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# Cell-Mediated Delivery of Fibroblast Growth Factor-2 and Vascular Endothelial Growth Factor onto the Chick Chorioallantoic Membrane: Endothelial Fenestration and Angiogenesis

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**Key Words**

Angiogenesis · Chorioallantoic membrane · FGF2 · VEGF

**Abstract**

Fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor (VEGF) exert their angiogenic activity by interacting with endothelial cells in a distinct manner. In this study, we investigated the morphological features of endothelial cells of the chick embryo chorioallantoic membrane (CAM) microvasculature after stimulation with FGF2 or VEGF. In order to provide a continuous delivery of the growth factor, we utilized a recently developed gelatin sponge/CAM assay in which a limited number of FGF2- or VEGF-transfected cells were adsorbed onto gelatin sponges and applied on the top of the CAM on day 8 of development. Their angiogenic activity was compared to that exerted by a single bolus of the corresponding growth factor. All the angiogenic stimuli induced a comparable vasoproliferative response, as demonstrated by the appearance of similar numbers of immature blood vessels within the sponge on day 12. No angiogenic response was observed in CAMs implanted with the corresponding parental cell lines or vehicle. Electron microscopy demonstrated that VEGF-overexpressing cells modified the phenotype of the endothelium of the blood vessels at the boundary between the implant and the surrounding CAM mesenchyme. The endothelial lining of 30% of these vessels showed segmental attenuations, was frequently interrupted and became fenestrated, mimicking what is observed in tumor vasculature. In contrast, the vessels consisted of continuous endothelium sealed by tight junctions in all the other experimental conditions. These results indicate that FGF2 and VEGF interact with endothelial cells of the CAM in a distinct manner. Both growth factors induce a potent angiogenic response, but only VEGF delivered in a continuous manner by its transfectants can modify the phenotype of the otherwise quiescent endothelium of CAM blood microvessels. The gelatin sponge/CAM assay may constitute a new model to study the mechanisms leading to endothelial fenestration in tumor growth.

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## Introduction

Fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) are the best-characterized angiogenic cytokines and the most potent angiogenesis inducers [1, 2]. The requirement for FGF2 in physiological and developmental angiogenesis is not completely clarified whereas the importance of VEGF, with its two main functions (induction of sprouting and proliferation of endothelial cells and enhancement of vascular permeability), has been clearly demonstrated. Angiogenesis is also of importance in pathologic conditions, including tumor growth. To this respect, FGF2 is expressed by neoplastic cells, endothelial cells and infiltrating cells within human tumors of different origin [3–7]. Antisense FGF2 and FGF receptor-1 cDNAs inhibit neovascularization and growth of human melanomas in nude mice [8]. VEGF is expressed by a variety of human tumors [9]. Blocking anti-VEGF antibodies [10], antisense VEGF cDNA [11] and dominant negative VEGF receptor mutant [12] inhibit tumor growth in different experimental models.

Tumor vessels differ from normal vessels in their architecture, blood flow, and permeability [13]. Endothelial fenestration has been observed in both core and peripheral tumor microvasculature [14, 15]. However, the molecular mechanisms responsible for endothelial fenestration and the relationship between tumor angiogenesis, increased endothelial cell fenestration and permeability remain unclear. Recent in vitro and in vivo studies have suggested that VEGF may induce such fenestrations [14, 16, 17]. Its topical administration or interstitial injection, in fact, resulted in fenestration and an increase in the permeability of small venules and capillaries of the cremaster muscle and skin, whose endothelial cells are normally not fenestrated [16]. Roberts and Palade [16] have also demonstrated that FGF2-induced neovasculature has open junctions in the endothelium. The degree of fenestration was higher with VEGF compared to FGF2 although the angiogenic response was greater with FGF2. Esser et al. [17], however, showed that in vitro incubation with VEGF, but not FGF2, affected the number of fenestrations in cloned bovine adrenal cortex endothelial cells.

