RGD-Dependent Vacuolation and Lumen Formation Observed during Endothelial Cell Morphogenesis in Three-Dimensional Fibrin Matrices Involves the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ Integrins

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Recent data have revealed the involvement of the $\alpha_v\beta_3$ integrin in angiogenesis. However, few studies to date have provided a convincing role for this receptor in *in vitro* assays of endothelial cell morphogenesis where defined steps can be examined. Here, we present data showing that two integrins, $\alpha_v\beta_3$ and $\alpha_5\beta_1$, regulate human endothelial cell vacuolation and lumen formation in three-dimensional fibrin matrices. Cells resuspended in fibrin formed intracellular vacuoles that coalesced into luminal structures. These morphogenic events were completely inhibited by the simultaneous addition of anti- $\alpha_v\beta_3$ and anti- α_5 integrin antibodies. Complete blockade was also accomplished with a combination of the cyclic Arg-Gly-Asp (cRGD) peptide and anti- α_5 integrin antibodies. No blockade was observed with the control Arg-Gly-Glu (RGE) peptide alone or in combination with control antibodies. Finally, we were able to demonstrate regression of vacuoles and lumens several hours after the addition of cRGD peptides combined with anti- α_5 integrin antibodies. These effects were not observed with control peptides alone or in combination with control antibodies. We report here the novel involvement of both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins in vacuolation and lumen formation in a fibrin matrix, implicating a role for multiple integrins in endothelial cell morphogenesis. (Am J Pathol 2000, 156:1673–1683)

Angiogenesis, the development of new capillaries from preexisting networks, is important for organ development, wound healing, and various pathological conditions such as tumor growth.^{1–4} This process has been characterized by changes in vascular cell permeability alongside endothelial cell (EC) migration, proliferation, and differentiation. Less well defined are the steps regulating changes in EC shape, or morphogenesis, that occur during the formation of capillary networks.^{5–19} A num-

ber of *in vivo* and *in vitro* studies on angiogenesis have reported the presence of EC intracellular vacuoles, cellular structures that appear to regulate EC lumen formation.^{5–10,12–17} However, the molecular mechanisms determining EC vacuolation and lumen formation during EC morphogenesis remain to be defined. The process of angiogenesis is clearly orchestrated by a combination of cytokines, proteases, extracellular matrix (ECM), and integrins.^{1–4} Studying the role of these molecules will provide clues to the cellular control of morphogenesis.

One key cytokine associated with angiogenesis is vascular permeability factor/vascular endothelial growth factor (VEGF). While capable of stimulating EC proliferation, cell shape changes, adhesion, and migration, VEGF is also a potent inducer of vascular permeability.^{20–22} An increase in microvascular permeability in the tumor microenvironment is responsible for the exudation of plasma proteins such as fibrinogen, fibronectin, and vitronectin, which form a provisional ECM.³ Several investigators have successfully identified and measured increases in the permeability of tumor vessels as compared to normal vessels,^{23–26} and histochemical analysis of human tumors has revealed substantial fibrin deposits in tumor stroma.^{27–33} A fibrin matrix forms when the plasma protein fibrinogen is cleaved by thrombin. Fibronectin has affinity for fibrin and becomes covalently cross-linked into this matrix by transglutaminase enzymes such as factor XIII.³⁴ The fibrin/fibronectin matrix deposited as a result of VEGF-induced permeability may contribute to angiogenesis by providing structure and signals within the provisional ECM to regulate EC differentiation and vessel development. An approach to investigation of EC interactions within fibrin matrices has involved the establishment of *in vitro* models of EC morphogenesis using three-dimensional fibrin gels.^{16,19,35–37} Such models are useful for dissecting the mechanisms that regulate EC morphogenesis in fibrin matrices.

