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Technical Advance

Quantitation and Physiological Characterization of Angiogenic Vessels in Mice

Effect of Basic Fibroblast Growth Factor, Vascular Endothelial Growth Factor/Vascular Permeability Factor, and Host Microenvironment

Marc Dellian, Brian P. Witwer, Hassan A. Salehi, Fan Yuan, and Rakesh K. Jain

From the E. L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

A prerequisite for the development of novel angiogenic and anti-angiogenic agents is the availability of routine* in vivo *assays that permit 1) repeated, long-term quantitation of angiogenesis and 2) physiological characterization of angiogenic vessels. We report here the development of such an assay in mice. Using this assay, we tested the hypothesis that the physiological properties of angiogenic vessels are governed by the microenvironment and vessel origin rather than the initial angiogenic stimulus. Gels containing basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) were implanted in transparent windows in the dorsal skin or cranium of mice. Vessels could be continuously and non-invasively monitored and easily quantified for more than 5 weeks after gel implantation. Newly formed vessels were first visible on day 4 in the cranial window and day 10 in the dorsal skinfold chamber, respectively. The number of vessels was dependent on the dose of bFGF and VEGF. At 3000 ng/ml, bFGF- and VEGF-induced blood vessels had similar diameters, red blood cell velocities, and microvascular permeability to albumin. However, red blood cell velocities and microvascular permeability to albumin were higher in the cranial window than in the dorsal skinfold chamber. Leukocyte-endothelial interaction was nearly zero in both sites. Thus, newly grown microvessels resembled vessels of granulation and neoplastic tissue in many aspects. Their physiological properties were mainly determined by the microenvironment, whereas the initial angiogenic response was stimulated by growth factors. (Am J Pathol 1996, 149:59–71)

The formation of new blood vessels (angiogenesis) plays an important role in development, physiological repair processes, and various diseases such as diabetic retinopathy and the growth of solid tumors.[1] The past decade has witnessed discovery of more than 20 angiogenic and anti-angiogenic factors.[2] Among these, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) are the most potent and widely investigated angiogenic factors.[2] bFGF is a mitogen of cells from several different tissues and increases both cell migration and proliferation.[2]

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Address reprint requests to Rakesh K. Jain, Department of Radiation Oncology, Massachusetts General Hospital, Boston, MA 02114.

VEGF/VPF, expressed in a variety of cells including tumor cells[3–6] and up-regulated in cells of hypoxic tissues,[7–9] has been shown to increase permeability of blood vessels and to be a specific mitogen for endothelial cells.[3,5] The role of these two cytokines during growth and metastasis of solid tumors has been studied extensively.[10] Each tumor has its own angiogenic profile.[11] Some tumors depend on and secrete large amounts of bFGF,[12,13] whereas others release VEGF.[14–16] It seems, therefore, reasonable to hypothesize that the angiogenic vessels resulting from different growth factors have different physiological characteristics.[17] Furthermore, the characteristics of these vessels are also likely to depend on the origin of the endothelial cells and the local microenvironment,[18] because host cells such as macrophages and fibroblasts in the stroma participate in angiogenesis by releasing positive and negative regulators.[10,19]

A prerequisite to delineate the role of host factors *versus* extrinsic angiogenic factors is the availability of assays that would permit 1) repeated, long-term quantitation of angiogenesis and 2) physiological characterization of angiogenic vessels. A variety of methods have contributed to our current understanding of angiogenesis and allowed the discovery of factors controlling neovascularization.[20] A number of questions, however, remain unanswered including the role of different growth factors in angiogenesis, the time interval at which the individual factors are effective, and the extent to which they affect the physiological properties of blood vessels. To date, these questions have been addressed partly *in vitro* by examining proliferation, migration, and differentiation of endothelial cells derived from different tissues and exposed to different growth factors. Relatively few methods exist for analysis of the complex events during angiogenesis *in vivo*. Direct observation of newly growing vessels is possible in routinely used assays such as the rabbit or mouse cornea and the chicken embryo chorioallantoic membrane, although efforts for quantification of the response are aggravated by three-dimensional vessel growth and high variability.[20] Many *in vivo* models are based on indirect evaluation of angiogenesis from excised tissue[21] or measurement of blood flow.[22] Procedures based on the quantification of hemoglobin content[21] or vascular density in histological sections[23–24] are invasive and are thus limited to a single time point only. Nguyen et al[25] have recently developed an elegant, quantitative angiogenesis assay for the chicken embryo chorioallantoic membrane. Because angiogenesis is affected by the host, an assay allowing prolonged investigation of the angiogenic response to different growth factors and physiological properties of newly grown blood vessels at different sites in mammals would be a clear advantage.

To this end, we have established a method for repeated, non-invasive observation and quantification of angiogenesis in mice. We have used this approach to test the hypothesis that the physiological properties of angiogenic vessels are governed by the microenvironment rather than the initial angiogenic stimulus. Similar to the chorioallantoic membrane assay,[25] a background level of angiogenesis was observed in control animals in our pilot studies. This nonspecific angiogenesis reflects a host response to implants and cannot be eliminated in *in vivo* models, with the exception of the cornea, which physiologically inhibits vessel growth. Therefore, the effect of exogenous growth factors (eg, bFGF and VEGF/VPF) on angiogenesis and physiological properties of the newly formed blood vessels can be assessed only by the differences in parameters between treated and control groups. In our study, a collagen gel containing angiogenic factors (bFGF or VEGF/VPF) was implanted in transparent chronic windows in the dorsal skin or on the pia mater (Figure 1A). Newly formed vessels grew vertically toward the window and could be directly observed and noninvasively quantified in this focal plane. This model allowed a comparison of physiological properties of newly formed blood vessels modified by bFGF and VEGF/VPF, respectively, at the two different anatomical sites. These properties included functional vascular density and diameters, red blood cell (RBC) velocity, leukocyte-endothelial interaction, and vascular permeability to albumin.

Materials and Methods

Animal Models

Experiments were performed in male severe combined immunodeficient (SCID) mice (6 to 8 weeks old, 25 to 35 g) following institutional guidelines, bred and housed in our defined flora animal colony. A dorsal skinfold chamber[26,27] or cranial window[28,29] was implanted under anesthesia (75 mg of ketamine hydrochloride/25 mg of xylazine per kg body weight subcutaneously) as described earlier.[27,29] Animals were allowed to recover from the chamber implantation surgery (2 days after dorsal skinfold chamber, >8 days after cranial window preparation) before the implantation of gels saturated with growth factors.