The chick embryo chorioallantoic membrane (CAM) assay is a well-established assay for studying the effects of growth factors on blood vessel, but none has yet been reported to induce cell fenestration of the CAM endothelium [18–20]. Our recent modification of this assay enables the delivery of a very small number of tumor cells onto the CAM (approximately 20,000 cells/implant) and the assessment of their angiogenic capacity [21]. These experimental conditions grant the slow, continuous delivery of the growth factor(s) released by these few cells [21], thus more closely resembling the initial stages of tumor-cell-induced angiogenesis [22]. The goal of the present study was to investigate the morphological features of the CAM vasculature after stimulation with FGF2 or VEGF continuously released by tumor cells overexpressing them. Gelatin sponges adsorbed with FGF2-overexpressing pZipFGF2 MAE cells or VEGF-overexpressing V12-MCF-7 cells were implanted on top of the developing CAM. pZipFGF2 MAE cells were originated by transfection of mouse aortic endothelial (MAE) cells with a retroviral expression vector harboring the human FGF2 cDNA. They are angiogenic in vivo and cause the formation of opportunistic vascular tumors with morphological features resembling Kaposi's sarcoma when injected in nude mice [23]. V12-MCF-7 cells were obtained by transfection of the human $VEGF_{121}$ cDNA into the human breast carcinoma MCF-7 cell line. VEGF overexpression enhances tumor growth and vascular density of V12-MCF-7 xenografts and promotes a strong angiogenic response in vivo in the rabbit cornea [24, 25]. Modifications of the CAM vasculature induced by the transfectants were compared to those induced by the delivery of a single bolus of human recombinant FGF2 (hrFGF2) or of human recombinant $VEGF_{165}$ (hrVEGF). Parental cell lines and vehicle were used as controls.

## Materials and Methods

*Cell Lines*

Balb/c mouse aortic endothelial 22106 cells (MAE cells), pZipFGF2 MAE cells [23], parental and $VEGF_{121}$-transfected MCF-7 cells (clone V12) [24] were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum in the absence (parental cells) or presence (transfected cells) of 500 μg/ml of G 418 sulfate (Sigma, St. Louis, Mo., USA).

*Animals*

Fertilized White Leghorn chicken eggs (20 eggs/ group) were incubated under conditions of constant humidity at a temperature of 37 °C. On the 3rd day, a square window was opened in the shell after removal of 2–3 ml of albumen to detach the shell from the developing CAM. The window was sealed with a glass of the same size and the eggs were returned to the incubator.

*Implantation of Gelatin Sponges onto the CAM*

Gelatin sponges (Gelfoam Upjohn, Kalamazoo, Mich., USA) were cut to a size of 1 $mm^3$ and placed on top of the CAM on day 8 under sterile conditions [21]. They were then adsorbed with 3 μl of cell suspension (18,000 cells/sponge) or with hrFGF2 or $hrVEGF_{165}$ (R & D Systems, Abingdon, UK), both at 1 μg/sponge. Sponges

loaded with RPMI-1640 alone were used as negative control. On day 12, CAMs were photographed in ovo with a stereomicroscope equipped with a Camera System MC 63 (Zeiss, Oberkochen, Germany). In some experiments, blood vessels entering the sponges within the focal plane of the CAM were counted by two observers in a double-bind fashion at a magnification of ×50 [26]. Then, all CAMs were processed for light and electron microscopy. Briefly, the embryos and their membranes were fixed in ovo in 3% phosphate-buffered glutaraldehyde. The sponges and the underlying and immediately adjacent portions of CAM were dehydrated in serial alcohol solutions, postfixed in 1% phosphate-buffered $OsO_4$, and embedded in Epon 812. One-micrometer semithin and ultrathin sections were cut on a LKB V ultramicrotome according to a plane parallel to the surface of the CAM. The semithin sections were stained with a 0.5% aqueous solution of toluidine blue (Merck, Darmstadt, Germany) and observed under a Leitz-Dialux 20 light microscope (Leitz, Wetzlar, Germany). The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined under a 9A Zeiss electron microscope.

*Microscopic Quantitation of the Angiogenic Response*

The angiogenic response was assessed by a planimetric method of point counting [21]. Briefly, every third section within 30 serial sections from an individual specimen was examined by a 144-point mesh inserted in the eyepiece of the microscope. Six randomly chosen fields were evaluated for each section at a magnification of ×250. The total number of the intersection points occupied by vessels cut transversely (diameters from 3 to 10 μm) inside the sponge were counted. Means ± SD were determined for each analysis. The vascular density was indicated by the final mean number of occupied intersection points. The statistical significance of the differences between the mean values of the intersection points in the experimental and control CAMs was determined by Student's t test for unpaired data.

