Angiogenesis in Collagen I Requires $\alpha_2\beta_1$ Ligation of a GFP*GER Sequence and Possibly p38 MAPK Activation and Focal Adhesion Disassembly*

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Angiogenesis depends on proper collagen biosynthesis and cross-linking, and type I collagen is an ideal angiogenic scaffold, although its mechanism is unknown. We examined angiogenesis using an assay wherein confluent monolayers of human umbilical vein endothelial cells were overlain with collagen in a serum-free defined medium. Small spaces formed in the cell layer by 2 h, and cells formed net-like arrays by 6–8 h and capillary-like lumens by 24 h. Blocking of $\alpha_2\beta_1$ but not $\alpha_1$ or $\alpha_2\beta_1$ integrin function halted morphogenesis. We found that a triple-helical, homotrimeric peptide mimetic of a putative $\alpha_2\beta_1$ binding site: $\alpha_1(1496-507)$ GARGERGFP*GER (where single-letter amino acid nomenclature is used, *P = hydroxyproline) inhibited tube formation, whereas a peptide carrying another putative site: $\alpha_1(127-138)$ GLP*GERGFP*GAP or control peptides did not. A chemical inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), SB202190, blocked p38 MAPK activity was increased in collagen-treated cultures, whereas targeting MAPK kinase (MEK), focal adhesion kinase (FAK), or phosphatidylinositol 3-kinase (PI3K) had little effect. Collagen-treated cells had fewer focal adhesions and 3- to 5-fold less activated FAK. Thus capillary morphogenesis requires endothelial $\alpha_2\beta_1$ integrin engagement of a single type I collagen integrin-binding site, possibly signaling via p38 MAPK and focal adhesion disassembly/FAK inactivation.

In adult mammals, angiogenesis, the growth of new capillaries from the existing vasculature, is the exclusive mechanism by which new vessels are formed, and is involved in normal homeostasis as well as in various diseases, including growth and metastasis of solid tumors, rheumatoid arthritis, and diabetic retinopathy (1). It is a complex process by which endothelial cells degrade their matrix, migrate, proliferate, and differentiate into new vessels (2). Angiogenesis depends upon the interaction of endothelial cells with extracellular matrix proteins via cell adhesion molecules, and the activities of growth factors and cytokines (3). Because type I collagen is a ubiquitous component of many tissues that undergo angiogenesis during embryonic development, it may play a role in promoting blood vessel development and contribute to pathological angiogenesis. In fact, type I collagen is among the most ideal scaffolds for the induction of angiogenesis in vitro. Thus, bovine aortic endothelial cells synthesize type I collagen (4, 5), and its expression precedes angiogenesis and is limited to the vicinity of cells forming capillary tubes in endothelial cell monolayers (6, 7). Furthermore, endothelial cells grown between or dispersed within collagen gels form branching networks of tubes (8–10), and in HUVEC monolayers, angiogenesis rapidly proceeds in the presence of type I collagen and sulfated glycosaminoglycans (11–13). In vivo, angiogenesis is disrupted in the chick embryo by inhibiting collagen triple helix formation or fibrillogenesis using α,β-dipropyl or β-aminopropionitrile, respectively (14). Therefore, a definitive role for collagen exists, but the mechanisms by which it exerts its pro-angiogenic effect remain elusive.

Roles for several intracellular signaling pathways are proposed for collagen-induced angiogenesis. Thus, in long term collagen gel cultures, the ERK1/2 and p38 MAPK pathways are implicated in mediating angiogenesis of the endothelial cell line MSS31, although their relative contributions to specific phases of angiogenesis are unknown (15). It is of interest that in mice null for p38α MAPK, primary angiogenesis proceeds normally, but remodeling of the capillary plexus and placental angiogenesis are defective (16), whereas lack of Mekk3, the p38 MAPK upstream activator, causes deficiency in both primary as well as placental angiogenesis (17). Additionally, links between the collagen-associated integrins and p38 MAPK have been established, albeit in a non-angiogenic context (18, 19). In three-dimensional collagen gels, PMA treatment induces HUVEC survival and tube formation by activating MAPK, PI3K, and Akt/protein kinase B pathways, which are postulated to act