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One way to gain a better understanding of EC morphogenesis involves identification of the EC receptors involved. Both *in vivo* and *in vitro* studies have reported the involvement of integrins in this process.^{3,4,15,16} These receptors are transmembrane receptors that maintain cell adhesion to ECM while also controlling cell proliferation, motility, trafficking, differentiation, and apoptosis, along with cell shape changes, cytoskeletal organization, phosphorylation states, and gene transcription (reviewed in refs. 38–40). Thus, while mediating cell adhesion to ECM, integrins also transduce intracellular signals. Understanding how integrins, growth factors, and a provisional fibrin matrix coordinate efforts to stimulate EC morphogenesis and development of a vascular supply within the microenvironment of a tumor or injured tissue is critical to uncovering mechanisms that regulate the angiogenic process. Currently, there is little information concerning the involvement of particular integrins during EC morphogenesis within fibrin matrices.

In this study, human ECs suspended in a three-dimensional fibrin matrix were stimulated by cytokines to undergo morphogenesis *in vitro* and form intracellular vacuoles and lumens. Anti-integrin antibodies and peptides revealed that blockade of both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins was required to interrupt the EC morphogenic process. Antagonists to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ also induced regression of preformed vacuolar and luminal structures. These novel findings further our understanding of how integrins regulate differentiation and EC morphogenesis in a fibrin matrix.

Materials and Methods

Three-Dimensional Fibrin System

Human umbilical vein endothelial cells were grown to confluence in M199 (Gibco-BRL, Grand Island, NY) supplemented with 20% fetal calf serum (Gibco-BRL) and bovine brain extract as described.⁴¹ Before experiments, cells were rinsed in phosphate-buffered saline, trypsinized, and resuspended in Dulbecco's minimum essential medium (DMEM) (Gibco-BRL) at a density of 5×10^6 cells/ml. Fibrinogen (Calbiochem, La Jolla, CA) was suspended at 20 mg/ml in DMEM. Final concentrations in each 25- μ l gel included 10 mg/ml fibrinogen and 1×10^6 cells/ml in DMEM solidified with 1 μ g thrombin (Amersham Pharmacia Biotech, Alameda, CA). Cultures were equilibrated at 37°C with 5% CO₂ before the addition of 100 μ l media. DMEM was supplemented with 20% fetal calf serum, 40 ng/ml recombinant vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) (Upstate Biotechnologies, Lake Placid, NY); 200 units of aprotinin (American Diagnostica, Piscataway, NJ), 50 μ g/ml ascorbic acid (Sigma); and 50 ng/ml 12-O-tetradecanoyl phorbol 13-acetate (TPA). Cultures were maintained for the times indicated in each experiment before being photographed or fixed, stained, and quantitated.

Time-Course Experiments

EC cultures were resuspended three-dimensionally in fibrin gels as described above, using DMEM without phenol red (Gibco-BRL). Random fields were selected, and sequential photographs of individual wells were taken at 0.5, 5, 7, 12, and 24-hour time points. In this experiment, we were able to observe the development of luminal and vacuolar structures in the same culture at an identical focal plane.

Blockade of Endothelial Cell Vacuolation and Lumen Formation Using Integrin Antibodies

To determine the integrins involved in EC vacuolation, various antibodies directed toward human integrin subunits and heterodimers were added (20 μ g/ml). The antibodies included anti- β_1 (MAb13; Becton-Dickinson, Bedford, MA),⁴² anti- α_5 (MAb16; Becton-Dickinson),⁴² anti- α_2 (A2-IIE10; Upstate Biotechnologies, Lake Placid, NY),⁴³ anti- $\alpha_v\beta_3$ (LM609; Chemicon, Temecula, CA),⁴⁴ and anti- $\alpha_v\beta_5$ (P1F6; Chemicon).⁴⁵ Furthermore, in some experiments, a cyclic Arg-Gly-Asp (cRGD) peptide (GPenGRGDSPCA; Gibco-BRL) and its control, Gly-Arg-Gly-Glu-Ser-Pro (GRGESp; Gibco-BRL), was added to both the gel and medium at 500 μ g/ml.