*Quantification of the Surface Density of Endothelial Fenestrae and Plasmalemmal Endothelial Vesicles*

A quantitative evaluation of the surface density of endothelial capillary fenestrae and of plasmalemmal endothelial vesicles in arterioles, capillaries and venules was carried out as follows. Twenty electron micrographs at a final magnification of ×30,000 were chosen for each experimental condition from specimens processed for electron microscopy on day 12. The mean number of fenestrae/100 $\mu m^2$ of endothelial surface and of plasmalemmal vesicles/$\mu m^2$ was counted with an electronic pen connected to a graphic tablet (Digicad Plus Kontron Elektronic GmbH, Germany) and to a VIDAS 2.5 computerized image analyzer (Kontron Elektronic). The mean value in each endothelial profile, the final mean value for all profiles of each condition and the standard deviation were calculated. The statistical significance of the difference between the mean values in all the conditions was determined by Student's t test for unpaired data.

## Results

### *FGF2- and VEGF-Transfectants Induce a Similar Angiogenic Response in the Chick Embryo CAM*

pZipFGF2 MAE and V12-MCF-7 cells (18,000 cells/embryo) were delivered on the top of 8-day CAMs by means of a gelatin sponge implant [21]. Macroscopic

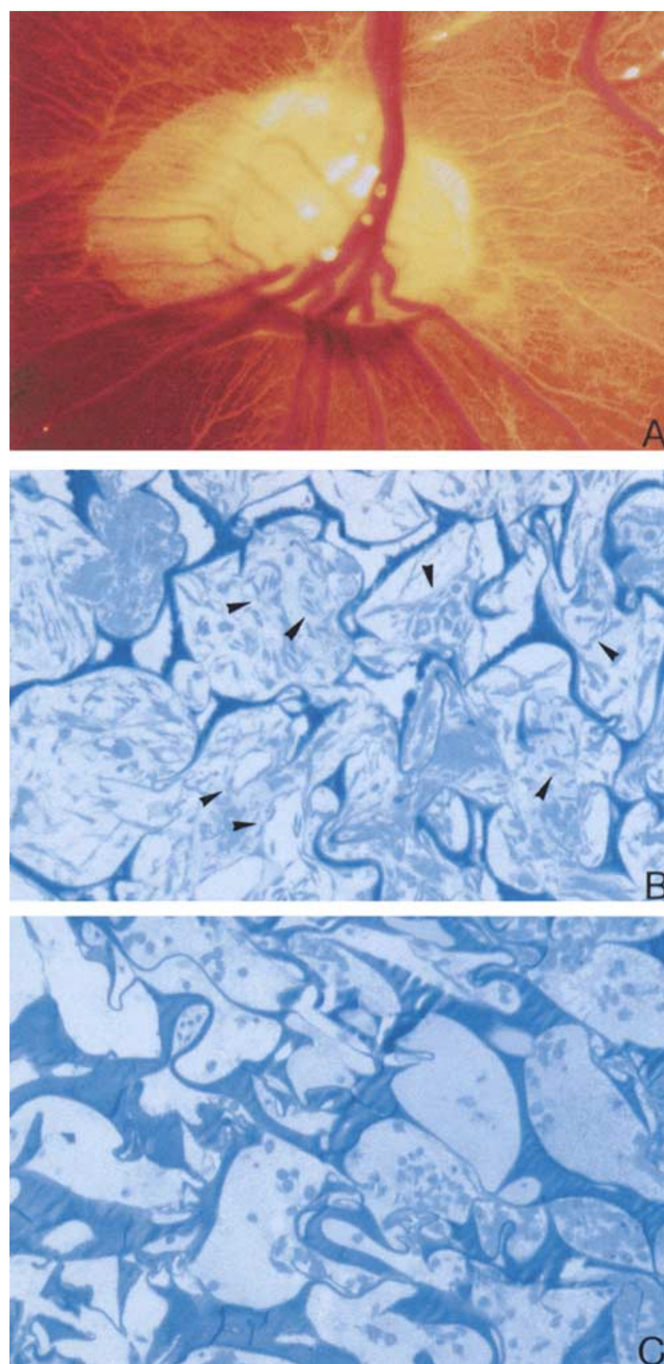


**Fig. 1.** Effect of V12-MCF-7 cells on CAM neovascularization. Cells were delivered at 18,000 cells/embryo on the top of the CAM on day 8 using a gelatin sponge implant. **A** Macroscopic observation of the CAM, performed on day 12, shows the gelatin sponge surrounded by allantoic vessels that develop radially towards the implant in a 'spoked-wheel' pattern. **B** Histologic analysis of the CAM grafted with V12-MCF-7 cells. A highly vascularized tissue is recognizable among the sponge trabeculae, consisting of newly formed blood vessels (arrowheads). The vessels are absent in implants treated with RPMI-1640 alone (**C**). Original magnification: **A** ×50; **B**, **C**, ×400.