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‡ The abbreviations used are: HUVECs, human umbilical vein endothelial cells; EBM, endothelial cell basal medium; ERK, extracellular regulated protein kinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MAPKAP, MAPK-activated protein; PI3K, phosphatidylinositol 3-kinase; SFDM, serum-free defined medium; SPR, surface plasmon resonance; SSP, single stranded peptide; THP, triple helical peptide; VEGF, vascular endothelial growth factor; PMA, phorbol 12-myristate 13-acetate; FGF-2, fibroblast growth factor-2; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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by up-regulating VEGF receptor expression and cell-cell adhesion molecules such as vascular endothelial cadherin (10). Within other matrix scaffolds, FGF-2-dependent angiogenesis correlates with a sustained, αβ2 integrin-dependent wave of ERK activity, necessary for endothelial cell migration (20), whereas VEGF promotes angiogenesis, but requires src kinase activity in cultures using a non-radioactive assay (Cell Signaling). Two average background subtraction functions of ImageQuant software. Phosphorylation were determined using the integrate volume and local average measurement of HUVEC network formation, i.e. the formation of tube-like structures and their intervening spaces, as the total empty area in a given culture. Area measurements were performed using Image 1.62 (National Institutes of Health). All assays were repeated at least three times with at least two wells per treatment group. In some experiments the progression of morphogenesis was also quantitated by measuring the width of cellular processes at the midpoint, as a measure of the stage of tube formation, i.e. well-formed tubes have narrow diameters, whereas poorly or partially formed tubes do not. p values were generated using the Student’s t test.

Fluorescent Staining of Angiogenic Cultures—100 µg of collagen I was coated onto permanes 2-well plates and allowed to dry at 4 °C prior to use. Slides were rehydrated with warm PBS for 5 min, before plating 10^6 HUVEC/cm² in SFDM; gels were prepared as outlined above. After 30 min or 1 h of collagen gel exposure, both the monolayer cultures, and the treated cultures (following gel removal) were rinsed in PBS, fixed in 4% paraformaldehyde for 10 min, rinsed in PBS, permobilized for 5 min with TBS/0.1% triton X 100, and blocked with TBS/1% BSA for 30 min. Cells were incubated with 1:100 anti-phospho-FAK in TBS for 1 h. In some cases, cells were dual-stained with 1:200 anti-human vinculin (hVIN1, Sigma) or 1:50 anti-talin (Research Diagnostics, Inc.). Following incubation with primary antibodies, cells were rinsed, incubated with the appropriate secondary antibodies, and rinsed again. Photographs were taken using Kodak 1600 color slide film and an Olympus BX50 upright microscope.

Analysis of the Phosphorylation State of Focal Adhesion Kinase—Two 12-well plates were set up for angiogenesis assays per time point; collagen gels were prepared as outlined above. One plate received collagen while the other SFDM; following polymerization, plates were extracted using 40 µl (10 µM/cm²) of 1x SDS loading buffer at various times after collagen addition. Gels were removed as outlined above, and cells were rinsed twice with cold PBS prior to extraction. Equivalent microgram quantities of total lysate were loaded and run on 12% pre-cast polyacrylamide gels (ICN Biomedicals). Proteins were then transferred onto polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and incubated with primary antibody, 1:500 phospho-FAK, 4 h at room temperature, and 1:250 fluorescein isothiocyanate-conjugated secondary antibody, 1 h at 25 °C. Blots were visualized using a Molecular Dynamics Fluorimagar. Relative levels of phosphorylation were determined using the integrate volume and local average background subtraction functions of ImageQuant software.

Analysis of p38 MAPK Activity—We determined relative p38 MAPK activity in cultures using a non-radioactive assay (Cell Signalling). Two 12-well plates were set up for angiogenesis assays as outlined above; one plate received collagen while the other SFDM. At various times following the addition of the collagen, gels and media were removed, cells were rinsed, incubated with the appropriate secondary antibody, and rinsed again; coverslips were glycerol-mounted. Photographs were taken using Kodak 1600 color slide film and an Olympus BX50 upright microscope.

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were twice with PBS, resuspended in PBS, and mixed 1:1 with 4% paraformaldehyde. Samples were read on a Beckman Coulter XL/MCL analyzer using a 488-nm argon ion laser and system II software. **Triple Helical Peptide Synthesis**—Chemicals, including 9-fluorenylmethoxycarbonyl-amino acid derivatives, of analytical reagent grade or better were from Novabiochem (San Diego, CA) or Fisher. Amino acids, except for Gly, were L-isomers. The monoalkyl chains decanoic acid (CH₃-(CH₂)₈-CO₂H, designated C₁₀) and palmitic acid (CH₃-(CH₂)₁₄-CO₂H, designated C₁₄) were purchased from Aldrich. THPs and single-stranded peptides (SSP) were synthesized and purified as described (28–30). Three THPs and one SSP were constructed: α₁(1256–270 THP, [M + H]⁺) 3721.8 Da (theoretical, 3722.0 Da); α₁(1217–138 THP, [M + H]⁺) 3498.8 Da (theoretical, 3502.9 Da); α₁(1496–507 THP, [M + H]⁺) 3595.5 Da (theoretical, 3595.5 Da); and α₁(1499–510 SSP, [M + H]⁺) 3499.9 Da (theoretical, 4170.7 Da). Circular dichroism spectra were recorded over the range λ = 190–250 nm on a JASCO J-815 spectropolarimeter using a 10-mm path-length quartz cell. We obtained thermal transition curves by recording the molar ellipticity (θ) at λ = 225 nm while continuously increasing the temperature from 5 to 80 °C at 0.2 °C/min, using a JASCO PTC-346W temperature control unit. For samples exhibiting sigmoidal melting curves, the reflection point in the transition region (first derivative) is defined as the melting temperature (T_m). T_m values were 44.5, 56.0, and 52.5 °C for α₁(1256–270 THP, α₁(1217–138 THP, and α₁(1496–507 THP, respectively.