Induction of Regression: Collapse of Vacuolation and Lumen Formation

To determine whether integrins expressed by ECs were involved in the maintenance of vacuolar and luminal structures, cultures were allowed to develop to either 12- or 24-hour time points before we attempted to collapse the structures. Medium was removed and replaced with fresh medium containing either the cRGD or RGE peptides alone or combined with anti- α_5 or anti- α_2 integrin antibodies. Cultures were allowed to regress for 3 hours, at which time medium was removed and wells were fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO) overnight.

Quantitation of Endothelial Vacuolation and Lumen Formation

Cultures were fixed at various time points by removing the medium and replacing it with 3% glutaraldehyde in PBS overnight at 4°C. Wells were washed with water and stained with 1% toluidine blue (Sigma) containing 2% sodium borate (Sigma) for 5 minutes at room temperature. Cultures were destained and photographed and/or quantitated.

Percentage vacuolation was determined by analyzing 200 cells for each condition. A cell was considered to be vacuolating if >30% of the cell's area contained a lumen or vacuole. As cultures progressed to later stages, we observed that the cells coalesced. If a network of cells was encountered during quantitation, the number of nuclei in the network determined the number of vacuolating cells or lumen-forming networks, as toluidine blue prominently stains nuclei.

Embedding and Sectioning of Fibrin Gels

Fibrin gels at various time points were fixed overnight in 3% glutaraldehyde in PBS. After sequential ethanol washes, gels were stained with 0.1% toluidine blue in ethanol. Gels were embedded and prepared for sectioning with a Polybed 812-BDMA Embedding Kit (Polysciences, Warrington, PA) according to the Glauert method,⁴⁶ as instructed by the manufacturer. Sections were stained with 1% toluidine blue and photographed.

Results

Endothelial Cell Vacuolation in Three-Dimensional Fibrin Matrices Regulates Lumen Formation

Human umbilical vein endothelial cells were used to investigate endothelial morphogenesis in a three-dimensional fibrin matrix. ECs were suspended in a solution of fibrinogen, and a fibrin matrix was formed after the addition of thrombin. Cultures were allowed to undergo morphogenesis over a 24-hour period. Photographs of the same culture and focal plane taken at various times (Figure 1) revealed that suspended cells began forming vacuoles at approximately 3–5 hours of culture. These vacuoles grew in size over time and merged to form luminal structures. Cultures fixed at various times during morphogenesis were embedded in a plastic matrix. Cross-sectional analysis revealed the presence of intracellular vacuoles and lumens (Figure 2). The development of intracellular vacuoles and the transition of these into larger luminal structures are evident.

Endothelial Cell Vacuolation and Lumen Formation in Three-Dimensional Fibrin Matrices Is RGD-Dependent and Involves Both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ Integrins

The influence of cRGD and control peptides either added alone or in combination with blocking antibodies directed to the α_5 or α_2 integrin subunits were assessed in time-course experiments. Combining cRGD peptide with anti- α_5 integrin blocking antibodies resulted in complete blockade of vacuolation and subsequent lumen formation at all time points. As shown in Figure 3, 85–90% of the ECs within the fibrin matrix developed vacuoles and lumens. These morphogenic events became apparent at approximately 3 hours of culture and reached maximum levels at 12 hours. Adding cRGD peptide alone blocked approximately 50% of vacuolation and lumen formation. Combining cRGD with anti- α_2 integrin blocking antibodies did not further increase the effects of cRGD. The addition of RGE peptide either alone or combined with anti- α_2 antibodies had no effect when compared with control, while anti- α_5 blocking antibodies had a slight effect at later time points. Toluidine blue-stained cultures of control *versus* cRGD/anti- α_5 -treated cultures were photographed at various time points (Figure 4). Extensive

intracellular EC vacuolation and lumen formation was observed with time in the control cultures, whereas complete blockade of these morphogenic changes occurred in the presence of cRGD and α_5 antibodies.