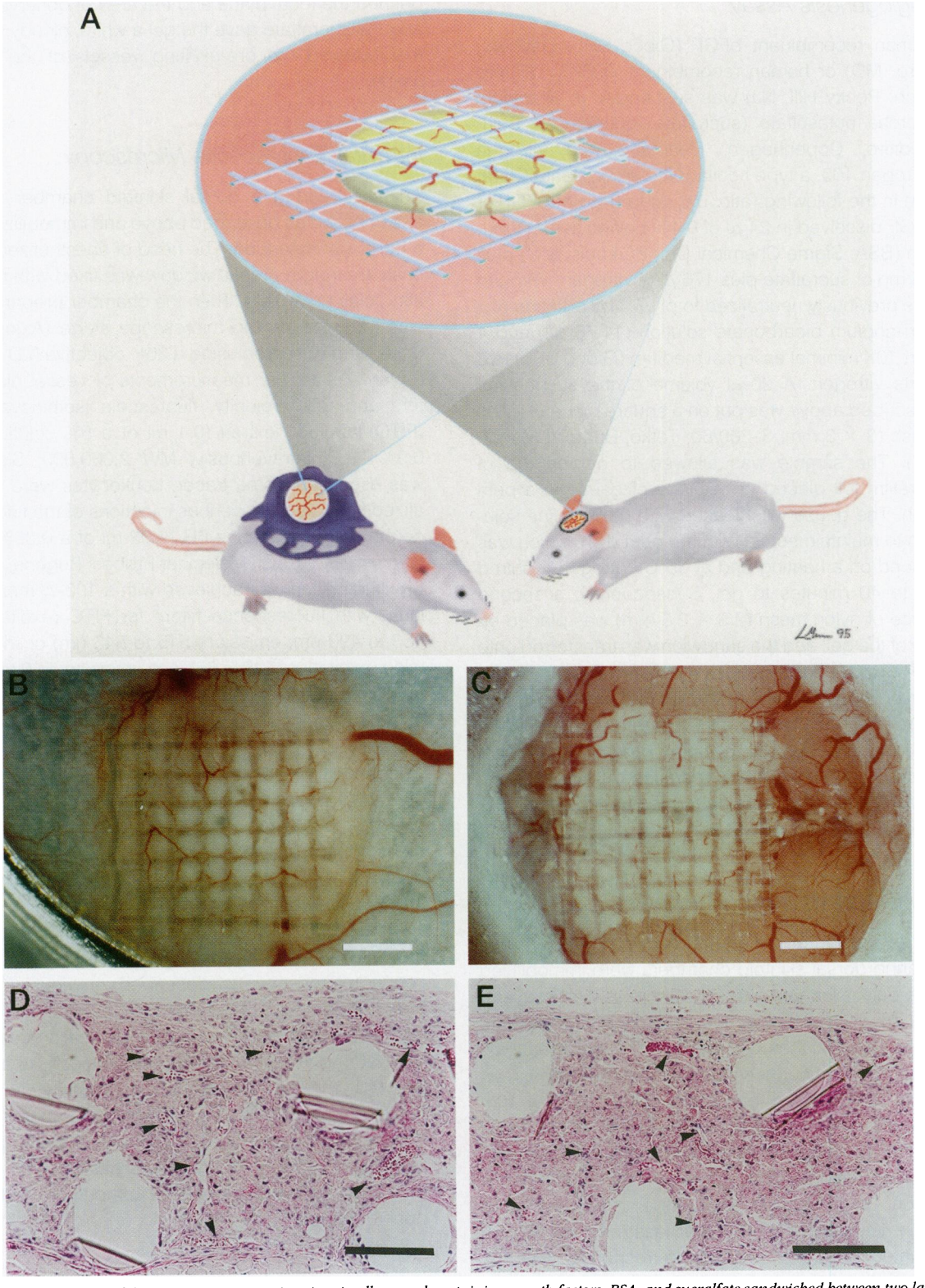

Figure 1. A: *Scheme of the angiogenesis assay in mice. A collagen gel containing growth factors, BSA, and sucralfate sandwiched between two layers of nylon mesh (top) is implanted into a subcutaneous or cranial window preparation of mice.* B *and* C: *Microscopic pictures of the angiogenesis response to a gel containing 3000 ng/ml bFGF implanted in the dorsal skinfold chamber* (B) *and the cranial window* (C) *on day 25 and day 12 after implantation, respectively. Newly formed blood vessels are visible on top of the nylon mesh. Bar, 1 mm.* D *and* E: *Histological section (hematoxylin and eosin) through a collagen gel containing 3000 ng/ml bFGF excised on day 25* (D) *after implantation in the dorsal skinfold chamber and day 12* (E) *after implantation in the cranial window. Numerous blood vessels* (arrows) *growing from the underlying striated muscle of the skin* (D) *or the pia mater* (E) *toward the glass coverslip* (top) *were visible. Large holes represent position of nylon fibers of the mesh. Bar, 100 μm.*

Angiogenesis Assay

Human recombinant bFGF (Gibco BRL, Gaithersburg, MD) or human recombinant $VEGF_{165}$ (Pepro Tech, Rocky Hill, NJ) was suspended in aluminum sucrose octasulfate (sucralfate; courtesy of Bukh Meditec, Copenhagen, Denmark) and vitrogen (vitrogen 100, a type I collagen; Celtrix, Santa Clara, CA) in the following ratio: 0.6 ng to 6 μg of bFGF or VEGF dissolved in 24 μl of 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) plus 6.5 mg of sucralfate plus 176 μl of vitrogen. Vitrogen was previously neutralized to pH 7.4 by addition of 1 part sodium bicarbonate solution (11.76 g/l) and 1 part 10X minimal essential medium (Gibco BRL) to 8 parts vitrogen. A 20-μl volume of the suspension described above was put on a square piece of nylon mesh (3 × 3 mm, 3–300/50; Tetko, Briarcliff Manor, NY). The sample was allowed to gel on top of parafilm foil placed on a piece of gauze in a petri dish. The gauze was soaked with 0.9% saline solution to maintain humidity in the petri dish, which was placed on a heating pad at 35°C. Vitrogen required 20 to 40 minutes to gel. Subsequently, a second piece of nylon mesh (2.5 × 2.5 mm) was placed on top of the gel, and the sandwich was transferred onto the subcutaneous tissue of the dorsal skinfold chamber or the pia mater of the cranial window (Figure 1A). The chambers were then carefully closed again with a glass coverslip, avoiding pressure on the gel and air bubbles in the preparation. All preparations and implantations were performed under sterile conditions.