**Surface Plasmon Resonance Measurements of Integrin-THP Interactions**—Binding of THP and SSP peptides to α₂β₁, α₁β₃, and α₁ integrins was determined at 25 °C using SFR from measurements of the accompanying increase in refractive index through time using a Biacore™ 2000 Biosensor (Biacore, Inc., Piscataway, NJ). Initially, the α₂β₁, α₁β₃, and α₁ integrins were captured on individual channels of a CM5 sensor chip by amine coupling. Solutions containing THPs and SSPs in elution buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl₂, pH 7.4) at the required concentrations were injected over each surface, and the response was measured as a function of time. The surface was regenerated after each injection by a single 10-s injection of 1 M NaCl. After subtraction of the contribution of bulk refractive index changes and non-specific interactions with the CM5 chip surface, typically less than 5%, the individual association (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data to a 1:1 Langmuir binding model using BIAevaluation™ (Biacore, Inc.). These values were then used to calculate the dissociation constant (K_d). The values of average squared m values obtained were not significantly improved by fitting data to models that assumed bivalent or heterogeneous interactions. Conditions were chosen so that the contribution of mass transport to the observed values of K_d was negligible.

**RESULTS**

To examine mechanisms of type I collagen-induced angiogenesis, we first modified the classic method for the induction of angiogenesis by a type I collagen gel deposited apically on a confluent HUVEC monolayer plated on a collagen gel (9). Thus, we simplified the system by replacing the basal collagen gel with a collagen film to prevent cellular invasion and developed a serum-free medium (SFDM) that optimally supported angiogenesis.

**Development of In Vitro Angiogenesis System**

Development of a Novel Serum-free Defined Medium—Typically, endothelial cells are grown and used in angiogenesis assays in 10% serum with either endothelial cell growth factor or endothelial cell growth supplement, a heterogeneous mixture derived from bovine hypthalamic extracts (24); in such media angiogenesis occurs over 24–72 h. To develop a SFDM substitute, we tested the effectiveness of three base media: EBM, Medium 199, and Dulbecco’s modified Eagle’s medium: Ham’s F-12 (1:1) in supporting tube formation. We found that EBM, a commercially available serum-free media for endothelial cells, although more complex and more expensive, was no more effective than Medium 199. The Dulbecco’s modified Eagle’s medium/Ham’s F-12 mixture, often used because it is a richer nutrient medium, was also no better than Medium 199. Next, we found that insulin, transferrin, and selenium, often included in serum-free formulations to enhance cell survival and growth, had no benefit. Finally, we evaluated the effect of FGF-2 and VEGF and found that VEGF alone at either 5 or 10 ng/ml was less effective than 5 ng/ml FGF-2 alone in supporting tube formation (10); however, a combination of 5 ng/ml of both growth factors was optimal in agreement with previous studies (31). Thus, our final SFDM was composed of Medium 199, 1% BSA (w/v), and 5 ng/ml FGF-2 and VEGF. Notably, use of this formulation resulted in the formation of widespread tube-like structures typically within 8–12 h.

**Time Course of Angiogenesis in SFDM**—Observation of live cultures revealed immediate changes within the cell layer following polymerization of the collagen gel, when cells appear flattened as compared with non-collagen-treated cells. Within 2 h small spaces appeared in the cell layer, enlarging to form a net-like structure by 6–8 h as cells condensed and elongated forming capillary-like tubes that persisted to 24 h (Fig. 1). At this time, intracellular vacuoles were apparent by phase contrast microscopy, and it is proposed that such structures are precursors to lumen formation (32, 33). Ultrastructural analysis of cell layers at 24 h revealed structures reminiscent of capillaries enclosing lumen-like cavities formed by several HUVEC (Fig. 2). The lumen-like cavities occasionally contained filopodia, debris, and cellular protrusions yet always excluded the fibrillar collagen gel (Fig. 2, B–D). Thus our system resulted in the rapid formation of capillary-like tubes and therefore replicated the end point or capillary morphogenesis phase of angiogenesis.