**Table 1.** Macroscopic and microscopic evaluation of the angiogenic activity in the chick embryo CAM

| Treatment | Macroscopic evaluation | Microscopic evaluation | |
|---|---|---|---|
| | number of vessels at the sponge-CAM boundary (means ± SD) | number of intersection points (means ± SD) | microvessel density, % |
| RPMI-1640 | 5 ± 2 | 0 | 0 |
| hrFGF2 | 38 ± 6* | 28 ± 3* | 19.4 |
| hrVEGF | 40 ± 5* | 29 ± 5* | 20.1 |
| MAE cells | 7 ± 1 | 0 | 0 |
| pZipFGF2 MAE cells | 42 ± 6* | 32 ± 3* | 22.2 |
| MCF-7 cells | 8 ± 2 | 0 | 0 |
| V12-MCF-7 cells | 42 ± 5* | 30 ± 6* | 20.1 |

FGF2 and VEGF (1 μg/embryo), MAE, pZipFGF2 MAE, MCF-7 and V12-MCF-7 cells (18,000 cells/embryo) were adsorbed into gelatin sponges and grafted onto the CAM on day 8 (20 embryos/group). RPMI-1640 was used as a negative control. The angiogenic response was assessed on day 12 macroscopically by counting the number of blood vessels entering the sponge and histologically by a planimetric method of 'point counting'. * $p < 0.001$ vs. RPMI-1640, MAE and MCF-7 cells.

observation on day 12 showed that pZipFGF2 MAE and V12-MCF-7 cell implants were surrounded by allantoic vessels that developed radially towards the implant in a 'spoked-wheel' pattern (fig. 1A). Quantification of the macroscopic blood vessels entering the sponge indicated that the two cell types elicited a similar angiogenic response (table 1). No angiogenic response was detectable in the parental MAE and MCF-7 cell implants (table 1), indicating that neovascularization observed after implantation of the corresponding transfectants was due to the transduced growth factor. Similarly, hrFGF2 and hrVEGF (both at 1 μg/implant) caused a strong angiogenic response in the CAM that was quantitatively similar to that elicited by transfected cells (table 1). No vascular reaction was detectable around sponges loaded with RPMI-1640 alone (negative control; table 1).

Microscopic examination revealed a highly vascularized tissue around the trabeculae of the sponges loaded with the transfectants or the recombinant growth factors (fig. 1B). It consisted of newly formed blood vessels, mainly capillaries 3–10 μm in diameter growing perpendicularly to the plane of the CAM, within an abundant network of collagen fibers. In agreement with the pleiotropic activity of FGF2, infiltrating fibroblasts were observed in the sponges adsorbed with pZipFGF2 MAE cells or hrFGF2. The vessels were absent among trabeculae of CAMs implanted with parental MAE and MCF-7 cells and of control implants (fig. 1C). In agreement with the macroscopic measurements, microscopic quantification of the vessels infiltrating the sponge by a morphometric method of 'point counting' (table 1) demonstrated that FGF2 and VEGF transfectants, as well as hrFGF2 and hrVEGF, exerted a similar angiogenic response [21, 27]. No angiogenic response was detectable in the sponges treated with parental MAE and MCF-7 cells and with vehicle alone.

**Table 2.** Endothelial fenestration in CAM vessels on day 12 of incubation

| Treatment | Vessels counted | Fenestrated vessels, % | Fenestrae/100 $\mu m^2$ (mean) |
|---|---|---|---|
| RPMI-1640 | 40 | 0 | 0 |
| hrFGF2 | 65 | 0 | 0 |
| hrVEGF | 84 | 0 | 0 |
| MAE cells | 52 | 0 | 0 |
| pZipFGF2 MAE cells | 50 | 0 | 0 |
| MCF-7 cells | 75 | 0 | 0 |
| V12-MCF-7 cells | 80 | 30* | 6.5 ± 1.5* |

The mean number of fenestrae/100 $\mu m^2$ of endothelial surface was counted as described. * $p < 0.001$ vs. other groups.