Quantification of Angiogenesis

For evaluation of the angiogenic response, animals bearing dorsal skinfold chambers were immobilized in a polycarbonate tube of 25 mm in diameter and placed on the stage of a microscope (Axioplan, Zeiss, Oberkochen, Germany). The collagen gels were observed under transillumination using green filters to enhance the vessels' contrast with a 10× objective. Angiogenesis in the cranial windows was quantified under ketamine/xylazine anesthesia with epi-illumination under a stereomicroscope (SMZ-1; Nikon, Tokyo, Japan). The number of squares in the top nylon mesh containing at least one vessel was counted, and the angiogenic response was expressed as the percentage of squares with at least one vessel. Counting of the vessels was easily done with low inter-investigator variability, because newly formed blood vessels grew vertically toward the coverslip; the top mesh directly below the coverslip defined the focal plane and the area of observation, and the sucralfate gave the gel a white, cloudy color that covered the pre-existing vessels of the host tissue.

Intravital Fluorescence Microscopy

Animals bearing a dorsal skinfold chamber were anesthetized as described above and immobilized in a polycarbonate tube. The head of anesthetized animals bearing a cranial window was fixed with modeling clay on a plate. Then the chamber preparation was mounted on the microscope stage (Axioplan, Zeiss) and observed using a 20× objective (LD Achroplan, Zeiss). For measurements of vessel diameters and RBC velocity, fluorescein isothiocyanate (FITC)-labeled dextran (0.1 ml of a 5% solution in 0.9% NaCl intravenously; MW 2,000,000; Sigma) was used as plasma tracer. Leukocytes were visualized *in vivo* by intravenous injections of the fluorescent marker rhodamine 6G (0.02 ml of a 0.05% solution in 0.9% NaCl; Molecular Probes, Eugene, OR). Epi-illumination was achieved with a 100-W mercury lamp with fluorescence filters for FITC (excitation, 450 to 490 nm; emission, 515 to 545 nm) or rhodamine (excitation, 525 to 555 nm; emission, 580 to 635 nm). Images of confluent microvessels in five areas of the collagen gel were acquired using a CCD camera (AVC D7, Sony, Tokyo, Japan), displayed on a video monitor (PVM-11, Electrohome), and recorded on video tapes using a video recorder (SVO-9500MD, Sony) for off-line analysis. For measurement of functional vascular density, which is defined later, a 10× objective was used to acquire images of the vasculature visualized by FITC-dextran.

Measurements of the effective microvascular permeability to albumin were performed by fluorescence microscopy as described previously.[30] In brief, rhodamine-labeled BSA (MW 67,000; Molecular Probes) was dissolved in phosphate-buffered saline (6.5 mg/ml) and injected intravenously as a bolus (40 mg/kg). Fluorescence intensity in the selected area with a 20× objective was quantified every minute over a period of 20 minutes by a photomultiplier (9203B, Thorn EMI, Rockaway, NJ). The data were stored on a personal computer for subsequent analysis.

Quantification of Microhemodynamic Parameters

RBC velocities and vessel diameters were analyzed using a four-slit apparatus (model 208C, IPM, San

Diego, CA) and an image shearing monitor (model 907, IPM), respectively, as described earlier in detail.[27] Leukocyte flux (all passing leukocytes) and rolling leukocytes were quantified as the number of cells passing a defined cross section of the vessel per unit time. From these data, the percentage of rolling leukocytes was calculated.[31] Adherent leukocytes were given as the number of leukocytes sticking for 30 seconds per square millimeter of vessel wall surface.[31] Functional vascular density, defined as the total length of vessels (with plasma flow) per unit area of observation, was measured using NIH Image software after digitization of images from video tapes on a Macintosh computer.[27]

Histology

The gel and underlying tissue were excised from the chamber preparation and fixed in 4% buffered formaldehyde. Sections from the tissue embedded in paraffin were stained with hematoxylin and eosin (H&E).

Experimental Groups

The response to different doses of bFGF (30, 300, 3,000 and 30,000 ng/ml) and VEGF (30 or 300 and 3,000 ng/ml) in comparison with controls was investigated in 42 animals bearing a dorsal skinfold chamber and 42 animals with a cranial window preparation (six animals per group). Microcirculatory parameters and microvascular permeability were assessed in separate groups of animals (six animals per group) after implantation of gels containing BSA only, bFGF, or VEGF (3,000 ng/ml).

Statistics

Results are presented as mean ± SEM. Values of several independent groups were compared with the Kruskal-Wallis and the Mann-Whitney *U* test using StatView (Abacus, Berkeley, CA). The Spearman's coefficient was calculated to analyze correlation. *P* values smaller than 5% were considered to be significant.

Results

Quantitative Response to bFGF and VEGF

In a first group of experiments, we evaluated dynamics of angiogenic response to different doses of bFGF and VEGF in the dorsal skinfold chamber and the cranial window. After implantation of the gel, vascular sprouts were first visible with high doses of bFGF or VEGF (3000 ng/ml) on day 4 (cranial window) and day 10 (dorsal skinfold chamber), respectively. Peak response to high doses of growth factors (3000 ng/ml) was seen on day 12 in the cranial window and day 25 in the dorsal skinfold chamber. Figure 1, B and C, shows the newly formed vascular network in the dorsal skinfold chamber and cranial window on the days of peak response. As confirmed from histological sections of the gels (Figure 1, D and E), blood vessels grew toward the glass coverslip from the underlying tissue and crossed both layers of the nylon mesh in the gel. In addition to ingrowing vessels, a similar cellular infiltration into the collagen gel including fibroblasts and leukocytes was seen at both sites. Quantitative results obtained in the dorsal skinfold chamber and the cranial window are depicted in Figure 2. Newly formed blood vessels were visible earlier with high doses of bFGF or VEGF (3000 ng/ml) than in controls and also covered 100% of the squares in the top mesh more rapidly (on day 13 in the cranial window and day 27 in the dorsal skinfold chamber). In the dorsal skinfold chamber 30, 300, 3,000, and 30,000 ng/ml bFGF (Figure 2A) or 30, 300, and 3,000 ng/ml VEGF/VPF (Figure 2B) clearly induced a more intense angiogenesis than controls. The effect was dose dependent with high doses approaching 100% of the squares earlier than low doses; 3,000 ng/ml bFGF and VEGF and 30,000 ng/ml bFGF resulted in a comparable angiogenic response. Similar results were obtained in the cranial window (Figure 2, C and D). The results obtained were highly reproducible in the subsequent experiments for observation of physiological properties of newly grown blood vessels. Inter-observer differences in the count of squares filled with at least one blood vessel ranged between 5 and 10%.