Cyclic RGD peptides are known to inhibit the function of many α_v integrins while showing minimal effects on other RGD-binding integrins such as $\alpha_5\beta_1$.^{47,48} To assess the extent to which different α_v integrins expressed by ECs were involved in vacuolation and lumen formation, various integrin-blocking antibodies directed toward integrin heterodimers and subunits were added (Figure 5). Although none of the various anti-integrin antibodies alone were able to block lumen formation, the combination of anti- α_5 and anti- $\alpha_v\beta_3$ integrin antibodies completely inhibited vacuolation and subsequent lumen formation. The addition of anti- $\alpha_v\beta_3$ with anti- α_5 and anti- $\alpha_v\beta_3$ did not further inhibit this effect. The addition of anti- $\alpha_v\beta_3$ with anti- α_5 had little effect compared to control. Combining anti- α_2 antibodies with anti- $\alpha_v\beta_3$ did not further the effects of anti- $\alpha_v\beta_3$ alone, ruling out the involvement of $\alpha_2\beta_1$ in the fibrin system. These data confirm that together the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins regulate EC vacuole and lumen development in three-dimensional fibrin matrices. Moreover, blockade of vacuole formation completely inhibited luminal development. This evidence indicates that lumen formation is preceded by vacuolation in this system and that intracellular vacuolation is a key step in lumen formation.

Regression of Pre-Existing Endothelial Cell Vacuoles and Lumens in Fibrin Matrices Occurs after the Addition of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ Antagonists

To determine whether blocking integrin function or ligation affected preformed vacuolar or luminal structures, various monoclonal anti-integrin blocking peptides and antibodies were added to developed cultures (Figure 6). At both 12- and 24-hour time points, we observed that combinations of cRGD peptide and anti- α_5 integrin antibodies induced nearly complete collapse of existing structures, resulting in minimal vacuolation and lumen formation. The addition of cRGD peptides alone or in combination with anti- α_2 integrin antibodies reduced vacuolation to approximately 50%. The RGE peptide combined with anti- α_5 integrin antibodies induced a small amount of regression, reducing vacuolation to approximately 70% at both time points. Addition of the control peptide, RGE, alone or combined with anti- α_2 integrin antibodies did not induce regression and had no effect on vacuolation or lumen formation. Photographs were taken of stained cultures at the 24-hour time point (Figure 7). The addition of cRGD peptides and α_5 antibodies induced complete regression and collapse of existing luminal structures as compared to control. The RGE peptide and α_2 antibodies had no effect. These data indicate that $\alpha_v\beta_3$ and $\alpha_5\beta_1$ not only regulate formation of vacuoles and lumens, but are necessary for the maintenance of these structures.

Figure 1. Time course of endothelial cell morphogenesis in a three-dimensional fibrin matrix. ECs (25,000 per well) were resuspended in 25 μ l of 10 mg/ml fibrinogen and added to 1 μ g thrombin to solidify the matrix. Medium was added and photographs were taken under transmitted light of the same culture and at an identical focal plane at the various time points (0.5, 5, 7, 12, and 24 hours) listed on each figure (**upper right**). **Arrowheads** indicate markers present at all time points to assist in orientation. Scale bar, 100 μ m.

Discussion

Here we present data showing that two integrins, $\alpha_v\beta_3$ and $\alpha_5\beta_1$, are involved in EC vacuolation and lumen formation in a three-dimensional fibrin matrix. Cells changed shape from solid and spherical to form vacuoles that converged into luminal structures. Combining anti- $\alpha_v\beta_3$ and anti- α_5 integrin-blocking antibodies completely

inhibited the formation of vacuoles and subsequent lumens. No blockade was observed with various combinations of control antibodies directed toward α_2 or $\alpha_v\beta_5$ integrins. Furthermore, blockade was also accomplished with a combination of the cyclic Arg-Gly-Asp (cRGD) peptide and anti- α_5 integrin antibodies. Blockade was not observed with either control Arg-Gly-Glu (RGE) peptide or anti- α_2 integrin antibodies. Finally, we were able to

Figure 2. Endothelial cell lumen development in a fibrin matrix is regulated by intracellular vacuole formation. Fibrin gels were embedded in plastic, cross-sectioned, stained with toluidine blue, and photographed at the various time points indicated (0, 5, 10, and 24 hours). Scale bar, 20 μm .

demonstrate regression of preformed structures by adding cRGD peptide and anti- α_5 integrin antibodies, while control experiments with RGE peptide and anti- α_2 integrin antibodies did not induce regression. These data indicate that EC morphogenesis in three-dimensional fibrin involves the coordinated efforts of both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.