**Defining Cell Surface and Matrix Determinants Required for Collagen-induced Angiogenesis**

**Temporal Requirement for Collagen**—To determine whether the collagen gel initiates angiogenesis, or is required throughout the process, collagen gels were added to cultures, then removed and replaced with SFDM from 2–12 h following polymerization and were examined by microscopy an additional 24 h (not shown). We found that, when apical gels were removed at times less than 8 h after their addition, cellular reorganization was arrested and cells returned to a near-confluent monolayer as previously reported (34). However, in cultures where collagen gels were present for at least 8 h and capillary-like networks had formed, some remnants of the net-like structure remained following gel removal and persisted even at 24 h. Thus collagen must be constantly present during HUVEC tube formation. However, once morphogenesis is complete, parts of the capillary-like network remain quite stable morphologically, even in the absence of collagen.

**Requirement for the α₂β₁ Integrin**—We next examined the role of various integrins, the cell surface receptors likely to mediate HUVEC-collagen interactions. First, we tested the roles of the α₁, α₂, and β₁ subunits and the α₁β₁ and α₂β₁ heterodimers and found that addition of a function-blocking α₁β₁ integrin (VLA2) antibody at any time during the assay disrupted tube formation by about 70% (Fig. 3, B and E), albeit partial cell layer reorganization can be seen by 24 h. Notably, three times as much control IgG or twice as much function-
blocking $\alpha_1$ antibody had no effect (Fig. 3). Interestingly, addition of the VLA2 antibody at times up to 8 h after addition of the collagen gel halted morphogenesis at the stage at which the antibody was added; after 12 h of exposure to the collagen gel, reorganization had progressed to a stage where addition of the antibody was ineffective (not shown). Function-blocking antibodies against the $\alpha_1$ subunits alone inhibited morphogenesis by 50–60% at doses of 10 $\mu$g/ml (not shown). Similarly, doses as low as 5 $\mu$g/ml of function-blocking $\beta_1$ antibodies disrupted angiogenesis (not shown). We saw no effect of Reopro, the Fab portion of the $\alpha_1$V3 antibody, or LM609, a function-blocking $\alpha_1V3$ heterodimer, when tested at doses up to 25 $\mu$g/ml (Fig. 3D). Thus the endothelial cell-collagen interactions during angiogenesis depend upon sustained $\alpha_2\beta_1$ integrin engagement, with both subunits playing essential roles. In contrast, neither $\alpha_2\beta_1$ nor $\alpha_1\beta_3$ receptors have a detectable role.

HUVECs Express $\alpha_2\beta_1$, $\alpha_1$, and $\alpha_1\beta_3$ integrins—To test whether the lack of activities of the anti-$\alpha_1$ and -$\alpha_1\beta_3$ receptor antibodies could be attributed to the lack of expression of those integrins, we examined their expression by flow cytometry. Staining of cell populations with saturating or near-saturating concentrations of anti-integrin function-blocking antibodies revealed significant expression levels of $\alpha_2\beta_1$, $\alpha_1$, and $\alpha_1\beta_3$ receptors independent of passage number, in at least three experiments (not shown). Therefore, despite the presence of substantial amounts of $\alpha_2\beta_1$, $\alpha_1$, and $\alpha_1\beta_3$ receptors on HUVEC, the $\alpha_2\beta_1$ receptor alone promotes collagen-induced angiogenesis.

Requirement of the $\alpha_2\beta_1$ Integrin-binding Sites within Type I Collagen—Our finding that collagen-driven angiogenesis depends upon sustained HUVEC $\alpha_2\beta_1$ integrin engagement of collagen led us to ask whether specific sequences within type I collagen were also necessary. Candidate sequences include the two preferred putative $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrin binding domains recently identified on type I collagen (35, 36; Ref. 37 for review). Thus, we synthesized THPs carrying either of these sequences, and control peptides, and tested their effects by adding them immediately after collagen gel addition and following the cultures for the next 24 h (Fig. 4). Peptides carrying the $\alpha_1(I)496–507$ sequence GARGERGER inhibited tube formation at...
Although some morphogenesis was seen by 24 h. An analogous sis relies on engagement of a single type I collagen triple helix by endothelial cell IgG (25 μg/ml) (25). Fluent HUVEC monolayers received collagen gels and SFDM plus conduced HUVEC reorganization and tube formation by shown, magnification, ×1100. Values are the average of data from four experiments; p values are <0.01 for comparison of αβ1- treated cultures with control IgG-treated cultures.