Functional Vascular Density

By intravital microscopy, the density of vessels in tissue can be most accurately quantified by measurement of functional vascular density (Figure 3A), ie, the total length of perfused microvessels per unit area of observation.[32] We measured simultaneously functional vascular density and the percentage of squares with at least one vessel in bFGF-induced angiogenesis to evaluate the accuracy of the simple method based on counting the squares with at least one vessel. The results shown in Figure 3B revealed excellent correlation between the two parameters.

Intensity of the angiogenesis response was compared between the two different locations by mea-

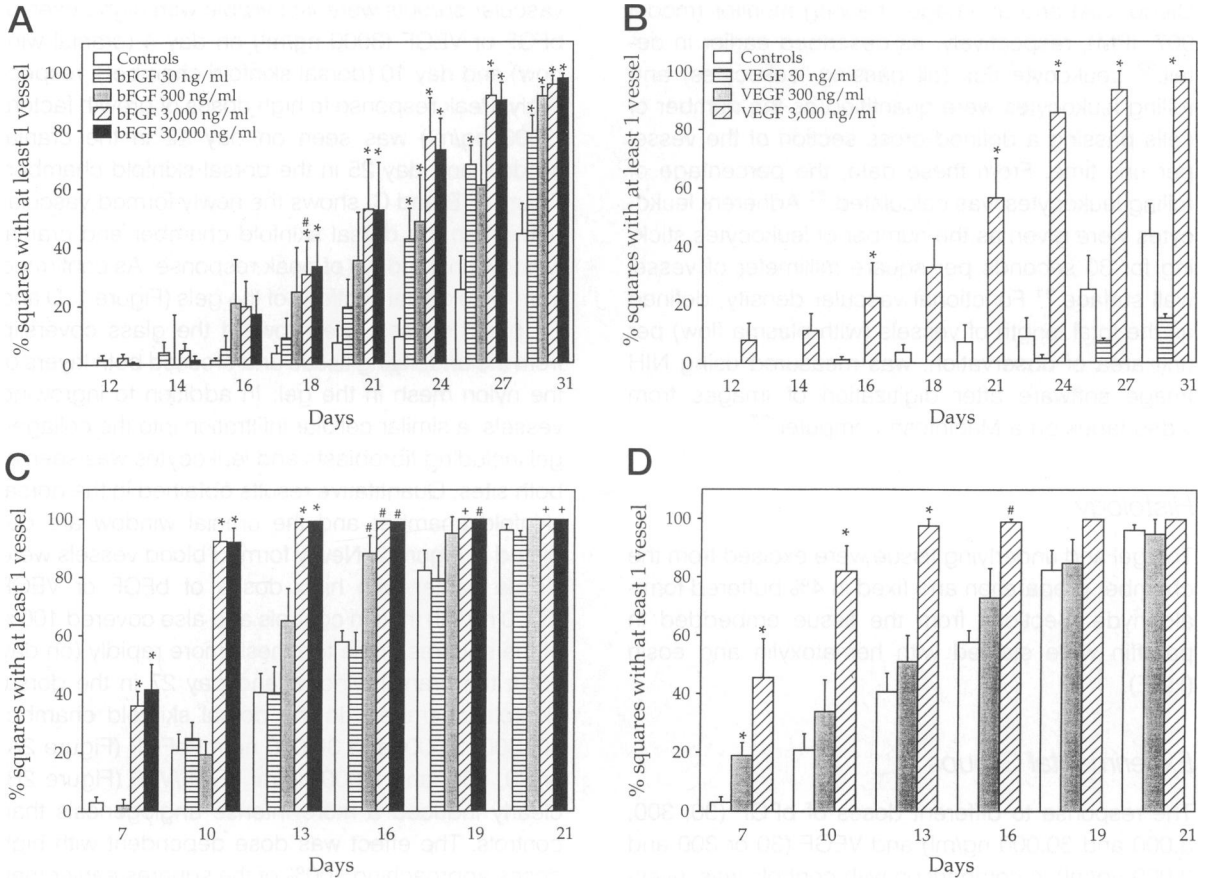

Figure 2. *Effect of bFGF and VEGF dose on angiogenesis* in vivo *as quantified by counting the squares in the top mesh containing blood vessels. Each group consisted of six animals.* **A:** *Angiogenesis response to different doses of bFGF in the dorsal skinfold chamber as compared with controls.* *P < 0.05 versus *corresponding controls;* #P < 0.05 versus *30 ng/ml bFGF.* **B:** *Effect of VEGF (30, 300, and 3000 ng/ml) on angiogenesis in the dorsal skinfold chamber.* *P < 0.05 versus *corresponding controls.* **C:** *Angiogenesis response to different doses of bFGF in the cranial window.* *P < 0.05 versus *controls and 30 and 300 ng/ml bFGF;* #P < 0.05 versus *controls and* versus *30 ng/ml bFGF;* +P < 0.05 versus *controls.* **D:** *Angiogenesis in the cranial window after 300 and 3000 ng/ml VEGF compared with controls.* *P < 0.05 versus *controls and* versus *300 ng/ml VEGF;* +P < 0.05 versus *controls.*

suring functional vascular density (Figure 4). At the time of peak response, day 25 in the dorsal skinfold chamber and day 12 in the cranial window, 3000ng/ml bFGF- and VEGF-induced vasculature demonstrated significantly higher vascular density than controls. Functional vascular density of vessels induced by bFGF was higher in the dorsal skinfold chamber but comparable for VEGF-induced vessels in two locations. Results measured in the cranial window on day 25 showed no changes in vascular density compared with day 12, with the exception of controls, which reached values similar to bFGF- and VEGF-induced vasculature. This indicates that vascular density did not increase or decrease later (eg, regression of vessels) after reaching peak response, which is also reflected in saturation of the count of squares filled with at least one vessel (approaching 100%) as shown in Figure 2.