In conclusion, our data indicate that FGF2- and VEGF-transfectants elicit a quantitatively similar angiogenic response in the CAM which was also similar to that exerted by the recombinant growth factors.

*Morphological Features of the CAM Vessels within and outside the Sponge Implant*

In agreement with previous observations [28], capillaries of control CAMs on day 12 of incubation consist of a continuous endothelium with closely apposed and sometimes slightly overlapping cells with a well-developed rough endoplasmic reticulum and very few plasmalemmal vesicles (fig. 2A). The two transfectants and the recombinant growth factors caused profound morphological alterations of the blood vessels both within and outside the sponge at the interface between the sponge and the CAM mesenchyme.

*Vessels within the Sponge.* Four days after implantation of the angiogenic stimulus, newly formed blood vessels, consisting mainly of immature capillaries, are recognizable among the trabeculae of the gelatin sponge implants. They lacked a basement membrane and consisted of two endothelial cells arranged in parallel with a slit-like lumen bordered by high endothelial cells equipped with the usual organelles (fig. 2B). They were sometimes encircled by mast cells with cytoplasmic matrix filled with numerous electron-dense secretory granules. No morphological differences were observed between blood vessels induced by FGF2 or VEGF transfectants or by the recombinant growth factors.

*Vessels outside the Sponge.* Following implantation of the different angiogenic stimuli, the endothelium of blood vessels at the interface between the gelatin sponge and the surrounding CAM mesenchyme showed an extensive rough endoplasmic reticulum, prominent Golgi complexes and more small electron-dense mitochondria when compared to control CAMs. These features were shared by CAMs loaded with FGF2 and VEGF transfectants as well as with hrFGF2 or hrVEGF. However, CAMs implanted with VEGF transfectants also showed segmental attenuations and frequently interruptions of the endothelial lining of the vessels outside the sponge. These vessels were characterized by open interendothelial junctions and numerous fenestrations, abnormal convoluted lumina and incomplete basement membrane, along with more membrane vesicles and clusters of swollen, deformed and fused caveolae. (fig. 3A, B). Morphometric analysis performed on day 12 of incubation showed that 30% of blood vessels were fenestrated in this experimental group with approximately 6–7 fenestrae/100 $\mu m^2$ of endothelial surface (table 2). Endothelial fenestration was not observed in all the other experimental groups, including CAMs treated with hrVEGF. In contrast, a significant increase in the number of plasmalemmal vesicles was evident in the capillary endothelium of the CAMs implanted with VEGF transfectants as well as of those treated with hrVEGF (table 3).