Concentrations from 9 to 46 μM by 8–12 h in triple-helical form, although some morphogenesis was seen by 24 h. An analogous sequence in single-stranded form at concentrations up to 50 μM had no effect. In contrast, a comparable α/αβ1 integrin binding site at α1(I)127–138 had no effect on angiogenesis when tested in triple-helical form from 9 to 46 μM. A triple-helical peptide, α1(I)256–270 THP, carrying a non-relevant type I collagen sequence, also had no effect at concentrations up to 44 μM. These data suggest that type I collagen-driven angiogenesis relies on engagement of a single αβ1 integrin binding site within the type I collagen triple helix by endothelial αβ1 integrins.

Purified Integrins Interact with THPs Carrying Potential Integrin-binding Sequences—SPR was used to assess whether THPs containing sequences proposed to bind either αβ1 or αβ1 integrins bind to purified integrins (Table I). In the presence of 1 mM MgCl2, the triple-helical peptide α1(I)127–138 THP bound ~7 times more strongly to the α2 integrin subunit than to αβ1 integrins or αβ3 integrins, which had equivalent binding strengths. Likewise, the triple-helical peptide α1(I)496–507 THP bound 5 times more strongly to the α2 integrin subunit than to αβ1, and negligibly to αβ3. A single-stranded peptide containing an analogous sequence, α(I)499–510 SSP and a THP, including a non-relevant sequence, the α(I)256–270 THP, bound negligibly to all integrins demonstrating the need for triple-helical conformation and the GER sequence for significant binding. Thus the putative αβ1 and αβ1 integrin binding sequences in THP form bind to the appropriate αβ1 and αβ1 integrins.

Probing the Roles of the αβ1 Integrin-associated Signaling Pathways, FAK (Tyr-397) and p38 MAPK

Effects of Signaling Pathway Inhibitors—To identify intraacellular signaling pathways involved in αβ1-mediated angiogenesis, we tested the effects of a battery of signaling pathway inhibitors on tube formation (Fig. 5 and Table II). Some of these inhibit the function of pathway intermediates typically associated with integrin signaling, such as FAK and p38 MAPK, whereas others target additional pathways implicated in angiogenesis. We targeted FAK indirectly using geldanamycin (0.5–5 μg/ml) (38, 39); PI3K with LY294002 (1–10 μM, not shown) (40, 41) and wortmannin (10–50 nM) (38); p38 MAPK with SB202190 (5–25 μM) (42) and SB203580 (15, 18, 43) (1–25 μM); protein kinase C using Ro31-8220 (44) (100–500 nM) and calphostin C (45–47) (100–500 nM, not shown); and MEK with PD98059 (10, 15, 48, 49) (10–50 μM). Cultures were scored qualitatively for the extent of cell layer reorganization leading to HUVEC network formation; cell elongation and formation of capillary-like network; and cell attachment and survival of the tube-like network (Table II). Only SB202190, which inhibits the ability of p38 MAPK to activate the ATF-2 transcription factor (50), significantly affected endothelial tube formation, resulting in cultures that reorganized to form intercellular spaces in the cell layer in response to a collagen gel but never progressed to the cell elongation and endothelial tube formation phases (Fig. 5, B and C). Quantitation of the angiogenic area of SB202190-treated cell layers revealed only a minor effect in comparison with the controls despite a significant difference obvious by visual inspection of the cultures. We therefore measured the widths at the midpoint of the cellular processes comprising the vascular-like network in control and treated cultures, confirming that the inhibitor-treated cultures fail to undergo the elongation/narrowing phase of tube formation, i.e. the cellular processes in the treated cultures remain at a relatively constant width of 80–100 μm over time compared with the controls that decreased in width from 40 to 20 μm (Fig. 5C). Notably, another inhibitor of p38 MAPK, SB203580, which inhibits activation of MAPKAP kinase 2 and stress-induced activation of HSP27, was inactive (51). Inhibitors of FAK and PI3K slightly inhibited cell survival without affecting tube formation (Table II; not shown). To ensure that the observed effects were due to a specific inhibition of the target enzymes and not toxicity, we used a viability assay on treated cultures. Only SB202190 (at 10 and 25 μM) and wortmannin (at 50 nM) were mildly toxic based on a decrease in calcein and an increase in ethidium homodimer staining after 12 h of treatment (not shown). However, SB202190 at 5 μM showed no toxicity yet blocked tube development (Fig. 5). Thus, p38 MAPK activation may promote endothelial tube development, whereas the other signaling pathways examined may play no significant role.