Vessel Diameters, RBC Velocity, and Leukocyte-Endothelial Interaction

Based on the results of the experiments on dose response of angiogenesis, we selected 3000 ng/ml bFGF and VEGF for subsequent investigations on physiological properties of bFGF- and VEGF-derived blood vessels. Intravital videomicroscopy visualized geometric and hemodynamic characteristics of newly grown vessels (Figure 5). Whereas sluggish blood flow or even stasis was frequently observed in the early stages of angiogenesis in both anatomical locations, all blood vessels were perfused by day 20 (dorsal skinfold chamber) and day 7 (cranial window), respectively. As depicted in Figures 6 and 7, vessel diameters were comparable, but RBC velocity was almost twofold higher in newly formed vessels in the cranial window as compared with the dorsal

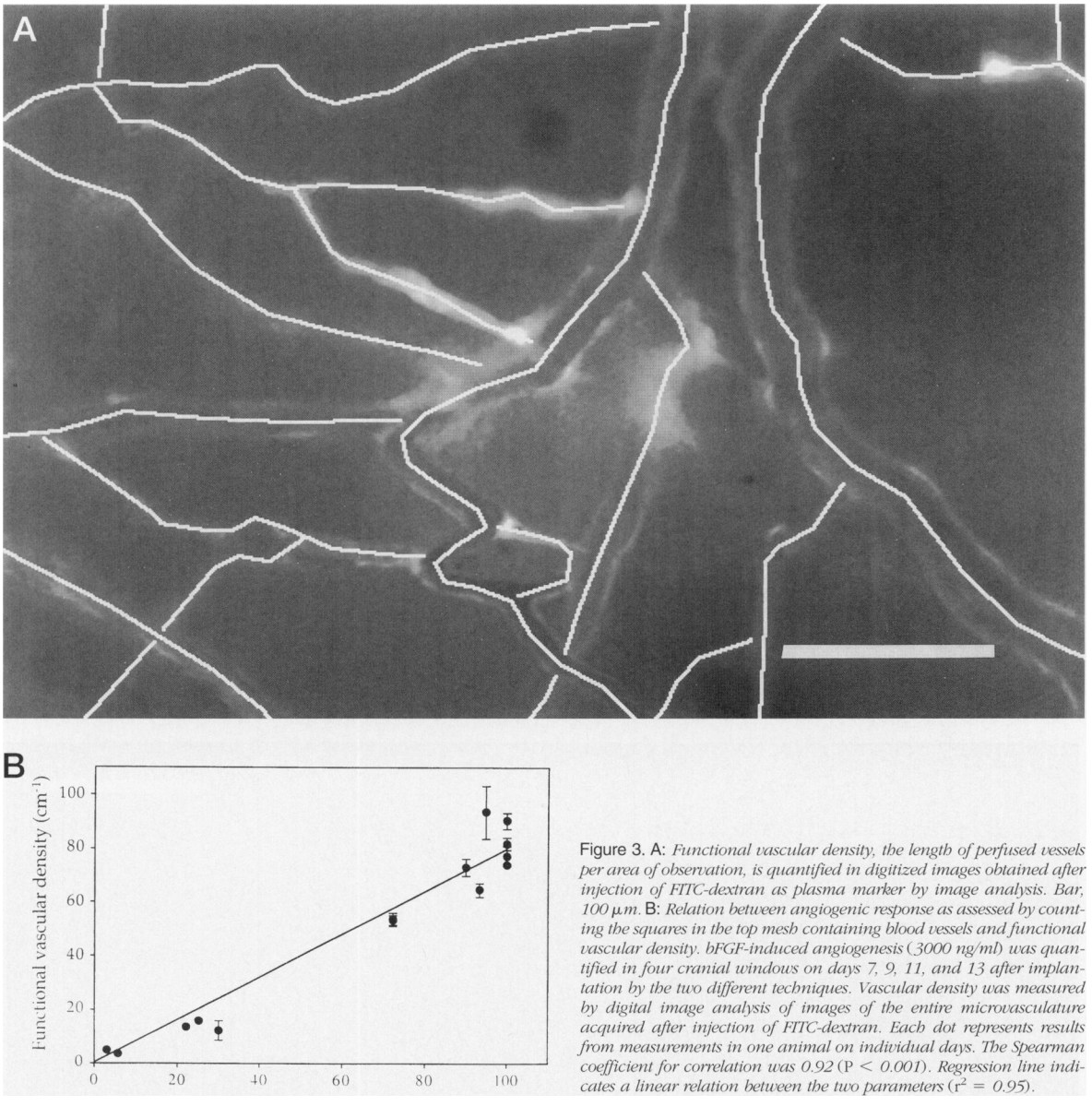

Figure 3. A: *Functional vascular density, the length of perfused vessels per area of observation, is quantified in digitized images obtained after injection of FITC-dextran as plasma marker by image analysis. Bar, 100 μm.* B: *Relation between angiogenic response as assessed by counting the squares in the top mesh containing blood vessels and functional vascular density. bFGF-induced angiogenesis (3000 ng/ml) was quantified in four cranial windows on days 7, 9, 11, and 13 after implantation by the two different techniques. Vascular density was measured by digital image analysis of images of the entire microvasculature acquired after injection of FITC-dextran. Each dot represents results from measurements in one animal on individual days. The Spearman coefficient for correlation was 0.92 ($P < 0.001$). Regression line indicates a linear relation between the two parameters ($r^2 = 0.95$).*

skinfold chamber. To evaluate the effect of the age of the newly formed vessels on differences in RBC velocities, vessel density, and vessel diameter observed between the two locations, we measured these parameters in separate groups of animals with cranial windows on day 25. Results were similar to those obtained in the cranial window on day 12 (Figures 4, 6 and 7). The data presented in Table 1 show that leukocyte-endothelial interaction was comparable and almost absent in bFGF- and VEGF/VPF-induced blood vessels at the time of peak response. Slightly higher numbers of adherent leukocytes were found in the cranial window on day 25 as compared with day 12 ($P < 0.01$). The response of the young endothelium to activation by superfusion with tumor necrosis factor-α (100 ng in 10 μl) was tested in bFGF-induced microvessels in the dorsal skinfold chamber on day 25 (n = 5). Three hours after superfusion, when maximal increase of expression of leukocyte adhesion molecules was expected,[33] increases from 0 to 106 ± 33 adherent leukocytes/mm^2 and from 0 to 73 ± 10% rolling leukocytes were observed ($P < 0.01$), whereas leukocyte flux remained at a low level (3.6 ± 0.8 cells/30 seconds).