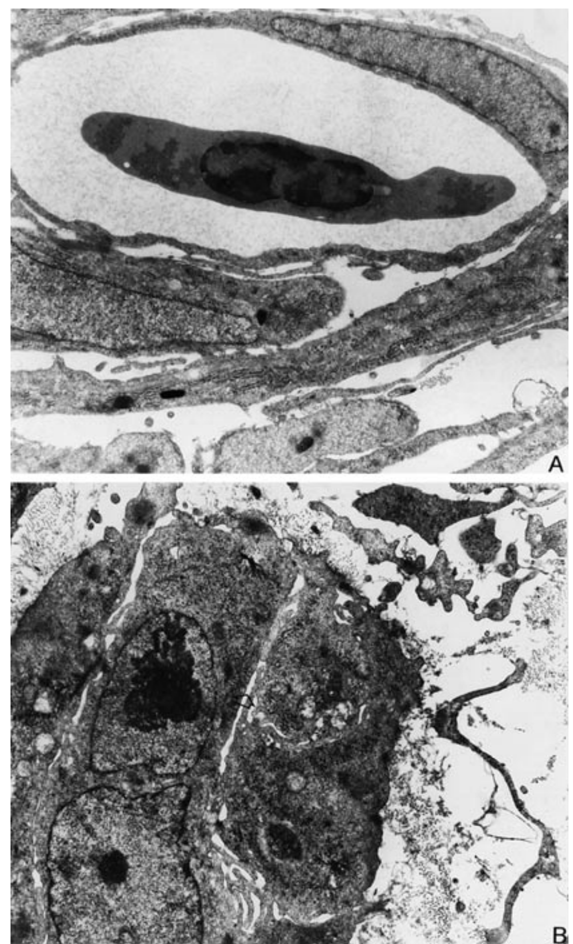


**Fig. 2. A** A capillary from a 12-day control CAM containing a nucleated red blood cell. The endothelial cells exhibit an abundance of free ribosomes and rough endoplasmic reticulum. Few pinocytotic vesicles are apparent. **B** A newly formed capillary from a 12-day CAM treated with V12-MCF-7 cells. The newly formed blood vessel shows a slit-like lumen (arrowhead). Basement membrane cannot be identified around the endothelial cell. Original magnification: **A**, **B** × 15,000.

No morphological alterations were induced in the vessels outside the sponge of CAMs implanted with parental MAE and MCF-7 cells or the vehicle.

## Discussion

Here we demonstrate that tumor cell lines overexpressing FGF2 or VEGF induce a quantitatively similar vasoproliferative response when adsorbed on gelatin sponges

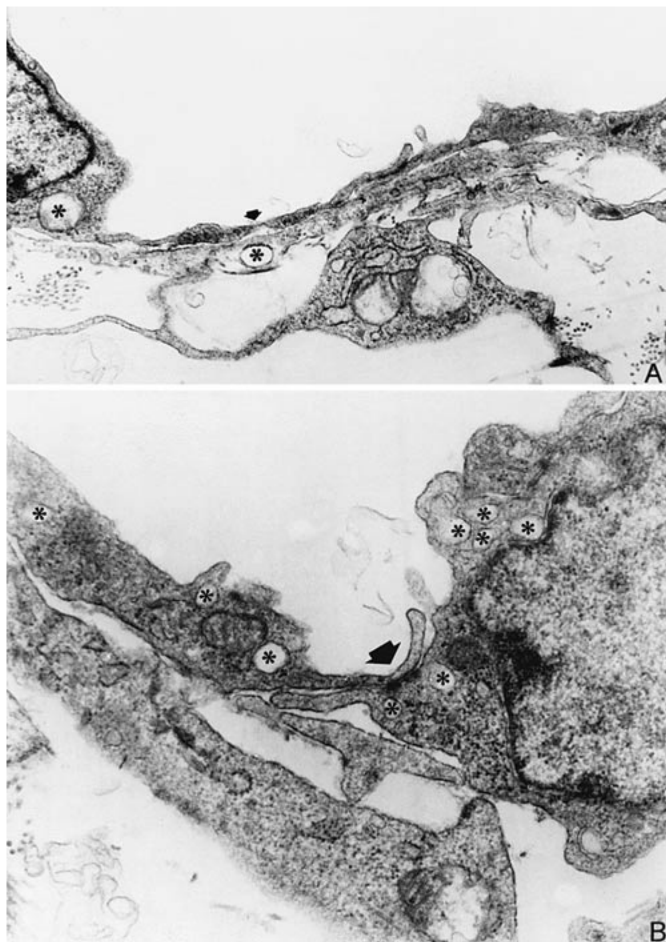

**Fig. 3.** Capillaries at the boundary between gelatin sponge and surrounding mesenchyme from 12-day CAMs treated with V12-MCF-7 cells. **A** A segmental attenuation of the endothelial lining, intracytoplasmic vacuoles (asterisks) and an endothelial fenestration closed by a diaphragm (arrow) are apparent. **B** An open intercellular junction (arrow) and numerous intracytoplasmic vacuoles (asterisks) are recognizable. Original magnification: **A** × 24,000; **B** × 15,000.

**Table 3.** Numerical densities of plasmalemmal vesicles within the segmental CAM microvascular endothelia on day 12 of incubation

| Treatment | Arterioles | Capillaries | Venules |
|---|---|---|---|
| RPMI-1640 | 3.0 ± 1.0 | 8.5 ± 2.5 | 4.2 ± 1.5 |
| hrFGF2 | 2.5 ± 1.0 | 7.0 ± 1.0 | 5.0 ± 2.5 |
| hrVEGF | 3.2 ± 1.5 | 15.5 ± 4.5* | 5.5 ± 1.8 |
| MAE cells | 3.3 ± 0.5 | 8.2 ± 2.5 | 4.7 ± 1.6 |
| pZipFGF2 MAE cells | 2.8 ± 0.6 | 7.5 ± 1.0 | 5.2 ± 2.0 |
| MCF-7 cells | 3.1 ± 1.0 | 8.1 ± 1.5 | 5.0 ± 2.1 |
| V12-MCF-7 cells | 3.5 ± 1.7 | 18.4 ± 4.5* | 6.0 ± 2.0 |