Contact with Collagen Increases p38 MAPK Activity—Because p38 MAPK inhibition resulted in an arrest of tube formation, we reasoned that HUVEC contact with the collagen gel might result in increased p38 MAPK activity. Thus, we performed p38 MAPK assays using activated p38 MAPK immunoprecipitated from control and collagen-induced cultures. We determined p38 MAPK activity based on relative levels of phos-
Fig. 4. A triple-helical peptide (THP) representing a putative αβ1 and αβ3 integrin binding site inhibits tube formation. Cultures stimulated with an apical collagen gel received SFDM, 50 µM α1(1496–507) GAP*GERGEK*NH2 single-stranded peptide (SSP), 9 µM α1(1496–507)GAP*GERGEK*NH2, 9 µM α1(1256–270)GAP*GERGEK*NH2, or up to 46 µM α1(1256–270)GAP*GERGEK*NH2. Vascular tube formation was assessed by luxiferin assay. (A)演员 addicted to hair, (B) hairdressing. (C) A non-relevant THP: α1(1256–270)GAP*GERGEK*NH2, and (D) vehicle (SFDM). Each peptide was tested three times at each concentration, although there was some variation in the time course of angiogenesis in each of the experiments, which prohibited us from pooling the data. However, on average α1(1496–507)GAP*GERGEK*NH2 inhibited tube formation by 60 ± 7%, the data shown are from one experiment.

**TABLE I**

| Interaction                        | k_d | k_d | k_d |
|-----------------------------------|-----|-----|-----|
| α1(127–138)THP → α1β1*            | 6.98 ± 0.51 × 10³ | 4.81 ± 2.13 × 10⁻² | 7.00 ± 3.55 × 10⁻⁶ |
| α1(256–270)THP → α1β1*            | _b   | _b   | _b   |
| α1(496–507)THP → α1β1*            | 4.58 ± 2.22 × 10³ | 6.10 ± 2.96 × 10⁻² | 1.69 ± 1.46 × 10⁻⁵ |
| α1(499–510)SSP → α1β1*            | _b   | _b   | _b   |
| α1(127–138)THP → α1β2*            | 1.39 ± 0.57 × 10⁴ | 4.85 ± 1.34 × 10⁻³ | 4.05 ± 2.62 × 10⁻⁷ |
| α1(256–270)THP → α1β2*            | _b   | _b   | _b   |
| α1(496–507)THP → α1β2*            | 7.83 ± 2.47 × 10³ | 4.75 ± 5.59 × 10⁻⁵ | 1.10 ± 0.27 × 10⁻⁶ |
| α1(499–510)SSP → α1β2*            | _b   | _b   | _b   |
| α1(127–138)THP → α1β3*            | 1.22 ± 0.23 × 10⁴ | 8.81 ± 5.36 × 10⁻² | 6.93 ± 3.13 × 10⁻⁶ |
| α1(256–270)THP → α1β3*            | _b   | _b   | _b   |
| α1(496–507)THP → α1β3*            | _b   | _b   | _b   |
| α1(499–510)SSP → α1β3*            | _b   | _b   | _b   |

*p ≤ 0.004.

Negligible binding.

*p ≤ 0.064.

The αβ3, and αβ3 integrins were amine-coupled on the surface of a CM5 sensor chip, and the α1(127–138)THP, α1(256–270)THP, and α1(496–507)THP, and α1(499–510)SSP peptides were then injected over the chip surface. The response at equilibrium (R_equilibrium) and values of association (k_d) and dissociation (k_d) rate constants were derived from global fits of response against time data to a 1:1 Langmuir binding model. The dissociation constants (K_d) were obtained from the ratio k_d/k_d. Data are the average of two separate analyses; errors are ± S.E.

pho-ATF2 by immunoblotting, revealing 1.5- to 3-fold more p88 MAPK activity in collagen-treated than in control cultures at all times (Fig. 6).

**Contact with Collagen Causes Focal Adhesion Disassembly and a Decrease in Activated FAK**—We examined the distribution of focal adhesions in collagen-treated cultures because of the significant role that focal adhesions and their associated proteins play in mediating integrin–matrix interactions. Thus, we localized activated FAK (Tyr-397) and the focal adhesion-associated proteins vinculin and talin by immunocytochemistry following 30- or 60-min exposure to the apical collagen gel (not shown). We demonstrate that cells in control monolayers contained large numbers of focal adhesions positive for vinculin and talin rich in activated FAK at all times (not shown), whereas, cells stream out of the monolayer and show substantially reduced numbers of focal adhesions positive for vinculin and talin and contain less activated FAK after contact with collagen gels. To quantitatively examine levels of activated FAK, we extracted total protein from control cultures and those exposed to collagen from 15 min to 2 h. Immunoblotting for phospho-FAK (Y397) confirmed our immunohistochemistry observations revealing a 3- to 5-fold decrease in levels of phosphorylated FAK in collagen-treated cultures at all times, as compared with controls (Fig. 6). Thus focal adhesion disassembly and inactivation of FAK occur during the early phase of collagen-induced angiogenesis.