Microvascular Permeability

Besides modulating endothelial proliferation and angiogenesis, growth factors may influence vascular permeability, as demonstrated for VEGF.[34] The ef-

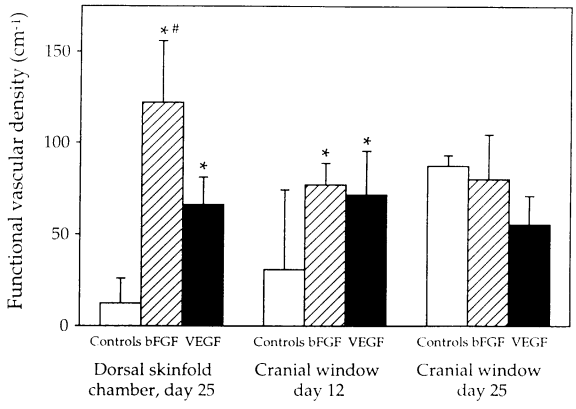

Figure 4. *Functional vascular density (mean ± SEM) of the newly formed vasculature in controls, bFGF (3000 ng ml), and VEGF (3000 ng ml) releasing gels in the dorsal skinfold chamber (day 25) and in the cranial window (day 12 and day 25). Control animals were implanted with gels without the growth factors. Each group consisted of six animals. *P < 0.05 versus corresponding controls; #P < 0.01 versus VEGF in the dorsal skinfold chamber and versus bFGF in the cranial window on day 12.*

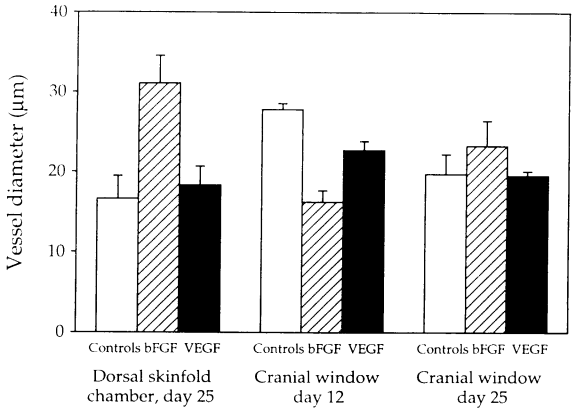

Figure 6. *Vessel diameters (mean ± SEM) of controls and bFGF- and VEGF-induced microvessels (3000 ng ml) in the dorsal skinfold chamber (day 25) and in the cranial window (day 12 and day 25). Control animals were implanted with gels without the growth factor (n = 6).*

fective microvascular permeability to BSA was measured in newly formed blood vessels in the dorsal skinfold chamber and the cranial window (Figure 8). No difference was observed in the microvascular permeability between bFGF- and VEGF-induced vessels or controls at the same location. However, angiogenic vessels in the cranial window exhibited an approximately fourfold higher permeability to BSA than vessels in the dorsal skinfold chamber ($P <$

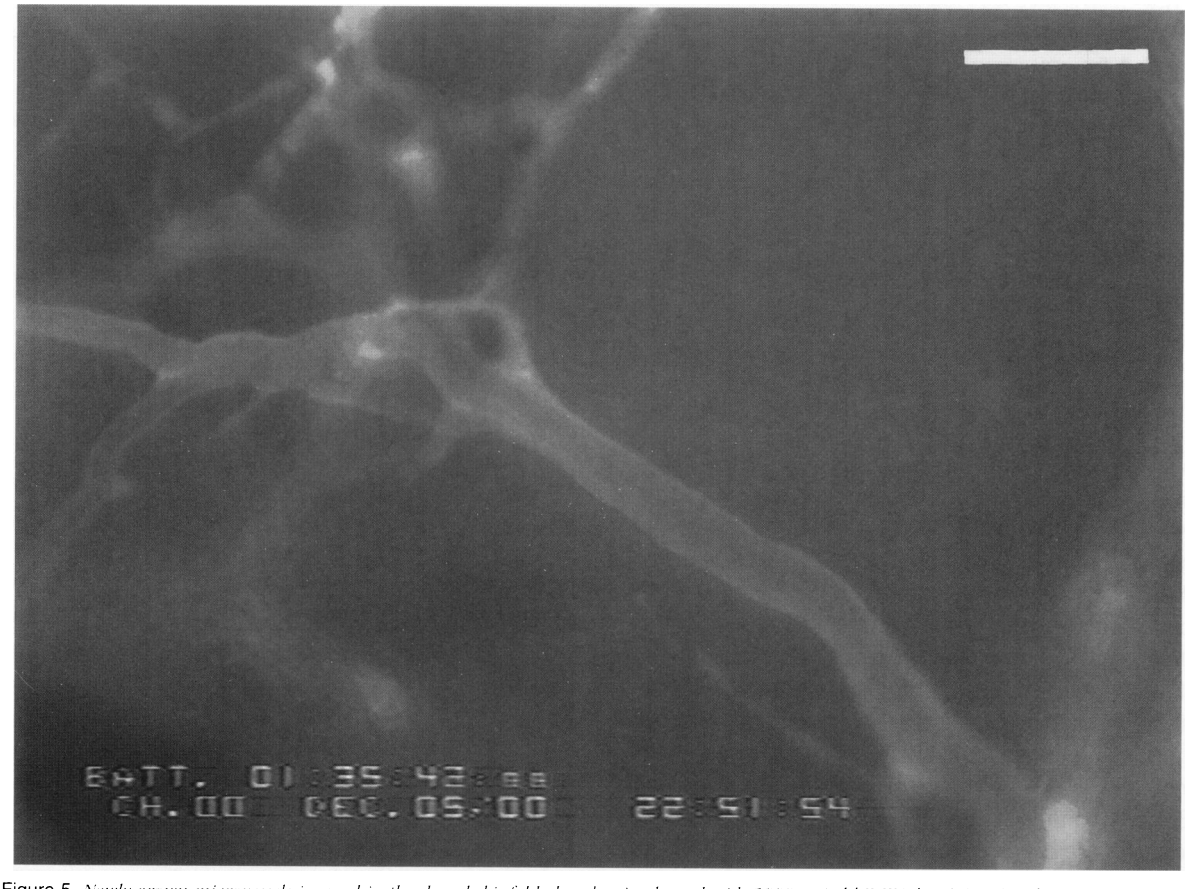

Figure 5. *Newly grown microvessels in a gel in the dorsal skinfold chamber implanted with 3000 ng ml bFGF (day 25). FITC-dextran was used as intravascular marker. Bar, 50 μm.*