The mean number of plasmalemmal vesicles (number/$\mu m^2$) was counted as described. * $p < 0.001$ vs. other groups.

implanted on top of the chick embryo CAM on day 8 of incubation, comparable to that elicited by hrFGF2 and hrVEGF. As compared to the application on the CAM of large amounts of the pure recombinant angiogenic cytokine in a single bolus, cell implants overexpressing angiogenic cytokines enable the continuous delivery of the growth factor produced by a limited number of cells (18,000 cells/implant) and thus more closely mimic the initial stages of tumor angiogenesis and metastasis. It can be calculated that pZipFGF2 MAE cells secrete approximately 100 pg/96 h/$10^6$ cells, as evaluated by immunoassay [29] and can be presumed to have released approximately 2–3 pg FGF2 throughout the experimental period when applied onto the CAM according to our protocol. Continuous release of very small amounts of an angiogenic growth factor by a few tumor cells is able to trigger an angiogenic stimulus in the CAM quantitatively similar to that elicited by 1 μg of the recombinant molecule.

The parental MAE and MCF-7 cell lines were not angiogenic in our experimental conditions, in keeping with the lack of angiogenic activity of parental MAE cells in the CAM and in the rabbit cornea assays [24] and the limited angiogenic potential of MCF-7 cells implanted in the rabbit cornea when compared to VEGF transfectants [25].

The results of our study indicate that the angiogenic response within the sponge induced by the recombinant growth factors and their cell transfectants was quantitatively similar, as shown by morphometric evaluation of newly formed blood vessels by a 'point counting' method. These newly formed blood vessels within the sponges are similar in the various experimental groups (all characterized by immature capillaries with a slit-like lumen and lacking a basement membrane) and distinct from the blood vessels of the control CAMs. Young capillaries with a slit-like lumen have also been observed during in vitro angiogenesis using cultured aorta fragments [30] and during in vivo angiogenesis induced by a fibrin gel in a subcutaneous chamber [31]. This feature is typical of the first steps of tumor-related angiogenesis [32, 33].

More interestingly, our data show that the various angiogenic stimuli have a different effect on the morphology of the blood vessels at the boundary between the sponge and the surrounding CAM mesenchyme. CAMs treated with FGF2 transfectants, hrFGF2 or hrVEGF were morphologically 'activated' compared to the control vessels, with no signs of endothelial fenestration. In contrast, the vessels outside the sponge of CAMs implanted with VEGF transfectants displayed an endothelial lining with segmental attenuations and frequent interruptions.

Moreover, open interendothelial junctions, numerous fenestrations, abnormal convoluted lumina, clusters of swollen, deformed, and fused caveolae, and incomplete basement membrane were recognizable. These features are similar to those observed in the peripheral microvasculature of human and murine tumors grown subcutaneously in nude and syngeneic mice [14, 16]. Interestingly, in both of these murine models and in our gelatin sponge/CAM assay, approximately 30% of peripheral blood vessels displayed endothelial fenestration.

Our data are in agreement with previous observations on the ability of $VEGF_{165}$-transfected CHO cells grafted in mice to induce vascularized tumors with fenestrated endothelium, open interendothelial junctions, and clustered fused caveolae [34]. Also, topical administration or intradermal injection of $hrVEGF_{165}$ to a microvascular bed supplied with a continuous endothelium, namely the cremaster muscle and skin, induces the rapid formation (within 10 min) of endothelial fenestration [14]. Interestingly, when slow-release pellets containing hrVEGF were implanted on the rat cremaster muscle and the muscle was excised after 10–20 days, VEGF-induced neovasculature had open junctions and fenestrated endothelium [16]. In these and our experimental conditions, chronic secretion of VEGF induced and maintained fenestrations in microvascular endothelium. This finding further substantiates the role of VEGF in inducing and maintaining fenestrated endothelium in normal physiology [35]. These authors have shown that VEGF is continuously expressed in epithelial cells of adult organs with fenestrated endothelium such as choroid plexus and kidney glomeruli. Also, VEGF has been shown to induce endothelial abnormalities, consisting of the appearance of interendothelial gaps and the formation of fenestrations in cerebral capillaries normally sealed by tight junctions [36]. This latter observation is in keeping with our data showing that VEGF transfectants, but not FGF2 transfectants, modify the morphological characteristics of the blood vessels of the CAM whose endothelia are sealed by tight junctions [37]. Interestingly, we found a higher immunoreactivity to VEGF receptor-2 in the capillary endothelium of the CAMs loaded with V12-MCF-7 cells as compared to those treated with parental cells (data not shown). This suggests that VEGF might be involved in the induction or maintenance of fenestrations in adjacent endothelial cells expressing these receptors. Experiments are in progress to evaluate the expression of the different VEGF receptors in CAM blood vessels and their relative contribution to angiogenesis and endothelial fenestration.

