Tumour cell adhesion to microvessel wall. A Nikon Eclipse TE-300 inverted fluorescence microscope with ×20 magnification lens (NA = 0.75) was used to observe the mesentery. A filter set from Omega Optical (Brattleboro, VT, USA), consisting of an excitation filter (510DF23), a dichroic mirror (525DRLP) and an emission filter (535DF35), was used to observe CellTracker Green fluorescence. To minimize tissue damage and quench, the intensity of the microscope xenon light source was kept as low as possible. Further protection was provided by using an experimental protocol in which the time of tissue exposure to the excitation light was kept as short as possible for the intensity measurement. Generally, the exposure time for an individual image recording was 0.2–1.0 s. Cell adhesion to the microvessel wall was observed every minute using a low-light-level CCD Camera (COHU, San Diego, CA, USA), and the images were recorded into a computer through an A/D board by an InCyt Im1™ imaging system (Intracellular Imaging Inc., Cincinnati, OH, USA). Our imaging and recording setting induced negligible image quench in ~1.5 h (less than 1%). Cell adhesion was represented by the total cells (the fluorescence intensity of total cells) in a vessel segment (60–80 μm wide × 300–400 μm long).

For WT cells not labelled with fluorescence, the mesenteric tissue was observed using a ×20 magnification objective lens (NA = 0.75, super, Nikon) under a bright field. The adhesion process was imaged by a high-performance digital 12-bit CCD camera (SensiCam QE, Cooke Corp., Romulus, MI, USA) using InCyt Im1™ software. Adherent cells were counted offline in a vessel segment of 300–400 μm length and expressed as the number of adherent cells per 5000 μm² plane area (length × diameter) of the vessel segment. The measuring area was set at least 150 μm downstream from the cannulation site of the vessel to avoid entrance flow effects.

Apparent solute permeability (P) of a microvessel. A detailed method used to measure P of fluorescently labelled solutes is described by Fu & Shen (2004). The same microscope described above with a ×10 magnification lens (NA = 0.3, Nikon) was used to measure P. The filter set from Omega optical, which was used for sodium fluorescein, FITC–BSA and FITC–α-lactalbumin, consisted of an excitation filter (485DF22), a dichroic mirror (505DRLP) and an emission filter (535DF35). Fluorescence intensity (I_f) in the capillary lumen and surrounding tissue was measured by aligning the vessel segment within an adjustable measuring window consisting of a rectangular diaphragm in the light path. The dimensions of this measurement window were generally ~200 μm width (roughly 5 times the microvessel diameter) and 300–400 μm length. The window was set at least 100 μm from the cannulation site of the vessel to avoid solute contamination from that site. The value of I_f measured using a photometer (HC135-11, Hamamatsu, Hamamatsu City, Shizuoka, Japan) was recorded into a computer using InCyt Pm1™ Photometry software (Intracellular Imaging Inc.). Compared with the previous strip chart recorder, this type of recording greatly improved the spatial and temporal resolution of the intensity versus time curve, which was used to calculate solute permeability P, as follows:

\[ P = \frac{1}{\Delta I_{f0}} \left( \frac{dI_{f}}{dt} \right) \frac{r}{2} \]

where \(\Delta I_{f0}\) is the step increase in fluorescent light intensity as the test solute fills the microvessel lumen, \(\left( \frac{dt}{dI_{f}} \right)_{0}\) is the initial rate of increase in fluorescence intensity after solute fills the lumen and begins to accumulate in the tissue, and r is the microvessel radius (Adamson et al. 1988).

 Calibration experiments

In determining adhesion, in vitro calibration experiments were used to test the assumption that the fluorescence intensity of the fluorescently labelled cells is a linear function of the number of cells. Figure 2 shows our results for three concentrations: 1, 2 and 10 million cells ml⁻¹. These intensity versus cell number curves are linear for all the tested concentrations, although they have different slopes because the optimized settings for imaging were different for different concentrations. However, when

![Figure 1. Perfusion flow velocity as a function of the driving pressure](image-url)
normalized by the intensity for a group of cells with the highest cell number (40 cells in our test) at each concentration, the curves of the intensity versus cell number for different concentrations almost overlap each other (Fig. 2).