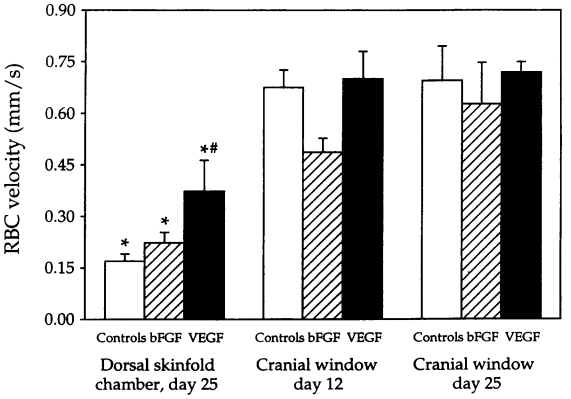

Figure 7. *RBC velocity in microvessels of controls and induced by bFGF (3000 ng/ml) and VEGF (3000 ng/ml) in the dorsal skinfold chamber (day 25) and the cranial window (day 12 and day 25). Each group consisted of six animals.* *P < 0.05 versus *cranial window (day 12 and day 25);* #P < 0.05 versus *corresponding controls.*

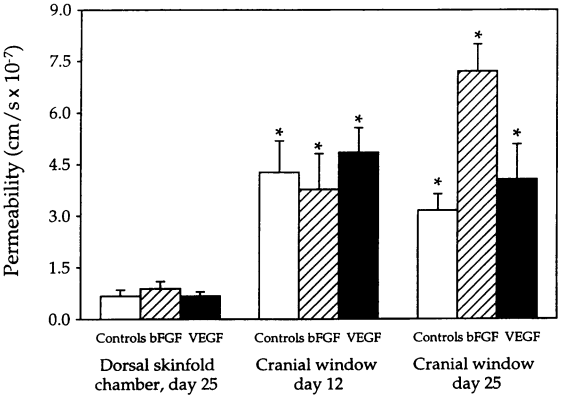

Figure 8. *Effective microvascular permeability to BSA of control vessels and vessels induced by bFGF and VEGF (3000 ng/ml) in the dorsal skinfold chamber and the cranial window (n = 6).* *P < 0.01 versus *corresponding group of the dorsal skinfold chamber.*

0.01). The age of the vessels has no effect on the vascular permeability (Figure 8).

Discussion

Response to Different Doses of bFGF and VEGF/VPF

The new model presented here allows investigation of angiogenesis by direct observation of newly growing vessels in mice. Induction of blood vessels preferentially exposed to one individual growth factor *in vivo* was possible by implantation of a gel slowly releasing the bFGF or VEGF in transparent tissue chamber preparations in mice. The assay was composed of a collagen gel, which provided support and the matrix for ingrowing vessels. Sucralfate in the collagen gel presumably protected and slowly released the heparin-binding growth factors bFGF and VEGF/VPF.[35] The results show that angiogenic response can be easily and non-invasively quantified over a prolonged period of time by directly counting the number of vessels. The size of the nylon mesh allows for measurements with high accuracy due to the low vascular density, as demonstrated by the relation between the count of squares in the top mesh containing at least one vessel and functional vascular density. Because the number of vessels growing into each square in the top nylon mesh was low, saturation of the parameter used to quantify angiogenesis occurred late. The assay also makes the investigation of the newly formed vessels' physiological properties possible. Both bFGF and VEGF/VPF induced a dose-dependent angiogenic response in the two locations. The site of bFGF action on angiogenesis is controversial. Vascular endothelial cells may not express receptors to bFGF *in vivo*. Thus, our results could not distinguish the direct *versus* indirect effect of bFGF on angiogenesis. Newly formed blood vessels were visible earlier in the cranial window than in the dorsal skinfold chamber (day 4 *versus* day 10), which may reflect variations in the distribution of growth factor receptors between skin and brain, an effect of the lower temperature of the skin, or other differences between the

Table 1. *Leukocyte Flux and Rolling and Adherent Leukocytes in Angiogenic and Host Vessels*

	Dorsal skinfold chamber (day 25)				Cranial window (day 12)				Cranial window (day 25)		
	Host*	Controls	bFGF	VEGF	Host*	Controls	bFGF	VEGF	Controls	bFGF	VEGF
Leukocyte flux (1/30 seconds)	8.4 ± 1.0	0.0 ± 0.0	1.5 ± 0.3	0.4 ± 0.2	38.8 ± 8.0	1.1 ± 0.5	1.5 ± 0.5	1.1 ± 0.4	3.3 ± 0.3	2.5 ± 0.9	1.2 ± 0.4
Rolling leukocyte flux (1/30 seconds)		0	0	0	0.9 ± 1.0	0	0	0	0	0	0
Rolling leukocytes (%)	62.9 ± 7.2	0	0	0		0	0	0	0	0	0
Adherent leukocytes (1/mm²)	54.3 ± 37.0	0	0	0	16.4 ± 18.9	0	0	0	5.0 ± 4.2	12.6 ± 5.3	6.7 ± 5.1

*Data from Fukumura et al.[44]

two sites that are as yet unknown. It seems remarkable, however, that it required 4 days in the cranial window and 10 days in the dorsal skinfold chamber before vessels became first visible. Angiogenesis in a rat sponge model induced by VEGF revealed a similar time course as observed in the cranial window, with maximal response registered 10 days after implantation.[36] The lack of differences between 3,000 and 30,000 ng/ml bFGF may be explained by saturation of its receptors. A background level of angiogenesis was observed in control animals similar to that reported by Nguyen et al[25] for the chorioallantoic membrane. This may be related to angiogenesis induced by BSA in control gels, extraction of growth factors from adjacent tissue by the sucralfate, which is known to bind and protect bFGF,[35] or growth factors released by infiltrating host cells.