The fact that a single 1-μg bolus of hrVEGF did not induce a discontinuous phenotype in the endothelial cells of the CAM blood vessels is in apparent contrast with the effect elicited by VEGF transfectants in the same experimental system. This different response might depend on the use of the $hrVEGF_{165}$ isoform for the bolus, while V12-MCF-7 cells produce $VEGF_{121}$ [24]. Indeed, various VEGF isoforms exist that differ in terms of structural, biochemical, and biological properties [2]. For instance, $VEGF_{121}$ does not bind heparin and is freely diffusible whereas $VEGF_{165}$ binds heparin and can be sequestered in the extracellular matrix [2]. In addition, the two VEGF isoforms share different receptor recognizing patterns [38]. It is conceivable that any of these molecular characteristics attributed to the two VEGF isoforms could account for the observed phenotypic differences in CAM vessels stimulated by $hrVEGF_{165}$ versus those stimulated by $VEGF_{121}$ transfectants. Nevertheless, previous experiments performed in the CAM assay by using $VEGF_{121}$ homodimer or $VEGF_{121/165}$ heterodimer as a single bolus demonstrated the absence of endothelial fenestrations [19], in keeping with our results obtained with the $VEGF_{165}$ homodimer. Therefore, another explanation for this apparent discrepancy may be the different bioavailability and pharmacokinetics of the growth factor delivered onto the CAM in large amounts as a single bolus compared to its continuous release in low amounts by producing cells. Cell transfectants may themselves produce cofactor(s) that contribute to the biological activity of the growth factor. These cofactor(s), if any, are however unable to cause per se endothelial fenestration, as shown by the lack of activity of parental MCF-7 cells. Further experiments will be required to clarify this point.

VEGF increases vascular permeability [39]. Four days after implantation of sponges containing hrVEGF or VEGF transfectants we noted a higher number of plasmalemmal endothelial vesicles in the capillaries, but not in the arterioles and venules, of the CAM vasculature tree. Nevertheless, it should be noticed that the dense supply of lymphatic vessels and the high osmotic pressure of the allantoic fluid [40] may prevent edema formation in the CAM. No significant increase in the number of plasmalemmal vesicles was instead observed in endothelial capillaries of FGF2-treated CAMs, in keeping with the inability of this growth factor to increase vascular permeability.

FGF2 upregulates VEGF expression in endothelial cells and this effect may mediate the angiogenic activity exerted by FGF2 in the murine cornea [41]. However, FGF2 overexpression did not increase VEGF production in pZipFGF2 MAE cells [29]. Also, in our experimental

conditions, the impact of FGF2 secreted by transfected cells on blood vessels of the CAM was clearly distinct from that exerted by VEGF, suggesting that its biological activity is independent of endogenous VEGF. Our data are in accord with those of Esser et al. [17], who found that VEGF but not FGF2 induced fenestration in adrenal cortex capillary endothelial cells.

In conclusion, our data extend previous observations on the different ability of FGF2 and VEGF to modulate endothelial cell morphology. In particular, an increased endothelial fenestration appears to be restricted to the blood vessels of the CAM stimulated by a limited number of tumor cells overexpressing VEGF. This, together with the observation that FGF2 and VEGF transfectants and the corresponding recombinant growth factors elicit a quantitatively similar angiogenic response, indicates that endothelial fenestration and angiogenesis are not strictly related. The gelatin sponge/CAM assay may constitute a new model for the study of the mechanisms leading to endothelial fenestration in tumor growth.

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