**Experimental protocol**

**Cell adhesion.** To measure the cell adhesion rate in normal permeability conditions, a single straight postcapillary venule was cannulated with a micropipette filled with 1% BSA–Ringer solution containing ~5 million cells ml\(^{-1}\). The venule was perfused with a driving pressure of 10 cmH\(_2\)O to maintain a normal flow velocity of ~1000 \(\mu\)m s\(^{-1}\), or by a driving pressure of 2 cmH\(_2\)O to maintain a reduced flow velocity of ~150 \(\mu\)m s\(^{-1}\). The adhesion process was recorded at ~2 frames s\(^{-1}\) in a ~1 min interval for ~60 min in each experiment. A single experiment was carried out in one microvessel per animal.

To test the effect of VEGF on cell adhesion to the microvessel wall, the perfusate also contained 1 nM VEGF, which has been shown to significantly increase microvessel permeability to both water and solutes (Bates & Curry, 1996; Fu & Shen, 2004; also present study). Cells were injected simultaneously with 1 nM VEGF in 1% BSA–Ringer into a single vessel in the same way as in the control experiment and at the same perfusion velocity.

**Measurement of \(P\).** To test the effect of 1 nM VEGF on permeability of solutes of different sizes, for each test solute, after making several control measurements with a \(\theta\) pipette when the washout lumen was filled with Ringer perfusate containing BSA (10 mg ml\(^{-1}\)) and the dye lumen was filled with the same perfusate, to which the test solute was added, we replaced the pipette with a new \(\theta\) pipette of both washout and dye solutions also containing VEGF (1 nM). After 10–15 s perfusion with washout solution containing VEGF, pressure at the dye side was increased to a higher value while pressure at the washout side was decreased to a lower value for dye perfusion. The perfusion of dye solution containing 1 nM VEGF lasted 5–15 s, depending on solute size. From the initial step increase in fluorescence intensity of the test dye solution and its accumulation in the measurement window, we can calculate the solute permeability, \(P\), which was measured every 15–30 s including both dye (5–15 s) and washout perfusion (10–15 s). The alternating perfusion of dye and washout solutions lasted ~5 min.

**Analysis and statistics**

Measurements of \(P\) during the control period in a vessel were averaged to establish a single value for the control \(P\). This value was then used as a reference for all subsequent measurements on that vessel. Owing to the difference in the cell concentration, fluorescence labelling and vessel size for different experiments, in the cell adhesion measurement we defined a base intensity, \(I_0\), for each vessel, which was an averaged value of three measurements in the first 5 min of cell perfusion. The time course of cell adhesion \(I(t)\) was normalized as \(I(t)/I_0\). To present adhesion data at a specific time after 5 min, individual measurements were grouped with 5 min intervals at 10 min (6–10 min), 15 min (11–15 min), etc. Results for both permeability and cell adhesion are presented as the means ± S.E.M. unless specified otherwise.

Statistical significance of the treatment over time was tested with a non-parametric Wilcoxon signed rank test applied to the averaged adhesion data. The Mann–Whitney \(U\) test was applied to between-group data to test for adhesion differences at specific times. Significance was assumed for probability levels \(P < 0.05\).

**Results**

**Effect of VEGF on MDA-MB-435s cell adhesion to the microvessel wall**

Adhesions of MDA-MB-435s cells in representative microvessels are shown in Fig. 3, at ~15 min (Fig. 3A) and ~60 min (Fig. 3B) at a reduced perfusion velocity of ~150 \(\mu\)m s\(^{-1}\) in either control conditions (1% BSA–Ringer solution) or during treatment with 1 nM...
VEGF. Bright spots in these images represent adherent fluorescently labelled tumour cells.