Physiological Properties of Newly Formed Blood Vessels

Based on results of experiments on dose response, we selected 3000 ng/ml bFGF and VEGF for the subsequent investigation of properties of blood vessels by intravital microscopy. The morphological characteristics of newly formed vessels resembled those found in granulation tissue or solid tumors in many aspects. Their mostly disordered architecture differed largely from the regular, well defined geometric patterns observed in normal tissues. Functional vascular density was lower than values found in many other tissues, which may reflect a low nutritional demand of the tissue, which mainly consisted of collagen gel. Vascular diameters were irregular, revealed a large heterogeneity, and were slightly higher than those reported for granulation tissue,[37,38] similar to tumors.[39,40] RBC velocity and vessel diameters in newly formed vessels in the dorsal skinfold chamber were comparable to data reported for normal subcutaneous tissue.[39,40] The higher RBC velocity measured in newly grown microvessels in the cranial window may reflect the origin of these vessels from pial vessels, which have higher RBC velocities.[29]

Investigation of leukocyte-endothelial interaction by intravital microscopy allows evaluation of the expression and function of leukocyte adhesion molecules on the endothelium. The lack of interaction between leukocytes and microvascular endothelium on the days of peak response in both sites may be explained by immaturity of the endothelium, similar to that observed in granulation tissue[41] and tumor microvasculature.[42–45] Slightly higher values of adhesive leukocytes as observed in the assay in the cranial window on day 25 are comparable to those reported for 6-week-old granulation tissue in the rabbit ear chamber.[42] However, the number of leukocytes interacting with the endothelium was strikingly below values found in vasculature of normal subcutaneous tissue.[44,46] Activation of the endothelium by superfusion of the gels with tumor necrosis factor-α[33,44] induced only a modest increase in the number of leukocytes interacting with the endothelium of the newly formed blood vessels. In contrast, a more than 10-fold increase of adherent leukocytes was found in subcutaneous postcapillary venules after superfusion with tumor necrosis factor-α.[44] These observations suggest that adhesion receptors for leukocytes were down-regulated or not adequately expressed in the young endothelium. Moreover, leukocyte flux was also reduced in comparison with normal tissues despite comparable RBC velocity and blood flow, similar to that in tumors[44] (M. Dellian, unpublished observation). The new finding of a reduced number of leukocytes passing through newly grown vessels may be caused by rheological factors at the sites where angiogenic vessels branch off capillaries or venules of normal tissue. However, additional studies are required to investigate the regulation of adhesion molecule expression in this endothelium in response to various inflammatory stimuli and mechanisms causing reduced leukocyte flux. In particular, members of the integrin and selectin families of adhesion molecules play an important role in angiogenesis.[47–49] Integrins are a family of transmembrane receptors mediating binding of cells to the extracellular matrix or to surface proteins on other cells.[50,51] Thus, either a repertoire of specific adhesion molecules may be expressed during angiogenesis of newly formed blood vessels or adhesion molecules are preferentially located at the abluminal membrane of endothelial cells. This pattern may predominantly promote interaction of endothelial cells with the extracellular matrix and other cells in the matrix, eg, fibroblasts. Expression of adhesion molecules during angiogenesis possibly differs from the large repertoire found in mature endothelium, when interaction with blood cells is involved in vascular function. Finally, bFGF has been shown to down-regulate the expression of various adhesion molecules in endothelial cells *in vitro* (Melder, Koenig, Safabakhsh, Witwer, Munn, and Jain, unpublished data).[52] It is, therefore, possible that cytokines sequestered in the gels had down-regulated adhesion molecules in the endothelium and hence lowered the leukocyte-endothelial interaction.

Increased Permeability of Newly Grown Microvessels

Besides modulating endothelial proliferation, growth factors may also modify vascular permeability, as demonstrated for VEGF/VPF.[3,34,53] Increased microvascular permeability during angiogenesis possibly facilitates the extravasation of proteins and thus formation of extracellular matrix for cell migration.[3] Our data confirm that newly grown blood vessels are more leaky than vessels of normal tissues reported in the literature,[54] independent of the growth factor in the collagen gel. These permeability values were comparable with values from tumors implanted into the dorsal skinfold chamber[30,55] or the cranial window.[29] No differences were found in effective microvascular permeability to BSA between newly formed blood vessels induced by bFGF or VEGF. Acute local administration of VEGF has been shown to transiently increase vascular permeability[34] through augmentation of transendothelial transport[3] and endothelial fenestration,[53] but bFGF does not modify permeability in acute experiments.[3] In contrast, it is unknown how chronic exposure to mitogenic factors affects morphological and functional characteristics of the newly grown blood vessels; they may thus exhibit common properties. Even though some vessels in our study were induced by VEGF, its levels might have been insufficient to elevate permeability at the time of measurements. Studies in the literature show that, after application of VEGF to the chorioallantoic membrane, its presence was demonstrated over a period of 3 days.[56] Although the release of VEGF from sucralfate may be slower in our assay, low levels can be assumed on days 12 and 25, when permeability measurements were performed (N. Ferrara, personal communication). Our measurements may therefore reflect a secondary, complex stimulus rather than a direct effect of the growth factors originally present in the gels. Angiogenesis was induced by bFGF and VEGF, and endothelial cells originated from vessels of the striated muscle of the skin or from the pia mater. Subsequently, factors released from ingrown fibroblasts, mast cells, and macrophages contributed to angiogenesis and stroma generation[57] and maintained the additional growth of vessels.[58] Thus, the properties of the newly grown vessels are at later stages more likely affected by the microenvironment and the anatomical location[59,60] than by the growth factors initiating angiogenesis.

As our results have shown, permeability differed between the two anatomical sites with fourfold higher values in the cranial window. Thus, vascular permeability seems to be mainly determined by the anatomical location and immaturity. The higher permeability observed in the cranial window suggests lack of a blood-brain barrier, which is in agreement with a presumed immaturity of the vascular wall. The higher RBC velocity also found in angiogenic vessels in the cranial window associated with higher vascular permeability may in part be explained by elevated microvascular pressure with increased convective flow at this site. The elevated permeability seems not to be related to the younger age of the vessels in the cranial window (day 12 after implantation *versus* day 25 in the dorsal skinfold chamber). This is indicated by similar permeability of angiogenic vessels in the cranial window on day 12 compared with aged vessels on day 25. Finally, we found no direct correlation between angiogenesis and vascular permeability at the two different sites, similar to our recent results in tumors.[29]

In summary, we have established a new technique for non-invasive, long-term monitoring of angiogenesis *in vivo*. The growth of new blood vessels modified by bFGF and VEGF/VPF could be easily, rapidly, and accurately quantified in the assay. We have shown that physiological properties of newly grown vessels are mainly determined by the anatomical location and the microenvironment, whereas the initial response of angiogenesis is stimulated by growth factors. Leukocyte-endothelial interaction in angiogenic vessels is significantly lower than in vessels of normal tissues. The model will thus be a useful tool for future investigations on the mechanisms of angiogenesis and anti-angiogenesis. The method can be used for 1) assessment of the potency of different growth factors and anti-angiogenic compounds and their inter-relation *in vivo* and 2) evaluation of the effects of these compounds on the properties of newly formed blood vessels.

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