A Case for Dual Integrin Control of Angiogenesis

ECs express the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins.^{49,50} The $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins have been reported to be involved in angiogenesis both *in vivo* and *in vitro*.^{15,50–57} Antibody and peptide blocking data in all studies revealed that blocking integrin function resulted in the blockade of angiogenesis. Recent evidence has revealed that the $\alpha_v\beta_3$ integrin is important for angiogenesis, based on the ability of LM609, a monoclonal antibody directed to the $\alpha_v\beta_3$ heterodimer or cyclic RGD peptides, to block angiogenesis.^{51–55} However, concern about $\alpha_v\beta_3$ being primarily responsible for the development of angiogenesis has been raised from recent analyses of α_v integrin knockout mice. Although removal of the α_v integrin subunit was lethal, considerable vascular development occurred.⁵⁸ One interpretation of this may be that the $\alpha_v\beta_3$ integrin functions to modulate the angiogenic response but is not solely responsible for it, and the addition of $\alpha_v\beta_3$ antagonists provides inhibitory signals that limit the response. It is clear from *in vitro* models that multiple integrins can reg-

ulate EC morphogenesis, and that it appears to depend on the matrix environment.^{15,50} For example, $\alpha_2\beta_1$ regulates morphogenesis in a collagen type I matrix,¹⁵ $\alpha_6\beta_1$ regulates morphogenesis in a laminin-rich matrix,⁵⁰ and $\alpha_v\beta_3$ and $\alpha_5\beta_1$ regulate morphogenesis in a fibrin matrix as reported here. Thus it appears that multiple integrins, presumably through common signaling pathways in ECs, can regulate the same critical steps in EC morphogenesis, such as migration, sprouting, invasion, vacuole formation, and lumen development. In support of these conclusions are *in vivo* studies defining the involvement of multiple integrins (eg, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_1\beta_1$, and $\alpha_2\beta_1$) during angiogenesis.^{51–57} The matrix environment through which EC morphogenesis proceeds *in vivo* is likely to be variable, depending on whether the process is occurring during embryonic development, during physiological events such as the female reproductive cycle, or following tissue injury (eg, wound repair and tumorigenesis). During these latter events, a prominent provisional matrix consisting of fibrin and fibronectin is observed.²⁰ Thus, depending on the circumstances, varying matrix environments may alter the relative contributions of particular integrins in the control of angiogenesis or vasculogenesis.

The finding that multiple integrins regulate EC morphogenesis raises the concept that cooperative interactions and signaling through two or more integrins may be important in these events. Cooperation between integrins has previously been reported for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ in leukocytes,⁵⁹ as well as with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in endothelial cells,⁶⁰ a transfected Chinese hamster ovary cell line,⁶¹

Figure 3. Time-course experiments demonstrating that the development of vacuoles and lumens by endothelial cells in fibrin matrices is an RGD- and α_5 integrin-dependent process. Cultures were prepared in the presence of either cRGD or RGE peptides (500 $\mu\text{g/ml}$) alone or combined with anti- α_5 or anti- α_2 integrin monoclonal antibodies (20 $\mu\text{g/ml}$). Control indicates the absence of peptides or antibodies. Cultures were fixed, stained, and quantitated at the various time points shown. Values represent the percentage of cells forming vacuoles and lumens. In each data set, four groups of 50 cells each were analyzed. Data shown are mean values $\pm$ SEM.

and K562 cells.⁶² In one study on AIDS-related Kaposi sarcoma, HIV-1 Tat protein binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins stimulated EC migration, basement membrane degradation, and morphogenesis *in vitro*.⁶⁰ A second study reported that cooperativity between the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins was involved in controlling cell migration.⁶¹ Another study using K562 cells transfected with $\alpha_v\beta_3$ determined that antibody blockade of the $\alpha_v\beta_3$ integrin also completely blocked $\alpha_5\beta_1$ -mediated phagocytosis.⁶² Pharmacological data from the same study suggested that ligation of $\alpha_v\beta_3$ blocked $\alpha_5\beta_1$ signaling through a protein kinase C pathway. Taken together, these studies support that $\alpha_v\beta_3$ - $\alpha_5\beta_1$ integrin cooperation and signaling may play a role in the regulation of many cellular activities, including morphogenesis.