Figure 4A presents the time course of the number of adherent cells as indicated by fluorescence intensity in a vessel segment, in control conditions (1% BSA–Ringer solution) and during treatment with 1 nM VEGF, with normal perfusion velocity (1000 μm s⁻¹). In conditions of normal flow and vessel permeability, there was a monotonic, statistically significant increase of cell adhesion from its basal value, starting at 15 min (P < 0.003 after 20 min perfusion). In contrast, VEGF treatment produced a rise in cell adhesion starting earlier, at 10 min (P < 0.001 after only 15 min perfusion). Linear regression of data for intensity (cell adhesion amount) versus time revealed that cell adhesion increased almost linearly with time, with R² = 0.97 and 0.88 for control and VEGF treatment, respectively. The cell adhesion rate (the slope of the intensity versus time curve) during VEGF treatment was 1.9-fold that of the control value.

Breast cancer cells express VEGF strongly (Brown et al. 1995; Lee et al. 2003), while mesentery microvascular endothelium expresses abundant VEGF receptor KDR/Flk-1 (Mukhopadhyay et al. 1998). To test whether the interaction between VEGF and its receptor, KDR/Flk-1, plays a role in breast cancer cell adhesion, we pretreated the cells with anti-VEGF for 60 min at 4°C before perfusing the microvessel, or pretreated the microvessel with a VEGF receptor (KDR/Flk-1) inhibitor, SU-1498, for 45 min before perfusing the cells. Figure 4B shows our results. Anti-VEGF (filled triangles) and SU-1498 (filled diamonds) almost completely abolished cancer cell adhesion to the vessel wall in conditions of normal permeability, starting from 5 min (adhesion during 0–5 min; P < 0.05).

**Effect of VEGF on WT cell adhesion to the microvessel wall**

Figure 5 shows the adhesion of ErbB2-transformed mouse mammary carcinoma cells. Figure 5A shows cell adhesion in normal permeability conditions, while Fig. 5B shows adhesion during treatment with VEGF. Compared with
control conditions, 1 nM VEGF significantly increased WT cell adhesion, starting from 5 min (adhesion during 0–5 min, \( P < 0.05 \)). As with MDA-MB-435s adhesion, WT cell adhesion increased almost linearly with time, with \( R^2 = 0.94 \) and 0.97 for control and VEGF treatment, respectively. The cell adhesion rate (the slope of the number of adherent cells versus time curve) during VEGF treatment was 1.8-fold that of control values.

As was also the case for MDA-MB-435s, pretreatment of the microvessel with SU-1489 completely abolished WT cell adhesion in normal permeability conditions (diamonds in Fig. 5A; \( P < 0.001 \) starting at 5 min).

Integrins, such as \( \alpha6\beta4 \) and \( \alpha6\beta1 \), and their ligands in the ECM, laminin-5, -4, -2 and -1, have been suggested as key players for breast cancer cell adhesion (Spinardi et al. 1995; Giannelli et al. 2002; Guo & Giancotti, 2004; Guo et al. 2006). We therefore examined the integrin effect on cell adhesion in our in vivo single-vessel perfusion model. In normal permeability conditions and during VEGF treatment, a control antibody, W632 (filled triangles in Fig. 5A and B), insignificantly decreased the WT cell adhesion (\( P > 0.05 \)). However, anti-\( \alpha6 \) (filled circles in Fig. 5A and B) and anti-laminin-5 (open squares in Fig. 5A and B) significantly decreased the WT cell adhesion by 40–50\% in both conditions (\( P < 0.05 \)).

**Effect of Flow on MDA-MB-435s cell adhesion to the microvessel wall**

The effect of flow on tumour cell adhesion in a single microvessel is shown in Fig. 6. In control conditions, Fig. 6A demonstrates that after 25 min, normal perfusion induced a 30–37\% higher adhesion than that induced by a reduced perfusion (\( P < 0.05 \)). Similarly, Fig. 6B shows that during VEGF treatment, perfusion at a velocity of \( \sim 1000 \mu \text{m s}^{-1} \) induced a 20–47\% higher adhesion than that induced by a perfusion velocity of \( \sim 150 \mu \text{m s}^{-1} \) after 30 min (\( P < 0.05 \)). Figure 6C shows the ratio of mean adhesion amount during VEGF treatment to that in control conditions. Treatment with VEGF increased adhesion beyond that observed in control conditions (ratio \( > 1 \)), but that increase was independent of flow rate. There was no significant difference between the normal and reduced flows at any time (\( P > 0.75 \)).