**DISCUSSION**

To study mechanisms of endothelial cell-collagen interactions in angiogenesis, we developed a novel SFDM and simplified an in vitro model representing the final stage of angiogenesis, capillary tube development. This formulation, consisting of a protein carrier (BSA) and low concentrations of FGF-2 and VEGF, was simpler and less expensive than other formulations (8, 52–54). Under these conditions confluent monolayers of HUVEC formed capillary-like tubes by 12–24 h in response to
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The effects of various pharmacological agents on HUVEC reorganization, tube formation, and long term attachment/survival were scored subjectively. + denotes that a particular event occurred, i.e. there was no effect of the inhibitor; − indicates that the event did not occur. The table summarizes data from experiments such as those in Fig. 5.

| Compound          | Reorganization | Tube formation | Attachment/survival |
|-------------------|----------------|----------------|--------------------|
| Geldanamycin      | +              | +              | −                  |
| LY 294002         | +              | −              | −                  |
| SB 202190         | +/−            | −              | −                  |
| SB 203580         | +              | +              | +                  |
| Ro 318220         | +              | +              | +                  |
| Wortmannin        | +              | +              | +                  |
| Me₃SO             | +              | +              | +                  |
| SSFM              | +              | +              | +                  |

**Fig. 6.** Levels of activated focal adhesion kinase (FAK) decrease and p38 mitogen-activated protein kinase (MAPK) activity increases during collagen-induced HUVEC tube formation. **Upper panel,** cultures were extracted, blotted for phospho-FAK (Y397), and quantitated as detailed under “Materials and Methods.” **Lower panel,** total protein extracts were immunoprecipitated using an immobilized anti-phospho-p38 MAPK antibody and blotted for phospho-ATF2. Data are representative of three to five separate extractions.

Furthermore, our system differs in its simplicity as many other systems require PMA (10, 33, 48), serum (34, 56), or conditioned medium (9). A notable exception may be the recently described system for angiogenesis of microvascular endothelial cultures, which form cellular cords within 6 h of collagen induction in the presence of serum, although lumen formation was not reported (56).

We demonstrated that collagen must be constantly present for angiogenesis to proceed, and removal of the apical gel at early times halted morphogenesis, causing disassembly of the tube-like network, as observed in a similar model (34). However, in cultures where the collagen gels were removed following HUVEC exposure for 8–10 h, some of the intercellular spaces persisted for up to 24 h, although a significant portion of cells returned to a monolayer. A possible explanation is that, during morphogenesis, a provisional matrix may be deposited delineating the intercellular spaces in the monolayer, preventing subsequent cellular repopulation.

We examined the role of integrins known to bind type I collagen and established a primary role for α₁β₁ integrins. We also found that angiogenesis relies on HUVEC interactions with triple-helical collagen in a region encompassing amino acids 496–507 on the α₁(I) chain, using THPs that retain the secondary and tertiary structure of native collagen. This region is one of two previously identified, potential α₁β₁ and α₂β₁ binding sites containing the I domain binding sequence, GER (35, 36). Despite the homology between these sequences, only a THP carrying the sequence at amino acids 496–507 of type I collagen inhibited angiogenesis. Our SPR results indicated that...
the two putative integrin binding sequences were able to bind both $\alpha_\beta_1$ and $\alpha_\beta_2$ integrins in peptide form, but in the context of the type I collagen fibril, potentially only the region around amino acids 496–507 of type I collagen may be accessible to HUVEC, e.g. the site around 127–138 could be buried within the collagen fibril. Although collagenous sequences containing the GFR triplet support both cell attachment and platelet aggregation, this is the first time they have been demonstrated to be essential for angiogenesis, a more complex morphogenic event (35, 57). These data are also the first report of preferential use of one of the two putative $\alpha_\beta_1$ integrin binding sites, because these were previously shown to support integrin binding and cell attachment equivalently (35, 36, 57).