Integrin Control of Endothelial Cell Vacuolation and Lumen Formation

The cytoskeletal alterations associated with EC morphogenesis have yet to be elucidated. Recently, however, much progress has been made in this area.⁶³ What is clear is that outside-in signaling through integrins, which tether the cell's actin cytoskeleton to the ECM, is one mechanism of controlling cell shape changes.⁶⁴ Recent work in our laboratory involving EC morphogenesis in

three-dimensional collagen matrices demonstrated $\alpha_2\beta_1$ -dependent vacuole, lumen, and network formation,¹⁵ which was associated with up-regulation of gelsolin, vasodilator-stimulated phosphoprotein (VASP), and profilin, three proteins that regulate actin cytoskeletal reorganization and signaling.⁶⁵ In the former study, anti- $\alpha_2\beta_1$ integrin antibodies completely blocked vacuole formation and completely interfered with subsequent lumen and capillary tube formation. Here our data support the same conclusion that when vacuole formation is blocked, subsequent luminal development is prevented. In the previous study using collagen matrices, EC intracellular vacuole formation was shown to be a pinocytic process that required both actin microfilaments and microtubules. In addition, a number of proteins associated with vesicular trafficking in cells including caveolin and annexin II were found to be associated with the vacuole compartment.¹⁵ These data, combined with the work in this report, indicate that intracellular vacuoles represent a novel intracellular compartment regulating the lumen formation step in EC morphogenesis.

Previous data¹⁵ and the data reported here show that integrins are required for the formation of intracellular vacuoles. Interestingly, the integrin-blocking reagents show no overlapping inhibitory influence in that anti- $\alpha_v\beta_3$ and - $\alpha_5\beta_1$ antibodies block EC morphogenesis in a fibrin

Figure 4. Complete blockade of endothelial cell vacuole and lumen formation by a combination of cRGD peptides and anti- α_5 integrin antibodies. Cultures (cRGD/anti- α_5) were prepared in the presence of cRGD peptides (500 $\mu\text{g/ml}$) combined with anti- α_5 integrin monoclonal antibodies (20 $\mu\text{g/ml}$). Control indicates the absence of peptides or antibodies. Cultures were fixed at the time points shown (3, 5, 7, 12, and 24 hours), stained, and photographed. Scale bar, 50 μm .

Anti-Integrin Blocking Antibodies Present

Figure 5. Endothelial cell vacuolation and morphogenesis are dependent on the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Endothelial cells suspended in a fibrin matrix were allowed to undergo morphogenesis in the presence of various monoclonal anti-integrin antibodies (20 $\mu\text{g/ml}$) for 24 hours. Antibodies used included MAb16 (α_5), clone A2-IIIE10 (α_2), clone LM609 ($\alpha_v\beta_3$), and clone P1F6 ($\alpha_v\beta_5$). Control indicates the absence of antibodies (None). Values represent the percentage of cells forming vacuoles and lumens. For each data set, four groups of 50 cells each were analyzed to determine the extent of vacuolation/lumen formation. Data shown are mean values $\pm$ SEM.

Blocking Conditions

Figure 6. Regression of preformed endothelial cell lumens follows the addition of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin antagonists. Collapse of developed vacuolar and luminal structures is initiated at both the 12- and 24-hour time points by adding cRGD peptides combined with anti- α_5 integrin antibodies. Endothelial cells suspended in a fibrin matrix were allowed to undergo morphogenesis for either a 12- or 24-hour period, at which time 500 $\mu\text{g/ml}$ of the peptides indicated and/or 20 $\mu\text{g/ml}$ of the various antibodies were added. Cultures were fixed 3 hours later and stained, and vacuolation was quantitated. Values represent the percentage of cells forming vacuoles and lumens. For each data set, four groups of 50 cells each were analyzed to determine the extent of vacuolation/lumen formation. Control indicates the absence of antibodies. Data shown are mean values $\pm$ SEM.