**Effect of VEGF on apparent solute permeability (P) of rat mesenteric microvessels**

As in our previous studies (Fu & Shen, 2004), paired measurements of \( P \) in individual rat mesenteric microvessels (Fig. 7) indicate that \( P \) for three solutes of different sizes showed a transient increase during treatment with VEGF, peaked at \( \sim 30 \text{s} \) and returned to baseline values in \( \sim 2 \text{min} \). The value of \( P \) for sodium fluorescein (mean ± S.E.M.) in five vessels, measured at the peak of the response to VEGF (25 ± 5 s), was \( 6.4 ± 0.74 \times 10^{-5} \text{ cm s}^{-1} \), compared with a baseline \( P \) of \( 2.9 ± 0.24 \times 10^{-5} \text{ cm s}^{-1} \). This represents a mean peak increase in \( P \) for sodium fluorescein of 2.2 ± 0.13-fold compared with the baseline in the same vessel (\( P < 0.01 \)). The value of \( P \) for \( \alpha\)-lactalbumin, again measured at the peak of the response to VEGF (26 ± 2 s), was \( 2.5 ± 0.54 \times 10^{-5} \text{ cm s}^{-1} \), compared with a baseline \( P \) of \( 0.63 ± 0.051 \times 10^{-5} \text{ cm s}^{-1} \). This represents a...
mean peak increase in $P$ for $\alpha$-lactalbumin of $3.9 \pm 0.6$-fold compared with the baseline in the same vessel ($P < 0.01$). The value of $P$ for BSA, measured at the peak of the response to VEGF (28 ± 4 s), was $0.37 \pm 0.02 \times 10^{-5}$ cm s$^{-1}$, compared with a baseline $P$ of $0.052 \pm 0.004 \times 10^{-5}$ cm s$^{-1}$. This represents a mean peak increase in $P$ for BSA of $7.1 \pm 0.8$-fold compared with the baseline in the same vessel ($P < 0.01$).

**Discussion**

Vascular endothelial growth factor has been shown to produce a biphasic increase in the permeability of normal microvessel walls, with an initial transient increase lasting no more than a few minutes (Bates & Curry, 1996; Fu & Shen, 2003, 2004), followed by a chronic sustained increase 24 h after stimulation (Bates, 1997). In the present experiment (Fig. 5), VEGF significantly increased the WT cell adhesion within 5 min of its onset, which is consistent with these findings. Only about 40% of WT cell adhesion (also MDA-MB-435s adhesion; Fig. 4B) occurred within 1 min in control conditions with normal permeability. In contrast, during VEGF treatment, more than 70% of WT cell adhesion occurred within 1 min. This acute VEGF-induced microvessel hyperpermeability is most probably in part due to an increase in the gap between endothelial cells forming the vessel wall (Seeger et al. 1993; Michel & Neal, 1999; Fu & Shen, 2003; Lee et al. 2003), as well as because of partial degradation of endothelial surface.

![Figure 6. Comparison of perfusion velocity effects on MDA-MB-435s cell adhesion at different times](image)

**Figure 6.** Comparison of perfusion velocity effects on MDA-MB-435s cell adhesion at different times

A, control conditions with 1% BSA–Ringer perfusate. B, treatment with 1 nM VEGF perfusate. C, comparison of the effect of VEGF on MDA-MB-435s cell adhesion at different times with normal and reduced perfusion velocities. *$P < 0.05$, **$P < 0.01$.

![Figure 7. Paired measurements of apparent solute permeability ($P$) in individual rat mesenteric microvessels in the control conditions when $P$ was first measured with Ringer solution perfusate containing 1% BSA, then when $P$ was measured in the same vessel during reperfusion with the same solution also containing 1 nM VEGF for ~5 min](image)

The figure shows the comparison between the control (CTR) and peak values (VEGF) during the treatment of VEGF. The peak was at ~30 s after VEGF perfusion. Measurements are for three solutes: sodium fluorescein (NaF), $\alpha$-lactalbumin and BSA; $n = 5$ for each solute. **$P < 0.01$ compared with the control conditions.