We further demonstrated that angiogenesis depends on both the $\alpha_\beta_1$ and $\beta_1$ subunits but not the $\alpha_\alpha$ or $\alpha_\beta_2$ integrins, despite the abundance of each of these on the cell surface. Our data correlated with those demonstrating a requirement for $\alpha_\alpha$ integrins for murine mammary epithelial branching morphogenesis to proceed in collagen (58) and with a study using a sulfonyamide inhibitor, E7820, which blocks tube formation in collagen by down-regulating the amount of $\alpha_\alpha$ and $\beta_1$ integrins (59). On the other hand, mice lacking $\alpha_\alpha$ integrins show a defect in mammary gland branching morphogenesis as well as decreased platelet attachment to collagen under both static and shear conditions but no defect in wound healing or wound-associated angiogenesis (60), although compensation by other collagen-binding integrins could explain the latter observation. Interestingly, a number of reports suggest that HUVECs do not express $\alpha_\alpha$ integrins, although VEGF can induce its expression (61, 62), as may have been the case in our model. Our data differ from that of a study using microvascular endothelial cells in a serum-replete medium, demonstrating a requirement for both $\alpha_\alpha$ and $\alpha_\beta$ integrin subunits for endothelial cord formation in collagen, however, it is unclear whether these cultures form bona fide tubes (56). In light of recent reports demonstrating the lack of a primary role for the $\alpha_\beta_1$/$\beta_1$ integrins in angiogenesis, i.e. increased angiogenesis is seen in the absence of $\beta_1$ and $\beta_2$ integrins and normal angiogenesis in the absence of $\alpha_\beta_2$ integrins (63, 64), it was not surprising that block of $\alpha_\beta_2$ function had no effect on tube formation in our system.

We began identifying pathway(s) necessary for tube formation in a collagen gel, using a panel of pharmacological inhibitors. One such inhibitor, SB202190 targets p38 MAPK, allowed some degree of cell layer reorganization but blocked tube formation. This data suggest that there exist both early and late phases of angiogenesis and that p38 MAPK may be necessary for the later, tube formation phase. Interestingly, another p38 MAPK inhibitor, SB203580, that inhibits a murine endothelial cell line from forming tubes in collagen (15) had no effect on tube formation in our system or in a PMA-induced three-dimensional collagen gel system (10). A possible explanation for the discrepancy is that SB202190 inhibits p38 activation of ATF-2 and p38/p44, whereas SB203580 inhibits HSPP27 and MAPKAP kinase 2 activation in response to cellular stress, inter-leukin-1, and endothotoxin (51). Another possibility is that SB202190 may inhibit an angiogenic pathway distinct from p38 MAPK; future work will explore this. Consistent with a role for p38 MAPK, we showed its increased activity in collagen-induced HUVEC compared with untreated monolayers. A similar result is seen in murine mammary epithelium where $\alpha_\beta_2$ integrin contact with collagen activates p38 MAPK allowing for cell migration (19, 65). We saw no effect on tube formation by treatment with the MEK inhibitor, PD98059, nor did a group using immortalized endothelial cells in a sandwich-type collagen assay (66), whereas others demonstrate inhibition of tube formation in three-dimensional collagen assays (10, 15, 48).

One potentially relevant difference between these studies is the type of angiogenesis assay employed. Thus, the assays used here and by others recapitulate the tube formation stage of angiogenesis, discounting contributions of cell migration and proliferation (66). However, in three-dimensional culture systems, cells embedded within collagen gels or allowed to enter them from monolayers, form tubes after several days, allowing for collagen degradation, cell migration, and proliferation to contribute to tube morphogenesis (10, 15, 48). Therefore it is possible that MEK is necessary for HUVEC migration and/or proliferation but does not target tube formation. Recently, integrin-mediated suppression of PKA leading to an increase in actin stress fiber formation and tube formation is described in a system similar to ours (56). Such a mechanism could be at play in parallel, synergistically or at an earlier time to the one described in our study, and it is of interest to determine how the two might fit together.

We also examined the contribution of FAK, a molecule known to mediate integrin-dependent processes, in our system. Thus, we examined the levels of active, tyrosine 397-phosphorylated FAK (p-FAK Y397), using immunohistochemistry, and found less focal adhesions and less focal adhesions positive for p-FAK in collagen-exposed HUVECs compared with monolayer controls at all times, and immunoblot analysis confirmed this result. Potentially, FAK may play a permissive role during angiogenesis, i.e. focal adhesion disassembly accompanied by FAK deactivation may help eliminate cell-substratum contacts to allow cell layer reorganization and tube morphogenesis.

Our findings provide a starting point for understanding the mechanism(s) of type I collagen-driven angiogenesis. We have demonstrated a requirement for $\alpha_\beta_1$ and $\beta_1$ integrins and roles for p38 MAPK and FAK and pinpointed a single region of interaction between $\alpha_\beta_2$ integrins and triple-helical collagen I. Further work may elucidate whether $\alpha_\beta_2$ ligation of type I collagen leads to p38 MAPK activation, and in turn, how such a signal promotes capillary tube morphogenesis.

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Angiogenesis in Collagen I Requires α2β1 Ligation of a GFP*GER Sequence and Possibly p38 MAPK Activation and Focal Adhesion Disassembly
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