Figure 7. Antagonists of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ induce regression of preformed endothelial cell vacuoles and lumens. Preformed luminal structures (24 hours) were allowed to collapse for 3 hours in the presence of no antibodies or peptides (control), cRGD and anti- α_5 integrin antibodies (cRGD/anti- α_5), or RGE and anti- α_2 integrin antibodies (RGE/anti- α_2). The cultures were then fixed, stained, and photographed. Scale bar, 50 μ m.

matrix, but not in collagen,¹⁵ while the opposite is true for anti- $\alpha_2\beta_1$ antibodies. We have shown here that $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins together control EC vacuolation and lumen formation in fibrin. Thus this work provides new evidence for a previously undescribed role for the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins in EC vacuolation and lumen formation.

It is not entirely clear which ECM ligands are involved in $\alpha_v\beta_3$ - and $\alpha_5\beta_1$ -mediated EC morphogenesis in fibrin matrices. The fibrin matrix environment in the current study contains fibrin as well as serum-derived adhesive molecules such as fibronectin and vitronectin. Which of these molecules is most relevant to the EC morphogenic events remains to be determined. The $\alpha_5\beta_1$ integrin is known to bind fibronectin,⁶⁶ but also has recently been reported to bind fibrinogen.⁶⁷ In addition, $\alpha_v\beta_3$ is known to bind fibrinogen, fibronectin, and vitronectin,⁶⁴ all of which are present in our assays. The important point to be made is that the three-dimensional fibrin matrix system described in this report mimics the fibrin-fibronectin provisional matrices known to be prominent with the wound microenvironment where angiogenesis occurs. In any case, our study indicates that $\alpha_v\beta_3$ and $\alpha_5\beta_1$, both receptors for this provisional matrix, together control EC morphogenesis in this matrix environment.

Involvement of Integrins in Vascular Regression

A final point of this discussion deals with the relevance of this work as it relates to mechanisms of vascular regression in either tumor- or wound-derived provisional matrices. We present here evidence that not only are $\alpha_v\beta_3$ and $\alpha_5\beta_1$ involved in EC morphogenesis (ie, the formation of vacuoles and lumens), but ligation of these integrins is also necessary for maintenance of the cell's architecture and three-dimensional structure. Both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ have been discussed as targets for anti-angiogenic therapy.^{68,69} Some of the regression effects that we describe may be relevant to the known anti-angiogenic influence of $\alpha_v\beta_3$ integrin antagonists *in vivo*.^{51–53} These antagonists are known to induce regression of angiogenic blood vessels through an apoptotic mechanism, and they may act in part to induce regression of angiogenic vessels present in a fibrin-rich provisional matrix environment.

The $\alpha_5\beta_1$ integrin has been reported as a ligand for fibronectin⁶⁶ and fibrinogen⁶⁷ and regulates cell-mediated retraction of fibronectin-fibrin matrices.⁷⁰ This information, combined with our data, supports the possibility that $\alpha_v\beta_3$ antagonists in combination with $\alpha_5\beta_1$ antagonists may be more effective at inducing vascular regression than $\alpha_v\beta_3$ antagonists alone. Further work is needed to investigate this issue.

It is clear that EC morphogenesis and angiogenesis are complicated processes. The involvement of particular integrins in these events is dictated by multiple signals, including EC integrin expression levels, ECM content, and the source of the angiogenic stimulus. The system described here is an experimental method of investigating the pathways transmitted through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins that result in EC vacuole formation and morphogenesis in three-dimensional fibrin matrices. Elucidating the molecular events responsible for EC morphogenesis in fibrin matrices will provide important clues to a better understanding of how blood vessel formation and regression are regulated.

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