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Kajimura (2005) found that blood microvessel permeability and flow-induced hydrodynamic forces can also enhance tumour cell adhesion. Basson et al. (2003) used a microperfusion technique to vary the flow rate in frog mesenteric microvessels and demonstrated that permeability to potassium ions increased as velocity increased. In a very different preparation, Turner & Pallone (1997) reported that the permeability of isolated perfused descending vasa recta to small hydrophilic solutes increases with increasing perfusion rate. In addition to increasing microvessel permeability, flow-induced hydrodynamic factors can also enhance tumour cell adhesion. Basson et al. (2000) found in an in vitro system that increasing pressure stimulated malignant human colon cancer adhesion to matrix proteins by a cation-dependent, β1 integrin-mediated mechanism. Thamilselvan et al. (2004) demonstrated that non-laminar flow-induced shear stress increased colon cancer cell adhesion to extracellular proteins by a mechanism requiring both actin cytoskeletal reorganization and force activation of Src kinase. Tzima et al. (2005) found that blood flow-induced shear stress can trigger a mechanosensory complex that involves VEGFR2, platelet–endothelial cell adhesion molecule-1 and vascular endothelial cadherins. This mechanism would be expected to induce integrin activation. Our results show that increasing perfusion velocity and perfusion pressure did increase tumour cell adhesion both in control conditions and during VEGF treatment. However, the increase due to hydrodynamic factors was only about 35% when the perfusion velocity increased by ~6.7-fold and the driving pressure by about fivefold. We hypothesize that a greater rise in adhesion was not observed because of the inherently low magnitude of shear stress in our preparation. Shear stress in our perfused microvessel is only ~2.6 dyn cm\(^{-2}\) for a perfusion velocity of ~1000 μm s\(^{-1}\) and ~0.39 dyn cm\(^{-2}\) for a lower velocity of ~150 μm s\(^{-1}\). Cell adhesion with a perfusion velocity of ~1000 μm s\(^{-1}\) was almost completely abolished by an inhibitor of VEGFR2 (Figs 4B and 5A).

Leukocyte rolling at physiological flow rates has been demonstrated to be a prerequisite for stable adhesion to the endothelium (Butcher, 1991; Dong & Lei, 2000). A real-time \textit{ex vivo} experiment in excised dura mater (Glinskii et al. 2003) showed that prostate carcinoma cells exhibited rolling-like movement prior to engaging into stable adhesive interaction, while other neoplastic cells became stably adhered without rolling. In a flow chamber set-up, Giavazzi et al. (1993) observed that HT-29M colon carcinoma, OVCAR-3 ovarian carcinoma and T-47D breast carcinoma cells rolled on interleukin-1-activated human umbilical vein endothelial cells, but no rolling was observed for the A375M and A2058 melanomas and the MG-63 osteosarcoma, even at a very high shear stress. In \textit{vivo} observation of tumour cell adhesion in hepatic microcirculation showed that rolling of colon carcinoma cells was very rare. Cells almost adhered abruptly without reducing their velocity prior to adhesion (Haier et al. 2003). A similar effect was observed in the present single-vessel perfusion experiment. We observed no rolling adhesion behaviour for either MDA-MB-435s or WT cells. Unlike leukocyte extravasation after firm adhesion to the microvessel wall, in our 60 min \textit{in vivo} single-vessel experiments there was no tumour cell extravasation from the microvessel after adhesion.

In summary, our well-controlled, \textit{in vivo}, single-vessel perfusion study showed that VEGF increased microvessel permeability and enhanced both human and mouse breast cancer cell adhesion in the postcapillary venule of a rat mesentery. Anti-VEGF and an inhibitor of VEGFR2 almost completely abolished this adhesion. Anti-integrin α6 and anti-laminin-5 also reduced the WT cell adhesion in both normal and increased permeability conditions.
Our findings implicate a new target in antimetastatic therapy.

References


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